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The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling

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Abstract

The V2 vasopressin receptor (V2R) activates the mitogen activated protein kinases (MAPK) ERK1/2 through a mechanism involving the scaffolding protein βarrestin. Here we report that this activating pathway is independent of Gαs, Gαi, Gαq or Gβγ and that the V2R-mediated activation of Gαs inhibits ERK1/2 activity in a cAMP/PKA-dependent manner. In the HEK293 cells studied, the βarrestin-promoted activation was found to dominate over the PKA-mediated inhibition of the pathway, leading to a strong vasopressin-stimulated ERK1/2 activation. Despite the strong MAPK activation and in contrast with other GPCR, V2R did not induce any significant increase in DNA synthesis, consistent with the notion that the stable interaction between V2R and βarrestin prevents signal propagation to the nucleus. βarrestin was found to be essential for the ERK1/2 activation, indicating that the recruitment of the scaffolding protein is necessary and sufficient to initiate the signal in the absence of any other stimulatory cues. Based on the use of selective pharmacological inhibitors, dominant negative mutants and siRNA, we conclude that the βarrestin-dependent activation of ERK1/2 by the V2R involves c-Src and a metalloproteinase-dependent trans-activation event. These findings demonstrate that βarrestin is a genuine signalling initiator that can, on its own, engage a MAPK activation machinery upon stimulation of a GPCR by its natural ligand.

Keywords: V2 vasopressin receptor; GPCR; Extra-cellular signal-regulated kinases 1 and 2; MAPK; βarrestin

1. Introduction

The V2 vasopressin receptor (V2R) is a G protein-coupled receptor (GPCR) that is mainly expressed in the principal cells of the renal collecting duct. It plays an important role in the regulation of multiple renal functions, most notably water absorption but also NaCl and urea absorption, K^+ secretion as well as H^+ secretion/HCO_3^- absorption [1]. Although most of its actions have been attributed to the production of cAMP through positive coupling to the stimulatory G protein, Gαs, recent studies have shown that the V2R can also activate the extra-cellular signal-regulated kinases 1 and 2 (ERK1/2) [2,3]. Although the precise role for the V2R-regulated ERK1/2 activity in vivo remains unknown, the recent observations that the V2R could be implicated in the development of polycystic kidney diseases [4] and is found in various tumor cells [5], suggest that this receptor might play a role in abnormal cell growth or differentiation, raising the

Abbreviations: AT1aR, angiotensin 1a receptor; ATII, angiotensin II; AVP, arginine-8 vasopressin; β_2AR, β_2-adrenergic receptor; COS, simian kidney cells; CTX, cholera toxin; DMEM, Dulbecco's modified Eagle's medium; δOR, δ-opioid receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptors; ERK1/2, extra-cellular signal-regulated kinases 1 and 2; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GRK2 (also called βARK), GPCR kinase 2; HEK293, human embryonic kidney cells; HRP, horseradish peroxidase; IP, intracellular inositol phosphate; ISO, (−)-isoproterenol; JNK3, c-Jun N-terminal kinase 3; MAPK, mitogen activated protein kinases; MEK1, ERK1/2 kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphatidyl-inositol-3-kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; PTX, pertussis toxin; RGS, regulator of G protein signalling; RTK, receptor tyrosine kinases; siRNA, small interference RNA; TCA, trichloroacetic acid; V2R, V2-vasopressin receptor.

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interest for a potential role of the mitogen-activated protein kinases (MAPK) in these cases.

Many GPCR have previously been shown to activate various kinases of the MAPK family. Depending on the receptor studied and the cellular context considered, several different pathways have been unraveled [6,7]. These can be distinguished in two broad classes: the first one involves the production of second messengers through classical G protein activation whereas the second results from the recruitment of scaffolding proteins such as βarrestin. Although each of these pathways can be sufficient on its own to activate the MAPK, in many cases, they were found to contribute simultaneously to the overall activation process, albeit to different extent [8]. For both second messenger- and scaffolding-mediated MAPK activation, non-receptor tyrosine kinases such as c-Src and/or receptor tyrosine kinases (RTK) such as epidermal or platelet-derived growth factor receptors (EGFR and PDGFR, respectively) have often been found as central effectors [9,10]. Although the phenomenon of RTK transactivation is common in GPCR-stimulated MAPK, many distinct mechanisms have been described. Trans-activation can result from: the activation of metalloproteinases leading to the generation of mature cognate RTK ligands through ectodomain shedding that activate the system in an autocrine or paracrine manner, the intracellular activation of the RTK through interactions with protein complexes that can include non-receptor tyrosine kinases such as c-Src or Pyk2, or the inhibition of protein tyrosine phosphatases that constitutively inhibits RTK [11].

For the V2R, βarrestin has been shown to play an important role in the AVP-stimulated ERK1/2 activation observed in human embryonic kidney cells (HEK293) [3,12]. However, the mechanisms underlying this βarrestin-mediated process and its relative contribution to the overall activation remain poorly defined. In addition to its well characterized role in receptor desensitization and internalization, βarrestin has been shown to act as a scaffolding protein that can assemble several of the proteins involved in the MAPK signalling pathway [13]. For example, βarrestin has been seen in complexes with the non-receptor tyrosine kinase c-Src [14,15] as well as Raf-1, the ERK1/2 kinase MEK1 [16,17] and c-Jun N-terminal kinase 3 (JNK3) [18]. βarrestin can therefore be considered as a switch that can direct the signalling flow from the G protein-mediated second messenger production toward the MAPK signalling cascade. More recently, the use of artificial systems [19] or of synthetic ligands [20,21] lead to the suggestion that βarrestin is a genuine signalling molecule that can function on its own, independently of G protein activation.

The present study was therefore undertaken to determine the relative contribution of βarrestin in the V2R-stimulated ERK1/2 activity, to assess whether such βarrestin-mediated MAPK

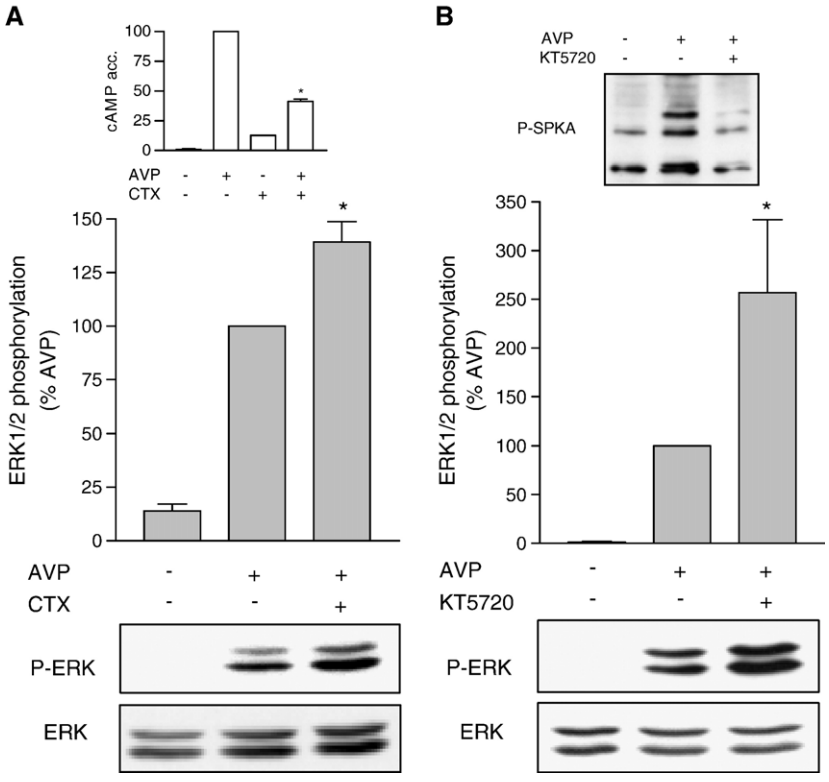

Fig. 1. Inhibition of the Gαs/PKA pathway potentiates the V2R-mediated ERK1/2 activation. Serum-starved HEK293 cells stably expressing Myc-V2R were treated or not with the indicated inhibitors at 37 °C prior to AVP stimulation. Cells were then lysed in Laemeli sample buffer, subjected to SDS-PAGE and MAPK activity was detected by western blot using phospho-specific anti-ERK1/2 antibodies (P-ERK). Expression levels of the MAPK were controlled using antibodies directed against the total kinase population (ERK) and data expressed as a % P-ERK/ERK of the level observed in AVP stimulated conditions. A) Cells were pre-treated 16 h with 300 ng/ml CTX. *Inset*, AVP-induced cAMP accumulation. B) Cells were pre-treated for 30 min with 100 nM of the PKA inhibitor KT5720. *Inset*, PKA phosphorylation was detected by western blot using phospho-(Ser/Thr) PKA substrate antibodies (P-SPKA). Data represent the mean±S.E. of at least three independent experiments. * indicates $p<0.05$.

engagement requires G protein activation and to characterize the signalling cascade linking βarrestin to ERK1/2 activation.

We report that V2R stimulation leads to a dual regulation of ERK1/2 involving a Gαs-dependent inhibition and a G protein-independent βarrestin-mediated activation of the MAPK. The βarrestin-dependent pathway was found to involve c-Src as well as a trans-activation event occurring through metalloproteinase-dependent ectodomain shedding and to supersede the inhibitory action of the G protein. In addition to shed new light on the complex regulation of MAPK by GPCR, our results show that βarrestin is a genuine signalling molecule that can sustain the activation of ERK1/2 by the V2R independently of G protein activation.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, glutamine, fungizone, G418 and phosphate-buffered saline (PBS) were from Wisent Inc. Cell culture plates and dishes were all purchased from BD Biosciences. Hank's balanced salt solution, RPMI 1640 Amino Acid solution, HEPES, arginine-8 vasopressin (AVP), SNC80, (−)-isoproterenol (ISO), angiotensin II (ATII), phorbol 12-myristate 13-acetate (PMA) and 3-isobutyl-1-methyl-xantine (IBMX) were from Sigma Chemical Co. while the epidermal growth factor (EGF) was from Calbiochem. [^{3}H]adenine, [^{3}H]myo-inositol, [^{3}H]thymidine and the enhance chemiluminescence lightening (ECL) were obtained from PerkinElmer. Cholera (CTX) and pertussis (PTX) toxins as well as all the inhibitors for protein kinases, phospholipases and RTK were from Calbiochem, whereas 1,10-phenanthroline was from Sigma. Antibodies recognizing ERK1/2 (ERK) and their phosphorylated forms (P-ERK) as well as anti-myc 9E10, anti-HA 12CA5 and anti-βarrestin2 (H9) IgGs were all from Santa Cruz Biotechnology Inc., whereas antibodies recognizing AKT/Protein kinase B (AKT) and its phosphorylated form (P-AKT) as well as the phospho-(Ser/Thr) protein kinase A (PKA) substrate antibody (P-SPKA) were purchased from Cell Signalling Technology. The H-Ras and c-Src dominant negative mutants (RasS17N and c-Src(K296R/Y528F), respectively) in pUSEamp as well as anti-H-Ras (RAS10), anti-Src (GD11 and EC10) IgG were from Upstate Biotechnology. Anti-mouse and anti-rabbit HRP-conjugated IgG were from GE Healthcare.

2.2. Expression vectors

The plasmid encoding Flag-tagged angiotensin 1a receptor (AT1aR) was a generous gift from Sylvain Meloche (Université de Montréal, Montréal). Plasmids encoding the β-adrenergic receptor kinase (βARK) carboxyl terminal (C)-tail conjugated to the extra-cellular and transmembrane domain of the CD8 protein (T8βARKctail) [22] and the regulator of G protein signalling (RGS) domain of GPCR kinase 2 (RGS_{GRK2}) [23] were generously provided by J. S. Gutkind (National Institutes of Health, Bethesda) and J.L. Parent (Université de Sherbrooke, Sherbrooke), respectively, and were described elsewhere. T8βARKctailHA and RGS_{GRK2}HA were generated by PCR, where the HA sequence YPYDVPDYA was added in frame at the carboxyl terminus of T8βARKctail and RGS_{GRK2}, respectively, and confirmed by sequencing. Plasmids encoding myc-tagged V2R [24], δ-opioid receptor (δOR) [25] and β_2-adrenergic receptor (β_2AR) [26] were described previously.

2.3. Cell culture and transfections

HEK293 and simian kidney (COS) cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml fungizone. Stable transfections were performed using the calcium phosphate precipitation method [27] and neomycin-resistant cells were selected in the presence of G418 (450 μg/ml). Resistant clones were screened for V2R expression by radioligand binding. Transient transfections were performed using the FuGENE 6 Transfection Reagent (Roche Diagnostics), according to the

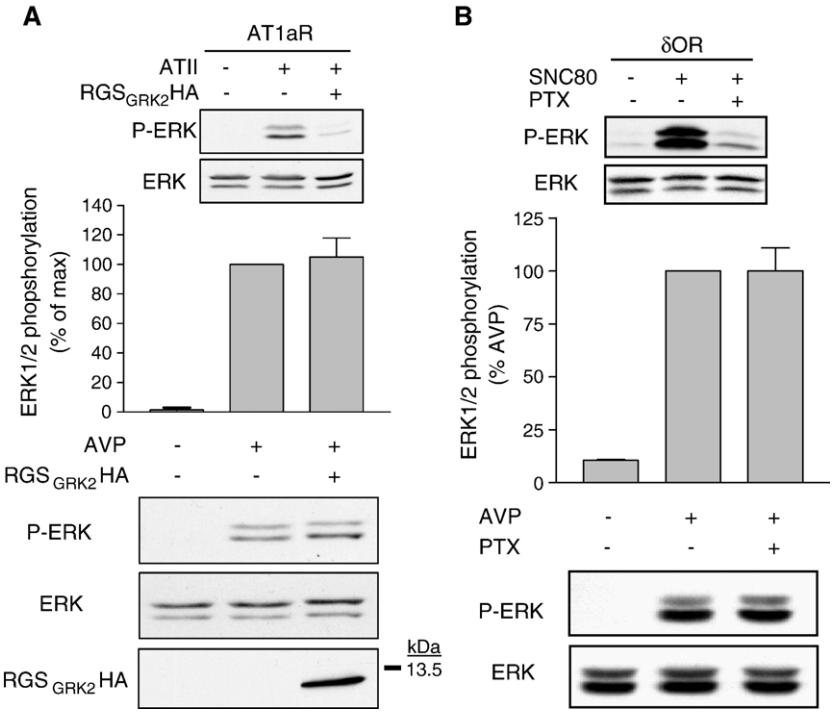

Fig. 2. Gαq- and Gαi-independent V2R-mediated ERK1/2 activation. A) HEK293 cells transiently expressing myc-V2R were co-transfected or not with RGS_{GRK2}HA and serum-starved prior to AVP stimulation. ERK1/2 phosphorylation was detected and quantified as described in Fig. 1. RGS_{GRK2} and V2R expression levels were controlled in western blot using anti-HA 12CA5 (lower panel) or anti-myc 9E10 antibodies (data not shown). *Inset*, ATII-induced ERK1/2 phosphorylation in cells transiently expressing Flag-AT1aR, and co-transfected or not with RGS_{GRK2}HA. B) Serum-starved cells stably expressing myc-V2R were pre-treated or not with 100 ng/ml PTX at 37 °C prior to AVP stimulation. *Inset*, SNC80-induced ERK1/2 phosphorylation in cells stably expressing myc-δOR and treated or not with PTX. Data represent the mean±S.E. of at least three independent experiments.

manufacturer's protocol, and cells were harvested 48 h after transfection. The previously described siRNAs for βarrestin 1 and 2 [28] were purchased from QIAGEN and transfected at 400 nM final (300 nM βarrestin1 siRNA and 100 nM βarrestin2 siRNA) using the RNAiFect transfection Reagent (QIAGEN), according to the manufacturer's protocol.

2.4. Western blotting

Cells were grown in 6 well plates and rendered quiescent by serum starvation for 24 h prior to incubation in the presence or absence of the specified inhibitors for the indicated time, followed by the different stimulations: 2 min with 1 μM AVP, 10 μM ISO, 1 μM ATII and 10 ng/ml EGF; or 5 min with 10% FBS and 10 min with 100 nM PMA. When assessing ectodomain shedding trans-activation, cells were incubated with 1 μM AVP, the supernatant was taken after 2 min of stimulation and transferred on wild-type HEK293 cells, which were then incubated for 2 min before being harvested. Cells were then placed on ice, washed twice with ice-cold PBS and solubilized directly in 150 μl of Laemmli sample buffer containing 50 mM of dithiothreitol. The samples were sonicated for 15 s, then heated 5 min at 95 °C and microcentrifuged 5 min before resolution of the proteins on SDS-PAGE. ERK1/2 phosphorylation was detected by protein immunoblotting using mouse monoclonal anti-P-ERK and anti-mouse HRP-conjugated antibodies for chemiluminescence detection. After quantification of phosphorylation by densitometry, nitrocellulose membranes were stripped of immunoglobulins and reprobed using rabbit polyclonal anti-ERK. ERK phosphorylation was normalized according to the loading of proteins by expressing the data as a ratio of P-ERK over total ERK. AKT phosphorylation was detected and quantified similarly, using rabbit polyclonal anti-P-AKT and anti-AKT antibodies coupled to anti-rabbit HRP-conjugated IgG. Phosphorylation of PKA substrates was detected using a rabbit polyclonal anti-P-SPKA antibody detecting proteins containing a phospho-Ser/Thr residue with arginine at the minus 3 position, followed by an anti-rabbit HRP-conjugated IgG. Detection of myc-, HA-, and Flag-tagged constructs was performed using mouse monoclonal anti-myc 9E10, anti-HA 12CA5 and anti-Flag M2 antibodies, respectively, and anti-mouse HRP-conjugated IgG. Detection of c-Src(K296R/Y528F) and H-Ras were carried out using mouse monoclonal anti-Src (EC10) and anti-H-Ras (RAS10) followed by an anti-mouse HRP-conjugated IgG. Detection of βarrestin1 and 2 was achieved using the mouse monoclonal anti-βarrestin2 (H9) antibody that recognizes both βarrestin isoforms, followed by anti-mouse HRP-conjugated IgG.

2.5. Intracellular cAMP accumulation measurement

Agonist-induced cAMP accumulation in HEK293 cells was measured as previously described [3]. Briefly, cells were grown in 6 well plates and incubated for 16 h in the presence of [^{3}H]adenine (2 μCi/ml) in complete DMEM medium with or without 300 ng/ml CTX. Cells were then washed twice with PBS containing 1 mM 3-isobutyl-1-methyl-xantine before being incubated for 15 min in the presence of 1 μM AVP at 37 °C. Reactions were stopped by adding 1 ml of ice-cold 5% trichloroacetic acid (TCA) and 1 mM of unlabeled cAMP to decrease enzymatic degradation of [^{3}H]cAMP. Cells were scraped off the plate and centrifuged at 800 ×*g* 20 min at 4 °C to clear the lysates. The [^{3}H]cAMP was then separated by sequential chromatography over Dowex and Alumina columns as described previously [29]. cAMP accumulation was then calculated as ([^{3}H]cAMP cpm/([^{3}H]cAMP cpm+ [^{3}H]ATP cpm))×1000 and expressed as a percentage of the maximal AVP-stimulated cAMP production.

2.6. Intracellular inositol phosphate (IP) accumulation measurement

Agonist-induced IP accumulation in cells was measured as previously described [30]. In brief, cells were grown in 24 well plates and incubated for 24 h in the presence of [^{3}H]myo-IP (1 μCi/ml) in IP-free media consisting of Hank's balanced salt solution supplemented with amino acids from a RPMI 1640 stock solution, 5 mM glutamine, 45 mM sodium bicarbonate, 100 mM HEPES, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml fungizone. Cells were then incubated for 15 min in the presence of 20 mM LiCl prior to stimulation with 1 μM AVP for 15 min at 37 °C. Reactions were stopped on ice, media aspirated and cells incubated for 16 h in the presence of 10 mM ice-cold formic acid at 4 °C. Samples were neutralized with 100 mM ammonium hydroxide and [^{3}H]IP was isolated by anion exchange chromatography as described previously [31]. IP accumulation was expressed as [^{3}H]IP cpm/1000 and as a percentage of the maximal AVP-stimulated IP production.

2.7. [^{3}H]Thymidine incorporation

[^{3}H]Thymidine incorporation was measured as described previously [32]. Cells were grown in 24 well plates and starved for 16 h prior to incubation in the presence of the indicated ligands for 24 h at 37 °C, followed by the addition of 1 μCi/ml of [^{3}H]thymidine for 3 h at 37 °C. Reactions were stopped on ice and cells were washed twice with cold PBS before being fixed with 1 ml 10% TCA for 30 min at 4 °C. Cells were then washed twice with 1 ml 5% TCA and solubilized in 250 μl 0.1 N NaOH/0.1% SDS. Incorporated [^{3}H]thymidine was measured by scintillation counting and expressed as fold over basal [^{3}H]thymidine incorporation.

2.8. Data analysis

Immunoreactivities were determined by densitometric analysis of the films using NIH Image software. Statistical significances of the differences were carried out using unpaired Student's *t*-test where $p<0.05$ was considered statistically significant.

3. Results

3.1. Role of Gα proteins

To determine if a G protein-dependent signalling component contributes to the V2R-mediated ERK1/2 activation, the potential role of different Gα proteins was first examined. To test Gαs's implication, we assessed the effect of down-regulating this G protein α subunit by sustained CTX treatment. Indeed, prolonged treatment with CTX, which induces activation of Gαs by inhibiting its GTPase intrinsic activity, leads to a significant down-

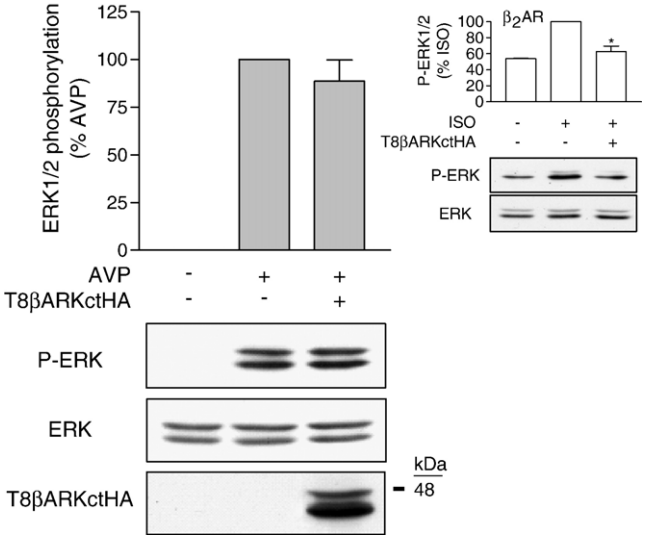

Fig. 3. Gβγ-independent V2R-mediated ERK1/2 activation. HEK293 cells transiently expressing myc-V2R were co-transfected or not with T8βARKct-HA and serum-starved prior to AVP stimulation. Expression levels of the transfected constructs were controlled in western blot using anti-HA 12CA5 (lower panel) or anti-myc 9E10 antibodies (data not shown). *Inset*, ISO-induced ERK1/2 phosphorylation in COS cells transiently expressing myc-β_2AR and co-transfected or not with T8βARKct-HA. Data represent the mean±S.E. of three independent experiments. * indicates $p<0.05$.

regulation of Gαs after 16 h in HEK293 cells [33]. We show in Fig. 1A that, whereas treatment with CTX led to a significant inhibition of V2R-promoted cAMP accumulation (Fig. 1A, *inset*), it produced the opposite effect on the AVP-induced ERK1/2 activity, as reflected by the potentiation of the AVP-stimulated ERK1/2 phosphorylation. Inhibition of the Gαs down-stream effector PKA by the selective inhibitor KT5720 [34] (Fig. 1B, *inset*) led to a similar increase in AVP-induced ERK1/2 phosphorylation (Fig. 1B). These results suggest that not only Gαs is not implicated in the stimulatory pathway linking the V2R to ERK1/2, but that it promotes inhibition of the MAPK through stimulation of cAMP production and the subsequent activation of PKA.

Even if the V2R has been shown to preferentially interact with Gαs, several studies suggested that the V2R can also induce IP production and Ca^{2+} mobilization through Gαq coupling [35,36]. To directly investigate if Gαq signalling could be implicated in the V2R-mediated ERK1/2 activation, we used the RGS domain of GRK2 (RGS_{GRK2}), which has been shown to selectively inhibit Gαq/11-dependent signalling [23]. As shown in Fig. 2A, whereas RGS_{GRK2} efficiently inhibited ERK1/2 phosphorylation mediated by the prototypical Gαq-coupled AT1aR (Fig. 2A, *inset*), over-expression of the Gαq-selective RGS domain had no effect on the AVP-stimulated MAPK activity. Interestingly, however, the RGS_{GRK2} was found to inhibit AVP-induced IP accumulation (data not shown), confirming a possible coupling of the V2R to Gαq that does not contribute to the ERK1/2 stimulatory pathway.

Since several Gαs-coupled receptors, notably the β_2AR [37], were shown to promote MAPK activation following a switch of coupling from Gαs to Gαi/o, we also assessed the potential role of Gαi/o in the V2R-promoted ERK1/2 activation. As shown in Fig. 2B, whereas the selective inhibition of Gαi/o signalling by PTX treatment efficiently blocked MAPK activation by the Gαi-coupled δOR, it had no effect on the V2R-promoted ERK1/2 phosphorylation.

Taken together, these results suggest that the V2R stimulates ERK1/2 activity in a Gα-independent fashion.

3.2. Role of Gβγ subunits

Since Gα and Gβγ subunits have been shown to act on different effectors [38], we then investigated the role of Gβγ subunits in the V2R-promoted ERK1/2 activation. For this purpose, we used the previously characterized Gβγ-scavenger consisting of the C-tail of GRK2 (βARK) conjugated to the extracellular and trans-membrane domains of the CD8 protein that provides a membrane anchor for βARK's C-tail (T8βARKctail) [22]. Over-expression of the T8βARKctail did not affect AVP-stimulated ERK1/2 activity, whereas this Gβγ scavenger efficiently inhibited the Gβγ-dependent ERK1/2 activation by the β_2AR [22] (Fig. 3). Associated with the lack of involvement of the Gα subunits, these data suggest that the V2R-promoted ERK1/2 activation occurs independently of heterotrimeric G protein signalling.

3.3. Enzymes intermediates

We next assessed the involvement of several enzymes previously shown to be involved in different cascades linking GPCR to MAPK [6]. In particular, the roles of phospholipase C (PLC), protein kinase C (PKC), phosphatidyl-inositol-3-kinase (PI3K)

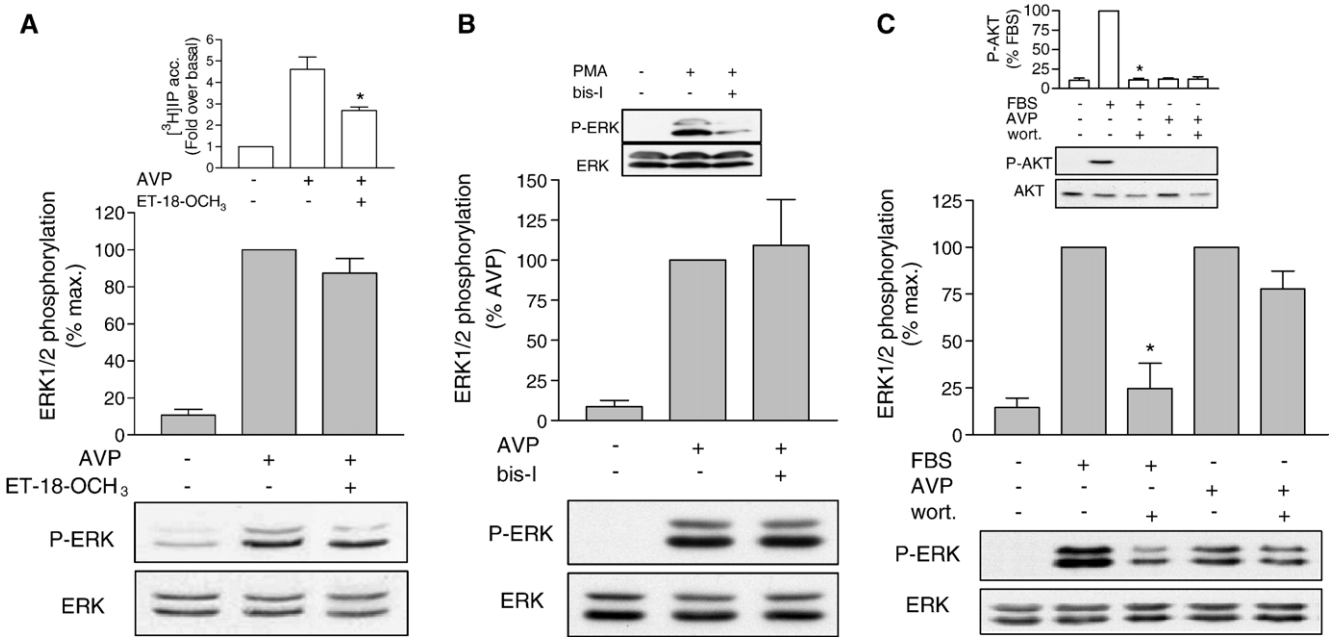

Fig. 4. PLC-, PKC- and PI3K-independent V2R-mediated ERK1/2 activation. Serum-starved HEK293 cells stably expressing myc-V2R were pre-treated or not with the indicated inhibitors at 37 °C prior to stimulation with AVP or FBS. A) Cells were pre-treated for 1 h with 10 μM of the PLC inhibitor ET-18-OCH_3. *Inset*, AVP-induced inositol phosphate (IP) accumulation. B) Cells were pre-treated for 30 min with 500 nM of the PKC inhibitor bisindolylmaleimide I (bis-I). *Inset*, PMA-induced ERK1/2 phosphorylation. C) Cells were pre-incubated for 30 min with 500 nM of the PI3K inhibitor wortmannin (wort.). *Inset*, AKT phosphorylation was measured and quantified by western blot using phospho-specific anti-AKT (P-AKT) and anti-AKT (AKT). Data represent the mean ± S.E. of at least three independent experiments. * indicates $p < 0.05$.

and the protein tyrosine kinase (PTK) c-Src were assessed using the selective inhibitors ET-18-OCH_3 [39], bisindolylmaleimide I [40], wortmannin [41] and PP2 [42], respectively. As shown in Fig. 4, neither the inhibition of PLC (Fig. 4A) nor PKC (Fig. 4B) had any significant effect on the AVP-stimulated ERK1/2 activity. In contrast, the PLC inhibitor significantly reduced the AVP-promoted inositol phosphate accumulation (Fig. 4A, *inset*) and the PKC inhibitor completely blocked PMA activation of ERK1/2 (Fig. 4B, *inset*), confirming the efficiency of the compounds. For PI3K, not only wortmannin treatment did not have a significant effect on the AVP-induced ERK1/2 phosphorylation (Fig. 4C), but AVP failed to induce the phosphorylation of the direct down-stream effector of PI3K, AKT (Fig. 4C, *inset*), strongly suggesting that this kinase is not involved in the V2R-mediated ERK1/2 activation. However, wortmannin completely blocked the FBS-stimulated AKT and ERK1/2 phosphorylation. In contrast to the lack of effect of PKC, PLC and PI3K inhibition, selective blockade of Src family PTK by PP2 completely prevented the AVP-stimulated ERK1/2 activity (Fig. 5). Over-expression of the dominant negative c-Src(K296R/Y528F) mutant [43] also led to a strong inhibition of the V2R-promoted MAPK activation (Fig. 5), suggesting an important role for c-Src in the sought signalling pathway.

3.4. Metalloproteinase trans-activation

In several cases, GPCR-stimulated MAPK activation through a c-Src-family tyrosine kinases dependent process was found to involve pro-ligand ectodomain shedding (eg: HB-EGF) resulting from metalloproteinase activation [44]. To determine whether such metalloproteinase activation is involved in the V2R-mediated ERK1/2 response, we assessed the effect of the metalloproteinase inhibitor phenanthroline on the AVP-induced ERK1/2 phosphorylation. As shown in Fig. 6A, whereas phenanthroline treatment did not affect the EGF-promoted ERK1/2 activation, it almost completely blocked the AVP-stimulated ERK1/2 activity. The role of a metalloproteinase-mediated ligand processing and shedding was next examined by assessing whether the supernatant of AVP-stimulated V2R-expressing cells could induce the activation of ERK1/2 in wild-type HEK293 cells, which do not express endogenous V2R. As shown in Fig. 6B, the supernatant of AVP-treated V2R-expressing cells significantly increased the ERK1/2 activity in wild-type cells. In contrast, a supernatant taken from V2R-expressing cells that were not stimulated with AVP had only marginal effects on ERK1/2 activity. This trans-activation was blocked by phenanthroline (data not shown), suggesting a role for metalloproteinases in this process and ruling out the possibility of a direct effect of AVP present in the transferred supernatant on wild-type HEK293 cells. This is further supported by the observation that AVP stimulation of wild-type cells failed to induce any detectable ERK1/2 phosphorylation (Fig. 6B, *inset*). Given that the EGFR was often shown to be trans-activated by GPCR through an ectodomain shedding mechanism, we then tested if this RTK is involved in the pathway linking the V2R to MAPK activation. As shown in Fig. 6C, this does not seem to be the case since treatment with the selective EGFR inhibitor AG1478 failed to block the AVP-stimulated ERK1/2 activity whereas it efficiently inhibited EGF-induced ERK1/2 phosphorylation (Fig. 6C, *inset*). Despite our efforts to identify the growth-factor receptor system involved, the identity of the pro-ligand being processed remains elusive.

Both Ras-dependent and -independent ERK1/2 stimulatory pathways have been described down-stream of GPCR and trans-activated RTK [45,46]. We thus investigated the potential involvement of Ras in the V2R-promoted MAPK activation by over-expressing the dominant negative mutant of H-Ras, RasS17N [47]. In contrast to its robust inhibition of EGF-induced ERK1/2 phosphorylation, the RasS17N did not affect MAPK activation by the V2R (Fig. 7A), excluding Ras from the pathway.

Even if the V2R has been reported to have non- or even anti-mitogenic effects in different cell types [2,32], our finding that it promotes ERK1/2 activation through an ectodomain shedding trans-activation mechanism suggests that, in this case, it could produce a mitogenic response similar to that induced by the direct stimulation of many RTK. As can be seen in Fig. 7B, stimulation of the β_2AR, previously characterized as mitogenic [2], led to a significant increase in DNA synthesis. In contrast, AVP stimulation of the V2R failed to promote DNA synthesis (Fig. 7B). Taken together, these results demonstrate that the metalloproteinase-mediated trans-activation of ERK1/2 promoted by the V2R activation is Ras-independent and non-mitogenic, clearly distinguishing it from classical direct RTK activation.

3.5. The crucial role of βarrestin

Given that βarrestin was often found to play an important role in GPCR-stimulated ERK1/2 activation [6], its involvement in promoting V2R-mediated MAPK activation was further

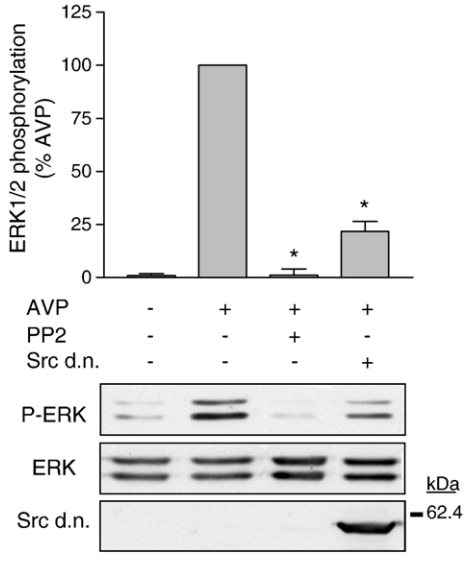

Fig. 5. c-Src is an intermediate in the V2R-mediated ERK1/2 activation pathway. HEK293 cells transiently expressing myc-V2R were co-transfected or not with the c-Src dominant negative mutant c-Src(K296R/Y528F) (Src d.n.) and serum-starved prior to AVP stimulation. Where indicated, cells were pre-treated for 1 h with 50 μM of the Src inhibitor PP2. Expression levels of the transfected constructs were controlled in western blot using either anti-Src EC10 (lower panel) or anti-myc 9E10 antibodies (data not shown). Data represent the mean ± S.E. of at least three independent experiments. * indicates $p < 0.05$.

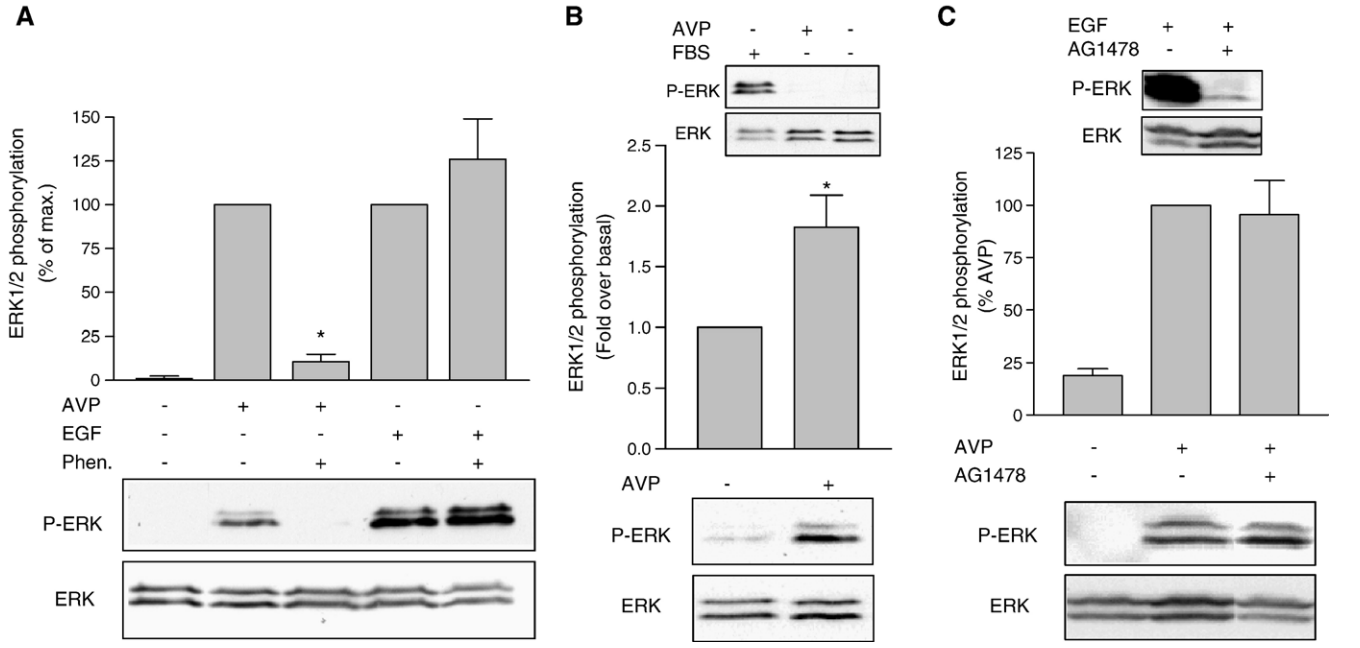

Fig. 6. V2R-mediated ERK1/2 activation involves a metalloproteinase-dependent ectodomain shedding trans-activation event. Serum-starved HEK293 cells were incubated or not for 30 min with the indicated inhibitors at 37 °C prior to stimulation. A) Cells stably expressing myc-V2R were pre-treated with 500 μM 1,10-phenanthroline before AVP or EGF stimulation. B) Wild-type cells were incubated for 2 min with the transferred supernatant from myc-V2R-expressing cells previously stimulated or not for 2 min with AVP. *Inset*, FBS- and AVP-induced ERK1/2 phosphorylation. C) Cells stably expressing myc-V2R were pre-treated with 500 nM of the EGFR inhibitor AG1478 before AVP stimulation. *Inset*, EGF-induced ERK1/2 phosphorylation. Data represent the mean±S.E. of at least two independent experiments. * indicates $p<0.05$.

investigated. First, we assessed the effect of a dominant negative mutant of βarrestin (βarrV53D) on the kinetic of AVP-induced ERK1/2 phosphorylation. As shown in Fig. 8A, the transient V2R-promoted MAPK activation was greatly inhibited by the over-expression of βarrV53D. Similarly, siRNA-directed silencing of the two βarrestin isoforms almost completely blocked ERK1/2 activation by AVP (Fig. 8B), confirming the crucial role of βarrestin in the signalling cascade linking the V2R to ERK1/2 activation.

4. Discussion

Our results show that V2R exerts dual and opposite regulatory influences on ERK1/2 activity. Whereas a G protein-independent pathway involving βarrestin, c-Src, and a metalloproteinase-mediated ectodomain shedding trans-activation event underlies ERK1/2 activation, the concomitant activation of Gαs dampens this stimulatory pathway in a PKA-dependent manner.

Similar observation of a single GPCR having opposing effects on MAPK signalling was previously reported for the Gαs-coupled β_2AR [37]. In that case, the β_2AR-promoted stimulation of Gαs and cAMP production was found to inhibit ERK1/2 activity, whereas the engagement of Gαi by the receptor stimulated the MAPK pathway. Interestingly, coupling to Gαi was found to require prior activation of Gαs, indicating a temporal switch from inhibitory to stimulatory MAPK pathway. This switch mechanism was found to rely on the PKA-mediated phosphorylation of the β_2AR, which enhances the affinity of the receptor for Gαi [48]. Such a switching mechanism cannot be invoked to explain the dual regulation of MAPK by the V2R since AVP-stimulated ERK1/2 activation was found to be independent of both Gαs and Gαi (Fig. 2A). Moreover, the V2R is not a substrate for PKA [49].

Inhibition of ERK1/2 activity through the Gαs–cAMP–PKA cascade results, in many cell types, from the inhibition of the Ras-mediated MAPK activation via phosphorylation and inhibition of the c-Raf-1 isoform [22]. This mechanism [50,51] could be responsible for the inhibitory branch observed in the present study (Fig. 1). The cAMP-mediated inhibitory branch of the MAPK activity could have prevalence in some physiological systems since AVP was previously found to inhibit the EGF-stimulated MAPK activity in a cAMP-dependent manner in Madin–Darby canine kidney cells [52]. In cells of neuronal and hematopoietic origins, cAMP production can also lead to the activation of ERK1/2 through PKA phosphorylation of Rap-1 and the subsequent activation of B-Raf [53,54]. This last pathway, however, is unlikely to contribute to the response promoted by the V2R in HEK293 cells given that the B-Raf-mediated activation appears to be restricted to specific cell types [55].

Coupling to Gαq could be invoked to explain the stimulatory component of the V2R-regulated ERK1/2 activity since, as was previously reported [35,36], the V2R can stimulate IP production (Fig. 4A *inset*). However, this Gαq-dependent pathway does not seem to be required in the activation of ERK1/2 by the V2R since inhibition of Gαq by over-expression of RGS_{GRK2} (Fig. 2A) or the selective inhibition of the classical Gαq effectors PLC and PKC (Fig. 4A,B) did not affect the AVP-stimulated MAPK activity. It should however be noted that Gαq-coupled receptors such as the M1 muscarinic receptor or the oxytocin receptor can activate ERK1/2 via a Gβγ-dependent pathway that

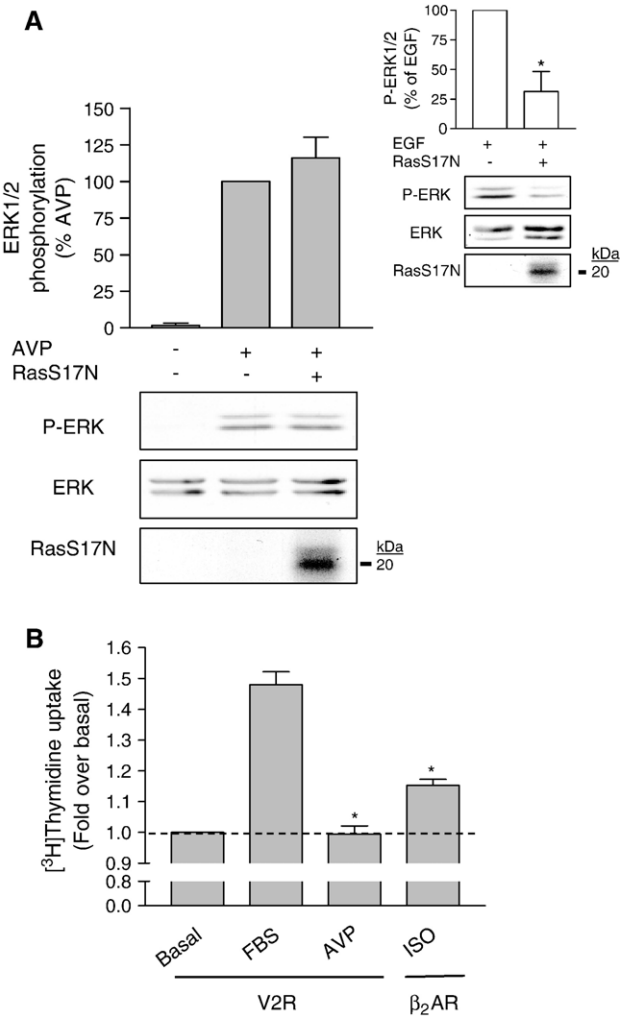

Fig. 7. The ERK1/2 activity stimulated by the V2R is Ras-independent and non-mitogenic. A) HEK293 cells transiently expressing myc-V2R were co-transfected or not with the H-Ras dominant negative mutant RasS17N and serum-starved prior to AVP stimulation. Expression levels of the transfected constructs were controlled in western blot using either anti-H-Ras RAS10 (lower panel) or anti-myc 9E10 antibodies (data not shown). *Inset*, EGF-induced ERK1/2 phosphorylation. B) HEK293 cells stably expressing myc-V2R or myc-β_2AR were serum-starved for 16 h prior to stimulation for 24 h at 37 °C with either AVP, ISO, or FBS. Cells were then incubated for 3 h in the presence of 1 μCi/ml [^{3}H]thymidine before being washed and harvested. Data represent the mean±S.E. of at least three independent experiments. * indicates $p<0.05$.

requires protein tyrosine kinase activity [56,57]. This pathway does not seem to be used by the V2R since over-expression of the Gβγ scavenger T8βARKctail (Fig. 3) was without effect on the AVP-promoted ERK1/2 activation. Taken together our data indicate that the V2R promotes ERK1/2 activation independently of its coupling to heterotrimeric G proteins.

Although the general paradigm of GPCR function entails that they transmit extra-cellular signals to intra-cellular signalling networks via heterotrimeric G proteins, a growing number of observations indicate that they can, in some cases, function independently of their cognate G protein partners [58–60]. For example, mutants of the AT1aR that failed to couple to G proteins have been found to activate the MAPK pathway. Similarly, an ATII analogue that does not promote G protein coupling could efficiently stimulate ERK1/2 phosphorylation. In both cases, the MAPK activation required the presence of βarrestin2 [21]. Independence from G protein coupling was also revealed by the observation that an inverse agonist towards the β_2AR-stimulated adenylyl cyclase activity promoted partial ERK1/2 activation in a βarrestin-dependent manner [20]. To our knowledge however, the V2R is the first example of a wild-

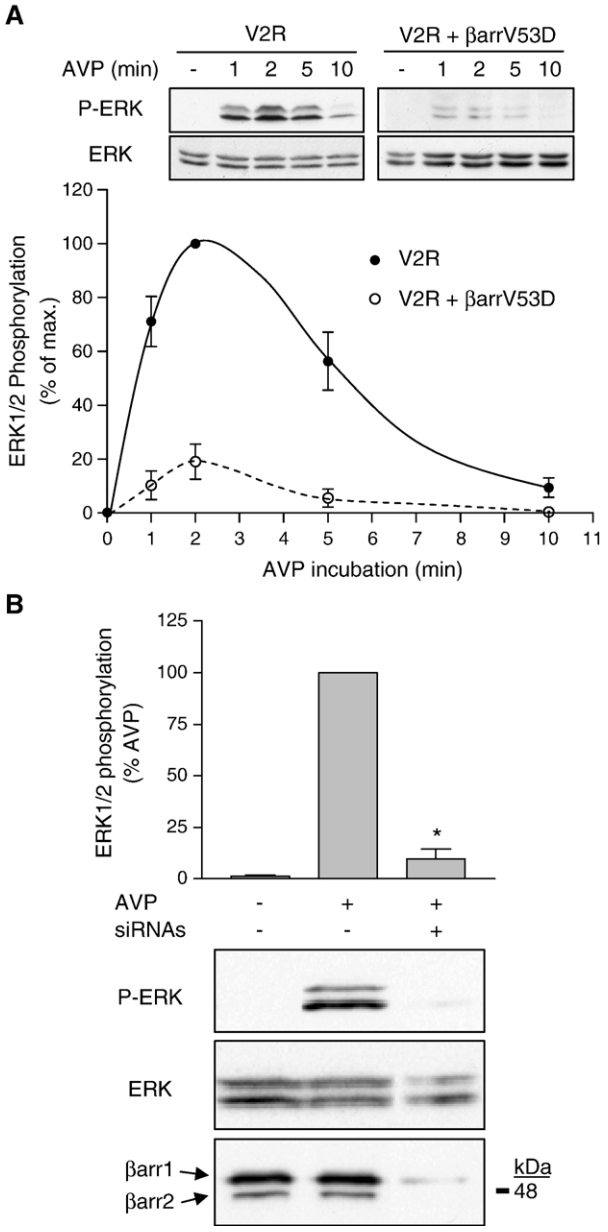

Fig. 8. V2R-mediated ERK1/2 activation requires βarrestin. A) HEK293 cells transiently expressing myc-V2R were co-transfected or not with the dominant negative mutant of βarrestin (βarrV53D) and serum-starved prior to AVP stimulation for the indicated time. Receptor expression level was controlled by western blot (data not shown). B) HEK293 cells stably expressing myc-V2R were transfected with siRNAs targeting both βarrestin isoforms and serum-starved prior to AVP stimulation. To assess βarrestin knock-down, lysates were subjected to western blotting using the anti-βarrestin2 (H9) antibody recognizing both βarrestin isoforms. Data represent the mean±S.E. of three independent experiments. * indicates $p<0.01$.

type GPCR promoting strong G protein-independent activation of ERK1/2 in response to its natural ligand AVP.

When considering its interaction with βarrestin, the V2R is a typical class B receptor undergoing high agonist-induced GRK phosphorylation that leads to stable βarrestin recruitment and a persistent co-localization of the two proteins in endosomes following endocytosis [61]. Such stable association between βarrestin and GPCR has been shown to promote the formation of a multiprotein complex, which can include the receptor, βarrestin, Raf-1, MEK1/2 and activated ERK1/2, leading to endosome-localized ERK1/2 activity [2,16,62]. Such βarrestin-mediated cytosolic retention of activated ERK1/2 could then explain the lack of V2R-mediated mitogenic effects (Fig. 7B and [2]), as this would prevent nuclear translocation of the activated MAPK and the ensuing stimulation of DNA synthesis.

Our observation that c-Src is required for the V2R-dependent activation of ERK1/2 (Fig. 5) is consistent with several studies documenting the role of this tyrosine kinase in the βarrestin-promoted signalling leading to MAPK activation by GPCR [14,17]. Given that Ras is not required for the AVP-stimulated ERK1/2 activation (Fig. 7A) and that c-Src can directly phosphorylate and activate Raf-1 [63], βarrestin is likely to act as a regulator of c-Src-mediated Raf-1 activation by scaffolding the two signalling proteins. The role of βarrestin would then be similar to that of the connector enhancer of the kinase suppressor of Ras-1 (CNK1), which has been shown to mediate the c-Src-dependent tyrosine phosphorylation and activation of Raf-1 through the scaffolding of these two kinases [64]. By allowing such a cross-talk between c-Src and Raf-1, βarrestin would then allow the activation of the Raf-1-MEK1/2-ERK1/2 module by the V2R. This MAPK module seems indeed implicated in ERK1/2 activation by the V2R since the selective inhibition of MEK1/2 completely abolished the AVP-stimulated MAPK activity (data not shown).

The complete inhibition of the V2R-mediated ERK1/2 activation by the dominant negative mutant of βarrestin, βarrV53D, or the RNAi-mediated cellular depletion of βarrestin1/2 (Fig. 8) suggest that the activation is entirely βarrestin-dependent, leaving no other alternative pathway that could connect the V2R to the MAPK. This contrasts with the recent report that V2R can promote ERK1/2 activation through both PKA and βarrestin2-dependent pathways that appear to function as independent path to the MAPK [12]. Although the reason for the difference is not clear, the use of different HEK293 isolates that could harbour distinct signalling partners could be invoked. Indeed, the cellular context has been shown to play a determinant role in directing the specific pathways leading to MAPK activation [65]. In any case, our study clearly indicates that βarrestin is essential to promote strong ERK1/2 activation.

The observations that the general metalloproteinase inhibitor phenanthroline completely inhibited the AVP-stimulated ERK1/2 and that conditioned medium derived from V2R-stimulated cells could activate ERK1/2 in naïve cells strongly suggest the involvement of a metalloproteinase-mediated ligand processing event (Fig. 6). Although c-Src-dependent metalloproteinase cleavage of pro-HB-EGF has previously been shown to be involved in GPCR-mediated ERK1/2 activation [66,67], the EGFR is most likely not the RTK linking the V2R to the MAPK pathway since EGFR inhibition did not affect AVP-stimulated ERK1/2 phosphorylation (Fig 6C). The contribution of another RTK is however supported by the blockade of AVP-induced ERK1/2 activation observed upon non-selective inhibition of RTK (data not shown). Although GPCR-promoted ectodomain shedding has only been experimentally demonstrated for EGFR ligands [66,68], the recent discovery of membrane-tethered pro-ligands for the PDGFR [69] suggests that this receptor also has the potential to be trans-activated via an inside–out model, and that similar pro-ligands might exist for other RTK that have yet to be discovered. The lack of PDGF-stimulated ERK1/2 activity in the HEK293 cells used in the present study rules out the contribution of the PDGFR (data not shown) and calls for future studies aiming to identify the ligand and RTK involved.

In conclusion, our study demonstrates that V2R regulates a delicate balance between activating and inhibiting signals that control ERK1/2 activity. The net effect of AVP stimulation in HEK293 cells favours the activating branch through a G protein-independent pathway involving βarrestin, c-Src and an ectodomain shedding trans-activation event that dominates the Gαs–cAMP–PKA-dependent inhibitory pathway. The intrinsic plasticity conferred by such a dual regulatory mechanism most likely contributes to the integration of multiple signalling cues that can arise from different physiological conditions.

Acknowledgments

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