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Angiotensin II phosphorylation of extracellular signal-regulated kinases in rat anterior pituitary cells

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Suárez, Cecilia, Graciela Díaz-Torga, Arturo Gonzalez-Iglesias, Jorge Vela, Alejandro Mladovan, Alberto Baldi, and Damasia Becu-Villalobos. Angiotensin II phosphorylation of extracellular signal-regulated kinases in rat anterior pituitary cells. *Am J Physiol Endocrinol Metab* 285: E645–E653, 2003. First published May 20, 2003; 10.1152/ajpendo.00015.2003.—We studied the effects of ANG II on extracellular signal-regulated kinase (ERK)1/2 phosphorylation in rat pituitary cells. ANG II increased ERK phosphorylation in a time- and concentration-dependent way. Maximum effect was obtained at 5 min at a concentration of 10–100 nM. The effect of 100 nM ANG II was blocked by the AT_1 antagonist DUP-753, by the phospholipase C (PLC) inhibitor U-73122, and by the MAPK kinase (MEK) antagonist PD-98059. The ANG II-induced increase in phosphorylated (p)ERK was insensitive to pertussis toxin blockade and PKC depletion or inhibition. The effect was also abrogated by chelating intracellular calcium with BAPTA-AM or TMB-8 by depleting intracellular calcium stores with a 30-min pretreatment with EGTA and by pretreatment with herbimycin A and PP1, two c-Src tyrosine kinase inhibitors. It was attenuated by AG-1478, an inhibitor of epidermal growth factor receptor (EGFR) activation. Therefore, in the rat pituitary, the increase of pERK is a G_q- and PLC-dependent process, which involves an increase in intracellular calcium and activation of a c-Src tyrosine kinase, transactivation of the EGFR, and the activation of MEK. Finally, the response of ERK activation by ANG II is altered in hyperplastic pituitary cells, in which calcium mobilization evoked by ANG II is also modified.

calcium; phospholipase C; protein kinase C; estrogen; mitogen-activated protein kinase; epidermal growth factor receptor

IT HAS BEEN DESCRIBED that all the components of the renin-angiotensin II system (RAS) are present in the pituitary, where angiotensin II (ANG II) is produced locally (5) and principally synthesized in gonadotropes (6). Two ANG II receptor subtypes, AT_1 and AT_2, first distinguished on a pharmacological basis, have been identified by expression cloning from various species, and the main biological functions exerted by ANG II are mediated by the AT_1 receptor subtype. The rat anterior pituitary predominantly expresses the AT_{1B} isoform of the AT_1 receptor (18, 21, 33), with a low expression of the AT_{1A} isoform and the AT_2 receptor subtype (33, 36). AT_{1B} receptors are found mainly in lactotropes and to a lesser extent in corticotropes and thyrotropes (21).

AT_1 receptors belong to the family of G protein-coupled receptors (GPCR). In the rat pituitary, activation of AT_{1B} receptors coupled to a $G_{q/11}$ protein increases phospholipase C-β (PLCβ) activity, resulting in inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) formation, followed by a biphasic increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Coupling of the AT_1 receptor to a G_i protein has also been described (11, 29). In both central and peripheral cells, AT_1 receptors also mediate ANG II-stimulated increase in the expression of protooncogenes (9, 11, 28, 38, 45) and stimulation of the JAK/STAT pathway, which has originally been associated with growth factors and cytokines. In addition, mitogen-activated protein kinases (MAPKs), which are critical components in cellular processes such as growth, differentiation, and apoptosis, are activated by ANG II binding to AT_1 receptors in various cell types (9, 16, 27, 42).

MAPKs, also termed extracellular signal-regulated kinases or ERKs, belong to a family of protein serine/threonine kinases that are believed to function as integrators of mitogenic signals originating from several distinct classes of cell surface receptors, mainly receptor tyrosine kinases (RTKs) but also GPCRs. In response to an extracellular stimulus, their activated forms, $p42^{MAPK}$ (pERK2) and $p44^{MAPK}$ (pERK1), are generated by phosphorylation of specific threonine and tyrosine residues catalyzed by an MAPK kinase family member, also known as MAPKK or MEK. The cascade from growth factor RTKs to ERK has been elucidated (23); however, the ERK activation pathway originating from GPCRs is beginning to unfold. It has been described that ANG II stimulates ERKs in hepatic cells (35), neurons (16), vascular smooth muscle cells (9), cardiac fibroblasts (27), bovine adrenal glomerulosa cells (42), and several other cell types, but no description of activation of ERKs by ANG II in pituitary cells has been documented.

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We have therefore investigated the effects of ANG II on ERK activation, as well as the signal transduction cascades involved, in rat pituitary cells. Furthermore, because we have previously described that ANG II response is greatly modified in estrogen-induced pituitary hyperplasia (7, 12, 13), we compared results in control and in hyperplastic pituitary cells.

MATERIALS AND METHODS

Materials. DUP-753 was a generous gift from DuPont Merck Pharmaceutical (Wilmington, DE), and the AT_2 antagonist PD-123319 was a gift from Parke-Davis (Ann Arbor, MI). Chelerytrine was from Alomone Labs (Jerusalem, Israel), and tyrphostin AG-1478 and PP1 were from Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Animals. Female regularly cycling 60-day-old Sprague-Dawley rats were housed in an air-conditioned room with lights on at 0700 and off at 1900. They had free access to laboratory chow and tap water. Rats were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Rats in diestrus were used as the control group.

Pituitary hyperplasia was developed by subcutaneous implantation of a 20-mg diethylstilbestrol pellet; rats were killed after 8 wk of treatment.

Cell culture. Cell dispersion and culture were performed as previously described (13). After 4 days in culture, cells were washed twice with DMEM-F12 [Dulbecco's modified Eagle's medium with F-12 Nutrient Mixture (GIBCO), supplemented with 1% BSA, 2 mM glutamine, 25,000 U/l nystatin, and 25 ng/l gentamicin] to remove all traces of serum. Fresh medium (without serum and with the stimuli that required a 24-h preincubation when necessary) was added, and cells were incubated at 37°C for 24 h before stimulation with appropriate drugs.

Immunoblotting. Pituitary cells (500,000 cells/well) were then stimulated with agonists at 37°C in serum-free DMEM for the indicated times. The reaction was terminated by the replacement of the medium with 60 μl of 62.5 mM Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue. After 5 s of sonication, samples were boiled for 5 min at 95°C, and 20 μl were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane (Bio-Rad, Buenos Aires, Argentina). Membranes were probed with mouse polyclonal phosphospecific ERK antibody (1:1,000, Santa Cruz Biotechnologies, Santa Cruz, CA). After incubation with secondary anti-mouse antibody conjugated with horseradish peroxidase (1:2,500, Santa Cruz Biotechnologies), immunoreactive proteins were detected by enhanced chemiluminescence. Membranes were stripped in 62.5 mM Tris, 2% SDS, and 100 mM mercaptoethanol, pH 6.7, for 40 min at 50°C and then incubated with rabbit polyclonal anti-ERK1 antibody (1:1,200, Santa Cruz Biotechnologies) and then with antirabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnologies) and revealed as described. Bands were quantitated using the ImageQuant software.

Intracellular calcium measurement. Measurements were made as previously described (14).

Statistical analysis. Results are expressed as means ± SE. pERK1 and pERK2 band intensity was normalized in all cases to total ERK1, and the pERK/ERK1 ratio is presented. For those figures that include pretreatment and acute treatment, two-way ANOVA for repeated measures for the effects of pretreatment and treatment was performed. In all cases, if F of interaction was found significant, individual means were compared by Tukey's honestly significant difference or Fisher's protected least significant difference tests; if it was not significant, groups of means were analyzed by the same tests. Time course and concentration dependence of ERK phosphorylation were analyzed by one-way ANOVA for repeated measures. $P < 0.05$ was considered significant.

RESULTS

ANG II type 1 receptor stimulation leads to ERK phosphorylation in pituitary cells. In cultured anterior pituitary cells, ANG II (100 nM) induced maximal ERK phosphorylation at 5 min, and the response declined at 10–15 min (Fig. 1*A*). ERK phosphorylation was dependent on the concentration of the agonist (Fig. 1*B*). Increased phosphorylation was detectable at 0.1 nM, half-maximal at ~1.6 nM, and maximal at 10–100 nM. Therefore, subsequent assays on ANG II-induced ERK phosphorylation were performed with 100 nM ANG II stimulation for 5 min. To determine which receptor subtype mediates pERK increase, pituitary cells were pretreated for 30 min with either the AT_1 antagonist DUP-753 (losartan) or the AT_2 antagonist PD-123319. ERK phosphorylation by ANG II was abolished by 10 μM DUP-753, whereas the same concentration of PD-123319 did not alter the effect of the octapeptide (Fig. 2*A*), indicating that ANG II-induced ERK phosphorylation is mediated mainly by the AT_1 receptor in pituitary cells.

Pituitary cells also express RTKs linked to growth factors. Thus addition of epidermal growth factor (EGF; 10 ng/ml) for 5 min caused a marked increase in ERK1/2 phosphorylation (Fig. 2*B*). Moreover, the MEK1 kinase inhibitor PD-98059 (50 μM) abolished ERK phosphorylation induced by both ANG II (100 nM) and EGF ($P = 0.0041$ and 0.016, respectively; Fig. 2*B*).

ANG II-induced ERK phosphorylation requires PLC activation through a pertussis toxin-insensitive G protein. It has been reported that AT_1 receptors can be coupled to either G_q or G_i proteins, which activate PLCβ or inhibit adenylate cyclase, respectively. To determine which G protein-mediated signaling is involved in ERK phosphorylation by ANG II in pituitary cells, the effects of pretreatment with pertussis toxin (PTX) and with U-73122, a specific PLCβ inhibitor, were studied. Pretreatment with PTX (150 ng/ml) for 24 h had no effect on the response evoked by ANG II [Fig. 3*A*; effect of ANG II vs. basal after buffer or PTX pretreatment: $P = 0.0023$, F of interaction = 0.089 not significant (NS)]. In contrast, pretreatment with 10 μM U-73122 reduced ANG II-induced ERK phosphorylation (effect of ANG II vs. basal after buffer or U-73122 pretreatment: $P = 0.0044$ and 0.15, respectively; Fig. 3*B*). U-73122 (10 μM) induced a significant increase in $[Ca^{2+}]_i$, evidenced 5 s after the stimulus, with a maximum response at 45 s (Fig. 3*C*). When 100 nM ANG II was added 7 min after U-73122, there was no response to ANG II (compared with buffer-pretreated cells). U-73122 also blocked the K^+ (25 mM)-induced $[Ca^{2+}]_i$ increase.

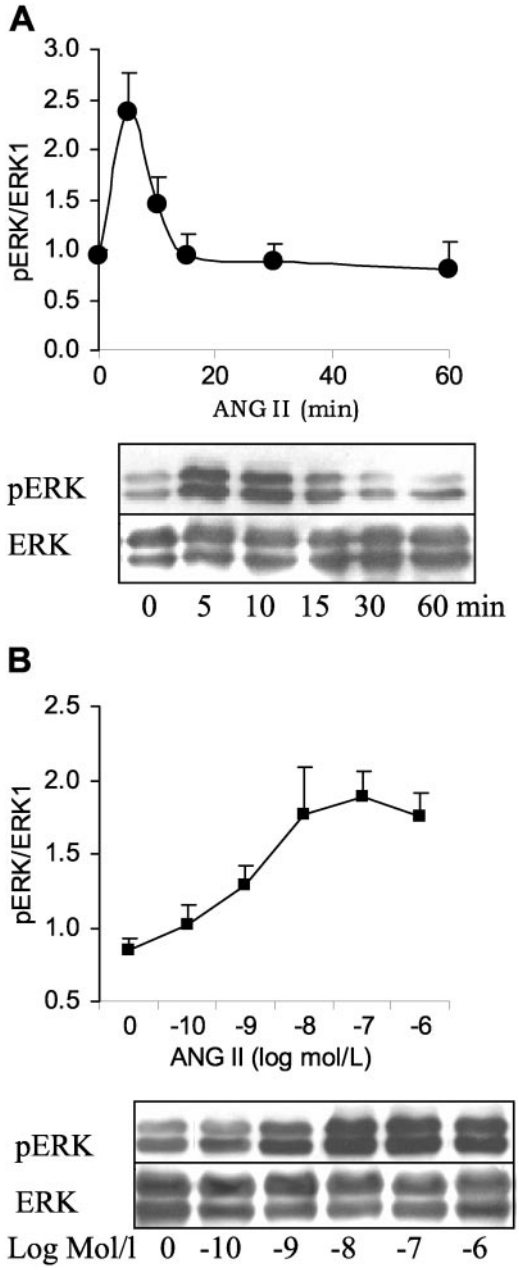

Fig. 1. ANG II stimulates phosphorylation (p) of extracellular signal-regulated kinase (ERK)1/2 in cultured rat pituitary cells. *A*: pituitary cells were stimulated with ANG II (100 nM) for the indicated time (min); $n = 7$ independent experiments. For this and following figures, results shown are means ± SE. *Bottom*: representative Western blot. *B*: pituitary cells were stimulated with ANG II at the indicated concentrations for 5 min, $n = 6$. *Bottom*: representative Western blot.

Role of PKC in ANG II-induced ERK phosphorylation. In cultured pituitary cells, PLCβ activation by ANG II leads to production of two second messengers, IP_3 and DAG, which induce the release of Ca^{2+} from intracellular stores and PKC activation, respectively. Because PKC activation by a phorbol ester has been reported to stimulate MAPK in other cells (9), we examined whether phorbol ester-sensitive PKC is essential for ANG II-induced ERK phosphorylation in pituitary cells. Depletion of PKC by a 24-h pretreatment with 1 μM phorbol 12-myristate 13-acetate (PMA 24) moderately decreased basal ERK phosphorylation ($P = 0.32$) and completely inhibited ERK phosphorylation induced by a 10-min stimulation with 1 μM PMA (PMA 10; Fig. 4*A*), confirming the completeness of the PKC depletion. However, ANG II-induced ERK phosphorylation was not inhibited by PMA 24 pretreatment ($P = 0.027$ and 0.0027 with and without pretreatment, respectively), suggesting a dominant role of a PKC-independent mechanism in ANG II-induced ERK phosphorylation in pituitary cells. This was confirmed using the specific PKC inhibitor chelerythrine (15). A

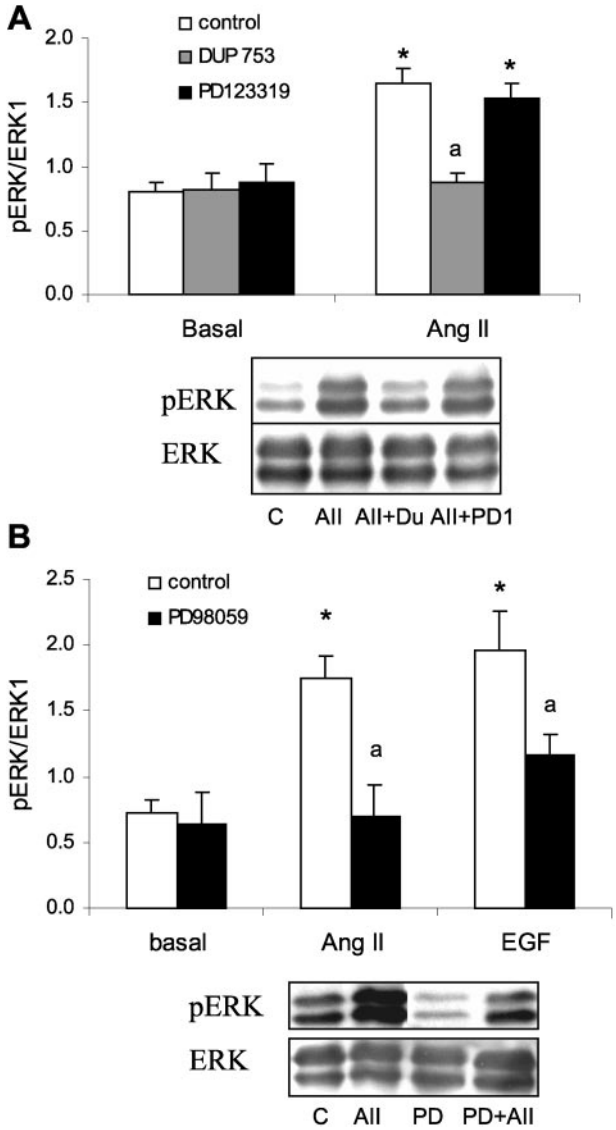

Fig. 2. *A*: pituitary cells were pretreated with buffer (control), the AT_1 antagonist DUP-753 (DU, losartan; 10 μM), or the AT_2 antagonist PD-123319 (PD1, 10 μM) for 30 min and stimulated with buffer (basal) or 100 nM ANG II (AII) for 5 min, $n = 7$. *Bottom*: representative Western blot. *B*: effect of the MAPK kinase (MEK) inhibitor PD-98059 (PD, 50 μM, 1 h) on ANG II (100 nM, 5 min) or epidermal growth factor (EGF; 10 ng/ml, 5 min)-induced phosphorylation of ERK, $n = 7$. $^*P < 0.05$ vs. respective basal, $^aP < 0.05$ vs. ANG II control. *Bottom*: representative Western blot.

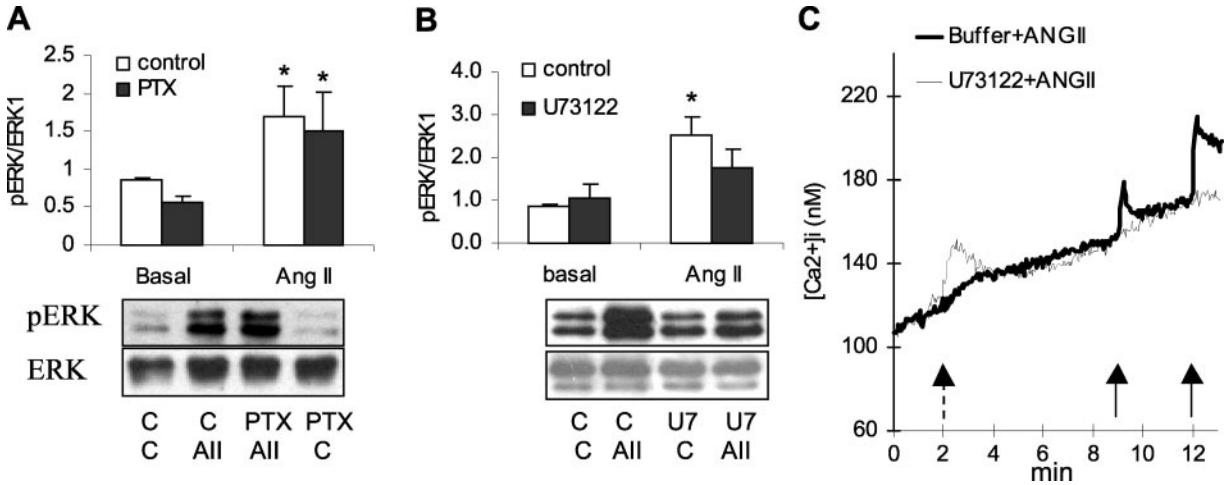

Fig. 3. A: pituitary cells were pretreated with 150 ng/ml pertussis toxin (PTX) or buffer (control) for 24 h and then stimulated with 100 nM ANG II for 5 min, $n = 6$. *Bottom*: representative Western blot. *B*: pituitary cells were pretreated with buffer (control) or the phospholipase C inhibitor U-73122 (U7; 10 μM) for 30 min and stimulated with 100 nM ANG II for 5 min, $n = 4$. *$P < 0.05$ vs. respective basal. *Bottom*: representative Western blot. *C*: effect of U-73122 (10 μM) on ANG II (100 nM)-induced increase in $[Ca^{2+}]_i$ (nM). First arrow indicates U-73122 or buffer administration, second arrow corresponds to ANG II, and third arrow to KCl (25 mM).

30-min pretreatment with 1 μM chelerythrine did not modify ERK phosphorylation induced by 100 nM ANG II (effect of ANG II vs. basal after buffer or chelerythrine pretreatment: $P = 0.00076$, F of interaction = 0.35, NS; Fig. 4*B*).

Calcium-dependent ERK phosphorylation by ANG II. In pituitary cells, ANG II causes a rapid and transient elevation of cytosolic Ca^{2+} released from the IP_3-sensitive intracellular stores (7). This is followed by a sustained elevation of $[Ca^{2+}]_i$ through voltage-sensitive calcium channel-mediated Ca^{2+} influx. Because it has been described that intracellular Ca^{2+} elevation is a sufficient stimulus for ERK activation in other cell types (2), we sought to determine whether ERK activation by ANG II was Ca^{2+} dependent in pituitary cells. As expected, cells pretreated for 10 min with 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM; 10 μM) or 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8, 100 μM; ICN Pharmaceuticals, Irvine, CA), two intracellular Ca^{2+} chelators, showed a reduction in the $[Ca^{2+}]_i$ spike increase in response to ANG II (Fig. 5, *A* and *B*). Both chelators also reduced the pERK increase induced by the octapeptide (Fig. 5*C*) (effect of ANG II vs. basal in buffer or BAPTA-pretreated cells: $P = 0.008$ and 0.16, respectively; in buffer or TMB-8-pretreated cells: $P = 0.00022$ and 0.10, respectively). In contrast, acute (1-min) extracellular Ca^{2+} chelation with 5 mM EGTA lowered basal $[Ca^{2+}]_i$ but did not prevent the spike increase in $[Ca^{2+}]_i$ induced by ANG II (Fig. 6*A*) or ANG II-induced phosphorylation of ERK [$P = 0.0016$, F interaction (2,12) = 0.29, NS; Fig. 6*C*].

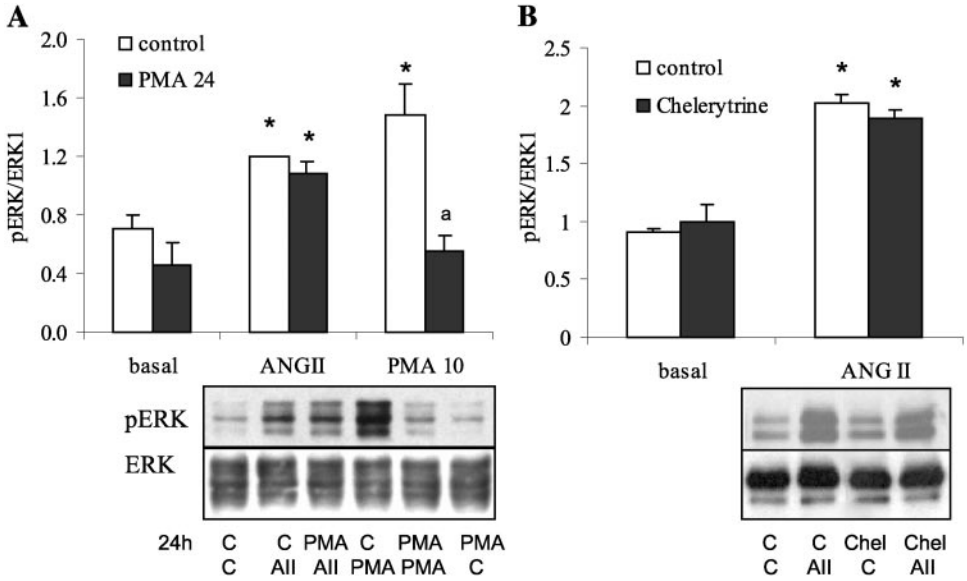

Fig. 4. A: pituitary cells were pretreated with 1 μM phorbol 12-myristate 13-acetate (PMA) or buffer (control) for 24 h (PMA 24) and stimulated with buffer (basal), 100 nM ANG II, or 1 μM PMA for 10 min (PMA 10). *$P < 0.05$ vs. respective basal, [a]$P < 0.05$ vs. PMA 10 control; $n = 4$. *Bottom*: representative Western blot. *B*: pituitary cells were pretreated with buffer (control) or the PKC inhibitor chelerythrine (Chel, 1 μM) for 30 min and stimulated with 100 nM ANG II for 5 min. *$P < 0.05$ vs. respective basal; $n = 3$. *Bottom*: representative Western blot.

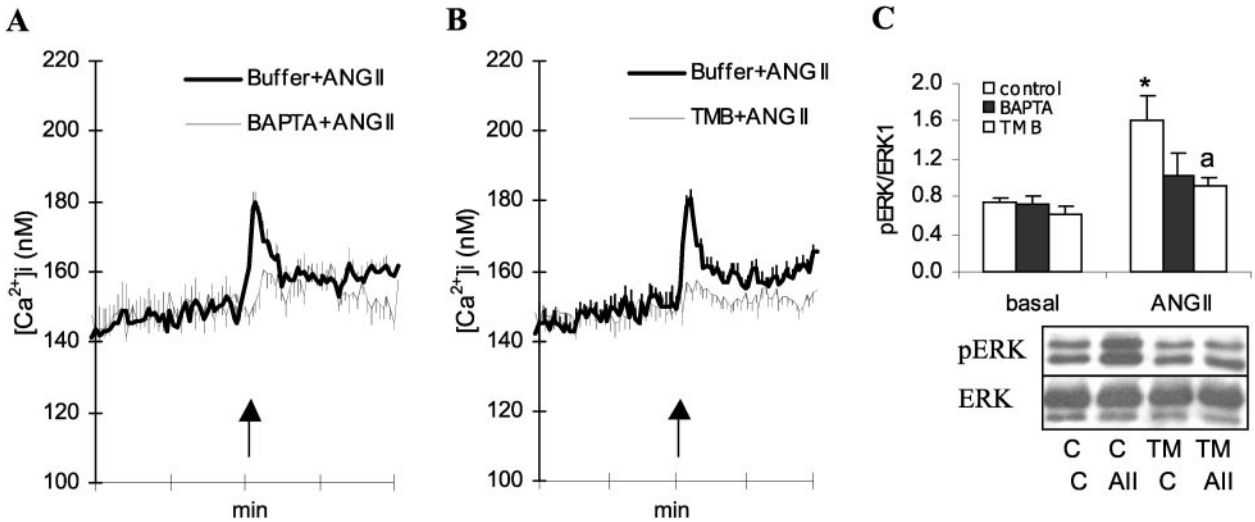

Fig. 5. $[Ca^{2+}]_i$ (nM) in cells pretreated with 10 μM 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) or buffer (n = 6; *A*), or with 100 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8, TM) or buffer (n = 8; *B*) for 10 min and then challenged with 100 nM ANG II (arrow). *C*: effect of 30-min pretreatment with BAPTA-AM or TMB-8 on ERK phosphorylation induced by 100 nM ANG II, 5 min. *$P < 0.05$ vs. respective basal, [a]$P < 0.05$ vs. ANG II control; n = 8. *Bottom*: representative Western blot.

If EGTA pretreatment lasted for 10 min (or more), basal $[Ca^{2+}]_i$ also decreased and 100 nM ANG II could not evoke a consistent Ca^{2+} response (Fig. 6*B*), indicating that intracellular Ca^{2+} stores had been depleted. Consequently, 100 nM ANG II failed to increase ERK phosphorylation (effect of ANG II vs. basal, with buffer or 30-min EGTA pretreatment: P = 0.0021 and 0.80, respectively; Fig. 6*C*).

Role of Src-tyrosine kinase and EGFR activation on ERK phosphorylation. ANG II has been shown to cause a rapid increase in tyrosine phosphorylation of multiple cellular proteins before ERK activation in different systems. We therefore examined the role of c-Src kinases in ANG II-induced ERK activation.

Herbimycin A, a c-Src tyrosine kinase inhibitor, reduced the phosphorylation of ERK1/2 induced by ANG II (Fig. 7*A*) (effect of ANG II vs. basal in buffer and in herbimycin A-pretreated cells: P = 0.016 and 0.56, respectively). To confirm this effect, we tested a more selective inhibitor of the Src-family tyrosine kinases, PP1 (10 μM). In this case, a complete inhibition of ANG II-induced ERK1/2 phosphorylation was evidenced

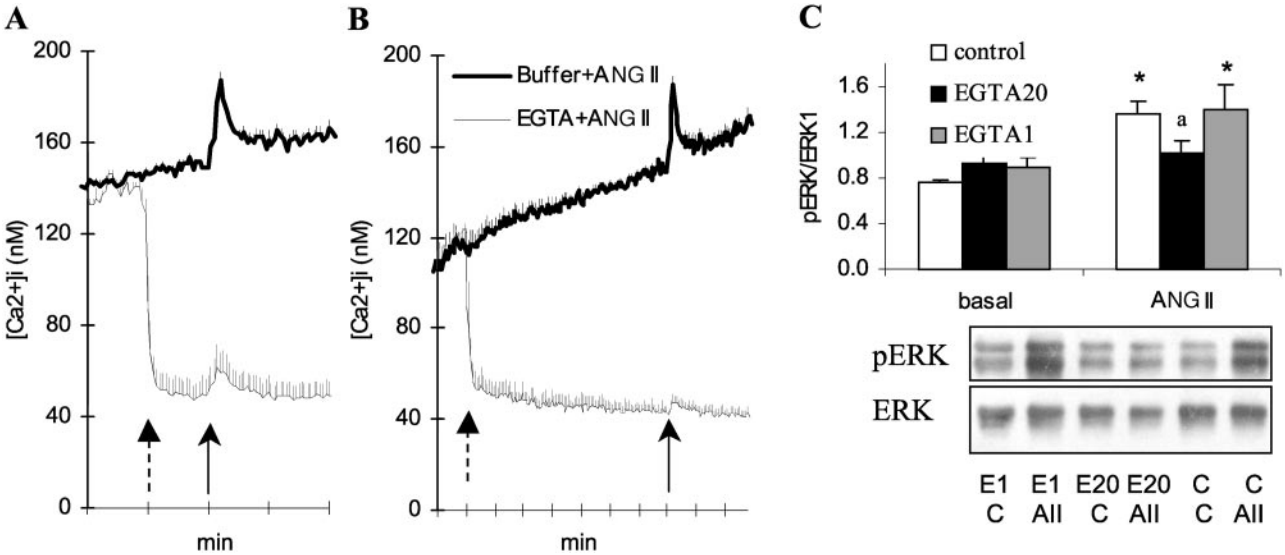

Fig. 6. $[Ca^{2+}]_i$ (nM) in pituitary cells pretreated with EGTA (5 mM, dotted arrow) for 1 min (*A*) or 7 min (*B*) and then challenged with 100 nM ANG II (2nd arrow). Tics on *x*-axis indicate 1 min; n = 7. *C*: cells were pretreated for 1 min (EGTA 1, E1; n = 5) or 20 min (EGTA 20, E20; n = 6) with 5 mM EGTA and then challenged with 100 nM ANG II, and pERK was measured. *$P < 0.05$ vs. respective basal, [a]$P < 0.05$ vs. ANG II control. *Bottom*: representative Western blot.

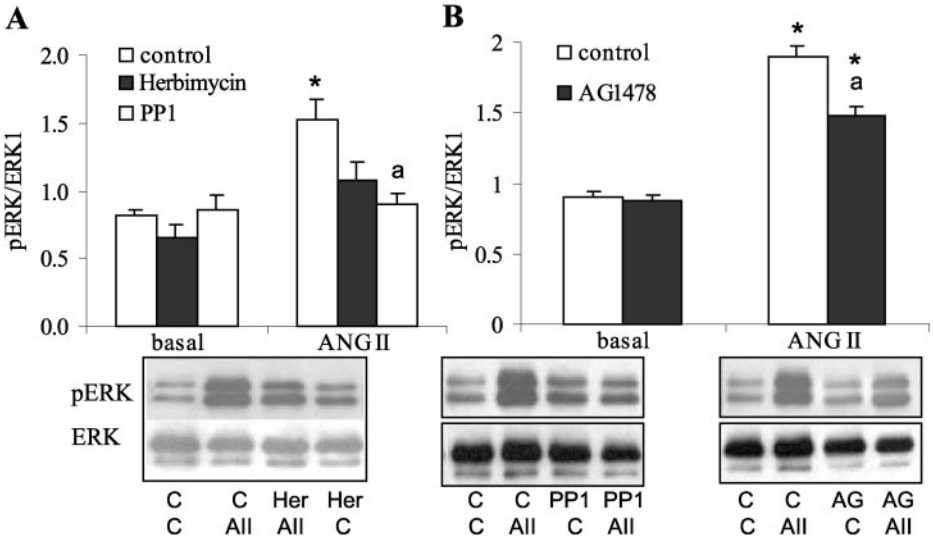

Fig. 7. *A*: effect of a 24-h pretreatment with buffer (control), 1 μM herbimycin A (Her; $n = 9$), or 10 μM PP1 ($n = 3$) on 100 nM ANG II-induced ERK phosphorylation. $^{*}P < 0.05$ vs. respective basal, $^{a}P < 0.05$ vs. ANG II control. *B*: pituitary cells were pretreated with buffer (control) or the EGF receptor inhibitor AG-1478 (AG; 400 nM) for 30 min and stimulated with 100 nM ANG II for 5 min. $^{*}P < 0.05$ vs. respective basal, $^{a}P < 0.05$ vs. ANG II control; $n = 4$. *Bottom*: representative Western blots.

(Fig. 7*A*) (effect of ANG II vs. basal in buffer or PP1-pretreated cells: $P = 0.00098$ and 0.98, respectively), suggesting that c-Src phosphorylation has an important role in the activation of ERK1/2 in pituitary cells.

It is well established that transactivation of the EGFR contributes to GPCR-mediated ERK1/2 activation in certain cell types. Pituitary cells express EGFR, and, as we showed, EGF stimulation caused a marked increase in ERK1/2 phosphorylation. To examine the involvement of the EGFR in ANG II-induced ERK1/2 activation, cells were pretreated with the selective EGFR kinase inhibitor AG-1478. AG-1478 at 400 nM attenuated ANG II (100 nM)-induced ERK1/2 phosphorylation (Fig. 7*B*) (effect of ANG II in buffer-pretreated compared with AG-1478-pretreated cells: $P = 0.010$), indicating its dependence on transactivation of the EGFR. AG-1478 at 400 nM blocked EGF (10 ng/ml)-induced ERK1/2 phosphorylation (data not shown).

ANG II-induced ERK phosphorylation and $[Ca^{2+}]_i$ mobilization in cells from hyperplastic pituitaries. In cells from estrogen-induced pituitary hyperplasia, the profile of pERK increase in response to ANG II (100 nM) was different from that of control cells (Fig. 8*A*). Maximum increase was lower and less abrupt. The increase was evidenced at 5 min, peaked at 10 min, and was still significant at 15 min. In correlation, $[Ca^{2+}]_i$ mobilization evoked by ANG II (100 nM) in hyperplastic cells did not present an early peak phase but rather a prolonged plateau phase (Fig. 8*B*).

DISCUSSION

Activation of ERK by ANG II has been previously demonstrated in several cell types including vascular smooth muscle cells (VSMCs) (9), cardiac fibroblasts (27), bovine adrenal fasciculata/reticularis cells (42), and Chinese hamster ovary (CHO) cells stably transfected with the human AT_1 receptor (1). However, this is the first description of ANG II-induced ERK phosphorylation in pituitary cells. Studies from numerous laboratories have shown that ANG II affects pituitary secretion, and pituitary ANG II receptor number varies in diverse physiological conditions (34). Furthermore, ANG II may modulate estrogen-induced vascular changes (30) and proliferation (19) of pituitary cells.

Signaling mechanisms in ANG II-induced ERK phosphorylation in pituitary cells exhibited similarities and differences with endogenously expressed or

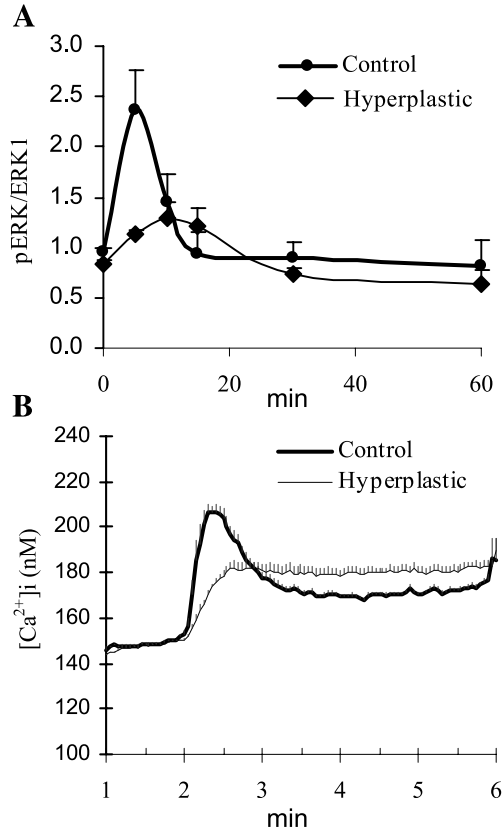

Fig. 8. *A*: time course of 100 nM ANG II-induced ERK phosphorylation in control and hyperplastic pituitary cells; $n = 6$. *B*: mobilization of $[Ca^{2+}]_i$ (nM) in response to 100 nM ANG II applied in *minute 2* in control and hyperplastic pituitary cells; $n = 7$.

transfected AT_1 receptors in diverse cell types. Therefore, ERK activation by ANG II may be a cell-specific phenomenon, and discrepant results may be related to different amounts of expressed receptors or differential cell expression of elements involved in the mechanism of agonist-induced ERK activation.

In pituitary cells, the ANG II-induced increase in pERK was a concentration-dependent process that reached an apparent maximum after 5 min of stimulation with 10–100 nM of the octapeptide. The estimated half-maximal effective concentration (EC_{50}, 1.59 nM) was in the range of the K_d for the AT_1 receptor in the pituitary (3). Fifteen minutes after ANG II stimulation, ERK phosphorylation returned to basal levels. This pattern of response is similar to that found in VSMCs (9) and cardiac fibroblasts (27). On the other hand, in bovine adrenal glomerulosa (BAG) cells, the response returned to basal levels at 60 min, and maximal phosphorylation was elicited by concentrations as low as 500 pM (42). Moreover, in CHO fibroblasts overexpressing the rat AT_{1A} receptor, ANG II induced a biphasic elevation of ERK activity (44).

The effect of ANG II was blocked by the AT_1 receptor antagonist losartan and not by the AT_2 antagonist PD-123319, indicating that ANG II-induced ERK phosphorylation is mediated by the AT_1 receptor in pituitary cells. Similar results have been described in most tissues studied, with the exception of neonatal neurons, in which AT_1 and AT_2 receptors have opposite actions on ERK activation (16).

The cloned AT_1 receptor can be coupled to either G_q or G_i proteins (29). ANG II-induced ERK phosphorylation was PTX insensitive in pituitary cells, and it was suppressed by the PLCβ inhibitor U-73122. Taken together, these observations indicate that, in pituitary cells, the AT_1 receptor transduces its signal through a PTX-insensitive G_q protein as it has been described in VSMCs (9, 42), rather than through a G_i protein as was proposed for hepatic C9 cells (35). U-73122 also prevented an ANG II-induced spike in $[Ca^{2+}]_i$, consistent with its role as a specific inhibitor of PLC, but surprisingly it acutely increased $[Ca^{2+}]_i$ and prevented the depolarization-induced rise in $[Ca^{2+}]_i$. It has been described that U-73122 can directly activate ion channels and can itself promote the release of Ca^{2+} from intracellular stores (24) through inhibition of the internal Ca^{2+} pump in hepatic microsomes (4). Therefore, these results would caution that U-73122 may not be selective at concentrations required for maximal blockade of PLC and that the selectivity of U-73122 may be dependent on cell type.

It has been proposed that a G_q protein-coupled receptor can activate ERK through PKC activation (43). PKC is known to activate ERK, presumably by inducing the phosphorylation of the EGFR and subsequent activation of Ras/Raf/MEK/ERK (35). Because the present study showed that ANG II-induced ERK phosphorylation was probably a PLC-dependent process and the octapeptide has been shown to stimulate PKC activity in pituitary cells through PLC-mediated DAG production (37), we tested the hypothesis that ANG II could activate ERK pathways by activated PKC. The present results clearly showed that PKC depletion by prolonged PMA treatment or pretreatment with the specific PKC inhibitor chelerythrine had no effect on ERK phosphorylation by ANG II. Therefore, our data point to the dominant role of a PKC-independent pathway in ANG II-induced ERK activation in pituitary cells. This is in contrast to results obtained in VSMCs (25), BAG cells (42), AT_1-expressing CHO cells (1), or hepatic cells (35), in which ANG II-induced ERK activation was dependent on PKC. Nevertheless, other studies indicate that calcium signaling, rather than PKC, plays a critical role in ERK activation induced by the octapeptide (9, 27, 31, 46). We therefore sought to determine the role of Ca^{2+} in ANG II-induced ERK phosphorylation.

Intracellular Ca^{2+} chelators and long exposure to EGTA, which significantly lowered the intracellular Ca^{2+} pool, prevented the ANG II-induced increase in $[Ca^{2+}]_i$ and in pERK. This points to a key role of Ca^{2+} in ANG II-mediated ERK phosphorylation. On the other hand, short-term pretreatment with EGTA, which did not deplete internal stores, did not prevent the elevation of pERK in response to ANG II. A similar Ca^{2+} dependency has been described in VSMCs (9) and cardiac myocytes (31). In contrast, in hepatic cells, Ca^{2+} chelators (BAPTA and EGTA) did not impair ERK activation by ANG II (35).

To determine whether tyrosine kinase activity was required for ERK phosphorylation in response to ANG II, cells were pretreated with herbimycin A, an inhibitor of Src family tyrosine kinases, or with PP1, a more selective c-Src tyrosine kinase inhibitor. Herbimycin A lowered, and PP1 inhibited, the increase of pERK in response to ANG II. This indicates that a c-Src tyrosine kinase is involved in ANG II-induced ERK phosphorylation even though other component(s) might participate in this mechanism. These results are consistent with the finding that, in VSMCs from c-Src-deficient transgenic mice, a decrease in ANG II-induced ERK activity was described (17). Several studies emphasize the importance of c-Src in ANG II-stimulated ERK activity in VSMCs (9) and cardiac cells (27). However, other groups reported that herbimycin A failed to block ANG II-induced increase in ERK activity in vascular and aortic smooth muscle cells (20, 39) and in an opossum kidney cell line (41).

There is now abundant evidence for the frequent involvement of EGFR transactivation in GPCR- and particularly in ANG II-mediated ERK activation (22, 32), and it has been suggested that c-Src activation precedes EGFR transactivation. In pituitary cells, inhibition of EGFR phosphorylation with AG-1478 attenuated ERK1/2 phosphorylation, suggesting that this may be a common mechanism shared by several GPCRs coupled to G_q, such as AT_1. Similar involvement of EGFR transactivation in the effect of ANG II has been described in VSMCs (8, 10), hepatic C9 cells (35), and cardiac fibroblasts (26). Nevertheless, incomplete inhibition of ANG II-induced ERK activation by AG-1478 was observed in our experiments, as was

described in VSMCs (10). This may indicate that the activation induced by ANG II is not exclusively mediated by the AG-1478-sensitive pathway in our tissue.

The present study demonstrates that, in pituitary cells, ANG II-induced ERK phosphorylation is dominantly mediated by a G_q protein-coupled, PLCβ- and Ca^{2+}-dependent mechanism. The signaling response to ANG II also involves a nonreceptor tyrosine kinase, the c-Src kinase, and transactivation of the EGFR. When other results in the literature are considered, it is apparent that the pathways leading to ERK phosphorylation from the AT_1 receptor, and the role played by PKC, Ca^{2+}, and tyrosine kinases in these pathways, vary considerably among different cell types, consistent with multiple pathways converging on ERK. The mechanism used by a given receptor to stimulate ERK is likely to be dependent on the subtype of G protein and downstream components expressed by a given cell type. Therefore, the quest for agonist-induced ERK activation is orchestrated and shaped in any particular cell by a myriad of components expressed in a cell-, tissue-, and time-dependent fashion. Under this scenario, it might be possible that additional cell- and tissue-specific signaling molecules may contribute synergistically to ANG II-specific component(s) in the ERK activation process.

Finally, we described an altered activation pattern of ERK in cells from estrogen-induced pituitary hyperplasia. In a previous work (12), we showed that estrogen alters calcium influx and mobilization in response to ANG II in pituitary cells. Our present results indicate that, in control pituitary cells, the effect of ANG II on ERK phosphorylation was dependent on the elevation of $[Ca^{2+}]_i$. Consistent with these results, we found that, in hyperplastic cells, ERK activation was altered in correlation with an altered increase in $[Ca^{2+}]_i$ in response to ANG II. In VSMCs, it has also been demonstrated that estrogen attenuates AT_1 receptor-mediated ERK activation and that estrogen antagonizes the effect of the AT_1 receptor via the activation and induction of phosphatases through nongenomic as well as genomic signaling (40). These results may be of paramount importance if we consider that two of the most highly recognized factors implicated in the pathogenesis of hypertension, atherosclerosis, congestive heart failure, and associated cardiovascular disease are the RAS and estrogen and that a major effect of estrogen on these diseases results from its influence on the RAS.

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DISCLOSURES

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