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AMILORIDE SENSITIVE ACTIVATION OF S_6 KINASE BY ANGIOTENSIN II IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

Timothy Scott-Burden, Thérèse J. Resink, Ursula Baur, Maria Bürgin and Fritz R. Bühler

Department of Research
Hypertension Laboratory
University Hospital
4031 Basel / Switzerland

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Summary. Angiotensin II was shown to activate S_6-kinase in cultured vascular smooth muscle cells (VSMC) in a dose- (10^{-9}-10^{-6} M) and time-dependent manner. Pretreatment of quiescent cells with 12-O-Tetradecanoylphorbol-13-acetate had no effect on the activation levels of the kinase at the hormone levels used. However, stimulation of S_6-kinase activity by angiotensin II was markedly inhibited by the inclusion of amiloride hydrochloride in serum-free medium during activation procedures. Angiotensin was not mitogenic for VSMC at even the highest doses used (10^{-6} M). These findings support the notion that raised intracellular pH results in the activation of protein synthesis in quiescent cells.

Introduction. The activation of the cellular protein synthetic machinery is part of a series of coordinated events that occur rapidly when quiescent cells are exposed to growth factors in culture (1-4). This process has been shown to involve the activation of cytosolic S_6-kinase, so called since it is responsible for the multiple phosphorylation of the S_6 polypeptide associated with the small 40S ribosomal subunit (5-7). The control of S_6-kinase activity itself has been shown to occur via the well documented phosphorylation/dephosphorylation mechanism of regulation associated with important metabolic enzymes (8-10).

We have shown that a number of growth factors can activate S_6-kinase in cultured vascular smooth muscle cells (VSMC) which is consistent with their growth promoting activity in such systems (11,12). Furthermore, thrombospondin, a large molecular weight glycoprotein, can also elicit such a protein synthetic activational response even in the absence of mitogenic activity (11).

For some time, evidence has been accumulating to suggest that stimulation of Na^{2+}-influx may be an event common to all growth factor proliferative

Abbreviations: AII, angiotensin II; TPA, 12-O-Tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto; MEM, minimal essential medium; PKC, protein kinase C; VSMC, vascular smooth muscle cells; EGF, epidermal growth factor.

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activation (13-15). Furthermore, the amiloride sensitive alkalinization of Chinese hamster lung fibroblasts (CC139) has been correlated with phosphorylation of peptide S_6 in the 40S ribosomal subunit (16,17).

Angiotensin II (AII) has been shown to markedly increase intracellular pH of VSMC and this process was sensitive to the presence of amiloride but insensitive to prolonged pretreatment with 12-0-Tetradecanoylphorbol-13-acetate (TPA) (18,19). The latter treatment indicated both protein kinase C-dependent and -independent pathways for the stimulation of Na^+/H^+-exchange by AII in cultured VSMC.

We report here on our findings in relation to the amiloride sensitive activation of S_6-kinase by AII and TPA in quiescent rat VSMC.

MATERIALS AND METHODS

Materials. All tissue culture material and chemicals were obtained from Gibco AG, Basel, Switzerland except for fetal calf serum (FCS) which was purchased from Fakola AG, Switzerland. All radioactive isotopes were supplied by Amersham, Zürich, Switzerland and included [γ-^{32}P]-ATP (3000 Ci/mmol) and [methyl-^{3}H]-Thymidine (92 Ci/mmol). Angiotensin II (AII), 12-0-Tetradecanoylphorbol-13-acetate (TPA) and amiloride hydrochloride were from Sigma Chemical Co, St. Louis, Mo., USA. Lab. Tek. eight chamber slides were supplied through Bayer AG (Miles), Zürich, Switzerland. All other chemicals and reagents were obtained either from E. Merck, Darmstadt, FRG or Sigma Chemical Co., St. Louis, Mo., USA.

Methods. VSMC from thoracic aortas of SHR rats were isolated using procedures described previously (19). SHR animals were used since a greater yield of fast growing cells (vs control WKY rats) could be obtained. Cultures were used between 5th and 14th passage and cell numbers determined following enzymatic disaggregation of cell layers (12). Cells were maintained in minimal essential medium (MEM) containing Earles salts, 20 mM glutamine, 20 mM TES-NaOH, 20 mM HEPES-NaOH (both pH 7.3), and 100 U/ml penicillin and 100 U/ml streptomycin as bacteriostatic agents. Media was supplemented with 10% v/v heat-inactivated fetal calf serum (FCS) except for the 48 hr period prior to assays involving S_6-kinase activation or nuclear labelling (see below).

Activation of S_6-kinase in cells maintained on serum-free medium for 48 hrs was performed essentially as described previously (10) with minor modifications (11). For activation assays performed in the presence of amiloride, VSMC were changed to serum-free medium (MEM) containing 20 mM NaCl and 100 mM choline chloride (ccMEM) for 20 min prior to activation. Amiloride dissolved in DMSO was added to this medium from 100x concentrated stocks and control cultures in ccMEM were treated with equivalent volumes of DMSO. Pretreatment of cultures with TPA (100 nM) was performed during the 48 hr period on serum-free medium. S_6-kinase phosphorylation assays were performed using $40S_4$ ribosomal subunits as substrate (specific activity of [γ-^{32}P]ATP at 2 x 10^4 dpm/pmol) as described previously (11). Based on extractions of cell layers containing between 1.0 and 3.2x10^6 cells/well, assays were linear with respect to time and protein concentration and 40S subunits were used at saturating levels (10). Stimulation of cells with 10% FCS resulted in the incorporation of between 4 and 4.65 pmol $PO_4/10^6$ cells into S_6 polypeptide while unstimulated cells (serum-free) exhibited values of 0.42 to 0.48 pmol $PO_4/10^6$ cells. These values were obtained from ± 140 phosphorylation assays.

Mitogenesis assays (nuclear labelling) were performed using VSMC plated onto Lab. Tek. eight chamber slides previously rinsed with 1% v/v gelatine (12). Sparsely confluent cultures were maintained on serum-free media (with one change) for 48 hrs. Quiescent cells were exposed to various agonists (as

indicated in the text) in serum-free medium for 32 hrs in the presence of [^{3}H]-thymidine (1 μCi/ml). Thereafter, cells were fixed and slides processed as previously described (11).

RESULTS AND DISCUSSION

When quiescent cells were exposed to AII there was a rapid (Fig. 1) and dose-dependent (Fig. 2A) activation of cytosolic S_6-kinase as evidenced by the increased ability of lysates from treated cells to incorporate $^{32}PO_4$ into the integral 40S polypeptide, S_6 (11). The time course for activation of S_6-kinase by AII was similar to that reported for growth factors such as EGF (10,11) in that there was a increase in activation for up to 15 min followed by a progressive loss in the continued ability of AII to elicit such a response on prolonged exposure of VSMC to the hormone (Fig. 1). The kinetics of S_6-kinase activation by AII in VSMC contrasted with those for TPA (Fig. 1) which elicited a slower and more sustained response.

Prior treatment of VSMC for 48 hrs with TPA did not affect the dose-dependent activation of S_6-kinase by AII (Fig. 2B). However, prolonged pre-exposure of VSMC to TPA prevented subsequent induction of S_6-kinase activation by the same (Fig. 2B).

The data pertaining to the inability of TPA to negate subsequent S_6-kinase activation by AII strongly suggest that AII can activate S_6-kinase as a direct consequence of AII interaction with its own receptor and in a manner that does not involve prior activation of protein kinase C (PKC). We and others have shown that AII elicits a marked stimulation in phosphoinositide hydrolysis

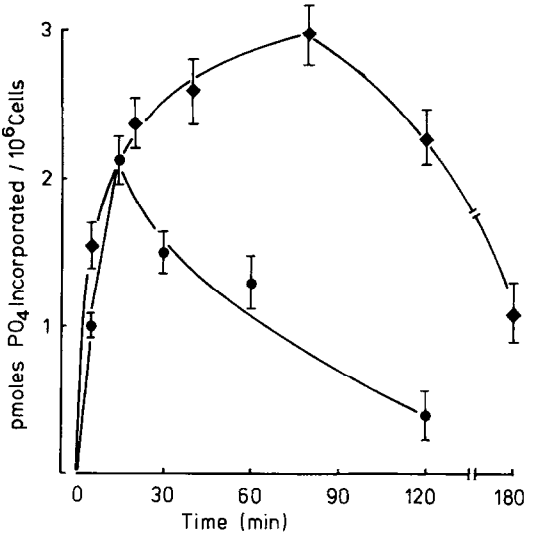

Figure 1. Time course for the activation of S_6-kinase in quiescent VSMC by AII ● (10^{-7} M) and TPA ◆ (100 nM). Data represent the mean ± SD of triplicate phosphorylation assays from activations performed on three separate occasions for AII and twice for TPA.

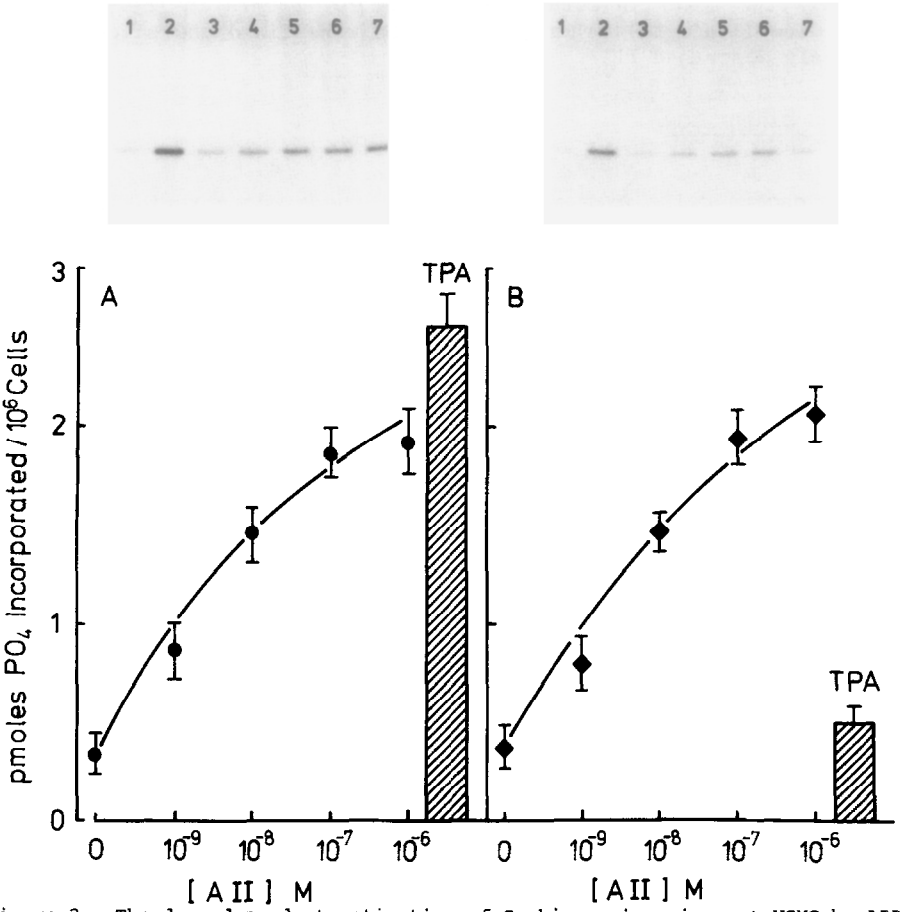

Figure 2. The dose-dependent activation of S_6-kinase in quiescent VSMC by AII without (panel A) and with (panel B) pretreatment of cultures for 48 hrs with TPA (100 nM). The histograms indicate values obtained for the activation of S_6-kinase in these VSMC cultures by TPA (100 nM). Data represent mean ± SD for triplicate phosphorylation assays from activations performed on four separate occasions for (A) and three separate occasions for (B). The duration of activations (at 37°C) was 15 min for AII and 45 min for TPA. Superimposed autoradiograms illustrate representative results for the S_6-kinase phosphorylation assay, and were obtained following exposure at -70°C of Kodak X-Omat S films to dried SDS-polyacrylamide (17.5%) gels for 12 hrs (panel A) and 8 hrs (panel B). Numbered lanes in Panel A and B represent S_6-kinase activation under the following experimental conditions: 1) serum-free, 2) 10% FCS, 3-6) AII at the indicated increasing doses, 7) 100 nM TPA.

leading to production of second message molecules (inositol trisphosphate and diacylglycerol) (20,21). However, our data suggest that AII does not mediate its action on S_6-kinase via a PKC controlled pathway and is capable of directly activating S_6-kinase in a rapid transitory manner (Fig. 1) (18,22).

At the levels of AII used in our experiments we observed no proliferative effect on VSMC cultured either in the absence or presence of low levels of serum (1% v/v) (data not shown). Furthermore, even at the highest levels (10^{-6} M) used in these studies AII was not mitogenic for VSMC as assessed by nuclear labelling experiments. The results obtained using this procedure to assay for

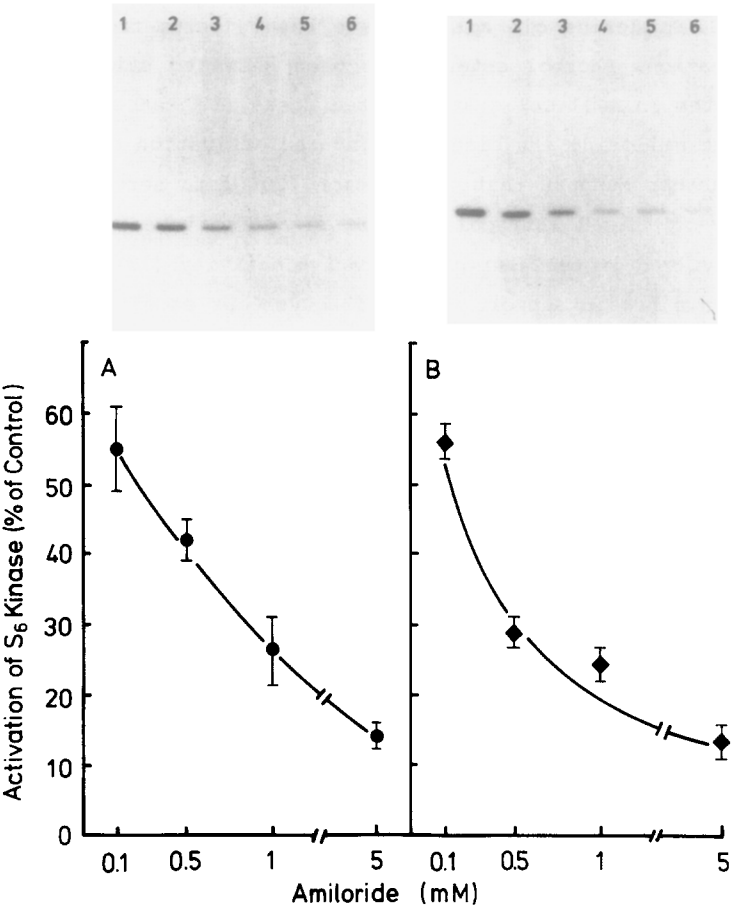

Figure 3. Amiloride inhibition of the S_6-kinase activation induced by AII (panel A) and TPA (panel B). Data express percentage activation of S_6-kinase following exposure of quiescent VSMC to either AII and amiloride or TPA and amiloride, where S_6-kinase activation responses to either AII or TPA in the absence of amiloride were arbitrarily taken as 100%. Absolute 100% values (pmol PO_4 incorporated per 10^6 cells) for AII and TPA were 2.01 ± 0.18 and 3.04 ± 0.27, respectively. Final compound concentrations were 10^{-7} M AII, 100 nM TPA and amiloride as indicated. Duration of activations (at 37°C) was 15 min for AII (panel A) and 60 min for TPA (panel B). Values in figures are given as mean ± SD of quadruplicate phosphorylation assays from two separate activations for both AII and TPA. Autoradiograms of representative experiments are superimposed and numbered lanes indicate conditions of activation; Panel A 1) AII alone, 2-5) AII plus amiloride at the indicated increasing concentrations and 6) serum-free. Panel B 1) TPA alone, 2-5) TPA plus amiloride at levels indicated and 6) serum-free.

mitogenesis were as follows: serum-free, 10.3 ± 4.0%; 10% FCS, 92.3 ± 2.5%; 5 ng/ml EGF, 23.4 ± 3.8%; and 10^{-6} M AII, 12.0 ± 3.1%. In these experiments EGF was included as a control, low level VSMC mitogen (12,23).

As above mentioned, AII has recently been shown to cause a rapid and marked alkalinization of VSMC (18,19). We therefore investigated the activation of S_6-kinase by this hormone both in the presence and absence of amiloride, which is a well known inhibitor of Na^{2+}-influx into cells (24).

Our findings (Fig. 3) clearly demonstrate that amiloride exerted a powerful and dose-dependent inhibition of both AII- and TPA-induced S_6-kinase activa-

tion in VSMC. Amiloride did not decrease basal (serum-free) levels of S_6-kinase activation. Phorbol esters have been shown to stimulate the Na^+/H^+ exchange system in A431 cells at elevated doses (100-500 nM) (25,26) and our findings that amiloride inhibited TPA-induced activation of S_6-kinase in VSMC (Fig. 3B) further support that observation. Our data serve to emphasize the universality of raised intracellular pH as a departure point for the many processes involved in cellular proliferative activity (27).

The findings herein support those of Pouysségur et al (16) in relation to phosphorylation of the 40S ribosomal S_6 polypeptide (16,17) and are in agreement with the general concepts (1-7) concerning the chain of events that lead to eventual cellular proliferation. However, we have shown that although AII is capable of activating the protein synthetic machinery (i.e. S_6-kinase activation) in VSMC, this hormone does not cause a mitogenic/proliferative response. Thus, it is evident that a combination of events must occur before cells transit from the quiescent to the proliferative state, activation of protein synthesis being only one of these. These results are in accord with those of Thomas et al (28) and are supported by our previous findings with regard to S_6-kinase activation by thrombospondin at submitogenic levels (11).

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