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Parathyroid hormone increases the sensitivity of inositol trisphosphate receptors by a mechanism that is independent of cyclic AMP

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1 In fura 2-loaded HEK-293 cells stably expressing human type 1 parathyroid hormone (PTH) receptors, PTH potentiated the Ca^{2+} mobilization evoked by carbachol by >4 fold without itself increasing the intracellular [Ca^{2+}].

2 PTH potentiated the Ca^{2+} release evoked by a cell-permeant analogue of inositol 1,4,5-trisphosphate (InsP_3BM).

3 Prolonged incubation with InsP_3BM emptied the Ca^{2+} stores as effectively as PTH in combination with a maximal concentration of carbachol, indicating that PTH did not increase the size of the InsP_3-sensitive Ca^{2+} pool.

4 Responses to PTH were unaffected by disruption of the cytoskeleton.

5 The EC_{50} for carbachol-evoked Ca^{2+} release and InsP_3 formation were indistinguishable ($\sim$40 μM), consistent with even the highest concentrations of carbachol generating insufficient InsP_3 to release the entire InsP_3-sensitive Ca^{2+} pool.

6 Inhibition of cyclic AMP-dependent protein kinase A (PKA), using H89 or CMIQ, did not affect potentiation of carbachol-evoked Ca^{2+} signals by PTH.

7 SQ22536 or DDA, inhibitors of adenylyl cyclase, inhibited PTH-evoked cyclic AMP formation and IBMX, an inhibitor of cyclic nucleotide phosphodiesterase, increased the amount of cyclic AMP detected after stimulation by PTH. None of these drugs affected the potentiation of Ca^{2+} signals by maximal or submaximal concentrations of PTH.

8 We conclude that PTH potentiates the Ca^{2+} release evoked by receptors that stimulate InsP_3 formation by sensitizing InsP_3 receptors through a cyclic AMP-independent mechanism.
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Abbreviations: $[Ca^{2+}]_i$, cytosolic free [Ca^{2+}]; cAMP, adenosine 3′5′-cyclic monophosphate; CCE, capacitative Ca^{2+} entry; CCh, carbachol; CMIQ, 4-cyano-3-methylisoquinoline; CREB, cAMP-response element-binding protein; DDA, 2′,5′-dideoxyadenosine; EPAC, exchange protein directly activated by cAMP; *h*, Hill coefficient; HEK/PTH-R1 cells, human embryonic kidney 293 cells stably transfected with human type 1 PTH receptor; IBMX, 3-isobutyl-1-methylxanthine; InsP_3, inositol 1,4,5-trisphosphate; InsP_3BM, InsP_3 hexakis(butyryloxymethyl) ester; PKA, cAMP-dependent protein kinase; PTH, parathyroid hormone; $t_{\frac{1}{2}}$, half-time

Introduction

Parathyroid hormone (PTH) plays a major part in controlling the plasma Ca^{2+} concentration and bone re-modelling. Although the hormone comprises 84 amino acid residues, the first 34 residues (PTH 1–34) are sufficient to elicit the full range of biological responses. Each of the two subtypes of receptor for PTH belongs to the family of G protein-coupled receptors to which the receptors for glucagon, secretin, calcitonin and vasoactive intestinal peptide also belong (Jüppner *et al*., 1991). Each of these receptors shares an ability to stimulate both formation of adenosine 3′,5-cyclic monophosphate (cAMP) and the release of Ca^{2+} from intracellular stores, although the relationship between these two signalling processes is not yet clear.

The means whereby PTH stimulates release of Ca^{2+} from intracellular stores is unknown, although it is clear that type 1 and type 2 PTH receptors (Abou-Samra *et al*., 1992; Jüppner *et al*., 1991) are each capable of stimulating Ca^{2+} mobilization. Some reports have suggested that PTH stimulates formation of inositol 1,4,5-trisphosphate (InsP_3) and thereby release of Ca^{2+} from intracellular stores (Abou-Samra *et al*., 1992), but others failed to detect any effect of PTH on InsP_3 formation (Seuwen & Boddeke, 1995; Short & Taylor, 2000). Neither ryanodine receptors, cyclic ADP ribose, nicotinic acid adenine dinucleotide phosphate (NAADP) or sphingosine-1-phosphate are likely to mediate the effects of PTH on intracellular Ca^{2+} stores (Short & Taylor, 2000; Tong *et al*., 1996). Our work with human type 1 PTH receptors expressed in HEK-293 cells (Short & Taylor, 2000) and a subsequent study using an osteosarcoma cell line (Buckley *et al*., 2001) have suggested that the most pronounced effect of PTH is to massively potentiate the Ca^{2+} signals evoked by other receptors that stimulate InsP_3 formation, without PTH itself directly evoking significant

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Ca^{2+} mobilization. Because PTH potentiated the Ca^{2+} signals evoked by even supramaximal concentrations of carbachol, an agonist that stimulates InsP_3 formation, we speculated that PTH might work by facilitating transfer of Ca^{2+} from an InsP_3-insensitive store to an InsP_3-sensitive one (Short & Taylor, 2000). The present work addresses this possibility directly.

The nature of the intracellular signals that link PTH receptors to the behaviour of intracellular Ca^{2+} stores is also unclear. The possibility that cAMP-dependent protein kinase (PKA) mediates these effects has been considered because PTH stimulates cAMP formation, and PKA is known to phosphorylate InsP_3 receptors (Bruce *et al*., 2002), but available evidence suggests that PKA is unlikely to be required for PTH to potentiate the Ca^{2+} signals evoked by other InsP_3-linked receptors (Buckley *et al*., 2001; Short & Taylor, 2000). EPAC, a guanine nucleotide exchange factor, is another intracellular target of cAMP (de Rooij *et al*., 1998), and it has recently been shown to stimulate phospholipase C-ε, leading to InsP_3 formation (Schmidt *et al*., 2001). This pathway is unlikely to mediate the effect of PTH on Ca^{2+} release because under conditions where PTH massively potentiates responses to agonists that cause InsP_3 formation, it barely stimulates Ca^{2+} release itself and causes no detectable formation of InsP_3 (Buckley *et al*., 2001; Short & Taylor, 2000). Nevertheless, it is important to resolve whether cAMP is required for the actions of PTH. Earlier results are inconclusive: 8-Br-cAMP (Short & Taylor, 2000) or forskolin (Buckley *et al*., 2001) failed to mimic PTH, but 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterase, potentiated responses to PTH (Buckley *et al*., 2001).

In this study, we use HEK-293 cells expressing human type 1 PTH receptors to resolve two important issues. Does PTH work by increasing the size of the intracellular Ca^{2+} pool available to InsP_3 (Short & Taylor, 2000)? Secondly, what role does cAMP play in mediating the effects of PTH? We conclude that PTH increases the sensitivity of the InsP_3 receptor without affecting the size of the InsP_3-sensitive Ca^{2+} stores and by a mechanism that does not require cAMP.

Methods

Materials

Brefeldin A, colchicine, cytochalasin D, nocodazole, CCh, 3-isobutyl-1-methylxanthine (IBMX), SQ22536 (9-(tetrahydro-2′-furyl)adenine) and all protease and phsophatase inhibitors were obtained from Sigma (Poole, U.K.) Jasplakinolide, latrunculin B, paclitaxel, vinblastine sulphate, ionomycin, H-89 dihydrochloride and CMIQ (4-cyano-3-methylisoquinoline) were from Calbiochem (Nottingham, U.K.). Fura 2 AM was obtained from Molecular Probes (Leiden, The Netherlands). *D-myo*-[2-^{3}H]inositol (16 Ci mmol^{-1}) was from Amersham Pharmacia Biotech (Little Chalfont), and $^{45}Ca^{2+}$ (620 Ci mol^{-1}) was from ICN Biomedicals (Thame, U.K.). Rabbit polyclonal anti-CREB and anti-phospho CREB antibodies were from New England Biolabs (Hitchin, U.K.) and HRP-conjugated donkey anti-rabbit IgG secondary antibodies were from AbCam (Cambridge, U.K.). Human parathyroid hormone (residues 1–34) was from Bachem (Saffron Walden, U.K.); throughout the text, we refer to this active fragment of the hormone as PTH. Thapsigargin was from Alomone Labs (Jerusalem, Israel). The membrane-permeant ester of InsP_3, D-*myo*-Ins(1,4,5)P_3 hexakis(butyryloxymethyl) ester (InsP_3BM) prepared by Professor Andrew Holmes (Department of Chemistry, University of Cambridge, U.K.) (Li *et al*., 1997) was a gift from Dr Martin Bootman (Babraham Institute, Cambridge, U.K.).

Cell culture

HEK 293 cells stably transfected with the human type 1 PTH receptor (HEK/PTH-R1 cells) (Short & Taylor, 2000) were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with L-glutamine (2 mM), foetal calf serum (10%) and G-418 (800 μg ml^{-1}). Cells were incubated in a humidified atmosphere (95% air, 5% CO_2, 37°C), the culture medium was replaced every third day, and cells were passaged when they reached ~80% confluence. For single cell imaging, cells were plated onto 22-mm round glass coverslips coated with 0.1% poly-L-lysine (Sigma) and used after 2 days in culture.

Single cell imaging of $[Ca^{2+}]_i$

Cells on coverslips were loaded with fura 2 in HEPES-buffered saline (HBS) by incubation with fura 2 AM (2 μM, prepared in anhydrous DMSO) for 45 min at 20°C, followed by a further 45-min incubation in the absence of fura 2 AM to allow de-esterification of the indicator. HBS had the following composition (mM): NaCl 135, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6 and glucose 11.5, pH 7.3. Measurements of $[Ca^{2+}]_i$ in single cells were performed at 20°C as previously described (Short & Taylor, 2000), with fluorescence ratios collected at intervals of 5 s. After correction for autofluorescence, the fluourescence ratios (F_{340}/F_{380}) were calibrated to $[Ca^{2+}]$ using a look-up table prepared from Ca^{2+} standard solutions (Molecular Probes, Calcium Calibration Kit with 1 mM $MgCl_2$). For experiments with drugs that disrupt the cytoskeleton, the cells were pre-incubated with drugs for up to 150 min and the drugs were then present in all media used subsequently; time-matched incubations with only the solvent were used for controls.

In some experiments, we asked whether a treatment had selectively affected CCh-evoked Ca^{2+} release from intracellular stores relative to Ca^{2+} entry across the plasma membrane. The peak increase in $[Ca^{2+}]_i$ evoked by CCh in Ca^{2+}-free HBS was used to report Ca^{2+} release from intracellular stores, and because that signal terminated within 60 s (Figure 1A), we used the average $[Ca^{2+}]_i$ recorded in normal HBS during the following 50 s to report Ca^{2+} entry.

For most experiments, the responses of 30 single cells from a coverslip were averaged, and statistical analyses then applied to the average results from independent measurements of at least three coverslips.

Measurements of $^{45}Ca^{2+}$ efflux

Confluent cultures of HEK/PTH-R1 cells in 35-mm culture dishes were loaded with $^{45}Ca^{2+}$ (10 μCi ml^{-1} in HBS) for 2 h at 20°C. The cells were washed (6 × 1 ml) with Ca^{2+}-free HBS (HBS in which 1 mM EGTA replaced the $CaCl_2$). Thereafter,

the medium (1 ml with appropriate additions) was removed and replaced at intervals of 45 s. The amount of $^{45}Ca^{2+}$ released into the medium during each 45-s interval was then determined after addition of scintillation cocktail (Emulsifier Safe, Packard, Groningen, The Netherlands) to each fraction followed by liquid scintillation counting. At the end of each experiment, the $^{45}Ca^{2+}$ remaining within the cells was determined by addition of Triton X-100 (1%). The $^{45}Ca^{2+}$ released during each 45-s interval was expressed as a fractional release rate (i.e. the amount of $^{45}Ca^{2+}$ released during that interval as a fraction of the $^{45}Ca^{2+}$ content of the stores at the beginning of the interval).

Measurement of ^{3}H-InsP$_3$ formation

HEK/PTH-R1 cells (5×10^5 cells per dish) were plated onto 35-mm dishes and cultured under identical conditions to those described above, but with D-*myo*-[2-^{3}H]inositol (10 μCi ml^{-1}) present for the final 48 h. After stimulation under conditions identical to those used for $^{45}Ca^{2+}$ efflux experiments, the incubations were terminated by aspiration of the medium and addition of cold perchloric acid (3%). The acid extracts were neutralized and the ^{3}H-inositol phosphates separated by anion exchange chromatography (Short & Taylor, 2000).

cAMP assay

Confluent cultures of HEK/PTH-R1 cells in 6-well plates were pre-incubated at 20°C with HBS containing IBMX (1 mM, 30 min) before addition of appropriate stimuli for 45 s in Ca^{2+}-free HBS (to exactly mimic the conditions used to record $^{45}Ca^{2+}$ efflux). Incubations were terminated by lysis of the cells and the amounts of cAMP then determined using an immunosorbant assay kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Immunoblotting assay of CREB phosphorylation

HEK/PTH-R1 cells grown to confluence in 75-cm^2 flasks were washed with HBS before incubation at 20°C in HBS first with appropriate inhibitors and then in their continued presence with appropriate stimuli for 10 min. Reactions were terminated by placing the flasks on ice and washing the cells with cold phosphate-buffered saline containing NaF (10 mM) and Na_3VO_4 (100 μM). The cells were scraped into lysis buffer (0.5 ml): NaCl 50 mM, $Na_4P_2O_7$ 30 mM, NaF 50 mM, $ZnCl_2$ 5 mM, Na_3VO_4 100 μM, okadaic acid 200 nM; *p*-nitrophenyl-phosphate 10 mM, PMSF 0.2 mM, benzamidine 0.5 mM, 4-(2-aminoethyl)benzensulfonyl fluoride 2 mM, EDTA 1 mM; bestatin 130 μM, E-64 1.4 μM, leupeptin 1 μM, aprotinin 0.3 μM; dithiothreitol 1 mM, β-glycerophosphate 20 mM, 1% triton X-100, Tris 10 mM, pH 7.05) and vortexed vigorously for 60 s. Cell lysates were centrifuged ($14{,}000\times g$, 10 min) and the supernatant used for protein determination (Bradford, 1976) and immunoblotting. Standard methods were used for SDS–PAGE (8.5%) and immunoblotting (Harlow & Lane, 1998) using the primary antibodies (for phospho-CREB and CREB independent of its phosphorylation state) diluted 1 : 1000 for 12 h at 4°C, and the secondary antibody at 1 : 2000 for 1 h at 20°C. Blots were developed using Supersignal chemiluminescent reagents (Pierce and Warriner, Chester, U.K.) and Hyperfilm (Amersham Pharmacia Biotech, U.K.).

Statistical analysis

Results are expressed as the mean ± standard error of the mean (s.e.mean) and statistical significance was assessed using unpaired Student's *t*-test with $P<0.05$ considered significant.

Results

PTH potentiates CCh-evoked Ca^{2+} signals

Figure 1 shows the characteristic response of HEK/PTH-R1 cells to stimulation with CCh and PTH. In Ca^{2+}-free HBS, 83 ± 3% ($n=10$ fields) of cells responded to addition of a maximally effective concentration of CCh (1 mM) with a substantial (485 ± 29 nM, $n=8$) increase in $[Ca^{2+}]_i$ (Figure 1A). A subsequent challenge with CCh caused only a slight (135 ± 9 nM, $n=3$) increase in $[Ca^{2+}]_i$, indicating that the first challenge had substantially depleted the Ca^{2+} stores available to CCh. Addition of ionomycin after two CCh challenges evoked a substantial increase in $[Ca^{2+}]_i$ (318 ± 58 nM, $n=3$), confirming that despite the poor response to the second CCh challenge, which we previously showed not to result from desensitization of the signalling pathway (Short & Taylor, 2000), the intracellular Ca^{2+} stores had not been completely emptied. In keeping with previous results (Short & Taylor, 2000), PTH alone (100 nM) rarely caused an increase in $[Ca^{2+}]_i$ (Figure 1B) (the average increase in $[Ca^{2+}]_i$ was only 12 ± 10 nM, $n=7$), but it massively potentiated the response to CCh. The response to a second CCh challenge was increased by more than 4 fold when PTH was present (563 ± 57 nM *versus* 135 ± 9 nM) (Figure 1B). This increased response to CCh was accompanied by a decrease in the amplitude of the Ca^{2+} rise evoked by subsequent addition of ionomycin (from 318 ± 58 nM to 156 ± 22 nM) (Figure 1A–C).

PTH does not affect Ca^{2+} removal from the cytosol

The ability of PTH to massively increase the amplitude of the Ca^{2+} signals evoked by CCh in Ca^{2+}-free HBS (Figure 1) and the loss of any response to PTH after incubation with thapsigargin (not shown) are consistent with PTH increasing the amount of Ca^{2+} released from intracellular stores by CCh. However, the increase might also have resulted from inhibition of either Ca^{2+} extrusion across the plasma membrane or sequestration of Ca^{2+} into intracellular stores: either could increase the amplitude of the increase in $[Ca^{2+}]_i$ detected by fura 2. Indeed PTH has been suggested to inhibit the plasma membrane Ca^{2+} pump of hepatocytes (McKenzie *et al*., 1990).

Because Ca^{2+} continuously cycles across the membranes of the intracellular stores, we argued that if PTH inhibited Ca^{2+} re-uptake into intracellular organelles, then in the absence of extracellular Ca^{2+}, the intracellular stores would lose Ca^{2+} more rapidly in the presence of PTH. However, although the Ca^{2+} stores lost ~30% of their Ca^{2+} content during an 11-min incubation in Ca^{2+}-free HBS, the amount of Ca^{2+} retained by the stores (assessed by measuring the transient

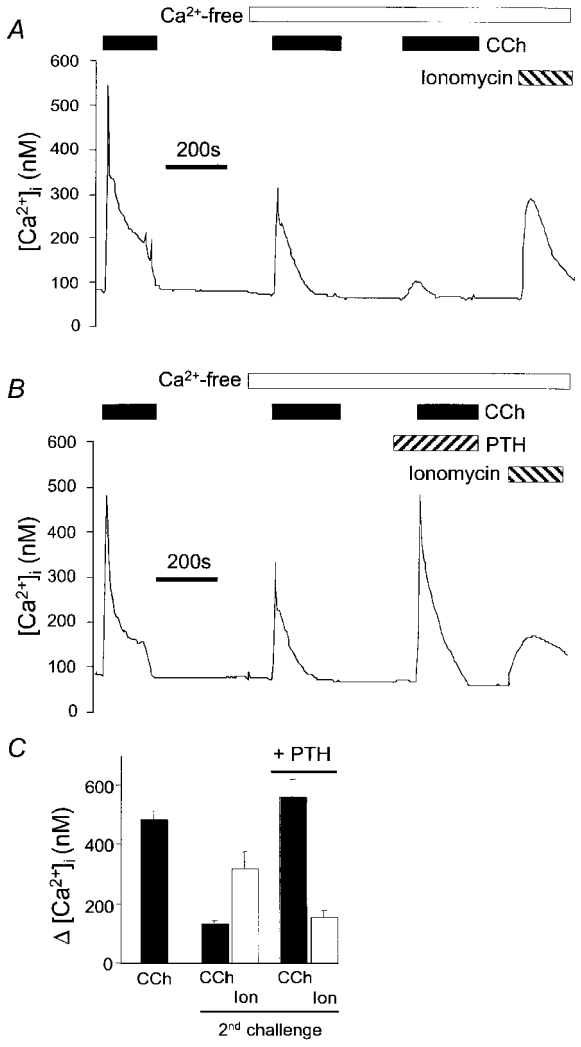

Figure 1 PTH potentiates the Ca^{2+} signals evoked by CCh in Ca^{2+}-free HBS. (A,B) HEK/PTH-R1 cells were first stimulated with CCh (1 mM) in normal HBS and then twice with CCh in Ca^{2+}-free HBS either alone (A) or in the presence of PTH (100 nM, B). The amount of Ca^{2+} remaining within the stores at the end of the experiment was determined by addition of ionomycin (1 μM). Each trace shows the average results from 30 individual cells on a single coverslip, and is typical of at least three independent experiments. (C) Average results ($n=3-8$) from experiments similar to those in (A) and (B) show the amplitude of the increase in $[Ca^{2+}]_i$ evoked by the first challenge in Ca^{2+}-free HBS, the responses to the second CCh challenge (solid bars) either alone or with PTH and the response to addition of ionomycin (open bars) after both CCh challenges.

increase in $[Ca^{2+}]_i$ after addition of 1 μM ionomycin) was similar in the absence (656 ± 36 nM, $n=4$) and continuous presence of 100 nM PTH (651 ± 42 nM, $n=4$).

After complete emptying of the intracellular Ca^{2+} stores by incubation with thapsigargin (1 μM for 10 min), restoration of extracellular Ca^{2+} caused the anticipated increase in $[Ca^{2+}]_i$ mediated by capacitative Ca^{2+} entry (CCE). The CCE signal was unaffected by PTH. The peak CCE signal was 124 ± 6 nM ($n=3$ coverslips, each with 30 cells) under control conditions, and 126 ± 3 nM when Ca^{2+} was restored in the presence of PTH. Furthermore, after rapid removal of extracellular Ca^{2+}, there was no significant difference in the rate at which $[Ca^{2+}]_i$ returned to its basal level in the absence (half-time, $t_{\frac{1}{2}}=24\pm4$ s, $n=3$) and presence of PTH ($t_{\frac{1}{2}}=31\pm2$ s). These results suggest that PTH neither regulates Ca^{2+} extrusion from the cell nor its uptake into intracellular stores.

PTH effects recorded using $^{45}Ca^{2+}$ efflux

The experiments shown in Figure 2 provide a complementary means of assessing the effects of PTH on the responses evoked by CCh. The amount of $^{45}Ca^{2+}$ released into the medium by intact HEK/PTH-R1 cells loaded with $^{45}Ca^{2+}$ is increased when they are stimulated with CCh in Ca^{2+}-free HBS, as $^{45}Ca^{2+}$ released from the stores is actively extruded from the cells (Figure 2A). The half-maximal effect (EC_{50}) of CCh in this assay (36 ± 6 μM; Figure 2B) is similar to that determined earlier from measurements with fura 2 ($EC_{50}=71\pm6$ μM) (Short & Taylor, 2000). More importantly, a second challenge with a maximal concentration of CCh in Ca^{2+}-free HBS caused only small release of $^{45}Ca^{2+}$ (Figure 2C*i*) but this response was increased by 4.2 ± 0.6 fold ($n=5$) in the presence of PTH (100 nM) (Figure 2C*ii*), which alone had no effect on $^{45}Ca^{2+}$ release (Figure 2A). These results demonstrate that under conditions where an inhibition of Ca^{2+} removal from the cytosol would diminish the amount of $^{45}Ca^{2+}$ detected in the medium (because $^{45}Ca^{2+}$ would be less effectively extruded from the cells), PTH massively potentiates the response to CCh. The EC_{50} for the effect of PTH on responses to CCh was 9.3 ± 1.7 nM ($h=1.18\pm0.26$, $n=3$). PTH also potentiated the responses evoked by ATP (Figure 2D), another agonist that stimulates $InsP_3$ formation (Short *et al*., 2000); this occurred despite there being no detectable response to either PTH alone or ATP alone (to which only about 40% of cells responded in single cell imaging experiments; not shown).

We conclude that the ability of PTH to potentiate responses to CCh and ATP results from an increased release of Ca^{2+} from intracellular stores, which our earlier work has shown not to result from enhanced formation of $InsP_3$ (Short & Taylor, 2000). Subsequent experiments sought to establish the links between the PTH receptor and the intracellular Ca^{2+} stores.

PTH potentiates responses to $InsP_3$

To examine more directly the role of $InsP_3$ receptors in the responses to PTH, we used a cell-permeant form of $InsP_3$ ($InsP_3$BM) (Li *et al*., 1997), which others have previously shown to release Ca^{2+} from $InsP_3$-sensitive stores (Li *et al*., 1998; Thomas *et al*., 2000). During a 10-min incubation with $InsP_3$BM (100 μM), $[Ca^{2+}]_i$ increased modestly after a latency of several minutes (Figure 3B), but addition of PTH (100 nM) after 5 min rapidly evoked a massive increase in $[Ca^{2+}]_i$ (Figure 3A,C). Subsequent addition of CCh caused only a very small increase in $[Ca^{2+}]_i$ despite there still being Ca^{2+} within the intracellular stores (Figure 3A,C).

These results demonstrate that $InsP_3$ alone, without activation of receptors linked to phospholipase C, is sufficient to allow PTH to evoke Ca^{2+} release from intracellular stores, consistent with our earlier demonstration that heparin, an antagonist of $InsP_3$ receptors, prevents PTH from potentiating responses to CCh (Short & Taylor, 2000).

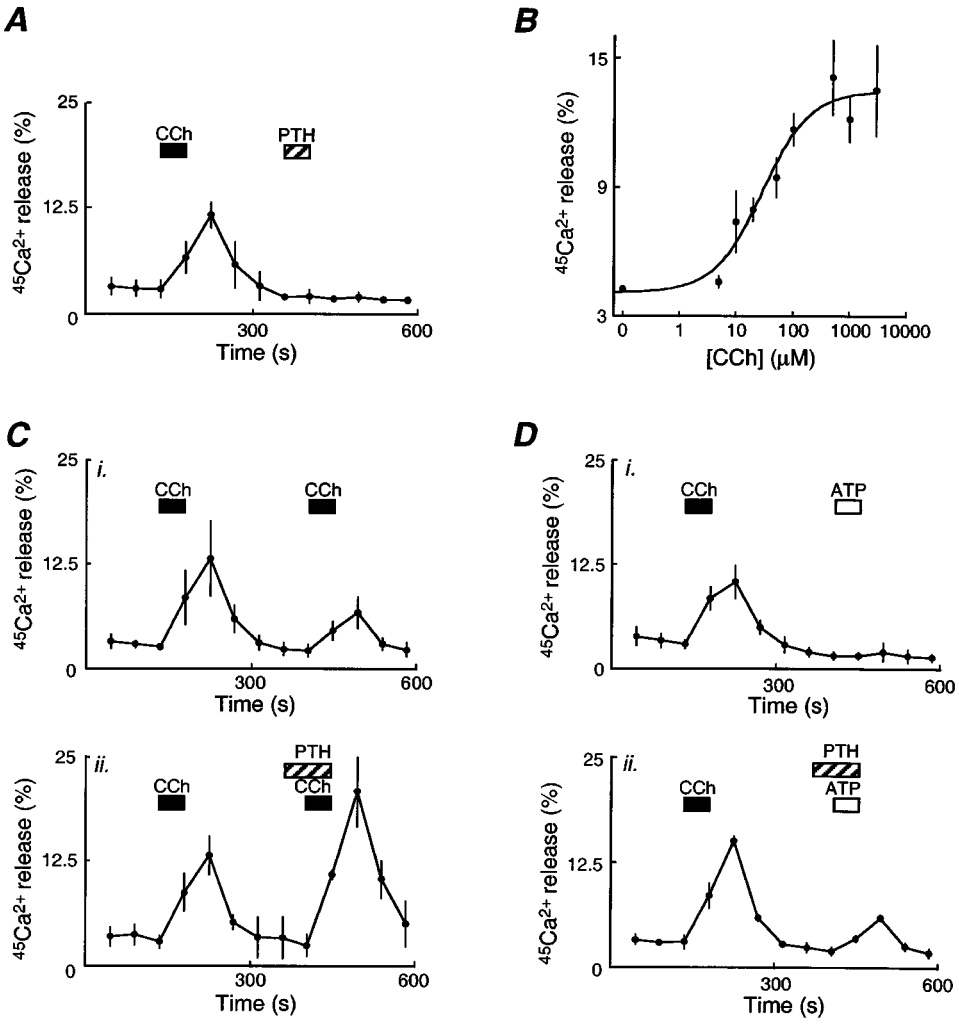

Figure 2 PTH effects recorded using a $^{45}Ca^{2+}$ efflux assay. HEK/PTH-R1 cells loaded with $^{45}Ca^{2+}$ were stimulated as shown with CCh (1 mM) or ATP (1 mM) and/or PTH (100 nM) in Ca^{2+}-free HBS; the amounts of $^{45}Ca^{2+}$ released during each 45-s interval are shown as fractional release rates (see Methods). (A) Shows the effects of stimulating cells with CCh or PTH alone. (B) Shows the concentration-dependence of the peak $^{45}Ca^{2+}$ release evoked by the first CCh challenge. (C,D) shows the effects of CCh or ATP alone (*i*) or in combination with PTH (*ii*). All results are mean±s.e.mean from five (A–C) or three (D) independent experiments.

Because PTH increases the Ca^{2+} release evoked by a maximal concentration of CCh, we had assumed that PTH was unlikely to be working by sensitizing the InsP_3 receptor to InsP_3. We instead suggested that PTH might facilitate transfer of Ca^{2+} from an InsP_3-insensitive to an InsP_3-sensitive organelle (Short & Taylor, 2000). That conclusion supposes that a maximal concentration of CCh generates enough InsP_3 to maximally activate InsP_3 receptors.

Measurements of CCh-evoked ^{3}H-InsP_3 formation under conditions identical to those used for measurements of $^{45}Ca^{2+}$ release demonstrated that both responses were similarly sensitive to CCh. The EC_{50} for $^{45}Ca^{2+}$ release was 36±6 μM (n=3) and 30 μM CCh caused 44±6% of the InsP_3 formation evoked by a maximal CCh concentration. We conclude, therefore, that a maximal concentration of CCh generates no more InsP_3 than is required to maximally activate InsP_3 receptors, and possibly less. It therefore becomes important to resolve whether PTH exerts its effects on Ca^{2+} mobilization by increasing the sensitivity of InsP_3 receptors or by increasing the size of the InsP_3-sensitive Ca^{2+} stores.

Responses to PTH are unaffected by disruption of the cytoskeleton

If PTH were acting by causing fusion of discrete intracellular Ca^{2+} stores, we might expect disruption of intracellular membrane trafficking to inhibit responses to PTH. None of the drugs used to disrupt microtubules (colchicine, nocodazole, vinblastine, paclitaxel), actin microfilaments (cytochalasin D, latrunculin B, jasplakinolide) or the Golgi apparatus (brefeldin A) at the concentrations shown in Figure 4 affected the basal $[Ca^{2+}]_i$, the amplitude of the first response to CCh in Ca^{2+}-free HBS (i.e. release of Ca^{2+} from stores), or the sustained phase of the response to CCh in normal HBS (i.e. Ca^{2+} entry) (Figure 4A). Each drug was used for longer and

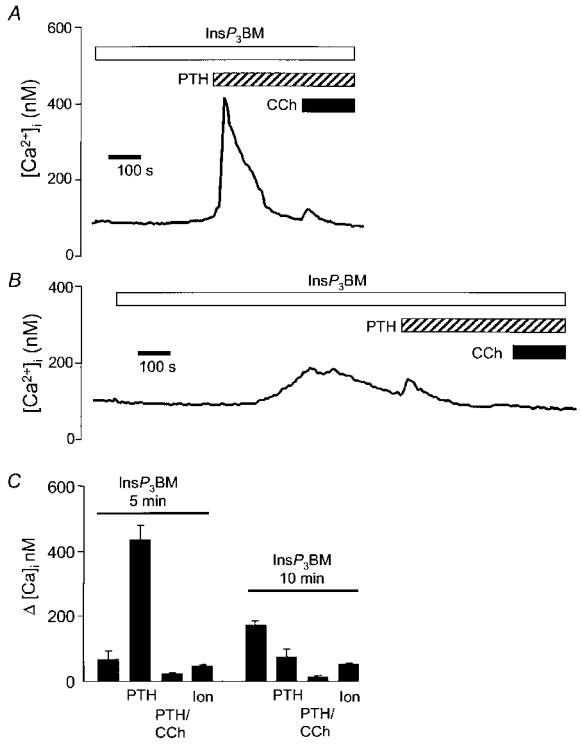

Figure 3 PTH potentiates responses to $InsP_3BM$. (A,B) Fura 2-loaded HEK/PTH-R1 cells were incubated with $InsP_3BM$ (100 μM) in Ca^{2+}-free HBS before addition of PTH (100 nM) and CCh (1 mM). (C) Summarizes the results (mean $\pm$ s.e.mean, $n=3-5$), with the first column showing the $[Ca^{2+}]_i$ immediately before addition of PTH, which was added 5 or 10 min after addition of $InsP_3BM$. The subsequent response to CCh with PTH and finally to ionomycin are also shown.

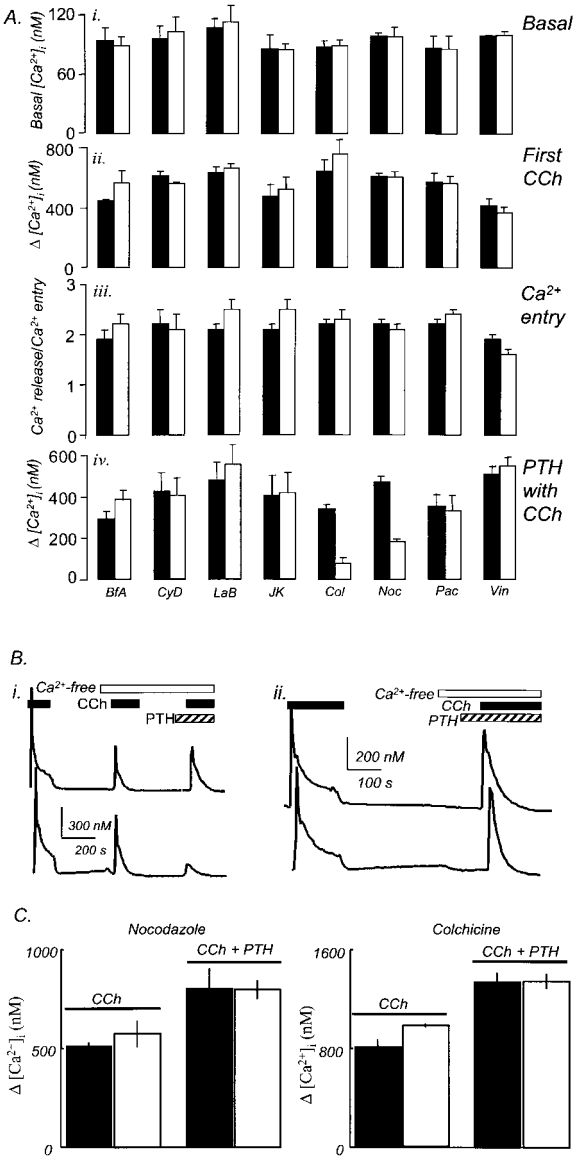

Figure 4 The cytoskeleton is not required for PTH to potentiate CCh-evoked Ca^{2+} mobilization. (A) Basal $[Ca^{2+}]_i$ (*i*), the change in $[Ca^{2+}]_i$ evoked by the first challenge with CCh in Ca^{2+}-free HBS (*ii*), the ratio of the amplitudes of the Ca^{2+} release to Ca^{2+} entry signals (*iii*, see Methods), and the response to a second CCh challenge in the presence of PTH (*iv*) are shown in the presence of drugs that interfere with the cytoskeleton (mean $\pm$ s.e.mean, $n \geqslant 3$). Each treatment (open bar) is shown with its paired control (solid bar). BfA, brefeldin A (100 μM); CyD, cytochalasin D (10 μM; LaB, latrunculin B (10 μM), JK, jasplakinolide (5 μM), Col., colchicine, (100 μM); Noc, nocadazole (10 μM); Pac, paclitaxel (100 μM); Vin, vinblastine (100 μM). In each case, cells were pre-treated with the drug for 150 min before stimulation in the continued presence of the drug. (B) Nocadazole was present for the lower traces of both panels: its ability to prevent PTH from potentiating the second response to CCh (*i*) (as shown in (A) probably reflects loss of Ca^{2+} from the stores, because it does not prevent PTH from potentiating the first response to CCh (*ii*). (C) The effects of nocadazole and colchicine are shown for cells stimulated with either CCh alone or CCh with PTH as the first challenge. (Note the lesser fold potentiation by PTH in these experiments relative to other experiments in which the stores were partially depleted by prior stimulation with CCh before the challenge with CCh and PTH). Open bars denote responses in the presence of nocodazole (left) or colchicine (right).

at a concentration at least as high as that shown by others (Fogarty *et al*., 2000; Patterson *et al*., 1999; Ribeiro *et al*., 1997; Rosado & Sage, 2000a; Thomas *et al*., 2000) to cause disruption of its target; indeed with nocadozole and colchicine the morphology of the HEK/PTH-R1 cells was clearly affected by the treatments. Others have suggested a requirement for the actin and tubulin cytoskeletons for receptors to release Ca^{2+} from intracellular stores (Ribeiro *et al*., 1997) and for actin in regulation of capacitative Ca^{2+} entry (Patterson *et al*., 1999; Rosado & Sage, 2000b). But our results in HEK cells, in keeping with those from HeLa cells (Thomas *et al*., 2000), have failed to provide any evidence to implicate the cytoskeleton in receptor-regulation of the global Ca^{2+} signals resulting from either Ca^{2+} mobilization or Ca^{2+} entry.

Of the drugs used, only colchicine (100 μM) and nocodazole (10 or 100 μM) significantly ($P<0.05$) inhibited the ability of PTH to potentiate the increase in $[Ca^{2+}]_i$ evoked by a second challenge with CCh in Ca^{2+}-free HBS (Figure 4A*iv*). However, the inhibition appeared to result simply from a faster loss of Ca^{2+} from intracellular stores that became apparent only when the cells were incubated in Ca^{2+}-free HBS. Although responses to CCh in normal HBS or immediately after transfer to Ca^{2+}-free HBS were unaffected by either drug (Figure 4C), the response to a second challenge to CCh in Ca^{2+}-free HBS was reduced by $>60\%$, as was the amount of Ca^{2+} remaining within the

stores (assessed using ionomycin or thapsigargin) (not shown). By measuring the effect of PTH on the first response to CCh immediately after transfer to Ca^{2+}-free HBS, it became clear that even prolonged incubation with a high concentration of nocodazole or colchicine had no effect on the ability of PTH to potentiate the response to CCh (Figure 4B,C).

We conclude that neither microtubules, actin microfilaments nor an intact Golgi apparatus are acutely required for PTH to potentiate the Ca^{2+} signals evoked by CCh.

PTH sensitizes InsP_3 receptors rather than increasing the size of the InsP_3-sensitive Ca^{2+} pool

We used prolonged incubation with InsP_3BM to assess whether InsP_3 alone, given sufficient time, was capable of completely emptying the stores to which CCh in combination with PTH had access. During a 13-min incubation in Ca^{2+}-free HBS, the Ca^{2+} content of the intracellular stores of unstimulated cells (assessed by addition of ionomycin) declined by about 50%, but in the presence of InsP_3BM (100 μM), they lost about 90% of their Ca^{2+} content (Figure 5). The most important point, however, is that whether cells were stimulated only with InsP_3BM or with CCh in combination with PTH, the residual Ca^{2+} content of the stores (i.e. that released by ionomycin) was similar (Figure 5). Furthermore, as the duration of the incubation of cells with InsP_3BM (100 μM) was prolonged, the ability of PTH to evoke a response substantially declined (Figure 3B). These results suggest that InsP_3 alone, when present for long enough at a high enough concentration, is capable of emptying stores to the same degree as PTH with CCh. We conclude that CCh alone is incapable of stimulating formation of sufficient InsP_3 to cause complete emptying of the InsP_3-sensitive stores and that PTH, by sensitizing InsP_3 receptors to InsP_3, potentiates responses to even a maximal concentration of CCh.

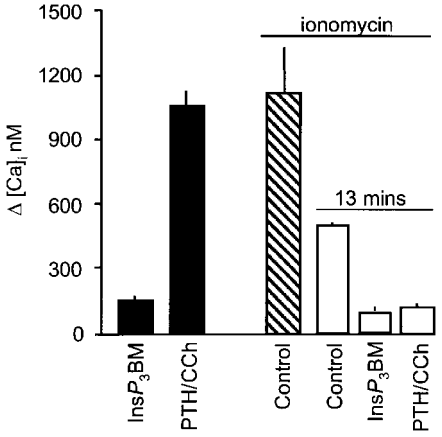

Figure 5 InsP_3BM empties stores as completely as PTH with CCh. The peak increases in $[Ca^{2+}]_i$ after stimulation of fura-2-loaded cells with InsP_3 BM (100 μM) or CCh (1 mM) with PTH (100 nM) are shown by solid bars. The peak Ca^{2+} signal evoked by addition of ionomycin (1 μM) was used to assess the amount of Ca^{2+} within intracellular stores after 0 (hatched bar) or 13 min in Ca^{2+}-free medium, and after stimulation in the same medium with InsP_3BM, or CCh and PTH. Results are means$\pm$s.e.mean from at least three coverslips, with >30 cells analysed on each.

PKA does not mediate the effects of PTH on Ca^{2+} mobilization

In other cells, type 2 InsP_3 receptors, the predominant subtype in HEK-293 cells (Wojcikiewicz, 1995), are phosphorylated by PKA leading to an increase in their InsP_3 sensitivity (Bruce *et al*., 2002; Burgess *et al*., 1991). In light of our conclusion that PTH works by sensitizing the InsP_3 receptor, we re-examined the possible involvement of PKA in mediating the effects of PTH.

Pre-incubation (30 min) with supramaximal concentrations of established inhibitors of PKA (H89, 10 μM; CMIQ, 10 μM) had no significant effect on the ability of either a maximal (100 nM) or, more, importantly, a submaximal (10 nM) concentration of PTH to potentiate the CCh-evoked release of $^{45}Ca^{2+}$ from intact HEK/PTH-R1 cells (Figure 6A,B). Parallel measurements under similar conditions established that H89 had inhibited phosphorylation of CREB by PTH (Figure 6C), and in other cells a much lower concentration of H89 has been shown to prevent PKA-mediated phosphorylation of InsP_3 receptors (Bruce *et al*., 2002). In fura 2 assays too, there was no effect of PKA inhibitors (H89 and CMIQ) on the effects of PTH (100 nM) on CCh-evoked Ca^{2+} mobilization (not shown).

Cyclic AMP does not mediate the effects of PTH on Ca^{2+} signals

We had earlier suggested that the membrane-permeant analogue of cAMP, 8-Br-cAMP failed to mimic the effects of PTH on CCh-evoked Ca^{2+} mobilization (Short & Taylor, 2000). We confirmed that observation with fura 2-loaded cells and extended it by demonstrating that even 200 μM 8-Br-cAMP affected neither CCh-evoked Ca^{2+} release nor the ability of PTH to potentiate CCh-evoked Ca^{2+} release (Figure 7A).

PTH ($EC_{50}=3.7\pm0.4$ nM, $n=3$) caused a more than 1000 fold increase in cAMP formation in HEK/PTH-R1 cells. SQ22536 (1 mM), an inhibitor of adenylyl cyclase (Reid *et al*., 1990), substantially reduced the amount of cAMP produced in response to stimulation by concentrations of PTH that were either maximal (100 nM) or submaximal (10 nM) for potentiated Ca^{2+} signals, although in neither case was the cAMP response abolished (Figure 7C*i*). Despite causing a 4 fold reduction in the amount of cAMP produced by a submaximal concentration of PTH, SQ22536 had no effect on the ability of this concentration of PTH to potentiate the $^{45}Ca^{2+}$ release evoked by CCh (Figure 7C*ii*). Because $^{45}Ca^{2+}$ release ($EC_{50}=9.3\pm1.7$ nM) and cAMP formation ($EC_{50}=3.7\pm0.4$ nM) are similarly sensitive to PTH, it is impossible to argue that the cAMP produced in response to a maximal concentration of PTH is more than sufficient to evoke a maximal Ca^{2+} signal. The ability of SQ22536 to decrease the amount of cAMP made in response to maximal PTH without significantly affecting the Ca^{2+} signal (Figure 7C) therefore provides further evidence that cAMP does not mediate the effect of PTH on Ca^{2+} mobilization. In measurements of single fura-2-loaded cells too, SQ22536 had no significant effect on responses to maximal or submaximal concentrations of PTH (not shown). DDA (200 μM), another inhibitor of adenylyl cyclase, also had no significant effect on the ability of PTH (100 nM) to potentiate

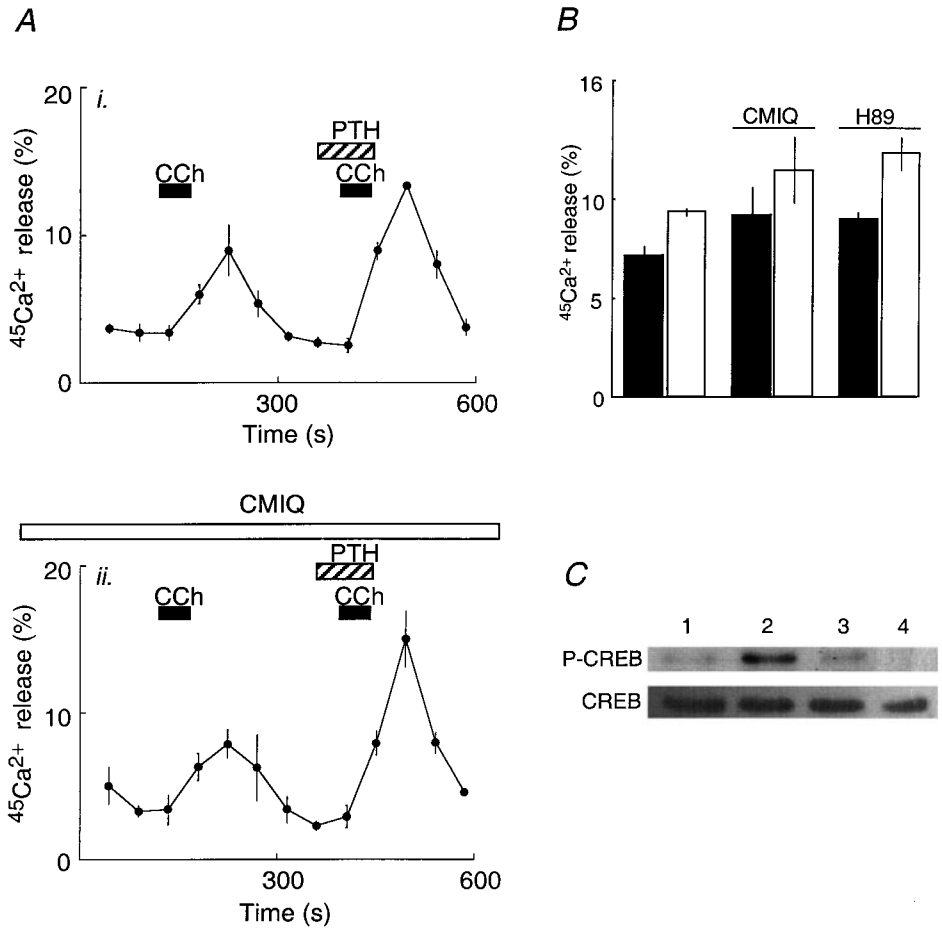

Figure 6 Effects of PTH are not mediated by PKA. (A) $^{45}Ca^{2+}$ efflux from HEK/PTH-R1 cells was recorded during stimulation with CCh (1 mM) and PTH (100 nM) (*i*) or in the presence of CMIQ (10 μM, present throughout and for 30 min before the first CCh addition) (*ii*). (B) Results from similar experiments show the effects of maximal (100 nM, open bars) or submaximal (10 nM, solid bars) concentrations of PTH on the $^{45}Ca^{2+}$ release evoked by a second CCh challenge under control conditions or in the presence of CMIQ (10 μM) or H89 (10 μM). Both inhibitors were present throughout and for 30 min before the first CCh challenge. Results (means $\pm$ s.e.mean, $n=3$) show the effect of PTH on $^{45}Ca^{2+}$ release (i.e. the $^{45}Ca^{2+}$ release in the presence of PTH and CCh after subtraction of that evoked by CCh alone). (C) Western blots show the effects PTH alone (100 nM, lane 2) or PTH in the presence of 1 μM (lane 3) or 10 μM (lane 4) H89 on the levels of phospho-CREB (upper panel) and CREB independent of its phosphorylation state (lower). Lane 1 shows the control cells.

CCh-evoked increases in $[Ca^{2+}]_i$ in fura 2-loaded cells (Figure 7B). Finally, combined application of DDA and SQ22536 reduced the amount of cAMP produced in response to 10 nM PTH by 91$\pm$2%, a significantly greater inhibition than that obtained with either inhibitor alone (Figure 7C*i*). Nevertheless, when measured under identical conditions the ability of 10 nM PTH to potentiate CCh-evoked $^{45}Ca^{2+}$ release was unaffected by combined application of DDA and SQ22536 (Figure 7C*ii*).

IBMX, by inhibiting degradation of cAMP, might be expected to potentiate responses to submaximal concentrations of PTH if the responses were mediated by cAMP. IBMX (1 mM) significantly potentiated the increase in intracellular cAMP concentration achieved during a 45-s incubation with 10 nM PTH (Figure 7D*i*), but in $^{45}Ca^{2+}$ flux assays performed under identical conditions, there was no significant potentiation of the effects of PTH on CCh-evoked $^{45}Ca^{2+}$ release (Figure 7D*ii*).

Discussion

The receptors for PTH, in common with other members of this sub-family of G-protein-coupled receptors, stimulate both adenylyl cyclase activity and an increase in $[Ca^{2+}]_i$ (Abou-Samra *et al*., 1992; Jüppner *et al*., 1991), but the relationship (if any) between these two events is unclear. We (Short & Taylor, 2000) (Figure 1) and others (Buckley *et al*., 2001) have observed that PTH massively potentiates the Ca^{2+} signals evoked by other receptors that stimulate phospholipase C. The earlier work (Buckley *et al*., 2001; Short & Taylor, 2000) established that the effect of PTH did not require Ca^{2+} entry, but left open the possibility that the larger Ca^{2+} signal evoked by other stimuli resulted from inhibition of Ca^{2+} removal from the cytosol. The present results establish that PTH increases the amount of Ca^{2+} released from intracellular stores by receptors that stimulate phospholipase C: PTH affects neither Ca^{2+} extrusion from

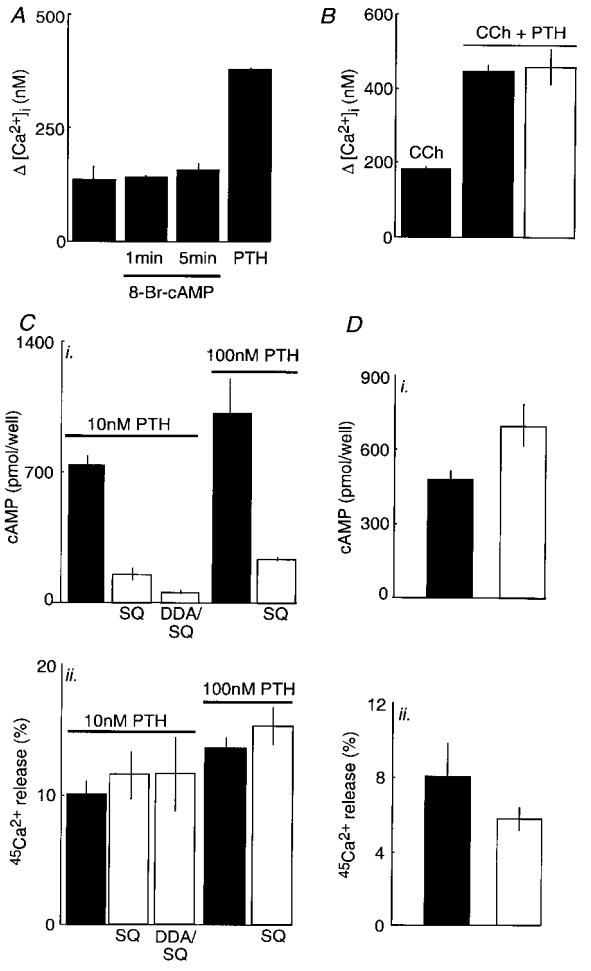

Figure 7 The effects of PTH are not mediated by cAMP. (A) The increase in $[Ca^{2+}]_i$ is shown for fura 2-loaded cells stimulated with CCh alone (1 mM), with CCh after pre-incubation with 200 μM 8-Br-cAMP for 1 or 5 min, or with CCh and PTH (100 nM). (B) Fura 2-loaded cells were stimulated with CCh alone (1 mM) or with PTH (100 nM) and CCh under control conditions (solid bars) or after incubation with DDA (open bar; 200 μM, present throughout and for 30 min before the stimulus. (C*i*) The amounts of cAMP formed during a 45-s incubation with 10 or 100 nM PTH are shown in the absence (solid bars) or presence (open bars) of SQ22536 (1 mM) alone or with DDA (200 μM) (both present throughout and for 30 min before addition of PTH). (C*ii*) Experiments performed under similar conditions show the effects of PTH on the peak $^{45}Ca^{2+}$ release evoked by a second CCh challenge in the presence of 10 or 100 nM PTH. Results are means$\pm$s.e.mean, $n=3-6$ (*i*) or 3–9 (*ii*). (D) The effect of IBMX (1 mM, present throughout and for 30 min before addition of PTH) on the amount of cAMP produced during a 45-s incubation with 10 nM PTH (*i*). (D*ii*) Shows the effect of PTH (10 nM) on the peak $^{45}Ca^{2+}$ release evoked by a second CCh challenge. Control (solid bars) and IBMX (open bars). Results are means$\pm$s.e.mean, $n=3$ (*i*) or 6 (*ii*).

the cell nor Ca^{2+} uptake into intracellular stores, and it similarly affects Ca^{2+} signals whether they are measured using fura 2 (Figure 1) or $^{45}Ca^{2+}$ efflux (Figure 2). We suggested earlier (Short & Taylor, 2000) that PTH increased Ca^{2+} release *via* $InsP_3$ receptors, but the evidence was indirect: PTH potentiated responses to different phospholipase C-linked receptors (Figure 2) and there was no response from cells injected with heparin (Short & Taylor, 2000). $InsP_3$BM allows the intracellular $InsP_3$ concentration to be increased without stimulating cell-surface receptors. Addition of PTH to cells loaded with sub-threshold concentrations of $InsP_3$ using $InsP_3$BM evoked massive Ca^{2+} signals (Figure 3), thereby directly establishing that PTH works by potentiating $InsP_3$-evoked Ca^{2+} release.

Because $InsP_3$BM alone, given sufficient time, is capable of emptying the intracellular Ca^{2+} stores to the same extent as CCh and PTH together (Figure 5), we conclude that PTH does not, as we previously suggested (Short & Taylor, 2000), recruit additional Ca^{2+} into the $InsP_3$-sensitive Ca^{2+} store, rather it must increase the sensitivity of the $InsP_3$ receptor to $InsP_3$. The lack of effect of agents that disrupt the cytoskeleton on responses to PTH (Figure 4), further supports the conclusion that regulated fusion of $InsP_3$-insensitive with $InsP_3$-sensitive Ca^{2+} stores does not underlie the effects of PTH on Ca^{2+} signalling. We conclude, therefore, that PTH increases the sensitivity of $InsP_3$ receptors and so allows a maximal concentration of CCh, which alone generates insufficient $InsP_3$ to activate all $InsP_3$ receptors, to completely empty the $InsP_3$-sensitive Ca^{2+} stores.

What is the signal that passes from the PTH receptor to the $InsP_3$ receptor? Because PTH stimulates cAMP formation and the sensitivity of type 2 $InsP_3$ receptors to $InsP_3$ is increased after their phosphorylation by PKA (Burgess *et al*., 1991; Hajnóczky *et al*., 1993), we re-considered the possibility that PKA might mediate the effects of PTH on Ca^{2+} signals. High concentrations of either of two inhibitors of PKA, H89 and CMIQ, had no effect on the ability of PTH to potentiate Ca^{2+} signals (Figure 6B), despite blocking the effect of PTH on CREB phosphorylation (Figure 6C), confirming previous suggestions (Buckley *et al*., 2001; Short & Taylor, 2000) that PKA does not mediate the effects of PTH on $InsP_3$-evoked Ca^{2+} mobilization. We confirmed that 8-Br-cAMP did not mimic the effects of PTH (Figure 7A). More importantly, substantial inhibition of adenylyl cyclase using SQ22536, DDA or both did not affect Ca^{2+} responses to maximal or submaximal concentrations of PTH (Figure 7C), suggesting that cAMP does not mediate the effects of PTH on $InsP_3$ receptors. That conclusion gains further support from results with IBMX, which significantly increased the amount of cAMP produced in response to a submaximal concentration of PTH without affecting the Ca^{2+} signal (Figure 7D).

We conclude that PTH potentiates the Ca^{2+} release evoked by receptors that stimulate $InsP_3$ formation by sensitizing the $InsP_3$ receptor to $InsP_3$. Although PTH stimulates cAMP formation and so activation of PKA, neither signal mediates the effects of PTH on $InsP_3$ receptors. Other receptors, including those that couple to Gs (Goraya and Taylor, unpublished observation) and Gi/Go (Werry *et al*., 2002), also potentiate $InsP_3$-mediated Ca^{2+} signals in HEK cells. In other cells too, Gi/Go-coupled receptors have been shown to enhance the Ca^{2+} signals evoked by receptors that stimulate $InsP_3$ formation by a mechanism that appears not to involve formation of additional $InsP_3$ and which may require G protein $\beta\gamma$ subunits (Yeo *et al*., 2001). We speculate that an early step in the signalling pathway, perhaps an α-GTP or $\beta\gamma$ subunit of a G protein (Neylon *et al*., 1998; Zeng *et al*., 1996) activated by the PTH receptor, may be the means whereby PTH regulates the sensitivity of $InsP_3$ receptors. Such a mechanism would allow G protein-coupled receptors to

directly tune the sensitivity of $InsP_3$ receptors and so to modulate their responses to the $InsP_3$ produced in response to activation of either the same or different receptors.

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