Regulation of Angiotensin II–induced Neuromodulation by MARCKS in Brain Neurons

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Abstract. Angiotensin II (Ang II) exerts chronic stimulatory actions on tyrosine hydroxylase (TH), dopamine β-hydroxylase (DβH), and the norepinephrine transporter (NET), in part, by influencing the transcription of their genes. These neuromodulatory actions of Ang II involve Ras-Raf-MAP kinase signal transduction pathways (Lu, D., H. Yang, and M.K. Raizada. 1997. J. Cell Biol. 135:1609–1617). In this study, we present evidence to demonstrate participation of another signaling pathway in these neuronal actions of Ang II. It involves activation of protein kinase C (PKC)β subtype and phosphorylation and redistribution of myristoylated alanine-rich C kinase substrate (MARCKS) in neurites. Ang II caused a dramatic redistribution of MARCKS from neuronal varicosities to neurites. This was accompanied by a time-dependent stimulation of its phosphorylation, that was mediated by the angiotensin type 1 receptor subtype (AT₁). Incubation of neurons with PKCβ subtype specific antisense oligonucleotide (AON) significantly attenuated both redistribution and phosphorylation of MARCKS. Furthermore, depletion of MARCKS by MARCKS-AON treatment of neurons resulted in a significant decrease in Ang II–stimulated accumulation of TH and DβH immunoreactivities and [3H]NE uptake activity in synaptosomes. In contrast, mRNA levels of TH, DβH, and NET were not influenced by MARKS-AON treatment. MARCKS peptidomimetic, which contains PKC phosphorylation sites, inhibited Ang II stimulation of MARCKS phosphorylation and reduced the amount of TH, DβH, and [3H]NE uptake in neuronal synaptosomes. These observations demonstrate that phosphorylation of MARCKS by PKCβ and its redistribution from varicosities to neurites is important in Ang II–induced synaptic accumulation of TH, DβH, and NE. They suggest that a coordinated stimulation of transcription of TH, DβH, and NET, mediated by Ras-Raf-MAP kinase followed by their transport mediated by PKCβ-MARCKS pathway are key in persistent stimulation of Ang II’s neuromodulatory actions.

Key words: MARCKS • brain neurons • AT₁ receptors • neuromodulation • protein kinase C subtypes

Evidence has been accumulating that angiotensin II (Ang II) exerts diverse physiological actions in both peripheral and neural tissues. In the periphery, its actions include vasoconstriction, hypertrophy, cell multiplication, and tissue remodeling, whereas the central nervous system (CNS) actions of Ang II include neuroendocrine hormone secretion, regulation of sympathetic activation and dampening of baroreceptor function (Saavedra, 1992; Steckeling et al., 1992; Dzau, 1993; Timmermans et al., 1993; Naftilan, 1994; Raizada et al., 1994; Wright and Harding, 1994). Sympathetic activation of the CNS neurons by Ang II is associated with the stimulation of turnover, synthesis, and release of catecholamines (Steckeling et al., 1992; Gelband et al., 1998). All of these diverse physiological effects of Ang II are mediated by its interaction with the angiotensin type 1 receptor subtype (AT₁ receptor), and that such diversity may stem from coupling of the receptor to various signal transduction pathways. For example, the vascular smooth muscle cell AT₁ receptor is linked to the JAK-STAT signaling system that mediates Ang II’s effects on DNA replication, hypertrophy, and extracellular matrix; whereas neuronal AT₁ receptor is cou-
pled to the Ras-Raf-MAP kinase signaling pathway, which regulates enhanced neuromodulatory actions of Ang II including stimulation of tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and norepinephrine transporter (NET) (Marrero et al., 1995; Lu et al., 1996, 1997; Yang et al., 1996; Yu et al., 1996; Gelband et al., 1998).

In addition to its coupling with JAK-STAT and Ras-Raf-MAP kinase pathways, the AT₁ receptor is also coupled to the phospholipase C–phosphoinositide–protein kinase C (PKC) signaling system (Sumners and Raizada, 1993; Freeman et al., 1995). Studies have established that AT₁ receptor stimulation increases inositol phosphate (IP) formation, stimulates PKC, and mobilizes Ca²⁺ from IP₃-sensitive pools in both brain neurons and peripheral cells (Sumners and Raizada, 1993; Freeman et al., 1995). This pathway is particularly important in neurons because both Ca²⁺ mobilization and PKC activation are involved in the synthesis, release, and re-uptake of many neurotransmitters (Zhu and Ikeda, 1994; Patel et al., 1995). Since Ang II regulates neurotransmitter synthesis and release, it was of great importance for us to further delineate the mechanism of PKC involvement in Ang II–induced neuromodulation. In this study we focused our attention on myristoylated alanine-rich C-kinase substrate (MARCKS) since it is a major PKC substrate, exists in high concentrations in neurons, and has been implicated in cytoskeletal rearrangement, membrane trafficking, and neurotransmitter release (Wang et al., 1989; Adema, 1995; Blakely, 1993; Manenti et al., 1994). In spite of well-defined roles of MARCKS in cell motility and membrane trafficking, little is known about its involvement in neurotransmitter synthesis and release, including its role in enhanced neuro-

![Figure 1](image-url)

**Figure 1.** Effects of Ang II on TH, DBH immunoreactivities, and [³H]NE uptake in synaptosomes and whole cells of brain neurons. (A–C) Neuronal cultures were incubated with 100 nM Ang II for indicated time periods, synaptosomes were prepared and subjected to quantitation of TH (A) and DBH (B) immunoreactivities, and [³H]NE uptake (C) activities essentially as described in Materials and Methods. A comparable amount of proteins in each sample were also electrophoresed and subjected to Western blotting with the use of antibodies to synaptophysin. Top in A and B are representative autoradiograms. Bottom in A and B are mean data from three experiments mean ± SE. Data in C are mean ± SE (n = 3). *, significantly different (P < 0.01) from zero time. (D and E) TH and DBH levels in whole cells and synaptosomes. After treatment with Ang II for 4 h essentially as described above, whole neuronal cells and synaptosomal preparation from them were subjected to TH (D) and DBH (E) Western blotting. Equal amounts of proteins (20 μg) were used for electrophoresis. Top in D and E are representative autoradiograms. Bottom, mean data from two experiments.
modulation. In addition, the role of MARCKS in any G protein–coupled receptor’s signal transduction propagation is even less understood. In view of these gaps in our understanding, coupled with our observation that AT$_1$ receptors stimulate PKC and neuromodulation, the objective in this investigation was to test the following hypothesis: Ang II interaction with the neuronal AT$_1$ receptor stimulates phosphorylation of MARCKS by PKC. Phosphorylated MARCKS redistributes itself in a way to facilitate the transport of TH, DJB$_2$, and NET along the neurites to synaptic vesicles where they participate in increased synthesis, release, and re-uptake of norepinephrine (NE). Observations presented in this study provide evidence in support of this hypothesis.

**Materials and Methods**

1-d-old Wistar Kyoto (WKY) rats were obtained from our breeding colony, which originated from Harlan Sprague-Dawley (Indianapolis, IN). DME, plasma-derived horse serum (PDHS), and $1 \times$ crystal trypsin were from Central Biomedia (Irwin, MA). Ang II and mAb to synaptophysin were purchased from Genemed Biotechnologies (San Francisco, CA). PEG$_{48-165}$ competes for PKC-mediated phosphorylation of MARCKS since three out of the four sequences (151, 155, and 162) in this molecule are known PKC phosphorylation sites (Amess et al., 1992). In contrast, mut$_{148-165}$ would not be a competitor, and thus serves as control for pep$_{48-165}$.

**Preparation of Neuronal Cultures**

Hypothalamus–brainstem areas of 1-d-old WKY rat brains were dissected and brain cells were dissociated by trypsin as described previously (Raizada et al., 1984, 1993). Dissociated brain cells were plated in poly-l-lysine–precoated tissue culture dishes (2 $\times$ $10^5$ cells/100-mm-dish dish or 3 $\times$ $10^5$ cells/35-mm-dish dish) in DME containing 10% PDHS and brain cells were dissociated by trypsin as described previously (Raizada et al., 1984, 1993). The cultures were allowed to grow for 15 d before their use in experiments. These cultures contain 85–90% neuronal cells and 10–15% astroglial cells (Raizada et al., 1984, 1993).

**Determination of TH, DJH$_2$ Immunoreactivities, and Specific $^3$H]NE Uptake in Synaptosomal Preparations of Neuronal Cells**

Neuronal cells, established in 100-mm-dish tissue culture dishes, were subjected to various pretreatments followed by incubation with 100 nM Ang II for indicated time periods. Cells from 10 culture dishes were collected, cell pellets homogenized in 0.32 M sucrose, and then homogenates were used for synaptosomal preparation essentially as described elsewhere for neuronal cultures (Kishi et al., 1991). Purity of the synaptosomal fraction was established with the use of synaptophysin antibody as marker (Kishi et al., 1991). Synaptosomal preparations containing 100 $\mu$g protein were subjected to 4–15% SDS-PAGE, separated proteins were transferred to nitrocellulose membrane, and then blotted with the use of 1 $\mu$g/ml anti-TH or anti-DJH$_2$ antibodies essentially as described previously (Lu et al., 1997). Antibodies bound to TH or DJH$_2$ were identified by HRP-labeled anti–rabbit antibody and visualized by chemiluminescence as described previously (Yang et al., 1996; Lu et al., 1997). Bands corresponding to TH and DJH$_2$ immunoreactivities were quantitated by SW/5000 Gel Analyzer after ascertaining that densities of each immunoreactive band was within the linear range as described previously (Lu et al., 1996, 1997; Yang et al., 1996).

Specific $^3$H]NE uptake was measured by incubating synaptosomal preparations containing 1 mg protein with 1 nM $^3$H]NE (2 $\mu$Ci) in the absence or presence of 1 $\mu$mol maprotiline essentially as described previously.

Figure 2. (A) Effect of PKC$_{\beta}$ AON on PKC$_{\alpha}$, PKC$_{\beta}$, and PKC$_{\gamma}$ immunoreactivities in brain neurons. Neuronal cultures were incubated with 2.5 $\mu$m PKC$_{\alpha}$ or PKC$_{\beta}$ AON or SON for indicated time periods. Cells were lysed and lysates were used for Western blot to determine levels of PKC$_{\beta}$, PKC$_{\alpha}$, and PKC$_{\gamma}$ as described in the Materials and Methods. Top, a representative autoradiogram. Bottom, data from three experiments mean ± SE. *, significantly different from control ($P < 0.05$). (B) Effects of PKC$_{\alpha}$ and PKC$_{\gamma}$ AON and SON on PKC$_{\alpha}$ and PKC$_{\gamma}$ immunoreactivities in brain neurons. Neuronal cultures were treated with 2.5 $\mu$m PKC$_{\alpha}$ or PKC$_{\gamma}$ AON or SON for 48 h at 37℃ essentially as described for PKC$_{\beta}$ above. Levels of PKC$_{\alpha}$ and PKC$_{\gamma}$ was determined by Western blot. Top, representative autoradiogram. Bottom, mean ± SE (n = 3).
body (1:200 diluted antiserum) in PBS containing 0.5% BSA overnight at some of brain neurons. Neuronal cultures were pretreated with 2.5 μM PKCβ AON or SON for 48 h, essentially as described in Fig. 2. This was followed by incubation of cells with 100 nM Ang II for 4 h. Synaptosomes were prepared and TH (A), DβH (B), and [3H]NE (C) uptake activities were determined as described in Materials and Methods. Top in A and B show representative autoradiograms. Bottom, mean data from three experiments ± SE. Data in C are mean ± SE (n = 3). *, significantly different (P < 0.05) from control. **, significantly different (P < 0.01) from Ang II–treated neurons.

Western Blotting of PKCα, β, and γ Subtypes and MARCKS

Western blotting was used to identify and quantitate the PKCα, PKCβ, PKCγ, and MARCKS proteins essentially as described previously (Yang et al., 1996). Briefly, cell-free lysates were prepared and electrophoresed on 10% SDS-PAGE and then proteins were transferred to nitrocellulose membranes. Membranes were treated with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 h, followed by incubation with rabbit anti–PKC subtype antibodies (1 μg/ml) or rabbit anti-MARCKS antibody (1 μg/ml), and then incubated with HRP-labeled anti-rabbit antibody, and enhanced chemiluminescence assay reagents. Densities in each band was quantitated by SW5000 Gel Analyzer (Lu et al., 1996, 1997).

Immunofluorescent Staining of Neurons for MARCKS

Neuronal cells, established in 35-mm-diam culture dishes, were fixed in methanol at −10°C, and then incubated with rabbit anti-MARCKS antibody (1:200 diluted antiserum) in PBS containing 0.5% BSA overnight at 4°C. Specificity of this antibody has been previously established (Watson and Lenox, 1996). After removal of primary antibody, cells were incubated with FITC-conjugated anti–rabbit IgG (1 μg/ml), and then processed for confocal microscopy (Yang et al., 1996; Lu et al., 1997). For colocalization of MARCKS with synaptophysin, cells were first stained with anti-MARCKS antibody followed by incubation with mAb to synaptophysin (1 μg/ml in PBS) for 1 h at 37°C. Cells were stained with rhodamine-conjugated anti–mouse IgG (1 μg/ml) and subjected to confocal microscopy (Yang et al., 1996; Lu et al., 1997).

Labeling of Neurons with [32P]Orthophosphate and Analysis of Phosphorylated MARCKS

Neuronal cells, established in 100-mm-diam culture dishes, were rinsed once with phosphate-free DME, followed by incubation in the phosphate-free DME containing 10% dialyzed PDHS for 4 h at 37°C. [32P]Orthophosphate (1 mCi/ml) was added and incubation was continued for an additional 20 h. After stimulation with 100 nM Ang II for desired time period, cultures were rinsed free of [32P]orthophosphate, cells were lysed in lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 0.8 μg/ml leupeptin), and then lysates were subjected to an immunoprecipitation protocol to separate 32P-labeled MARCKS (Lu et al., 1997). Briefly, cell lysates were mixed with 1 μg/ml anti–MARCKS antiserum at 4°C and incubated overnight with gentle shaking (Watson and Lenox, 1996). This was followed by incubation with 10 μg of agarose-conjugated anti–rabbit IgG for 4 h at 4°C. Immunoprecipitates were collected by centrifugation, washed six times with the lysis buffer, suspended in Laemmli’s buffer, electrophoresed in 4–15% SDS-PAGE and subjected to autoradiography to detect 32P-labeled bands representing phosphorylated MARCKS (Yang et al., 1996; Lu et al., 1997).

Treatment of Neuronal Cells with Antisense Oligonucleotides or Sense Oligonucleotides to PKC Subtypes or MARCKS

Neuronal cultures, established in 35-mm-diam tissue culture dishes, were incubated with 2.5 μM antisense oligonucleotides (AON) or sense oligonucleotides (SON) to PKC subtypes (α, β, or γ) or MARCKS for various time periods as described elsewhere (Aigner and Caroni, 1993; Balboa et al., 1996). Specific uptake was calculated by subtracting the total [3H]NE uptake from that in the presence of maprotiline.
MARCKS phosphorylation. Neuronal culture, pre-labeled with \[^{32}P\]orthophosphate and incubated with 100 nM Ang II for indicated time periods. \[^{32}P\]-labeled MARCKS was immunoprecipitated by MARCKS-specific antibody and subjected to SDS-PAGE followed by autoradiography as described in the Materials and Methods. Top, a representative autoradiogram. Bottom, mean data from three experiments ± SE. *, significantly different (P < 0.05) from time zero. (C) Effect of Ang receptor antagonists on MARCKS phosphorylation. Experimental conditions were essentially as described above in Fig. 4 B, except cultures were incubated without (1, 3, 5) or with 100 nM Ang II (2, 4, 6) in the presence of either 10 μM losartan (3 and 4) or 10 μM PD123,319 (5 and 6). *, significantly different (P < 0.05) from control. **, significantly different (P < 0.05) from Ang II–treated neurons. Bars in A: (a) 4 μm; (b–e) 4 μm.

Osmotic Loading of Neurons with pep\[^{148-165}\] and mut\[^{148-165}\]

Osmotic loading of neurons with pep\[^{148-165}\] or mut\[^{148-165}\] was carried out essentially as described previously by Ahmad et al. (1995). This method has recently been adapted by us for neuronal cell culture (Lu et al., 1998). In brief, neuronal cells were rinsed with PBS, pH 7.4, and incubated for 10 min with a loading solution (0.5 M sucrose, 10% polyethylene glycol 1000, 10% FBS, and 200 μg/ml pep\[^{148-165}\] or mut\[^{148-165}\] in DME buffered with 25 mM Hepes, pH 6.8). Cultures were rapidly rinsed with a hypotonic solution (6.5 vol H₂O: 3.5 vol DME, buffered with 25 mM Hepes, pH 6.8) (Reddy et al., 1991; Ahmad et al., 1995), incubated with DME containing 10% PDHS, and were subjected to the experimental protocol for the measurement of Ang II stimulation of synaptosomal TH, DjH and \[^{3}H\]NE uptake as described above.

Experimental Groups and Data Analysis

Each data point represented triplicate culture dishes and cells in these dishes were derived from multiple brains of 1-d-old WKY rats. Each experiment was replicated three times unless indicated otherwise. Densities of radioactive bands representing phosphorylated MARCKS or bands on immunoblots representing MARCKS, PKC subtypes, TH, or DjH were quantitated by SW5000 Gel Analyzer as described elsewhere (Yang et al., 1996; Lu et al., 1997). Observed densities (OD) of these bands were such that they were all in the linear range as previously established by calibrating protein concentrations with ODs. Data were normalized for equal loading with the use of equal amounts of proteins in each sample, and were presented as mean absorbance of at least three experiments ± SE.
The levels of immunoreactive synaptophysin was observed to demonstrate the specificity of Ang II’s actions. The levels of TH and DβH immunoreactivities were compared in the whole cell homogenates and synaptosomal preparations of Ang II–treated neurons to further confirm the effects of Ang II on the transport process. Fig. 1, D and E show that Ang II caused a 2.7- and 3.9-fold increase in TH levels in whole cells and synaptosomes, respectively. Similarly, a three- and fivefold increase in DβH levels were seen in whole cells and synaptosomes. Comparison of data indicated that the level of TH and DβH stimulation was 30–40% higher in synaptosomes compared with the whole cells.

Ang II interacts with the AT_1 receptor subtype to regulate NE neuromodulatory effects (Gelband et al., 1998). In view of the observations that the AT_1 receptor belongs to G protein–coupled receptor superfamily that is coupled to PLC-PKC signaling pathway, and that Ang II stimulates PKC in neurons in a calcium-dependent manner (Sumners and Raizada, 1993; Sumners et al., 1995; Gelband et al., 1998), our next aim was to determine which calcium-dependent PKC subtype was involved in Ang II stimulation of TH, DβH transport, and NE uptake. Fig. 2 shows that preincubation of neurons with AON to PKCβ caused a time-dependent decrease in PKCβ immunoreactivity. Maximal decrease of 86% was observed with 2.5 μM PKCβ AON in 48 h. In contrast, PKCβ SON showed no such decrease in PKCβ immunoreactivity during the same time period; also in contrast, both PKCβ AON and SON failed to influence immunoreactive levels of PKCα and PKCγ. However, AON to PKCα and PKCγ caused a maximum of 73% decrease in their respective PKC subtype immunoreactivities in 48 h (Fig. 2 B). Depletion of PKCβ by its AON was associated with a significant attenuation of Ang II–induced increase in TH and DβH immunoreactivities and [3H]NE uptake in synaptosomes (Fig. 3, A–C). This attenuation was specific since PKCβ SON or PKCα and PKCγ AONs showed no significant effects on Ang II stimulation of TH, DβH, and NE uptake. An example of the lack of effect of PKCα and PKCγ AONs on TH immunoreactivity is shown in Fig. 4.

Next, we studied the involvement of MARCKS in Ang II stimulation of NE neuromodulation. The rationale was based on the evidence that MARCKS is a substrate for PKC, is present in high concentrations in neurons, and is proposed to be involved in regulation of neurotransmitter release (Zhu and Ikeda, 1994; Patel et al., 1995). Fig. 5A shows representative images of immunocytochemical distribution of MARCKS and its colocalization with TH and synaptophysin in the neurons. MARCKS immunoreactivity was predominately localized in vesicular structures of neurites with little distribution in the cell soma (Fig. 5A, a and b). This distribution overlapped with the distribution of immunoreactive synaptophysin in the neurites. Treatment with Ang II resulted in a dramatic redistribution of MARCKS (Fig. 5A, c and d). MARCKS immunoreactivity began to diffuse out of the varicosities and within 30 min it was completely diffused throughout the neurites.

MARCKS immunoreactivity was colocalized with synaptophysin (Fig. 5A, e) and with TH (Fig. 5A, f) in Ang II–treated neurons. This further supports our contention that AT_1 receptor–induced MARCKS redistribution occurs in noradrenergic neurons. A hypothalamic–brainstem

**Figure 6.** Effect of PKCβ AON on Ang II–induced MARCKS phosphorylation. Neuronal cultures were pre-treated with 2.5 μM SON or AON for PKCβ as described in legend to Fig. 2. After 24 h, 1 mCi/ml [32P]orthophosphate was added to the cultures and incubation was continued for an additional 20 h. [32P]-labeled cells were incubated with 100 nM Ang II for 4 h. Immunoprecipitation of [32P]-labeled MARCKS, followed by its quantitation, was carried out as described in Materials and Methods. Top, a representative autoradiogram. Bottom, mean ± SE (n = 3). *, significantly different (P < 0.05) from control. **, significantly different (P < 0.05) from Ang II–treated neurons.

**Results**

Chronic stimulation of NE neuromodulation by Ang II is mediated by activation of the neuronal AT_1 receptor and is associated with increased expression of genes for TH, DβH, and NET in neuronal cultures (Lu et al., 1996; Yu et al., 1996; Gelband et al., 1998). These observations have led us to hypothesize that stimulation of NE synthesis, release, and uptake by Ang II is dependent upon the transcription of TH, DβH, and NET genes followed by transport of these activities to the synaptic terminals. Our first aim in this study was to determine if the transport process itself was influenced by Ang II. Synaptosomal preparations showed low but significant basal immunoreactive TH and DβH (Fig. 1, A and B). Similarly, [3H]NE uptake, a measure of NET activity, was also low (Fig. 1 C). Treatment with 100 nM Ang II resulted in a time-dependent increase in immunoreactive TH, DβH, and [3H]NE uptake by synaptosomes. Maximal stimulation of 8.8-, 7.4-, and 7.9-fold in TH, DβH, and [3H]NE uptake, respectively, was observed in 4 h, and stimulated levels were still evident 24 h after Ang II treatment. No significant change in the levels of immunoreactive synaptophysin was observed to demonstrate the specificity of Ang II’s actions. The levels of TH and DβH immunoreactivities were compared in the whole cell homogenates and synaptosomal preparations of Ang II–treated neurons to further confirm the effects of Ang II on the transport process. Fig. 1, D and E show that Ang II caused a 2.7- and 3.9-fold increase in TH levels in whole cells and synaptosomes, respectively. Similarly, a three- and fivefold increase in DβH levels were seen in whole cells and synaptosomes. Comparison of data indicated that the level of TH and DβH stimulation was 30–40% higher in synaptosomes compared with the whole cells.

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neuronal cells in primary culture from 1-d-old rat contain 20–40% TH-positive neurons (Sumners and Raizada, 1993; Raizada et al., 1994). This coupled with our estimation that ~30% of neurons express AT$_1$ receptor, would indicate that significant number of noradrenergic neurons would be Ang II responsive. In fact, our immunocytochemical observations on Ang II–induced redistribution of MARCKS depicted in Fig. 5 support this view.

In addition to its redistribution, Ang II also stimulated the phosphorylation of MARCKS. Fig. 5B shows a time-dependent increase in the incorporation of $^{32}$P in immunoprecipitated MARCKS. Low but significant phosphorylation of MARCKS was observed in control, untreated neurons. A twofold stimulation of phosphorylation was seen as early as 15 min with 100 nM Ang II and maximal stimulation of 4.7-fold was observed in 4 h. The stimulation was completely blocked by 10 μM losartan, an AT$_1$ receptor subtype–specific antagonist and not by 10 μM PD123319, an AT$_2$ receptor subtype–specific antagonist (Fig. 5 C). Neuronal cultures were preincubated with PKCβ AON for 48 h to specifically deplete them of PKCβ subtype. Ang II failed to stimulate phosphorylation of MARCKS in these PKCβ-depleted neurons (Fig. 6), further supporting our view that PKCβ subtype is involved in this effect.

Cultures were treated with MARCKS AON to deplete neurons of MARCKS immunoreactivity to determine the role of MARCKS in Ang II–induced NE neuromodulation. Typical distribution of MARCKS immunoreactivity in varicosities was seen in untreated, control neurons (Fig. 7 A, a). Pre-incubation of neurons for 48 h with AON to MARCKS significantly reduced intensity of MARCKS staining (Fig. 7 A, b). MARCKS SON showed no effect under these conditions (Fig. 7 A, c). Quantitation of immunoreactive MARCKS by Western blotting revealed that MARCKS AON caused a time-dependent decrease of this protein and a maximal decrease of 77% was observed in 48 h (Fig. 7 B). MARCKS SON treatment did not reduce immunoreactive MARCKS. Neurons were pre-treated with MARCKS AON or SON for 48 h, and then incubated with 100 nM Ang II for 4 h and levels of TH, DβH, and $[^3$H]NE uptake in synaptosomes were analyzed. Fig. 8 shows that depletion of MARCKS resulted in a 65–70% attenuation in the ability of Ang II to stimulate TH and DβH immunoreactivities and $[^3$H]NE uptake. This effect on NET, TH, DβH appeared to be posttranscriptional because MARCKS AON did not effect Ang II stimulation of mRNAs for these proteins (Fig. 9). Direct effect of Ang II on the release of $[^3$H]NE from neuronal synaptosomes was measured to determine if MARCKS is also involved in the release of catecholamines. Neurons were treated with MARCKS AON. Synaptosomes were prepared, pre-

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**Figure 7.** Effect of MARCKS AON and SON treatments on neuronal MARCKS. (A) Neuronal cultures were treated without (a) or with 2.5 μM MARCKS AON (b) or 2.5 μM MARCKS SON (c) for 48 h. MARCKS immunoreactivity was determined by the use of confocal microscopy. (B) After treatment with MARCKS AON or SON for indicated time periods, levels of MARCKS were determined by Western blotting as described in Materials and Methods. Top, a representative autoradiogram. Bottom, mean data ± SE (n = 3). *, significantly different (P < 0.01) from control. Bar, 4 μm.
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loaded with [3H]NE and used for Ang II–stimulated release experiments (Wakade et al., 1995). Basal release of [3H]NE was comparable in control, MARCKS SON– and MARCKS AON–treated neurons. Ang II caused a 3.7-fold increase in specific [3H]NE release from synaptosomes of control neurons. This accounted for ~29% of total synaptosomal [3H]NE. MARCKS AON treatment did not alter this level of [3H]NE release. This indicated that, in spite of a decrease in the intracellular levels of MARCKS, Ang II–stimulated release of [3H]NE was not affected.

Finally, neuronal cultures were osmotically loaded with pep148–165 or mut148–165 followed by stimulation with Ang II to further determine the involvement of MARCKS in the transport of TH, DβH, and NET. The rationale behind this experiment was based on the hypothesis that pep148–165 would compete with the native MARCKS for phosphorylation by PKCβ, and as a result Ang II–mediated phosphorylation of MARCKS would be decreased. Fig. 10 confirms this prediction and shows that Ang II–induced phosphorylation of MARCKS was decreased by 81% in neurons preloaded with pep148–165. Pre-loading with mut148–165 did not affect Ang II stimulation of MARCKS phosphorylation. Fig. 11 shows that pep148–165 treatment resulted in 83–87% decrease in the ability of Ang II to stimulate TH (Fig. 11 A), DβH (Fig. 11 B), and [3H]NE uptake (Fig. 11 C) in synaptosomes. mut148–165 peptide showed no such inhibitory effect. These data confirm that phosphorylation of MARCKS by PKCβ is an important step in the transport of TH, DβH, and NET from cell soma to synaptic terminal.

**Discussion**

The most significant observation of this study is our demonstration that Ang II stimulates redistribution and phosphorylation of MARCKS in a PKCβ-dependent process, and that this phosphorylation is involved in the transport of TH, DβH, and NET in hypothalamic/brainstem neurons. This is of particular interest since MARCKS has recently been shown to be highly expressed in limbic regions of the brain that retain neuroplastic properties beyond early development (McNamara and Lenox, 1997). Thus, MARCKS may play an important role in the mediation of fast axonal transport of catecholamine-synthesizing enzymes and NET to synaptic terminals. As a result, an increased turnover and release of NE is achieved under chronic stimulation of brain neurons by Ang II.

Ang II has been previously shown to stimulate catecholamines synthesis, turnover, and release both acutely and chronically (MacLean et al., 1990; Stadler et al., 1992; Sumners and Raizada 1993; Gelband et al., 1998). These effects are mediated by activation of the AT1 receptor subtype. Acute stimulation, which has been termed *evoked* response involves posttranscriptional processes (Lu et al.,

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**Figure 8.** Ang II stimulation of TH and DβH immunoreactivities, and [3H]NE uptake in MARCKS-depleted neurons. Neuronal cultures were pretreated with 2.5 μM MARCKS SON or AON for 48 h essentially as described in the legend to Fig. 7. Synaptosomal preparation was used to quantitate immunoreactive TH (A) or DβH (B) or specific [3H]NE uptake (C) as described in Materials and Methods. *Top* in A and B are representative autoradiograms. *Bottom* in A and B represented mean ± SE (n = 3). Data in C are mean ± SE (n = 3). *, significantly different (P < 0.01) from control. ***, significantly different (P < 0.01) from Ang II–treated neurons.

**Figure 9.** Effects of MARCKS AON treatment on TH, DβH, and NET mRNA levels in neurons. Neuronal cultures were pre-treated with 2.5 μM MARCKS AON or SON for 48 h. After incubation without or with 100 nM Ang II for 4 h, total RNA was isolated and mRNA levels for TH (A), DβH (B), and NET (C) were measured by reverse transcription PCR essentially as established by us previously (Lu et al., 1996; Yu et al., 1996).
motic loading of MARCKS pep148–165 and mut148–165 essentially as
Lu et al.
cellular NET and transcription of NET, TH, and D
is associated with both translocation of pre-existing intra-
1996), whereas a chronic or enhanced
response of Ang II. In this scheme, we propose that Ang II stimulates transport of TH, DβH, and NET along the neurites for them to be available for increased synthesis, release, and re-uptake of NE at the synaptic level. This stimulation involves activation of PKCβ: 
(a) Ang II stimulates PKC, which is involved in axonal transport (Komoly et al., 1991); (b) treatment of neurons with AON to PKCβ, which selectively depletes neurons of this subtype, causes attenuation of Ang II stimulation of TH, DβH, and NET transport. This effect is selective for PKCβ subtype since AONs to other major calcium-dependent PKC subtypes such as PKCα and PKCγ had no such effect. It is also pertinent to point out that Ang II–induced MARCKS phosphorylation and redistribution appears to be a relatively slower response compared with its relatively rapid effect on PKC. Although, we have suggested that PKCβ stimulation is directly involved in Ang II–induced MARCKS phosphorylation and redistribution. Alternate possibilities can not be ruled out at the present time. For example, it is possible that Ang II influences PKCβ-dependent neuronal activity. A change in this activity could activate an entire set of distinct neurons to stimulate redistribution of MARCKS, TH, and DβH. However, our observations that AT1 receptors are present on nonadrenergic neurons and that the AT1 receptor blockade attenuates NET activity in these neurons (Lu et al., 1996) argue against such an indirect effect.

Phosphorylation of MARCKS is instrumental in its redistribution. MARCKS possesses a basic phosphorylation site domain (PSD). Phosphorylation of this PSD domain prevents the electrostatic interaction of the effector region of the MARCKS to the plasma membrane (George and Blackshear, 1992; Taniguchi and Manenti, 1993; Kim et al., 1994). Whereas there is little evidence for a preferential phosphorylation of MARCKS by the PKCβ subtype, there is considerable evidence for its presence and involvement in synaptosomal activities (Guadagno et al., 1992; Heemskerk et al., 1993; Sheu et al., 1995; Cabell et al., 1996; Seki et al., 1996). Present data are novel since they provide evidence that PKCβ subtype is involved in Ang II stimulation of MARCKS phosphorylation, redistribution, and in NE neuromodulation. The evidence for this includes the following: (a) Ang II stimulation of phosphorylation of MARCKS is blocked by depletion of PKCB by AON treatment. No such attenuation was observed with AON to PKCα or PKCγ; (b) Ang II–induced redistribution of immunoreactive MARCKS from varicosities is also blocked by PKCβ-AON treatment; (c) Presence of PKC substrate domain is well documented in MARCKS and a peptide containing this domain blocks Ang II stimulation of MARCKS phosphorylation.

Finally, phosphorylation of MARCKS appears to be key in its redistribution and increased accumulation of TH, DβH, and NET in synaptic vesicles: (a) blocking the phosphorylation of MARCKS by pep148–165 inhibits Ang II–induced redistribution of MARCKS in neurites. As a result, MARCKS immunoreactivity remains localized in the varicosities (unpublished data); (b) AON to MARCKS, that significantly reduces endogenous levels of neuronal MARCKS, attenuates Ang II–induced accumulation of TH, DβH, and NET in synaptosomes; (c) Ang II induces redistribution of MARCKS. Such a PKC-activated redistribution has been proposed in membrane trafficking and cycling of MARCKS between plasma membrane and lysosomes in other systems (Allen and Aderem, 1995); and (d) blocking the phosphorylation of MARCKS by osmotic loading of the peptide148–165 attenuates Ang II enhancement of TH and DβH in synaptosomes. Collectively, these observations strongly support the notion that PKCβ-MARCKS are facilitating signaling pathway in enhanced regulation of NE neuromodulation by Ang II.
These observations are important in that they provide the first evidence that two distinct signaling pathways induced by AT$_1$ receptor activation, converge downstream to participate in the *enhanced* stimulation of neuromodulation. Such long-term enhancement of noradrenergic neurotransmission has been shown to involve an increase in critical proteins such as TH and D$eta$H and their transport to synaptic sites. Studies over the years have provided data in support of the close association of both TH and D$eta$H transport and the association of organelles in the process (Coyle and Wooten, 1972; Dahlstrom et al., 1982). Whereas it has been clear that microtubule-dependent movement is essential to transport associated with a variety of intracellular organelles, it is only more recently that a role for actin microfilaments in this process has been elucidated (Morris and Hollenbeck, 1995). In fact, there is extensive interaction between microtubules and the actin cytoskeleton, and it has been suggested that the specialized properties of these two cytoskeletal filament systems may act in a coordinated manner to achieve regional and even compartmentalized net transport of organelles and associated proteins for proper physiological regulation (Goldstein and Vale, 1992; Morris and Hollenbeck, 1995). MARCKS is a member of a small family of proteins that bind calmodulin in the presence of calcium and bind and cross-link actin in a mutually exclusive fashion. PKC-mediated phosphorylation prevents both calmodulin binding and actin cross-linking and shuttles MARCKS out of the plasma membrane (Wang et al., 1989; Rosen et al., 1990; Thelen et al., 1991). Recent studies have demonstrated that MARCKS is transported from plasma membrane to lysosomes upon PKC-mediated phosphorylation, and recycling of MARCKS to the plasma membrane appears to depend upon interaction with intact microtubules (Allen and Aderem, 1995). By virtue of the fact that MARCKS’ intracellular location and properties are responsive to cell signaling events, there is significant evidence that MARCKS may play an important role in translating extracellular signal–mediated cytoskeletal restructuring associated with cell movement, phagocytosis, and neurosecretion (Aderem, 1995; Blackshear, 1993; Wang et al., 1989).

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References

Aderem, A. 1995. The MARCKS family of protein kinase-C substrates. Biochem. Soc. Trans. 23:S87–S91.

Ahmad, F., P.M. Li, J. Meyerovitch, and B.J. Goldstein. 1995. Osmotic loading of neutralizing antibodies demonstrates a role for protein-tyrosine phosphatase 1B in negative regulation of the insulin action pathway. J. Biol. Chem. 270:20903–20908.

Aigner, L., and P. Caroni. 1993. Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. J. Cell Biol. 123:417–429.

Allen, L.A., and A. Aderem. 1995. Protein kinase C regulates MARCKS cy-
Asotra, K., and W.B. Macklin. 1994. Developmental expression of protein kinase C isoforms and their differential modulation by 4 beta-phorbol 12,13-dibutyrate. J. Neurosci. Res. 40:273–289.

Balboa, M.A., B.L. Firestein, C. Godson, K.S. Bell, and P.A. Insel. 1994. Protein kinase C alpha mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. Stimulation of phospholipase D is independent of activation of polyphosphoinositide-specific phospholipase C and phospholipase A2. J. Biol. Chem. 269:10511–10516.

Blackshear, P.J. 1993. The MARCKS family of cellular protein kinase C substrates. J. Biol. Chem. 268:1501–1504.

Cabel, C.H., G.M. Verghese, N.B. Ranki, D.J. Burns, and P.J. Blackshear. 1996. MARCKS phosphorylation by individual protein kinase C isoforms in insect S2 cells. Proc. Assoc. Am. Physicians. 108:37–46.

Coyle, J.T., and G.F. Wooten. 1972. Rapid axonal transport of tyrosine hydroxylase and dopamine-β-hydroxylase. Brain Res. 44:701–704.

Dahlstrom, A., S. Boog, M. Goldstein, and P.A. Larsson. 1982. Cytolfluorometric scanning: a tool for studying axonal transport in monoaminergic neurons. Brain Res. Rev. 6:91–68.

Drez, V.J. 1995. Vascular renin-angiotensin system and vascular protection. J. Cardiovasc. Pharmacol. 22(Suppl.1):1–9.

Freeman, E.J., G.M. Chisolm, and E.A. Tallant. 1995. Role of calcium and protein kinase C inactivation between the plasma membrane and lysosomes in fibroblasts. Proc. Assoc. Am. Physicians. 108:37–46.

Goldstein, L.S., and R.D. Vale. 1992. Cell biology. New cytoskeletal liaisons. EMBO J. 11:329–380.

Graff, J.M., T.N. Young, J.D. Johnson, P.J. Blackshear. 1989. Phosphorylation-kinase C substrates from punctate structures in macrophage filopodia. J. Biol. Chem. 264:21818–21823.

Guadagni, S., C. Borner, and I.B. Weinstein. 1992. Altered regulation of a major substrate of protein kinase C in rat 6 fibroblasts overproducing PKC beta I. J. Biol. Chem. 267:2697–2700.

Hammerschlag, R. 1994. Is the intracellular phase of fast axonal transport driven by oscillations of intracellular calcium? Neurochem. Res. 19:1431–1437.

Hartwig, J.H., M. Thelen, A. Rosen, P.A. Janmey, A.C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium calmodulin. Nature. 356:618–622.

Heemskerk, F.M., H.C. Chen, and F.L. Huang. 1993. Protein kinase C phospholipase D Ser/152, Ser/156 and Ser/163 but not Ser/160 of MARCKS in rat brain. Biochem. Biophys. Res. Commun. 190:236–241.

Kim, J., J.Y. Ishishita, C. Aden, and S. McLaughlin. 1994. Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. J. Biol. Chem. 269:28214–28219.

Kishi, M., O. Okochi, H.Y. Ma, and K. Kataoka. 1994. Pharmacological characteristics of choline transport system in mouse cerebral cortical neurons in primary culture. Jpn. J. Pharmacol. 55:223–232.

Komoly, S., Y. Liu, H.D. Webster, and K.F. Chan. 1991. Distribution of protein kinase C isozymes in dissociated neurons. J. Neurosci. Res. 29:379–389.

Liu, D., K. Yu, M.R. Paddy, N.E. Rowland, and M.K. Raizada. 1996. Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains. Endocrinology. 137:763–772.

Liu, D., H. Yang, and M.K. Raizada. 1997. Angiotensin II regulation of neuro-modulation: Downstream signaling mechanism via activation of mitogen-activated protein kinase J. Cell Biol. 135:1609–1617.

Liu, D., H. Yang, and M.K. Raizada. 1998. Angiotensin II-induced nuclear targeting of AT1 receptor in brain tissues. Endocrinology. 439:365–375.

Manenti, S., O. Sorokine, A. Van Dorsseelaer, and H. Taniguchi. 1994. Demyelination of the major substrate of protein kinase C (MARCKS) by the cyto-plasmic domain of the HIV-1 gp120 envelope glycoprotein. Nature. 351:270–273.

Marrero, M.B., B. Schieffer, W.G. Paxton, L. Heerdt, B.C. Berk, P. Delafontaine, and K.E. Bernstein. 1995. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. Nature. 375:247–250.

McNamara, R.K., and R.H. Lenox. 1997. Comparative distribution of myosin light chain phosphatases and their substrates in developing and adult skeletal muscle. J. Appl. Physiol. 83:830–8313.

Morris, R.L., and P.J. Hollowbeck. 1995. Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons. J. Cell Biol. 131:1315–1326.

Naftilan, A.J. 1994. Role of the tissue renin-angiotensin system in vascular remodeling and smooth muscle cell growth. Curr. Opin. Nephrol. Hypertens. 3:218–227.

Patel, A.J., A. Hung, W. Jacques-Berg, J. Kiss, and J. Rodriguez. 1995. Effects of protein kinase C modulation on NMDA receptor mediated regulation of neurotransmitter enzyme and c-fos protein in cultured neurons. Neurochem. Res. 20:561–569.

Raizada, M.K., T.F. Muther, and C. Sumners. 1984. Increased angiotensin II receptors in neuronal cultures from hypertensive rat brain. Am. J. Physiol. 247:Vol. 137:2151–2152.

Raizada, M.K., D. Lu, W. Tang, P. Kurian, and C. Sumners. 1993. Increased angiotensin II type-1 receptor gene expression in neuronal cultures from spontaneously hypertensive rats. Endocrinology. 132:1715–1722.

Raizada, M.K., D. Lu, and C. Sumners. 1994. AT1 receptors and angiotensin actions in the brain and neuronal cultures of normotensive and hypertensive rats. In Current Concepts. M. Mukhopadhyay, and M.K. Raizada, editors. Plenum Press. New York. 331–348.

Reddy, R., F. Zhou, L. Huang, F. Carbone, M. Bevan, and B.T. Rouge. 1991. pH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. J. Immunol. Methods. 141:157–163.

Rivera, D.T., G.M. Langford, G. Weiss, and D.J. Nelson. 1995. Calmodulin regulates fast axonal transport of squa axoplasm organelles. Brain Res. Bull. 37:47–52.