CMLS, Cell. Mol. Life Sci. 59 (2002) 2165–2171
1420-682X/02/122165-07

CMLS Cellular and Molecular Life Sciences

Research Article

ERKs are the point of divergence of PKA and PKC activation by PTHrP in human skin fibroblasts

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Received 2 August 2002; received after revision 10 September 2002; accepted 18 October 2002

Abstract. Parathyroid hormone-related peptide (PTHrP) receptors, coupled to trimeric G proteins, operate in most target cells through at least three different transduction routes: Gαs-mediated stimulation of adenylylcyclase (AC), Gαq-mediated activation of phospholipase Cβ (PLC) and mitogen-activated protein kinase (MAPK) activation. In this study we investigated the relative role of different pathways in human skin fibroblast proliferation. Using chemical inhibitors and activators of signal transduction, we demonstrated that: (i) AC/cAMP and PLC/1,4,5 inositol triphosphate/diacylglycerol second-messenger systems are simultaneously activated following PTHrP binding to its receptors; (ii) the mitogenic response to PTHrP derives from a balance between two counteracting pathways – an activating route mediated by protein kinase C (PKC) and an inhibitory route mediated by protein kinase A (PKA); (iii) PTHrP mitogenic effects are largely dependent on MAPKs, whose activity can be modulated by both PKA and PKC. Our results indicate that MAPKs are common targets of both transduction routes and, at the same time, their point of divergence in mediating PTHrP dual and opposite mitogenic effects.

Key words. PTHrP; fibroblast; proliferation; PKA; PKC; MAPK.

The search for the pathogenetic factor responsible for the development of hypercalcemia of malignancy led to the discovery of parathyroid hormone (PTH)-related peptide (PTHrP) [1]. PTHrP has been observed to be expressed by a wide variety of normal adult and fetal tissues where it plays predominantly paracrine or autocrine roles [2].

Due to its wide tissue distribution and degree of conservation through evolution, PTHrP has been proposed to have a significant developmental role, and is now known to influence the proliferation and differentiation of a variety of cell types, such as chondrocytes, osteoblasts and keratinocytes [3]. Although the molecular mechanisms involved in cellular turnover mediated by PTHrP have not been fully defined, due to the homology of the NH_2-terminal sequence between PTH and PTHrP, both can interact with a common receptor [4]. This receptor is grouped into the general category of seven transmembrane-domain receptors coupled to heterotrimeric guanosine triphosphate-binding proteins (GPCRs). The transduction of the PTHrP signal through the plasma membrane of target cells may activate several second-messenger pathways depending on the G protein involved, the receptor to which it is coupled and the cell type [4]. In fact, PTHrP

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binding to GPCR stimulates the formation of cyclic 3′,5′-adenosine monophosphate (cAMP) by activating adenylate cyclase (AC) through the action of stimulatory G-alpha proteins (Gαs) coupled to the receptor. In turn, cAMP binds to the regulatory subunit of protein kinase A (PKA), which releases the active catalytic subunits of the enzyme. PTHrP binding to GPCRs activates phospholipase Cβ (PLC) by Gαq, leading to the formation of diacylglycerol (DAG), which activates protein kinase C (PKC) and 1,4,5 inositol triphosphate (IP3) resulting in increased intracellular free Ca^{2+} [4].

Both AC and PLC signaling can occur through a common receptor [4]. However, in some cases, the binding of PTHrP to the receptor activates only one signaling pathway. In mouse keratinocytes [5], cardiac myocytes [6] and lymphocytes [7], the PTHrP receptor activates PLC but not AC. Conversely, in vascular smooth muscle cells, the PTHrP receptor activates AC but not PLC [8].

The basis of the cell-specific signaling patterns exhibited by the PTHrP receptor in skin fibroblasts is the subject of the present study.

cAMP and PKC pathways are known to modulate mitogen-activated protein kinases (MAPKs) that are normally activated by receptor tyrosine kinase and/or GPCRs. MAPKs, described as a downstream target of both cAMP and PKC cascades [9, 10], are a group of serine-threonine kinases including ERKs, JNK and p38, which mediate signal transduction from the plasma membrane to the nucleus and have a central role in mitogenic signaling pathways. Upon activation, MAPKs translocate to the nucleus where they can induce the expression of transcription factors involved in DNA synthesis and cell division [11]. MAPK activation through GPCRs involves the release of the G$\beta\gamma$ dimer that, via phosphatidyl inositol 3-kinase (PI3-kinase), may regulate the phosphorylation of the protein Shc. Upon activation, Shc binds to the adapter protein Grb2. Grb2 can then associate with the guanine nucleotide exchange factor Sos, which in turn activates Ras, anchored to the plasma membrane, by exchanging GTP for GDP. GTP-bound Ras then binds directly to a serine-threonine kinase, Raf, which in turn phosphorylates MEK leading to the subsequent activation of the p44 and p42 MAPKs, also termed ERK1 and ERK2, of the MAPK family [12]. This pathway is one of the main mechanisms involved in cell growth.

PTHrP receptors coupled to trimeric G proteins may therefore operate through one or more of these different pathways. This could be the reason for the pleiotropic effects of PTHrP described in the literature in different target cells [13]. However, the involvement of the G$\beta\gamma$ subunit-dependent pathway on MAPK activation in PTHrP signaling is not yet clearly proven.

We recently found that synthetic PTHrP 1–40 strongly affects human skin fibroblast proliferation, by inhibiting [^{3}H]-thymidine incorporation into DNA, dose dependently. This inhibitory effect was apparently mediated by the cAMP/PKA second-messenger pathway, since an inverse correlation was found between proliferation rate and extracellular cAMP release [14].

In light of the evidence that PTHrP can activate many signaling pathways in cells, the present study investigated the possible role of diverse signal transduction routes in the mitogenic effect of PTHrP observed in skin fibroblasts. The importance of PKA and PKC activation and the involvement of MAPKs of the ERK group in fibroblasts was studied through the use of chemical inhibitors and activators of signal transduction. MAPKs were investigated as a possible point of convergence/divergence of these activated signaling pathways.

Materials and methods

Human skin fibroblast cultures

Fibroblast cultures were established from healthy volunteers (age 20–30 years). The primary cultures of human skin fibroblasts were started with a 3-mm skin punch biopsy. Dermal pieces were placed in a 25-cm^2 tissue culture flask with Dulbecco's modified Eagle's medium (DMEM) (Sigma, Milan, Italy), containing 20% fetal calf serum (FCS) (Gibco, Milan, Italy) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) (Gibco). Flasks were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 3 days, the medium was then replaced with 10% FCS/DMEM and was changed every 3 days. When fibroblasts growing from the dermal pieces formed a confluent layer, the dermal pieces were removed and a 0.25% trypsin/EDTA mixture was added for 5 min to separate fibroblasts.

Detached cells were freezed in 90% FCS/DMSO. For our experiments, cells were thawed and seeded in a 25-cm^2 culture flask (Falcon, Perugia, Italy) and subcultured in 10% FCS/DMEM. All experiments presented here were performed on cells from the same subject, between passages 3–5.

In vitro treatments

In our experimental design (see below), primary human skin fibroblasts were seeded either on 24-well plates at 7×10^4 cells per well or on 12-well plates at 1.5×10^5 cells per well and cultured with 500 and 800 ml of medium, respectively. After reaching subconfluence, the cells were serum starved for 24 h with 0% FCS. Synthetic lyophilized PTHrP 1–40 (Sigma) was then added at a dose of 320 nM in serum-free medium and the cultures were incubated for an additional 24 h.

A set of experiments was performed pre-treating the cells with 10 µM U73122 (Sigma), a PLC inhibitor, for 3 h, 1 µM GF 109203X (Sigma), a highly selective cell-per-

meable PKC inhibitor, for 1 h, 10 µM SQ 22536 (Sigma), an AC inhibitor, for 3 h, or 25 µM PD 98059 (Calbiochem), a MEK inhibitor, for 1 h, before exposure to synthetic PTHrP 1–40.

Another set of experiments was performed exposing the cells for 24 h to forskolin (FK) (ICN, Milan, Italy), an AC activator, at a dose of 40 µM, or to phorbol 12-myristyl 13-acetate (PMA) (ICN), a PKC-activating agent, at a dose of 0.3 µM. In a parallel study, cells were exposed to 25 µM PD 98059 for 1 h, prior to treatment with FK or PMA. All substances were dissolved in dimethylsulfoxide (DMSO) (0.6% v/v), stored at appropriate stock concentrations and diluted to the desired concentrations before use. In all cases, appropriate controls were performed.

Proliferation assay

Cells were seeded in 24-well plates at a density of 7×10^4 cells per well and pulsed with 10 µCi/ml [methyl-^{3}H]-thymidine (New England Nuclear, Boston, Mass.; specific activity 80 Ci/mol) for the last 24 h of culturing and lysed in 0.1 M SDS, 0.1 N NaOH. The insoluble material formed by precipitation with 10% trichloroacetic acid (TCA) was collected and the radioactive signal was measured by liquid scintillation. The total amount of acid-precipitable [^{3}H]-thymidine incorporated per well was expressed as percent (%) of the control.

Cell lysates and Western blot analysis

After incubation with PTHrP, PMA or FK for 5 min, cells were rapidly lysed by addition of ice-cold lysis buffer (12.5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 25 mM β-glycerophosphate, 2 mM sodium vanadate, 10 µM phenylmethylsulfonylfluoride, 1 µg/ml leupeptin and 5 µg/ml apoprotin; Sigma). Cell lysates were centrifuged (at 4 °C) at 800 g for 10 min and then at 12,000 g for 15 min. Supernatants were removed, the protein concentration was determined by the Lowry method using bovine serum album (BSA) as a standard, and supernatants were stored at –80 °C.

Protein samples (40 µg) from whole-cell lysates were separated on 10% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (BioRad, Milan, Italy). Membranes were blocked with 1% BSA in Tris-buffered saline containing 2.5 mM EDTA and 0.1% (v/v) Tween-20 for 1 h at 20 °C. To detect activated ERKs, blots were incubated with anti-phospho-ERK antibodies overnight at 4 °C. Goat anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Milan, Italy) was used as secondary antibody. As a control, the levels of total ERKs were measured by stripping membranes and reprobing with antibody against non-phosphorylated ERK1/ERK2. Specific protein bands were detected by an enhanced chemiluminescence (ECL) Western blotting system (Amersham, Milan, Italy), as recommended by the manufacturer. Total ERK1/ERK2 and phospho-ERK1/ERK2 were quantified by scanning densitometry of blots. The specific activity of phospho-MAPKs was calculated as percent (%) of total MAPKs.

Statistical analysis

Results were expressed as the mean ± SD. Data were analyzed using Student's t test; differences were considered significant if $p < 0.05$.

Results

Effect of PTHrP on cAMP activation and cell proliferation

In a previous study, our group reported the antiproliferative effect of PTHrP on human skin fibroblasts with marked cAMP release [10]. To further evaluate the involvement of cAMP in cell growth inhibition, skin fibroblasts were treated with FK (an AC activator) at 40 µM for 24 h.

As shown in figure 1, FK induced a significant decrease in thymidine incorporation as did PTHrP (34 and 40%, respectively) compared to the control (untreated cells). Pre-treatment of skin fibroblasts with a specific inhibitor of AC (SQ 22536) before the stimulation with PTHrP not only completely suppressed the PTHrP growth inhibitory effect but also absolutely reversed it, leading to a marked and significant enhancement of cell proliferation. This result suggested the existence of a parallel PTHrP-activated route with promitogenic effects. SQ 22536 alone did not significantly affect the proliferation rate.

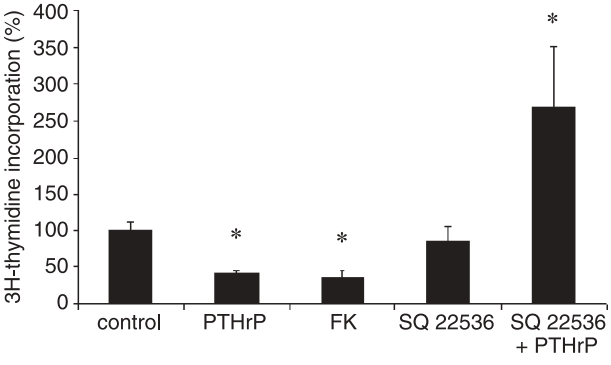

Figure 1. The PTHrP antiproliferative effect is mimicked by FK and reversed by pre-treatment with the AC inhibitor SQ 22536. Fibroblasts were treated with 320 nM PTHrP or 40 µM FK for 24 h or pre-treated with 10 µM SQ 22536 for 3 h before PTHrP exposure. Fibroblast proliferation was evaluated by [^{3}H]-thymidine incorporation into DNA, as described in Materials and methods. Results are given as the mean ± SD of three independent experiments, each performed in quadruplicate. * Significantly different from the control ($p < 0.05$).

PLC/IP3, DAG/PKC pathway involvement in PTHrP effects

To evaluate whether the PLC/IP3, DAG/PKC pathway is required for the PTHrP-induced cell proliferation, human skin fibroblasts were pre-treated with 10 µM U73122, a specific inhibitor of PLC, or with 1 µM GF 109203X, a highly selective PKC inhibitor, before the hormone stimulation. As shown in figure 2, blockade of this transduction pathway, at both levels of the cascade, resulted in a potentated antiproliferative effect of PTHrP. U73122 alone and GF 109203X alone did not significantly affect the proliferation rate.

To confirm that the PLC/IP3, DAG/PKC transduction pathway is responsible for the PTHrP promitogenic effect, PKC was activated by 0.3 µM PMa for 24 h. As illustrated in figure 2, PMA significantly stimulated [^{3}H]-thymidine incorporation into DNA compared to the control.

PTHrP mitogenic effects occur by an opposite regulation of ERK activity

In view of the role played by ERKs in the regulation of cell growth, studies were carried out to test whether the opposite effects of PKA and PKC could be due to an opposite regulation of ERK activity.

Figure 3 shows the results of ERK inhibition by PD 98059. The treatments with PD 98059 led to a lowering of [^{3}H]-thymidine incorporation, indicating that a basal activity of ERKs is present in unstimulated fibroblasts. This was confirmed by immunoblotting analysis of ERKs in control cultures (see below).

The treatment with 25 µM PD 98059 before PTHrP treatment resulted in a stronger inhibition of cellular growth than when PTHrP was used alone, but equivalent to that obtained with 25 µM PD 98059 (fig. 3 A). Although this dose did not completely suppress growth, no additive an-

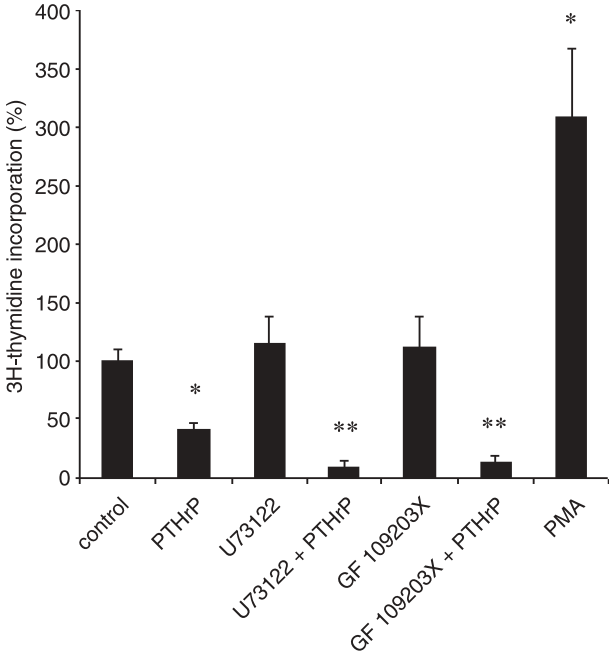

Figure 2. PLC or PKC inhibition enhances the PTHrP antiproliferative effect: the PLC/PKC pathway mediates PTHrP promitogenic effects. Fibroblasts were treated with the PKC activator PMA (0.3 µM) for 24 h or pre-treated with the PLC inhibitor U73122 (10 µM) for 3 h, or PKC inhibitor GF 109203X (1 µM) for 1 h, before 320 nM PTHrP exposure. Fibroblast proliferation was evaluated by [^{3}H]-thymidine incorporation into DNA, as described in Materials and methods. Results are given as the mean ± SD of three independent experiments, each performed in quadruplicate. * Significantly different from the control ($p < 0.05$); ** significantly different from PTHrP-only treated cells ($p < 0.05$).

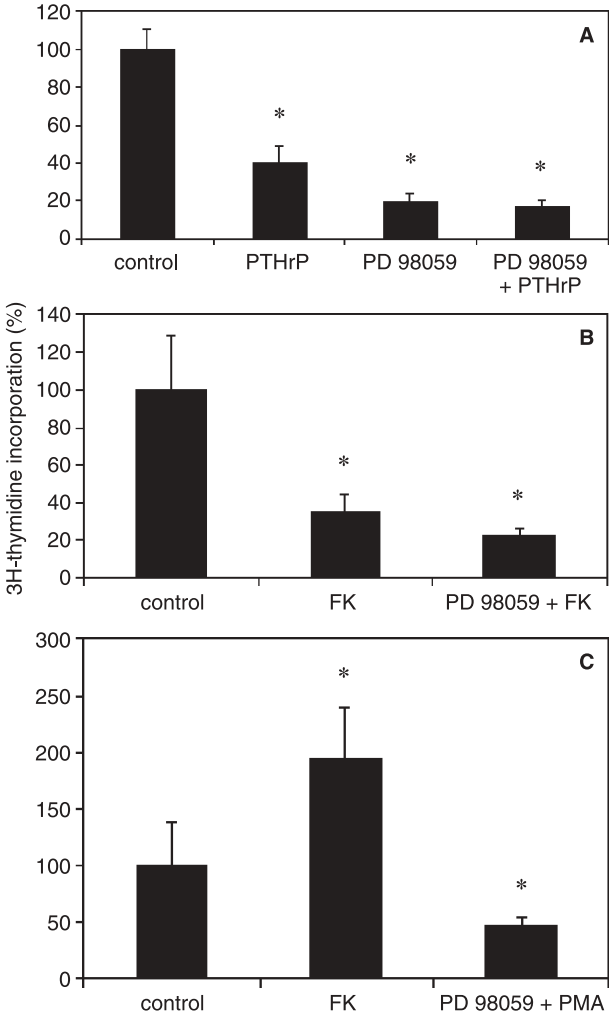

Figure 3. Effect of MEK inhibition on cell proliferation: MEK is activated by PKC and inhibited by the cAMP/PKA pathway. Fibroblasts were pre-treated with the MEK inhibitor PD 98059 (25 µm) for 1 h before exposure to 320 nM PTHrP (*A*), 40 µM FK (*B*) or 0.3 µM PMA (*C*). Fibroblast proliferation was evaluated by [^{3}H]-thymidine incorporation into DNA, as described in Materials and methods. Results are given as the mean ± SD of three independent experiments, each performed in quadruplicate. * Significantly different from the control ($p < 0.05$).

tiproliferative effect of PTHrP was measured. This implies that the PTHrP antiproliferative effect is likely mediated by inhibition of MAPKs of the ERK group only. When cells were treated with FK alone or pre-treated with PD 98059 before exposure to 40 µM FK, similar antiproliferative effects were seen (fig. 3B). These results let us to conclude that ERKs are likely inhibited by PTHrP-induced PKA activation.

The exposure of cells to PD 98059, before PMA treatment, resulted in a decrease in proliferation rate from 194% (PMA) to 47% (PD 98059 plus PMA) of the control. Thus, the promitogenic effect of PMA was completely reversed (fig. 3C).

Since the block of ERKs and the stimulation of PKC activities have antagonistic effects on cell proliferation, ERKs are likely stimulated by PKC. These conclusions were supported by immunoblotting results shown in figure 4.

PTHrP differential regulation of ERK activity by PKC and PKA

To investigate the signal-transducting pathways activated by PTHrP in skin fibroblasts, cells were incubated with PTHrP (320 nM), PMA (0.3 µM) or FK (40 µM) for 5 min and the activation of ERK1/2 was measured by Western blot and scanning densitometry of blots. The amount of active ERK was calculated as a percent of total ERK (specific activity) and expressed as a percent of the control. As shown in figure 4, PMA treatment resulted in a significant increase in ERK1/2 specific activity, while both PTHrP and FK significantly decreased the amount of active ERKs. These results confirmed that MAPKs of the ERK group are under the control of PTHrP, which acts through a positive regulation by PKC and a negative regulation by PKA. We can hypothesize ERKs as the point of convergence of these PTHrP-activated signaling pathways in skin fibroblasts.

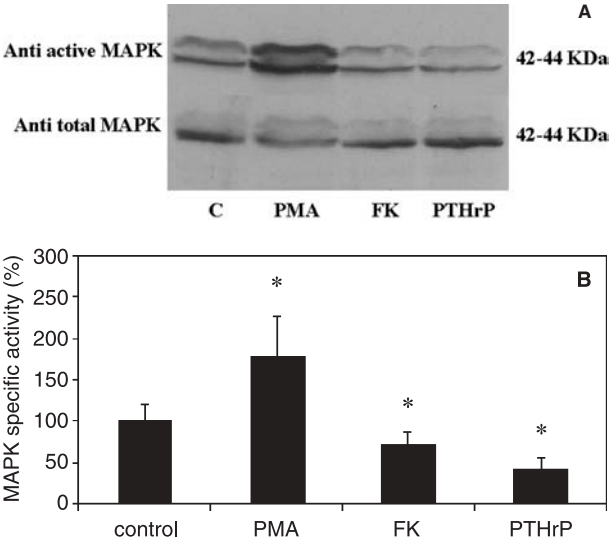

Figure 4. Opposite regulation of ERK activity by PKA and PKC. Fibroblasts were treated with 320 nM PTHrP or 40 µM FK or 0.3 µM PMA for 5 min. The amount of active ERKs was evaluated by Western blot and scanning densitometry of blots as described in Materials and methods. Phospho-MAPK specific activity was calculated as a percent of total MAPKs and expressed as percent of the control. (*A*) Western blots of MAPKs obtained in one of three similar series of experiments. (*B*) Scanning densitometry of blots. Results are given as the mean ± SD (n = 3). *Significantly different from the control ($p < 0.05$).

Discussion

Recent studies in our laboratory have demonstrated the role of PTHrP in modulating cell proliferation and extracellular matrix turnover (MMP2) in primary skin fibroblasts obtained from a single human donor [14]. Evidence for such a role is also confirmed by studies with PTHrP knockout mice that, in addition to chondrodystrophy, displayed several cutaneous abnormalities, including a fibrotic dermis [15]. These data are linked to pathophysiological events such as wound healing, skin aging and tumor progression in which either PTHrP, cell proliferation or MMP activity are implicated [14].

In the present study, we focused our interest on the PTHrP antiproliferative effect in an attempt to identify the intracellular signal transduction pathways activated by PTHrP in skin fibroblasts obtained from a second human donor. In our previous work, we found a dose-dependent release of cAMP into the medium and a negative association between cAMP levels and cell proliferation following exposure to PTHrP [14].

Our present findings integrate previous results and demonstrate that: (i) at least two signaling pathways are simultaneously activated after PTHrP treatment – the AC/cAMP/PKA and the PLC/IP3, DAG second-messenger systems; (ii) the final response to PTHrP mitogenic effects is a balance between two counteracting pathways – a promitogenic route mediated by PKC and an antiproliferative route involving cAMP and PKA activation.

This result does not exclude the possibility that additional, unexplored regulatory pathways are involved in the proliferative response to PTHrP. Moreover, previous and present findings derive from experiments conducted on fibroblasts from two human donors only, so they are not generalizable to the general population.

The downstream step of the cell proliferation and differentiation pathway is regulated by MAPK activation, and the lack of this control might result in oncogenesis [16, 17]. Since MAPKs play a central role in mitogenic signaling pathways and are downstream targets of both PKA and/or PKC activation, they appeared to be the point of divergence responsible for the opposing PTHrP effects on fibroblast proliferation. PKA and PKC activation are known to influence MAPK activity by a complex cell-

specific network of cross-talk among individual mitogenic pathways [10].

The MAPK cascade usually transduces signals from receptor tyrosine kinases to two members of the Ras family of small G proteins, Ras and Rap-1, which then stimulate the sequential activation of Raf, MEK and ERK1/2. Ras functions principally to activate isoform Raf-1, whereas Rap-1 stimulates isoform Raf-B activity [18].

However, cross-talk between the Ras/Raf/MEK/ERK cascade and the cAMP second-messenger system have been described [19, 20]. There is evidence that cAMP can either activate or inhibit the MAPK pathway in different cell types acting at the level of different Raf isoforms. Raf isoforms are tissue specific and differentially regulate MAPK activity in different cells. Regarding the inhibition of MAPK activation by cAMP, in a number of cells, including Rat-1 and NIH 3T3 fibroblasts, isoform Raf-1 has been reported to be phosphorylated by cAMP-dependent PKA [19]. This phosphorylation reduces the affinity of Raf-1 binding to Ras, resulting in the inhibition of MAPK activation and cell proliferation [21].

Moreover, both Raf-1-dependent and Raf-1-independent mechanisms have been described in the cAMP/PKA inhibition of MAPK activity. The activation of Rap-1 by PKA, especially in those cells where Raf-B is expressed at low levels (for example NIH 3T3), not only uncouples Raf-1 from Ras, but also triggers the association of Rap-1 with Raf-1, an inactive complex [22].

In contrast, a stimulatory effect of cAMP on ERK activity has been described in PC12 cells. It is linked to a direct activation of Rap-1, which then stimulates Raf-B [23]. In rat enterocytes, the cAMP/PKA pathway enhances MAPK activation, but the mechanism has not been fully elucidated [20]. Moreover, Raf-independent regulation of MAPK activity has been reported: PKA activation inhibits the association between MAPKs and their inactivating protein tyrosine phosphatases, resulting in enhanced MAPK activity [24].

As a general rule, the cAMP/PKA pathway stimulates Ras-independent and Rap-1-dependent ERK phosphorylation and cell proliferation in Raf-B-expressing cells, but inhibits growth in Raf-B-negative cells [22]. By integrating our findings with this general concept, we deduce that among the Raf isoforms, Raf-1 expression likely predominates in skin fibroblasts. This deduction is also supported by studies showing that NIH 3T3 cells, belonging to the fibroblast family, do not express Raf-B [25].

In contrast to the dual effect of the cAMP/PKA pathway on MAPK activity, the PKC transduction route always leads to MAPK activation [26, 27].

In skin fibroblasts, we found an unusual effect of PTHrP treatment: an opposite proliferative response mediated by the simultaneous activation of PKA and PKC.

Our study indicates that in skin fibroblasts, the cascades of activation downstream of cAMP and PKC converge at the level of MAPKs. In fact, MAPK activation, both directly evaluated by immunoblotting of phospho-ERKs and deduced by cell proliferation, was inhibited by cAMP/PKA and stimulated by PKC. Superimposable results were obtained by immunoblotting of phospho-MEK (not shown).

Moreover, the treatment of cells with PD 98059, a potent and selective MEK inhibitor, potentiates the antiproliferative effect of cAMP stimulated by FK (additive effect) and suppresses the mitogenic stimulus of PKC activated by PMA. In agreement with the net antiproliferative effect of PTHrP, the combination of PD 98059 and PTHrP resulted in a more marked inhibition of growth compared to PTHrP alone, because PTHrP activation of MEK via PKC could not occur.

The involvement of other members of the MAPK family, such as p38K or c-JNK, in PTHrP signaling cannot be answered by this study. In a recent report, PTH was shown to inhibit JNK activity by activation of the PKA signaling cascade in rat osteoblastic cells [28].

Because of the existence of two opposing signals acting simultaneously on fibroblasts, the action of PTHrP on cell growth may vary, even within the same cell type. In fact, different PTHrP receptor-associated enzymes and/or additional factors, acting upstream or downstream of PKA and PKC activation, involved in balancing these opposing signals may be expressed during the life span of a cell. A recent study suggests that a switch from the cAMP pathway to the PLC/intracellular calcium pathway, associated with PTHrP activation, occurs in malignant cells [29]. Moreover, the amount and activity of PKC in fibroblasts increases as a function of age [30].

Recently, age-related differences in N-terminal PTH-like region PTHrP 1–34 signaling have been described in rat enterocytes [31]. Thus, natural or pharmacologically induced resetting of PKC/PKA activity may lead to differential responses to PTHrP not only in skin fibroblasts but also in other PTHrP target cells.

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