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Apelin (65–77) Activates Extracellular Signal-Regulated Kinases via a PTX-Sensitive G Protein

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We report here that apelin (65–77) induces activation of extracellular-regulated kinases (ERKs) in Chinese hamster ovary (CHO) cells expressing the msr/apj receptor. This concentration-dependent activation was transient, peaking at 5 min. Pretreatment of CHO cells with pertussis toxin fully abrogated ERK phosphorylation, whereas overexpression of the β-adrenergic receptor kinase-1 C-terminal fragment did not alter ERK activation. Transfection with a dominant-negative mutant of Ras was without effect on ERK activation, whereas an inhibitor of many protein kinase C isoforms, GF109203X, strongly decreased it. These results demonstrate that stimulation of the murine msr/apj receptor promotes ERK activation via the α subunit of a pertussis toxin-sensitive protein in a Ras-independent pathway.

Key Words: **G protein-coupled receptors; phosphorylation cascades; endothelium; vertebrate; mouse.**

Despite their structural similarities, the family of G protein-coupled receptors (GPCRs) binds a large variety of ligands and regulates numerous intracellular transduction cascades in distinct cell subtypes (1–3). Although initially shown to fulfil highly specialized functions in differentiated tissues, G protein-coupled receptors were recently reported to participate in cell growth and oncogenesis (4–6). In addition, ectopic expression of wild-type receptors or overexpression of constitutively activated receptors can trigger neoplastic transformation of cultured fibroblasts (7, 8). Furthermore, a number of human diseases result from point mutations of specific serpentine receptors which lead to oncogenic activation and contribute to tumorigenesis (6, 9).

Transduction from the membrane receptor to the nuclear targets involves activation of the MAP kinase cascade (10), which proceeds through molecular mechanisms depending on the G protein to which receptors are coupled. Gi-coupled receptors transduce a mitogenic signal through the βγ subunits of Gi protein in a Src-dependent and Ras-dependent pathway (4, 11). On the other hand, Gq-coupled receptors initiate a proliferative response via the α subunit of Gq protein and the β isoform of phospholipase C, which activates the MAPK module via IP3, Pyk2, and Src in a Ras-dependent pathway (12, 13), whereas activation of protein kinase C by diacylglycerol (DG) leads to a direct activation of Raf-1 protein in a Ras-independent pathway (14, 15). Gs-coupled receptors can also activate MAPK by a Ras-independent pathway which involves the α subunit of Gs protein, cAMP, PKA, Rap-1, and ultimately B-Raf, which phosphorylates MEK (MAPKK) (16). Finally, some Go-coupled receptors can mediate MAPK activation via a PKC-dependent mechanism in a Ras-independent manner (17).

We recently identified a new G protein-coupled receptor, X-msr, that is expressed in endothelial precursors during early embryogenesis in *Xenopus laevis* (18). Taking advantage of its structural homology to the orphan human h-APJ receptor (19), we cloned the murine msr/apj receptor and showed that its expression pattern is similar to that of the amphibian receptor in embryonic tissues (20). Interestingly, a protein of 36 amino acid residues, designated apelin, was characterized as a putative endogenous ligand for the human APJ receptor (21). The sequence of *apelin* cDNA codes for a preproprotein of 77 amino acids and deletions in the amino-terminus reveal that the biological activity is conserved in the 13 carboxy-terminal residues of preproapelin, apelin (65–77).

In view of the structural properties that it shares with chemokine receptors (22), we speculated that the

Abbreviations used: ARK, adrenergic receptor kinase; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; DG, diacylglycerol; ERK, extracellular-regulated kinase; GPCR, G protein-coupled receptor; IP3, inositol 1,4,5-triphosphate; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; PI, phosphatidylinositol; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin.

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msr/apj receptor may be preferentially coupled to Gi protein, and consequently that apelin could inhibit adenylylcyclase and activate extracellular-regulated kinases (ERKs). As various forms of apelin were recently shown to inhibit adenylylcyclase activity (23), we decided to determine whether apelin could also activate the MAP kinase cascade.

MATERIALS AND METHODS

Chemicals. Pertussis toxin was from Sigma; GF109203X and PD098059 were from Calbiochem; FuGENE 6 Transfection Reagent was from Roche.

Antibodies and plasmids. The mouse monoclonal antibody against the phospho-p44/42 MAP kinase (E10) was purchased from Cell Signaling (Beverly, U.S.A.) and the rabbit polyclonal antibodies against ERK2 (C-14) was from Santa Cruz. The mouse monoclonal pan-ras antibody (Ab-2) was purchased from Oncogene. The rabbit polyclonal antiserum against chloramphenicol acetyltransferase (CAT) was raised in our laboratory by Dr. Maret. The following constructs were used in their expression vectors: βARK-ct in pRK5 (from R. J. Lefkowitz); N17Ras in pcDNA3 (from A. Eychene); V12Ras in pcDNA3 (from McKenzie); 12VCAT in pSCT (from A. C. Prats).

Cell culture and transfections. Chinese hamster ovary (CHO) cells were grown in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum (FCS). For stable expression of the msr/apj receptor in CHO cells, the 1290-bp msr/apj receptor gene fragment was inserted into the pREN expression vector downstream of the cytomegalovirus promoter and upstream of the internal entry ribosome site of encephalomyocarditis virus and the neomycin gene. CHO cells were transfected by calcium phosphate coprecipitation and selected for their resistance to the antibiotic G418. G418-resistant clones were screened for expression of the msr/apj receptor by the ability of apelin (65–77) to decrease cellular cAMP levels in the presence of forskolin.

For transient transfections, we slightly modified the protocol previously described (24). Confluent cells were trypsinized, washed and resuspended (2×10^6 cells/ml) in complete media. Fifteen microliters of Fugene 6 reagent was diluted in 1 ml OPTI-MEM, incubated at room temperature for 10 min and mixed with 5 μg of plasmid DNA. The mixture was then incubated at room temperature for 30 min and added to the cell suspension. CHO cells were incubated for 15 h in the presence of the transfection mixture.

Immunoblotting. CHO cells at subconfluence were serum-deprived for 18 h and then stimulated as indicated for 5 min at 37°C. Cells were washed once in PBS and lysed for 15 min on ice in a Hepes buffer (50 mM, pH 7.4) containing 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, 20 μM leupeptin and 1% Triton. The mixture was gently agitated for 15 min at 4°C and centrifuged at 13,000*g* for 15 min. Soluble proteins (75–100 μg) were fractionated on 10% SDS–polyacrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Activation of ERK1 and ERK2 by phosphorylation on threonine 202 and tyrosine 204 was revealed by immunoblotting with mouse monoclonal antibody against the phospho-p44/42 MAP kinase (E10), while total ERK2 protein was detected using rabbit polyclonal antibodies against ERK2 (C-14). A similar procedure was used for immunoblotting with monoclonal pan-ras antibody or polyclonal CAT antiserum. Immunoreactive proteins were visualized by incubation with a horseradish peroxidase-conjugated secondary antibody and the ECL detection system. The phosphorylation of p42 and p44 MAP kinases was quantified by scanning densitometry using a Personal apparatus. Data are expressed as percentages of the maximal value, obtained with 1 μM apelin (65–77) in CHO cells stably transfected to produce the msr/apj receptor.

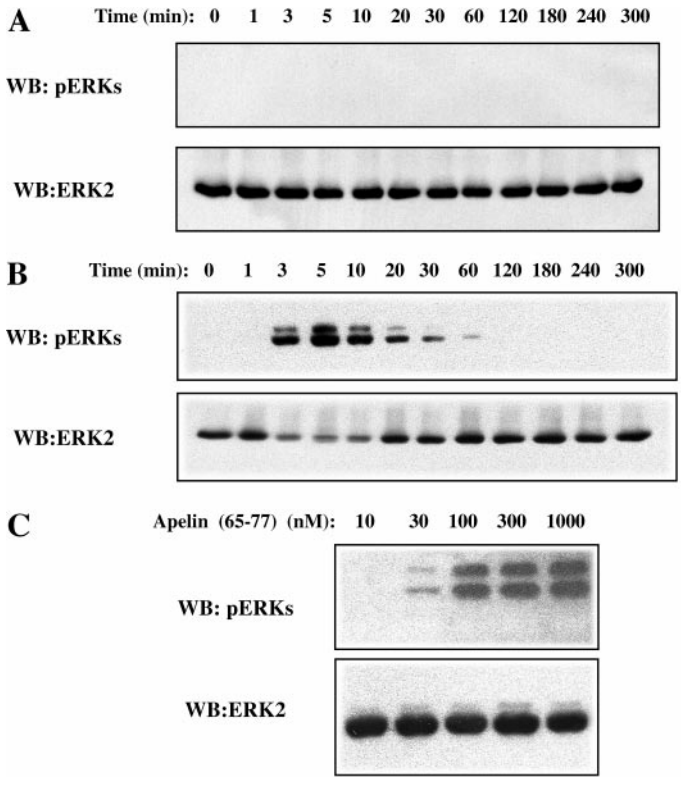

FIG. 1. Apelin (65–77) promotes a transient and concentration-dependent activation of ERKs in CHO cells expressing the msr/apj receptor. CHO cells stably transfected with the empty vector (A) or msr/apj receptor (B) were starved of serum for 20 h. Cells were stimulated with 1 μM apelin (65–77) for the indicated times, lysed and analyzed by SDS–PAGE and immunoblotting for Thr202/Tyr204 phosphorylated ERK1 and ERK2 (top) or total ERK2 protein (bottom), using specific antisera. (C) Activation of ERKs by apelin (65–77) is dose-dependent. Cells were incubated with varying concentrations of apelin (65–77) for 5 min and lysates were immunoblotted as described before. The results shown are representative of three independent experiments.

Phospholipase C assay. The activation of phospholipase C was determined by measuring cellular inositol phosphate accumulation. Cells grown on 24-well plates were labeled overnight with 6 μCi of myo-[^{3}H]inositol per milliliter in inositol-free DMEM medium, treated for 20 min with 20 mM LiCl and then challenged with 1 μM apelin (65–77) for 1 h at 37°C. Reactions were stopped by addition of 1 ml methanol containing 0.1% HCl. Water-soluble inositols were separated by anion-exchange chromatography using AG-X8 resin and quantified by liquid scintillation counting.

Measurement of Ras-GTP. The extent of Ras activation was assessed by measuring the amount of Ras-GTP as described (25). Cells were lysed for 15 min on ice in a Tris buffer (50 mM, pH 8) containing 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 2 μg/ml aprotinin, 20 μM leupeptin. The mixture was gently agitated for 15 min at 4°C and centrifuged at 13,000g for 15 min. Proteins (1 mg) were adsorbed for 40 min at 4°C to bacterially expressed GST-Ras binding domain of Raf. The amount of Ras–GTP proteins was revealed by immunoblotting with monoclonal pan-ras antibody.

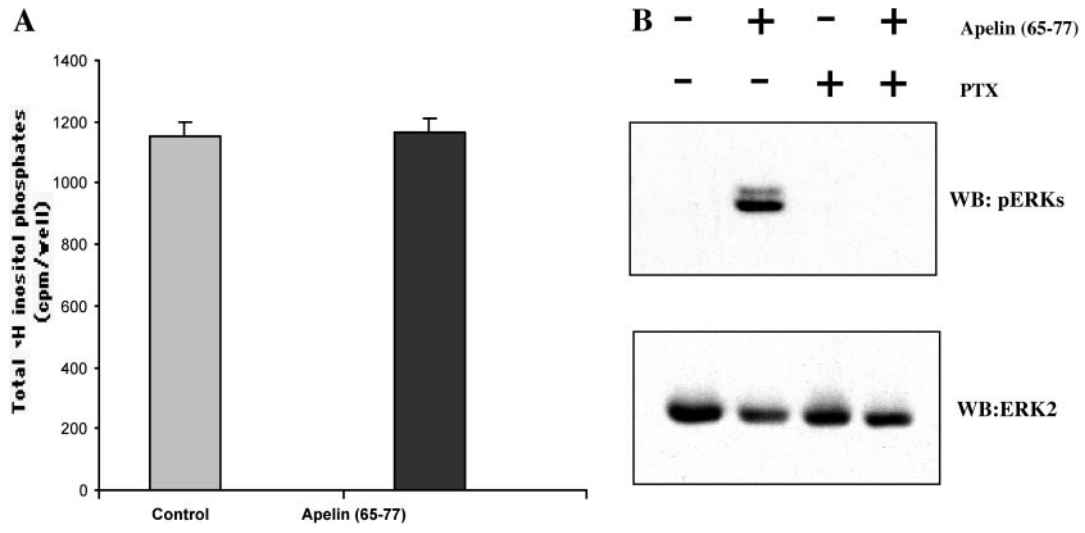

FIG. 2. The msr/apj receptor is not coupled to Gq/G11 protein and uses a pertussis toxin-sensitive protein to stimulate ERK activity. (A) In msr/apj receptor-transfected CHO cells grown on 24-well plates, the increase of total inositol phosphates (PLC activation) was measured with or without stimulation by 1 μM Apelin (65–77) for 60 min. Means ± SEM of duplicates from three independent experiments are shown. (B) CHO cells stably transfected with the msr/apj receptor were grown on 10-cm plates and starved of serum for 20 h. ERK activities in cells stimulated with 1 μM apelin (65–77) for 5 min were determined by immunoblotting to reveal phosphorylated ERK1 and ERK2 (top) or total ERK2 proteins (bottom) and compared with those obtained after pretreatment with pertussis toxin (25 ng/ml, 16 h).

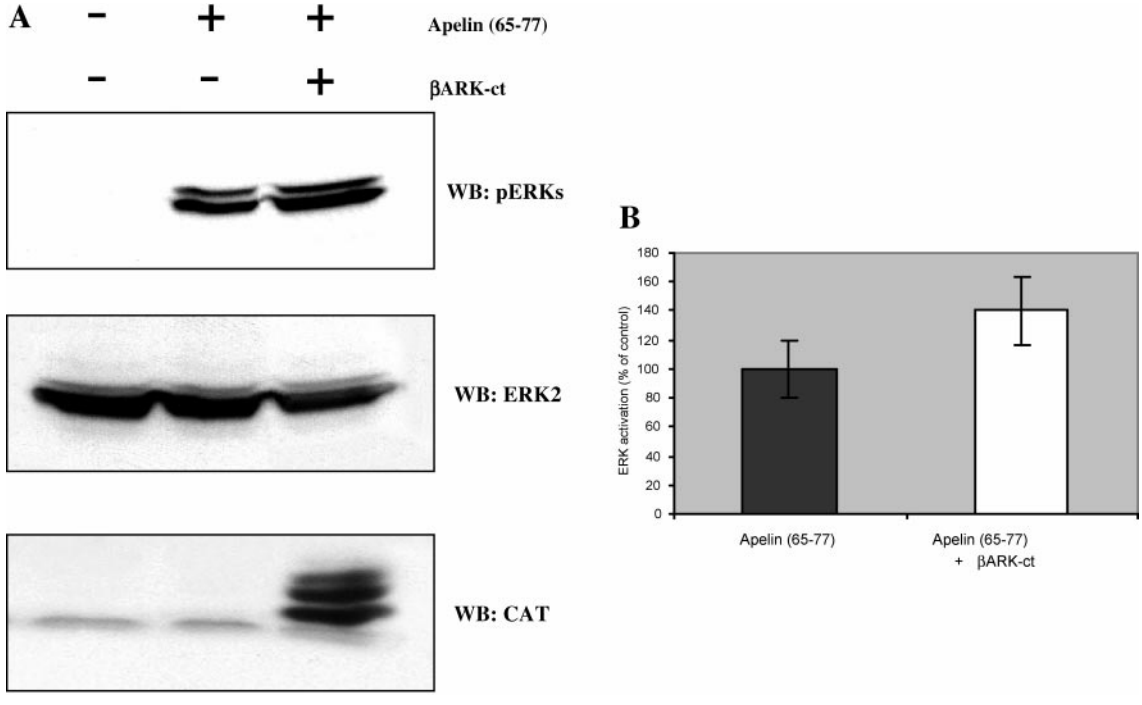

FIG. 3. The apelin-mediated ERK activation in CHO cells is independent of G$\beta\gamma$ subunits. *A*, CHO cells were cotransfected with a pRK5 vector encoding βARK-ct (5 μg/10-cm plate) and a pSCT vector encoding FGF-CAT (5 μg/10-cm plate). The next day, cells were starved of serum for 20 h and stimulated with 1 μM apelin (65–77) for 5 min. ERK activation was monitored by immunoblotting for phosphorylated ERK1 and ERK2 (top) and accurate loading was controlled by Western blotting with antibodies recognizing total ERK2 proteins (middle). To confirm protein expression from the transfected DNAs, the membranes were stripped and blots were reprobed with CAT antibody (bottom). (B) Densitometric analyses of the data from six separate experiments are expressed as percentages of the maximal response obtained with 1 μM apelin (65–77).

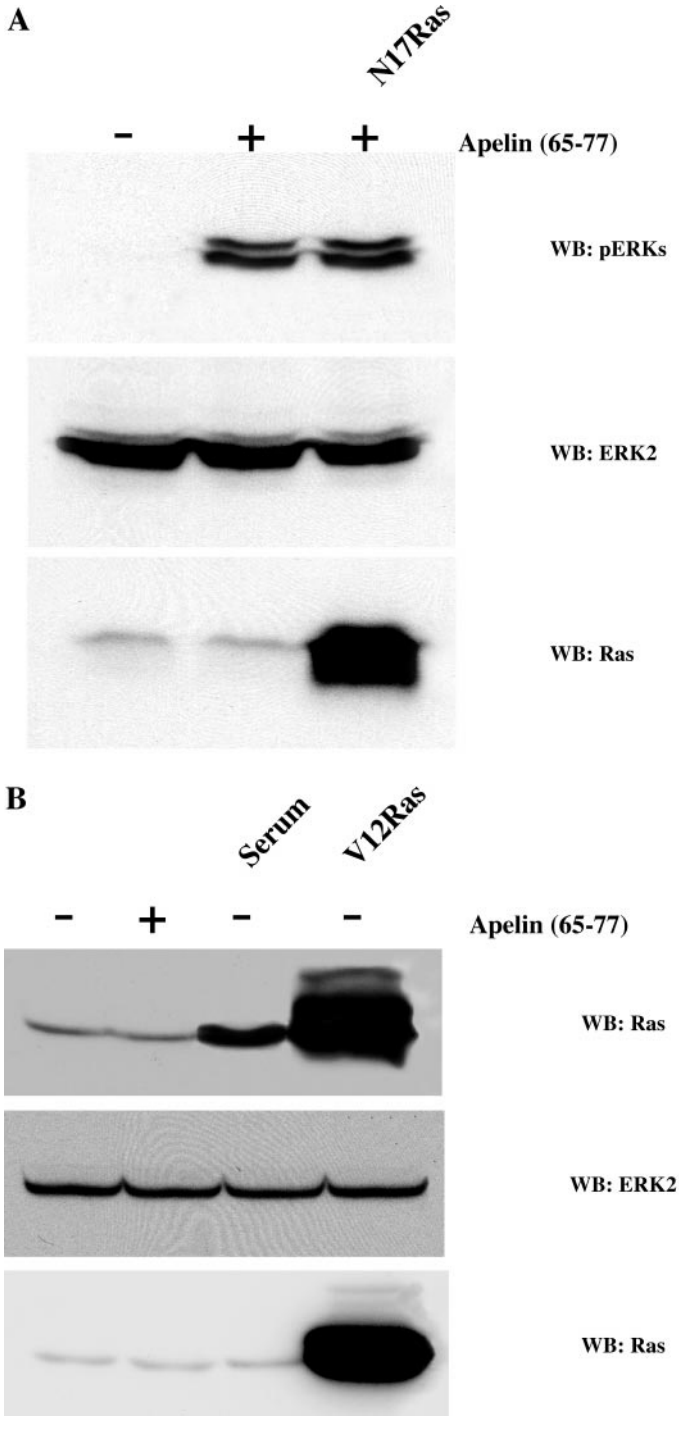

FIG. 4. The apelin-mediated ERK activation in CHO cells is Ras-independent. (A) CHO cells were transiently transfected with 5 μg of empty pcDNA3 vector or a pcDNA vector encoding the dominant-negative mutant N17Ras. The next day, cells were starved of serum for 20 h and stimulated with 1 μM apelin (65–77) for 5 min. Phosphorylated ERK1 and ERK2 (top) were detected by immunoblotting and accurate loading was controlled by blotting with antibodies recognizing total ERK2 proteins (middle). To confirm protein expression of the transfected DNA, the membranes were stripped and blots were reprobed with Ras antibody (bottom). (B) CHO cells were transiently transfected with 5 μg of empty pcDNA3 vector or a pcDNA vector encoding the activated mutant V12Ras. The next day, cells were starved of serum for 20 h and stimulated with 1 μM apelin (65–77) for 5 min or 20% calf fetal serum for 2 min. Cell lysates were adsorbed on GST-Ras binding domain of Raf and the amount of Ras-GTP proteins was revealed by immunoblotting with monoclonal pan-ras antibody (top). Separately, cell lysates were assessed for ERK expression by blotting with antibodies recognizing total ERK2 proteins (middle) and for Ras expression by blotting with monoclonal pan-ras antibody (bottom).

RESULTS

Apelin (65–77) Activates p42 and p44 ERKs

Treatment with 1 μM apelin (65–77) promoted a time-dependent formation of phosphorylated p42 and p44 ERK proteins in CHO cells expressing the msr/apj receptor (Fig. 1B), but not in CHO cells transfected with the empty vector (Fig. 1A). Maximum activation was observed between 5 and 10 min and was abolished by 60 min.

The phosphorylation of MAP kinases in response to apelin (65–77) was concentration-dependent (Fig. 1C) and densitometric analysis showed that the EC_{50} was in the range of 50 nM.

The msr/apj Receptor Activates ERKs via a PTX-Sensitive G Protein

Apelin (65–77) could not trigger an increase of inositol phosphate levels (Fig. 2A), implying that the msr/apj receptor does not couple to Gq/G11 proteins, which are insensitive to pertussis toxin (PTX). Furthermore, pretreatment of CHO cells with PTX, which selectively ADP-ribosylates Gi/Go proteins and uncouples them from their receptor (26, 27), fully abrogated the activation of MAP kinases induced by apelin (65–77) (Fig. 2B). Taken together, these results suggest that, in CHO cells, the msr/apj receptor preferentially couples to a PTX-sensitive protein.

Activation of ERKs Is Independent of the $\beta\gamma$ Complex

The C-terminal fragment of the β-adrenergic receptor kinase (βARKct), which binds free $\beta\gamma$ subunits, has been widely used for demonstrating the role of the $\beta\gamma$ complex of G proteins in signal transduction (28). As shown in Fig. 3A, transfection of βARKct produced no significant effect on the apelin-induced phosphorylation of p42 and p44 proteins (Fig. 3A). In the bottom panel, the detection of the three forms of FGF–CAT chimera resulting from alternative initiation of translation clearly reveals that transient transfection was effective and resulted in overexpression of the corresponding proteins.

In fact, densitometric analysis of immunoblots from five independent experiments (Fig. 3B) revealed a slight increase of MAPK activation in the presence of βARKct. These data therefore suggest that apelin (65–77) acts through the α subunit rather than the $\beta\gamma$

complex of a PTX-sensitive protein to stimulate MAP kinases in CHO cells.

Activation of the MAPK Cascade Occurs via a Ras-Independent Pathway

To characterize the involvement of Ras, we examined the effects of a dominant-negative mutant of Ras, N17Ras. An expression plasmid encoding N17Ras transfected into CHO cells was unable to inhibit apelin-dependent MAPK activation (Fig. 4A), although we detected a strong increase of Ras protein levels following transient transfection. Indeed, we consistently noticed an increase in the amount of p42 and p44 phosphorylation.

As Ras may be activated by a decrease in Ras GAP activity which would not be influenced by N17Ras (29), we directly analyzed the levels of Ras-GTP in the presence of apelin (Fig. 4B). Whereas calf fetal serum or transfection of V12Ras increased the amount of Ras-GTP, 1 μM apelin (65–77) did not modify the amount of Ras-GTP.

Activation of the MAPK Cascade Is PKC-Dependent

To determine the contribution of PKC to MAPK signaling, we applied the PKC inhibitor GF109203X. This decreased MAPK activation in a concentration-dependent manner and led to a strong reduction of MAPK activity at 10 μM (Fig. 5). Densitometric analysis of 4 distinct experiments revealed that the extent of inhibition was close to 70%.

PKC-Dependent Activation of ERK Signaling Involves MEK Activation

Two possible entry points from PKC on the MAPK module have been identified (10, 15, 16). Since both pathways involve activation of MEK protein, we decided to analyze the effect of the specific MEK inhibitor, PD098059. As shown in Fig. 6, ERK activation was fully sensitive to the MEK inhibitor which caused a concentration-dependent decrease of p42 and p44 phosphorylation and resulted in a greater than 90% inhibition of ERK activation.

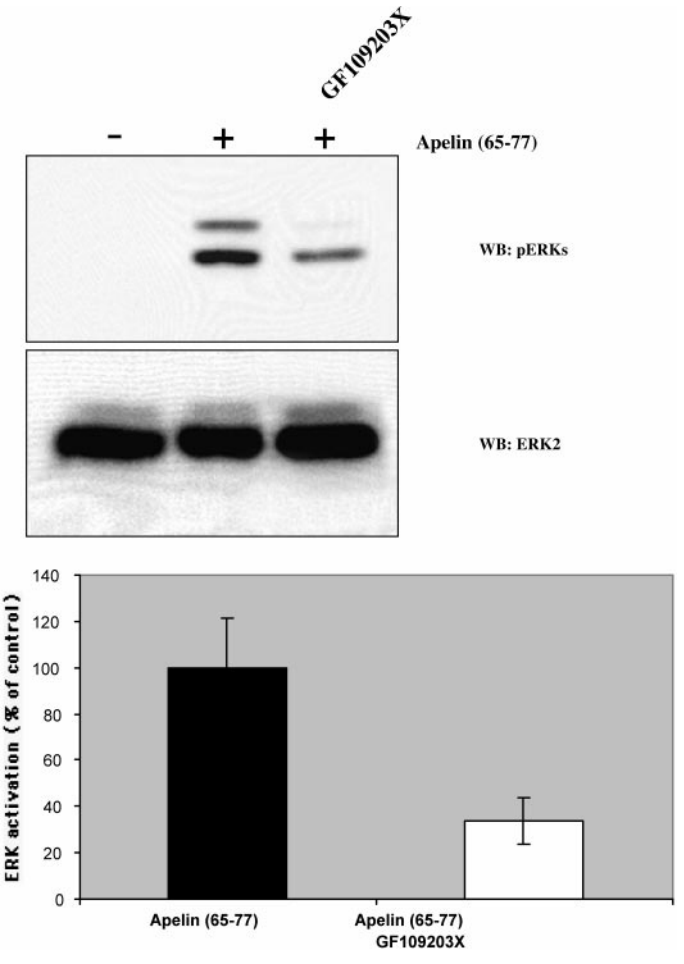

FIG. 5. The apelin-mediated ERK activation is PKC-dependent. CHO cells stably transfected with the msr/apj receptor were grown on 10-cm plates and starved of serum for 20 h. ERK activities in cells stimulated with 1 μM apelin (65–77) for 5 min were determined by immunoblotting to reveal phosphorylated ERK1 and ERK2 (top) or total ERK2 proteins (middle) and compared with those obtained after pretreatment with GF109203X (10 μM, 1 h). Densitometric analyses of four independent experiments (bottom) are expressed as percentages of the maximal response obtained with 1 μM apelin (65–77).

DISCUSSION

Our results clearly establish that apelin (65–77) stimulates, in a dose-dependent fashion, the phosphorylation of ERKs in CHO cells expressing the murine msr/apj receptor. We have characterized various intermediates between the membrane receptor and the MAP kinases by using a pharmacological approach with specific inhibitors and a dominant-negative approach based on transient transfection of inactive forms or quenchers of endogenous proteins.

At the G protein level, the blockade of the apelin-induced ERK activation by pertussis toxin suggests that a Gi or a Go protein exclusively transduces the extracellular stimulation of the msr/apj receptor to the intracellular kinase cascade, at least in CHO cells. Interestingly, a similar coupling has already been described for the inhibition of adenylylcyclase by apelin in the same cells (23). Although MAPK activation via Gi-coupled receptors is often dependent upon the $\beta\gamma$ subunits (10, 16), an activated αi2 subunit mutant constitutively stimulates MAPK (30) and the αi subunit is the mediator of ERK activation following stimulation of the δ-opioid receptor (31). In addition, Go-mediated MAPK activation of M1Ach and PAF receptors essentially involves the αo subunit (17). Similarly, the α subunit of a PTX-sensitive G protein is likely to be the transducer of ERK activation and, in

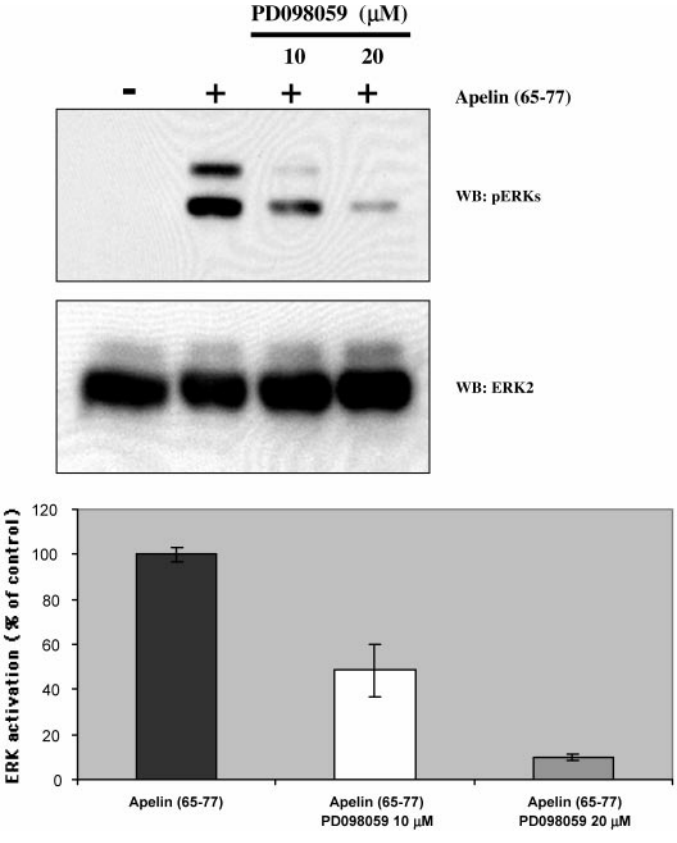

FIG. 6. The ERK activation induced by apelin requires MEK. CHO cells stably transfected with the msr/apj receptor were grown on 10-cm plates and starved of serum for 20 h. ERK activities in cells stimulated with 1 μM apelin (65–77) for 5 min were determined by immunoblotting to reveal phosphorylated ERK1 and ERK2 (top) or total ERK2 proteins (middle) and compared with those obtained after pretreatment with PD098059 (10 μM or 20 μM, 1 h). Densitometric analyses of four independent experiments (bottom) are expressed as percentages of the maximal response obtained with 1 μM apelin (65–77).

view of the established role of the Gi2 protein in adenylylcyclase inhibition (32), a relevant next step would be to investigate whether the msr/apj receptor is coupled, in CHO cells, to a Gi2 protein whose α subunit regulates two downstream effectors, adenylylcyclase and ERKs.

Connection to the MAPK module can occur via two different pathways, Ras-dependent or PKC-dependent respectively (10, 16). The insensitivity to the dominant-negative N17Ras and the lack of increase in the amount of Ras-GTP induced by apelin both clearly demonstrate that the msr/apj receptor uses a Ras-independent pathway to activate ERKs. On the other hand, the activity of the PKC inhibitor GF109203X reveals that a PKC isoform is involved in the apelin-induced activation of ERKs. Indeed, our results concerning apelin signaling are very similar to those described for the δ-opioid receptor (DOR) in Jurkat T lymphocytes (31). In this cell type, the activation of MAP kinases is also sensitive to pertussis toxin, is mediated by the αi subunit and is insensitive to N17Ras.

Interestingly, cAMP signaling can regulate the ERK pathway in as much as Raf-1 is inhibited following phosphorylation by PKA (33); decrease of cAMP levels may thus release PKA-dependent inhibition of Raf-1. Similarly, ERK activation can also result from a mechanism involving activation of Rap1GAP by a GTP-bound αi subunit (34) which counteracts the inhibitory action of Rap1 on Ras signaling by scavenging Raf1 (35). On the other hand, the Goα chain activates the MAP kinases in CHO cells through activation of B-Raf in a PKC-dependent and a Ras-independent manner (36). In the light of all these observations, it remains to be clarified whether the transduction of apelin signaling from the α subunit to the MAPK cascade is mediated via activation of B-Raf or Raf-1.

Activation of the ERKs is tightly linked to cell growth and transformation in various cell lines. In this context, it is striking that the αi-dependent ERK pathway activated by apelin is not restricted to one ligand-receptor couple (31) and has been characterized in other cell types, such as fibroblasts (37, 38) and T lymphocytes (31), in which it always leads to a proliferative response. Finally, this signaling pathway, mediated by the msr/apj receptor and the δ-opioid receptor, resembles that stimulated by the αi2 oncogenic gip2 mutant, which activates MAP kinases (30) and transforms fibroblasts in a Ras-independent manner (37). Accordingly, it is tempting to speculate that apelin can also activate the ERK pathway in the embryonic cell type expressing the msr/apj receptor and thus lead to the proliferation of endothelial progenitors during the formation of the cardiovascular system.

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