Transduction of a Functional Domain of the AT\textsubscript{1} Receptor in Neurons by HIV-Tat PTD

Jorge Vázquez, Chengwen Sun, Jianqing Du, Lucía Fuentes, Colin Sumners, Mohan K. Raizada

Abstract—Despite advances in transgenic and gene transfer technologies, in vivo structure–function studies of the angiotensin II type I receptor (AT\textsubscript{1}R) have revealed limited information on the diverse actions of angiotensin II. Our objective in the present study was to determine if protein transduction technology with the use of the HIV-Tat protein transduction domain could fill this gap. Recombinant HIV-Tat protein transduction domain fused to EGFP and to the third intracellular loop of the AT\textsubscript{1}R was expressed. Incubation of hypothalamus and brainstem neurons with this peptide indicated an efficient transport of the protein to most of the cells. This transduction was accompanied by an increase in neuronal firing rate, an effect similar to that observed with angiotensin II stimulation of the neuronal AT\textsubscript{1}R. The characteristics of the chronotropic effects of recombinant third intracellular loop and its synthetic counterpart were similar and comparable to the effects of angiotensin II on these neurons. In addition, in the presence of the protein kinase C inhibitor calphostin C, the peptide failed to increase firing rate. These observations demonstrated that transduction of neurons with the third intracellular loop of the AT\textsubscript{1}R produces chronotropic effects similar to those induced by angiotensin II. The data suggests that protein transduction technology could be useful for in vivo AT\textsubscript{1}R domain transduction. (Hypertension. 2003;41[part 2]:751-756.)

Key Words: receptors, angiotensin II \pmb{\&} protein kinases \pmb{\&} transduction technology

Angiotensin (Ang) II is one of the most potent vasoconstrictors and is known to exert diverse cellular and physiological effects on both peripheral tissues and the central nervous system.\textsuperscript{1,2} These actions are mediated by the interaction of Ang II with its specific angiotensin receptor type 1 (AT\textsubscript{1}R) subtype. The AT\textsubscript{1}R belongs to a superfamily of G protein–coupled receptors, which contains seven-transmembrane domains linked by alternating intra- and extracellular loops with a characteristic cytoplasmic C-terminal tail.\textsuperscript{3-6} It has been demonstrated that the AT\textsubscript{1}R is linked to multiple signal transduction pathways involving different G proteins and kinases, which impart its physiological diversity.\textsuperscript{1,3,7} Despite our understanding of the ability of this receptor to influence multiple signaling pathways, little is known about how the structural aspects of the AT\textsubscript{1}R provide such a physiological diversity for Ang II actions. Traditionally, structure–function studies involve mutation of the receptor, its expression in vitro and evaluation of a cellular function as a result of the mutation.\textsuperscript{9-13} Such studies have been key in mapping and identifying distinct domains of the AT\textsubscript{1}R and their linkage to diverse cellular functions. For example, they have led to our understanding of the receptor domains that are responsible for its coupling to various G proteins, signaling kinases, ligand binding, and internalization.\textsuperscript{10,11,13,14} However, these studies have been of limited success in identifying domains of the AT\textsubscript{1}R that are physiologically relevant in Ang II actions, such as the increase in blood pressure, hormone secretion, and neuromodulation. This is, in part, due to technological limitations in which it has been difficult to introduce mutant receptors in an in vivo situation. Germline transmission transgenic technology has been used to overexpress the AT\textsubscript{1}R in an attempt to address structure-function aspects of the receptor with limited success.\textsuperscript{15-19}

Recently, a new technology involving the use of protein transduction domains (PTDs) has been developed as an alternative for an efficient delivery of protein in vivo.\textsuperscript{20} It is based on the observation that an 11-amino-acid sequence from the HIV-Tat protein possesses the ability to transport any protein linked to it across the plasma membrane.\textsuperscript{21,22} This has enabled investigators to deliver large proteins, including receptors and enzymes, into cells under physiological conditions.\textsuperscript{23} Thus, fusion of PTDs to a functional protein dramatically increases its bioavailability in the cell and opens the possibility of studying the structural–functional aspects in an in vivo physiological setting.\textsuperscript{24,25} In the present study, we used this protein transduction technology to determine whether a functional domain of the AT\textsubscript{1}R can be delivered into neurons while retaining its cellular actions. We have chosen the third intracellular loop (3IL) of the AT\textsubscript{1}R for this...
“proof of concept.” The rationale for it is based on our previous observation that this domain, when microinjected into a neuron, mimics the effects of Ang II of K+ and Ca2+ currents.26 The data for the present study provide direct evidence that the HIV-Tat-PTD–mediated transfer of protein can be used for in vivo studies.

Methods

Materials

One-day-old Wistar-Kyoto rats (WKY) were obtained from our breeding colony, which originated from Charles River Farms (Wilmington, Mass). pEGFP vector was purchased from Clontech. Prokaryotic expression vector pET-15b and Ni-agarose were obtained from Novagen. Dialysis cassettes slide-A-lyzer were obtained from Pierce. Synthetic peptides were obtained from the protein synthesis core at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida. Oligonucleotides were purchased from Genomechanix. Pfu polymerase was from Stratagene. Isopropyl-β-D-thiogalactopyranoside, X-gal, and Dulbecco’s modified Eagle’s medium were obtained from GIBCO. Crystallized trypsin (1X) was from Cooper Biomedical. Plasma-derived horse serum, cytosine arabinoside, DNase I, poly-L-lysine (molecular weight 150,000), calphostin C, and all other chemicals were purchased from Sigma-Aldrich Chemicals.

Construction of Vectors to Express HIV-Tat-PTD N-Terminal and EGFP C-Terminal Fusions

An outline of our cloning strategy and protein expression is shown in Figure 1. The amino acid sequence that corresponds to the HIV-Tat-PTD was flanked by glycine residues to increase flexibility of the protein fusion (GYGRKKRRQRRQR).27 This was reverse translated and used to design an oligonucleotide with an in-frame N-terminal fusion with EGFP, and it possessed a unique BglII site separating both HIV-Tat-PTD and EGFP sequences to allow in-frame fusions of another protein with the PTD and the reporter gene. The primers for this polymerase chain reaction (PCR) were as follows: HIV-Tat-PTD-EGFP (F), 5’-CATATGGGTTATGGCAG GAAGAAGCG-GAGACAGCGAGGTAGATCTATGGTG AG-3’; EGFP (R), 5’-CGCTTTACTTGTACAGCTCGTC-3’. The PCRs were performed in the presence of 20 mmol/L Tris HCl (pH 8.8), 2 mmol/L MgSO4, 10 mmol/L KCl, 10 mmol/L (NH4)2SO4, 0.1% Triton X-100 and 0.1 mg/mL nuclease-free BSA mediated by Pfu polymerase. The PCR product was gel purified and cloned into the NdeI–XhoI sites of pET15-b to create the HIV-Tat-PTD-EGFP-pET15-b protein expression vector, which also provides an N-terminal 6 histidine tag to facilitate protein purification.

The amino acid sequence WKALKKAYKIQKNTPRNDDIFR from the AT-1 receptor 3IL W219-R240 was reverse translated and used to design complementary overlapping oligonucleotides. The primer sequences included a BglII site (underlined) and were as follows: AT1R 3IL (F), 5’-AGAGATCTTGGAAAG CTCTAAAGAAG-GCTTATAAAATTC AGAAGAA-3’; AT1R 3IL (R), 5’-CTAGATCT CCTAAAGATGTCATCATTTCTTGGCGT GTTCT-TCTGAATT-3’. PCR was performed under the conditions previously mentioned, and the product was gel purified and cloned into the BglII site in HIV-Tat-PTD-EGFP-pET15-b. The identity of the clones was confirmed by DNA sequencing.

Protein Expression and Purification

Recombinant proteins were expressed in Escherichia coli BL21 (DE3) by conventional protocols. The proteins were extracted following a modification of the protocol described by Vocero-Akbani et al.,23 in which the urea was removed by dialysis against PBS.

Figure 1. Schematic representations of the HIV-Tat-PTD fusion protein construct strategy. Genetic fusions of the PTD and EGFP were produced by PCR and cloned into the expression vector pET15-b. AT,R 3IL was obtained by extension of overlapping oligonucleotides and cloned into the PTD-EGFP expression vector. Recombinant proteins were expressed in E. coli BL-21 and purified using His Tag-Ni affinity under denaturant conditions. Proteins were dialyzed against PBS and used in the biological assays.

Figure 2. PTD-3IL–EGFP recombinant protein transduces neurons in vitro. Neuronal cells were established in culture for 14 days. They were incubated with 300 µg/mL of PTD-3IL-EGFP for 30 minutes at 37°C. A, Light micrograph of the neuronal culture. B, Fluorescence micrograph of the transduced neurons. C, Higher magnification of transduced neurons showing nuclear localization of the PTD-3IL-EGFP.
Synthetic Peptides
In the design of the synthetic peptide, we included some modifications to the HIV-Tat-PTD that optimized the α-helical content and optimized the placement of arginine residues, enhancing the transduction potential of this PTD. The amino acid sequence of the PTD-AT1bR 3IL was YARAAARQARAGWKALKKAYKIQKNT-PKNDIFIR, and the sequence of the PTD-scrambled peptide was YARAAARQARAGNKPFANRWYKDKLTKIDKRAQ. The PTD sequence is shown underlined.

Peptides were synthesized by solid phase with 9-fluorenylmethoxycarbonyl (FMOC) chemistry in an Applied Biosystems Peptide Synthesizer (Protein Core Facility of the ICBR, University of Florida). Each peptide was purified to >95% by high-performance liquid chromatography and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Preparation of Neuronal Cultures
Neuronal co-cultures were prepared from the hypothalamus and brainstem of 1-day-old WKY as previously described. At the time of use, cultures consisted of 90% neurons and 10% astrocyte glia, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein. Unless noted, experiments were performed using WKY neuronal cultures.

Transduction of HIV-Tat Fusion Proteins Into Neurons
To evaluate the ability of the recombinant HIV-Tat fusion proteins to transduce into neurons, different concentrations of HIV-Tat-EGFP (PTD-EGFP) and HIV-Tat-AT1bR 3IL-EGFP (PTD-3IL-EGFP) were added to the culture media of neuronal co-cultures from 1-day-old rat brain. After different incubation times, the media was removed, and the plates were washed once with PBS for fluorescent microscopic examination.

Electrophysiological Recordings
Spontaneous action potentials in neuronal cultures were recorded with the use of the whole-cell voltage clamp configuration in current clamp mode as described previously.

Data Analysis
Results were expressed as mean±SE. Statistical significance was evaluated with the use of a 1-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P<0.05$.

Results
PTD-3IL-EGFP Transduces Neuronal Cells
Incubation of neuronal cultures with 300 μg/mL of the recombinant protein PTD-3IL-EGFP caused a time-dependent increase in the intracellular fluorescence (Figure 2). Within minutes, the fluorescence was evident in the cytoplasmic compartment and increased in its intensity as a function of time. By 10 minutes, the fluorescence was predominantly seen in the cytoplasm, but some was also localized in the nucleus. The turnover rate of this protein appeared to be slow because the fluorescence was detected for >1 hour after transduction.

Transduction of TAT-PTD-AT1b-R 3IL-EGFP Increases Neuronal Firing Rate
Spontaneous action potentials presented by the neurons were of a random burst–firing pattern. Stimulation with Ang II (100 nmol/L) superfusion caused an increase in firing rate from 0.4±0.1 to 1.4±0.5 Hz. Similar to Ang II, superfusion with PTD-3IL-EGFP (7.8 μg/mL) recombinant protein resulted in an increase in the firing rate within 1 to 2 minutes, from 0.46±0.05 to 1.27±0.29 Hz. Such an increase was not observed when TAT-PTD-EGFP (7.8 μg/mL; control protein) was used in the superfusate (Figure 3). In addition, a lower dose of PTD-3IL-EGFP (3.5 μg/mL) stimulated only a 48% increase in firing rate compared with that of the 7.8 μg/mL dose.

Having established the transduction efficacy and functional effects on the neurons, we next determined if the synthetic PTD-AT1bR 3IL protein would mimic the stimulation of firing rate observed with the recombinant protein. The rationale for this approach was as follows: (1) the synthetic peptide allows one to perform modifications on the desired protein with relative ease without undergoing site-directed mutagenesis, cloning, and sequencing as required for the recombinant protein; and (2) it permits us to decrease the size of the transducible protein. Figure 4 shows that superfusion of...
neuronal cultures with 7.8 μg/mL PTD-3IL caused an increase in the spontaneous firing rate, from 0.59 ± 0.17 to 1.42 ± 0.37 Hz. A scrambled amino acid sequence of the AT1bR 3IL fused to PTD (7.8 μg/mL) was without any significant effect on the firing rate (Figure 4).

PTD-3IL Stimulation of Firing Rate Involves Protein Kinase C Signaling

Our previous studies have demonstrated that the AT1R-mediated stimulation of neuronal firing rate was partially mediated by the activation of protein kinase C (PKC) signaling.32 We determined the effect of PKC inhibition on the PTD-AT1bR 3IL–mediated stimulation of neuronal firing rate. This was performed in an attempt to determine the specificity of this peptide to mimic AT1R function. Neurons were superfused with the PKC inhibitor calphostin C (10 μmol/L) under the conditions that inhibit PKC-dependent firing rate of Ang II.32 In the presence of calphostin C, PTD-3IL did not alter neuronal firing rate (Figure 5).

Discussion

The most significant observation of this study is that the PTD of HIV-Tat can be successfully used to transduce neuronal cells with a functional domain of the AT1R. Thus, this technology provides a “proof of concept” that protein transduction is a potentially valid alternative to gene transfer and transgenic technology to study structure–function relationship in vivo of this G protein–coupled receptor.

Our previous studies have demonstrated that Ang II increases neuronal firing rate via AT1R-mediated increases in Ca2+ current and decreases in K+ currents.33 These actions involve the 3IL of this receptor subtype.26 In the present study, we used these properties of the 3IL to determine the efficacy of the HIV-Tat-PTD system. We were able to mimic the stimulatory action of Ang II on firing rate by combining the AT1bR 3IL with the Tat-PTD. The resulting protein was freely diffusible across the plasma membrane into the neurons without any apparent toxicity. As a result, it mimicked the action of Ang II on neuronal firing rate. This stimulation was specific and identical to that observed by Ang II activation of the neuronal AT1R: (1) scrambled peptide or EGFP coupled to a PTD did not stimulate firing rates, (2) synthetic and recombinant PTD-3IL were equally effective, and (3) increase of firing rate by PTD-3IL was partially inhibited by inhibition of PKC. A similar inhibition of Ang II-induced firing rate has been previously established for these neurons.32 This indicated that like Ang II, PTD-3IL uses the PKC signaling pathway to stimulate neuronal firing.

The AT1R mediates diverse physiological actions of Ang II.2,24,35 Thus, the specificity of these actions must reside in various structural domains of the receptor and their interac-

Figure 4. Synthetic PTD-3IL protein increases the firing rate of WKY neuronal cultures. A and C, Recordings of action potentials from a representative neuron under the following conditions. A, Superfusion of Tyrode’s solution (top) and superfusion of Tyrode’s solution containing 7.8 μg/mL synthetic PTD-3IL (bottom); C, Superfusion of Tyrode’s solution (top) and superfusion of Tyrode’s solution containing 7.8 μg/mL synthetic PTD-scrambled-3IL (PTD-3ILS) (bottom). B and D, Bar graphs showing neuronal firing rate (mean ± SE) under the respective treatment conditions in A and C. *P < 0.05 vs control recording.

Figure 5. PTD-3IL-EGFP recombinant protein does not increase neuronal firing rate in the presence of a PKC inhibitor. A, Representative recordings of action potentials made from a representative neuron after superfusion of Tyrode’s solution (control; top), the PKC inhibitor calphostin C (Cal 10 μmol/L; middle), and Rec-GFP-3IL in the presence of calphostin C (bottom). B, Bar graphs showing the neuronal firing rate (mean ± SE) under the treatment conditions described in A.
tions with intracellular signaling molecules. So far, studies have relied on the use of genetic approaches when deleted, chimeric, or mutated cDNA sequences have been used in transient expression assays in vitro in an attempt to characterize various functional domains of the AT1R.9–13,36 An alternative to this approach has been to use synthetic peptides for intracellular injections to study cellular functions.14,26 Both these methods have met with limited success for in vivo studies because of their inability to be used in physiological settings. The present study presents an alternative approach in which combination of a putative functional domain of the AT1R fused to a PTD enables it to be freely transported through the membrane and into the cell.

Neuronal cells transduced with PTD-3IL-EGFP showed that the cells took up the protein in minutes. It was predominantly localized into the cytoplasmic compartment but began to localize into the nuclear compartment during 1-hour incubation. This observation is not surprising because HIV-Tat contains a nuclear localization signal.37 However, it raises some very interesting questions. For example, does nuclear targeting of the AT1R 3IL play any role on neuronal firing rate on a long-term basis? It would be interesting to determine if AT1bR 3IL exerts long-lasting effects on firing rate. It is possible that cytoplasmic targeting of the AT1bR 3IL is linked to one set of responses (eg, stimulation of firing rate, neurotransmitter release), whereas its nuclear targeting regulates chronic responses of Ang II. There is some evidence for nuclear targeting of the AT1R in hepatocytes and in neurons.38–40 These are relevant issues that are easily resolved by the mutation of the nuclear localization signal of HIV-Tat-PTD, which would result in its exclusion from the nucleus and accumulation in the cytoplasmic compartment only.

**Perspectives**

Our research group has extensively used AT1R antisense to attenuate AT1R function. Thus, the rationale for using AT1bR subtype in this study was our anticipated expectation to study which physiological actions of Ang II attenuated by the AT1bR antisense could be restored by the AT1bR 3IL. We believe that the use of PTDs presents a valuable tool to explore the molecular mechanism both in vitro and in vivo, and this study has put us in a position to test the AT1R structure–function in vivo. In fact, examples already exist for the use of this technology in vivo and in whole-animal situations.25,28,41,42

**Acknowledgments**

This research was supported by the National Institutes of Health grant HL33610. Dr. Lucia Fuentes was a visiting postdoctoral fellow from the Department of Pharmacology, San Luis National University, Argentina. Dr. Jianqiong Du was a visiting professor from the Department of Physiology, Xi’an Jiaotong University Medical College, Xi’an, Shaanxi, Peoples Republic of China.

**References**


Transduction of a Functional Domain of the AT₁ Receptor in Neurons by HIV-Tat PTD
Jorge Vázquez, Chengwen Sun, Jianqing Du, Lucía Fuentes, Colin Sumners and Mohan K. Raizada

Hypertension. 2003;41:751-756; originally published online December 16, 2002;
doi: 10.1161/01.HYP.0000047878.13793.41
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/41/3/751

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/