Potentiation of ATP- and Bradykinin-Induced $[Ca^{2+}]_c$ Responses by PTHrP Peptides in the HaCaT Cell Line

Helen E. Burrell[1,4], Alec W.M. Simpson[1], Sharonpreet Mehat[1], David T. McCreavy[1], Brian Durham[2], William D. Fraser[2], Graham R. Sharpe[3] and James A. Gallagher[1]

In the epidermis, local and systemic factors including extracellular nucleotides and parathyroid hormone-related protein (PTHrP) regulate keratinocyte proliferation and differentiation. Extracellular nucleotides increase proliferation via activation of P2 receptors and induction of calcium transients, while endoproteases cleave PTHrP, resulting in fragments with different cellular functions. We investigated the effects of adenosine 5′-triphosphate (ATP) alone and in combination with synthetic PTHrP peptides on calcium transients in HaCaT cells. ATP induced calcium transients, while PTHrP peptides did not. C-terminal and mid-molecule PTHrP peptides (1–100 pM) potentiated ATP-induced calcium transients independently of calcium influx. 3-isobutyl-1-methylxanthine potentiated ATP-induced calcium transients, suggesting that a cyclic monophosphate is responsible. Cyclic AMP is not involved, but cyclic GMP is a likely candidate since the protein kinase G inhibitor, KT5823, inhibited potentiation. Co-stimulation with ATP and either PTHrP (43–52) or PTHrP (70–77) increased proliferation, suggesting that this is important in the regulation of cell turnover and wound healing and may be a mechanism for hyperproliferation in skin disorders such as psoriasis. Finally, PTHrP fragments potentiated bradykinin-induced calcium transients, suggesting a role in inflammation in the skin. Since PTHrP is found in many normal and malignant cells, potentiation is likely to have a wider role in modulating signal transduction events.

Journal of Investigative Dermatology (2008) **128,** 1107–1115; doi:10.1038/sj.jid.5701159; published online 15 November 2007

INTRODUCTION

Extracellular nucleotides, via activation of P2 receptors, are important regulators of cellular processes including proliferation, differentiation, and apoptosis. In cultured keratinocytes, activation of the G protein-coupled $P2Y_2$ receptor has been linked to proliferation via elevation of cytosolic-free calcium concentration ($[Ca^{2+}]_c$) (Pillai and Bikle, 1992; Dixon *et al.*, 1999; Burrell *et al.*, 2003; Greig *et al.*, 2003). $P2Y_2$ receptor mRNA is differentially expressed in normal keratinocytes (Burrell *et al.*, 2003) in agreement with *in situ* hybridization (Dixon *et al.*, 1999) and immunohistochemistry (Greig *et al.*, 2003). Extracellular nucleotides can act synergistically with other growth regulators in the skin, including neurotransmitters (Wang *et al.*, 1990), epidermal growth factor, and insulin (Huang *et al.*, 1989) to enhance proliferation, for example during wound healing. In bone, extracellular nucleotides act synergistically with parathyroid hormone (PTH), in both human (Buckley *et al.*, 2001) and rat (Kaplan *et al.*, 1995) osteoblasts. PTH also potentiates the carbachol-induced elevation of $[Ca^{2+}]_c$ in HEK-293 cells after stable transfection with the human PTH type I receptor (Short and Taylor, 2000; Tovey *et al.*, 2003). In the epidermis, a related protein, parathyroid hormone-related protein (PTHrP), is involved in the regulation of proliferation and differentiation and this led us to hypothesize that PTHrP may also synergize with extracellular nucleotides in keratinocytes.

PTHrP is a multifunctional autocrine/paracrine protein produced by normal and malignant cells (Burtis, 1992; Kaiser and Goltzman, 1993). Depending on the cell type, the PTHrP gene can be transcribed from three separate promoter regions into at least 15 different mRNA products, which form proteins containing 139, 141, or 173 amino acids (Heath *et al.*, 1995). These proteins are post-translationally modified by enzymes, which cleave the protein producing smaller fragments that may act at different receptors (Burtis, 1992; Orloff *et al.*, 1994). Expression of some of these enzymes is ubiquitous (Hatsuzawa *et al.*, 1990), while others are restricted (Johnson *et al.*, 1994). Post-translational processing (Yang *et al.*, 1994) and fragment function (Fenton *et al.*, 1991; Whitfield *et al.*, 1996) appear to be species-, tissue-, and isoform-specific,

[1]*Department of Human Anatomy and Cell Biology, University of Liverpool, Liverpool, UK;* [2]*Department of Clinical Biochemistry, University of Liverpool, Liverpool, UK and* [3]*Department of Dermatology, Broadgreen Hospital, Liverpool, UK*

[4]*Current address: Biomolecular Sciences, James Parsons Building, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK*

Correspondence: Dr Helen E. Burrell, Biomolecular Sciences, James Parsons Building, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK. E-mail: H.Burrell@ljmu.ac.uk

Abbreviations: ATP, adenosine 5′-triphosphate; $[Ca^{2+}]_c$, cytosolic-free calcium concentration; cGMP, cyclic guanosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; $InsP_3$, inositol 1,4,5-trisphosphate; PTHrP, parathyroid hormone-related protein

Received 2 August 2006; revised 22 August 2007; accepted 30 August 2007; published online 15 November 2007

and in the epidermis there are conflicting results. While the N-terminal (1–34) fragment triggers differentiation in normal keratinocytes (Holick *et al.*, 1988), the C-terminal fragment stimulates growth of quiescent cells but inhibits growth of actively cycling cells (Whitfield *et al.*, 1996). High levels of PTHrP increase epidermal proliferation (Foly *et al.*, 1998) with production predominantly in the proliferating basal (Blomme *et al.*, 1999) and early-spinous layers (Sharpe *et al.*, 1998) although localization can be altered since the PTHrP (34–68) peptide is predominantly expressed in the granular cells (Juhlin *et al.*, 1992). PTHrP expression in HaCaT cells is greatest in actively dividing cells (Lam *et al.*, 1997). In contrast, absence or low levels of PTHrP induces differentiation (Kaiser *et al.*, 1992, 1994; Foly *et al.*, 1998; Sharpe *et al.*, 1998) and once the cells have migrated to the granular layer, PTHrP production ceases (Danks *et al.*, 1989).

PTHrP (1–34) has significant sequence and functional similarities with PTH and shares a common receptor, the PTH/PTHrP type I receptor. Receptor activation initiates cellular responses via protein kinase C (Whitfield *et al.*, 1996), Ca^{2+}, and adenylyl cyclase (Orloff *et al.*, 1994) depending on the G-protein subtype utilized. There are links with both G_q and G_s subtypes of G proteins. However, the PTH/PTHrP type I receptor is not expressed by keratinocytes (Hanafin *et al.*, 1995; Sharpe *et al.*, 1998), but is expressed by underlying dermal fibroblasts. It is thought that keratinocytes produce PTHrP, which may affect the dermal fibroblasts to later induce a secondary effect on the keratinocytes (Hanafin *et al.*, 1995). The C-terminal (107–111) region also stimulates protein kinase C activity (Whitfield *et al.*, 1996) and has been postulated to bind to a separate and distinct receptor, which is as yet uncharacterized.

The aims of this study are to use the HaCaT keratinocyte cell line to identify whether PTHrP fragments can also synergize with extracellular nucleotides and to elucidate the mechanism by which any responses occur. These fragments have been designed around cleavage sites for the modification enzymes that are known to exist in skin *in vivo* (Pearton *et al.*, 2001). Consequently, they are likely to be naturally occurring. N-, C-, and mid-molecule PTHrP fragments have been used to identify the biologically active regions in cultured human keratinocytes.

RESULTS

Effects of PTHrP peptides on ATP-induced $[Ca^{2+}]_c$ responses

Adenosine 5′-triphosphate (ATP; 5 μM) induced acute Ca^{2+} transients, which lasted for approximately 200 seconds (Figure 1a). PTHrP fragments (Table 1) alone failed to acutely elevate $[Ca^{2+}]_c$ (Figure 1b; only PTHrP (43–52) shown for clarity). Acute Ca^{2+} responses due to simultaneous delivery of ATP and PTHrP (Figure 1c), or delivery of C-terminal or mid-molecule PTHrP prior to ATP (Figure 1b) had equal duration but greater amplitudes compared with ATP alone (Figure 1a; PTHrP (43–52) is shown for clarity). The acute increase in $[Ca^{2+}]_c$ from simultaneous addition of ATP and PTHrP (43–52) was approximately three times greater (803 ± 58.6 nM) than with 5 μM ATP alone (248 ± 41.9 nM) (mean ± SEM; Figure 2). Similar potentiation occurred with simultaneous addition of ATP and PTHrP (70–77), PTHrP (107–113), PTHrP (127–138), and PTHrP (145–172), while the background $[Ca^{2+}]_c$ remained unaltered in the short-term (Figure 2). The potentiation was concentration-dependent in the range from 1 to 100 pM PTHrP fragment (Figure 3) and occurred when PTHrP (43–52) was added 10 minutes prior to ATP (Figure 4), but acute changes in $[Ca^{2+}]_c$ were not observed with the N-terminal PTHrP (1–34) fragment (Figure 2). In contrast, simultaneous incubation of 5 μM ATP with 100 pM PTHrP (1–34) for 24 hours significantly elevated the mean baseline $[Ca^{2+}]_c$ (177 ± 3.6 nM) in comparison with untreated controls (109 ± 1.5 nM) (mean ± SEM; $P<0.01$ Student's *t*-test, $n=14$). This effect was not observed with either ATP or PTHrP (1–34) when added alone ($n=14$).

Thapsigargin (1 μM) was added after ATP, PTHrP, or ATP/PTHrP to identify whether potentiation occurred via different Ca^{2+} pools. While the duration was not significantly altered, the amplitude of the acute thapsigargin-induced Ca^{2+} responses was reduced after addition of ATP (Figure 1e), was unchanged after addition of PTHrP (data not shown), and was abolished after co-stimulation with ATP and PTHrP (Figure 1f) in comparison to thapsigargin alone (Figure 1d). Since Ca^{2+}-free conditions were used (treated with 1 mM EGTA), Ca^{2+} influx was prevented and stimulation with ATP resulted in Ca^{2+} store depletion, thus preventing subsequent thapsigargin responses. ATP in the presence or absence of PTHrP failed to acutely elevate $[Ca^{2+}]_c$ after cells were treated with 1 μM thapsigargin (data not shown). This suggests that a thapsigargin-sensitive Ca^{2+} pool is responsible for both the ATP and PTHrP-enhanced Ca^{2+} responses.

Potentiation of ATP-induced $[Ca^{2+}]_c$ response is independent of cAMP, but dependent on cGMP

ATP in the presence or absence of PTHrP fragments failed to stimulate cAMP production in HaCaT cells in comparison with the control (Figure 5). However, the cell-permeable adenylyl cyclase activator, forskolin (50 μM), significantly increased cAMP production as would be expected (Figure 5). Although 50 μM forskolin elevated cAMP (Figure 5), it significantly reduced the ATP-evoked elevation of $[Ca^{2+}]_c$ (Figure 4). In contrast, the addition of the nonspecific phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM), mimicked the response observed with PTHrP (only data for PTHrP (43–52) shown for clarity; Figures 4 and 6b), indicating the involvement of another cyclic monophosphate. Since cAMP does not appear to be involved in the peptide- or IBMX-induced potentiation of $[Ca^{2+}]_c$, we examined whether cyclic guanosine monophosphate (cGMP) could be involved. Addition of the highly selective protein kinase G inhibitor, KT5823 (0.25 μM), prevented PTHrP or IBMX from potentiating the ATP-induced elevation of $[Ca^{2+}]_c$ (Figure 6a and c respectively; 327 ± 134.6 and 162 ± 21.4 nM for KT5823 in the presence of PTHrP or IBMX respectively; mean increase in $[Ca^{2+}]_c$ ± SEM), indicating that cGMP was the likely mediator.

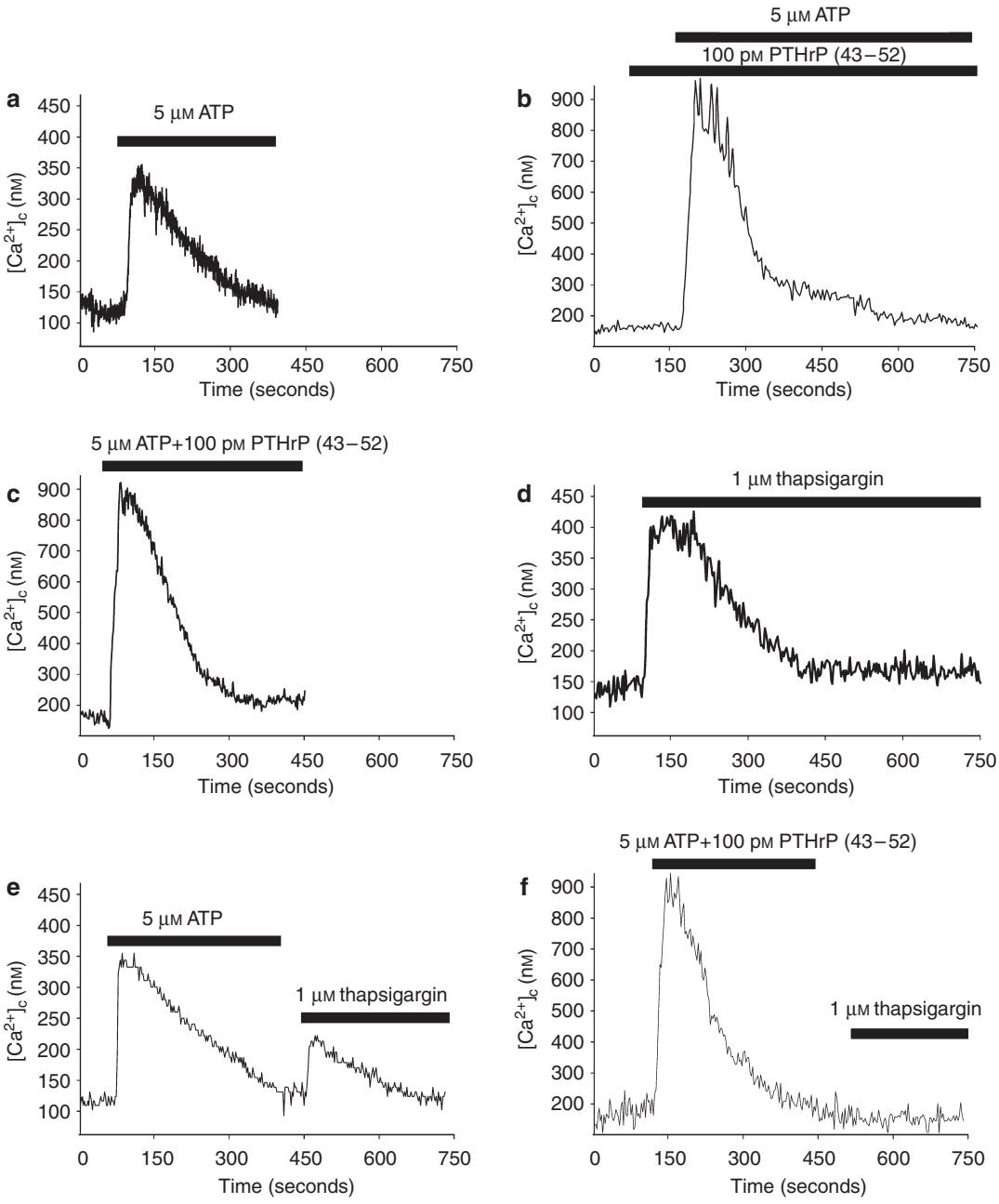

Figure 1. Effect of ATP (5 μM) and PTHrP peptides on $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded cells were stimulated with (**a**) ATP (5 μM) alone, (**b**) ATP (5 μM) in the presence of 100 pM PTHrP (43–52), (**c**) simultaneous delivery of both ATP (5 μM) and 100 pM PTHrP (43–52), (**d**) thapsigargin (1 μM) alone, (**e**) ATP (5 μM) followed by a wash and 1 μM thapsigargin, and (**f**) co-stimulation with 5 μM ATP and 100 pM PTHrP (43–52) followed by a wash and 1 μM thapsigargin (results are representative of $n = 6$ responses per treatment). The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve.

Effects of PTHrP on ATP-induced proliferation

ATP-induced proliferation was significantly increased by 100 pM PTHrP (43–52) and (70–77), but significantly decreased by 100 pM PTHrP (1–34), (127–138), and (145–172) (Figure 7). The greatest increase in proliferation was observed with ATP in combination with PTHrP (43–52). PTHrP fragments alone failed to induce a change in cell numbers in comparison with untreated cells (data not shown), indicating that fragments are not active when alone.

PTHrP (43–52) potentiates the bradykinin-induced $[Ca^{2+}]_c$ response

To identify whether the synergistic effects of PTHrP were confined to ATP, the effect on bradykinin was investigated. Bradykinin (10 μM) induced Ca^{2+} transients, which, like ATP, lasted for approximately 200 seconds (Figure 8a) and these were potentiated by 100 pM PTHrP (43–52) (Figure 8b) by approximately two-fold in comparison to bradykinin alone (Figure 9).

Table 1. Summary of PTHrP peptides

Sequence	Residues	Number of amino acids	pI	M_w
AVSEHQLLHDKGKSIQDL RRRFFLHHLIAEIHTA	1–34	34	8.66	4,118
SPNSKPSPNT	43–52	10	8.47	1,112
QETNKVET	70–77	8	4.53	1,032
TRSAWLD	107–113	7	5.75	931
SDTSTTSLELDS	127–138	12	3.49	1,339
GLKKKKENN	145–153	9	10.00	1,142

M_w, molecular weight; pI, isoelectric point.
Details include primary sequence, location, pI, and M_w.

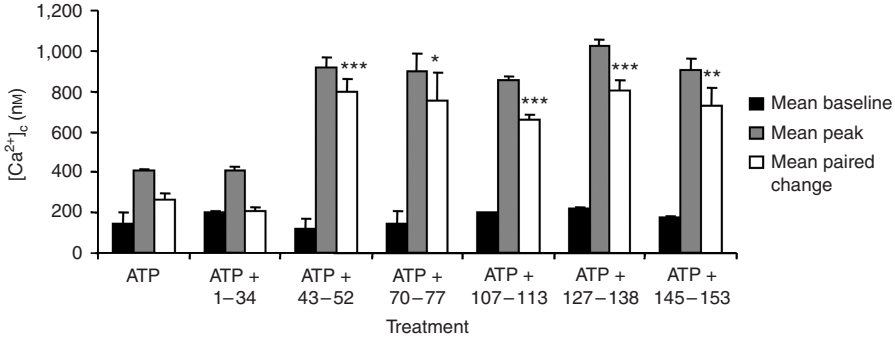

Figure 2. PTHrP peptides from throughout the PTHrP molecule, but not PTHrP (1–34), potentiate the ATP-induced $[Ca^{2+}]_c$ response in HaCaT cells. Separate fura-2-loaded HaCaT cells were simultaneously stimulated with 5 μM ATP and PTHrP fragments (100 pM) in Ca^{2+}-free phosphate-buffered saline. The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. Results are shown as mean baseline $[Ca^{2+}]_c$, mean peak $[Ca^{2+}]_c$, or mean paired change in $[Ca^{2+}]_c \pm$ SEM ($n = 6$ per treatment). Mean paired change in $[Ca^{2+}]_c$ was calculated by subtracting the baseline from the peak $[Ca^{2+}]_c$ for each cell. Statistical significance is denoted by $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ between the treatment and the control as determined by Mann–Whitney *U*-test.

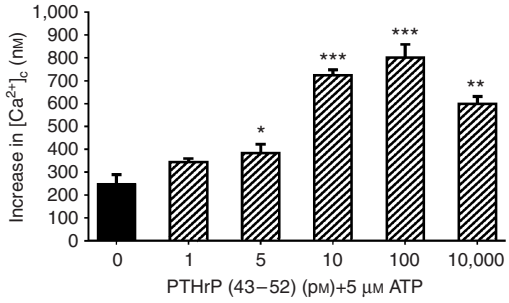

Figure 3. PTHrP (43–52) concentration-dependently increases $[Ca^{2+}]_c$ when in combination with 5 μM ATP. Single cells loaded with Ca^{2+}-free fura-2 were treated with varying concentrations of PTHrP (43–52) in combination with 5 μM ATP. The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. Results shown are mean ± SEM ($n = 6$ per treatment). Statistical significance between the treatment and the 0 pM PTHrP control is denoted as $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ and as determined by Mann–Whitney *U*-test.

DISCUSSION

Although extracellular nucleotides are important regulators of proliferation, differentiation, and apoptosis in the epidermis (Pillai and Bikle, 1992; Dixon *et al.*, 1999; Burrell *et al.*, 2003; Greig *et al.*, 2003), the mechanism by which keratinocyte proliferation is increased during wound healing is unclear. Extracellular nucleotides can act synergistically with other growth regulators to elevate proliferation (Huang *et al.*, 1989; Wang *et al.*, 1990). In this study, we have shown early evidence that multiple PTHrP fragments act synergistically with ATP (Figure 2) and alter proliferation in HaCaT cells (Figure 7). Previously, the function of specific PTHrP domains was unclear with some fragments, inducing proliferation and/or differentiation depending on the tissue or stage of the cell cycle (Whitfield *et al.*, 1996).

As HaCaT cells express multiple P2Y receptors, 5 μM ATP was used to only activate $P2Y_2$ receptors (Burrell *et al.*, 2003). Normal keratinocytes have been reported to respond to PTHrP (1–34) with elevated $[Ca^{2+}]_c$ (Orloff *et al.*, 1992), but we found no evidence for this (Figure 1b), indicating differences in the responsiveness between the HaCaT cell line and normal keratinocytes. Although mechanical stress induces ATP release from normal keratinocytes (Dixon *et al.*, 1999) and HaCaT cells (Burrell *et al.*, 2005), addition of PTHrP fragments alone cannot have induced sufficient ATP release to initiate subsequent $[Ca^{2+}]_c$ responses. The $[Ca^{2+}]_c$ responses reported here are therefore a consequence of the agonists and not mechanical stress.

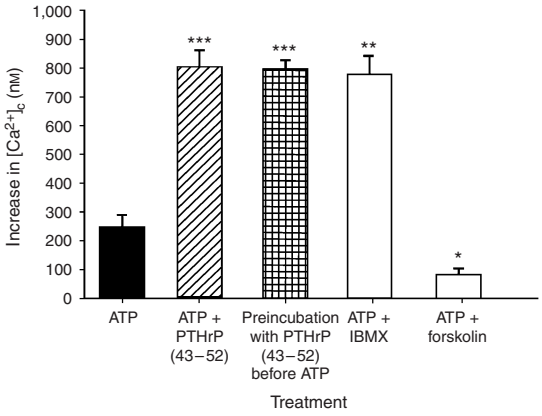

Figure 4. Effect of IBMX, forskolin, or pre-incubation with PTHrP (43–52) on ATP-induced elevation of $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded HaCaT cells were stimulated with IBMX (0.1 mM) or forskolin (50 μM) in the presence of 5 μM ATP or they were pre-incubated with 100 pM PTHrP (43–52) for 10 minutes prior to stimulation with ATP (5 μM). The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. The increase in $[Ca^{2+}]_c$ was then calculated by subtracting the baseline level from the peak concentration. Results shown are means ± SEM ($n = 6$ per treatment). Statistical significance between the treatment and ATP alone is denoted as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as determined by Mann–Whitney *U*-test.

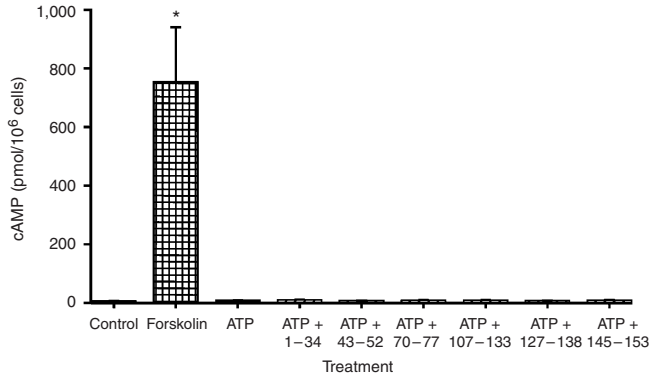

Figure 5. PTHrP fragments do not induce cAMP production in HaCaT cells. HaCaT cells were grown until confluency and serum-starved overnight in medium containing IBMX (0.1 mM) before treatment with PTHrP fragments in combination with 5 μM ATP. Forskolin (50 μM) was used as a positive control. Treatment lasted for 45 minutes before cells were collected in 65% ethanol. Samples were acetylated and cAMP analyzed by ^{125}I-radioimmunoassay. A measure of the radioactivity within samples was measured by a gamma counter, and converted into the cAMP concentration by comparison with a standard curve. Results are expressed as mean concentrations per 10^6 cells ± SEM ($n = 6$ per treatment). Statistical significance between forskolin and the control is denoted as $^*P < 0.05$ as determined by Mann–Whitney *U*-test.

The increase in $[Ca^{2+}]_c$ during potentiation of ATP-induced $[Ca^{2+}]_c$ responses by PTHrP can occur via a number of sources. $[Ca^{2+}]_c$ is controlled by the balance of elevation by release from intracellular stores, and Ca^{2+} influx with removal from the cytoplasm by Ca^{2+} efflux and sequestration. In our studies, Ca^{2+}-free conditions prevented Ca^{2+} influx, and Ca^{2+} efflux is unlikely to be maintained in the continued absence of Ca^{2+} influx since this would result in a steady decline in the cellular Ca^{2+} content and no such decline was observed. For sequestration to be involved in potentiation, a decrease in the rate of reuptake would be necessary. From our results, the converse is true since the duration of the transient when co-stimulated is approximately equal to that when stimulated with ATP alone (Figure 1c and a respectively), suggesting that sequestration is enhanced in co-stimulated cells. PTHrP, like PTH, may also facilitate translocation of Ca^{2+} between discrete intracellular stores (Short and Taylor, 2000). Ca^{2+} stores that lack inositol 1,4,5-trisphosphate ($InsP_3$) receptors (i.e. $InsP_3$-insensitive) and possess $InsP_3$ receptors (i.e. $InsP_3$-sensitive) have been suggested with the releasable Ca^{2+} pool regulated by linking these stores (Short and Taylor, 2000; Tovey *et al.*, 2003). Our results show that if this is the case, then both Ca^{2+} pools are thapsigargin-sensitive. Another hypothesis is that G proteins may also directly ''tune'' the sensitivity of $InsP_3$ receptors (Tovey *et al.*, 2003), although the mechanism remains unknown.

If potentiation involves the PTH/PTHrP type I receptor, $[Ca^{2+}]_c$ elevation could occur via activation of G_q (phospholipase C/$InsP_3$ pathway) or G_s (adenylyl cyclase/cAMP pathway) G proteins (Abou-Samra *et al.*, 1992). While $InsP_3$ directly elevates $[Ca^{2+}]_c$, cAMP activates protein kinase A, which phosphorylates the $InsP_3$ receptor at serines 1755 and 1589 (Ferris *et al.*, 1991; Haug *et al.*, 1999; Bruce *et al.*, 2002; Straub *et al.*, 2002) on the endoplasmic reticulum surface, triggering release of stored Ca^{2+}. We found no evidence for the involvement of G_s-coupled events (Figures 4 and 5) in agreement with previous studies where protein kinase A activation did not alter proliferation or $[Ca^{2+}]_c$ in HaCaT cells (Paramio and Jorcano, 1997), although events upstream of adenylyl cyclase may occur via direct cross-talk of G_s with the adjacent G_q protein (Jimenez *et al.*, 1999; Tovey *et al.*, 2003).

From the results with KT5823 and IBMX we suggest that the potentiation is due to cGMP, although the cGMP source remains unknown. IBMX, which is not G_q protein-coupled, prevents cyclic monophosphate breakdown and since cAMP can be discounted (Figure 5) it suggests the involvement of another cyclic monophosphate. Potentiation of Ca^{2+} responses by IBMX occurs in several cell types (Pannabecker and Orchard, 1987; Dasarathy and Fanburg, 1988; Buckley *et al.*, 2001). However, the involvement of cGMP was discounted in UMR-106 clonal rat osteoblasts as dibutyryl-cGMP (a cGMP analogue) did not mimic the effects of PTH (Buckley *et al.*, 2001). cGMP is elevated in specific intracellular pools with discrete effects on Ca^{2+} signaling (Zolle *et al.*, 2000). There are distinct particulate and cytosolic sources of cGMP within cells (Braughler *et al.*, 1979; Waldman *et al.*, 1984; Mittal, 1985; Ignarro *et al.*, 1986; Chinkers *et al.*, 1989; Drewett and Garbers, 1994) and at least five phosphodiesterases are involved in cGMP hydrolysis. These are expressed in different cellular regions (Braughler *et al.*, 1979; Waldman *et al.*, 1984; Mittal, 1985; Ignarro *et al.*, 1986; Beavo, 1988; Fisher *et al.*, 1998).

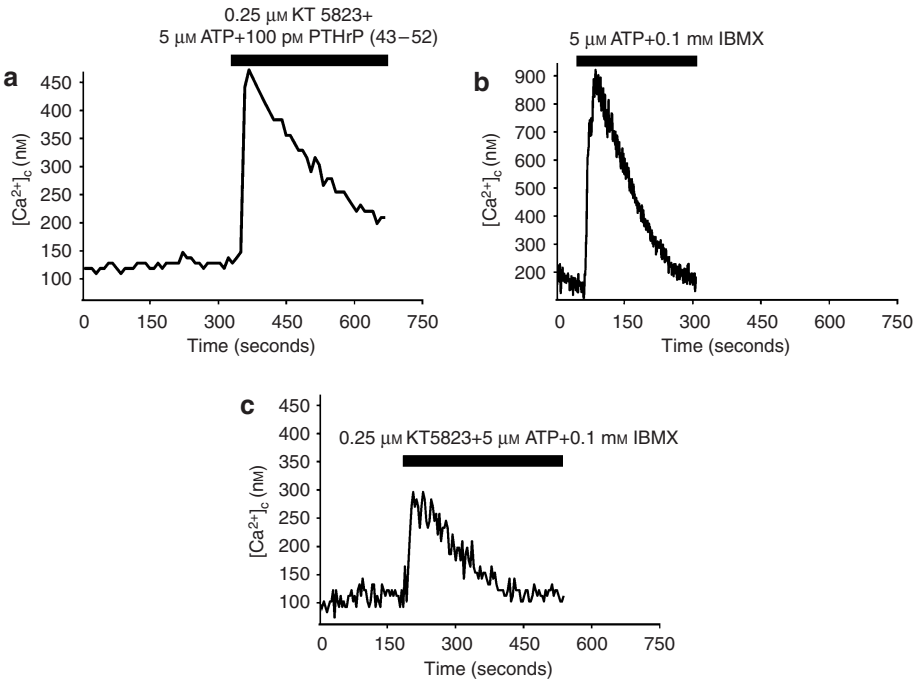

Figure 6. KT5823 abolishes the potentiation of the ATP- and IBMX-induced elevation of $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded HaCaT cells were stimulated with (**a**) KT5823 (0.25 μM), 5 μM ATP, and 100 pM PTHrP (43–52) simultaneously, (**b**) IBMX (0.1 mM) in the presence of 5 μM ATP, and (**c**) IBMX (0.1 mM) in the presence of 5 μM ATP and 0.25 μM KT5823. The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. Results are representative of $n = 10$ responses.

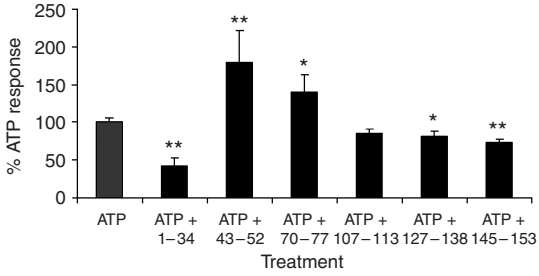

Figure 7. ATP-induced proliferation is potentiated by PTHrP (43–52) and (70–77), but decreased by PTHrP (1–34), (127–138), and (145–153) in HaCaT cells. HaCaT cells were seeded at a density of 1×10^5 cells/6 cm petri dish and allowed to settle for 24 hours prior to stimulation by 100 pM PTHrP fragments in combination with 5 μM ATP. Proliferation was determined using a Coulter counter (model ZM) to obtain mean cell numbers after a further 24 hours incubation with agonist. Results are expressed as % ATP response ± SD ($n = 8$ for controls and $n = 4$ for treatments). Figures are representative of four separate studies. Statistical significance between ATP alone and ATP in combination with PTHrP is denoted by $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test).

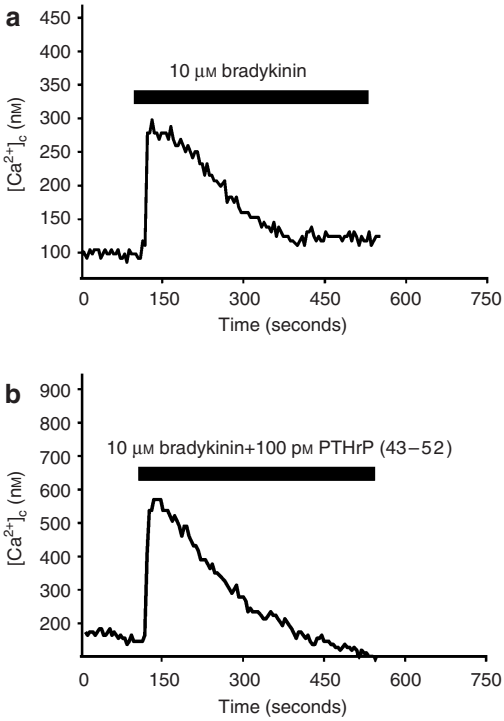

Figure 8. Effect of bradykinin and PTHrP peptides on $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded cells were stimulated with (**a**) bradykinin (10 μM) alone and (**b**) 100 pM PTHrP (43–52) with bradykinin (10 μM). The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. Result shown is representative of $n = 6$ responses.

Dibutyryl-cGMP is poor at inducing pool-specific effects of cGMP (Zolle *et al.*, 2000), which explains the results of Buckley *et al.* (2001). Potentiation of ATP-induced elevation of $[Ca^{2+}]_c$ by brain natriuretic peptide has also been linked to cGMP in ECV304 cells (Zolle *et al.*, 2000).

$[Ca^{2+}]_c$ elevation is linked to both proliferation and differentiation (Sharpe *et al.*, 1993). The significant decrease in cell number with ATP in combination with PTHrP (1–34),

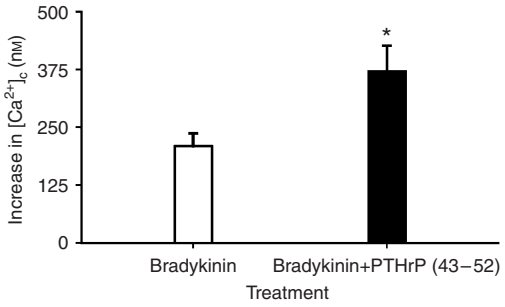

Figure 9. PTHrP (43–52) potentiates the bradykinin-induced $[Ca^{2+}]_c$ response in HaCaT cells. Bradykinin (10 μM) was added to fura-2-loaded HaCaT cells in the presence of 100 pM PTHrP (43–52). The increase in $[Ca^{2+}]_c$ was calculated by subtracting the baseline level from the peak concentration. Results are shown as mean increases in $[Ca^{2+}]_c \pm$ SEM ($n = 6$ per treatment). Statistical significance is denoted as $^*P < 0.05$ as determined by Mann–Whitney *U*-test.

(127–138), or (145–153) in comparison with ATP alone (Figure 7) may be due to the cells differentiating, as has been previously suggested (Holick *et al.*, 1989). On examination of the effects of ATP/PTHrP (1–34) on long-term elevation of $[Ca^{2+}]_c$, we found that the mean baseline $[Ca^{2+}]_c$ increased, which is also consistent with the onset of differentiation (Sharpe *et al.*, 1993). As ATP/PTHrP (43–52) or (70–77) increased proliferation above that induced by ATP alone (Figure 7), this suggests that PTHrP fragments may be important in the localized regulation of wound healing where accelerated proliferation is necessary to replace the epidermal barrier.

Finally, the effects of PTHrP in combination with a non-nucleotide substitute were studied. In contrast to normal keratinocytes (McGovern *et al.*, 1995), $[Ca^{2+}]_c$ is elevated by relatively few agonists in HaCaT cells with carbachol and substance P being ineffective (Rosenbach *et al.*, 1993). Bradykinin elevates $[Ca^{2+}]_c$ in HaCaT cells (Rosenbach *et al.*, 1993) and normal keratinocytes (Tuschil *et al.*, 1992; Rosenbach *et al.*, 1993; Coutant *et al.*, 1998; Koegel and Alzheimer, 2001) via phospholipase C (Talwar *et al.*, 1990) and $InsP_3$ production (Rosenbach *et al.*, 1993). As PTHrP (43–52) potentiated the bradykinin-induced elevation of $[Ca^{2+}]_c$ (Figure 8b), the potentiation is not confined to extracellular nucleotides. Since potentiation of the $[Ca^{2+}]_c$ response is linked to an increase in proliferation (Figure 7), we hypothesize that bradykinin, which is involved in the inflammatory response in the skin (Mullins, 1986) and is present in wounds, may increase the inflammation leading to accelerated proliferation above that of normal epidermal homeostasis particularly during wound healing.

In conclusion, these studies suggest that PTHrP peptides are functional in the epidermis where they act in synergy with G protein-coupled agonists such as ATP and the inflammatory-mediator, bradykinin, and are involved in the regulation of proliferation and differentiation. More importantly, since PTHrP is found in many normal and malignant cells (Burtis, 1992; Kaiser and Goltzman, 1993), the potentiation shown in this paper is likely to have a wider role in modulation of signal transduction events.

MATERIALS AND METHODS

Materials

DMEM, fetal calf serum, penicillin, streptomycin, and 0.05% trypsin in 0.02% EDTA were purchased from Invitrogen (Paisley, UK) and fura-2AM was from Molecular Probes via Invitrogen (Paisley, UK). EDTA (0.02%), nucleotides, IBMX, forskolin, triethylamine, acetic anhydride, and cAMP were purchased from Sigma (Poole, UK). The HaCaT cell line was a gift from Professor Fusenig (Division of Differentiation and Carcinogenesis *In Vitro*, Institute of Biochemistry, German Carcinogenesis Research Centre). Synthetic PTHrP peptides were purchased from Thistle Research (Glasgow, UK). ^{125}I-labeled cAMP, acetate buffer, primary antibody (rabbit), and donkey anti-rabbit secondary antibody solid phased to Sepharose 4B were purchased as a kit from Amersham (Buckinghamshire, UK). KT5823 was purchased from Tocris (Avonmouth, UK).

Cell culture

HaCaT cells were cultured as described previously (Burrell *et al.*, 2003). Cells were passaged at approximately 70% confluence and were pre-incubated with 0.02% EDTA for 5–10 minutes, before 5 minutes incubation with 0.05% trypsin in 0.02% EDTA. Cells were resuspended in fresh DMEM and split at a ratio of 1:10.

$[Ca^{2+}]_c$ Measurements

Cells were grown on 22 mm round coverslips for 24 hours at densities of approximately 1×10^4 cells/coverslip. Measurements were made from single cells from colonies of approximately 4–8 cells, loaded with the fluorescent calcium dye, fura-2 in calcium-free phosphate-buffered saline (EGTA-treated), using methods and equipment described previously (Sharpe *et al.*, 1993; Zolle *et al.*, 2000; Burrell *et al.*, 2003). Cells were washed in calcium-free phosphate-buffered saline to remove residual medium containing calcium, before stimulation by agonists. The ratio of emitted light (measured at 520 nm), after excitation at 340 and 380 nm, was plotted graphically and was converted into the $[Ca^{2+}]_c$ by calibrating the 340/380 nm ratio (Grynkiewicz *et al.*, 1985). The increase in $[Ca^{2+}]_c$ was determined by subtracting the baseline level from the peak level and a mean was calculated for each treatment.

Induction of cAMP

HaCaT cells were seeded onto 6 cm petri dishes and allowed to adhere and grow until confluence. Cultures were serum-starved overnight in medium containing 0.1 mM IBMX to prevent breakdown of cAMP by phosphodiesterases. Cells were treated for 45 minutes with agonists before the medium was removed and the cells scraped into 1 ml 65% ethanol. Forskolin (10 μg/ml), a cell-permeable adenylyl cyclase activator, was used as a positive control. The cells were stored at −20°C prior to cAMP analysis. Samples of cells (500 μl) were acetylated using 10 μl triethylamine and 5 μl acetic anhydride and were vortexed thoroughly between additions. A standard curve, to which samples were compared, was produced by serially diluting a stock solution of 20 mM cAMP in 0.05 M acetate buffer (pH 5.8) forming eight concentrations in the range 40–2,500 pM. These were acetylated as described above and analyzed in the same way as the samples. Additionally, three controls containing 0.25, 0.5, and 1 mM of cAMP were analyzed both at the beginning and at the end of the assay.

cAMP analysis

cAMP analysis was carried out in 12 × 75 mm borosilicate glass tubes. Acetylated standards, controls, or samples (50 µl) were added to 100 µl primary antibody (rabbit) and 100 µl of ^{125}I-labeled cAMP (100 µl of 5 µCi stock diluted in 20 ml 0.05 M acetate buffer, pH 5.8). They were then vortexed thoroughly, sealed, and incubated overnight for a minimum of 12 hours at 4°C, before 200 µl secondary antibody (donkey anti-rabbit solid phased to Sepharose 4B, diluted between 1:2 and 1:8 times) were added and shaken for a minimum of 2 hours. Samples were then centrifuged at 2,000 × *g* for 5 minutes and the pellet washed three times in 2 ml 0.09 % NaCl. Readings of the radioactivity were taken on a NE1600 gamma counter for 1 minute/sample and compared to the standard curve.

Proliferation assay

Proliferation was measured according to the protocol outlined previously (Burrell *et al.*, 2003). Cells were serum-starved overnight before being incubated for 24 hours in the presence of 100 pM PTHrP fragments, and 5 µM ATP before cell numbers were counted using a Coulter counter (model ZM). Results are expressed as % ATP response.

Statistical analysis

As the $[Ca^{2+}]_c$ measurements from keratinocytes are not normally distributed (Shapiro–Wilk *W*-test for normality), the non-parametric Mann–Whitney *U*-test was employed. For the proliferation data, the results were normally distributed (Shapiro–Wilk *W*-test for normality) and so unpaired Student's *t*-tests were carried out.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Abou-Samra AB, Juppner H, Force T, Freeman MW, Kong XF, Schipani E *et al.* (1992) Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci USA* 89:2732–6

Beavo JA (1988) Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Second Messenger Phosphoprotein Res* 22:1–38

Blomme EAG, Zhou H, Kartsogiannis V, Capen CC, Rosol TJ (1999) Spatial and temporal expression of parathyroid hormone-related protein during wound healing. *J Invest Dermatol* 112:788–95

Braughler JM, Mittal CK, Murad F (1979) Effects of thiols, sugars, and proteins on nitric oxide activation of guanylate cyclase. *J Biol Chem* 254:12450–4

Bruce JIE, Shuttleworth TJ, Giovannucci DR, Yule DI (2002) Phosphorylation of inositol 1,4,5-trisphosphate receptors in parotid acinar cells. A mechanism for the synergistic effects of cAMP on Ca^{2+} signaling. *J Biol Chem* 277:1340–8

Buckley KA, Wagstaff SC, McKay G, Gaw A, Hipskind RA, Bilbe G *et al.* (2001) Parathyroid hormone potentiates nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of G_q activation or cyclic monophosphate accumulation: a mechanism for localizing systemic responses in bone. *J Biol Chem* 276:9565–71

Burrell HE, Bowler WB, Gallagher JA, Sharpe GR (2003) Evidence for multiple P2Y receptors involved in proliferation in HaCaT cells. *J Invest Dermatol* 120:440–7

Burrell HE, Wlodarski B, Foster BJ, Buckley KA, Sharpe GR, Quayle JM *et al.* (2005) Human keratinocytes release ATP and utilize three mechanisms for nucleotide interconversion at the cell surface. *J Biol Chem* 280:29667–76

Burtis WJ (1992) Parathyroid hormone-related protein: structure, function and measurement. *Clin Chem* 38:2171–83

Chinkers MD, Garbers L, Chang MS, Lowe DG, Chin H, Goeddel DV *et al.* (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* 338:78–83

Coutant KD, Wolff-Winiski B, Ryder NS (1998) Fluvastatin enhances receptor-stimulated intracellular Ca^{2+} release in human keratinocytes. *Biochem Biophys Res Commun* 245:307–12

Danks JA, Ebeling PR, Hayman J, Chou ST, Moseley JM, Martin TJ *et al.* (1989) Parathyroid hormone-related protein: immunohistochemical localization in cancers and normal skin. *J Bone Miner Res* 4:273–8

Dasarathy Y, Fanburg BL (1988) Elevation of angiotensin converting enzyme by 3-isobutyl-1-methylxanthine in cultured endothelial cells: a possible role for calmodulin. *J Cell Physiol* 137:179–84

Dixon CJ, Bowler WB, Littlewood-Evans A, Dillon JP, Bilbe G, Sharpe GR *et al.* (1999) Regulation of epidermal homeostasis through $P2Y_2$ receptors. *Br J Pharmacol* 127:1680–6

Drewett JG, Garbers DL (1994) The family of guanylyl cyclase receptors and their ligands. *Endocrinol Rev* 15:135–62

Fenton AJ, Kemp BE, Kent GN, Moseley JM, Zheng MH, Rowe DJ *et al.* (1991) A carboxy-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts. *Endocrinology* 129:1762–8

Ferris CD, Cameron AM, Bredt DS, Huganir RL, Snyder SH (1991) Inositol 1,4,5-trisphosphate receptor is phosphorylated by cyclic AMP-dependent protein kinase at serines 1755 and 1589. *Biochem Biophys Res Commun* 175:192–8

Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* 273:15559–64

Foly J, Longely J, Wysolmerski JJ, Dreyer BE, Broadus AE, Philbrick WM (1998) PTHrP regulates differentiation in adult mice. *J Invest Dermatol* 111:1122–8

Greig AV, Linge C, Terenghi G, McGrouther DA, Burnstock G (2003) Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. *J Invest Dermatol* 120:1007–15

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–50

Hanafin NM, Chen TC, Heinrich G, Segre GV, Holick MF (1995) Cultured human fibroblasts and not cultured human keratinocytes express a PTH/PTHrP receptor mRNA. *J Invest Dermatol* 105:133–7

Hatsuzawa K, Hosaka M, Nakagawa T, Nagase M, Shoda A, Murakami K *et al.* (1990) Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4C1 cells. *J Biol Chem* 265:22075–8

Haug LS, Jensen V, Hvalby O, Walaas SI, Ostvold AC (1999) Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic nucleotide-dependent kinases *in vitro* and in rat cerebellar slices *in situ*. *J Biol Chem* 274:7467–73

Heath JK, Southby J, Fukumoto S, O'Keefe T, Martin TJ, Gillespie MT (1995) Epidermal growth factor-stimulated parathyroid hormone-related protein expression involves increased gene transcription and mRNA stability. *Biochem J* 307:159–67

Holick MF, Nussbaum S, Persons KS (1988) PTH-like humoral hypercalcemia factor (HHF) of malignancy may be an epidermal differentiation factor: synthetic hHHF(1–34)NH_2 inhibits proliferation and induces terminal differentiation of cultured human keratinocytes. *J Bone Miner Res* 3:S214 (abstract)

Holick MF, Nussbaum S, Persons K (1989) Synthetic fragments of PTH-related peptide (PTHrP) inhibit the proliferation and induce differentiation of cultured human keratinocytes. PTHrP may be an endogenous anti-proliferation and differentation factor for keratinocytes. *Clin Res* 37:529 (abstract)

Huang N, Wang D, Heppel LA (1989) Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors. *Proc Natl Acad Sci USA* 86:7904–8

Ignarro LJ, Harbison RG, Wood KS, Kadowitz PJ (1986) Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. *J Pharmacol Exp Ther* 237:893–900

Jimenez AI, Castro E, Mirabet M, Franco R, Delicado EG, Miras-Portugal MT (1999) Potentiation of ATP calcium responses by A(2B) receptor stimulation and other signals coupled to G_s proteins in type-1 cerebellar astrocytes. *Glia* 26:119–28

Johnson RC, Darlington DN, Hand TA, Bloomquist BT, Mains RE (1994) PACE4: a subtilisin-like endoprotease prevalent in the anterior pituitary and regulated by thyroid status. *Endocrinology* 135:1178–85

Juhlin L, Hagforsen E, Juhlin C (1992) Parathyroid hormone-related protein is localised in the granular layer of normal skin and in the dermal infiltrates of mycosis fungoides but is absent in psoriatic lesions. *Acta Derm Venereol* 72:81–3

Kaiser SM, Goltzman D (1993) Parathyroid hormone-related peptide. *Clin Invest Med* 16:395–406

Kaiser SM, Laneuville P, Bernier SM, Rhim JS, Kremer R, Goltzman D (1992) Enhanced growth of a human keratinocyte cell line induced by antisense RNA for parathyroid hormone-related peptide. *J Biol Chem* 267:13623–8

Kaiser SM, Sebag M, Rhim JS, Kremer R, Goltzman D (1994) Antisense-mediated inhibition of parathyroid hormone-related peptide production in a keratinocyte cell line impedes differentiation. *Mol Endocrinol* 8:139–47

Kaplan AD, Reimer WJ, Feldman RD, Dixon SJ (1995) Extracellular nucleotides potentiate the cytosolic Ca^{2+}, but not cyclic adenosine 3′, 5′-monophosphate response to parathyroid hormone in rat osteoblastic cells. *Endocrinology* 136:1674–84

Koegel H, Alzheimer C (2001) Expression and biological significance of Ca^{2+}-activated ion channels in human keratinocytes. *FASEB J* 15:145–54

Lam MH, Olsen SL, Rankin WA, Ho PW, Martin TJ, Gillespie MT *et al.* (1997) PTHrP and cell division: expression and localization of PTHrP in a keratinocyte cell line (HaCaT) during the cell cycle. *J Cell Physiol* 173:433–46

McGovern UB, Jones KT, Sharpe GR (1995) Intracellular calcium as a second messenger following stimulation of human keratinocytes. *Br J Dermatol* 132:892–6

Mittal CK (1985) Atriopeptin II and nitrovasodilator-mediated shifts in guanosine 3′,5′-cyclic monophosphate in rat thoracic aorta: evidence for involvement of distinct guanylate cyclase pools. *Eur J Pharmacol* 115:127–8

Mullins RJ (1986) Bradykinin causes a prolonged increase in skin microvascular permeability. *J Surg Res* 40:540–9

Orloff JJ, Ganz MB, Ribaudo AE, Burtis WJ, Reiss M, Milstone LM *et al.* (1992) Analysis of parathyroid hormone-related protein binding and signal transduction mechanisms in benign and malignant squamous cells. *Am J Physiol* 262:E599–607

Orloff JJ, Reddt D, de Papp AE, Yang KH, Soifer NE, Stewart AF (1994) Parathyroid hormone-related protein as a pro-hormone: post-translational processing and receptor interactions. *Endocrine Rev* 15:40–60

Pannabecker T, Orchard I (1987) Regulation of adipokinetic hormone-release from locust neuroendocrine tissue-participation of calcium and cyclic-AMP. *Brain Res* 423:13–22

Paramio JM, Jorcano JL (1997) Role of protein kinases in the *in vitro* differentiation of human epidermal HaCaT cells. *Br J Dermatol* 137:44–50

Pearton DJ, Nirunsuksiri W, Rehemtulla A, Lewis SP, Presland RB, Dale BA (2001) Proprotein convertase expression and localisation in epidermis: evidence for multiple roles and substrates. *Exp Dermatol* 10:193–203

Pillai S, Bikle DD (1992) Adenosine triphosphate stimulates phosphoinositide metabolism, mobilises intracellular calcium, and inhibits terminal differentiation of human epidermal keratinocytes. *J Clin Invest* 90:42–51

Rosenbach T, Liesgang C, Binting S, Czarnetzki BM (1993) Inositol phosphate formation and release of intracellular free calcium by bradykinin in HaCaT keratinocytes. *Arch Derm Res* 285:393–6

Sharpe GR, Dillon JP, Durham B, Gallagher JA, Fraser WD (1998) Human keratinocytes express transcripts for three isoforms ofparathyroid hormone-related protein (PTHrP), but not for the parathyroid hormone/PTHrP receptor: effects of $1,25(OH)_2$ vitamin D_3. *Br J Dermatol* 138:944–51

Sharpe GR, Fisher C, Gillespie JI, Greenwell JR (1993) Growth and differentiation stimuli induce different and distinct increases in intracellular free calcium in human keratinocytes. *Arch Derm Res* 284:445–50

Short AD, Taylor CW (2000) Parathyroid hormone controls the size of the intracellular Ca^{2+} stores available to receptors linked to inositol trisphosphate formation. *J Biol Chem* 275:1807–13

Straub SV, Giovannucci DR, Bruce JIE, Yule DI (2002) A role for phosphorylation of inositol 1,4,5-trisphosphate receptors in defining calcium signals induced by peptide agonists in pancreatic acinar cells. *J Biol Chem* 277:31949–56

Talwar HS, Fisher GJ, Vorhees JJ (1990) Bradykinin induces phosphoinositide turnover, 1,2-diglyceride formation, and growth in cultured adult human keratinocytes. *J Invest Dermatol* 95:705–10

Tovey SC, Goraya TA, Taylor CW (2003) Parathyroid hormone increases the sensitivity of inositol trisphosphate receptors by a mechanism that is independent of cyclic AMP. *Br J Pharmacol* 138:81–90

Tuschil A, Lam C, Haslberger A, Lindley I (1992) Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. *J Invest Dermatol* 99:294–8

Waldman SA, Rapoport RM, Murad F (1984) Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J Biol Chem* 259:14332–4

Wang D, Huang N, Heppel LA (1990) Extracellular ATP shows synergistic enhancement of synthesis when combined with agents that are active in wound healing or as neurotransmitters. *Biochem Biophys Res Commun* 66:251–8

Whitfield JF, Isaacs RJ, Jouishomme H, MacLean S, Chakravarthy BR, Morley P *et al.* (1996) C-Terminal fragment of parathyroid hormone-related protein, PTHrP (107–111), stimulates membrane-associated protein kinase C activity and modulates the proliferation of human murine skin keratinocytes. *J Cell Physiol* 166:1–11

Yang KH, Depapp AE, Soifer NE, Dreyer BE, Wu TL, Porter SE *et al.* (1994) Parathyroid hormone-related protein: evidence for isoform-specific and tissue-specific post-translational processing. *Biochemistry* 33:7460–9

Zolle O, Lawrie AM, Simpson AWM (2000) Activation of the particulate and not the soluble guanylate cyclase leads to the inhibition of Ca^{2+} extrusion through localized elevation of cGMP. *J Biol Chem* 275:25892–9