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Angiotensin II Type 1 Receptor-Modulated Signaling Pathways in Neurons

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Abstract

Mammalian brain contains high densities of angiotensin II (Ang II) type 1 (AT_1) receptors, localized mainly to specific nuclei within the hypothalamus and brainstem regions. Neuronal AT_1 receptors within these areas mediate the stimulatory actions of central Ang II on blood pressure, water and sodium intake, and vasopressin secretion, effects that involve the modulation of brain noradrenergic pathways. This review focuses on the intracellular events that mediate the functional effects of Ang II in neurons, via AT_1 receptors. The signaling pathways involved in short-term changes in neuronal activity, membrane ionic currents, norepinephrine (NE) release, and longer-term neuromodulatory actions of Ang II are discussed. It will be apparent from this discussion that the signaling pathways involved in these events are often distinct.

Index Entries: Angiotensin II type 1 receptor; neuron; protein kinase C; calcium/calmodulin; norepinephrine; intracellular signaling.

Distribution, Characteristics, and Functions of Brain Ang II Receptor Subtypes

It is widely recognized that mammalian brain contains all the components of the renin-angiotensin system, including specific receptors for the octapeptide angiotensin II (Ang II) (*1,2*). Furthermore, it has been demonstrated that Ang II elicits important receptor-mediated actions in the brain (*3,4*). The major concentrations of Ang II receptors are located in the hypothalamus and brainstem (*1,2*) and Ang II acts at specific neuronal receptors in these areas to modulate the activity of neural pathways. These actions ultimately lead to physiological and behavioral changes such as increased blood pressure, altered baroreflex modulation, increased water and sodium

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intake, and increased secretion of arginine vasopressin (AVP) (*5–14*).

Consistent with many peripheral tissues, mammalian brain contains two major subtypes of Ang II receptors: the Ang II type 1 (AT_1) and Ang II type 2 (AT_2) (*15*). These Ang II receptor subtypes were initially identified on the basis of differential affinities for peptide and nonpeptide ligands (*15–17*). Briefly, AT_1 receptors have a high affinity for the nonpeptide losartan, whereas AT_2 receptors have a high affinity for the nonpeptides PD123,177 and PD123,319 and for the peptide CGP42112A (*16,17*). Cloning studies have since demonstrated that the AT_1 and AT_2 receptors are quite different molecules. Both are similar in size (359 amino acids for the AT_1 363 amino acids for the AT_2; hydropathy analyses of the sequences showed that both have a seven-transmembrane spanning domain structure, consistent with G-protein-coupled receptors (*18–21*). However, AT_1 and AT_2 receptors are only 32–34% identical, based on amino acid sequence (*18–21*). Considering this, it is perhaps not surprising that AT_1 and AT_2 receptors have very different functions. AT_1 receptors, which are widespread in peripheral tissues, such as blood vessels, heart, kidney, adrenal cortex, and liver (*15*), are also localized in the hypothalamus and brainstem in areas such as the paraventricular nucleus of the hypothalamus (PVN), the median eminence, the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT), the solitary tract nucleus, and the dorsal vagal nucleus (*22–25*). The AT_1 receptors within these brain areas are involved in mediating the above-listed stimulatory effects of Ang II on cardiovascular regulation, fluid balance, and hormone secretion (*26–29*). It is also known that a critical component of the AT_1 receptor-mediated actions of Ang II in many, but not all, situations is the modulation of noradrenergic neuronal pathways (*29–31*). By contrast, the AT_2 receptors are localized mostly in different brain areas, such as the mediodorsal thalamic nuclei, ventral septum, inferior olive (IO), locus coeruleus (LC), and lateral septum (*22–25,32*), and do not appear to play a major role in the actions of Ang II on blood pressure, drinking, and vasopressin secretion (*26–29*). In fact, the physiological role(s) of AT_2 receptors is (are) not well understood.

In the following sections, we focus on the intracellular signaling pathways involved in the AT_1 receptor-mediated actions of Ang II in neurons. In the rodent, but not in the human, there are two subtypes of AT_1 receptor: AT_{1a} and AT_{1b} (*33,34*). The AT_{1a} and AT_{1b} receptor proteins are 95% homologous, and their binding characteristics and signaling mechanisms appear to be similar insofar as they have been studied (*33–37*). They are, however, only 35% homologous in the untranslated parts of the mRNAs, and this may help explain their distinct distributions in the brain. The AT_{1b} has a very restricted distribution in the adult rat brain occurring only in the anterior pituitary (*38*) and, under some circumstances, the arcuate nucleus of the female (*39*). The AT_{1a} receptor accounts for all other AT_1 receptor sites in the adult brain (*38*). In the fetus and neonate, only AT_{1a} mRNA could be identified in the pituitary gland (*40*). In many instances, we refer to experiments performed on neurons cultured from newborn rat hypothalamus and brainstem (*41*). For the sake of simplicity, these are referred to simply as "cultured neurons." We have not strictly determined which subtype of AT_1 receptor is present in these cultured neurons; however, the mRNA for both AT_{1a} and AT_{1b} is present according to results using reverse transcriptase-polymerase chain reaction (RT-PCR) methodology (U. V. Shenoy and C. Sumners, unpublished data), as well as that for the AT_2 receptor (*42*). Ang II receptor binding can be displaced by both AT_1 and AT_2 subtype-specific drugs (*41*). Thus, the subtype of AT_1 receptor involved in the Ang II-mediated effects discussed in the cultured neurons is unknown.

It is important to remember that the in vitro work discussed predominantly throughout this paper does not necessarily represent the intact adult rat brain, and we in no way intend to suggest that it does. However, this model yields useful data, especially when considered with data derived from other approaches.

AT_1 Receptor-Modulated Intracellular Signaling Pathways

Most information available on AT_1 receptor-mediated intracellular signal transduction pathways has been obtained from studies on peripheral tissues and cells. Many investigations have demonstrated that AT_1 receptors are coupled to either activation of phospholipase C (PLC) and a stimulation of phosphoinositide (PI) hydrolysis, or to an inhibition of adenylyl cyclase, depending on the cell or tissue type (*43–48*). These pathways are modulated presumably by G_q (or a G_q family member, such as G_{11} or G_{13}) and G_i, respectively. It is now apparent that AT_1 receptor signaling pathways are not so straightforward, because activation of AT_1 receptors can lead to stimulation of phospholipase D and phospholipase A_2 activities in certain cell types (*49–51*). In addition, Ang II causes an AT_1 receptor-mediated stimulation of the Ras/Raf/mitogen-activated protein (MAP) kinase and Janus kinase)/signal transducers and activators of transcription (JAK/STAT) pathways (*52,53*). These latter pathways may be activated by $G_{\beta\gamma}$ subunits. Stimulation of AT_1 receptors also leads to increased transcription of immediate-early genes, such as c-*fos* and c-*jun* and to production of the respective gene products Fos and Jun (*54,55*). These effects are likely mediated via PKC and MAP kinase (for review, see ref. *56*). Many of the same pathways are mediated by brain AT_1 receptors. For example, activation of AT_1 receptors in the median eminence leads to stimulation of PI hydrolysis (*57*), and our studies in cultured neurons have revealed that Ang II stimulates, via AT_1 receptors, PI hydrolysis with generation of inositol 1,4,5-triphosphate (IP_3) and subsequent increases in intracellular Ca^{2+} ($[Ca^{2+}]_{int}$) and activation of protein kinase C (PKC) (*41,58,59*). AT_1 receptor- mediated stimulation of PI hydrolysis has also been observed in NG108-15 neuroblastoma × glioma cells (*60,61*). Studies from brain and from cultured neurons also indicate that Ang II, via AT_1 receptors, stimulates increased levels of c-*fos* mRNA and induction of cFos and cJun proteins (*41,62–66*). More recently, Ang II has been shown to elicit AT_1 receptor-mediated stimulation of the Ras/Raf pathway, leading to increased MAP kinase activity, in cultured neurons (*67,68*). Thus, similar to peripheral tissues and cells, stimulation of neuronal AT_1 receptors leads to modulation of a variety of different intracellular signaling pathways. The ability of Ang II to affect multiple intracellular signaling molecules might indicate that this peptide can modulate different cellular functions through divergent signaling cascades. Indeed, as will be seen in the following sections, the intracellular pathways involved in the AT_1 receptor-mediated effects of Ang II on neuronal membrane ionic currents and norepinephrine (NE) turnover are exclusive in many cases.

Role of Intracellular Signaling Pathways in AT_1 Receptor Modulation of Neuronal Membrane Ionic Currents

Relatively few studies have addressed the modulatory actions of Ang II on neuronal membrane ionic currents and channels. These currents (and the underlying channels) are the basis of neuronal action potentials (APs). Therefore, an understanding of how they are modulated by Ang II is extremely important because the frequency and firing pattern of APs is the basic regulator of all physiological and behavioral events mediated by a given neuron. It is equally important to understand the intracellular signaling events responsible for mediating the effects of Ang II on membrane ionic currents. This is critical because perturbations of these signaling mechanisms would lead to altered influences of Ang II on neuronal activity and on the physiological and behavioral events mediated by a given neuron. Subsequent paragraphs discuss what is known about the AT_1 receptor-mediated effects of Ang II on neuronal membrane ionic currents/chan-

nels and, where available, neuronal activity. Furthermore, we discuss the intracellular signaling events that mediate these effects of Ang II on neuronal ionic currents.

AT_1 Receptor Modulation of Neuronal K^+ and Ca^{2+} Currents

Selective activation of neuronal AT_1 receptors by Ang II *in situ* or in brain slices elicits an increase in firing rate in specific brain regions such as the PVN, SFO, and the rostral ventrolateral medulla (RVLM) (*69–72*). Similar increases in neuronal excitation elicited by Ang II via AT_1 receptors have been observed in isolated single cells in plates of cultured neurons (*73*). Whole-cell and single-channel voltage-clamp recordings have been made to investigate the changes in membrane ionic currents that underlie the AT_1 receptor-mediated increases in neuronal excitation. In cultured neurons, Ang II, via AT_1 receptors, elicits a decrease in neuronal net outward ionic current (I_{no}) (*74*). In these cells, I_{no} is mainly comprised of Na^+, K^+, and Ca^{2+} current, and so to decrease I_{no}, Ang II must either attenuate K^+ current or potentiate Na^+ or Ca^{2+} current. Limited data are available on the actions of Ang II on Na^+ current in cultured neurons. However, Ang II (in the presence of 1 μ*M* PD123,319 to block AT_2 receptors) significantly decreases a voltage-dependent delayed rectifier K^+ current ($I_{K(v)}$, formerly referred to as I_K) and a transient A-type K^+ current (I_A), effects mediated via AT_1 receptors (*59,75*). Consistent with the latter effect is the recent finding that Ang II caused an AT_1 receptor-mediated decrease in single-channel open probability (NP_o) of an A-type K^+ channel in the same cultured neurons (*75*). Studies in cultured neurons have also shown that Ang II elicits an AT_1 receptor-mediated stimulation of voltage-dependent Ca^{2+} current (I_{Ca}) (*59*). The above observations on I_A agree with studies that determined that Ang II elicits an AT_1 receptor-mediated decrease in I_A in neurons from the SFO, SON, and PVN magnocellular areas contained in brain slices (*76–78*). In summary, the decreases in neuronal $I_{K(v)}$ and I_A and the increase in I_{Ca} elicited by Ang II are consistent with the observed increases in neuronal excitation discussed above (*69–72*). Furthermore, recent studies demonstrate that AT_1 receptors are localized on cultured catecholaminergic neurons (*79*) and that stimulation of AT_1 receptors on cultured neurons elicits release of NE (*see* Modulation of Brain NE Systems by AngII). Thus, the observed changes in $I_{K(v)}$, I_A, and I_{Ca} may underlie the increases in neuronal excitation that ultimately lead to NE release.

Signaling Pathways Involved in AT_1 Receptor-Modulated Neuronal K^+ and Ca^{2+} Currents

Activation/inhibition of membrane ionic currents and their underlying channels by G protein coupled receptors can occur through either direct (membrane delimited) coupling of the G protein to the channel or indirect modulation via protein kinases or phosphatases (*80,81*). PKC, Ca^{2+}, and IP_3 are known modulators of neuronal ion channels (*81–87*), and it is known that Ang II stimulates generation of IP_3 and increased $[Ca^{2+}]_{int}$ and PKC activity in neurons (*41,58,59*). Thus, studies have been performed to investigate the possible roles of IP_3, PKC, and Ca^{2+} in the modulation of neuronal $I_{K(v)}$, I_A, and I_{Ca} after AT_1 receptor stimulation. These studies are summarized as follows. With respect to $I_{K(v)}$ in cultured neurons, the AT_1 receptor-mediated inhibition of this current was partially reduced by intracellular application of anti-$G_{q/11\alpha}$ antibodies (*59*), and was totally abolished by the nonselective PLC inhibitor U73122 (10 μ*M*) (M. Zhu and C. Sumners, unpublished observations). U73343 (10 μ*M*), an inactive analog of U73122, did not modify the inhibition of $I_{K(v)}$ elicited by Ang II. Overall, these data suggest that the AT_1 receptor-mediated reduction of neuronal $I_{K(v)}$ involves a $G_{q/11\alpha}$ protein and activation of PLC, which is not surprising. However, this stimulation may not be so straightforward, and the role of $G_{q/11\alpha}$ needs to be more firmly established. This is because the anti-$G_{q/11\alpha}$ antibodies may be working by preventing the dissociation of

$G_{B\gamma}$ from $G_{q/11\alpha}$, so it might be argued that it is the $G_{\beta\gamma}$ that are responsible for the signal transduction. Further complications arise because recent reports have shown that Ang II (via AT_1 receptors) stimulates PI hydrolysis in vascular smooth muscle cells through a mechanism that involves activation of a soluble tyrosine kinase ($pp60^{c\text{-}src}$), which then phosphorylates, and activates, PLC (*88*). This proposed mechanism does not involve a $G_{q\alpha}$ protein and is unusual because G protein-coupled receptors normally activate $PLC\beta$ via $G_{q/11\alpha}$, whereas $PLC\gamma$ is normally activated by tyrosine kinase receptors (*88,89*). Such a mechanism may be involved in the negative modulation of $I_{K(v)}$ by AT_1 receptors in cultured neurons, because the reduction in neuronal $I_{K(v)}$ elicited by Ang II is abolished by intracellular perfusion of polyclonal anti-$PLC\gamma$ antibodies (*59*). The AT_1 receptor-mediated reduction in neuronal $I_{K(v)}$ elicited by Ang II was mimicked by intracellular injection of either IP_3 or superfusion of the PKC agonist phorbol-12-myristate-13-acetate (PMA) (*59*). This effect of Ang II was also partially reduced by treatment of cultures with either of the PKC antagonists calphostin C or PKC inhibitory peptide 19–31 (PKCIP) or by chelation of $[Ca^{2+}]_{int}$ with BAPTA (*59*). These data indicate involvement of both PKC and $[Ca^{2+}]_{int}$ in the negative modulatory effects of Ang II on $I_{K(v)}$ in cultured neurons, via AT_1 receptors. These data also suggest that another Ca^{2+}-dependent pathway, aside from PKC, is important in this response. Support for roles of both PKC and $[Ca^{2+}]_{int}$ in the negative modulation of $I_{K(v)}$ by Ang II also comes from experiments with a synthetic 25-amino acid peptide that corresponds to cytoplasmic loop 3 of the AT_{1a} receptor ($AT_{1a/i3}$) (*90*). Intracellular application of $AT_{1a/i3}$ elicited a decrease in neuronal $I_{K(v)}$ similar to that obtained with Ang II, an effect that was partially blocked by either PKCIP, BAPTA, or IP_3 receptor antibodies (*90*). Combined intracellular application of both PKCIP and BAPTA totally blocked the reduction in $I_{K(v)}$ elicited by $AT_{1a/i3}$, indicating that both PKC and $[Ca^{2+}]_{int}$ are essential for this response. The fact that all recordings of $I_{K(v)}$ were performed in the presence of the Ca^{2+} channel blocker $CdCl_2$, and that the AT_1 receptor-mediated decrease in $I_{K(v)}$ was partially reduced by anti-IP_3 receptor antibodies, indicates that IP_3-sensitive intracellular Ca^{2+} stores are important in this response. Because $[Ca^{2+}]_{int}$ is also involved in the negative modulation of neuronal $I_{K(v)}$ after AT_1 receptor activation, the possibility that another Ca^{2+}-dependent mechanism is involved in this response was investigated. Calcium/calmodulin-dependent protein kinase II (CAM kinase II) is a known modulator of ion channels and is activated by Ang II via AT_1 receptors in vascular smooth muscle cells (*91*). Thus, the idea that calmodulin and CAM kinase II are involved in the AT_1 receptor modulation of $I_{K(v)}$ in cultured neurons was tested. Preliminary studies have shown that the AT_1 receptor-mediated reduction in neuronal $I_{K(v)}$ is partially blocked by either the calmodulin antagonist W-7 (10 μM) or the specific CAM kinase II inhibitor KN-93 (10 μM) (M. Zhu and C. Sumners, unpublished observations).

In summary, these data indicate that the AT_1 receptor-mediated inhibitory effect of Ang II on neuronal $I_{K(v)}$ occurs via an intracellular pathway involving the activation of PLC and subsequent increases in the activity of PKC and CAM kinase II. These findings are summarized diagrammatically in Fig. 1. Although the data so far provide a basic framework for understanding how AT_1 receptor activation leads to inhibition of $I_{K(v)}$, many questions remain. For example, the nature of the PLC involved and mechanism of PLC activation are unclear. Other questions concern the mechanisms by which PKC and CAM kinase II modulate $I_{K(v)}$. For example, are they direct effects on the channel proteins? Furthermore, which biophysical properties of the K^+ channel do these enzymes modulate? These questions, and many others, remain the subject of intense investigation.

The intracellular signaling pathways that underlie the AT_1 receptor-mediated inhibition of I_A are less well defined. However, in preliminary studies, we have determined that the

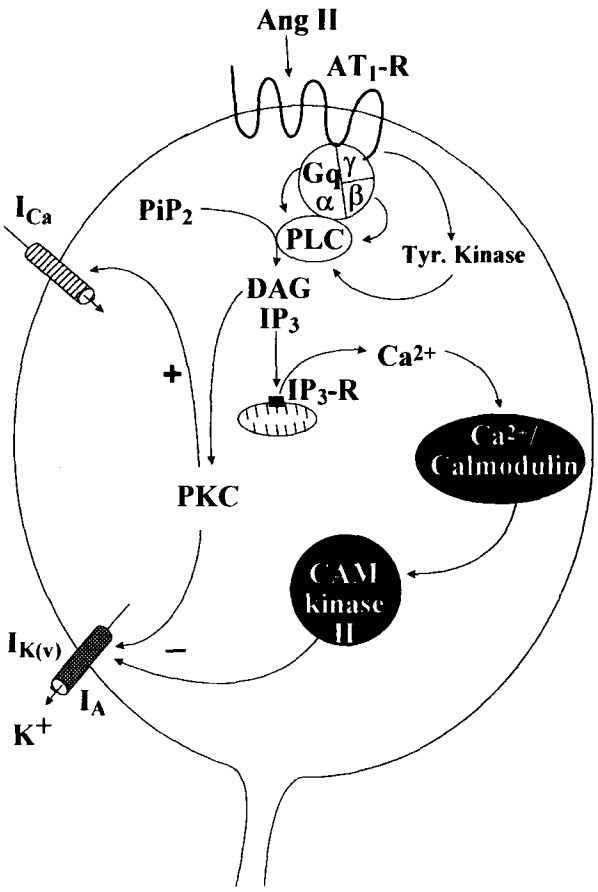

Fig. 1. AT_1 receptor-modulated neuronal potassium and calcium currents: putative mechanisms. $I_{K(v)}$, voltage-dependent delayed rectifier K^+ current; I_A, transient K^+ current; I_{Ca}, total Ca^{2+} current; PLC, phospholipase C; PiP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3-R, IP_3 receptor; PKC, protein kinase C; CAM kinase II, calcium/calmodulin-dependent protein kinase II. *Solid arrows,* stimulatory pathways; *dashed arrows,* inhibitory pathways for which there is support from published *(see* ref. *48,49,77–83)* or preliminary *(see text)* studies. *Areas shaded in black,* putative pathways.

PKC activator PMA decreases I_A in cultured neurons and also causes a dramatic decrease in the open possibility (NP_o) of A-type K^+ channels in these cells (C. H. Gelband and C. Sumners, unpublished observations). Thus, the effects of PMA are similar to those of Ang II (via AT_1, receptors) on I_A and A-type K^+ channels (*59,75*). This may indicate that the inhibitory effects of Ang II on neuronal I_A involve PKC, similar to its effect on $I_{K(v)}$.

The stimulatory effects of Ang II on neuronal I_{Ca} via AT_1 receptors also appear to occur via an indirect intracellular signaling pathway. In many respects, this pathway is similar to that which mediates the inhibitory effects of Ang II on $I_{K(v)}$. For example, the stimulation of neuronal I_{Ca} by Ang II and $AT_{1a/i3}$ involves both G_q and PLC (*59,90*). However, Ang II and $AT_{1a/i3}$-stimulated I_{Ca} is completely abolished by the PKC antagonists calphostin C or PKCIP (*59,90*). Thus, the AT_1 receptor mediated stimulation of I_{Ca} involves PKC alone, rather than dual regulation by PKC/CAM kinase II, as is the case for modulation of $I_{K(v)}$. These proposed pathways are summarized diagrammatically in Figure 1; similar to the modulation of $I_{K(v)}$, many questions remain concerning the precise mechanisms involved.

Role of Brain NE in the Centrally Mediated Actions of Ang II

In the periphery, a strong interaction between Ang II and catecholamines is well established. Ang II increases NE release from sympathetic nerves via AT_1 receptors and increases epinephrine release and synthesis in the adrenal medulla, amongst other actions (*92–94*). With the acceptance that a renin-angiotensin system exists in the brain, it quickly became apparent that the strong interaction between the Ang II and NE systems occurred centrally as well (*3,41*). The drinking and blood pressure responses to intracerebroventricular (ivt) injection of Ang II could be wholly or partially blocked by interruption of central catecholamine systems (reviewed in (*3*). Ang II receptors were found to be present in brain areas containing catecholamines, including localization of AT_1 receptors on noradrenergic cell bodies (*95,96*), and cells excited by Ang II and immunocytochemically

identified angiotensinergic neurons were found to project to many catecholamine-rich areas of the brain. At a more cellular level, Ang II was found to increase the release, synthesis, and reuptake of NE in brain areas important in the control of water balance and blood pressure, as well as in the anterior pituitary (*97*). Brain cell cultures of hypothalamus and brain-stem of 1-d-old rats contain the cell bodies and projection areas of most of the catecholamine and angiotensin cells in the brain important in the control of blood pressure and drinking behaviors. Use of these cultured neurons from rat brain confirmed that the strong interrelationship of NE and Ang II was true in this model. Readers should refer to more complete reviews of Ang II/NE interactions for further information and to references (*3*) and (*41*). The following section discusses more recent work on the role of AT_1 receptors, rather than Ang II receptors, in the interactions between Ang II and NE.

Modulation of Brain NE Systems by Ang II Via AT_1 Receptors

Further understanding of Ang II and NE interactions in the whole animal has come from studies of AVP release (*29,98*). AVP release is an important mediator of the blood pressure increase following ivt injection of Ang II. Examination of the mechanisms whereby Ang II stimulates AVP release from the SON and PVN via the pituitary gland into the blood, have revealed a complex interaction between Ang II, acting at AT_1 receptors, and NE at α_1 adrenergic receptors. Ang II acting on periventricular AT_1 receptors caused the release of both NE into the SON and PVN, and AVP from the SON and PVN via the pituitary into the blood. The action of Ang II on AVP release could be partially mimicked by injections of NE into the SON or PVN and partially blocked by inhibition of α_1 adrenoceptors in the SON and PVN. The findings suggested that stimulation of periventricular AT_1 receptors by Ang II caused NE release in the SON and PVN. In the PVN and SON, NE acted on α_1 adrenoceptors to stimulate AVP release via the pituitary into the blood. There are also AT_1 receptors in the PVN and SON whose stimulation resulted in AVP release that was not blocked by α_1 receptor blockade. This finding suggested that some AT_1 receptors in the SON and PVN do not indirectly stimulate AVP release via NE release; rather, they have a direct action on AVP release. However, some autoradiographic and *in situ* hybridization studies have not detected AT_1 receptors or AT_1 mRNA in the SON in the rat (*25,38,99*). This is hard to reconcile with studies in which AT_1 receptor stimulation by Ang II resulted in a physiological response in the SON (*29,98*), and with immunocytochemical studies (*62,100–102*), showing the presence of AT_1 receptors. Clearly, further studies are needed to clarify this situation.

Ivt injection of Ang II also stimulates the release of NE from the anterior hypothalamus, an AT_1 receptor-mediated effect which may be part of the pathway causing the increases in blood pressure after Ang II administration (*103*). Central AT_1 receptors are also implicated in the control of sympathetic outflow and increases in NE release into the blood from the adrenal medulla in response to immobilization stress, as central administration of the AT_1 receptor blocker losartan inhibited this response (*104*).

These actions of AT_1 receptors on noradrenergic functions are not unique to brain, as there are well-documented effects in the periphery. For example, in the caudal artery of the rat, Ang II elicits AT_1 receptor-mediated increases in NE efflux and NE-induced vasoconstriction (*105*).

Central injection of Ang II increases the activity of tyrosine hydroxylase (TH) and TH mRNA levels in hypothalami and brainstems of rats (*106*). TH is the rate-limiting enzyme in the synthesis of NE. Thus, Ang II not only increases release of NE but also increases its potential for synthesis in the hypothalamus and brainstem.

The actions of Ang II at AT_1 receptors on NE neurotransmission have also been examined in neurons cultured from the hypothalamus and brainstem of 1-d-old rats. In cultured neu-

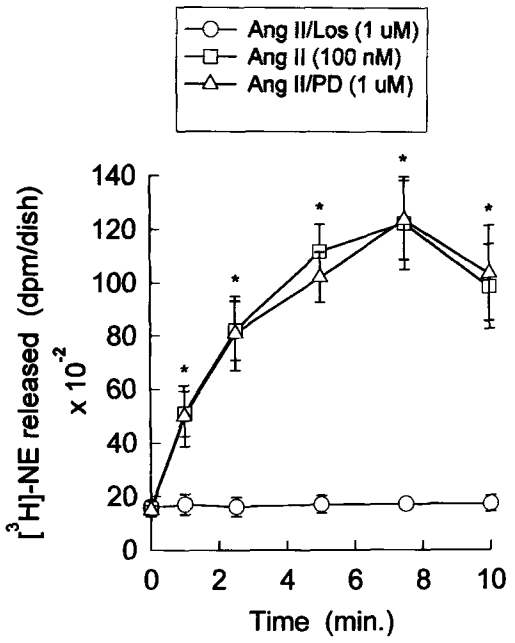

Fig. 2. Ang II stimulates NE release from cultured neurons via AT_1 receptors. Neurons cultured from newborn rat hypothalamus and brainstem were incubated with 0.1 μ*M* [^{3}H]-NE for 20 min in the presence of 200 μ*M* ascorbic acid and 100 μ*M* pargyline. Cultures were then washed three times with phosphate-buffered saline (PBS) (pH 7.4), followed by incubation with 0.5 mL/dish PBS ± Ang II (100 n*M*) for the indicated times at 37°C. Release of [^{3}H]-NE was assessed by counting the radioactivity contained in the PBS incubate from each treatment group. For the losartan (Los) and PD123319 (PD) treatments, cultures were preincubated with 1 μ*M* Los or 1 μ*M* PD for 30 s before Ang II and were also included with the Ang II. Data are means ±SEM from four experiments. *$p < 0.001$ compared with controls (*y*-axis), and Ang II/Los-treated neurons. Los or PD alone did not alter [^{3}H]-NE release (data not shown).

rons 100 n*M* Ang II caused a sixfold increase in NE release from cells preloaded with [^{3}H]-NE (Fig. 2). This response was significant 1 min after application of Ang II, peaked 8 min after Ang II application, and decreased thereafter. The AT_1 receptor antagonist, losartan (1 μ*M*), completely blocked the Ang II-mediated increase in NE release, whereas the AT_2 receptor antagonist, PD123,177, was without effect, indicating that AT_1 receptors mediate this effect (Fig. 2).

Since AT_1 receptor stimulation increased release of NE from cultured neurons, one might predict that it should also stimulate synthesis of NE. This is, in fact, the case, as demonstrated by the ability of Ang II, acting at AT_1 receptors to stimulate the synthesis of TH, an enzyme crucial in the anabolism of NE (*106*). This stimulation involves activation of PLC and PKC (*106*). Furthermore, the data presented in Fig. 3 indicate that there are increases in the levels of immunoreactive TH and dopamine β-hydroxylase (DβH) in synaptosomes prepared from neuronal cultures. Levels of both enzymes increased up to 4 h after Ang II treatment and were still raised at 24 h after Ang II, although the levels at 24 h were less than at 4 h. Previous studies in vivo and in neuronal cultures have shown that both the activities of and the mRNAs for TH and DβH increased after exposure to Ang II (*106*), so the findings in synaptosomes extend these to include the fact that the protein levels of the enzymes are increased and that the sites of increased TH and DβH include the nerve terminals (the predominant constituent of synaptosomes).

Once NE has been released into the synaptic cleft, it can itself modulate neuronal activity by acting on its specific receptors. The primary method for termination of the action of NE is its removal from the cleft by neuronal reuptake. Ang II, via AT_1 receptors, stimulates the neuronal reuptake of [^{3}H]-NE. Examined in neuronal cultures there are two phases to this response, a "fast" uptake, occurring in seconds and minutes, which appears to be a stimulation of extant norepinephrine transporter sites (NET). This is independent of AT_2 receptors (*107*). The mechanism by which AT_1 receptor stimulation can quickly enhance NE uptake is not understood. The second phase of AT_1 receptor-mediated enhancement of NE uptake occurs after longer periods of Ang II application (*107*). This point is illustrated by data obtained from synaptosomes prepared from

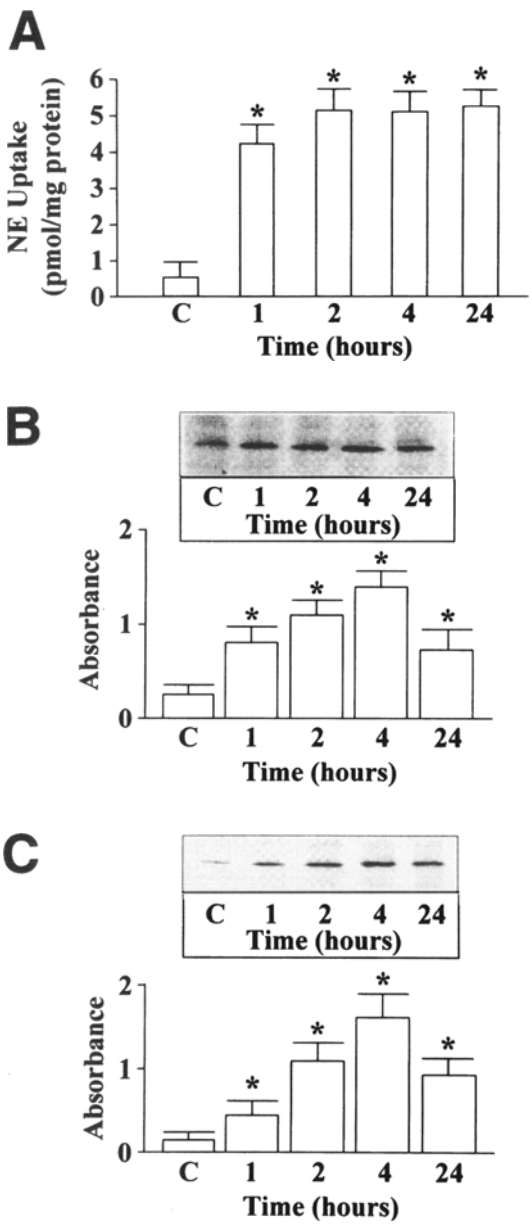

Fig. 3. Effects of Ang II on [^{3}H]-NE uptake, TH, and DβH immunoreactivities in synaptosomes from cultured neurons. Neuronal cultures were incubated with 100 n*M* Ang II for the indicated time periods. Cells from 10 100-mm culture dishes were homogenized in 0.32 *M* sucrose and synaptosomes were prepared essentially as described elsewhere *(107)*. Synaptophysin antibody was used to determine the purity of the synaptosomal preparations. **(A)** Synaptosomal preparation containing 1 mg protein was incubated with 1 n*M* [^{3}H]-NE in the absence or presence of the neuronal NE uptake blocker (1 μ*M*) maprotiline essentially as described previously *(105)*. Specific neuronal uptake of [^{3}H]-NE was calculated by subtracting uptake in the presence of maprotiline from the total [^{3}H]-NE uptake. Data are means ±SEM (n = 3). *, significantly different from control, $p < 0.01$. **(B,C)** A total of 100 μg of synaptosomal protein samples was subjected to 4–15% sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteins were transferred to nitrocellulose membranes. The membranes were then used for immunoblotting with 1 μg/mL anti-TH or anti-DβH polyclonal antibodies (Chemicon, Temecula, CA) essentially as described previously *(64)*. Antibodies bound to TH or DβH were identified by horseradish peroxidase-labeled antirabbit antibody and visualized by chemiluminescence. Bands corresponding to TH and DβH were quantitated using an SW5000 Gel Analyzer *(64,105)*. Top, representative autoradiograms. Bottom, mean data from three experiments ±SEM. *Significantly different from control, $p < 0.01$.

neuronal cultures (Fig. 3). In intact cells rather than synaptosomes, the longer-term AT_1 receptor-mediated stimulation of NE uptake has been shown to be caused by an increased number of newly synthesized NET that are active on the surface of the cells, as the increased uptake is blocked by RNA and protein synthesis inhibitors *(107)*. It has also been shown, by nuclear run on experiments, that there is increased transcription of the NET gene after AT_1 receptor stimulation, further confirmation of this mechanism of enhancement of NE uptake (M. K. Raizada, unpublished data).

In summary, the effects of Ang II acting at AT_1 receptors on NE in cultured neurons include increases in the release, synthesis and uptake of NE. They emphasize the strong neuromodulatory effect of Ang II on NE systems in nervous tissue.

Signaling Pathways Underlying the Neuromodulatory Actions of Angiotensin II

The intracellular events leading from stimulation of AT_1 receptors on the cell surface through

modulation of NE release, synthesis, uptake, and metabolism, and thence to behavioral events or increases in blood pressure are not well understood in vivo. However, some parts of this pathway have been discerned. It is known that ivt injection of Ang II causes increases in the expression of the immediate-early gene (IEG) product, Fos, in the SFO, median preoptic nucleus (MnPO), OVLT, SON, and PVN (*62–66*). In some of these brain areas, for example, the SON and PVN, the increase in immunoreactive Fos was often localized to cells that were also immunoreactive for the AT_1 receptor (*62*). The above mentioned brain areas are all associated with the drinking and blood pressure responses to central Ang II injection (*26–28*). These effects of Ang II can be blocked wholly or partially by interruption of NE pathways in the brain and mimicked by application of NE suggesting that they may be involved in the neuromodulatory effects of AT_1 receptor activation on NE pathways (*3,41*). These effects are AT_1 receptor mediated because either AT_1 receptor blockade by losartan or interruption of AT_1 receptor synthesis with antisense oligonucleotides abolished the induction of Fos (*62,64,65*).

Several other IEG products are also induced in the brain by AT_1 receptor stimulation. It has been shown that stimulation of central Ang II receptors resulted in increased expression of Fos, Fos B, Jun, Jun B, Jun D, Krox-20, and Krox-24 in the SFO, MnPO, PVN, and SON (*66*). These brain areas are important in the central effects of Ang II as mentioned above. The significance of the increases in IEG products in these brain areas is unclear, especially because some of them are probably secondary events, i.e., not occurring in AT_1 receptor-containing cells.

The importance of the finding that AT_1 receptor stimulation increases IEG expression is that it might help in understanding how activation of AT_1 receptors on the cell surface can lead to changes in the transcription of a gene for a protein, such as DβH. The products of iegs usually form complexes that act as transcription factors. There are many different IEG products, and the manner in which they interact with each other and with their DNA binding sites to affect transcription rates is quite complex. However, it can be simplified for the purpose of discussion. There are at least two basic ways that a transcription factor can have increased activity. The amounts of proteins that form the transcription factor complex can be changed, or its activity state can be altered, for example by phosphorylation. Phosphorylation of the complex-forming proteins either allows more complexes to form (for example those that bind AP-1), stimulates the DNA binding activity of previously formed complexes (e.g., those that bind SRE) or increases the activity of DNA bound complexes on transcriptional activity (*56*). In cultured neurons it has been shown that AT_1 receptor activation uses two, and by inference three, of these mechanisms to increase transcription of TH, DβH, and NET. Figure 4 illustrates these steps, and the following hypothesis describes this pathway. At the cell surface AT_1 receptor stimulation by Ang II leads to release of the βγ subunits from the G protein associated with the receptor. This βγ subunit interacts with the Ras-Raf-MAP kinase pathway, classically described for growth factor signaling, to yield phosphorylated (activated) Map kinase (erk1, erk2). Activated MAP kinase increases the synthesis of Fos and Jun. The mechanisms have not yet been elucidated in these cells, but probably involve phosphorylation of Elk-1 in preformed, DNA bound ternary complexes on the c-*fos* serum response element (SRE) (*56*). AT_1 receptor activation also increases the activity of PLC and calcium-dependent PKC, via $G_{q/11\alpha}$ (*41*). PKC activates Fos regulating kinase (FRK) by phosphorylation, which in turn phosphorylates Fos. Simultaneously c-Jun N-terminal kinase (JNK) is activated by AT_1 receptor stimulation probably in a $G_{\beta\gamma}$ subunit dependent fashion. Fos and Jun form heterodimers to become the AP-1 binding complex, and phosphorylation of the Fos and Jun in this complex results in increased ability to bind to DNA at the AP-1 site and increased transcription of the gene regulated by that AP-1 site (*108–110*). The genes for TH and DβH have been shown to contain either the AP-1 binding site or Fos-dependent sites in their promotor regions (*111–113*).

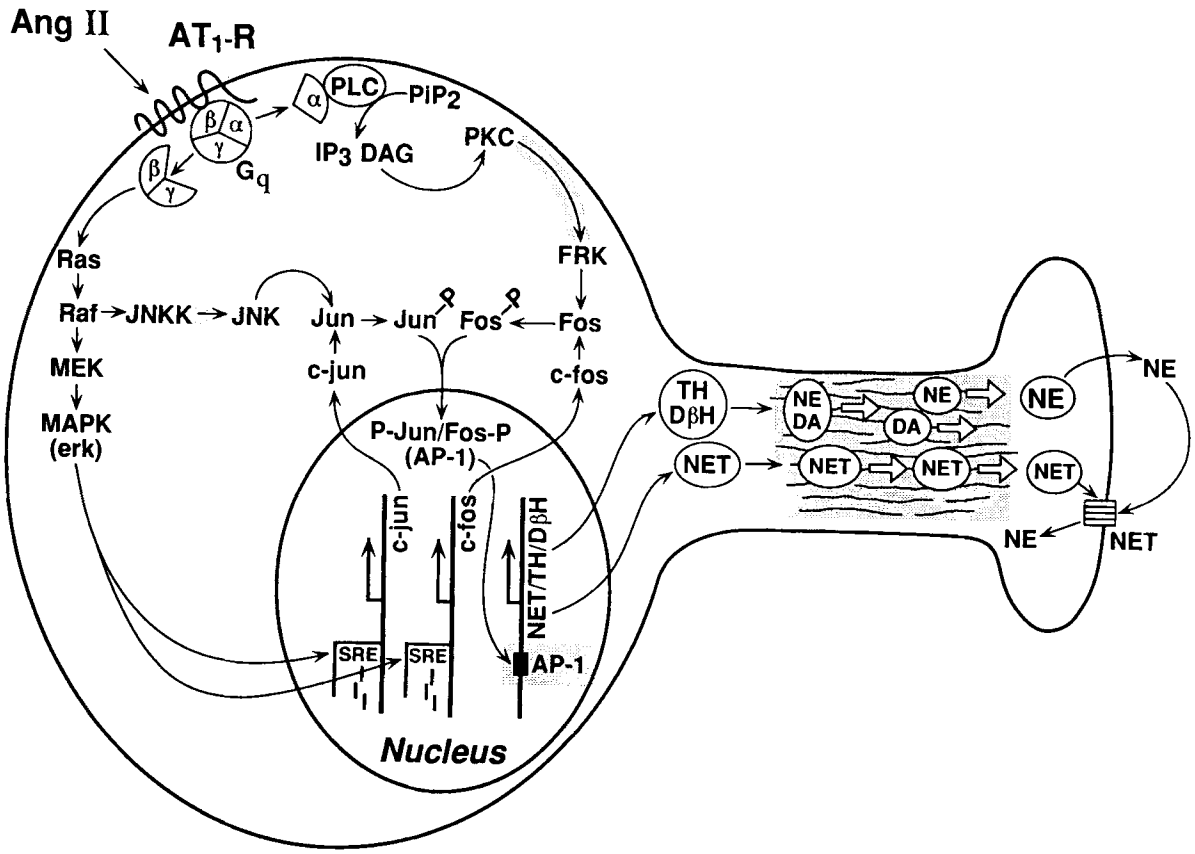

Fig. 4. AT_1 receptor modulation of neuronal NE: putative intracellular mechanisms. PLC, phospholipase C; PiP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; FRK, Fos regulating kinase; JNK, c-Jun N-terminal kinase; MEK, MAP kinase kinase; MAPK (Map kinase [erk1, erk2]); SRE, serum response element; NET, norepinephrine transporter; TH, tyrosine hydroxylase; DβH, dopamine β-hydroxylase; NE, norepinephrine; DA, dopamine. *Areas of gray shading,* putative pathways.

The evidence to support this hypothesis is as follows. AT_1 receptor activation increases MAPK activity in neuronal cells (*67,68*) as well as other cells (*114,115*). Use of either a MEK inhibitor, which totally blocks MAPK activity, or antisense oligonucleotides against MAPK, which reduce MAPK activity by about 70%, block the AT_1 receptor-mediated increase in transcription of TH, DβH, and NET genes (*116*). AT_1 receptor stimulation increase the content of Fos and Jun in neuronal cells (*41,116*) and increases the activity of FRK in a PKC- and calcium-dependent fashion (*117*). Ang II also increases JNK activity via AT_1 receptors, although the mechanism involved in this activation remains unknown (*117*). Finally, the binding activity at both AP-1 and SRE binding sites are increased following AT_1 receptor activation, as demonstrated by gel-shift experiments (*116*).

It is clear that the work described above falls far short of a complete understanding of all of the cellular events that occur during AT_1 receptor-mediated modulation of NE systems. However, they do provide some insight into the way in which one of the many potential transcription factors activated by AT_1 receptor stimulation exert effects on genes important in NE neurotransmission.

Summary

The data reviewed in this article indicate that Ang II activity at neuronal AT_1 receptors can modulate the electrical activity of neurons via changes in K^+ and Ca^{2+} currents, as well as elicit both short-term and long-term neuromodulatory effects on NE transmission. Examination of the signal transduction processes involved in these varied responses has shown that central AT_1 receptors couple to multiple intracellular pathways. In some instances, the various signal transduction pathways converge to affect a single AT_1 receptor-mediated event (e.g., TH synthesis), whereas in other cases the pathways exclusively alter a single neuronal property (e.g., I_{Ca}). Clearly, many facets of AT_1 receptor-mediated effects need to be determined before a complete understanding of when, and under what conditions, the different signal transduction pathways are active.

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