Parathyroid Hormone Activates Mitogen-activated Protein Kinase via a cAMP-mediated Pathway Independent of Ras*

(Received for publication, July 8, 1996, and in revised form, September 12, 1996)

Mark H. G. Verheijen and Libert H. K. Defize‡
From the Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

In a previous study, we demonstrated that parathyroid hormone (PTH) inhibits mitogen-activated protein (MAP) kinase activation in osteosarcoma cells via a protein kinase A-dependent pathway. Here, we show that PTH can induce a transient activation of MAP kinase as well. This was observed in both Chinese hamster ovary R15 cells stably expressing high levels of rat PTH/PTH-related peptide receptor and parietal yolk sac carcinoma cells expressing the receptor endogenously. PTH was a strong activator of adenylate cyclase and phospholipase C in Chinese hamster ovary R15 cells. PTH-induced MAP kinase activation did not depend on activation of Gs, phorbol ester-sensitive protein kinase C, elevated intracellular calcium levels, or release of Gβγ subunits. It could, however, be mimicked by addition of forskolin or 8-bromo-cAMP to these cells. Prolonged treatment with forskolin caused sustained protein kinase A activity, whereas MAP kinase activity returned to basal levels. Subsequent treatment with PTH or 8-bromo-cAMP did not result in MAP kinase activation, whereas phorbol ester- or insulin-induced MAP kinase activation was unaffected. Finally, expression of a dominant negative form of Ras (RasA-N17), which completely blocked insulin-induced MAP kinase activation, did not affect activation by PTH or cAMP. In conclusion, PTH regulates MAP kinase activity in a cell type-specific fashion. The activation of MAP kinase by PTH is mediated by cAMP and independent of Ras.

Mitogen-activated protein (MAP)1 kinases are protein serine and threonine kinases that play an important role in the regulation of cell growth and differentiation (1–3). The activity of MAP kinase is under the control of external stimuli that mediate their effects by binding to cell surface receptors. Protein tyrosine kinase receptors transduce the signal by autophosphorylation of tyrosine residues, allowing the receptor to interact with Src homology 2 domain-containing proteins, such as Grb2, which will recruit son of sevenless, resulting in the activation of Ras. This will cause the successive activation of Raf-1, MAP kinase (MEK), and MAP kinase (4).

* This work was supported by Netherlands Organization for Scientific Research Grant 9003 46 102. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 011-31-302510211; Fax: 011-31-302516464; E-mail: bas@hubrecht.nl.
‡ To whom correspondence should be addressed. Tel.: 011-31-302510211; Fax: 011-31-302516464; E-mail: bas@hubrecht.nl.

1 The abbreviations used are: MAP, mitogen-activated protein; PTH, parathyroid hormone; PTHR, PTH-related peptide; GPCR, G protein-coupled receptor; MEK, MAP kinase kinase; LPA, lysophosphatidic acid; CHO, Chinese hamster ovary; PYS, parietal yolk sac; TPA, 12-O-tetradecanoylphorbol-13-acetate; PTX, pertussis toxin; HA, hemagglutinin; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; FCS, fetal calf serum; Gaα, Gα subunit of retinal transducin.

G protein-coupled receptors (GPCRs) regulate MAP kinase activity, depending on the identity of the G protein, the receptor, and the cell type involved. Gα-coupled receptors, such as the M2 muscarine acetylcholine receptor, the α1-adrenergic receptor, or the receptors for lysophosphatidic acid (LPA) or thrombin, stimulate MAP kinase in a Ras-dependent manner (5–7). Recent reports have shown that MAP kinase activation through Gα involves the release of Gβγ subunits (8–11), which via an as yet unidentified tyrosine kinase induce the phosphorylation of Shc, leading to the formation of a Shc-Grb2-son of sevenless complex (12, 13) and activation of Ras.

For receptors coupled to Gαq such as the M1 acetylcholine receptor, the α1-adrenergic receptor, or the bombesin receptor, both βγ subunit-dependent (10) and βγ subunit-independent (8, 11) activation of MAP kinase has been reported. The βγ subunit-induced activation is mediated by Ras (10), whereas the Gα–α-induced activation is mediated by phorbol ester-sensitive protein kinase C (PKC) in a Ras-independent manner (11). Gαq-coupled receptors, such as the β-adrenergic receptor or the pituitary adenylate cyclase-activating polypeptide receptor, can either trigger or inhibit MAP kinase activation (14–16). This is cell type-dependent and can in most cases be mimicked by addition of cell-permeable CAMP analogues. With respect to the inhibition of MAP kinase activation, it was demonstrated that activation of the CAMP-dependent protein kinase A (PKA) interfered with Ras-mediated activation of MAP kinase at the level of Raf-1 (17). However, the mechanism behind the CAMP-mediated activation of MAP kinase, as in PC12 cells, is largely unclear (16, 18).

PTH and PTHR bind to a common receptor, which has been shown to couple to at least two signal transduction systems: (i) a Gα-q-mediated increase in CAMP, leading to activation of PKA (19–21); and (ii) a Gα–α-mediated activation of PLC-β, leading to increases in intracellular inositol triphosphate and calcium levels and activation of PKC (19, 21–23). The identity of downstream effectors and their role in cellular responses to PTH and PTHR are unclear. We have recently demonstrated that PTH inhibits growth factor-induced MAP kinase activation in osteosarcoma cells via a pathway that is dependent on PKA (24).

Here we show for two cell types that triggering of the PTH/PTHrP receptor can also lead to activation of MAP kinase. This activation was not dependent on three well established Gα–α mediated events, i.e.: (i) elevation of intracellular calcium levels, (ii) activation of PKC, and (iii) the release of Gβγ subunits. We provide evidence that the effect is mediated by elevation of intracellular cAMP levels and occurs in a Ras-independent manner.

EXPERIMENTAL PROCEDURES

Materials—Rat PTH(1–34) was purchased from Peninsula Laboratories Europe (St. Helens, United Kingdom). 8-Bromo-cAMP and thapsigargin were obtained from Biomol (Plymouth Meeting, PA). Thrombin, insulin, LPA, isoproterenol, 12-O-tetradecanoylphorbol-13-acetate (TPA), forskolin, pertussis toxin (PTX), and the peptides Leu-Arg-Arg-

This paper is available on line at http://www-jbc.stanford.edu/jbc/
PTH Activates MAP Kinase Independent of Ras

Ala-Ser-Leu-Gly (Kemptide), myelin basic protein, and protein kinase inhibitor were from Sigma. [γ-32P]ATP and ECL were purchased from Amersham Corp. Indo-1 acetoxyethyl ester was obtained from Molecular Probes (Eugene, OR). Polyclonal antibodies against p42 MAP kinase were kindly provided by Drs. J. L. Bos and B. M. T. Burgering (Utrecht University, Utrecht, The Netherlands).

Cell Culture and Transfections—CHO-R15, CHO-β2, and CHO-K1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal calf serum (FCS). PYS-2 cells were grown in medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 containing 7.5% FCS. Transient transfections were performed using the calcium phosphate precipitation method. One day prior to transfection, the CHO-R15 cells were plated at a density of 8 × 10⁴ cells/cm² in six-well tissue culture clusters. The following day they were cotransfected with plasmid DNA encoding p44 HA-MAP kinase (2 µg/well), Go subunit of retinal transducin (Gαo) (1 µg/well), or RasKα17 (3 µg/well). Puer-Rous sarcoma virus plasmid was added to bring the total amount of plasmid DNA to 10 µg/well.

Calcium Measurements—Nearly confluent CHO-R15 monolayers, attached to rectangular glass coverslips, were incubated in Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum for 18 h. The cells were loaded with indo-1 by exposing them to 10 mM indo-1 ester for 40 min at 37 °C. [Ca²⁺]-dependent fluorescence was recorded at an excitation wavelength of 355 nm and an emission of 405 nm (25). Since we were not able to perform proper calibration of the calcium responses in CHO-R15 cells, we related the calcium response to PTH(1–34) to that to thrombin (1 arbitrary unit).

Activation and Phosphorylation of MAP Kinase—The CHO and PYS-2 cells were plated in concentrations of, respectively, 2.5 × 10⁴ and 1 × 10⁶ cells/cm² in six-well tissue culture clusters and grown for 24 h. The cells were incubated in medium containing 0.5% FCS for 18 h and subsequently treated with agents as indicated. MAP kinase phosphorylation was measured by Western blotting with anti-p42 MAP kinase antibodies as described previously (24). Phosphorylated p42 MAP kinase was detected as a band with reduced mobility compared with unphosphorylated p42 MAP kinase (26). Experiments were repeated at least three times, and representative results are shown.

For determination of MAP kinase activity, endogenous p42 MAP kinase and epitope-tagged p44 HA-MAP kinase were immunoprecipitated with protein A-Sepharose beads coupled to, respectively, anti-p42 MAP kinase antibodies and monoclonal antibody 12CA5, as described previously (24, 27). After the kinase reaction with myelin basic protein as a substrate, the reaction mix was subjected to SDS-polyacrylamide gel electrophoresis. Phosphorylation of myelin basic protein was measured using a PhosphorImager and ImageQuant software (Molecular Dynamics).

PKA Activation—CHO-R15 cells were plated at a concentration of 2.5 × 10⁴ cells/cm² in 96-well tissue culture clusters and grown for 24 h. The cells were incubated in medium containing 0.5% FCS for 18 h and subsequently treated with agents as indicated. Activation of PKA was measured as described previously (24, 28). Briefly, digitonin-permeabilized cells were incubated with a salt solution containing [γ-32P]ATP and Kemptide as a substrate, with or without protein kinase inhibitor as an inhibitory peptide. After a 10-min incubation, the reaction was stopped with 25% trichloroacetic acid, and the trichloroacetic acid-soluble material was spotted on phosphocellulose filters. The difference in radioactivity incorporated in the filters between samples treated with protein kinase inhibitor versus non-protein kinase inhibitor-treated samples was defined as PKA activity.

RESULTS

PTH Activates MAP Kinase in CHO-R15 Cells—Whereas in most cell types the PTH/PTHrP receptor is a potent activator of Gαs, the activation of Gαq is suggested to be dependent on receptor density (29). To investigate the effect of PTH on MAP kinase activity in a situation in which the receptor couples strongly to both Gαs and Gαq (21, 29), we used Chinese hamster ovary cells stably transfected with the rat PTH/PTHrP receptor (CHO-R15). Binding studies with a radiiodinated PTH analogue, PTH(1–34), revealed that these cells express approximately 300,000 PTH/PTHrP receptors per cell. Activation of MAP kinase was detected using a gel mobility shift assay (26).

Treatment of the cells with 10⁻⁷ M PTH(1–34) induced a transient phosphorylation of MAP kinase, which was maximal after 5–10 min and returned to a basal level within 60 min (Fig. 1A). PTH(1–34) induced MAP kinase phosphorylation in a dose-dependent manner, starting at 10⁻¹¹ M (Fig. 1B).

Fig. 1. PTH induces phosphorylation of MAP kinase in CHO-R15 cells. A, cells were treated with 100 nM PTH(1–34) for indicated periods. B, cells were treated with the indicated concentrations of PTH(1–34) for 10 min. Samples were treated as described under “Experimental Procedures.” Immunoblots against p42 MAP kinase are shown. Phosphorylated MAP kinase (pp42) is detected as a band with reduced mobility compared with that of unphosphorylated MAP kinase (p42).

PTH-induced MAP Kinase Activation Is Not Dependent on Gαs, Gβγ Subunit Release, Phorbol Ester-sensitive PKC, or Increase in Intracellular Calcium—Well-established pathways used by GPCRs in the activation of MAP kinase involve Gαi or PKC (5–7, 11, 30, 31). We tested their involvement in the PTH-induced MAP kinase activation by a prolonged treatment of the cells with PTX and TPA to respectively inhibit Gαi and down-modulate PKC. As expected, PTX inhibited the thrombin-induced MAP kinase activation by approximately 80%, whereas prolonged TPA treatment completely inhibited the TPA-induced MAP kinase activation. Neither of the treatments affected the activation of MAP kinase by PTH (data not shown). Combined treatment with PTX and TPA completely inhibited the activation of MAP kinase by both thrombin and TPA but had no effect on the activation of MAP kinase by PTH(1–34) (Fig. 2). Thus it appears that the PTH-induced MAP kinase activation is not dependent on Gαi or TPA-sensitive PKC.

Stimulation of CHO-R15 cells with PTH(1–34) induced a transient increase in intracellular calcium, as shown in Fig. 3A. Several reports demonstrate that an increase in intracellular calcium can be sufficient to activate MAP kinase (32–35). We first examined whether PTH mobilizes calcium from an intracellular or extracellular source by incubating the cells with thapsigargin. This depletes calcium from intracellular stores (36). As is demonstrated in Fig. 3A for FCS, the rapid component, due to release of calcium from intracellular stores, was completely inhibited by a 40-min thapsigargin pretreatment, whereas the slower component, due to calcium influx, was not. The calcium response to PTH(1–34) was completely inhibited by thapsigargin, suggesting that PTH induces release of calcium from intracellular stores. Thapsigargin treatment by itself induced transient MAP kinase phosphorylation (Fig. 3B), showing that also in CHO-R15 cells, a strong increase in intracellular calcium can be sufficient to activate MAP kinase. To determine whether an increase in calcium levels is necessary for the activation of MAP kinase by PTH, we prevented both calcium release and calcium influx by preincubating the cells with EGTA followed by thapsigargin. 30 min after the addition of these reagents, the phosphorylation of MAP kinase

2 M. H. G. Verheijen and L. H. K. Defize, unpublished observations.
had returned to basal levels. Subsequent addition of PTH(1–34) stimulated MAP kinase phosphorylation to a similar level as in control cells, indicating that an increase in intracellular calcium levels is not required for MAP kinase activation by PTH.

Another GPCR-mediated event, described as being involved in the activation of MAP kinase, is the release of Gβγ subunits (8). To examine a possible role of Gβγ subunits in the PTH-induced MAP kinase activation in CHO-R15 cells, we expressed Goα to sequester Gβγ subunits after they are released from G proteins on receptor stimulation (8). Fig. 4 shows that Goα expression efficiently inhibits the activation of a cotransfected, hemagglutinin-tagged MAP kinase (HA-MAP kinase) (27) by LPA, showing that Gβγ subunits are indeed efficiently sequestered (9, 13). However, PTH(1–34)-induced MAP kinase activation is not affected, suggesting that this is not dependent on the release of Gβγ subunits.

_Elevation of Intracellular cAMP Levels Induces MAP Kinase Activation in CHO Cells and Is Involved in the PTH-induced MAP Kinase Activation—PTH is a strong activator of adenylate cyclase in CHO-R15 cells (data not shown). Elevation of intracellular cAMP levels inhibits MAP kinase activation in many cell types (17), and we have previously shown that it is involved in the inhibition of MAP kinase by PTH in osteosarcoma cells (24). We were therefore surprised to find that elevation of intracellular cAMP levels by addition of forskolin or the cell-permeable cAMP analogue 8-bromo-cAMP induced a transient phosphorylation of MAP kinase in CHO-R15 cells (Fig. 5A). The kinetics and the quantity of this phosphorylation were comparable with the phosphorylation induced by PTH(1–34) (Fig. 1A). Also, in wild-type CHO cells (CHO-K1), elevation of cAMP levels resulted in MAP kinase phosphorylation (Fig. 5B). To examine whether Goα-induced cAMP formation is sufficient for MAP kinase activation, we measured the activation of MAP kinase by a typically Gα-coupled receptor, the β-adrenergic receptor. Addition of isoproterenol to CHO cells stably transfected with the β-adrenergic receptor (CHO-β2) (37) resulted in a phosphorylation of MAP kinase with the same quantity and kinetics as the phosphorylation induced by PTH in CHO-R15 cells (Fig. 5C). 8-Bromo-cAMP also induced phosphorylation of MAP kinase in these cells (not shown). Thus, elevation of cAMP levels through activation of Goα is sufficient to explain MAP kinase activation by PTH in CHO-R15 cells. To determine whether elevation of cAMP could account for MAP kinase activation by PTH, we measured activation of MAP kinase after

---

**Fig. 2.** PTH-induced MAP kinase activation is not dependent on either Gα or phorbol ester-sensitive PKC. CHO-R15 cells were incubated overnight with 100 ng/ml TPA and 200 ng/ml PTX. The following day, the cells were incubated with 100 nM PTH(1–34) (10 min), 1 unit/ml thrombin (5 min) or 100 ng/ml TPA (10 min), as indicated. MAP kinase activity was assayed using myelin basic protein as a substrate, as described under "Experimental Procedures." Results are expressed as percentage of the response in control untreated cells. Activation of MAP kinase by PTH(1–34) and LPA in control cells was 5.4- and 11.3-fold, respectively. The experiment was repeated twice; a representative result is shown.

**Fig. 3.** PTH-induced MAP kinase activation is not dependent on an increase in intracellular calcium. A, indo-1-loaded CHO-R15 cells were left untreated or pretreated with 1 μM thapsigargin for 40 min and subsequently stimulated with 500 nM PTH(1–34) and 0.5% FCS. The increase in intracellular calcium was measured as described under "Experimental Procedures." B, CHO-R15 cells were left untreated (lane 1), treated with 100 nM PTH(1–34) for 10 min (lane 2) or 1 μM thapsigargin for 5 min (lane 3), or pretreated with 5 mM EGTA for 5 min and subsequently treated with 1 μM thapsigargin for 5 min (lane 4). Thapsigargin-induced MAP kinase phosphorylation is less in EGTA-treated cells, which is consistent with work of others (32). Furthermore, CHO-R15 cells were pretreated with 5 mM EGTA for 5 min and subsequently treated with 1 μM thapsigargin for 40 min with (lane 6) or without (lane 5) 100 nM PTH(1–34) during the last 10 min. MAP kinase activation was determined by a mobility shift.

**Fig. 4.** PTH-induced MAP kinase activation is not dependent on release of Gβγ subunits. CHO-R15 cells were transiently transfected with hemagglutinin-tagged MAP kinase, with or without Goα. Activation of HA-MAP kinase by 100 nM PTH(1–34) (10 min) or 1 μM LPA (5 min) was measured as described under "Experimental Procedures." Results are expressed as percentage of the response in control cells. Activation of MAP kinase by PTH(1–34) and LPA in control cells was 5.4- and 11.3-fold, respectively. The experiment was repeated twice; a representative result is shown.
prolonged incubation (2 h) with forskolin. This resulted in a sustained PKA activation (Fig. 6B), whereas MAP kinase activity had returned to basal levels (Fig. 6A). Subsequent stimulation with insulin or TPA resulted in phosphorylation of MAP kinase to a comparable level as in nonpretreated cells, whereas addition of 8-bromo-cAMP or PTH(1–34) no longer induced phosphorylation of MAP kinase (Fig. 6A). 8-Bromo-cAMP or PTH had no or little effect on PKA activity in cells preincubated with forskolin (Fig. 6B). Similar results were obtained when cells were incubated overnight with cholera toxin (data not shown). Importantly, preincubation with forskolin had no effect on the PTH(1–34)-induced calcium release obtained when cells were incubated overnight with cholera toxin (data not shown). Importantly, preincubation with forskolin had no effect on the PTH(1–34)-induced calcium release (Fig. 6C), showing that PTH/PTHrP receptor functioning was not impaired. Taken together, these results suggest that the activation of MAP kinase by PTH is mediated solely by elevation of intracellular cAMP levels.

**cAMP and PTH Activate MAP Kinase via a Ras-independent Pathway**—Common pathways for the activation of MAP kinase by both protein tyrosine kinase receptors and GPCRs involve the activation of Ras (17). To examine the involvement of Ras in the activation of MAP kinase by PTH, we interfered with Ras-mediated signaling by overexpression of a dominant-negative form of Ras, Ras<sup>Asn-17</sup> (38). This was performed either by infection of the cells with recombinant vaccinia virus expressing Ras<sup>Asn-17</sup>, to interfere with endogenous Ras molecules, or by cotransfection of Ras<sup>Asn-17</sup> and HA-MAP kinase. Stimulation with insulin and TPA were used as controls for, respectively, Ras-dependent and -independent activation of MAP kinase (38, 39). Infection with wild-type vaccinia virus had no effect. As expected, expression of Ras<sup>Asn-17</sup> completely inhibited the activation of MAP kinase by insulin, whereas TPA-induced MAP kinase activation was not affected (Fig. 7A). Interestingly, forskolin and PTH(1–34) also were still able to induce MAP kinase activation. Similar results were seen with transfected RAS<sup>Asn-17</sup> on the activation of cotransfected HA-MAP kinase (Fig. 7B). These results suggest the existence of a Ras-independent MAP kinase-activating pathway in CHO cells, which involves elevated cAMP levels and can be triggered by the PTH/PTHrP receptor.

**PTH Activates MAP Kinase in PYS-2 Cells**—To test the relevance of our findings for PTH/PTHrP receptor signaling, we tested several cell lines that express the receptor endogenously. Fig. 8 shows that PTH(1–34) can also induce MAP kinase activation in one such cell line, parietal yolk sac carcinoma (PYS-2) cells (40). This activation was not affected by prolonged TPA treatment, suggesting that TPA-sensitive PKC is not involved. PTH is a strong inducer of cAMP formation in PYS-2 cells (40), and forskolin induced an activation of MAP kinase in these cells as well, suggesting a comparable mechanism for PTH-induced MAP kinase activation in PYS-2 and CHO-R15 cells.

**DISCUSSION**

In the present study, we show that stimulation of the PTH/PTHrP receptor induces activation of MAP kinase in CHO-R15 and PYS-2 cells. PTH-induced MAP kinase activation was not dependent on PLC-mediated events but appeared to be mediated by elevation of cAMP levels and to occur in a Ras-independent fashion.

Signaling via the PTH/PTHrP receptor involves the activation of at least two G proteins, G<sub>q</sub> and G<sub>q</sub>. We have recently shown that PTH inhibits the activation of MAP kinase in UMR 106 and ROS 17/2.8 cells through activation of PKA (24). Although PLC-β-mediated events can induce MAP kinase activation (11, 30, 31, 34), the activation of PLC-β by PTH was apparently not sufficient to affect MAP kinase activity in these cells. It has been reported that the efficiency of coupling of the PTH/PTHrP receptor to G<sub>q</sub> and PLC-β is related to receptor
density (29). Because the PTH/PTHrP receptor couples strongly to PLC-β in CHO-R15 cells, most likely because of the high levels of receptor expression, we examined whether this might explain the differences between the action of PTH on MAP kinase in CHO-R15 and osteoblast-like cells.

Our data suggest that typical PLC-β-mediated events, such as release of calcium from intracellular stores and activation of PKC, are not involved in the activation of MAP kinase by PTH. Thapsigargin treatment prevented the PTH-induced calcium response but had no effect on the activation of MAP kinase by PTH, suggesting that the calcium increase is not necessary for the PTH-induced MAP kinase activation. Nevertheless, the small increase in calcium observed with PTH in untreated cells could still be sufficient for MAP kinase activation. However, since a combined treatment with PTX and TPA completely blocked the thrombin-induced MAP kinase activation (Fig. 2), whereas the thrombin-induced calcium increase was not affected (not shown), and since the calcium response to PTH is weaker then the one observed with thrombin, this suggests that the calcium response to PTH is not sufficient to activate MAP kinase and that a strong response, like that with thapsigargin, is needed to activate MAP kinase. This is supported by the observation that prolonged cAMP elevation completely abolished MAP kinase activation by PTH without affecting the calcium response. An essential role of PKC was excluded by the observation that down-modulation of phorbol ester-sensitive PKC did not affect the activation of MAP kinase by PTH. These data suggest that the PTH-induced MAP kinase activation is not depending on PLC-β activity.

Other described intermediates between MAP kinase activation and GPCRs are Gβγ subunits. The action of Gαi on MAP kinase is established to be fully dependent on Gβγ subunits (8–11, 13). Studies concerning the role of Gβγ subunits in MAP kinase activation via Gαi have produced contradictory results. It was demonstrated that activation of MAP kinase by the Gαi-coupled β-adrenergic receptor in COS-7 cells is mediated by Gβγ subunits and is fully dependent on Ras (14). However, others reported that expression of the Gα1 coupled D1A dopamine receptor in COS-7 cells did not lead to activation of Ras (9). Studies on the activation of MAP kinase by Gα1-coupled receptors have produced contradictory results as well. It was reported that activation of MAP kinase by the M1 acetylcholine receptor occurred in a Gβγ- and Ras-dependent manner (10), while others showed that triggering of the M1 acetylcholine receptor, the α1B-adrenergic receptor, or the bombesin receptor resulted in MAP kinase activation that was independent of Gβγ subunits (8, 9, 11) and mediated by PKC in a Raf-dependent but Ras-independent manner (11). In this study, we show that sequestering of Gβγ by overexpressed Gαi blocked LPA-induced MAP kinase activation but did not inhibit the action of PTH, suggesting that the activation of MAP kinase by PTH is not dependent on the release of Gβγ subunits. As for most pathways involved in receptor-mediated MAP kinase activation, the Gβγ subunit-mediated activation of MAP kinase also depends on Ras (9–12, 13, 14). Here we show that inhibition of Ras-mediated signaling by overexpression of Ras<sup>Q61K</sup> completely blocks insulin-induced MAP kinase activation, whereas it does not interfere with PTH-induced MAP kinase activation. This suggests that Ras is not involved in the activation of MAP kinase by PTH. Since expression of Ras<sup>Q61K</sup> inhibits the activation of Ras and not the basal levels of Ras activity (43), it is still possible that PTH acts in cooperation with basal Ras activity, as has been suggested for the activation of MAP kinase by PKC (17).

The time course and extent of MAP kinase activation by

**Fig. 7.** MAP kinase activation by PTH or other cAMP-elevating agents is not dependent on Ras. A, uninfected CHO-R15 cells or CHO-R15 cells infected overnight with either wild-type (vv-wt) or vv-Ras<sup>Q61K</sup> expressing vaccinia virus (vv-Ras<sup>Q61K</sup>) were stimulated with 1 μg/ml insulin, 100 nM PTH(1–34), 10 μM forskolin, or 1 ng/ml TPA for 10 min. Activation of endogenous MAP kinase is shown as -fold increase of MAP kinase activity in unstimulated cells. Both experiments were repeated three times, and a representative result is shown. B, CHO-R15 cells were transiently transfected with HA-MAP kinase, with or without Ras<sup>Q61K</sup>. Cells were stimulated with 1 μg/ml insulin, 100 nM PTH(1–34), or 1 mM 8-bromo-cAMP for 10 min. Activation of HA-MAP kinase is shown as -fold increase of MAP kinase activity in unstimulated cells. Both experiments were repeated three times, and a representative result is shown.

**Fig. 8.** PTH activates MAP kinase in PYS-2 cells. PYS-2 cells were stimulated with 100 pM PTH(1–34), 100 nM PTH(1–34), 10 μM forskolin, or 100 ng/ml TPA for 10 min. Activation of MAP kinase is shown as -fold increase of MAP kinase activity in untreated cells. The data represent the means ± S.D. (bars) of triplicate samples.
cAMP and PTH were identical, and, importantly, both were shown to be independent of Ras. Sustained elevation of cAMP levels by forskolin or cholera toxin prevented activation of MAP kinase by cAMP or PTH, whereas insulin- or TPA-induced MAP kinase activation was not affected. This suggests that elevation of cAMP levels is the sole mediator of PTH on MAP kinase. The PTH-induced calcium transient was, under these conditions, similar to that in control cells. This shows that PTH/PTHrP receptor activation was not impaired, at least with respect to PLC-β activation. Taken together, these results strongly suggest that PTH-induced MAP kinase activation is mediated by elevation of intracellular cAMP levels.

Numerous reports have documented effects of cAMP on MAP kinase activity. The mechanism involved heavily depends on the cell type studied. It is well established that elevation of intracellular cAMP levels inhibits MAP kinase activation in fibroblasts, arterial smooth muscle cells, adipocytes, and osteoblasts (17, 24). It was demonstrated that PKA interfered with MAP kinase activation in the level of Raf-1 (44). Activation of MAP kinase by cAMP was first reported in COS and PC12 cells (8, 16). A number of recent reports demonstrated the same phenomenon in other cell types, e.g., Swiss-3T3, melanoma, pituitary, and ovarian granulosacells (45–48).activation of MAP kinase by cAMP or PTH, whereas insulin- or TPA-induced MAP kinase activation was not affected. This suggests that elevation of cAMP levels is the sole mediator of PTH on MAP kinase. The PTH-induced calcium transient was, under these conditions, similar to that in control cells. This shows that PTH/PTHrP receptor activation was not impaired, at least with respect to PLC-β activation. Taken together, these results strongly suggest that PTH-induced MAP kinase activation is mediated by elevation of intracellular cAMP levels.

Numerous reports have documented effects of cAMP on MAP kinase activity. The mechanism involved heavily depends on the cell type studied. It is well established that elevation of intracellular cAMP levels inhibits MAP kinase activation in fibroblasts, arterial smooth muscle cells, adipocytes, and osteoblasts (17, 24). It was demonstrated that PKA interfered with MAP kinase activation in the level of Raf-1 (44). Activation of MAP kinase by cAMP was first reported in COS and PC12 cells (8, 16). A number of recent reports demonstrated the same phenomenon in other cell types, e.g., Swiss-3T3, melanoma, pituitary, and ovarian granulosacells (45–48). It was demonstrated that PKA interfered with MAP kinase activation in the level of Raf-1 (44). Activation of MAP kinase by cAMP was first reported in COS and PC12 cells (8, 16). A number of recent reports demonstrated the same phenomenon in other cell types, e.g., Swiss-3T3, melanoma, pituitary, and ovarian granulosacells (45–48).

Acknowledgments—We thank Drs. J. L. Bos and B. M. T. Burgering for the cAMP-responsive adenovirus and generous supply of antibodies, A. B. Abou-Samra (Massachusetts General Hospital and Harvard Medical School, Boston, MA) for the CHO-R15 cells, P.-O. Couraud (Université Paris VII, Paris, France) for the CHO-b2 cells, H. R. Bourne (University of California, San Francisco, CA) for the Ge2 expression construct and J. Pouyssegur (Université de Nice, France) for providing the p44 HA-MAP kinase expression construct. We thank J. den Hertog for critically reading the manuscript.

REFERENCES

1. Pagès, G., Lenormand, P., L’Allemain, G., Chambard, J. C., Meloche, S., and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8319–8323
2. Troppmair, J., Bruder, J. T., Munoz, H., Lloyd, P. A., Kyriakis, J., Banerjee, P., Arvuch, J., and Rapp, U. R. (1994) J. Biol. Chem. 269, 7030–7035
3. Marshall, C. J. (1995) Cell 86, 179–185
4. Vandergeer, P., Hunter, T., and Lindberg, R. A. (1994) Annu. Rev. Cell. Biol. 10, 251–337
5. Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dweyer, L., and Johnson, G. L. (1993) J. Biol. Chem. 268, 19196–19199
6. Alblass, J., van Corven, E. J., Hordijk, P. L., Milligan, G., and Moolaenar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
7. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolaenar, W. H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1257–1261
8. Faure, M., Voyer-Yanaseckaya, T. A., and Bourne, H. R. (1994) J. Biol. Chem. 269, 7651–7654
9. Koch, W. J., Hawes, B. E., Allen, L. F., and Leukowitz, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 12706–12710
10. Crespo, P., Xu, N., Simonds, W. F., and Gutfink, J. S. (1994) Nature 369, 418–420
11. Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M., and Leukowitz, R. J. J. Biol. Chem. 270, 17148–17153
12. Carubou, S. M., Ramos-Murales, F., Fischer, S., Schweighoffer, F., Strusberg, A. D., and Couraud, P. O. (1994) J. Biol. Chem. 269, 24805–24809
13. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Tsuchara, K., Portiri, E., Sakaue, M., Luttrell, L. M., and Leukowitz, R. J. (1995) Nature 376, 781–784
14. Crespo, P., Cachero, T. G., Xu, N., and Gutfink, J. S. (1995) J. Biol. Chem. 270, 25559–25565
15. Hordijk, P. L., Verlaan, I., Jalink, K., van Corven, E. J., and Moolaenar, W. H. (1994) J. Biol. Chem. 269, 3534–3538
16. Fodin, M., Ferald, P., and Van Obbergen, E. (1994) J. Biol. Chem. 269, 6207–6214
17. Burgering, B. M. T., and Bos, J. L. (1995) Trends Biochem. Sci. 20, 18–22
18. Erhardt, P., Troppmair, J., Rapp, U. R., and Cooper, G. M. (1995) Mol. Cell. Biol. 15, 5524–5530
19. Fujimori, A., Cheng, S., Avioli, L. V., and Civitelli, R. (1992) Endocrinology 130, 29–36
20. Pardridge, N. C., Kemp, B. E., Veroni, M. C., and Martin, T. J. (1981) Endocrinology 108, 220–225
21. Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, F., Richards, J., Bonventre, J. V., Potts, J. T., Kronenberg, H. M., and Segre, G. V. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2732–2736
22. Reid, I. R., Civitelli, R., Halsted, L. R., Avioli, L. V., and Hruska K. A. (1987) Am. J. Physiol. 253, E45–E51
23. Offermanns, S., Ida-Klein, A., Segre, G. V., and Simon, M. I. (1996) Mol. Endocrinol. 10, 566–574
24. Verheijen, M. H. G., and Defize, L. H. K. (1995) Endocrinology 133, 3331–3337
25. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
26. Leerkes, S. J., and Marshall, C. J. (1992) EMBO J. 11, 569–574
27. Meloche, S., Pagès, G., and Pouyssegur, J. (1992) Mol. Biol. Cell 3, 63–71
28. Heasley, L. E., and Johnson, G. L. (1991) Mol. Pharmacol. 35, 331–338
29. Guo, J., Iida-Klein, A., Huang, X., Abou-Samra, A. B., Segre G. V., and Bringhurst, F. R. (1995) Endocrinology 136, 3884–3893
30. Ohmichi, M., Sawada, T., Kanda, Y., Koike, K., Hirota, K., Miyake, A., and Sakiel, A. R. (1994) J. Biol. Chem. 269, 3783–3788
31. Sundaresan, S., Colin, I. M., Pestell, R. G., and Jameson, J. L. (1996) Endocrinology 137, 304–319
32. Chao, T. S. O., Byron, K. L., Lee, K. M., Villereal, M., and Rosner, M. R. (1992) J. Biol. Chem. 267, 19876–19883
33. Burgering, B. M. T., de Vries-Smits, A. M. M., Medema, R. H., van Weeren, P. C., Tertoolen, L. G. J., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 7248–7256
34. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Ploeman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 747–745
35. Rosen, L. B., and Greenberg, M. E. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1113–1118
36. Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2466–2470
37. Nantel, F., Marullo, S., Krief, S., Strosberg, A. D., and Bouvier, M. (1994) J. Biol. Chem. 269, 13148–13155
38. de Vries-Smits, A. M. M., Burgering, B. M. T., and Bos, J. L. (1995) Methods Enzymol. 255, 221–229
39. de Vries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J., and Bos, J. L. (1992) Nature 357, 602–604
40. Liapi, C., Gerbaud, P., Anderson, W. B., and Evain Brion, D. (1987) J. Cell. Physiol. 133, 405–408
41. Deleted in proof
42. Deleted in proof
43. Medema, R. H., de Vries-Smits, A. M. M., van der Zon, G. C. M., Maassen, J. A., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 155–162
44. Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994) Mol. Cell. Biol. 14, 6696–6703
45. Faure, M., and Bourne, H. R. (1995) Mol. Biol. Cell 6, 1025–1035
46. Engloro, W., Rezzonico, R., Durand-Clément, M., Lallemand, D., Ortonne J. P., and Ballotti, R. (1995) J. Biol. Chem. 270, 24315–24320
47. Hazlerigg, D. G., Thompson, M., Hasting, M. H., and Morgan, P. J. (1996) Endocrinology 137, 210–218
48. Das, S., Maizels, E. T., DeManno, D., Clair, E. S., Adam, S. A., and Hunzicker-Dunn, M. (1996) Endocrinology 137, 967–974
49. Burgering, B. M. T., Prunk, G. J., van Weeren, P. C., Chardin, P., and Bos, J. L. (1993) EMBO J. 12, 4211–4220
50. Vaillancourt, R. R., Gardner, A. M., and Johnsen, G. L. (1994) Mol. Cell. Biol. 14, 6522–6530
51. Young, S. W., Dickens, E. M., and Tavare, J. M. (1994) FERS Lett. 338, 212–216
52. Yao, H., Labudda, K., Rim, C., Capodieci, P., Loda, M., and Stork, P. J. S. (1995) J. Biol. Chem. 270, 20748–20753
53. Chan, S. D. H., Strewler, G. J., King, K. L., and Nissenson, R. A. (1990) Mol. Endocrinol. 4, 639–646
54. van de Stolpe, A., Karperien, M., Lowik, C. W. G. M., Jüppner, H., Segre, G. V., Abou-Samra, A. B., de Laat, S. W., and Defize, L. H. K. (1993) J. Cell. Biol. 120, 235–243