Differential Involvement of the Ras and Rap1 Small GTPases in Vasoactive Intestinal and Pituitary Adenylyl Cyclase Activating Polypeptides Control of the Prolactin Gene*

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In pituitary cells, transcriptional regulation of the prolactin (PRL) gene and prolactin secretion are controlled by multiple transduction pathways through the activation of G protein coupled receptors and receptor tyrosine kinases. In the somatolactotrope GH4C1 cell line, we have previously identified crosstalk between the MAPKinase cascade ERK1/2 and the cAMP/protein kinase A pathway after the activation of the VPAC2 receptor by vasoactive intestinal polypeptide (VIP) or pituitary adenylyl cyclase-activating polypeptide (PACAP38). In the present study, we focus on the involvement of the GTPases Ras and Rap1 as downstream components of signal transmission initiated by activation of the VPAC2 receptor. By using pull-down experiments, we show that VIP and PACAP38 preferentially activate Rap1, whereas thyrotropin releasing hormone (TRH) and epidermal growth factor (EGF) mainly activate Ras GTPase. Experiments involving the expression of the dominant-negative mutants of Ras and Rap1 signaling (RasN17 or Rap1N17) indicate that both GTPases Ras and Rap1 are recruited for the ERK activation by VIP and PACAP38, whereas Rap1 is poorly involved in TRH or EGF-induced ERK activation. The use of U0126, a selective inhibitor of MAPKinase kinase, provides evidence that MAPKinase contributes to the regulation of the PRL gene. Moreover, cotransfection of RasN17 or Rap1N17 with the PRL proximal promoter luciferase reporter construct indicates that Rap1 may be responsible for VIP/PACAP-induced activation of the PRL promoter. Interestingly, Ras would be involved as a negative regulator of VIP/PACAP-induced PRL gene activation, in contrast to its stimulatory role in the regulation of the PRL promoter by TRH and EGF.

Regulation of pituitary involves activation of G protein-coupled receptors (GPCRs) or receptor tyrosine kinases by specific ligands and the subsequent activation of multiple transduction pathways (1, 2). Among the variety of hypothalamic neuropeptides controlling somatolactotroph cells, growth hormone releasing hormone and vasoactive intestinal polypeptide (VIP) bind to GPCRs that couple to the heterotrimeric Gs protein, resulting in activation of the adenyl cyclase and the generation of the intracellular second messenger cAMP which stimulates the cAMP-dependent protein kinase A (3, 4). To date, three receptors for pituitary adenyl cyclase activating polypeptide (PACAP) and VIP have been cloned: PAC1 receptor, for which PACAP is 100- to 1000-fold more potent than VIP, can couple to activation of phospholipase C, adenyl cyclase (AC), VPAC1, and VPAC2 receptors, which bind PACAP and VIP with the same affinity and are both coupled to activation of AC. All three PACAP receptor subtypes have been found in the anterior pituitary. Normal somatotrophs express both VIP receptors, although the data are more controversial and do not allow ascribing a PACAP-receptor subtype to normal lactotrophs (5). However, the clonal somatolactotroph cells GH4C1 express only the VPAC2 receptor subtype (4).

In pituitary cells, the stimulation of the cAMP-dependent pathway by growth hormone releasing hormone and VIP has been correlated to the synthesis and secretion of the growth hormone (GH) and prolactin (PRL) (6–9) and to proliferation (6, 10). Regulation of the PRL gene expression by PACAP38 is exerted via a cAMP/PKA-mediated pathway and is strongly dependent upon the intact cAMP-response element (CRE)-like element of the PRL proximal promoter (11, 12). Moreover, Kievit et al. have recently provided evidence that the induction of PRL transcription by cAMP involves Rap1-induced MAPKinase activity leading to stimulation of the transcriptional co-activators cAMP-response element-binding protein (CREB)-binding protein and p300 (13). PRL gene transcription is also regulated by thyrotropin releasing hormone (TRH); this regulation involves a PKC and Ca2+-dependent activation of MAPKinase which leads to phosphorylation of an Ets transcription factor (14). Besides activation of GPCRs, transcription of the PRL gene is also responsive to growth factors such as the epidermal growth factor (EGF). Although, the contribution of the classical MAPKinase pathway (Ras/Raf/MEK/ERK) in this regulation by EGF has not been clearly established (15, 16), the MAPKinase ERK1/2 seems to be essential for the modulation of PRL gene transcription by a number of ligands that bind to plasma membrane receptors of somatolactotroph cells, including TRH (17, 18), VIP/PACAP (19), PACAP27 (20), and insulin-like growth factor 1 (8).
The mechanisms underlying the crosstalk between cAMP and MAPK kinase signaling have also been intensively investigated in the case of the regulation of cell proliferation (see Ref. 21 for review). Cell type-specific actions of cAMP on cell proliferation have been first attributed to differential interactions between the GTPase Ras and its downstream kinase Raf1. However, new data suggest that another GTPase, Rap1, could play a pivotal role in the cAMP-dependent cell growth control. Moreover, Rap1 activated by cAMP or nerve growth factor promotes PC12 cell differentiation (22, 23). Thus, both GTPases Ras and Rap1 seem to be crucial components of the downstream transmission of the cAMP signal in the regulation of biological outcomes such as proliferation, neuritogenesis, or gene induction.

We have demonstrated previously that, in the VIP/PACAP-responsive neuroendocrine clonal cell line GH4C1, there is crosstalk between cAMP/PKA and MAPK kinase ERK1/2 signaling pathways. Moreover, ERK activation seemed to be involved in VIP-induced PRL expression as shown by the inhibition of VIP-induced elevation of PRL mRNA level by U0126 (a selective inhibitor of MAPK kinase kinase) (19). In the present study, we focus on the involvement of the GTPases Ras and Rap1 in ERK activation and its effects upon the transcription of the PRL gene by VIP and PACAP38, in comparison to the involvement of both GTPases in the control of the PRL gene expression by TRH and EGF. We note these findings: (i) Rap1 is preferentially activated by VIP and PACAP38, whereas by contrast, Ras is principally activated by TRH and EGF; (ii) both Ras and Rap1 are involved in ERK activation by VIP/PACAP, whereas Rap1 is poorly implicated in the activation of ERK by TRH or EGF; (iii) Rap1 is responsible for the positive control of VIP/PACAP-induced ERK-sensitive regulation of the proximal PRL promoter, whereas activation of Ras seems to be a negative regulator of VIP/PACAP-induced PRL promoter activation; and (iv) Ras and Rap1 both contribute to TRH-induced PRL promoter activation, whereas Ras activation appears as a major component of PRL promoter regulation by EGF.

MATERIALS AND METHODS

Materials—The peptides VIP, PACAP38, and TRH were purchased from Neosystem (France). Forskolin, bacitracin, and Nonidet P-40 were obtained from WVR International (France). EGF, U0126, and TransFast transfection reagent were obtained from Promega (France). Glutathione agarose beads and all other reagents were purchased from Sigma-Aldrich (France).

Cell Culture—The somatolactotroph GH4C1 cell line (a generous gift of Dr. Sobel, France) grown in Ham’s F-10 medium supplemented with 15% horse serum (Eurobio, France), 2.5% fetal calf serum (Life Technologies, France), penicillin (50 units/ml), and streptomycin (50 μg/ml) was maintained at 37 °C in a water-saturated atmosphere of 7% CO2. Cells were subcultured weekly. Before each experiment, cells were serum-starved for the last 15 h in Ham’s F-10 medium, 4–5 days after the last passage.

Ras and Rap1 Activation Assay—Ras and Rap1 GTP-loading were measured by pull-down experiments using the fusion proteins GST-Raf1-RBD and GST-RalGDS-RBD, as described previously (24, 25). Treated cells were solubilized at 4 °C for 20 min in respective lysis buffers: 50 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM MgCl2, 10 μg/ml leupeptin and apropin for Ras-GTP determination, and 50 mM Tris, pH 8, 200 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2.5 mM MgCl2, 10 μg/ml leupeptin and apropin for Rap1-GTP determination. Lysates were clarified by centrifugation at 10,000 × g for 20 min at 4 °C. 1–2 μg of total proteins from supernatants using DC protein assay (Bio-Rad, France) were incubated for 1 h at 4 °C with glutathione-agarose beads, freshly coupled to GST-Raf1RBD to isolate Ras-GTP or to GST-RalGDS-RBD to isolate Rap1-GTP. Beads were washed four times in respective lysis buffers in the absence of SDS and deoxycholic acid and then resuspended in Laemmli’s sample buffer. Denatured proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (PerkinElmer, France). Immunodetection of Ras and Rap1 were performed using anti-Ras and anti-Rap1 antibodies (Transduction Laboratory, Interchim, France) and anti-mouse IgG coupled to alkaline phosphatase as secondary antibodies. Blots were developed with the enhanced chemiluminescence Western-Star detection system (Tropix, Applied Biosystems, France) and quantified with a Molecular Imager (Bio-Rad, France). In all experiments described, the total content of Ras or Rap1 and the phosphorylation status of ERK1/2 were systematically controlled.

Assay of ERK Activation Using a Transient Expression System—GH4C1 cells cultured 2 days in 6-well tissue culture plates were co-transfected with 2.5 μg of pCDNA3/HA-tagged ERK1 (26) in combination with increasing quantities (0.25–2 μg of DNA) of pMT2/HARasN17 (dominant-negative mutant of Ras; Ref. 27) or pRK5/Rap1N17 (dominant-negative mutant of Rap1; Ref. 28) using the TransFast transfection reagent according to the manufacturer’s protocol (Promega, France). Total DNA was maintained constant by the addition of the respective empty vectors. 48 h after transfection, serum-starved cells were treated for 2 min and solubilized in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholic acid, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 1 mM NaF, 10 μg/ml leupeptin and apropin). Denatured proteins (40 μg) were separated on 10% SDS-PAGE gels. Immunodetection of phosphorylated HA-ERK1 was performed as described above by using a rabbit polyclonal phospho-specific MAPK antiserum (New England Biolabs, Ozyme, France). In all experiments described, levels of transfected mutants Ras and Rap1 were controlled. We observed a linear expression of both mutants up to 2- to 3-fold of the endogenous Ras or Rap1 proteins in the presence of increasing quantities of plasmids (not shown). Expression level of HA-ERK1 was assayed using an anti-HA antibody (12CA5, Roche, France).

rPRL Promoter Assay—An rPRL reporter construct pGL3 (–270–PRL) containing the sequence representing the –270 to +60 region of the rat PRL gene was obtained by PCR and inserted upstream of the firefly luciferase coding sequence in the pGL3 vector (Promega, France). GH4C1 cells, cultured in 24-well tissue culture plates for 2

Fig. 1. Activation of endogenous Ras. Serum-starved GH4C1 cells were incubated 2 min in the presence or absence of 0.1 μM VIP or PACAP38 (PAC) or 10 μM forskolin (FK). 1 μM TRH or 1 nM EGF. Cells were lysed, and 1 μg of cell lysates was incubated in the absence (–) (GST alone) or in the presence (+) of GST-Raf1RBD fusion protein coupled to glutathione agarose beads to isolate Ras-GTP. Isolated protein was resolved on a denaturing polyacrylamide gel and transferred to PVDF membrane. Ras-GTP was visualized with a monoclonal antibody (top). To verify that total Ras was not affected by the different treatments, 30 μg of cell lysates were resolved on a denaturing polyacrylamide gel and transferred to PVDF membrane; Ras was visualized using the monoclonal antibody (bottom). Two different representative experiments (left and right panels) are given, chosen from at least three independent determinations for each agonist.
FIG. 2. Activation of endogenous Rap1. Serum-starved GH4C1 cells were incubated 2 min in the presence or absence of 0.1 μM VIP or PACAP38 (PAC) or 10 μM forskolin (FK). 1 μM TRH or 1 μM EGF. Cells were lysed, and 2 mg of cell lysates were incubated in the presence (–) or the presence (+) of GST-RalGDS-RBD fusion protein coupled to glutathione agarose beads to isolate Rap1-GTP. Isolated protein was resolved on a denaturing polyacrylamide gel and transferred to PVDF membrane. Rap1-GTP was visualized with a monoclonal antibody (top). To verify that total Rap1 was not affected by the different treatments, 30 μg of cell lysates were resolved on a denaturing polyacrylamide gel and transferred to PVDF membrane; Rap1 was visualized by using the monoclonal antibody (bottom). A representative experiment is given from at least three independent determinations for each agonist.

Differential activation of Ras and Rap1 GTPases in GH4C1 cells treated by different agonists

Table I

| Agonists | VIP | TRH | EGF |
|----------|-----|-----|-----|
|          | Ras-GTP | Rap1-GTP | Ras-GTP | Rap1-GTP | Ras-GTP | Rap1-GTP |
| Activation of monomeric G proteins (%) of control | 255 ± 82 (n = 5) | 452 ± 55* (n = 5) | 650 ± 95 (n = 3) | 204 ± 58* (n = 3) | 1295 ± 338 (n = 3) | 165 ± 31* (n = 3) |
| Corresponding activation of ERK-2 (%) of control | 780 ± 132 (n = 5) | 772 ± 141 (n = 3) | 1411 ± 106 (n = 3) | 1619 ± 80 (n = 3) | 1793 ± 138 (n = 3) | 1909 ± 298 (n = 3) |

*p < 0.05 between Ras-GTP and Rap1-GTP for each agonist.

FIG. 3. Expressed HA-ERK1 can be activated in GH4C1 cells. GH4C1 cells were transfected or not (NT) with 2.5 μg of DNA of HA-ERK1. Two days after transfection, serum-starved cells were incubated 2 min in the presence or absence of 0.1 μM VIP or PACAP38 (PAC) or 10 μM FK, 1 μM TRH, or 1 μM EGF. 40 μg of cell lysates were resolved on 10% denaturing gel and transferred to PVDF membrane. P-ERKs were detected by using a phospho-specific polyclonal antibody (upper panel). The expression level of HA-tagged ERK1 was detected in the presence of anti-HA monoclonal antibody 12C5 (lower panel). Numbers given on the bottom of the upper panel represent (as a percent of control) the respective levels of phosphorylated HA-ERK1 and endogenous ERK2 in the presence of the different agonists. A representative experiment from at least three independent determinations is given.

days, were cotransfected with 100 ng of the pGL3 (~270rPRL) vector and 4 ng of the renilla luciferase reporter vector pRL-TK (Promega, France) as an internal standard, using Transfast reagent according to the manufacturer’s protocol. 48 h after transfection, serum-starved cells were treated with agonists for 6 h, after which cells were washed, lysed, and analyzed for luciferase activities according to the manufacturer’s protocol (Promega, France). In some experiments, cells were cotransfected with increasing quantities (0.05–0.5 μg DNA) of pB2-HA-RasN17 or pB2-Rap1N17 (total DNA was maintained constant by the addition of the respective empty vectors).

rPRL mRNA Determination—GH4C1 cells were plated in 35-mm dishes and grown for 4–5 days before treatment. Serum-starved cells were treated in Ham’s F10 medium containing 10−5 M bacitracin and the substances to be tested for 6 h. Total RNA was extracted with an RNeasy mini kit (Qiagen S.A., France). RNA samples (10 μg of total RNA) were denatured and electrophoresed on 1.2% agarose-formaldehyde gel, transferred to GeneScreen nylon-based membrane (PerkinElmer, France), and cross-linked by UV. The rat prolactin probe and hybridization were performed as described previously (29). Blots were stripped (30 min in boiling 0.1× SSC/0.1% SDS) and hybridized with 18 S probe (used as a control to normalize results). Northern blots were quantified with a Molecular Imager. Assays were performed in triplicate.

Data and Statistical Analysis—Expression of different mutants affects basal level of HA-ERK1 or basal level of the rPRL promoter activity in untreated cells. To assess specific effects of the mutants on cells treated by different agonists (VIP, PACAP, TRH, EGF, FK), results were expressed as a percent of maximal effect of each agonist remaining in the presence of a variable quantity (x) of mutant. Experimental values were first expressed as a percent of control conditions (untreated cells in the absence of mutant), then the calculation was performed as follows: \( \frac{Tx - Ux}{X} \times 100 \) %, where Tx and Ux are the respective values for treated and untreated cells in the presence of quantity x of mutant, and MTx and MUx are maximal values of treated and untreated cells, respectively, in the absence of mutant.

Experiments were performed at least three times. Data shown in figures and tables are representative experiments or means ± S.E. of several independent experiments or triplicate determinations, as stated in the legends. Statistical analysis was determined by using a Mann-Whitney test, with \( p < 0.05 \) denoting significant differences.

RESULTS

VIP and PACAP Stimulate Endogenous Ras and Rap1—in the clonal GH4C1 pituitary cells, we have shown previously that VIP and PACAP38 were able to activate the MAPK kinase ERK1/2 through a pathway dependent on cAMP/PKA (19). Because the small GTP-binding protein family Ras emerged as a key element in the control of the ERK pathway by cAMP (21), we investigated the ability of VIP and PACAP to activate Ras and Rap1. To examine the GTP-binding status of Ras and Rap1, we utilized glutathione S-transferase (GST) fusion proteins containing respectively the Ras-binding domain (RBD) of Raf1 and the Rap1-binding domain of RalGDS to precipitate GTP-bound Ras and Rap1 as described previously (24, 30).
FIG. 4. Involvement of Ras and Rap1 in ERK activation. GH4C1 cells seeded in 6-well cell culture plates were cotransfected with 2.5 μg of HA-ERK1 and increasing quantities of pMT2/HA-RasN17 (■) or pRK5-Rap1N17 (○) as indicated. Two days after transfection, serum-starved cells were incubated for 2 min in the presence of different agonists; cells were then lysed, and 40 μg of cell lysates were analyzed by Western blot as described under "Materials and Methods." a, untreated cells. b, 0.1 μM VIP-treated cells. c, 0.1 μM PACAP38-treated cells. d, 10 μM forskolin-treated cells. e, 1 μM TRH-treated cells. f, 10 nm EGF-treated cells. A representative experiment for each agonist is given from at least three independent determinations.
Cells were treated for 2 min with doses of different agonists that were previously determined to fully activate ERK1/2 in these cells (Ref. 19 and data not shown), then pull-down experiments were carried out on cell lysates. Although total Ras protein is constant in cell lysates whatever the treatment (Fig. 1, lower panel), Fig. 1 (upper left panel) shows that the active form of Ras (Ras-GTP) is increased following 0.1 μM VIP, 0.1 μM PACAP38, or 10 μM forskolin (FK) treatments. Fig. 1 (upper right panel) also shows that 0.1 μM VIP is less effective than 1 μM TRH or 1 nM EGF to activate Ras. In the same experimental conditions, although total Rap1 was not affected by any treatment (Fig. 2, lower panel), we observed an increase in the GTP-bound form of Rap1 in the presence of VIP, PACAP, and FK and, to a lesser extent, after TRH and EGF treatments (Fig. 2, upper panel). In all experiments performed, Rap1 was always activated more by VIP than Ras, whereas TRH and EGF induced a stronger activation of Ras than Rap1 (Table I).

Ras and Rap1 Are Both Involved in VIP- and PACAP-induced ERK Activation—To analyze directly whether Ras or Rap1 mediate the VIP- and PACAP-induced ERK1/2 activation, we investigated ERK regulation in GH4C1 cells transiently cotransfected with dominant-negative mutants of Ras and Rap1 (respectively, RasN17 and Rap1N17) and a tagged form of ERK1 (HA-ERK1). The chimeric kinase HA-ERK1, constructed by Meloche et al. (26), is functionally expressed in GH4C1 cells as shown by a similar increase in the phosphorylated form of HA-ERK1 and of endogenous kinases ERK1/2 in GH4C1 cells as shown by a similar increase in the phosphorylated form of HA-ERK1 and of endogenous kinases ERK1/2 after treatment with the different agonists (VIP, PACAP, FK, TRH, and EGF) (Fig. 3). Moreover, in GH4C1 cells transfected with increasing quantities of RasN17 or Rap1N17 mutant DNA constructs, previously used as dominant-negative mutants of Ras and Rap1, respectively (27, 28), we observed a linear increase in the expression of the mutants up to 2- to 3-fold the endogenous Ras and Rap1 levels (not shown). In these conditions, basal levels of phosphorylated HA-ERK1 decrease progressively in cells transfected with increasing amounts of RasN17, whereas it is not modified in the presence of Rap1N17 (Fig. 4a). When cells transfected with increasing amounts of RasN17 or Rap1N17 were subjected to either 0.1 μM VIP, 0.1 μM PACAP38, or 10 μM FK, activation of HA-ERK1 was inhibited in a dose-dependent manner by both mutants (Fig. 4b–d). On the contrary, activation of HA-ERK1 either by 1 μM TRH or 1 nM EGF is mainly depressed by RasN17, whereas Rap1N17 is less effective (Fig. 4e and f).

MAPKinase Activation Is Required for VIP- and PACAP-stimulated Transcriptional PRL Activity—Previous studies...
have provided evidence that the effects of TRH (14), insulin-like growth factor 1 (8), cAMP (13), and VIP (19) on PRL gene transcription may be mediated, at least in part, through the activation of MAPKinase ERK1/2. To confirm the contribution of ERK1/2 in VIP-induced PRL transcriptional activity, levels of MAPKinase ERK1/2 were then lysed, and luciferase activity was measured by a dual luciferase reporter assay system, as described under Materials and Methods. Data were expressed as a percent of control values (untreated cells). A representative experiment from at least three independent experiments is given. Each assay was performed in triplicate. *, p < 0.05, compared with untreated cells; **, p < 0.05, compared with respective control without U0126.

**Ras and Rap1 Are Differently Involved in VIP/PACAP Effects on PRL Promoter Activity**—To examine the respective role of Ras and Rap1 in the regulation of the PRL gene, GH4C1 cells were transiently cotransfected with the proximal promoter-luciferase reporter construct and increasing amounts of the dominant-negative mutants RasN17 or Rap1N17. In untreated or TRH-treated cells, both mutants partially reduced luciferase activity (Fig. 7a and c). In contrast, when cells were treated with 0.1 μM VIP or PACAP38 for 6 h, luciferase activity increased in the presence of RasN17, whereas it was progressively decreased by Rap1N17 (Fig. 7b and e). However, no significant effect of RasN17 was observed for 10 μM FK-treated cells (Fig. 7d). In contrast, luciferase activity was fully blocked by RasN17 in EGF-treated cells, whereas Rap1 has only a limited effect (Fig. 7f).

In all experiments performed, comparison of maximal effects of Ras or Rap1 dominant-negative mutants suggests that both Ras and Rap1 are involved in ERK activation by VIP. However, whereas Rap1 is involved in the positive control of the PRL gene by VIP, Ras seems to exert a negative control on this regulation. Both GTPases seem to be involved in ERK activation and in the positive control of the PRL promoter by TRH. Finally, Ras seems to play an important role in the regulation of ERK and the PRL promoter by EGF, whereas Rap1 is less effective (Table II).

**DISCUSSION**

In this report, we show that VIP and PACAP38 preferentially activate Rap1 as compared with Ras. Both Ras and Rap1 GTPases are recruited for the activation of ERK1/2 by VIP/PACAP. However, Ras and Rap1 are differently involved in the control of the PRL gene transcription by VIP/PACAP. Although Rap1 is involved in the positive regulation of the PRL gene by VIP and PACAP38, activation of Ras by the same ligands seems to exert a negative influence on the PRL gene regulation. Rap1 was first identified as an antagonist of oncogenic Ras signaling (32). However, recent analyses have shown that Rap1 functions in a signaling pathway distinct from Ras while using similar effectors (33-35). The involvement of Ras and Rap1 in the positive regulation of MAPKinase ERK1/2 by cAMP seems to be ligand-dependent and/or cell-type specific (21). In PC12 cells, cAMP-induced ERK activation is strictly dependent upon Rap1 and B-Raf activations (22). In melanocytes and HEK293 cells, activation of both Ras and Rap1 by cAMP-elevating agents has been described; however, only one GTPase is involved in ERK activation (36, 37). In GH4C1 pituitary cells, increase in cellular cAMP level induced by FK treatment or by activation of the VPAC2 receptor elicits activation of both Ras and Rap1 GTPases, both of which seem to be involved in ERK activation. However, we systematically observed a more efficient activation of Rap1 than Ras by cAMP-elevating agents. On the contrary, TRH and EGF preferentially activate Ras, which is the factor predominantly required for ERK activation. In GH3 cells, activation of Rap1 after FK treatment has been observed previously (13), although the same authors mention that they did not observe any cAMP-dependent activation of Ras. However, activation of Ras by TRH and EGF has been observed previously in the same cell line (16). Taken together, these results indicate that Ras and Rap1 can be activated through GPCRs and receptor tyrosine kinases involving different second messengers such as cAMP, Ca^{2+}, and diacylglycerol in pituitary cells but with variable efficiency.

It seems that both the magnitude and duration of MAPKinase activation can govern the nature of the cellular response. The involvement of Ras and Rap1 in the kinetics of MAPKinase activation has been studied in PC12 cells. Recently, Bouschet et al. have shown that induction of PC12 cell differentiation by PACAP38 requires a sustained activation of ERK1/2 that is mainly dependent upon Rap1 activation. However, the efficiency of ERK activation requires the permissive co-stimulation of PKA, PKC, and Ras (28). In the case of nerve growth factor, the sustained activation of ERK follows the recruitment of the docking protein FRS2 which stabilizes the Crk/C3G/Rap1-B-Raf complex and results in neuronal differentiation (23). In GH4C1 cells, kinetics of ERK1/2 activation are similar whatever the nature of the triggering receptor. ERK1/2 activation is maximal between 1.5 to 5 min and remains above resting level at least 1–2 h (data not shown). However the
Recent studies have shown the ability of Ras and Rap1 to induce the same cellular response in thyroid cells, depending on their association with specific effectors. TSH requires a cAMP-dependent Ras/PI3Kinase or Rap1/B-Raf activation to initiate the cellular response. EGFR activation is another important signaling pathway, and recent studies have shown that Ras and Rap1 can also be involved in the regulation of the PRL promoter activity. GH4C1 cells seeded in 24-well cell culture plates were cotransfected with 100 ng/well of pGL3(-270 PRL) vector, 4 ng/well of pRL-TK, and increasing quantities of pMT2/HA-RasN17 or pRK5-Rap1N17, as indicated. After 2 days in culture, serum-starved cells were incubated for 6 h in the absence or presence of 0.1 μM VIP, 0.1 μM PACAP38, 10 μM forskolin, 1 μM TRH, or 1 nM EGF. Cells were then lysed, and luciferase activity was measured by a dual luciferase reporter assay system. Representative experiments from at least three independent experiments for each agonist are given. Each assay was performed in triplicate. *, p < 0.05, compared with respective control conditions in the absence of RasN17 or Rap1N17.
Finally, as it has been suggested in PC12 cells (54), activation of the PRL promoter to FK and VPAC2, VIP/PACAP and EGF, or TRH, ERK1/2 could explain differences in the response of the PRL promoter. Moreover, the recruitment of specific pools of Ras, Rap1, or MAPK could be involved in the positive regulation of the PRL promoter but is not required for EGF-mediated control of the rPRL promoter (15, 44). In contrast, we provide evidence that, in GH4C1 cells, the Ras/MAPK pathway is primarily involved in the effect of EGF on the PRL promoter. In agreement with our data, activation of Ras/RAf1/MAPK pathway by EGF has been observed previously in GH3 cells (16). Moreover, the contribution of Ras and MAPK in the EGF-induced activation of the PRL promoter has been suggested previously for the VPAC2 receptor by Conrad et al. (45) and it has been shown recently that phosphorylation of the Ets2 repressor or Pit1 (a splice isoform of Pit1) which is involved in the positive control of the PRL promoter, may be involved in the recruitment of repressor factors such as the Ets family members downstream of Ras (48). Surprisingly, although both Ras and Rap1 GTPases are activated by VIP and PACAP, they seem to be mutually antagonistic in regulating PRL promoter activity. Rap1 would be involved in the positive control of the PRL promoter by cAMP-elevating agents, whereas activation of the Ras pathway would negatively modulate this control. This could explain why the magnitude of VIP/PACAP-induced PRL promoter activity is always lower than the activation of the Ras promoter by EGF or TRH. It has been shown previously that Ras and PKA pathways are mutually antagonistic in regulating PRL promoter activity (49). The inhibitory effect of VPAC2-induced Ras activation on the PRL promoter activity may be explained by a negative feedback of activated Ras on a pathway downstream to Rap1 other than ERK1/2. However, the inhibitory effect of VPAC2-induced Ras activation may also occur at the level of PRL transcription. The increased sensitivity of the proximal PRL promoter to cAMP, observed after mutation of Ets binding sites, suggests the existence of a repressor site on the proximal PRL promoter (13). Moreover, activation of Ras by the VPAC2 receptor could lead to the recruitment of repressor factors such as the Ets2 repressor or Pit1 (a splice isoform of Pit1) which would disrupt the cooperative interactions between Pit1 and Ets1 required for cAMP activation of the PRL promoter (50, 51). Moreover, it has been shown recently that phosphorylation of Pit1 can regulate its interaction with Ets1 (52).

The inhibitory effect of Ras on PRL promoter activity is only observed after stimulation of the VPAC2 receptor and is not reproduced by FK treatment. Activation of the α and βγ subunits of the heterotrimeric Gs protein by ligand binding to the VPAC2 receptor might be an important event for specific recruitment of downstream scaffolding proteins which would contribute to more complex regulation of its target genes. This has been suggested recently for the β2-adrenergic receptor (53). Moreover, the recruitment of specific pools of Ras, Rap1, or ERK1/2 could explain differences in the response of the PRL promoter to FK and VPAC2, VIP/PACAP and EGF, or TRH. Finally, as it has been suggested in PC12 cells (54), activation of the PRL promoter by EGF and cAMP could lead to the recruitment of repressor factors such as the Ets factors recently identified downstream of Ras (48).

### Table II

| Agonist    | Control | HA-ERK1 maximal inhibition (in percent) | HA-ERK1 maximal inhibition (as percent) | Luciferase activity (in percent) | Luciferase activity (as percent) |
|------------|---------|----------------------------------------|----------------------------------------|---------------------------------|---------------------------------|
| GHRH       | 85 ± 5  | 69 ± 7 (n = 3)                          | –                                      | 74 ± 24 (n = 3)                 | 74 ± 24 (n = 3)                 |
| VIP        | 57 ± 5  | 56 ± 10 (n = 3)                         | –                                      | 49 ± 10 (n = 5)                 | 49 ± 10 (n = 5)                 |
| VPAC2      | 75 ± 5  | 72 ± 6 (n = 3)                          | –                                      | 62 ± 9 (n = 3)                  | 62 ± 9 (n = 3)                  |
| PACAP      | 75 ± 5  | 72 ± 6 (n = 3)                          | –                                      | 62 ± 9 (n = 3)                  | 62 ± 9 (n = 3)                  |

* p < 0.01; ** p < 0.05 between pMT2-RasN17 and pRK5-Rap1N17 for each agonist.

**p = 0.05 between pMT2-RasN17 and pRK5-Rap1N17 for each agonist.**
of the Ras and Rap1 signaling pathways by different ligands could induce different combinations of transcription factors or coregulators, resulting in different functional effects on the transcription of the PRL gene. Further experiments are required to determine the molecular mechanisms whereby the activation of Ras induced by VPAC2 leads to an inhibition of PRL gene transcription, whereas activation of Ras by EGF or TRH stimulates PRL promoter activity. Taken together, these data suggest that the balance in the activity of Ras and Rap1-mediated signals may be an important determinant of the highly specialized ability of neurohormones or growth factor to regulate their target genes in pituitary cells.

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