

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

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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.

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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 P2Y₂ 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). P2Y₂ 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,

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Abbreviations: ATP, adenosine 5'-triphosphate; $[Ca^{2+}]_c$, cytosolic-free calcium concentration; cGMP, cyclic guanosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; InsP₃, inositol 1,4,5-trisphosphate; PTHrP, parathyroid hormone-related protein

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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* (Pearson *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 μ M) 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 \pm 58.6 nM) than with 5 μ M ATP alone (248 \pm 41.9 nM)

(mean \pm 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 μ M ATP with 100 pM PTHrP (1–34) for 24 hours significantly elevated the mean baseline $[Ca^{2+}]_c$ (177 \pm 3.6 nM) in comparison with untreated controls (109 \pm 1.5 nM) (mean \pm 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 μ M) 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 μ M 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 μ M), significantly increased cAMP production as would be expected (Figure 5). Although 50 μ M 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 μ M), prevented PTHrP or IBMX from potentiating the ATP-induced elevation of $[Ca^{2+}]_c$ (Figure 6a and c respectively; 327 \pm 134.6 and 162 \pm 21.4 nM for KT5823 in the presence of PTHrP or IBMX respectively; mean increase in $[Ca^{2+}]_c \pm$ SEM), indicating that cGMP was the likely mediator.

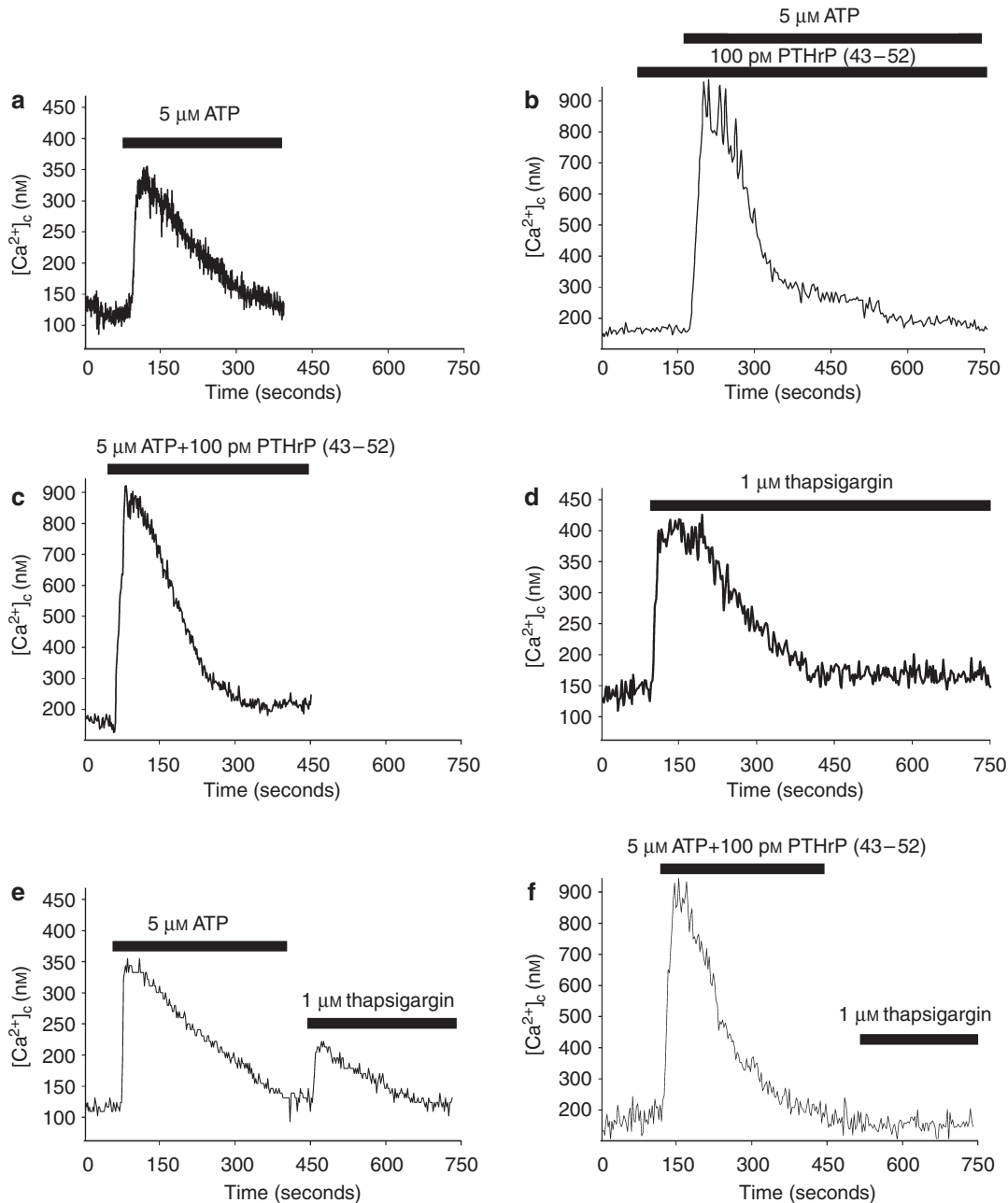


Figure 1. Effect of ATP (5 μ M) and PTHrP peptides on $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded cells were stimulated with (a) ATP (5 μ M) alone, (b) ATP (5 μ M) in the presence of 100 pM PTHrP (43–52), (c) simultaneous delivery of both ATP (5 μ M) and 100 pM PTHrP (43–52), (d) thapsigargin (1 μ M) alone, (e) ATP (5 μ M) followed by a wash and 1 μ M thapsigargin, and (f) co-stimulation with 5 μ M ATP and 100 pM PTHrP (43–52) followed by a wash and 1 μ M 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 μ M) 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 RRRFFLHHLIAEIHITA	1–34	34	8.66	4,118
SPNSKPSNT	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.

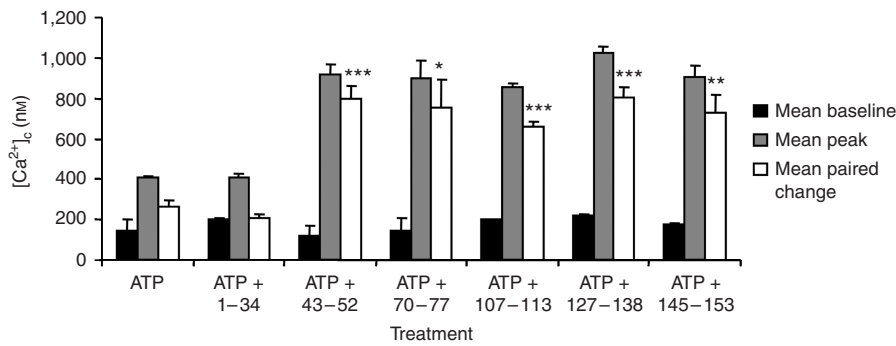


Figure 2. PTHrP peptides from throughout the PTHrP molecule, but not PTHrP (1–34), potentiate the ATP-induced $[Ca^{2+}]_c$ response in HaCaT cells. Separate fura-2-loaded HaCaT cells were simultaneously stimulated with $5 \mu M$ ATP and PTHrP fragments ($100 \mu M$) in Ca^{2+} -free phosphate-buffered 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.

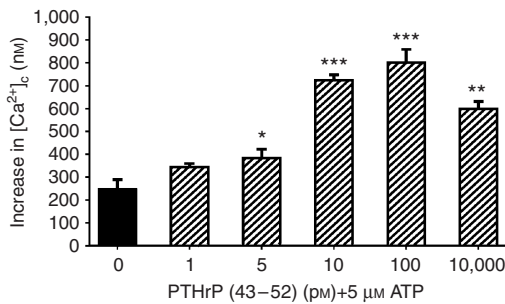


Figure 3. PTHrP (43–52) concentration-dependently increases $[Ca^{2+}]_c$ when in combination with $5 \mu M$ ATP. Single cells loaded with Ca^{2+} -free fura-2 were treated with varying concentrations of PTHrP (43–52) in combination with $5 \mu M$ ATP. The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$ (nM) by comparison with a standard curve. Results shown are mean \pm SEM ($n = 6$ per treatment). Statistical significance between the treatment and the $0 \mu M$ 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, $5 \mu M$ ATP was used to only activate P2Y₂ 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.

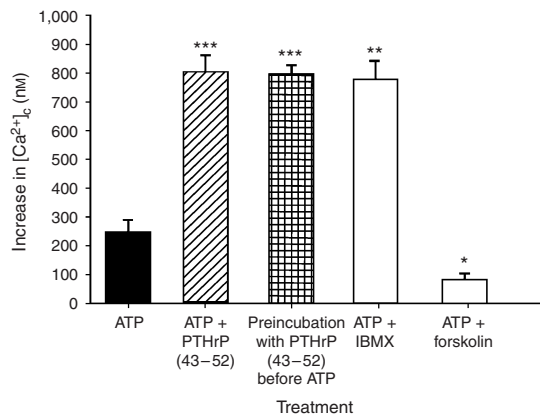


Figure 4. Effect of IBMX, forskolin, or pre-incubation with PTHrP (43-52) on ATP-induced elevation of $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded HaCaT cells were stimulated with IBMX (0.1 mM) or forskolin (50 μ M) in the presence of 5 μ M ATP or they were pre-incubated with 100 pM PTHrP (43-52) for 10 minutes prior to stimulation with ATP (5 μ M). 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 \pm 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.

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,

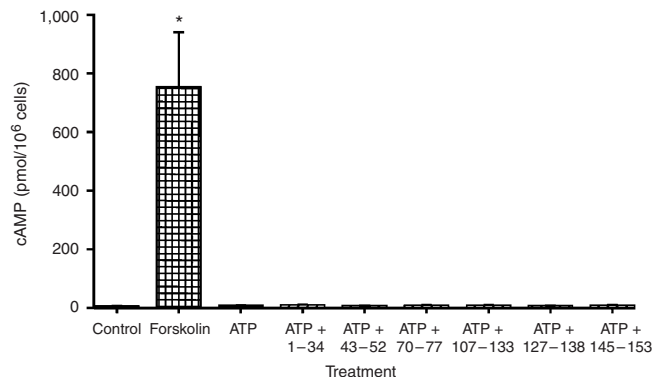


Figure 5. PTHrP fragments do not induce cAMP production in HaCaT cells. HaCaT cells were grown until confluency and serum-starved overnight in medium containing IBMX (0.1 mM) before treatment with PTHrP fragments in combination with 5 μ M ATP. Forskolin (50 μ M) was used as a positive control. Treatment 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 \pm 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.

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 (Braugher 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 (Braugher et al., 1979; Waldman et al., 1984; Mittal, 1985; Ignarro et al., 1986; Beavo, 1988; Fisher et al., 1998).

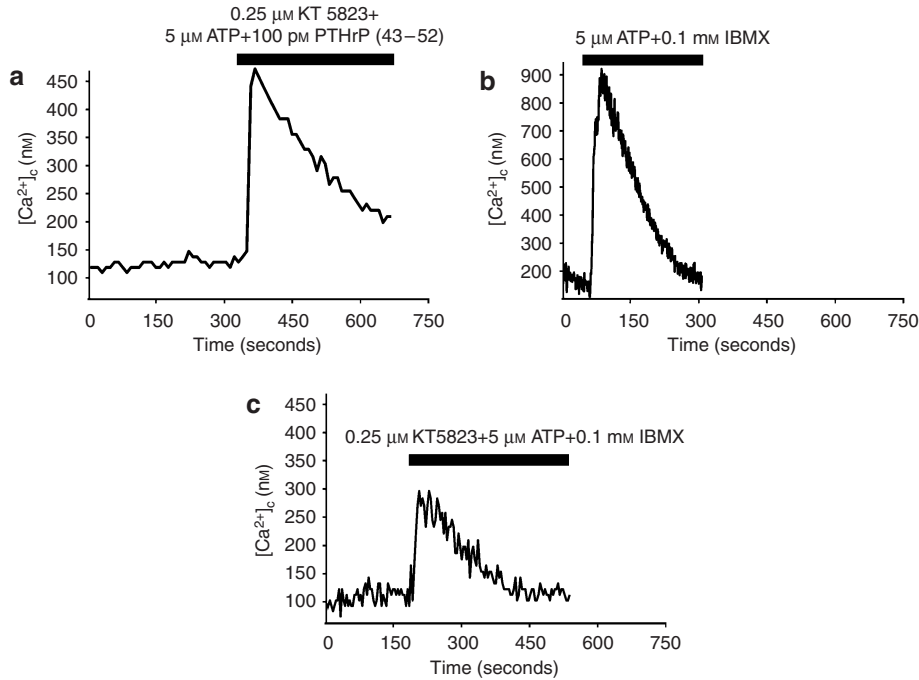


Figure 6. KT5823 abolishes the potentiation of the ATP- and IBMX-induced elevation of $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded HaCaT cells were stimulated with (a) KT5823 (0.25 μ M), 5 μ M ATP, and 100 pM PTHrP (43–52) simultaneously, (b) IBMX (0.1 mM) in the presence of 5 μ M ATP, and (c) IBMX (0.1 mM) in the presence of 5 μ M ATP and 0.25 μ M 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.

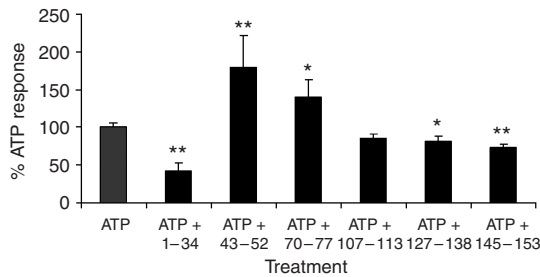


Figure 7. ATP-induced proliferation is potentiated by PTHrP (43–52) and (70–77), but decreased by PTHrP (1–34), (127–138), and (145–153) in HaCaT cells. HaCaT cells were seeded at a density of 1×10^5 cells/6 cm petri dish and allowed to settle for 24 hours prior to stimulation by 100 pM PTHrP fragments in combination with 5 μ M 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 \pm 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).

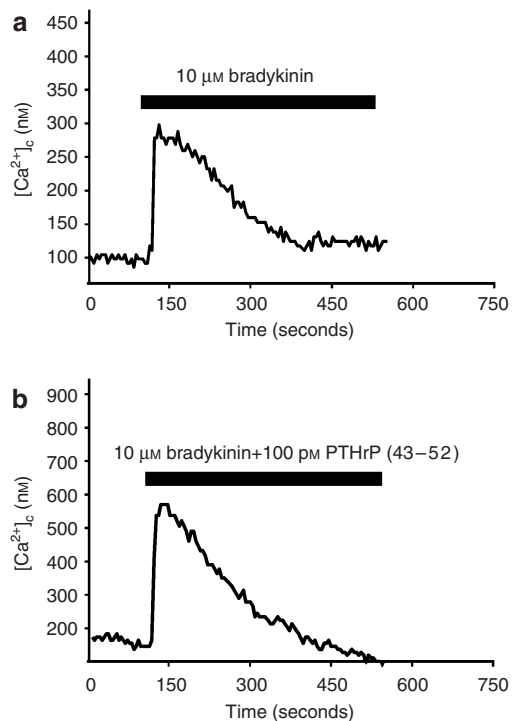


Figure 8. Effect of bradykinin and PTHrP peptides on $[Ca^{2+}]_c$ in HaCaT cells. Fura-2-loaded cells were stimulated with (a) bradykinin (10 μ M) alone and (b) 100 pM PTHrP (43–52) with bradykinin (10 μ M). The ratio of emitted light (520 nm) after excitation at 340 and 380 nm was converted into $[Ca^{2+}]_c$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

Dibutyl-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),

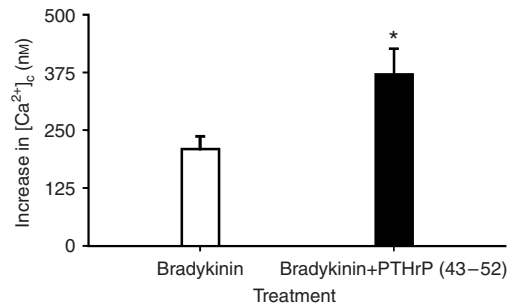


Figure 9. PTHrP (43-52) potentiates the bradykinin-induced $[Ca^{2+}]_c$ response in HaCaT cells. Bradykinin ($10 \mu\text{M}$) was added to fura-2-loaded HaCaT cells in the presence of 100 pM PTHrP (43-52). The increase in $[Ca^{2+}]_c$ was calculated by subtracting the baseline level from the peak concentration. Results are shown as mean increases in $[Ca^{2+}]_c \pm \text{SEM}$ ($n=6$ 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 (Gryniewicz *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 ($10 \mu\text{g/ml}$), 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 ($500 \mu\text{l}$) were acetylated using $10 \mu\text{l}$ triethylamine and $5 \mu\text{l}$ 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 $40\text{-}2,500 \text{ pM}$. 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 μ l) were added to 100 μ l primary antibody (rabbit) and 100 μ l of ^{125}I -labeled cAMP (100 μ l of 5 μ Ci 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 μ l 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 $2,000 \times g$ 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 μ M 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.

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