Kidney International, Vol. 46 (1994), pp. 1499–1501

Desensitization of angiotensin receptor function

HIROYUKI SASAMURA, VICTOR J. DZAU, and RICHARD E. PRATT

Division of Cardiovascular Medicine, Falk Cardiovascular Research Center, Stanford University School of Medicine, Stanford, California, USA

Desensitization of angiotensin receptor function. Angiotensin II is an eight amino acid peptide which plays a major role in the regulation of cardiovascular homeostasis. The physiologic effects of angiotensin (Ang) II are mediated by a G-protein coupled receptor, termed AT_1, which activates phospholipase C. A major factor regulating angiotensin II receptor function is the rapid desensitization following agonist stimulation. However, despite years of investigation, the mechanism by which the angiotensin receptor is regulated remains unclear. The cloning of the AT-1 receptor and the availability of cell lines which stabily express this receptor has helped elucidate these mechanisms. In this paper, we review the data from our laboratory concerning the post-translational regulation of the angiotensin receptor function.

Angiotensin (Ang) II is the effector molecule of the renin-angiotensin-aldosterone system. An eight amino acid peptide, Ang II exerts a wide range of actions in the kidney, heart, vascular system, adrenal gland and central nervous systems. In the past, it was thought that the main physiologic consequences of this peptide was an increase in vascular tone as well as salt and water retention. However, it is now apparent that this peptide exerts numerous other actions such as the regulation of gene transcription and the regulation of vascular, cardiac, mesangial and adrenal growth, both physiologic as well as pathologic [reviewed in 1].

Ang II exerts its actions via a family of membrane bound receptors that are widely distributed. Based on ligand binding experiments, two major types of specific Ang II binding sites have been defined and designated AT_1 and AT_2 [2, 3]. To date, a vast majority of the physiologic actions of Ang II in the normal, adult animal appear to be due to the actions of the AT_1 class of receptors. Several investigators have succeeded in the molecular cloning of the AT_1 receptors from numerous species [4–7]. The combined results indicate that the Ang II receptor belongs to the super family of seven transmembrane, G-protein coupled receptors. In some species, we and others have demonstrated the presence of two subtypes of the AT_1 receptor which have been termed AT_{1a} and AT_{1b} [5, 6]. These two subtypes are highly homologous, in the mouse differing by only 22 amino acids out of 359. No obvious difference in ligand binding characteristics signal transduction or rapid desensitization could be shown; however, differences in tissue distribution exist. The AT_{1a} was found to be distributed in a wide range of tissues while the AT_{1b} was restricted to the adrenal gland and kidney.

Over the years multiple lines of investigation from many laboratories have shown that the regulation of the Ang II receptor function is a complex yet physiologically important process. It has been demonstrated in whole animal, isolated vessels and in cultured cells that the Ang II receptor undergoes rapid tachyphylaxis or desensitization since repeated administration of ligand fails to elicit a response comparable to the initial response. Prior to the molecular cloning of the receptor, the mechanism for this desensitization was unclear. Based on studies with other seven-transmembrane receptors, we have hypothesized that protein kinase C, which is activated as part of the angiotensin receptor signaling cascade, would mediate the agonist-induced desensitization of this receptor. However, this is a controversial point [8–11]. With the availability of the cloned cDNA from many species, the processes involved in the tachyphylaxis of the receptor can be easily examined.

Characterization of the murine angiotensin receptors

Recent advances in murine embryology have made it possible to develop strains of mice overexpressing mutant genes or harboring targeted gene disruptions. Therefore, aiming towards future experiments, we have chosen to investigate the murine angiotensin receptor. A murine genomic library was screened with a PCR derived cDNA probe complementary to the mouse AT_1 cDNA [5]. Multiple positive genomic clones were isolated and shown by restriction mapping to encode two distinct clones which were termed AT_{1a} and AT_{1b}. Sequence analysis demonstrated that these clones were highly homologous, differing in only 22 out of 359 amino acids (93.9% homology). The amino acid differences were distributed through out the molecule but were particularly prevalent in the carboxy terminal intracellular domain. Of particular interest are differences involving serine residues which may reflect differences in phosphorylation and subsequent desensitization [5]. Binding studies were performed on membranes from COS-7 cells transfected with expression vectors encoding the two isoforms which confirmed their designation as AT_1 receptors. No significant differences were noted in the binding or competition profiles for the two isoforms. Using a RT-PCR based assay, the tissue distribution of the two isoforms was examined. AT_{1a} transcripts were the predominant form in most tissues including the kidney, heart, liver and brain while the AT_{1b} was the predominant form in the adrenal gland.

Expression plasmids coding for epitope-tagged AT_1 receptor were constructed by inserting the coding region of the mouse AT_{1a} and AT_{1b} receptors [5] into the eukaryotic expression vector pBC12BI [12]. Cells expressing the receptors were cloned after selection with G418 (800 mg/ml) and analyzed by binding studies. Scatchard analysis demonstrates that the expression of the two receptors was similar, (AT_{1a}, B_{max} = 235,000 sites/cell, K_d =

1.9 nM; AT_{1b} B_{max} = 216,000 sites/cell, K_d = 1.2 nM). We next examined the signal transduction cascade initiated by these two receptor isoforms. Measurements of intracellular calcium concentrations were performed by loading cells with 10 μM Fura-2-AM for 20 minutes at 37°C. Values of intracellular calcium concentration were calculated according to the method of Grykiewicz, Poenie and Tsien [13]. Measurements of angiotensin II-induced IP3 levels were performed by stimulating cells with angiotensin II and IP_3 levels in the supernatant were assayed using a competitive binding assay (NEN). Both isoforms elicited transient increases in IP_3 levels and intracellular calcium. Moreover, the magnitude and kinetics were virtually identical.

Desensitization of receptor function

The availability of stabily transfected 293 cells, expressing the AT_1 receptor subtypes has provided a unique model system to examine the mechanism of desensitization of these receptors. Previous studies have shown that the angiotensin receptor exhibits agonist-induced desensitization [14], which could account for the commonly observed phenomenon of tachyphylaxis to the actions of angiotensin II [15]. However, the mechanisms of angiotensin receptor desensitization have not been clarified. By comparison to other seven transmembrane receptor systems, we hypothesize that PKC should be involved in the desensitization of this receptor. However, this point is controversial. For example, in a study of neonatal rat cardiac ventricular myocytes, Abdellatif et al. [10] reported that PKC did not play a major role in the agonist-induced desensitization of the angiotensin receptor, whereas Pfeilschifter and co-workers [9–11] found that PKC appeared to be involved in the agonist-induced desensitization of the angiotensin receptor in rat mesangial cells. Since these investigators were using different tissue sources, different doses of Ang II and different methods to inhibit PKC (depletion of PKC by chronic pretreatment with the phorbol ester PMA, versus the use of PKC inhibitors such as H-7 and sphingosine), these results are not directly comparable.

Using stabily transfected cell lines, we have examined agonist-induced desensitization. Cells were stimulated with Ang II (10^{-7} M). Various times later, the agonist was removed by rinsing cells with 100 mM NaCl, 50 mM glycine (pH 3.0) followed by rinsing with PBS. Cells were then stimulated again with Ang II (10^{-7} M) for 15 seconds, harvested and the extracts assayed for IP_3. The results demonstrated that within 5 to 10 minutes of the initial stimulation, Ang II no longer stimulated an increase in IP_3, demonstrating that complete desensitization had occurred. The dose response relationship for the desensitization was examined and the ED_{50} shown to be 3×10^{-9} M. The agonist induced desensitization could be mimicked by the PKC activating phorbol ester, PMA. Furthermore, we found that PMA-induced desensitization could be blocked by staurosporin (PKC antagonist) or by down-regulation of PKC by prolonged pretreatment with high dose PMA. Thus, our results demonstrate that PKC activation can result in the desensitization of the AT_1 receptors. Similar results were obtained for the AT_{1b} isoform.

We then examined if PKC mediated the desensitization of the Ang II receptors induced by agonist. At low doses of Ang II (10^{-10} M), inhibition of PKC with staurosporine or down-regulation of PKC with 24 hour treatment with PMA blocked agonist induced desensitization of the AT_{1a} receptor. However, at high doses of Ang II (10^{-7} M), inhibition or down regulation of PKC had no effects. These results suggest the involvement of both PKC dependent and PKC independent pathways in the desensitization of AT_1 receptor isoforms. Preliminary studies indicate that the characteristics of desensitization of AT_{1a} subtype are qualitatively similar to those for the AT_{1b} subtype.

Agonist-induced loss of surface receptors

We next asked if the desensitization of the AT_1 receptors was due to the internalization or sequestration of the receptors. Angiotensin stimulation results in a loss of cell surface receptors as determined by binding to the hydrophilic antagonist ^{125}I-Sar1 Ile8-Ang II. However, the rate of disappearance of the receptors from the cell surface is considerably slower than the rate of agonist induced desensitization. These results suggest that loss of cell surface receptors does not contribute to the rapid agonist induced desensitization, but may play a role in maintaining the desensitized state. This loss of cell surface receptors following agonist stimulation has been observed for other G-protein coupled receptors. While it has been proposed to contribute to desensitization, the role of receptor induced internalization in receptor regulation remains unknown.

Discussion

The results of this study demonstrate that the regulation of angiotensin receptor function is a complex process involving uncoupling of the receptor from its effector as well as the sequestration/internalization of the receptor (Fig. 1). Agonist-induced desensitization occurred rapidly, within minutes of incubation with angiotensin II, and was maximally effective at high (saturating) concentrations of the agonist. The processes of desensitization and internalization/sequestration appear to be different processes. The desensitization is temporally distinct from internalization/sequestration, which occurs at a significantly slower rate. Moreover, desensitization could be induced by PKC activation and (at low doses of angiotensin) PKC appears to be the mechanism by which desensitization occurs (Fig. 1).

What might be the physiologic relevance of the dual regulatory pathways of the angiotensin receptor desensitization? Circulating levels of angiotensin II are in the pM range, significantly lower than the K_d of the receptor and in the range where the PKC-dependent mechanism may play a role in the desensitization. On the other hand, we and others have speculated that the local generation of angiotensin II within tissues may result in significantly higher levels of angiotensin II. This is a controversial area, moreover, the levels of angiotensin that may result from these local systems are not known. However, it is conceivable that the local concentration of peptide may be greater than in the systemic circulation and may reach levels where the PKC-independent mechanism may play an important role in the regulation of receptor signaling.

In summary, our results indicate that both of the recently discovered subtypes of the AT_1 receptor cause signal transduction by the inositol phospholipid/calcium signaling pathway. Desensitization of the two subtypes of the AT_1 receptor occurs within minutes of agonist treatment, and is maximal at concentrations of the agonist higher than 10 nM. Although PKC-dependent mechanisms were involved in agonist-induced desensitization at low

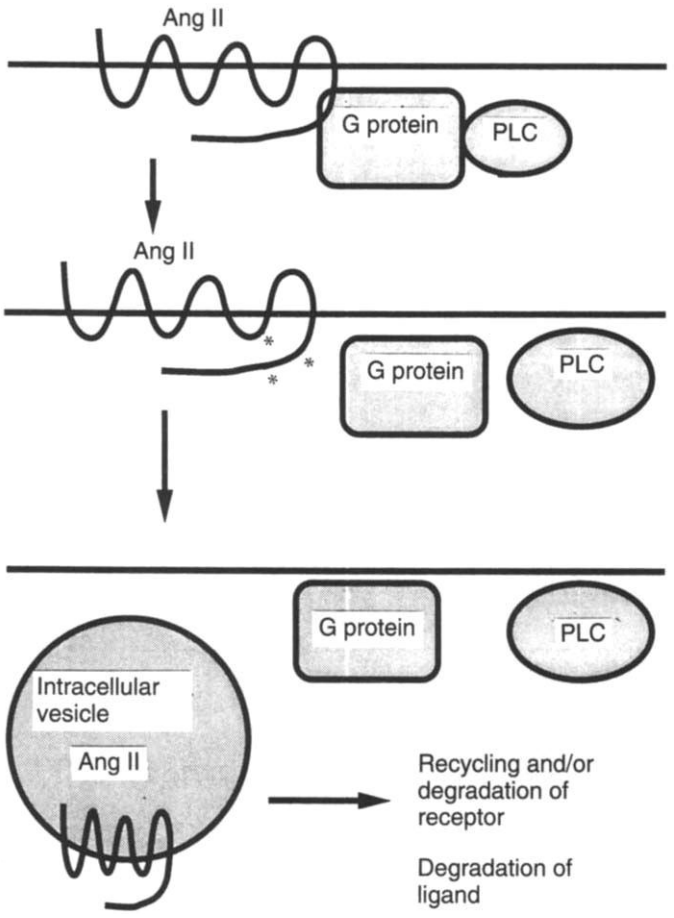

Fig. 1. *Schematic representation of the desensitization of the Ang II receptor.* The interaction of the receptor and ligand results in the activation of phospholipase C (PLC) via the coupling of the receptor with G proteins (stage 1). The receptor becomes uncoupled very rapidly following ligand addition by PKC dependent and independent mechanisms (perhaps via phosphorylation of the receptor (*), stage 2). This is followed by the internalization of the receptor with the subsequent recycling and/or degradation of the receptor and the degradation of the ligand (stage 3).

concentrations of agonist, the high dose agonist-induced desensitization was mediated by a non-PKC pathway. Also, loss of surface AT_1 receptor binding occurred for the two receptor isoforms by non-PKC dependent mechanisms. It is interesting to note that we have not found any differences in the regulation of AT_{1a} and AT_{1b} receptor subtypes in this study using transfected 293 cells. Our data have not excluded cell or tissue specific mechanisms of receptor subtype regulation. The significance of the presence of two AT_1 receptor subtypes in the mouse is still unclear at this time.

Acknowledgments

This work was funded by NIH grants HL35610, HL35252, and HL42663. The authors thank Drs. Lutz Hein and Brian Kobilka for helpful discussions and Ms. Wendy Lee for technical assistance.

Reprint requests to Richard E. Pratt, Ph.D., Division of Cardiovascular Medicine, Falk Cardiovascular Research Center, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-2874, USA.

References

1. Dzau VJ, Pratt RE: Renin-angiotensin system, in *The Heart and Cardiovascular System*, edited by Fozzard HA, New York, Raven Press, 1992, pp. 1817–1849
2. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, Timmermans P: Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Comm* 165:196–203, 1985
3. Whitebread S, Mele M, Kamber B, de Gasparo M: Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Comm* 163:284–291, 1989
4. Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T: Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature* 351:230–233, 1991
5. Sasamura H, Hein L, Krieger JE, Pratt RE, Kobilka BK, Dzau VJ: Cloning, characterization, and expression of two angiotensin receptor (AT-1) isoforms from the mouse genome. *Biochem Biophys Res Comm* 185:253–259, 1992
6. Sandberg K, Ji H, Clark AJ, Shapira H, Catt KJ: Cloning and expression of a novel angiotensin II receptor subtype. *J Biol Chem* 267:9455–9458, 1992
7. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE: Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature* 351:233–236, 1991
8. Pfeilschifter J, Ochsner M, Whitebread S, De Gasparo M: Down-regulation of protein kinase C potentiates angiotensin II-stimulated polyphosphoinositide hydrolysis in vascular smooth-muscle cells. *Biochemical J* 262:285–291, 1989
9. Pfeilschifter J, Fandrey J, Ochsner M, Whitebread S, de Gasparo M: Potentiation of angiotensin II-stimulated phosphoinositide hydrolysis, calcium mobilization and contraction of renal mesangial cells upon down-regulation of protein kinase C. *FEBS Lett* 262:307–311, 1990
10. Abdeffatif MM, Neubauer CF, Lederer WJ, Rogers TB: Angiotensin-induced desensitization of the phosphoinositide pathway in cardiac cells occurs at the level of the receptor. *Circ Res* 69:800–809, 1991
11. Pfeilschifter J: Protein kinase C from rat renal mesangial cells: its role in homologous desensitization of angiotensin II-induced polyphosphoinositide hydrolysis. *Biochim Biophys Acta* 969:263–270, 1988
12. Cullen B: Use of eukaryotic expression technology in the functional analysis of cloned genes. *Meth Enzymol* 152:684–704, 1987
13. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450, 1985
14. Brock TA, Rittenhouse SE, Powers CW, Ekstein LS, Gimbrone MA, Alexander RW: Phorbol ester and 1-oleoyl-2-acetylglycerol inhibit angiotensin activation of phospholipase C in cultured vascular smooth muscle cells. *J Biol Chem* 260:14158–14162, 1985
15. Peach MJ: Renin-angiotensin system: Biochemistry and mechanisms of action. *Physiol Rev* 57:313–370, 1977