

Fig. S1. Ca<sup>2+</sup> signals evoked directly by high concentrations of PTH(1-34) are unaffected by inhibition of adenylyl cyclase, protein kinase A, cyclic nucleotide phosphodiesterases, or EPACs. (A) Targets of the inhibitors used. Cyclic AMP produced by adenylyl cyclase (AC) can stimulate protein kinase A (PKA), exchange proteins activated by cAMP (EPACs) or IP<sub>3</sub> receptors (IP<sub>3</sub>R). DDA (2',5'-dideoxyadenosine) and SQ 22536 inhibit adenylyl cyclase, while NKH477 directly stimulates it. IBMX inhibits the phosphodiesterases (PDE) that degrade cAMP. H89 inhibits PKA. ESI-09 is an antagonist of EPACs. 8-Br-cAMP is a membrane-permeant analogue of cAMP. (**B-D**) Cells were incubated with SQ22536 (1 mM) and DDA (200  $\mu$ M) (B, SQ/DDA, 20 min) to inhibit adenylyl cyclase, H89 (C, 10  $\mu$ M, 20 min) to inhibit protein kinase A, or IBMX (D, 1 mM, 5 min) to inhibit cyclic nucleotide phosphodiesterases before addition of the indicated concentrations of PTH(1-34). Results show the peak increases in [Ca<sup>2+</sup>]<sub>c</sub> evoked by PTH(1-34). (E) Increases in [Ca<sup>2+</sup>]<sub>c</sub> evoked by 3  $\mu$ M PTH(1-34) alone or with the antagonist of EPACs, ESI-09 (10  $\mu$ M, 5 min). Results (B-E) are means ± s.e.m., n = 3.



Fig. S2. Exchange proteins activated by cAMP (EPACs) do not mediate the effects of PTH(1-34) on carbachol-evoked Ca<sup>2+</sup> signals. (A) HEK-PR1 cells were incubated with the EPAC antagonist, ESI-09 (10  $\mu$ M for 5 or 15 min), before addition of the indicated concentrations of carbachol (CCh) in Ca<sup>2+</sup>-free HBS. Results show the peak increase in [Ca<sup>2+</sup>]<sub>c</sub> evoked by carbachol. (B) Similar analyses of cells treated with 10  $\mu$ M ESI-09 for 5 min, and then stimulated with carbachol (20  $\mu$ M) added 1 min after the indicated concentrations of PTH(1-34). (C) Experiments similar to those in A, show responses to carbachol after preincubation (15 min) with ESI-09 (0, 10 or 50  $\mu$ M). (D) Effects of ESI-09 (10  $\mu$ M, added 5 min before 8-Br-cAMP) on the Ca<sup>2+</sup> signals evoked by carbachol after incubation with the indicated concentrations of the membrane-permeant analogue of cAMP, 8-Br-cAMP (5 min). Results (A-D) show means ± s.e.m., n = 3. (E) Target of the inhibitor used.



Fig. S3. Potentiation of carbachol-evoked  $Ca^{2+}$  signals by brief treatment with PTH(1-34) is unaffected by inhibition of protein kinase A or adenylyl cyclase. (A, C) Cells were incubated with H89 (A, 10 µM, 20 min, to inhibit PKA) or SQ22536 (1 mM) and DDA (200 µM) (C, SQ/DDA, 20 min, to inhibit AC) before addition of PTH(1-34) for 1 min and then carbachol (20  $\mu$ M) in Ca<sup>2+</sup>-free HBS. Results show the peak increase in [Ca<sup>2+</sup>]. evoked by carbachol. (**B**, **D**) Effects of the same treatments on the increase in intracellular levels of cAMP (as percentages of <sup>3</sup>H-ATP, <sup>3</sup>H-ADP and <sup>3</sup>H-cAMP) evoked by PTH(1-34) (3 µM) for 1 min or 60 min (B) or by the indicated concentrations of PTH(1-34) for 1 min (D). Results (A-D) are means ± s.e.m. from at least 3 experiments. (E) The effect of inhibiting adenylyl cyclase on  $Ca^{2+}$  signals modulated by globally delivered cAMP would be readily detectable. Panel D demonstrates that SQ/DDA caused a 61% inhibition of the amount of cAMP produced in response to stimulation with PTH(1-34) for 1 min. Similar inhibition by SQ/DDA was observed after stimulation for 60 min (~70%, supplementary material Table S6). Comparison of responses with and without SQ/DDA allows computation of the concentrations of PTH(1-34) needed to stimulate production of equivalent amounts of cAMP with and without SQ/DDA. Assuming (for  $Ca^{2+}$  signals regulated by globally distributed cAMP) that the relationship between cAMP and  $[Ca^{2+}]_c$  is unaffected by inhibition of AC (Fig. 5E, black line), we can predict the effects of SQ/DDA on potentiation of carbachol-evoked Ca<sup>2+</sup> signals by PTH(1-34). The analysis has been performed for control cells (black line) and for cells treated with SQ/DDA (red), and compared with the observed control response (blue). The results demonstrate that if the effects of PTH(1-34) were mediated by a global cAMP signal, SQ/DDA would cause an easily resolved inhibition of the potentiated  $Ca^{2+}$  signals. (F) Targets of the inhibitors used.



Fig. S4. Potentiation of carbachol-evoked  $Ca^{2+}$  signals by sustained stimulation with PTH(1-34) in the presence of IBMX is unaffected by inhibition of protein kinase A. (A, B) Cells were stimulated with PTH(1-34) for 1 min (A) or 60 min (B) before addition of carbachol (20 µM) in Ca<sup>2+</sup>-free HBS. Cells were pretreated with IBMX (1 mM, 5 min, to inhibit cyclic nucleotide phosphodiesterases) alone or with H89 (10 µM, 20 min, to inhibit PKA) as indicated. Results (means ± s.e.m., n = 3) show the peak increase in  $[Ca^{2+}]_c$  evoked by carbachol. (C) Targets of the inhibitors used.

## Table S1. Properties of the PTH analogues used.

PTH(1-34) PTH(2-38) Tyr <sup>1</sup> PTH(1-34) PTHrP(1-36) PTHrP(1-34) PTH(1-31)	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF VSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALG YVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTAQI AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA SVSEIQLMHNLGKHLNSMERVEWLRKKLQDV						
	Gs (AC)	Gq (PLC)	Signalling to AC from internalized PTH <sub>1</sub> R	References			
PTH(1-34)	Full agonist	Full agonist	Yes	Bisello et al., 2002; Castro et al., 2002; Cupp et al., 2013; Ferrandon et al., 2009; van der Lee et al., 2013.			
PTHrP(1-36) PTHrP(1-34)	Full agonist	Full agonist	No <sup>1</sup>	Bisello et al., 2002; Cupp et al., 2013; Dean et al., 2008; Ferrandon et al., 2009.			
PTH(2-38)	Full agonist	Almost Inactive	No	Cupp et al., 2013.			
PTH(1-31)	Full agonist	Strong partial agonist	Yes	Cupp et al., 2013; Takasu et al., 1999.			
PTH(3-34) <sup>2</sup>	Very weak partial agonist	Inactive	No	Takasu et al., 1999; Cupp et al., 2013; van der Lee et al., 2013.			
Tyr <sup>1</sup> PTH(1-34)	Full agonist	Almost Inactive	Weak <sup>3</sup>	Cupp et al., 2013.			

Sequences of the peptides used in this study are shown in the top panel using single-letter amino acid codes. The lower panel summarizes relevant properties of the PTH analogues. <sup>1</sup>PTHHrP(1-36) evokes internalization of PTH<sub>1</sub>R, but not of functional AC signalling complexes. <sup>2</sup>Not used in this study, but included for comparison with earlier work (Tovey et al., 2008). <sup>3</sup>Tyr<sup>1</sup>PTH(1-34) is a weak partial agonist for receptor internalization; it is not known whether internalized AC signalling complexes accompany receptor internalization.

	Carba 20 j	achol uM	Carbachol 1 mM			
_	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	pEC <sub>50</sub> (/M)	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	pEC <sub>50</sub> (/M)		
1 min	$230\pm10$	$7.5\pm0.2$	$234\pm29$	$7.8\pm0.1$		
5 min	$179\pm37$	$7.1 \pm 0.1$	$228\pm9$	$7.6\pm0.1$		
15 min	$94\pm7^{*}$	$7.9\pm0.1^{*}$	$120\pm7^{*}$	$8.7\pm0.1^{*}$		
30 min	$142 \pm 38^*$	$8.8\pm0.1^*$	$105\pm30^{*}$	$8.8\pm0.1^{\ast}$		
60 min	$108\pm7^{*}$	$8.1\pm0.2^*$	$110 \pm 16^{*}$	$8.2\pm0.2^{*}$		

Table S2. Potentiation of carbachol-evoked Ca<sup>2+</sup> signals by PTH(1-34).

Experiments similar to those shown in Fig. 1B were used to establish the concentrationdependent effects of PTH(1-34) added for the indicated times before carbachol (20  $\mu$ M or 1 mM) on carbachol-evoked Ca<sup>2+</sup> signals. The EC<sub>50</sub> (half-maximally effective concentration) for responses to carbachol alone was 32  $\mu$ M, pEC<sub>50</sub> = 4.5 ± 0.5 (where pEC<sub>50</sub> = -logEC<sub>50</sub>). Maximal  $\Delta$ [Ca<sup>2+</sup>]<sub>c</sub> describes the difference between the peak Ca<sup>2+</sup> signal evoked by carbachol alone and that evoked by carbachol in the presence of a maximally effective concentration of PTH(1-34). Results show means ± s.e.m., n = 4. \**P* < 0.05 relative to paired comparisons at 1 min.

	1 min				30 min			
-	peak [Ca <sup>2+</sup> ] <sub>c</sub>		cAMP		peak [	$[Ca^{2+}]_c$	cAMP	
	pEC <sub>50</sub> (/M)	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	Maximal $pEC_{50}$ Maximal $\Delta[Ca^{2+}]_c$ (/M)cAMP(nM)(%)		pEC <sub>50</sub> (/M) [ΔpEC <sub>50</sub> ]	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	pEC <sub>50</sub> (/M) [ΔpEC <sub>50</sub> ]	Maximal cAMP (%)
PTH(1-34)	$7.7\pm0.1$	$205 \pm 13$	$7.0\pm0.1$	$1.1 \pm 0.1$	$8.8 \pm 0.2^{*}$ [1.1 ± 0.3]	105 ± 13*	$7.5 \pm 0.1^{*}$ [0.5 ± 0.2]	$1.9 \pm 0.03*$
Tyr <sup>1</sup> PTH(1-34)	$6.7\pm0.03$	$233\pm10$	$6.1\pm0.2$	$1.3 \pm 0.2$	$\begin{array}{c} 7.4 \pm 0.1 * \\ [0.7 \pm 0.1] \end{array}$	$148 \pm 6*$	$\begin{array}{c} 6.7 \pm 0.2 * \\ [0.6 \pm 0.4] \end{array}$	$2.6\pm0.1*$
PTHrP(1-36)	$7.6\pm0.1$	$281\pm34$	$7.3\pm0.1$	$0.2 \pm 0.01$	$\begin{array}{c} 8.6 \pm 0.2 * \\ [1.0 \pm 0.3] \end{array}$	139 ± 2*	$\begin{array}{c} 7.3 \pm 0.2 \\ [0.0 \pm 0.3] \end{array}$	$0.3\pm0.01$
PTH(2-38)	$6.9\pm0.1$	$216\pm21$	$6.5\pm0.04$	$0.7\pm0.1$	$\begin{array}{c} 7.3 \pm 0.1 * \\ [0.4 \pm 0.2] \end{array}$	115 ± 13*	$\begin{array}{c} 7.2 \pm 0.2 * \\ [0.7 \pm 0.2] \end{array}$	$1.2 \pm 0.1*$

Table S3. Stimulation of cAMP accumulation and potentiation of carbachol-evoked Ca<sup>2+</sup> signals by analogues of PTH.

Cells were stimulated with PTH analogues for 1 or 30 min before measuring intracellular levels of cAMP (as percentages of <sup>3</sup>H-ATP + <sup>3</sup>H-ADP + <sup>3</sup>H-cAMP) and changes in  $[Ca^{2+}]_c$  evoked by addition of carbachol (20 µM) in  $Ca^{2+}$ -free HBS. Results show maximal responses and pEC<sub>50</sub> values as means ± s.e.m., n = 3.  $\Delta[Ca^{2+}]_c$  is the difference between the peak  $Ca^{2+}$  signals evoked by carbachol alone or after treatment with the PTH analogue. Numbers in brackets show the difference in pEC<sub>50</sub> value (where pEC<sub>50</sub> = -log EC<sub>50</sub>) from measurements taken after incubation for 1 min or 30 min ( $\Delta pEC_{50} = pEC_{50}^{1 min} - pEC_{50}^{30 min}$ ). \**P* < 0.05 relative to paired comparisons at 1 min.

	PTH(1-34)		NKI	H477	8-Br-cAMP		
	$\frac{\Delta[Ca^{2+}]_c}{(nM)}$	pEC <sub>50</sub> (/M)	$\frac{\Delta[Ca^{2+}]_c}{(nM)}$	pEC <sub>50</sub> (/M)	$\frac{\Delta[Ca^{2+}]_c}{(nM)}$	pEC <sub>50</sub> (/M)	
1 min 2 min 5 min 15 min 30 min	$243 \pm 16$ ND ND 127 $\pm 14^{\circ}$	$7.5 \pm 0.1$ ND ND $8.7 \pm 0.1^{\circ}$	$ \begin{array}{c} \text{ND} \\ 257 \pm 3 \\ 225 \pm 9 \\ 155 \pm 10^{\circ} \\ 124 \pm 24^{\circ} \end{array} $	ND $5.0 \pm 0.3$ $5.0 \pm 0.1$ $5.0 \pm 0.1$ $6.0 \pm 0.1^{\circ}$	$NDND207 \pm 7ND113 \pm 5^{^{+}}$	$ \begin{array}{c} \text{ND} \\ \text{ND} \\ 3.5 \pm 0.1 \\ \text{ND} \\ 3.7 \pm 0.1 \end{array} $	

Table S4. Ca<sup>2+</sup> signals evoked by carbachol after acute and sustained increases in intracellular cAMP.

Increases in  $[Ca^{2+}]_c$  evoked by 20 µM carbachol after incubations for the periods shown with PTH(1-34), NKH477 or 8-Br-cAMP. Results (means ± s.e.m., n = 3) show the maximal potentiated increase in  $[Ca^{2+}]_c$  ( $\Delta[Ca^{2+}]_c$ ) after subtraction of the response to carbachol alone (20 µM) and the sensitivity (pEC<sub>50</sub>) for each stimulus. \**P* < 0.05 relative to paired comparisons with the shortest incubation. ND, not determined.

## Table S5. Effects of acute and sustained exposure to cAMP on carbachol-evoked Ca<sup>2+</sup> signals.

	Peak [Ca <sup>2+</sup> ] <sub>c</sub> (nM)		
	1, 2 or 5 min 30 m		
Carbachol alone NKH477 then carbachol PTH(1-34) then carbachol 8-Br-cAMP then carbachol 8-Br-cAMP + NKH477 then carbachol NKH477 + PTH(1-34) then carbachol PTH(1-34) + 8-Br-cAMP then carbachol	$122 \pm 6 \\ 331 \pm 20 \\ 385 \pm 26 \\ 386 \pm 21 \\ 355 \pm 27 \\ 356 \pm 12 \\ 360 \pm 28$	$113 \pm 9$ $256 \pm 12^{\circ}$ $268 \pm 10^{\circ}$ $222 \pm 24^{\circ}$ $257 \pm 22^{\circ}$ $295 \pm 25^{\circ}$ $208 \pm 8^{\circ}$	

Effects of PTH(1-34) (1  $\mu$ M, 1 or 30 min), NKH477 (200  $\mu$ M, 2 or 30 min) and 8-Br-cAMP (10 mM, 5 or 30 min) alone or in combination on the peak increases in [Ca<sup>2+</sup>]<sub>c</sub> evoked by carbachol (20  $\mu$ M). Results are means ± s.e.m., n = 3. \**P* < 0.05 relative to paired comparisons with the shortest period of stimulation.

	1 min				60 min			
	peak $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub>		cAMP		peak $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub>		cAMP	
	pEC <sub>50</sub> (/M)	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	pEC <sub>50</sub> (/M)	Maximal ΔcAMP (%)	pEC <sub>50</sub> (/M)	Maximal $\Delta$ [Ca <sup>2+</sup> ] <sub>c</sub> (nM)	pEC <sub>50</sub> (/M)	Maximal ΔcAMP (%)
Control	$7.4\pm0.2$	$364\pm29$	$7.1\pm0.1$	$0.40\pm0.01$	$8.5\pm0.20$	$153\pm14$	$7.5\pm0.01$	$0.6\pm0.01$
+ SQ/DDA	$7.5\pm0.1$	$341\pm43$	$7.2\pm0.5$	$0.16\pm0.01*$	$8.9\pm0.10$	$193\pm32$	$7.9\pm0.4$	$0.18\pm0.03*$
+ IBMX + IBMX+SQ/DDA	$\begin{array}{c} 7.7 \pm 0.1 \\ 7.8 \pm 0.1 \end{array}$	$\begin{array}{c} 300\pm26\\ 259\pm8 \end{array}$	7.4 ± 0.3 ND	$\begin{array}{c} 0.80 \pm 0.1 * \\ ND \end{array}$	$9.6 \pm 0.1*$ $9.2 \pm 0.05*$	$\begin{array}{c} 177 \pm 18 \\ 178 \pm 14 \end{array}$	$7.7 \pm 0.1$ $8.1 \pm 0.05*$	$9.0 \pm 0.02*$ $1.9 \pm 0.2*$

Table S6. Substantial inhibition of adenylyl cyclase affects neither the acute nor sustained effects of PTH(1-34) on carbachol-evoked Ca<sup>2+</sup> signals.

Cells were stimulated with PTH(1-34) for 1 min or 60 min alone or after preincubation with IBMX (1 mM, 5 min) to inhibit cyclic nucleotide phosphodiesterases, or SQ22536 (1 mM) and DDA (200  $\mu$ M) (SQ/DDA, 20 min) to inhibit adenylyl cyclase. Intracellular levels of cAMP or peak  $\Delta$ [Ca<sup>2+</sup>]<sub>c</sub> and their sensitivities to PTH(1-34) (pEC<sub>50</sub>) are shown as means ± s.e.m., n = 3. \**P* < 0.05 relative to paired time-matched comparisons without inhibitors.

## **Supplementary references**

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