Wortmannin inhibits the activation of MAP kinase following vasopressin V1 receptor stimulation

Naoya Nishioka[a,b], Syu-ichi Hirai[a], Keiko Mizuno[a], Shin-ichi Osada[a], Atsushi Suzuki[a], Kenji Kosaka[b], Shigeo Ohno[a,*]

[a]*Department of Molecular Biology, School of Medicine, Yokohama City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236, Japan*
[b]*Department of Psychiatry, School of Medicine, Yokohama City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236, Japan*

Received 9 October 1995; revised version received 15 November 1995

Abstract Treatment of rat 3Y1 fibroblasts with vasopressin (AVP) results in a transient activation of MAP kinase as potent as with EGF and serum. An antagonist of vasopressin receptor V1, but not an antagonist of V2, inhibited the AVP-induced activation of MAP kinases, indicating that AVP activates MAP kinases through V1 receptor. Prolonged TPA treatment of cells resulted in partial MAP kinase activation, indicating the presence of PKC-independent pathway. The pathway was inhibited by wortmannin, an inhibitor of PI3-kinase. The results suggest that wortmannin-sensitive molecules such as PI3-kinase, are involved in the V1 receptor-mediated activation of the MAP kinase pathway independent of TPA-sensitive PKC.

Key words: Vasopressin; MAP kinase; Wortmannnin; 3Y1 cell

1. Introduction

The antidiuretic hormone vasopressin (AVP) is a cyclic nonapeptide involved in the homeostasis of body fluid osmolarity and blood pressure [1]. In addition, AVP exerts a variety of physiological effects on a variety of tissues and cells. These include platelet aggregation, metabolic stimulation, and cell proliferation [1,2]. These actions are mediated through specific cell surface receptors that are pharmacologically divided into V1 and V2 receptors. V1 and V2 receptors belong to a family of seven transmembrane G-protein coupled receptors [3–5]. V2 receptor is found in a limited numbers of tissues, such as kidney, while V1 receptor is found in many tissues and cells [6]. Thus, it seems that the action of AVP in a variety of cells, as listed above, involves the stimulation of V1 receptor. Previous studies have shown that V2 receptor stimulates adenylate cyclase, while V1 receptor activates phospholipase C (PLC), resulting in the production of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG), mobilization of intracellular Ca^{2+}, influx of extracellular Ca^{2+}, and activation of protein kinase C (PKC) [7]. V1 receptor stimulation also causes the activation of phospholipase D (PLD) and phospholipase A_2 (PLA_2), which might be involved in the activation of PKC and intracellular Ca^{2+} mobilization, respectively [7]. In addition to these immediate changes in cellular metabolism, AVP, most likely through V1 receptor, stimulates cell growth responses that accompany the induction of protein phosphorylation and gene expression[8]. However, the molecular mechanism leading to these effects remains to be explored.

Mitogen-activated protein kinases (MAP kinases) are activated in a wide variety of cells upon stimulation of tyrosine kinase-type growth factor receptors [9]. One role of activated MAP kinases appears to be the linkage of cytoplasmic signaling events to nuclear events including the induction of DNA synthesis [8]. In addition to tyrosine kinase-type growth factor receptors, receptors coupled to G-proteins, such as thrombin receptors and PAF receptors, activate MAP kinase [10]. Recent experiments based on the overexpression of $\beta\gamma$ subunits of G-protein in COS cells suggest that G-protein coupled receptors activate MAP kinases through the $\beta\gamma$ subunits of G-protein [11,12]. Thus, the signaling events downstream of G-protein coupled receptors, including those for AVP, should be reconsidered in light of novel signaling molecules including MAP kinases.

In the present study, we show that AVP activates MAP kinases through V1 receptor in rat 3Y1 fibroblasts. This signaling involves a novel pathway that is independent of TPA-sensitive PKC and seems to be mediated through PI3-kinase.

2. Experimental procedures

2.1. Materials

AVP ([Arg^8]-vasopressin) was obtained from Sigma Co.; DDAVP (desamino-[D-Arg^8]-vasopressin) and wortmannin were obtained from Peninsula Laboratories, Seikagaku Co. (Tokyo, Japan). OPC-21268 and OPC-31260 were obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan).

2.2. Cell culture and stimulation

Rat embryonic fibroblast 3Y1 cells (provided by Dr. G. Kimura, Kyushu University) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and incubated as previously described [13]. Cells were rendered quiescent by incubation in DMEM containing 0.5% FCS for 48 h. Cells were then treated with a variety of agents and collected after two rapid washes in ice-cold phosphate-buffered saline.

2.3. Mobility shift assay

Cells were ruptured in lysis buffer by sonication as described previously [14]. Cell lysates were adjusted for cell number and subjected to electrophoresis on 10% SDS-polyacrylamide gels. Immunoblotting was performed on nitrocellulose membranes with detection by an ECL system from Amersham. The antibody used was an anti-rat MAP-K R2 (Erk1-CT) polyclonal antibody from UBI. The density of the ERK2 band was quantified by a densitometer. The ERK2 response is expressed as (shift band/non-shift band+shift band) × 100 (%).

*Corresponding author. Fax: (81) (45) 785-4140.
E-mail: ohnos@med.yokohama-cu.ac.jp

Abbreviations: MAP kinase, mitogen-activated kinase; ERK1,2, extracellular signal-regulated kinase-1,2; PI3 kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; AVP, arginine-vasopressin; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2.

SSDI 0014-5793(95)01382-2

2.4. In-gel kinase assay

In-gel kinase assays were performed according to the method of Kawashima et al. with slight modification [15]. After electrophoresis, SDS was removed by washing the gel with 20% 2-propanol. After soaking in 6 M guanidine-HCl and 0.04% Tween 40-containing buffer, the gel was incubated at 22°C for 1 h with 10 ml of 40 mM HEPES (pH 7.5) containing 2 mM dithiothretiol, 0.1 mM EGTA, 5 mM $MgCl_2$, and 25 mM [γ-^{32}P]ATP (25 μCi) for the kinase assay. As a substrate, MBP (myelin basic protein) (0.5 mg/ml) was included in the separating gel prior to polymerization of the acrylamide. The samples were loaded onto 12% polyacrylamide gels, and the transfer of [γ-^{32}P]phosphate to MBP was visualized and quantified by a radio-image analyzer (Fuji Film BAS 2000).

3. Results

3.1. AVP activates MAP kinases in rat 3Y1 fibroblasts

During the course of experiments to identify extracellular signals that activate the transcription of a reporter gene driven by a TPA-response element (TRE) in serum-starved rat 3Y1

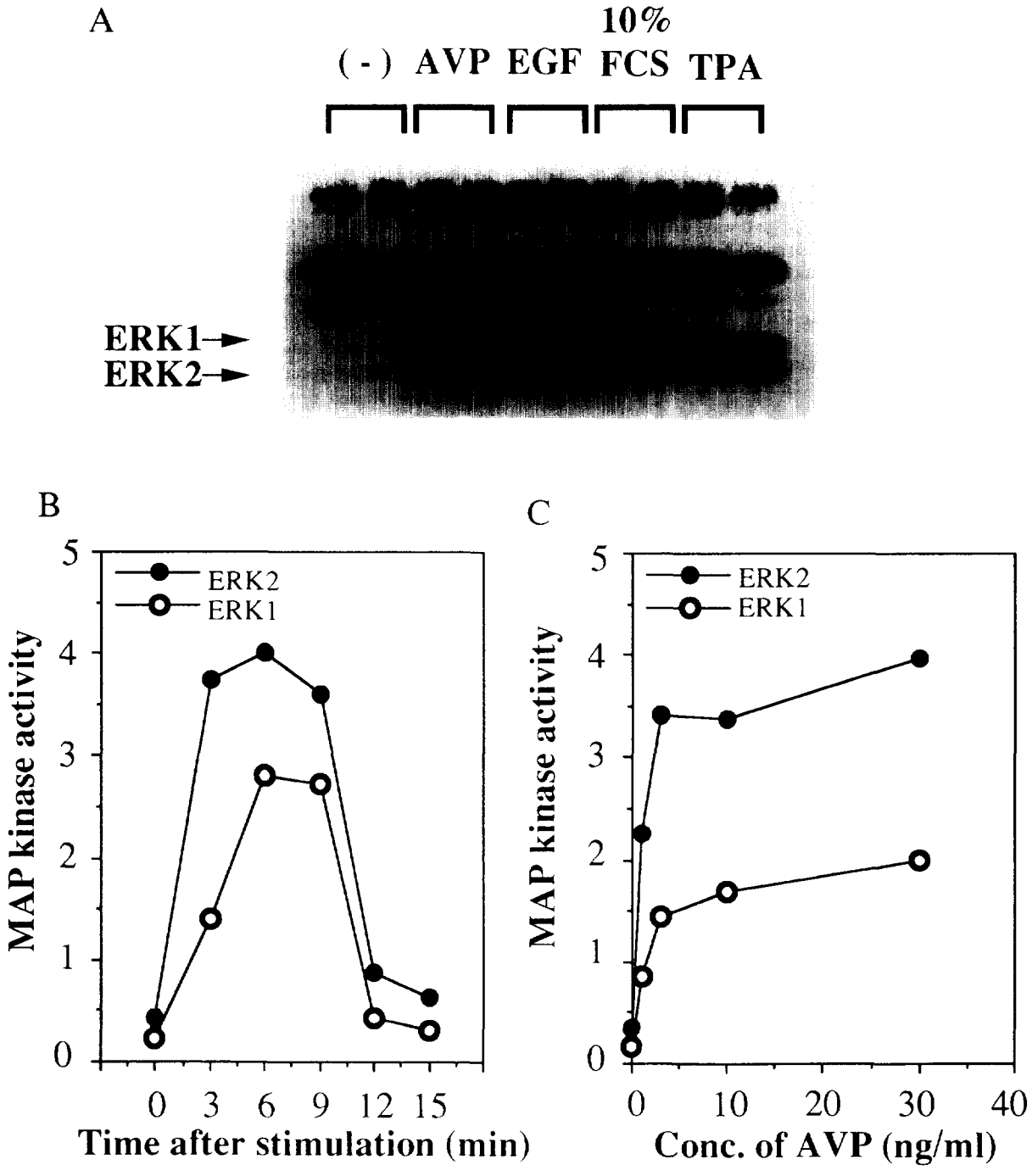

Fig. 1. AVP induces transient activation of MAP kinases. Cells were treated with a variety of agents (10 ng/ml AVP, 3 ng/ml EGF, 10% FCS, 30 ng/ml TPA) (A), with 10 ng/ml AVP for different times (0–15 min) or (B) at different AVP concentrations (0, 1, 3, 10, and 30 ng/ ml) for 5 min (C). The results of in-gel kinase assays are shown. Values shown (arbitrary units) are the averages of two samples.

fibroblasts, we identified AVP as a potent activator of a reporter gene containing TRE (data not shown). This is consistent with previous findings that AVP stimulates the expression of c-*fos* and c-*jun* in A7r5 cells[8], although the molecular events leading to the activation of gene expression remain to be clarified. In order to analyze immediate changes in the cells that might be involved in the activation of gene expression upon AVP stimulation, we next examined the effect of AVP on MAP kinase activation. Recent experiments on growth factor receptors suggest the involvement of MAP kinase in a variety of cell functions including gene expression mediated by SRE (serum response element) [16].

Serum-starved cells were stimulated with AVP and MAP kinase activities were evaluated by two different procedures, one involving the detection of MBP kinase activity in situ on a gel after denaturation and renaturation (in-gel kinase assay), and the other detecting the mobility shift of MAP kinases on polyacrylamide gels visualized after Western blotting with anti-MAP kinase antibody. Fig. 1A shows a typical example of the in gel kinase assay of total cell extract, clearly indicating the appearance of these two bands, upper and lower, with MBP kinase activity only when cells were stimulated with agents known to activate ERK1 and ERK2, such as EGF, serum, and TPA. These two bands most likely correspond to phosphorylated/activated $p44^{erk1}$ (ERK1) and $p42^{erk2}$ (ERK2), respectively, for the following reasons. First, immunoprecipitation of the cell extracts with an antibody directed against ERK1 followed by an in gel kinase assay resulted in the appearance of only two bands with a higher intensity in the upper band (data not shown). Second, Western blotting of total extracts as well as immunoprecipitates using anti-MAP kinase antibody resulted in the appearance of the two bands whose mobilities were retarded upon cell stimulation (data not shown). The results clearly show that AVP activates both ERK1 and ERK2, and is as potent as EGF, serum or TPA. EGF is one of the most effective mitogens in 3Y1 cells [17]. All stimuli examined, including AVP, caused stronger activation of ERK2 than ERK1.

MAP kinases are rapidly activated following stimulation of quiescent cells with AVP. The activity peaked 6 min after stimulation, with a 9 to 13-fold increase, and returned to near-basal levels after 12 min (Fig. 1B). Fig. 1C shows that MAP kinases are activated in an AVP-dose-dependent manner. The activity reached a maximal level at a dose of 10–30 ng/ml, suggesting that the effect of AVP is mediated through a specific receptor.

3.2. *AVP activates MAP kinases through V1 receptor*

In order to clarify which type of receptors are involved in MAP kinase activation in rat 3Y1 cells, we used a selective V1 antagonist (OPC-21268), V2 antagonist (OPC-31260), and a selective V2 agonist, DDAVP (desamino-[D-Arg^8]-vasopressin). Pretreatment of 3Y1 cells with OPC-21268, the V1 antagonist, inhibited the mobility shift of ERK2 in response to AVP (Fig.

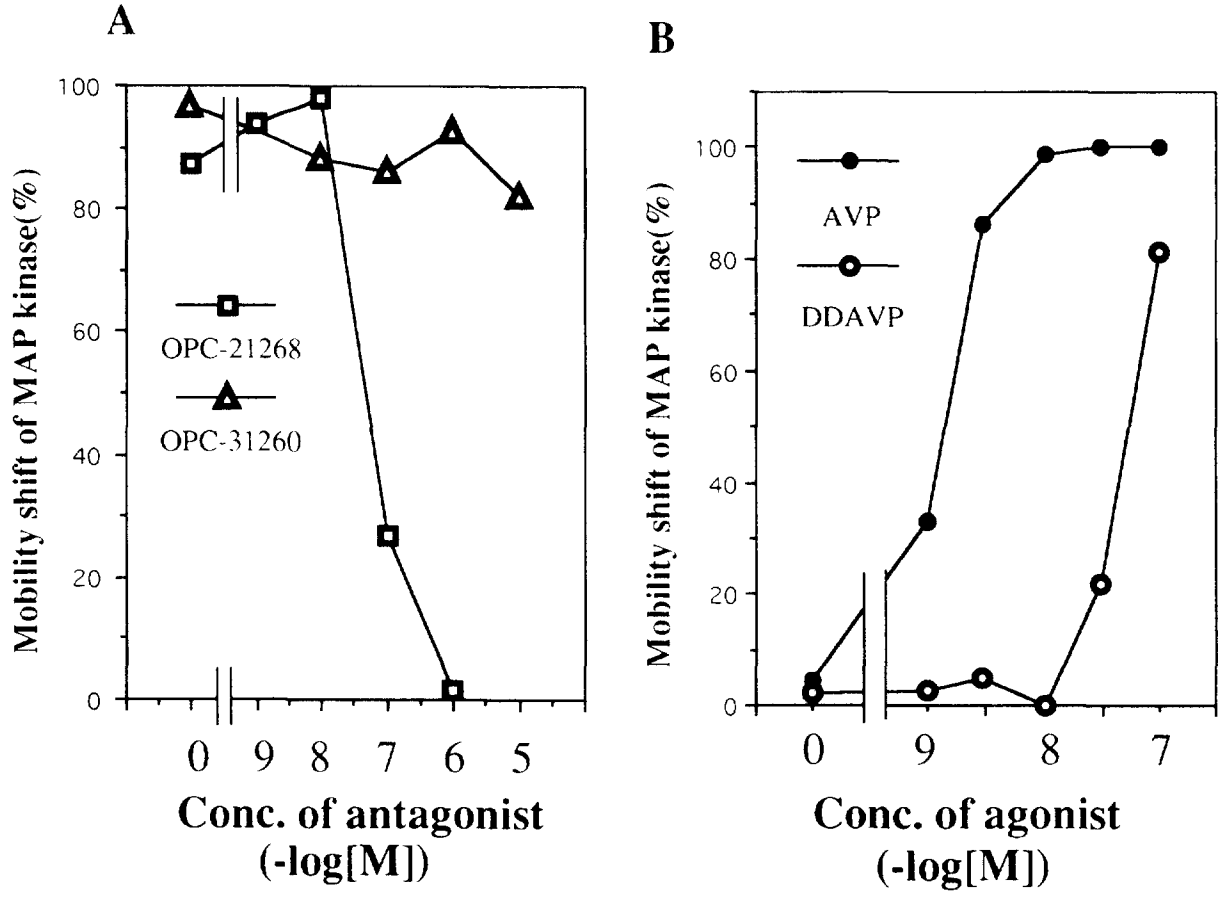

Fig. 2 The effects of receptor agonists and antagonists on AVP-induced activation of ERK2. Cells were pretreated with a V1 antagonist (OPC-21268) at a variety of concentrations (10^{-8} to 10^{-5} M) or a V2 antagonist (OPC-31260) at a variety of concentrations (10^{-9} to 10^{-6} M) for 10 min before 10 ng/ml AVP stimulation for 5 min (A). Cells were treated with AVP or DDAVP, a selective V2 agonist, at a variety of concentrations (0, 1, 3, 10, 30, and 100 ng/ml) for 5 min (B). The values shown indicate the percentage of the shifted bands of ERK2.

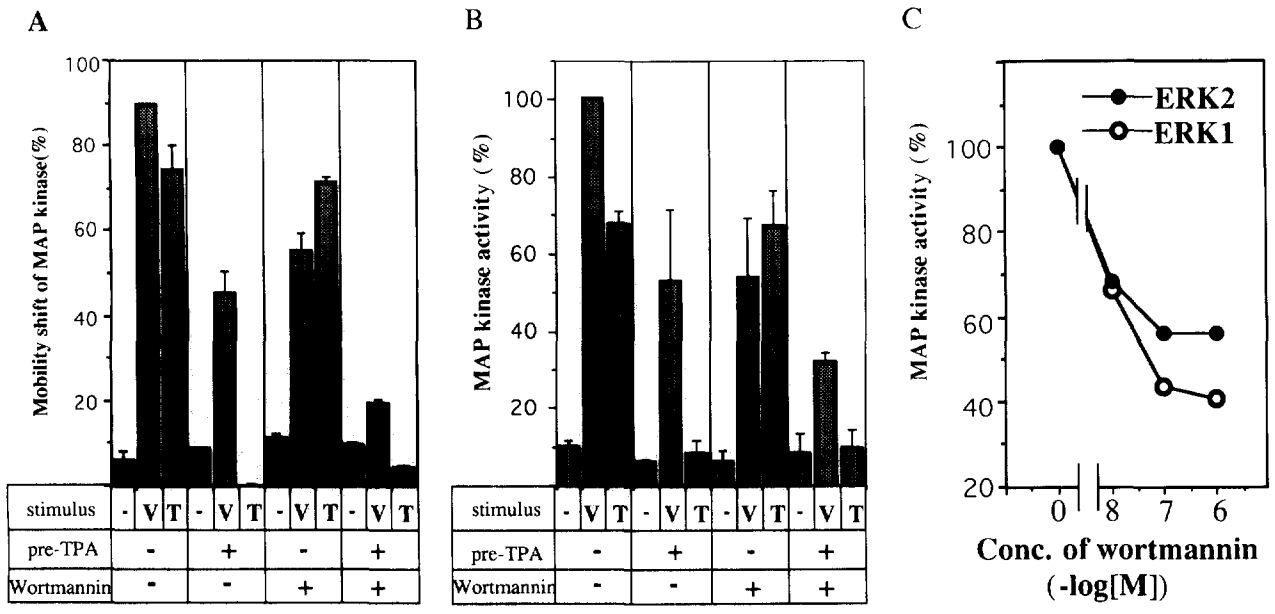

Fig. 3 The effects of prolonged TPA treatment and/or wortmannin on AVP induced-activation of MAP kinases. (A and B) A variety of pretreatments was performed on cells before AVP or TPA stimulation (- = no stimulation; V = 10 ng/ml AVP; T = 30 ng/ml TPA). Cells received no pretreatment, pretreatment with 100 ng/ml TPA for 1 day, pretreatment with 10^{-7} M wortmannin, or pretreatment with 100 ng/ml TPA for 1 day in addition to pretreatment with 10^{-7} M wortmannin. (A) The values shown indicate the percentage of the shifted bands of ERK2. The averages of two samples are shown. (B) The results of in-gel kinase assay of ERK2 are shown. Values for non-pretreated cells before AVP stimulation were taken as 100%. All data represent the mean ± standard deviation of three independent of experiments.(C) Cells were pretreated with wortmannin at a variety of concentrations (10^{-8} M to 10^{-6} M).The results of in-gel kinase assay are shown. The values from wortmannin-untreated cells were taken as 100%. Values shown are the averages of two samples.

2A). The OPC-21268 concentration that produced 50% inhibition of the ERK2-mobility shift (IC_{50}) was approximately 3×10^{-7} M. On the other hand, pretreatment of 3Y1 cells with OPC-31260, the V2 antagonist, caused no inhibition of the mobility shift of ERK2 in response to AVP (Fig. 2A). The IC_{50} of OPC-21268 for the displacement of specific AVP binding is reported to be 4×10^{-7} M and $>10^{-4}$ M for V1 and V2 receptors, respectively [18], and the IC_{50} of OPC-31260 for the displacement of specific AVP binding is reported to be 1.2×10^{-6} M and 1.4×10^{-8} M for V1 and V2 receptors, respectively [19]. Thus, our results suggest that the activation of ERK2 is mediated through the V1 receptor.

To confirm this, we next examined the effect of a V2 agonist, DDAVP. The EC50 of DDAVP for ERK2 activation was approximately 6×10^{-8} M (Fig. 2B). Since the reported EC_{50} of AVP for adenylate cyclase activity is 2×10^{-8} M [4], our result, considered together with those obtained for the antagonists, clearly indicates that AVP-induced activation of ERK2 is mediated through V1 receptor, not V2 receptor, in rat 3Y1 fibroblasts. Similar results were also obtained for the activation of ERK1 (data not shown).

3.3. Both TPA-sensitive, PKC-dependent and -independent pathways are involved in the AVP-induced activation of MAP kinases

Previous reports provide evidence for the activation of the PLC/PKC pathway downstream of the V1 receptor [7]. Thus, we next examined the effect of prolonged TPA treatment on AVP-induced MAP kinase activation. As previously reported, rat 3Y1 fibroblasts express at least three TPA-sensitive PKC species, PKCα, PKCδ, and PKCε, and prolonged TPA treatment of 3Y1 cells causes down-regulation of all three TPA-sensitive PKC species [17]. When cells were pretreated with TPA for 1 day, the activation of MAP kinases caused by AVP was partially inhibited (Fig. 3). This indicates that AVP activates MAP kinases through at least two independent pathways, one involving TPA-sensitive PKC and the other not involving TPA-sensitive PKC.

3.4. Wortmannin inhibits AVP-induced MAP kinase activation

For a long time, the prevalent hypothesis for the mechanism of G-protein mediated signal transduction was that the GTP-liganded α subunit activated effectors, while the $\beta\gamma$ subunit was only a negative regulator [20]. However, the $\beta\gamma$ subunit has recently been shown to be a positive regulator of MAP kinase [11,12]. In addition, the $\beta\gamma$ subunit may also activate PI3-kinase [21,22]. In order to examine whether PI3-kinase is required for the activation of MAP kinase in response to AVP in 3Y1 cells, cells were pretreated with wortmannin for 30 min. Wortmannin is known to specifically inhibit PI3-kinase in vivo and in vitro at a concentration of 10^{-7} M [10,23,24]. Incubation of cells with wortmannin resulted in an inhibition of AVP-induced MAP kinase activation (Fig. 3A,B,C). The concentration of wortmannin required for the inhibition (40–52%) of MAP kinases in response to AVP was 10^{-7} M (Fig. 3C). Since wortmannin had no inhibitory effect on the TPA-induced activation of MAP kinases, it does not affect any signaling components between PKC and MAP kinase (Fig. 3A,B). Thus, the effect of wortmannin is specific to AVP stimulation. Moreover, MAP kinase activation independent of prolonged TPA-treatment was also

inhibited by wortmannin, and wortmannin-insensitive MAP kinase activation was inhibited by prolonged TPA-treatment (Fig. 3A,B).

4. Discussion

Previous reports presented evidence that AVP, most likely through V1 receptor, stimulates the production of diacylglycerol, a potent activator of PKC, in hepatocytes, A7r5 cells, and A10 cells [7]. The activation of PKC upon AVP stimulation has also been examined in terms of the phosphorylation of MARCKS, a PKC substrate [25,26]. Our results confirm the presence of the PKC/MAP kinase pathway in 3Y1 cells (Figs. 1A and 3A,B), as reported for many other cell types. Furthermore, our results indicate that the pathway involving PKC mediates the activation of MAP kinase through V1 receptor. Our results also show that the signaling pathway from the V1 receptor to MAP kinase activation involves an additional pathway that is independent of TPA-sensitive PKC. Prolonged TPA treatment of cells eliminates TPA-sensitive PKC [17], and there are TPA-insensitive classes of PKC, atypical PKCζ and PKCλ, in 3Y1 cells [27]. However, whether these molecules are involved in the signaling pathway remains to be clarified.

The effect of wortmannin on the V1-receptor-mediated activation of MAP kinases suggests the involvement of PI3-kinase in the signaling pathway between V1-receptor and MAP kinases. Since wortmannin did not inhibit TPA-mediated MAP kinase activation (Fig. 3), the action of wortmannin does not involve the pathway from PKC to MAP kinase activation. Since MAP kinase activation, independent of prolonged TPA-treatment, was also inhibited by wortmannin, and since wortmannin-insensitive MAP kinase activation was inhibited by prolonged TPA-treatment (Fig. 3), there are at least two major pathways from V1-receptor to MAP kinases. One involves TPA-sensitive PKC, the other involves wortmannin-sensitive molecules such as PI3-kinase. These two pathways overlap to some extent, since prolonged TPA treatment followed by wortmannin treatment of cells resulted in a partial decrease in MAP kinase activation (Fig. 3). The presence of the former pathway is consistent with previous experiments showing that the stimulation of V1 receptors activates PLA_2, PLC, and PLD, resulting in the production of IP_3, and DAG, the mobilization of Ca^{2+}, and the activation of PKC [7], and that the stimulation of CHO cells overexpressing the V1a receptor causes inositol phosphate production and Ca^{2+} mobilization [5]. However, the latter pathway is a novel pathway and may correspond to the recently identified signaling pathway involving $\beta\gamma$ subunits of G-proteins and ras [21,22]. Recently, it was reported that the overexpression of $\beta\gamma$ subunits of G-protein in COS cells activates MAP kinase [11,12]. Moreover, a constitutive active mutant of PI3-kinase activates MAP kinase [28]. There are reports showing the generation of PIP_3, a product of PI3-kinase, upon stimulation of G-protein coupled receptors such as thrombin and PAF [29,30]. These results suggest that G-protein coupled receptor is able to activate PI3-kinase. There are multiple related PI3-kinases in mammalian cells, and it appears that p110γ, an isotype of PI3-kinases, is activated by G-protein [31,32]. Furthermore, one of the events downstream of PI3-kinase activation might involve the activation of MAP kinases. Our present observations suggest that V1 receptor stimulation activates MAP kinases through a novel pathway that most likely involves PI3-kinase. The signaling molecules involved in the pathway from PI3-kinase and MAP kinase remain to be clarified. At present, TPA-insensitive atypical PKCζ is an attractive candidate for such a molecule, since it is known to be activated directly by PIP_3, a product of PI3-kinase [33]. Some TPA-sensitive PKCs such as PKCε, have also been reported to be activated by PIP_3 [34]. In support of this notion, the overexpression of nPKCε (TPA-sensitive PKC) and also aPKCλ (TPA-insensitive PKC) in 3Y1 cells resulted in the enhanced expression of a reporter gene containing a synthetic TRE in response to AVP (Nishioka et al., unpublished observation). These observations raise the interesting possibility of the existence of a novel signaling pathway, in addition to the PLC/PKC pathway, downstream of the V1 receptor. Experiments to clarify these points are important for understanding the pleiotropic and long-term action of AVP through V1 receptor, which includes the regulation of gene expression and cell proliferation in a wide variety of cells.

Acknowledgements This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, from The Cell Science Research Foundation, and from the Uehara Memorial Foundation. We are grateful to Otsuka Pharmaceutical Co. (Tokushima, Japan) for generous gifts of OPC-21268 and OPC-31260.

References

[1] Share, L. (1988) Physiol. Rev. 68, 1248–1284.
[2] Liu, J.-P., Engler, D., Funder, J.W. and Robinson, P.J. (1992) Mol. Cell. Endocrinol. 87, 35–47.
[3] Brinbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brabet, P. and Rosenthal, W. (1992) Nature 357, 333–335.
[4] Lolait, S.J., O'Carroll, A.-M., McBride, O.W., Konig, M., Morel, A. and Brownstein, M.J. (1992) Nature 357, 336–339.
[5] Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berti-Matterna, L. and Clauser, E.T. (1994) J. Biol. Chem. 269, 3304–3310.
[6] Jard, S., Elands, J., Schmidts, A. and Barberis, C. (1988) Excerpta Medica 1183–1188.
[7] Thibonnier, M. (1992) Regul. Peptides 38, 1–11.
[8] Thibonnier, M., Bayer, A.L. and Leng, Z. (1993) Regul. Peptides 45, 79–84.
[9] Thomas, S.M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J.S. (1992) Cell 68, 1031–1040.
[10] Ferby, I.M., Waga, I., Sakanaka, C., Kume, K. and Shimizu, T. (1994) J. Biol. Chem. 269, 30485–30488.
[11] Crespo, P., Xu, N., Simonds, W.F. and Gutkind, J.S. (1994) Nature 369, 418–420.
[12] Faure, M., Voyno-Yasenetskaya, A., Bourne, H.R. (1994) J. Biol. Chem. 269, 7851–7854.
[13] Hata, A., Akita, Y., Suzuki, K. and Ohno, S. (1993) J. Biol. Chem. 268, 9122–9129.
[14] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.
[15] Kameshita, I. and Fujisawa, H. (1989) Anal. Biochem. 183, 139–143.
[16] Gille, H., Sharrocks, A.D. and Shaw, P.E. (1992) Nature 358, 414–417.
[17] Ohno, S., Mizuno, K., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S., Hirai, S.-I. and Suzuki, K. (1994) J. Biol. Chem. 269, 17495–17501.
[18] Yamamura, Y., Ogawa, H., Chihara, T., Kondo, K., Onogawa, T., Nakamura, S., Mori, T., Tominaga, M. and Yabuuchi, Y. (1991) Science 252, 572–574.
[19] Yamamura, Y., Ogawa, H., Yamashita, H., Chihara, T., Miyamoto, H., Nakamura, S., Onogawa, T., Yamashita, T., Hosokawa, T., Mori, T., Tominaga, M. and Yabuuchi, Y. (1992) Br. J. Pharmacol. 105, 787–791.
[20] Neer, E.J. (1995) Cell 80, 249–257.

[21] Stephens, L., Smrcka, A., Cooke, F.T., Jackon, T.R., Sternweis, P.C. and Hawkins, P.T. (1994) Cell 77, 83–93.
[22] Thomason, P.A., James, S.R., Casey, P.J. and Downes, C.P. (1994) J. Biol. Chem. 269, 16525–16528.
[23] Nakamura, I., Takahashi, I., Sasaki, T., Tanaka, S., Udagawa, N., Murakami, H., Kimura, K., Kabuyama, Y., Kurokawa, T., Suda, T. and Fukui, Y. (1995) FEBS Lett. 361, 79–84.
[24] Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) Trends Biochem. Sci. 20, 303–307.
[25] Fujise, A., Mizuno, K., Ueda, Y., Osada, S., Hirai, S.-I., Takayanagi, A., Shimizu, N., Owada, K., Nakajima, H. and Ohno, S. (1994) J. Biol. Chem. 269, 31642–31648.
[26] Liu, J.-P., Engler, D., Funder, J.W. and Robinson, P.J. (1994) Mol. Cell. Endocrinol. 105, 217–226.
[27] Akimoto, K., Mizuno, K., Osada, S., Hirai, S.-I., Tanuma, S., Suzuki, K. and Ohno, S. (1994) J. Biol. Chem. 269, 12677–12683.
[28] Hu, Q., Klippel, A., Musulin, A.J., Fantl, W.J. and Williams, L.T. (1995) Science 268, 100–102.
[29] Kucera, G.L. and Rittenhouse, S.E. (1990) J. Biol. Chem. 265, 5345–5348.
[30] Stephens, L., Jackson, T. and Hawkins, P.T. (1993) J. Biol. Chem. 268, 17162–17172.
[31] Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtcenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., Gierrscik, P., Seedorf, K., Hsuan, J.J., Waterfield, M.D. and Wetzker, R. (1995) Science 269, 690–693.
[32] Kapeller, R. and Cantley, L.C. (1994) BioEssays 16, 565–576.
[33] Nakanishi, H., Brewer, K.A. and Exton, J.H. (1993) J. Biol. Chem. 268, 13–16.
[34] Toker, A., Meyer, M., Reddy, K.K., Falck, J.R., Aneja, R., Aneja, S., Parra, A., Burns, D.J., Ballas, L.M. and Cantley, L.C. (1994) J. Biol. Chem. 269, 32358–32368.