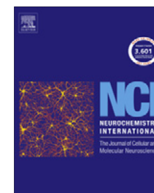
Neurochemistry International 60 (2012) 199–207

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci



AT_2 receptors recruit c-Src, SHP-1 and FAK upon activation by Ang II in PND15 rat hindbrain

Leonardo R. Seguin, Rodrigo S. Villarreal, Gladys M. Ciuffo *

Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL CONICET), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950, 5700 San Luis, Argentina

ARTICLE INFO

Article history:
Received 21 December 2010
Received in revised form 7 November 2011
Accepted 10 November 2011
Available online 20 November 2011

Keywords:
AT_2 receptors
SHP-1/c-Src interaction
Focal adhesion kinase
Hindbrain development

ABSTRACT

The functional role of AT_2 receptors is unclear and it activates unconventional signaling pathways, which in general do not involve a classical activation of a G-protein. In the present study, we aimed to investigate the transduction mechanism of AT_2 Ang II receptors in PND15 rat hindbrain membrane preparations, which represents a physiological developmental condition. To determine whether Ang II AT_2 receptors induced association to SHP-1 in rat hindbrain, co-immunoprecipitation assays were performed. Stimulation of Ang II AT_2 receptors induced both a transient tyr-phosphorylation and activation of SHP-1. The possible participation of c-Src in Ang II-mediated SHP-1 activation, we demonstrated by recruitment of c-Src in immunocomplexes obtained with anti AT_2 or anti-SHP-1 antibodies. The association of SHP-1 to c-Src was inhibited by PD123319 and the c-Src inhibitor PP2. Similarly, SHP-1 activity determined in AT_2-immunocomplexes was inhibited by PD123319 and the c-Src inhibitor PP2. Following stimulation with Ang II, AT_2 receptors recruit c-Src, which was responsible for SHP-1 tyr-phosphorylation and activation. Since AT_2 receptors are involved in neuron migration, we tested the presence of FAK in immunocomplexes. Surprisingly, AT_2-immunocomplexes contained mainly the 85 kDa fragment of FAK. Besides, p125FAK associated to SHP-1. In summary, we demonstrated the presence of an active signal transduction mechanism in PND15 rat hindbrain, a developmental stage critical for cerebellar development. In this model, we showed a complex containing AT_2/SHP-1/c-Src/p85FAK, suggesting a potential role of Ang II AT_2 receptors in cerebellar development and neuronal differentiation.

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1. Introduction

Angiotensin II (Ang II), initially described as a primary vasoconstrictor peptide, acts on its target tissues through binding to membrane receptors. Two main subtypes of Ang II receptors have been described, AT_1 receptors specifically blocked by Losartan and AT_2 receptors selectively displaced by CGP42112 or PD123319 (De Gasparo et al., 2000; Gallinat et al., 2000; Touyz and Berry, 2002). Both receptor subtypes belong to the superfamily of G-protein coupled receptors (Sasaki et al., 1991; Mukoyama et al., 1993; Kambayashi et al., 1994). However, both receptor subtypes induce opposite biological effects, such as vasoconstriction/vasodilatation and cell growth/anti-growth (Horiuchi et al., 1999; Steckelings et al., 2005) by acting via different intracellular signaling pathways.

Recently, a potential role for Ang II in development as a growth modulating factor was proposed (Landon et al., 2005; Deshayes et al., 2005; Hunyady et al., 2006; Godeny and Sayeski, 2006). The trophic effects of Ang II have been studied *in vitro* in primary cultures or in established cell lines (De Gasparo et al., 2000; Gallinat et al., 2000; Godeny and Sayeski, 2006).

AT_1 receptors interact with multiple heterotrimeric G-proteins, including $G_{q/11}$, G_i, G_{12} and G_{13} and leads to stimulation of MAPK (Godeny and Sayeski, 2006) and JAK/STAT signaling pathway, the phosphorylation of Elk-1, CREB and c-fos (Hernández-Vargas et al., 2005) and various isoforms of PKC (Hunyady et al., 2006; Higuchi et al., 2007).

The AT_2 receptor is much less well characterized, and its physiological role is not completely understood (Gendron et al., 2003; Porrello et al., 2009). The specific localization of AT_2 receptors in embryonic and neonatal tissue suggests a role in the regulation of developmental processes and organogenesis (Gallinat et al., 2000; Godeny and Sayeski, 2006; Arce et al., 2001, 2011). Earlier

Abbreviations: Ang II, angiotensin II; CREB, cAMP response element-binding; FAK, focal adhesion kinase; IGFR, insulin-like growth factor receptor; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; PND, post-natal day; PC, Purkinje cell; PTK, protein tyrosine kinase; JAK/STAT, janus kinase/signal transducer and activator of transcription; PKC, protein kinase c; PTPase, protein tyrosine phosphatase; SFK, c-Src family kinase; ITIM, immunoreceptor tyrosine-based inhibitory.

* Corresponding author. Tel./Fax: +54 2652 422644.
E-mail address: gciuffo@unsl.edu.ar (G.M. Ciuffo).

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doi:10.1016/j.neuint.2011.11.008

studies proposed a role of AT_2 receptors in apoptosis. In PC12 cells, stimulation of AT_2 receptors induced programmed cell death mediated by a PTPase (Yamada et al., 1996) and dephosphorylation of Bcl-2 (Horiuchi et al., 1997). Activation of the PTPase SHP-1 by AT_2 receptors was associated to cellular apoptosis in different cell lines (Bedecs et al., 1997; Shibasaki et al., 2001; Cui et al., 2002). SHP-1 (also named as SH-PTP-1, HCP, PTP-1C) is a non transmembrane PTPase that contains two Src homology 2 domains (SH2), allowing its association with multiple adaptor molecules (Wu et al., 2003; Chong et al., 2007).

Increasing evidence supports a role of AT_2 receptors mediated by protein tyrosine dephosphorylation (Nahmias et al., 1995; Gallinat et al., 2000; Gendron et al., 2003). We showed that Ang II stimulate a rapid and transient tyrosine dephosphorylation of several proteins in rat fetal tissue preparation (Alvarez et al., 2003). Studies performed in neuronal cell lines (e.g. N1E-115, NG108-15 and PC12W) showed that AT_2 receptors induce dephosphorylation and couple to SHP-1 (Bedecs et al., 1997; Shibasaki et al., 2001; Cui et al., 2002). Feng et al. (2002) demonstrated an AT_2-mediated ITIM-independent activation of SHP-1 that involves a $G\beta\gamma$-independent constitutive association to $G_{\alpha s}$ protein, suggesting a mechanism distinct from the classic heterotrimeric G-protein.

SHP-1 could be either phosphorylated or dephosphorylated in response to activation of G-protein coupled receptors (Feng et al., 2002; Cui et al., 2002; Shaw et al., 2003; Marrero et al., 2004; Min et al., 2004; Alvarez et al., 2008). SHP-1 and c-Src may have complementary substrate selectivity. Thus, substrates that are efficiently phosphorylated by c-Src kinase are in turn substrates for SHP-1 (Somani et al., 1997; Frank et al., 2004; Roskoski, 2004). Both, SHP-1 and c-Src can bind to membrane lipids (Sankarshanan et al., 2007; Ingley et al., 2008) and thus interact with membrane receptors participating on its signal transduction mechanism. We demonstrated the formation of a SHP-1/c-Src complex by activation of AT_2 receptors in fetal tissues (Alvarez et al., 2008). Guimond et al. (2010) demonstrated that Fyn, a member of the c-Src family kinase (SFK), participates in neurite outgrowth in NG108-15 by activation of AT_2 receptors. Focal Adhesion Kinase (FAK) is a substrate of c-Src which participates in extracellular signaling mediated by integrins in growth process, differentiation and cell migration (Wen et al.,1997; Watanabe et al., 2008; Otis et al., 2008).

Studies from knock-out mice reinforce the hypothesis that AT_2 receptors play a role in neuronal development. AT_2 knock-out mice suffer from perturbations in exploratory behavior and locomotor's activity (Hein et al., 1995; Ichiki et al., 1995), as well as anxiety-like behavior (Okuyama et al., 1999). Genetic deletion of AT_2 receptors lead to increased cell number in different brain structures in mice (von Bohlen und Halbach and Albrecht, 2006). Ang II participates in neuronal differentiation, neurite elongation, neuronal migration, excitability modulation and neuronal apoptosis (Gendron et al., 2003; Kilian et al., 2008).

Recently, we demonstrated that AT_2 receptors co-localize with molecular markers of Purkinje cells (PC) in developing cerebellar cortex. Since PCs are the sole output signal from the cerebellar cortex and considering the role of cerebellum in movement control, the specific receptor localization suggests a potential role for AT_2 receptors in cerebellar development (Arce et al., 2011). The aim of the present study was to characterize the signal transduction of Ang II AT_2 receptors in a critical stage of hindbrain development, PND15. Thus, we demonstrated that Ang II stimulation leads to physical association of AT_2 receptors with the PTPase SHP-1, c-Src and p85FAK. Complex formation as well as tyr-phosphorylation and activation of SHP-1 were inducible but not constitutive. The presence of p85FAK in immunocomplexes AT_2/SHP-1/c-Src suggests a potential role in cell adhesion/migration during hindbrain development.

2. Materials and methods

2.1. Animals

Wistar rats were kept in a dark–light cycle (12:12 h) and provided with standard rat food and water *ad libitum*. Rats were sacrificed by decapitation at PND15. Hindbrains were immediately removed and collected on hypotonic buffer (50 mM Tris–HCl, 5 mM EDTA, pH 7.4) at 4 °C and processed for membrane preparation. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.2. Drugs

Bovine serum albumin (BSA) fraction V, Triton X-100 and sodium orthovanadate were purchased from Sigma (St. Louis, MO, USA). Ang II, PP2 and PP3 were from Calbiochem (La Jolla, CA, USA); Losartan was a gift from Dr. R. Smith (Dupont, Wilmington, DE, USA) and PD123319 was Sigma (St. Louis, MO, USA). The antibodies anti phosphotyrosine PY99, anti SHP-1, anti AT_2, anti FAK, and anti c-Src were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies AP-coupled and HRP-coupled were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Jackson ImmunoResearch Inc. (West Grove, PA, USA). AP-substrate BCIP/NBT was from Promega (Madison, WI, USA) and ECL detection kit from Amersham (England, UK). Protein A/G agarose plus was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Membrane preparation

For membrane preparation, PND15 hindbrains were homogenized in 40 volumes of hypotonic buffer (50 mM Tris–HCl, 5 mM EDTA, pH 7.4) using a Teflon homogenizer for three times, 10 s each, as described (Alvarez et al., 2003). The homogenate was centrifuged at 1000*g* for 10 min at 4 °C to separate nuclear and cellular debris. The supernatant was centrifuged at 20000*g* for 60 min at 4 °C. The pellet was washed three times on hypotonic buffer and resuspended in isotonic buffer (50 mM Tris–HCl, 5 mM EDTA, 120 mM KCl, 10% glycerol, 0.32 M sucrose, pH 7.4) and used as source of receptors. Membranes were stored at −80 °C and stable for at least two months. Protein concentration was determined by Bradford assay.

2.4. Phosphorylation assays

Membrane preparations (400–600 μg protein) from PND15 rats were stimulated with Ang II (10^{-7} M) alone or in combination with different competitors at the indicated times and concentrations. The reaction was performed at final volume of 100 μl at 20 °C or RT in phosphorylation buffer (20 mM Hepes, 60 mM NaCl, 0.1% BSA, pH 7.2). After stimulation, the reaction was conducted in presence of ATP-$MgCl_2$ (0.25 mM ATP, 50 mM $MgCl_2{\cdot}6H_2O$) for 5 min. The phosphorylation reaction was stopped with 1 ml of stop buffer (20 mM Hepes, 60 mM NaCl, pH 7.2) at 4 °C. Membrane proteins were recovered by centrifugation at 20000*g* 4 °C, 15 min, the pellet was solubilized and used for immunoprecipitation.

2.5. Immunoprecipitation and immunoblotting

After phosphorylation assays, the pellets were solubilized in solubilization buffer (20 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, pH 7.2) containing 1% Triton X-100 for 60 min at 4 °C with gentle agitation and then centrifuged at 20000*g* for

15 min at 4 °C. Supernatants were used to carry out the immunoprecipitations, by adding the indicated primary antibody and incubating overnight at 4 °C with agitation, followed by addition of 20 l of protein A/G agarose plus for 3 h at 4 °C with gentle agitation. Immunocomplexes were recovered by centrifugation at 1000*g*, washed with solubilization buffer for three times and subjected to SDS–PAGE. Proteins were transferred onto PVDF membranes (Immobilon P, Millipore Corp., Bedford, MA, USA) or Nitrocellulose membranes (BioRad, Hercules, CA, USA) and blotted. Fig. S1 shows the immunocomplexes performed in the presence or absence of the primary antibody (anti-AT_2 or anti-SHP-1). No bands were obtained in immunocomplexes performed with protein A/G agarose alone.

Proteins were immunoblotted with the indicated antibodies (1:1000) followed by incubation with the secondary antibody (1:5000–10000 for HRP-coupled or 1:2000 for AP-coupled antibodies). The antibodies' specificity was proven before (Arce et al., 2011). Blots were developed with ECL for HRP-coupled antibodies or BCIP-NBT for Alkaline Phosphatase-coupled antibodies. Stripping of blots was achieved by incubation in buffer (62.5 mM Tris–HCl pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 10 min, 50 °C. Membranes were reprobed for loading control.

2.6. SHP-1 activity assay

SHP-1 activity was assayed on the immunocomplexes obtained with 2 μl of anti SHP-1 or anti AT_2 antibodies and processed as before. The immunocomplexes were resuspended in 40 μl of PTPase buffer (20 mM Hepes, 60 mM NaCl, 0.1% BSA, pH 7.2) and 30 μl of tyr-phosphopeptide DADE(pY)LIPQQG Sigma (St. Louis, MO, USA) were added at final concentration 10^{-4}M. The PTPase activity was assayed by measuring release of inorganic phosphate from tyr-phosphopeptides based on a malachite green detection system (Burshtyn et al., 1997). Following incubation for 30 min at RT, 300 μl of dye mixture solution was added and optical densities measured at 600 nm in spectrophotometer and quantified.

2.7. Statistical analysis

Values are expressed as mean ± SEM in the text and figures. Western blots were quantified with Scion Image and analyzed using PRISM package. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Ang II induce the association of SHP-1 to AT_2 receptors

In order to evaluate a possible physiological role of AT_2 receptors, highly expressed in PND15 rat hindbrain, we undertook the study of signal transduction mechanism of Ang II AT_2 receptors in this model. In a previous paper we demonstrated that Ang II induced physical association between AT_2 receptors and PTPase SHP-1 in rat fetal E20 membrane preparation (Alvarez et al., 2008). To determine whether Ang II AT_2 receptors induced association to SHP-1 in rat hindbrain, co-immunoprecipitation assays were performed. After stimulation with Ang II (10^{-7}M) in the presence or not of AT_1 antagonist Losartan (10^{-6}M) or AT_2 antagonist PD123319 (10^{-6}M) membranes were solubilized with solubilization buffer containing Triton X-100 and immunoprecipitated with anti AT_2 antibody. Immunocomplexes were separated by SDS–PAGE and blotted with anti SHP-1 antibody, revealing the presence of a polypeptide of apparent molecular weight 65 kDa corresponding to SHP-1 (Fig. 1A, upper panel). Equal protein loading was assessed by reprobing the membrane with anti AT_2 (Fig. 1B, lower panel) and the ratio SHP-1/AT_2 was plotted. The association between AT_2 receptors and SHP-1 phosphatase was inhibited by preincubation with PD123319 but not with Losartan (Fig. 1A).

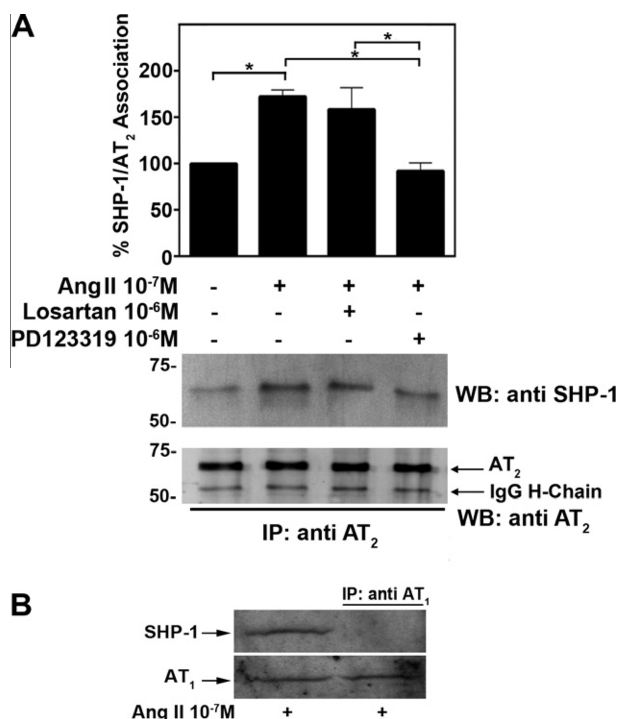

Fig. 1. Association of SHP-1 to AT_2 receptors. A. Co-immunoprecipitation of SHP-1 and AT_2 receptor. PND15 rat hindbrain membranes (400–600 μg protein) were stimulated with Ang II (10^{-7}M, 5 min) either alone or in the presence of Losartan (10^{-6}M, 10 min), or PD123319 (10^{-6}M, 10 min), solubilized with Triton X-100 and immunoprecipitated (IP) with anti rabbit AT_2 antibody. Western blot (WB) was performed using mouse anti SHP-1 antibody (upper panel) or rabbit anti AT_2 antibody (lower panel) to demonstrate equal protein loading. Representative image and histogram of densitometric data as mean ± SEM (n = 5) $^*P < 0.05$. B. Immunocomplexes obtained with anti-AT_1 antibody do not contain SHP-1. Lane 1: Rat hindbrain membranes were stimulated with Ang II (10^{-7}M, 5 min) and immunoblotted with anti SHP-1 (upper panel) or anti-AT_1 (lower panel) antibody as a control. Lane 2: Rat hindbrain membranes were stimulated with Ang II (10^{-7}M, 5 min), solubilized and immunoprecipitated with anti AT_1 antibody and developed with anti SHP-1 antibody (upper panel) or with anti AT_1 antibody (lower panel).

To confirm that AT_1 receptors do not associate with SHP-1, immunocomplexes obtained with anti AT_1 antibodies were tested for the presence of SHP-1. Fig. 1B shows that SHP-1 was present in hindbrain membrane preparations as well as AT_1 receptors (left panel). However, immunocomplexes obtained with anti AT_1 antibody, stimulated with Ang II (10^{-7}M), did not contain SHP-1. Taken together, these results indicate that SHP-1 associates to AT_2 but not to AT_1 receptors, following stimulation with Ang II (10^{-7}M). Physical association of AT_2 receptors to SHP-1 was not constitutive but it was induced by Ang II in PND15 rat hindbrain membranes.

3.2. Ang II induces activation of SHP-1 by AT_2 receptors

Activation of SHP-1 requires a tyr-phosphorylation that triggers tyrosine phosphatase activity (Frank et al., 2004). To test this hypothesis in the present model, hindbrain membrane preparations were stimulated with Ang II (10^{-7}M) and tyr-phosphorylation of SHP-1 was analyzed. After stimulation, membrane proteins were solubilized and immunoprecipitated with anti SHP-1 antibodies and the immunocomplexes separated by SDS–PAGE. Phosphorylation of the PTPase was assessed by western blot, using mouse monoclonal PY99 antibody. Ang II induced a rapid and transient tyr-phosphorylation of SHP-1 (Fig. 2A), maximal after

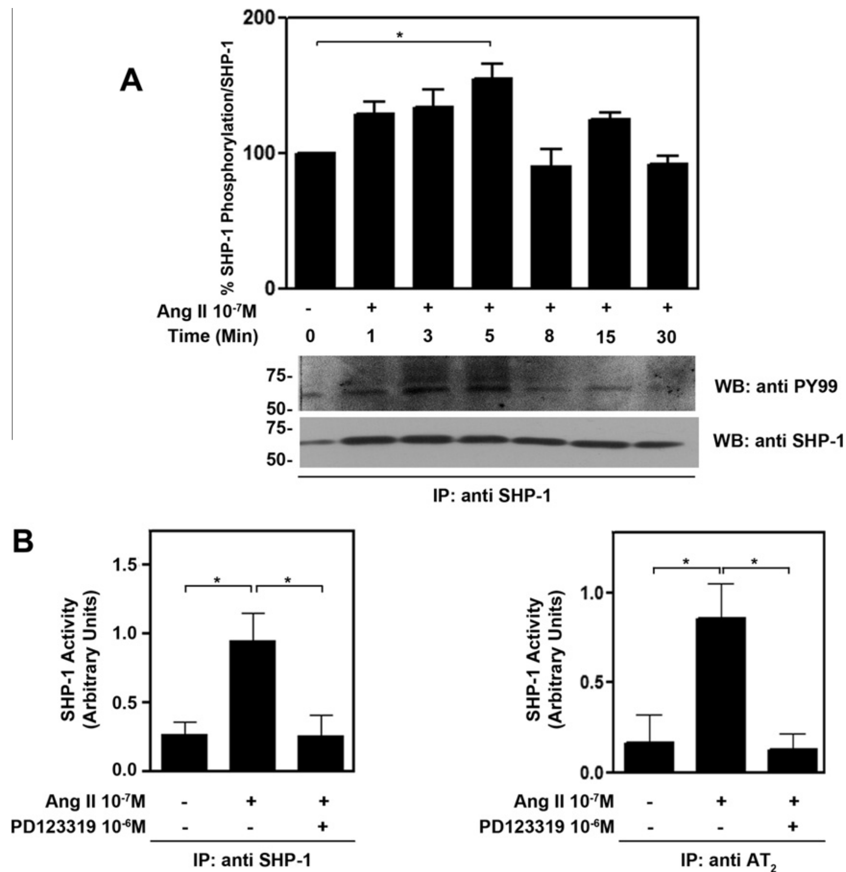

Fig. 2. Activation of SHP-1 is mediated by Ang II. A. Time course of SHP-1 tyr-phosphorylation. Membranes (400–600 μg protein) were stimulated with Ang II (10^{-7}M) for different times (1–30 min). Solubilized samples were immunoprecipitated with anti SHP-1 antibody and then blotted (WB) with anti p-Tyr PY99 antibody (upper panel). Blots were stripped and reprobed with anti SHP-1 antibody (lower panel). Representative image and histogram of the ratio of densitometric data as mean ± SEM ($n = 7$) $^{*}P < 0.05$. B. Detection of SHP-1 activity in immunocomplexes obtained with anti SHP-1 or anti AT_2 antibodies. Aliquots of membranes (400–600 μg protein) were stimulated with Ang II (10^{-7}M, 5 min) in the presence or absence of PD123319 (10^{-6}M, 10 min), solubilized and immunoprecipitated (IP). SHP-1 activity in immunocomplexes was determined by measuring the released orthophosphate (mean ± SEM, n = 7, $^{*}P < 0.05$).

5 min of stimulation which decreased at 8 min and recovered basal level after 15 or 30 min stimulation. Blots were stripped and reprobed with anti SHP-1 antibody and the ratio of SHP-1 phosphorylation/SHP-1 was plotted (Fig. 2A).

To confirm that tyr-phosphorylation is required for tyrosine phosphatase activity, we performed PTPase activity assays. Membranes were stimulated with Ang II (10^{-7}M) for 5 min in the presence or not of PD123329 (10^{-6}M), then solubilized and immunoprecipitated with anti SHP-1 or anti AT_2 antibodies. SHP-1 activity in immunocomplexes was determined by measuring the released orthophosphate as described in Section 2. Ang II induced phosphatase activity of SHP-1 which was blocked by AT_2 antagonist PD123319 in immunocomplexes obtained either with anti SHP-1 or anti AT_2 antibodies (Fig. 2B). Thus, SHP-1 activation correlates with SHP-1 tyr-phosphorylation induced by Ang II AT_2 receptors.

3.3. Association of c-Src to AT_2 receptors was mediated by Ang II

To test the possible participation of c-Src in Ang II-mediated SHP-1 activation, we assayed the recruitment of c-Src in immunocomplexes obtained with anti AT_2 antibodies. The presence of members of SFK was detected by western blot in immunocomplexes obtained with anti AT_2 antibody, by using a polyclonal antibody anti c-Src recommended for detection of SFK members. Rat hindbrain membrane preparations were stimulated with Ang II (10^{-7}M) in the presence or not of Losartan (10^{-6}M) or PD123319 (10^{-6}M), then solubilized and immunoprecipitated with anti AT_2 antibody. Fig. 3 shows that Ang II induced association of c-Src to AT_2 receptors. An excess of the AT_2 antagonist PD123319 prevents this association while Losartan did not altered recruitment of c-Src in the immunocomplexes (Fig. 3). This result confirms that c-Src or a member of the non-receptor tyrosine kinase family participated in the Ang II AT_2 receptor signaling pathway. Taken together with the Ang II-induced phosphorylation of SHP-1 shown in Fig. 2, we demonstrated that Ang II induced the formation of a complex between a SFK member, the AT_2 receptor and causes the phosphorylation/activation of the PTPase SHP-1.

3.4. c-Src participates in the activation of SHP-1 by AT_2 receptors

A close relationship between SHP-1 and c-Src has been previously proposed in different models (Somani et al., 1997; Frank et al., 2004; Roskoski, 2004). In rat fetal tissues, we demonstrated the participation of c-Src in the modulation of SHP-1 activity mediated by AT_2 receptor signaling (Alvarez et al., 2008). To evaluate this mechanism in the model of developing rat hindbrain, we searched for the presence of members of the SFK in SHP-1 immunocomplexes.

Following stimulation with Ang II (10^{-7} M, 5 min), immunocomplexes were obtained with antibodies against SHP-1 or AT_2 and the association between SHP-1 and c-Src was assayed (Fig. 4A and B). c-Src association in SHP-1 immunocomplexes increased significantly following stimulation with Ang II (Fig. 4A),

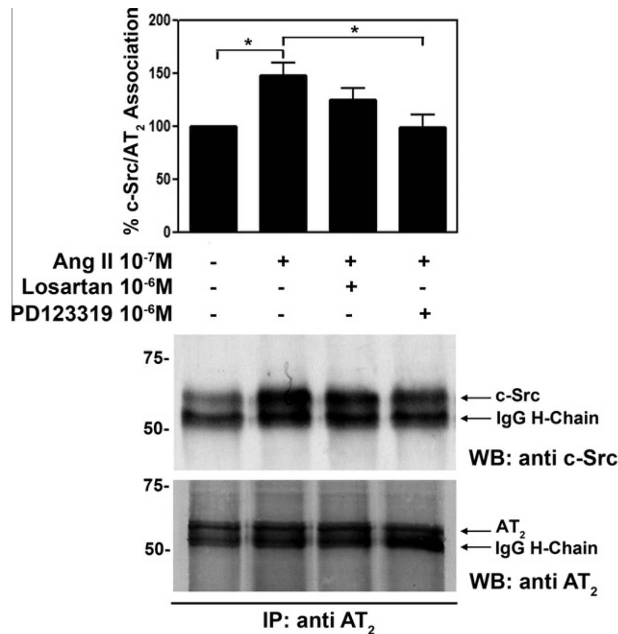

Fig. 3. Participation of c-Src in AT_2 signalling pathway. Co-immunoprecipitation of c-Src and AT_2 receptors. Rat hindbrain membranes (400–600 μg protein) were stimulated with Ang II (10^{-7}M, 5 min) in the presence or absence of Losartan (10^{-6}M, 10 min) or PD123319 (10^{-6}M, 10 min). Solubilized membranes were immunoprecipitated (IP) with anti AT_2 antibody and developed with polyclonal rabbit anti c-Src antibody (upper panel). Blots (WB) were reprobed with anti AT_2 antibody (lower panel) to confirm equal precipitation across all lanes. Representative image and histogram of densitometric data as mean ± SEM ($n = 6$) $^{*}P < 0.05$.

while preincubation with the AT_2 antagonist PD123319 (10^{-6}M) led to basal level this association. Moreover, the c-Src inhibitor PP2 (10 μM) led to basal level the association of SHP-1 to c-Src induced by Ang II, but the inactive analog PP3 has no effect on the increase caused by Ang II (Fig. 4A). Under baseline conditions there is an association between c-Src and SHP-1. Thus, the effect of c-Src inhibitors PP2 or PP3 alone was assayed (Fig. 4B). Under these conditions, PP2 alone was able to lower basal association. The use of *ex vivo* tissues in this study represents a physiological situation and thus high basal levels are expected. However, treatment with Ang II caused a significant increase in this association.

To demonstrate the participation of c-Src in SHP-1 activation induced by Ang II, we measured SHP-1 activity in AT_2 immunocomplexes. SHP-1 activity was induced (5.1-fold) by Ang II, an effect that was blocked by PD123319 (Fig. 4C) or the c-Src inhibitor PP2. These observations suggest that the presence of c-Src or a SFK member is required for SHP-1 activation (Fig. 4C). PP3 did not affect SHP-1 activity induced by Ang II. These results demonstrate that, following activation, AT_2 receptors recruit c-Src to cause SHP-1 tyr-phosphorylation and then its activation (Fig. 3A) mediated by AT_2 subtype in PND15 rat hindbrain.

3.5. SHP-1 tyr-phosphorylation requires recruitment of c-Src

Previously, we demonstrated the participation of a member of SFK in AT_2 signaling pathway in rat fetal tissues (Alvarez et al., 2008). To assay the possible participation of c-Src in SHP-1 activation in preparations from PND15 rat hindbrain, recruitment of c-Src in SHP-1 immunocomplexes was evaluated. The participation of c-Src in SHP-1 activation was corroborated by using the specific SFK inhibitor PP2 or its inactive analog PP3. Membranes were stimulated with Ang II (10^{-7}M) in the presence or not of PD123319 (10^{-6} M), PP2 (10 μM) or PP3 (10 μM), then solubilized and immunoprecipitated with anti SHP-1 antibody. SHP-1 activity assayed in

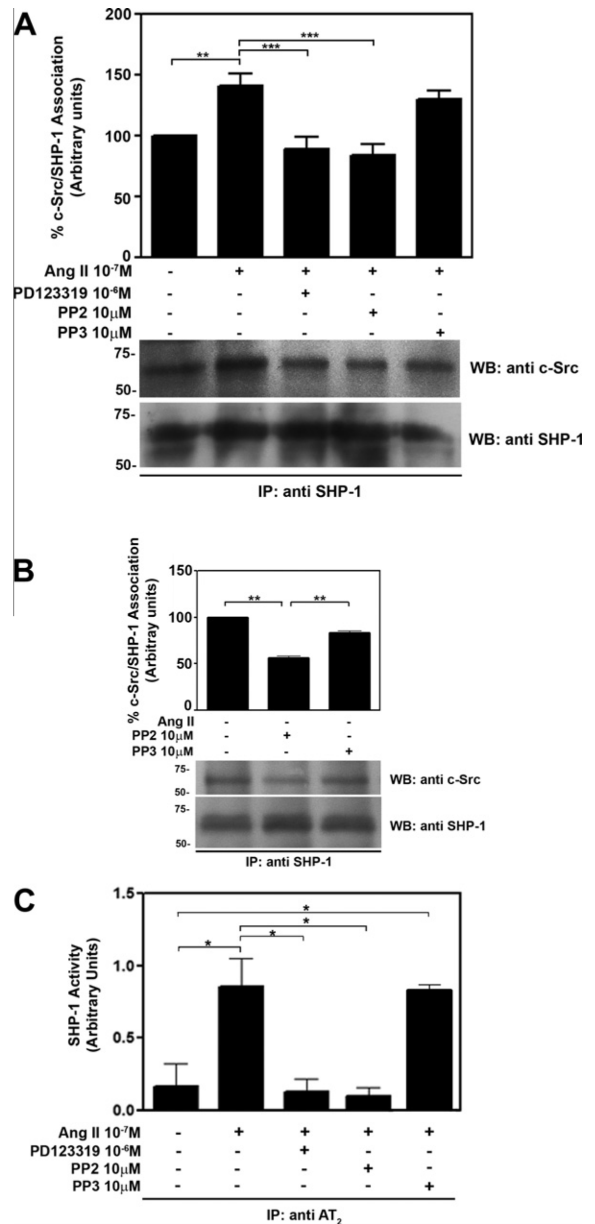

Fig. 4. Co-immunoprecipitation of SHP-1 and c-Src. A. c-Src was present in immunocomplexes obtained with anti SHP-1 antibody. Aliquots of membranes (400–600 μg protein) were pre-incubated with PD123319 (10^{-6}M, 10 min), PP2 (10 μM, 60 min) or PP3 (10 μM, 60 min) and then stimulated with Ang II (10^{-7}M, 5 min). Membranes were solubilized and immunoprecipitated (IP) with anti SHP-1 antibody. Western blots (WB) were performed using rabbit anti c-Src antibody or mouse anti SHP-1 antibody to demonstrate equal protein loading (representative image). Histogram showing the ratio of densitometric data as mean ± SEM ($n = 7$). $^{**}P < 0.01$, $^{***}P < 0.001$. B. PP2 reduces the basal association between SHP-1 and c-Src. Unstimulated membranes were immunoprecipitated (IP) with anti SHP-1 antibody and the western blot developed with polyclonal rabbit anti c-Src antibody (upper panel) o reprobed with mouse anti SHP-1 antibody (lower panel). C. Detection of SHP-1 activity in immunocomplexes obtained with anti AT_2 antibody. Aliquots of hindbrain membranes (400–600 μg protein) were stimulated with Ang II (10^{-7}M, 5 min) in the presence or not of PD123319 (10^{-6}M, 10 min), PP2 (10 μM, 60 min) or PP3 (10 μM, 60 min). Membranes were solubilized and immunoprecipitated (IP) with anti AT_2 antibody. Activity in immunocomplexes was determined by measuring the released orthophosphate (mean ± SEM, $n = 7$,$^{*}P < 0.05$).

immunocomplexes was selectively induced by Ang II AT_2 receptors since preincubation with PD123319 blocked SHP-1 activation. Besides, PP2 blocked SHP-1 activation. The specificity of PP2 inhibition was shown by using the inactive analog PP3 which did not interfere with Ang II-induced SHP-1 activation (Fig. 5A). After that,

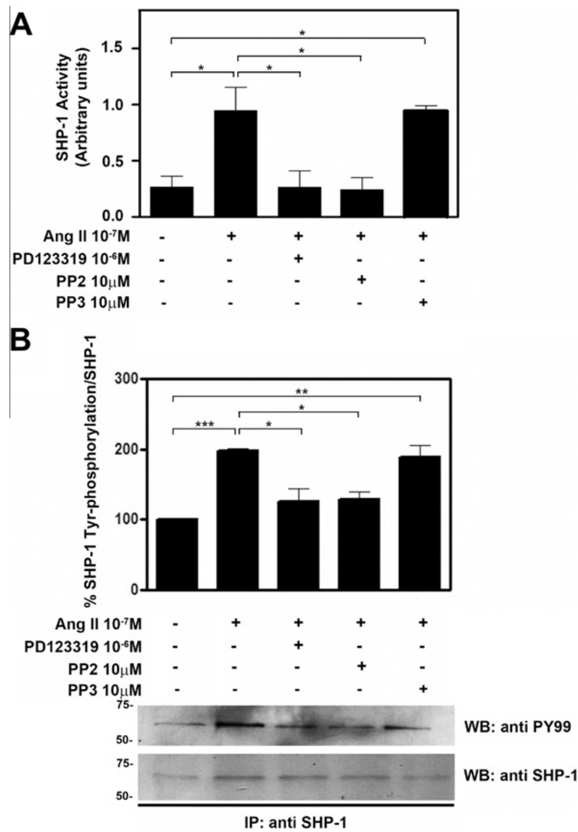

Fig. 5. SHP-1 activation is sensitive to PP2 inhibitor. A. Detection of SHP-1 activity in immunocomplexes obtained with anti SHP-1 antibody. Aliquots of membranes (400–600 μg protein) were pre-incubated with PD123319 (10^{-6}M, 10 min), PP2 (10 μM, 60 min) or PP3 (10 μM, 60 min) and then stimulated with Ang II (10^{-7} M, 5 min). Membranes were solubilized, immunoprecipitated and SHP-1 activity determined in immunocomplexes by measuring the released orthophosphate (mean ± SEM, $n = 5$,$*P < 0.05$). B. Level of tyr-phosphorylation of SHP-1 immunocomplexes. Immunocomplexes obtain in previous assay were centrifugated and blotted with anti PY99 antibody (upper panel) and anti SHP-1 antibody (lower panel) to demonstrate equal protein loading. Representative image and histogram of the ratio of densitometric data as mean ± SEM ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the immunocomplexes were recovered by centrifugation, separated by SDS–PAGE and blotted with anti PY99 antibody, stripped and reprobed with anti SHP-1 antibody. The tyr-phosphorylation level correlated with PTPase activity induced by Ang II. Preincubation with PD123319 or PP2 reverted tyr-phosphorylation of SHP-1 induced by Ang II (Fig. 5B). Altogether, these data confirm that c-Src is involved in SHP-1 activation, mediated by Ang II AT_2 receptors. The presence of SHP-1 (65 kDa), c-Src (60 kDa) and AT_2 receptor (66 kDa) in immnocomplexes were also verified by MALDI-TOF analysis (Supplementary table).

3.6. FAK participates in AT_2 immunocomplexes

It is well described the interaction between c-Src, the tyr-kinase FAK and others adaptor proteins in extracellular signal transduction (Mitra et al., 2006). To confirm the participation of FAK, membrane preparations were stimulated with Ang II (10^{-7}M, 5 min), solubilized and immunoprecipitated with antibody against AT_2 and the presence of FAK assayed by western blot. As control, we used hindbrain membrane preparations (PM) stimulated with Ang II (10^{-7} M) (Fig. 6A). Thus, we verified the presence of FAK (125 and 85 kDa) (Fig. 6A) in rat hindbrain. Surprisingly, immuno-

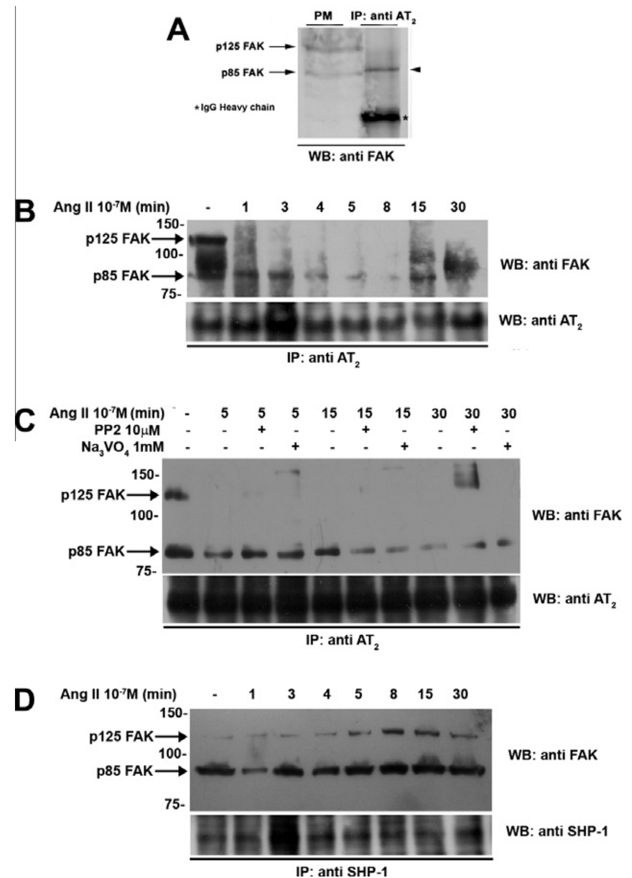

Fig. 6. p85FAK was present in immunocomplexes AT_2. A. Plasma membranes (PM) were stimulated with Ang II (10^{-7}M, 5 min) and then aliquots were used for western blots (WB) or immunoprecipitation (IP). Aliquots of PM were used for direct WB, and other aliquots were solubilized with Triton X-100 and immunoprecipitated with anti AT_2. Blot developed with anti FAK. p125FAK and p85FAK were present in the starting material (PM). AT_2 immunocomplexes contained the p85FAK fragment (arrowhead). B. Time-course of p85FAK association to AT_2 receptors. Following stimulation with Ang II (10^{-7}M) for different times, membranes were solubilized and immunoprepicitated with anti AT_2 antibody. The presence of FAK was assayed by western blot of the IP with anti AT_2 antibody. Blots were stripped and reprobed with anti- AT_2 antibody. Blots are representative of two independent assays. C. PP2 and orthovanadate affect the association of p85FAK to AT_2 receptors. Membranes were preincubated with PP2 (10 μM, 60 min) or orthovanadate Na_3VO_4 (1 mM, 10 min) before stimulation with Ang II (10^{-7}M) for 5, 15 or 30 min. The presence of FAK was revealed by western blot of the IP with anti AT_2 antibody. Blots were stripped and reprobed with anti-AT_2 antibody. Blots are representative of two independent assays. D. Time-course of p85 and p125FAK association to SHP-1. Following stimulation with Ang II (10^{-7}M) for different times, membrane were solubilized and immunoprepicitated with anti SHP-1antibody. The presence of FAK was assayed by western blot of the IP with anti $SHP\text{-}1_2$ antibody. Blots were stripped and reprobed with anti SHP-1 antibody. Blots are representative of two independent assays.

complexes obtained with anti AT_2 antibodies contained mainly the 85 kDa fragment of FAK (Fig. 6A) in AT_2-immunocomplexes.

To better understand the previous observation, we performed a time-course of the association of FAK to AT_2 receptor or SHP-1. Immunocomplexes obtained with anti-AT_2 antibody, showed the presence of p125FAK only in the control, unstimulated sample, while the p85 fragment associated to AT_2 at different times (Fig. 6B). A similar assay was performed at a few selected times in the presence of PP2 or orthovanadate (PTPase inhibitor) (Fig. 6C). The presence of the c-Src or PTPase inhibitors did not change the fact that only the p85 fragment associates to AT_2 receptor. However, maximal association seems to be at earlier times (1–4 min stimulation) (Fig. 6B). On the other hand, immunocomplexes obtained with anti SHP-1 evidence the presence of both, p85 and

p125 bands, while p85 was predominant. With respect to p125FAK, the association to SHP-1 increased between 8 and 15 min of stimulation, in correlation with lower SHP-1 phosphorylation (Fig. 6D). Wen et al. (1997) demonstrated the presence of a site sensitive to caspases in the full-length FAK C-terminal. The N-terminal cleaved fragment of FAK (85 kDa) contains an SH2 domain which accounts for recognition of c-Src, Fyn and other proteins (Wen et al., 1997). These results confirm the possible interactions between AT_2 signal transduction and the molecular machinery for cellular adhesion or migration in rat hindbrain.

4. Discussion

AT_2 receptors activate unconventional signaling pathways which in general do not involve a classical activation of a G-protein and its physiological role is still under discussion (Nouet et al., 2004; Porrello et al., 2009). Ang II AT_2 receptors show maximal expression in the cerebellum and brainstem from PND15 rats (Arce et al., 2001). By binding autoradiography and immunohistochemistry we showed a specific localization of Ang II AT_2 receptors in the Purkinje cell layer of the cerebellar cortex during development (Arce et al., 2011). Studies from knock-out mice reinforce the hypothesis that AT_2 receptors play a role in neuronal development. AT_2 knock-out mice suffer from perturbations in exploratory behavior and locomotor's activity (Hein et al., 1995; Ichiki et al., 1995), as well as anxiety-like behavior (Okuyama et al., 1999).

In the search of a potential role for Ang II AT_2 receptors, we selected PND15 rat hindbrain, which represent a physiological developmental condition, to investigate the transduction mechanism for AT_2 receptors. A number of evidences support the participation of SHP-1 in Ang II AT_2 receptors protein tyrosine dephosphorylation (Gallinat et al., 2000; Gendron et al., 2003). The present data confirm recruitment of c-Src and its participation in SHP-1 activation mediated by Ang II AT_2 receptors. Our results also indicate the recruitment of p85FAK in AT_2 immunocomplexes.

The participation of the PTPasa SHP-1 in protein tyr-dephosphorylation was evidenced by selective stimulation of Ang II AT_2 receptors which induced the association with SHP-1. We observed a marked increase in this association in response to Ang II. This observation opposed to those from different authors (Feng et al., 2002; Miura et al., 2005) who suggested a constitutive activation of AT_2 receptors and constitutive association to SHP-1. In our model, an *ex vivo* preparation which represents a physiological condition, we did not observe SHP-1 activation or association previous to stimulation by Ang II.

Stimulation of Ang II AT_2 receptors induced both a transient tyr-phosphorylation and activation of SHP-1 in PND15 rat hindbrain membrane preparations. The association between AT_2 receptors and SHP-1 activation was selectively blocked by the AT_2 antagonist PD123319. Tyr-phosphorylation of SHP-1 was maximal after 5 min stimulation and decreased to levels below to baseline at 8 min but recovered basal levels after 15 min stimulation.

Since AT_2 receptors belong to the superfamily of GPCR, we wonder for the mechanism involved in SHP-1 tyr-phosphorylation. Thus, we searched for the presence of c-Src in immunocomplexes obtained with anti AT_2 antibodies. Although a basal level of association between c-Src and SHP-1 was observed, Ang II induced a marked increase, selectively blocked by the AT_2 antagonist PD123319. In a previous study on rat fetal membrane preparation we showed the participation of c-Src in SHP-1 tyr-phosphorylation and its activation. The transduction mechanism of Ang II AT_2 receptors seems to vary between different cell lines and models where it was studied (Steckelings et al., 2005; Porrello et al., 2009). However, in these two models which represent a critical developmental stage, we observed a similar signaling pathway for Ang II AT_2 receptors.

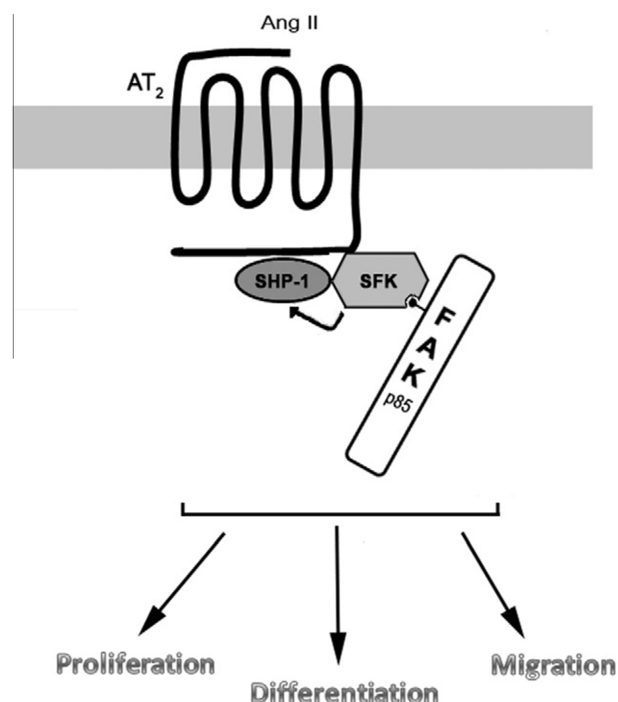

Fig. 7. Proposed model of AT_2 receptors signaling pathways. The figure summarizes the present findings on the mechanism used by Ang II AT_2 receptor. Stimulation of AT_2 receptors by Ang II leads to the formation of a complex AT_2/SHP-1/SFK/p85FAK. PP2 inhibitor blocks the association to AT_2 receptors and activation of SHP-1. p85FAK was also present in the complex. This FAK short-form is the result of caspase 3/6 cleavage. Blockade of Ang II AT_2 receptors during late pregnancy caused a serious disruption of the cerebellar cortex and lost of Ang II receptor expression in P0 animals (Sánchez et al., 2009). Previous data (Watanabe et al., 2008; Sánchez et al., 2009; Arce et al., 2011,) and the presence of FAK in AT_2 immunocomplexes in an *ex vivo* model suggest a potential participation of AT_2 receptor signaling in neuronal migration and proliferation/differentiation.

Participation of members of SFK in the transduction mechanism of AT_2 receptors has been recently shown in rat cerebellum (Clark et al., 2007) and neuronal cell lines (Guimond et al., 2010). Recently, Guimond et al. (2010) observed neurite outgrowth mediated by Ang II AT_2 receptors in NG108-15 cells, with participation of Fyn, member of the SFK. By using co-immunoprecipitation assays and activation of SHP-1, we demonstrate the formation of a tertiary complex, containing AT_2/SHP-1/c-Src, following stimulation with Ang II. AT_2 receptors recruit c-Src and SHP-1, being c-Src responsible for SHP-1 tyr-phosphorylation and its activation. Thus, a member of SFK was required in order to produce SHP-1 activation.

Blockade of Ang II AT_2 receptors during late pregnancy (gestational days 13–21) caused a serious disruption of the cerebellar cortex structure and a lost of Ang II receptor expression in PND0 animals born from PD123319-treated mothers (Sánchez et al., 2009). We also showed a specific localization of Ang II AT_2 receptors at the somata of Purkinje cells (Arce et al., 2011). Since treatment was performed during late pregnancy, a critical stage during Purkinje cells migration, we can speculate that blockade of AT_2 receptors prevents cell migration or differentiation. These observations are in agreement with previous studies about the potential role of Ang II AT_2 receptors in cell migration (Kilian et al., 2008; Otis et al., 2008). FAK is a large adaptor protein carrying binding sites for many proteins, including c-Src, RhoGTPases regulators and cytoskeletal proteins (Watanabe et al., 2008; Shani et al., 2009). It has been shown recently that FAK knock-out mice suffer from a serious damage on cerebellar foliation, Bergmann glia posi-

tioning and climbing fiber territory on Purkinje cells (Watanabe et al., 2008). Thus, we explored the presence of FAK in AT_2-immunocomplexes obtained in PND15 rat hindbrain. To our surprise, we found only the 85 kDa fragment in immunocomplexes. Cleavage of FAK by caspases 3 and 6 results in an 85 kDa fragment that has been related to apoptosis (Wen et al., 1997; Mian et al., 2008). A time-course of stimulation with Ang II showed association of AT_2 receptors to p85 as earlier as 1 min after stimulation, while the p125 protein associated to inactive SHP-1 after 8–15 min stimulation. Our present results confirm the possible interactions between AT_2 signal transduction and the molecular machinery for cellular focal adhesion in rat hindbrain. The association between SHP-1 and FAK might constitute the first evidence which supports an interaction between these proteins. FAK cleavage was almost completely prevented by the c-Src inhibitor PP2 or PTPase inhibitor orthovanadate. Altogether, these observations suggest a more complicated role of FAK in the signal transduction studied. Fig. 7 summarizes the experimental data obtained in the present study.

In the present study we provide evidences for an inducible signal transduction mechanism for Ang II AT_2 receptors in PND15 rat hindbrain, a developmental stage critical for cerebellar development. Here, we demonstrated that Ang II stimulation induces the formation of a complex containing AT_2/SHP-1/c-Src/p85FAK. Taken together, the present results and our previous data support a potential role of Ang II AT_2 receptors in cerebellar development and neuronal differentiation.

Acknowledgements

We wish to thank Dr. J.M. Saavedra and Dr. R. Smith for reagent contributions. G.M. Ciuffo is a member of the researcher's career of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina). Seguin LR and Villarreal RS have fellowships from CONICET, Argentina. This work was supported by grants from CONICET, PICT 01-9759 (ANPCYT) and Universidad Nacional de San Luis, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2011.11.008.

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