

1 **Sustained signalling by PTH modulates IP<sub>3</sub> accumulation and IP<sub>3</sub> receptors via cyclic**  
2 **AMP junctions**

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4 **Abha Meena,<sup>1,2</sup> Stephen C. Tovey,<sup>1</sup> and Colin W. Taylor<sup>1\*</sup>**

5 <sup>1</sup>Department of Pharmacology, University of Cambridge, Cambridge, CB2 1PD, UK.

6  
7 \*Correspondence to: Colin W Taylor, Department of Pharmacology, Tennis Court Road,  
8 University of Cambridge, Cambridge, CB2 1PD, UK. E-mail: [cwt1000@cam.ac.uk](mailto:cwt1000@cam.ac.uk)

9  
10 <sup>2</sup>Present address: Metabolic and Structural Biology Department, CSIR-Central Institute of  
11 Medicinal and Aromatic Plants, Lucknow, 226015, India.

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19  
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21 **Summary**

22 Parathyroid hormone (PTH) stimulates adenylyl cyclase (AC) via type 1 PTH receptors  
23 (PTH<sub>1</sub>R) and potentiates the Ca<sup>2+</sup> signals evoked by carbachol, which stimulates formation of  
24 IP<sub>3</sub>. We confirmed that in HEK cells expressing PTH<sub>1</sub>R, acute stimulation with PTH(1-34)  
25 potentiated carbachol-evoked Ca<sup>2+</sup> release. This was mediated by locally delivered cyclic  
26 AMP (cAMP), but unaffected by inhibition of protein kinase A (PKA), exchange proteins  
27 activated by cAMP, cAMP phosphodiesterases (PDE) or substantial inhibition of AC.  
28 Sustained stimulation with PTH(1-34) causes internalization of PTH<sub>1</sub>R-AC signalling  
29 complexes, but the consequences for delivery of cAMP to IP<sub>3</sub>R within cAMP signalling  
30 junctions are unknown. Here we show that sustained stimulation with PTH(1-34) or with  
31 PTH analogues that do not evoke receptor internalization reduced the potentiated Ca<sup>2+</sup> signals  
32 and attenuated carbachol-evoked increases in cytosolic IP<sub>3</sub>. Similar results were obtained  
33 after sustained stimulation with NKH477 to directly activate AC, or with the membrane-  
34 permeant analogue of cAMP, 8-Br-cAMP. These responses were independent of PKA and  
35 unaffected by substantial inhibition of AC. During prolonged stimulation with PTH(1-34),  
36 hyperactive cAMP signalling junctions, within which cAMP is delivered directly and at  
37 saturating concentrations to its targets, mediate sensitization of IP<sub>3</sub>R and a more slowly  
38 developing inhibition of IP<sub>3</sub> accumulation.

## 39 **Introduction**

40 Parathyroid hormone (PTH) is the major endocrine regulator of plasma  $\text{Ca}^{2+}$  and phosphate  
41 concentrations and, with PTH-related peptide (PTHrP), it regulates bone remodelling (Potts  
42 and Gardella, 2007). Many effects of PTH and PTHrP are mediated by type 1 PTH receptors  
43 ( $\text{PTH}_1\text{R}$ ), which are G-protein-coupled receptors (GPCR) (Mahon, 2012; Mannstadt et al.,  
44 1999). PTH receptors, along with other class II GPCRs, stimulate both adenylyl cyclase (AC)  
45 activity and an increase in the cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) (Taylor and Tovey,  
46 2012). The N-terminal fragments of PTH and PTHrP, PTH(1-34) and PTHrP(1-36), are  
47 sufficient for activation of  $\text{PTH}_1\text{R}$  (Mahon, 2012). However, PTH analogues differ in  
48 whether they favour  $\text{PTH}_1\text{R}$  coupling to G proteins or other signalling proteins, notably  
49 GPCR kinases and  $\beta$ -arrestins (Dean et al., 2008; Gesty-Palmer and Luttrell, 2011; Okazaki  
50 et al., 2008). Binding of  $\beta$ -arrestin to  $\text{PTH}_1\text{R}$  contributes to desensitization (Feinstein et al.,  
51 2011), but it also recruits additional signalling pathways (Gesty-Palmer et al., 2006) and  
52 initiates internalization of active  $\text{PTH}_1\text{R}\cdot\text{Gs}\cdot\text{AC}$  signalling complexes via  $\beta$ -arrestin- and  
53 dynamin-dependent endocytosis (Ferrandon et al., 2009; Gidon et al., 2014). These  
54 complexes then continue to generate cAMP from early endosomal compartments (Feinstein  
55 et al., 2011; Ferrandon et al., 2009; Wehbi et al., 2013). Similar agonist-evoked  
56 internalization of functional signalling pathways occurs for some other GPCRs (Calebiro et  
57 al., 2010; Irannejad et al., 2013). The significance for the present work is that internalized  
58  $\text{PTH}_1\text{R}$  signalling complexes and those at the plasma membrane may deliver cAMP to  
59 different intracellular compartments.

60 The links between cAMP and  $\text{Ca}^{2+}$  signalling by  $\text{PTH}_1\text{R}$  are complex (Taylor and Tovey,  
61 2012). In most, though not all, cells (Mahon, 2012),  $\text{PTH}_1\text{R}$  activates Gs, stimulation of AC  
62 and so formation of cAMP. When  $\text{PTH}_1\text{R}$  or Gq is expressed at high levels,  $\text{PTH}_1\text{R}$  can also  
63 stimulate phospholipase C (PLC) (Taylor and Tovey, 2012), which catalyses formation of  
64 inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), and so  $\text{Ca}^{2+}$  release from intracellular stores. Typically,  
65 such  $\text{Ca}^{2+}$  signals are evoked by higher concentrations of PTH than are required for  
66 stimulation of AC (Cupp et al., 2013; Okazaki et al., 2008; Takasu et al., 1999; Taylor and  
67 Tovey, 2012; van der Lee et al., 2013). Furthermore, some analogues of PTH favour coupling  
68 of  $\text{PTH}_1\text{R}$  to AC via Gs, while others favour PLC coupling (Cupp et al., 2013; Fujimori et al.,  
69 1991; Gesty-Palmer and Luttrell, 2011; Takasu et al., 1999) (supplementary material Table  
70 S1). Association of  $\text{PTH}_1\text{R}$  with the scaffold proteins,  $\text{Na}^+/\text{H}^+$  exchange regulatory factors-1  
71 and 2 (NHERF-1 and 2), both of which are expressed in HEK cells (Wang et al., 2010),  
72 favours coupling, via Gq or Gi/o, to  $\text{PLC}\beta$  (Wang et al., 2007). Cyclic AMP can also

73 stimulate IP<sub>3</sub> formation because binding of cAMP to an exchange protein-activated by cAMP  
74 (EPAC-1) allows it to activate the small G protein, rap 2B, which then stimulates PLC $\epsilon$   
75 (Schmidt et al., 2001).

76 We have shown that in HEK cells stably expressing human PTH<sub>1</sub>R (HEK-PR1 cells),  
77 PTH(1-34) stimulates AC. The cAMP produced directly sensitizes IP<sub>3</sub>R to the IP<sub>3</sub> produced  
78 when receptors, like endogenous M<sub>3</sub> muscarinic receptors, stimulate PLC. Hence,  
79 concentrations of PTH(1-34) that do not alone evoke increases in [Ca<sup>2+</sup>]<sub>c</sub> potentiate the Ca<sup>2+</sup>  
80 signals evoked by carbachol, which activates muscarinic receptors (Short and Taylor, 2000;  
81 Tovey et al., 2008; Tovey et al., 2003; Tovey and Taylor, 2013). This potentiation is  
82 mediated by cAMP, but it requires the cAMP to be delivered at high concentrations from AC  
83 to IP<sub>3</sub>R within a signalling complex that includes AC6 and IP<sub>3</sub>R2. Furthermore, from  
84 evidence that even substantial inhibition of AC failed to attenuate signalling from PTH<sub>1</sub>R to  
85 IP<sub>3</sub>R, we proposed that within each signalling complex, cAMP is presented at concentrations  
86 more than sufficient to maximally sensitize associated IP<sub>3</sub>Rs (Tovey et al., 2008). We  
87 describe the AC-IP<sub>3</sub>R complex as a 'signalling junction' to capture an analogy with the  
88 neuromuscular junction of focally innervated skeletal muscle (Fig. 1A), where release of  
89 acetylcholine from presynaptic terminals saturates postsynaptic receptors and leads to all-or-  
90 nothing contraction of the myofibril. Graded contractions of the muscle then result from  
91 graded recruitment of these all-or-nothing fibrillar responses. Because this mode of signalling  
92 to IP<sub>3</sub>R requires its close association with AC, we assessed whether the association is  
93 maintained during sustained stimulation with PTH(1-34) when PTH<sub>1</sub>R signalling pathways  
94 may be reconfigured. We show that sustained stimulation with PTH leads to diminished  
95 potentiation of carbachol-evoked Ca<sup>2+</sup> signals. This does not require internalization of  
96 PTH<sub>1</sub>R. We provide evidence that the hyperactive cAMP signalling junctions that mediate  
97 sensitization of IP<sub>3</sub>R by PTH also cause inhibition of IP<sub>3</sub> formation during sustained  
98 stimulation. Our results suggest that delivery of cAMP to its targets within signalling  
99 junctions allows rapid potentiation of IP<sub>3</sub>R activity followed by a more slowly developing  
100 inhibition of IP<sub>3</sub> accumulation.

101

## 102 **Results**

### 103 **PTH potentiates carbachol-evoked Ca<sup>2+</sup> release via cAMP-mediated sensitization of IP<sub>3</sub>** 104 **receptors**

105 In HEK-PR1 cells, concentrations of PTH(1-34) that did not alone stimulate Ca<sup>2+</sup> release  
106 potentiated the Ca<sup>2+</sup> signals evoked by carbachol (Fig. 1B) (Tovey et al., 2008). The effects of

107 PTH(1-34), added 1 min before addition of a submaximal concentration of carbachol (20  
108  $\mu\text{M}$ ), were concentration-dependent (Fig. 1C). Similar results, and with similar sensitivity to  
109 PTH(1-34), were obtained using a maximally effective concentration of carbachol  
110 (supplementary material Table S2).

111 At the highest concentrations used ( $>300$  nM), PTH(1-34) alone evoked small ( $< 40$  nM)  
112 increases in  $[\text{Ca}^{2+}]_c$  (Short and Taylor, 2000) (Fig. 1F) that were unaffected by inhibition of  
113 AC, cyclic nucleotide phosphodiesterases (PDEs), protein kinase A (PKA) or EPACs  
114 (supplementary material Fig. S1, which also illustrates the targets of the inhibitors used). We  
115 do not detect stimulation of PLC by PTH(1-34) in HEK-PR1 cells (Short and Taylor, 2000;  
116 Tovey et al., 2008; Tovey and Taylor, 2013), but in some settings  $\text{PTH}_1\text{R}$  can activate Gq and  
117 PLC (see Introduction). We showed previously that an analogue of PTH, PTH(1-31), that  
118 stimulates AC but was thought not to stimulate PLC, mimicked PTH(1-34) by potentiating  
119 carbachol-evoked  $\text{Ca}^{2+}$  signals (Tovey et al., 2008). Conversely, PTH(3-34), which was  
120 thought to selectively activate Gq (Fujimori et al., 1991; but see Takasu et al., 1999), was  
121 ineffective (Tovey et al., 2008). A recent study challenges the utility of both analogues (Cupp  
122 et al., 2013). In CHO cells expressing  $\text{PTH}_1\text{R}$ , PTH(1-31) was indistinguishable from PTH(1-  
123 34) in stimulating AC and PLC (Takasu et al., 1999); while PTH(3-34) stimulated AC (with  
124 very low potency), but not PLC (Cupp et al., 2013). In the same study, PTH(2-38) and  
125  $\text{Tyr}^1\text{PTH}(1-34)$  were as effective as PTH(1-34) in stimulating AC, but they failed to activate  
126 PLC (Cupp et al., 2013) (supplementary material Table S1). Selective activation of AC by  
127 PTH(2-38) and  $\text{Tyr}^1\text{PTH}(1-34)$  is consistent with evidence that N-terminal modifications of  
128 PTH attenuate coupling to PLC (Cupp et al., 2013; Takasu et al., 1999).

129 In HEK-PR1 cells, PTH(2-38) and  $\text{Tyr}^1\text{PTH}(1-34)$  mimicked PTH(1-34) in both  
130 stimulating AC and potentiating carbachol-evoked  $\text{Ca}^{2+}$  signals (Fig. 1C,D, supplementary  
131 material Table S3). Furthermore, the relationship between the change in intracellular cAMP  
132 concentration and the potentiated  $\text{Ca}^{2+}$  signals was indistinguishable for the three analogues  
133 (Fig. 1E). However, while the highest concentrations of PTH(1-34),  $\text{PTHrP}(1-34)$  and  
134 PTH(1-31) directly evoked small  $\text{Ca}^{2+}$  signals, there was no direct response to PTH(2-38) or  
135  $\text{Tyr}^1\text{PTH}(1-34)$  (Fig. 1F). These results demonstrate that only analogues reported to stimulate  
136 PLC directly evoked  $\text{Ca}^{2+}$  signals, and only at much higher concentrations than are required  
137 to potentiate carbachol-evoked  $\text{Ca}^{2+}$  signals. All the PTH analogues that stimulated AC also  
138 potentiated carbachol-evoked  $\text{Ca}^{2+}$  signals. These results reinforce our conclusion that cAMP  
139 mediates the ability of PTH(1-34) to potentiate carbachol-evoked  $\text{Ca}^{2+}$  signals (Tovey et al.,  
140 2008) (Fig. 1A). That conclusion is consistent with the observation that for all effective PTH

141 analogues, potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals was invariably evoked by lower  
142 concentrations of PTH (higher  $\text{pEC}_{50}$ , where  $\text{pEC}_{50}$  is the negative log of the half-maximally  
143 effective concentration) than was cAMP accumulation (Fig. 1C,D, supplementary material  
144 Table S3). The  $\text{Ca}^{2+}$  signals evoked by very high concentrations of PTH(1-34) probably result  
145 from stimulation of PLC. Our inability to detect  $\text{IP}_3$  formation under these conditions (Tovey  
146 et al., 2008; Tovey and Taylor, 2013) is unsurprising when the  $\text{Ca}^{2+}$  signals evoked by PTH  
147 are small and they are detected only under conditions when the  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release is  
148 also maximally potentiated via the cAMP produced in response to PTH.

149

### 150 **Potentiation of carbachol-evoked $\text{Ca}^{2+}$ release by PTH requires neither protein kinase A** 151 **nor EPACs**

152 We have provided evidence that the effects of PTH(1-34) on carbachol-evoked  $\text{Ca}^{2+}$  signals  
153 require neither PKA nor EPACs (Tovey et al., 2008). The latter conclusion came from  
154 experiments in which a membrane-permeant analogue of cAMP that selectively activates  
155 EPACs (8-Br-2'-*O*-Me-cAMP) did not mimic the effects of PTH(1-34) or 8-Br-cAMP on  
156 carbachol-evoked  $\text{Ca}^{2+}$  signals. That conclusion is strengthened by results with a new  
157 membrane-permeant antagonist of EPAC1/2 (ESI-09) (Almahariq et al., 2013). ESI-09 (10  
158  $\mu\text{M}$ , 5 min) had no significant effect on the  $\text{Ca}^{2+}$  signals evoked by carbachol alone, the  
159 concentration-dependent potentiation by PTH(1-34) on carbachol-evoked  $\text{Ca}^{2+}$  signals, or the  
160 small  $\text{Ca}^{2+}$  signals directly evoked by high concentrations of PTH(1-34) (supplementary  
161 material Figs. S1E, S2A,B). It was impracticable to use higher concentrations of ESI-09 or  
162 more prolonged treatments because they directly inhibited carbachol-evoked  $\text{Ca}^{2+}$  release  
163 (supplementary material Fig. S2A,C). Others have also recently reported non-specific effects  
164 of ESI-09 (Rehmann, 2013). A competitive antagonist of EPACs like ESI-09 might be  
165 ineffective if high concentrations of cAMP are locally delivered to  $\text{IP}_3$ Rs from AC (Tovey et  
166 al., 2008). However, potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals by 8-Br-cAMP, which is  
167 uniformly distributed in the cytosol, was also unaffected by ESI-09 (supplementary material  
168 Fig. S2D).

169 These results confirm that EPACs and PKA are not involved in the potentiation of  
170 carbachol-evoked  $\text{Ca}^{2+}$  signals by PTH(1-34) or the direct effects of high concentrations of  
171 PTH(1-34) on  $\text{Ca}^{2+}$  signals. The latter, with evidence that some analogues of PTH stimulate  
172 AC without directly evoking  $\text{Ca}^{2+}$  signals (Fig. 1D,F), suggest that cAMP/EPAC-mediated  
173 activation of PLC $\epsilon$  (Schmidt et al., 2001) does not contribute to PTH-evoked  $\text{Ca}^{2+}$  signals in  
174 HEK-PR1 cells. We conclude, and consistent with previous work (Tovey et al., 2008), that in

175 HEK-PR1 cells the effects of PTH(1-34) on carbachol-evoked  $\text{Ca}^{2+}$  release are mediated by  
176 cAMP, which sensitizes  $\text{IP}_3\text{Rs}$  to  $\text{IP}_3$  without need for activation of PKA or EPACs (Fig. 1A).

177

178 **Sustained stimulation with PTH reduces potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals**

179 PTH(1-34) stimulates delivery of cAMP to  $\text{IP}_3\text{Rs}$  within signalling junctions (Tovey et al.,  
180 2008; Tovey and Taylor, 2013). This, together with evidence that stimulation of AC at the  
181 plasma membrane is followed by internalization of functional  $\text{PTH}_1\text{R}\cdot\text{AC}$  signalling  
182 complexes (see Introduction), prompted us to examine responses of HEK-PR1 cells to  
183 carbachol after sustained stimulation with PTH(1-34).

184 Varying the duration of the incubation with PTH(1-34) before addition of carbachol  
185 established that sustained exposure to PTH(1-34) reduced the maximal amplitude of the  
186 carbachol-evoked  $\text{Ca}^{2+}$  signals by ~50%, while increasing the sensitivity to PTH(1-34) by  
187 almost 10-fold (Fig. 2A,B, supplementary material Table S2). These effects were apparent  
188 after 15-30 min, and not further increased by extending the incubation with PTH(1-34) to 60  
189 min. The inhibition (~50%) was similar whether maximal or submaximal carbachol  
190 concentrations were used to evoke the  $\text{Ca}^{2+}$  signals. Analyses of single cells showed that the  
191 reduced maximal response after prolonged incubation with PTH(1-34) was due to diminished  
192  $\text{Ca}^{2+}$  signals within individual cells rather than to fewer cells responding (Fig. 2C,D). The  
193 diminished amplitude of the potentiated  $\text{Ca}^{2+}$  signals was not due to loss of  $\text{Ca}^{2+}$  from  
194 intracellular stores. Neither  $\text{Tyr}^1\text{PTH}(1-34)$  nor  $\text{PTH}(2-38)$  directly stimulated  $\text{Ca}^{2+}$  release  
195 from intracellular stores (Fig. 1F), but responses to carbachol after brief and sustained  
196 stimulation with these analogues were similar to those evoked by equivalent treatments with  
197 PTH(1-34) (supplementary material Table S3). Furthermore, addition of ionomycin to cells in  
198  $\text{Ca}^{2+}$ -free HBS to assess the  $\text{Ca}^{2+}$  contents of the stores after incubation with PTH(1-34)  
199 showed that the increase in  $[\text{Ca}^{2+}]_c$  evoked by ionomycin was unaffected by acute or  
200 sustained stimulation with PTH(1-34) (Fig. 2E,F). The indistinguishable responses were not  
201 due to saturation of the  $\text{Ca}^{2+}$  indicator because restoration of extracellular  $\text{Ca}^{2+}$  after  
202 ionomycin evoked a much larger increase in fluo 4 fluorescence (Fig. 2E). Using similar  
203 methods to measure the residual  $\text{Ca}^{2+}$  content of the stores after stimulation with PTH(1-34)  
204 and carbachol, showed that more  $\text{Ca}^{2+}$  remained within the stores of cells stimulated with  
205 carbachol after prolonged treatment with PTH(1-34) (peak increase in  $[\text{Ca}^{2+}]_c = 181 \pm 12 \text{ nM}$ )  
206 than after brief treatment ( $97 \pm 4 \text{ nM}$ ,  $P < 0.05$ ) (Fig. 2F). This again indicates that  
207 diminished responses after sustained treatment with PTH(1-34) are not due to loss of  $\text{Ca}^{2+}$   
208 from intracellular stores. We conclude that sustained stimulation with PTH(1-34) reduces the

209 maximal potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals without affecting the  $\text{Ca}^{2+}$  content of  
210 the stores.

211

### 212 **Sustained stimulation with PTH reduces intracellular concentrations of $\text{IP}_3$**

213 The effects of acute and sustained stimulation with PTH(1-34) on the changes in cytosolic  $\text{IP}_3$   
214 concentration evoked by a submaximal concentration of carbachol (30  $\mu\text{M}$ ) were measured in  
215 single HEK-PR1 cells using a FRET-based  $\text{IP}_3$  sensor. Cells were first stimulated with 1 mM  
216 carbachol (3 min, S1) to identify responsive cells (Fig. 3A). After washing and a 30-min  
217 recovery interval, cells were then stimulated with 30  $\mu\text{M}$  carbachol (3 min, S2). The dual-  
218 stimulation protocol, with PTH(1-34) (100 nM) added 1 or 30 min before the second  
219 carbachol stimulus, allowed paired single-cell comparisons of treatments (S2/S1). This  
220 analysis reduced the variability arising from the limited dynamic range of the sensor. The  
221 control response shows that the FRET signal evoked by 30  $\mu\text{M}$  carbachol was less than that  
222 with 1 mM carbachol (Fig. 3A), and it was unaffected by prior exposure to 1 mM carbachol  
223 (Fig. 3B). These results confirm that the sensor was not saturated by the experimental  
224 stimulus. Addition of PTH(1-34) 1 min before the second challenge had no effect on the  
225 response to carbachol (Fig. 3C,E). This is consistent with evidence that acute stimulation with  
226 PTH(1-34) does not stimulate PLC in HEK-PR1 cells (Tovey et al., 2008; Tovey and Taylor,  
227 2013). However, a 30-min pretreatment with PTH(1-34) significantly reduced the increase in  
228 cytosolic  $\text{IP}_3$  evoked by carbachol (Fig. 3D,F,G). We conclude that sustained stimulation with  
229 PTH(1-34) reduces the stimulatory effect of carbachol on the cytosolic levels of  $\text{IP}_3$ .

230

### 231 **Internalization of AC signalling pathways does not mediate sustained effects of PTH on** 232 **carbachol-evoked $\text{Ca}^{2+}$ signals**

233 We used PTH analogues that differ in their abilities to evoke internalization of  $\text{PTH}_1\text{R}$  to  
234 assess whether endocytosis of functional AC-signalling pathways contributes to the sustained  
235 effects of PTH(1-34) on carbachol-evoked  $\text{Ca}^{2+}$  signals. PTH(1-34) evokes receptor  
236 internalization and sustained signalling from endosomal AC, PTH(2-38) does not evoke  
237 receptor internalization,  $\text{Tyr}^1\text{PTH}(1-34)$  is a weak partial agonist for receptor internalization,  
238 and  $\text{PTHrP}(1-36)$  evokes receptor internalization but no persistent AC signalling (see  
239 supplementary material Table S1). The acute and sustained effects of each analogue on  
240 carbachol-evoked  $\text{Ca}^{2+}$  signals were similar to those evoked by PTH(1-34) (Fig. 4A-D,  
241 supplementary material Table S3). For each PTH analogue, the maximal amplitude of the  
242  $\text{Ca}^{2+}$  signal evoked by carbachol was smaller after sustained stimulation, despite each causing



243 intracellular levels of cAMP to be greater after stimulation for 30 min relative to 1 min (Fig.  
244 4E-H, supplementary material Table S3). Although PTHrP(1-36) mimicked the effects of  
245 PTH(1-34) in potentiating carbachol-evoked  $\text{Ca}^{2+}$  signals, it stimulated lesser cAMP  
246 accumulation. This is unexpected because others suggest that PTHrP(1-36) (Dean et al.,  
247 2008), PTHrP(1-34) and PTHrP(1-37) (Cupp et al., 2013) are as efficacious as PTH(1-34) in  
248 stimulating accumulation of cAMP, albeit in cells with 10-fold greater levels of  $\text{PTH}_1\text{R}$   
249 expression (Dean et al., 2008). We have not further explored this issue. For most PTH  
250 analogues, the sensitivity to PTH of both cAMP accumulation and  $\text{Ca}^{2+}$  signalling increased  
251 during sustained stimulation ( $\Delta\text{pEC}_{50}$  values in supplementary material Table S3). This  
252 suggests that a component of the increased sensitivity of the  $\text{Ca}^{2+}$  signals is probably due to  
253 the increased sensitivity of AC activation to PTH during prolonged stimulation. The more  
254 important point for the present work is that for all the PTH analogues, sustained stimulation  
255 causes greater accumulation of cAMP, but lesser potentiation of carbachol-evoked  $\text{Ca}^{2+}$   
256 signals. Collectively, these results suggest that internalization of functional  $\text{PTH}_1\text{R}$  signalling  
257 complexes is unlikely to be responsible for the sustained effects of PTH on carbachol-evoked  
258  $\text{Ca}^{2+}$  signals. We therefore assessed the effects of more directly evoking sustained elevations  
259 in intracellular cAMP concentration on carbachol-evoked  $\text{Ca}^{2+}$  signals.

260 Brief stimulation (1-5 min) with 8-Br-cAMP, PTH(1-34) or NKH477, a soluble analogue  
261 of forskolin that directly activates AC (Ito et al., 1993), caused similar potentiation of  
262 carbachol-evoked  $\text{Ca}^{2+}$  signals (Fig. 5A-C) and their maximal effects were non-additive (Fig.  
263 5D). Because the three stimuli take different times to reach their targets, incubation periods  
264 (1-5 min) were optimized for each to achieve maximal potentiation of carbachol-evoked  $\text{Ca}^{2+}$   
265 signals. The results extend previous work (Tovey et al., 2008) by confirming that cAMP  
266 alone mediates potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals by PTH(1-34). However, the  
267 relationship between intracellular cAMP and  $\Delta[\text{Ca}^{2+}]_c$  is different for PTH(1-34), PTHrP(1-  
268 36) and NKH477 (Fig. 5E): the effects of PTH(1-34) on  $\text{Ca}^{2+}$  signals are associated with  
269 much larger accumulations of cAMP than are comparably potentiated  $\text{Ca}^{2+}$  signals evoked by  
270 PTHrP(1-36) or NKH477. This indicates that  $\text{IP}_3\text{R}$  cannot be responding to a uniformly  
271 delivered global increase in cytosolic cAMP.

272 Sustained exposure to PTH(1-34), NKH477 or 8-Br-cAMP caused similar decreases in the  
273 maximal potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals, and again the maximal effects of  
274 each were non-additive (Fig. 5A-D, supplementary material Table S4 and S5). For both  
275 PTH(1-34) and NKH477, prolonged stimulation reduced the apparent effectiveness of cAMP  
276 in potentiating carbachol-evoked  $\text{Ca}^{2+}$  signals (Fig. 5F and G). Neither the acute nor

277 sustained effects of PTH(1-34), NKH477 or 8-Br-cAMP were affected by inhibition of PKA,  
278 because treatment with H89 had no effect, under conditions where H89 inhibits PKA-  
279 mediated phosphorylation of proteins, (Tovey et al., 2008) (Fig. 6 and supplementary  
280 material Fig. S3A). There was also no effect of H89 on the amount of cAMP produced after  
281 stimulation with PTH(1-34) for 1 or 60 min. For cells treated with H89, amounts of  
282 intracellular cAMP detected after stimulation with 3  $\mu$ M PTH(1-34) for 1 or 60 min were  $95$   
283  $\pm 0.1$  and  $98 \pm 0.2\%$  of those detected in matched control cells ( $n = 3$ ) (Supplementary  
284 material Fig. S3B) . We conclude that sustained elevations of intracellular cAMP, whether  
285 evoked by activation of PTH<sub>1</sub>R or directly, attenuate the potentiation of carbachol-evoked  
286 Ca<sup>2+</sup> signals. Neither the potentiation of Ca<sup>2+</sup> signals by cAMP nor the diminished response  
287 after sustained elevation of cAMP requires activation of PKA.

288

### 289 **Brief and sustained stimulation with PTH potentiate carbachol-evoked Ca<sup>2+</sup> signals via** 290 **cAMP junctions**

291 Although cAMP mediates the effects of PTH on carbachol-evoked Ca<sup>2+</sup> signals (Tovey et al.,  
292 2008), sustained exposure to PTH causes a more substantial increase in intracellular cAMP  
293 than acute stimulation, but a lesser potentiation of carbachol-evoked Ca<sup>2+</sup> signals (Fig. 4,  
294 supplementary material Table S3). The reduced effectiveness of cAMP with increased  
295 duration of exposure is clear from comparison of the relationships between cAMP and  
296 potentiated Ca<sup>2+</sup> signals for cells stimulated acutely or chronically with PTH(1-34) or  
297 NKH477 (Fig. 5F,G).

298 Acute (1 min) potentiation of carbachol-evoked Ca<sup>2+</sup> signals by PTH(1-34) was unaffected  
299 by substantially inhibiting cAMP formation (by inhibiting AC with SQ/DDA) or degradation  
300 (by inhibiting cyclic nucleotide phosphodiesterases with IBMX), although both treatments  
301 had the expected effects on intracellular cAMP (Fig. 7A,B supplementary material Table S6,  
302 Fig. S3C,D). Figure S3E demonstrates that if the cAMP that regulates IP<sub>3</sub>R were uniformly  
303 distributed, the observed 60-70% inhibition of AC by SQ/DDA would cause a detectable  
304 inhibition of the effects of PTH(1-34) on carbachol-evoked Ca<sup>2+</sup> signals. The lack of effect of  
305 SQ/DDA on Ca<sup>2+</sup> responses is not, therefore, a limitation of our methods. Similar results  
306 were obtained when NKH477 was used to acutely stimulate AC. SQ/DDA and IBMX had the  
307 expected effects on intracellular concentrations of cAMP (Fig. 7C,D), but they had no effect  
308 on the potentiation of carbachol-evoked Ca<sup>2+</sup> signals (Fig. 7E,F). These results confirm  
309 previous work, where we argued that the inability of SQ/DDA or IBMX to affect potentiation  
310 of carbachol-evoked Ca<sup>2+</sup> signals by any concentration of acutely presented PTH(1-34),

311 despite substantial effects on intracellular concentrations of cAMP, suggests that cAMP is  
312 locally delivered at super-saturating concentrations to IP<sub>3</sub>R (Tovey et al., 2008; Tovey and  
313 Taylor, 2013). We propose that the concentration-dependent effects of PTH(1-34) then arise  
314 from recruitment of these signalling junctions, rather than from graded activity within each  
315 (Fig. 7G).

316 In cells stimulated with PTH(1-34) for 60 min, cAMP formation was reduced by ~70%  
317 after inhibition of AC by SQ/DDA, but there was no significant effect on the potentiation of  
318 carbachol-evoked Ca<sup>2+</sup> signals (supplementary material Table S6). Similar effects were  
319 observed after sustained stimulation with NKH477: cAMP accumulation was substantially  
320 inhibited by SQ/DDA without affecting the concentration-dependent effects of NKH477 on  
321 carbachol-evoked Ca<sup>2+</sup> signals (Fig. 8A,B). These results suggest that the sustained effects of  
322 PTH or direct activation of AC on carbachol-evoked Ca<sup>2+</sup> signals are, like those evoked by  
323 acute stimulation, mediated by hyperactive cAMP junctions.

324 IBMX massively increased the amount of cAMP produced after sustained stimulation with  
325 PTH(1-34) or NKH477. In parallel analyses, IBMX significantly increased the sensitivity of  
326 carbachol-evoked Ca<sup>2+</sup> signals to PTH(1-34) and NKH477 without affecting the maximal  
327 amplitude of the increase in [Ca<sup>2+</sup>]<sub>c</sub> (Fig. 8A,C-F, supplementary material Table S6). The  
328 latter remained smaller than the increase observed after acute stimulation, demonstrating that  
329 even massive increases in intracellular cAMP concentration cannot surmount the attenuation  
330 of potentiated Ca<sup>2+</sup> signals after sustained stimulation with PTH. As with all other analyses,  
331 inhibition of PKA (with H89) had no effect on the potentiation of carbachol-evoked Ca<sup>2+</sup>  
332 signals by PTH(1-34) in the presence of IBMX (supplementary material Fig. S4), re-  
333 affirming that PKA is not involved in the potentiation of carbachol-evoked Ca<sup>2+</sup> signals.

334 Whereas SQ/DDA had no effect on the acute potentiation of Ca<sup>2+</sup> signals by PTH(1-34)  
335 alone or with IBMX (Fig. 7, supplementary material Fig. S3), it partially reversed the  
336 increase in sensitivity to PTH(1-34) during sustained stimulation with PTH(1-34) and IBMX  
337 (Fig. 8E). These opposing effects of IBMX and SQ/DDA on the sensitivity of carbachol-  
338 evoked Ca<sup>2+</sup> signals to PTH(1-34) confirm the role of cAMP in mediating the effect.  
339 Sustained stimulation (60 min) with PTH(1-34) in the presence of IBMX generated levels of  
340 intracellular cAMP that were 22-fold greater than those evoked by acute (1 min) stimulation  
341 (supplementary material Table S6). Although SQ/DDA substantially inhibited AC, the  
342 amount of intracellular cAMP in cells stimulated with PTH(1-34) for 60 min with IBMX  
343 remained substantially greater than during acute stimulation (Fig. 8F, supplementary material  
344 Table S6). These results suggest that when the global intracellular cAMP concentration is

345 massively increased, it achieves levels that can sensitize the  $\text{Ca}^{2+}$  signals evoked by carbachol  
346 without need for cAMP signalling junctions. Under these conditions, cAMP will sensitize  
347 both junctional  $\text{IP}_3\text{R}$  and extra-junctional  $\text{IP}_3\text{R}$ . Recruitment of the latter would be expected  
348 to be attenuated by inhibition of AC, while junctional signalling would be unaffected (Fig.  
349 8G).

350

## 351 **Discussion**

### 352 **Signalling from $\text{PTH}_1\text{R}$ to $\text{Ca}^{2+}$ signals via AC- $\text{IP}_3\text{R}$ junctions**

353  $\text{PTH}(1-34)$  potentiates carbachol-evoked  $\text{Ca}^{2+}$  release by increasing the sensitivity of  $\text{IP}_3\text{R}$   
354 (Fig. 1A). The potentiated response is mediated by cAMP, it requires neither protein kinase A  
355 nor EPACs, and probably results from cAMP binding directly to  $\text{IP}_3\text{R}$  or closely associated  
356 proteins (Tovey et al., 2010; Tovey et al., 2008). Despite cAMP being the essential link  
357 between  $\text{PTH}_1\text{R}$  and  $\text{Ca}^{2+}$  signalling, acute responses to all concentrations of  $\text{PTH}(1-34)$  or to  
358 direct stimulation of AC (with NKH477) were insensitive to inhibition of either AC (with  
359 SQ/DDA) or cyclic nucleotide phosphodiesterases (with IBMX), although each inhibitor had  
360 the expected effect on global concentrations of intracellular cAMP (Fig. 7, supplementary  
361 material Table S6, Fig. S3). This, together with the inconsistent relationship between  
362 intracellular cAMP and  $\text{Ca}^{2+}$  signals for different analogues of PTH and direct stimulation of  
363 AC (Fig. 5E), establish that the responses are not mediated by global cAMP signals  
364 uniformly delivered to the cytosol. Instead, we suggest that cAMP is delivered to  $\text{IP}_3\text{R}$  within  
365 signalling junctions at concentrations more than sufficient to fully sensitize associated  $\text{IP}_3\text{R}$ .  
366 We propose that the concentration-dependent effects of  $\text{PTH}(1-34)$  then result from  
367 recruitment of these digital junctions, rather than from graded activity within individual  
368 junctions (Fig. 7G) (Tovey et al., 2008). This evidence that potentiation of carbachol-evoked  
369  $\text{Ca}^{2+}$  signals by  $\text{PTH}(1-34)$  requires local communication between AC and  $\text{IP}_3\text{R}$  motivated  
370 our analysis of sustained responses to  $\text{PTH}(1-34)$  during which functional AC signalling  
371 pathways are internalized (see Introduction).

372

### 373 **Sustained signalling from $\text{PTH}_1\text{R}$ via AC- $\text{IP}_3\text{R}$ junctions**

374 Sustained stimulation with  $\text{PTH}(1-34)$  potentiated carbachol-evoked  $\text{Ca}^{2+}$  signals, but the  
375 maximal amplitude of the response was smaller than with acute stimulation, and the  
376 sensitivity to  $\text{PTH}(1-34)$  was increased (Figs. 2A,B and 4). The latter may, at least in part, be  
377 due to an increase in the sensitivity of cAMP accumulation to  $\text{PTH}(1-34)$  during sustained  
378 stimulation (supplementary material Table S3). The diminished  $\text{Ca}^{2+}$  responses were not due

379 to fewer cells responding or to loss of  $\text{Ca}^{2+}$  from intracellular stores (Fig. 2), and they were  
380 unaffected by inhibition of PKA (Fig. 6). Acute and sustained  $\text{Ca}^{2+}$  responses to PTH  
381 analogues that differ in whether they evoke internalization of functional AC signalling  
382 complexes were similar to those evoked by PTH(1-34) (Fig. 4). Furthermore, acute and  
383 sustained responses to 8-Br-cAMP or direct activation of AC mimicked the responses evoked  
384 by PTH(1-34), and the maximal effects of sustained exposure to each stimulus were non-  
385 additive (Fig. 5A-D). Collectively, these results suggest that additional effects of active  
386  $\text{PTH}_1\text{R}$ , like stimulation of phosphatidylinositol 3-kinase and Akt (Yamamoto et al., 2007),  
387 are unlikely to contribute to the sustained effects of PTH on CCh-evoked  $\text{Ca}^{2+}$  signals.  
388 Instead, we conclude that attenuated potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals during  
389 sustained exposure to PTH(1-34) is mediated by a sustained increase in cytosolic cAMP that  
390 does not require PKA or internalization of  $\text{PTH}_1\text{R}$  signalling complexes.

391 The insensitivity of the sustained responses to PTH(1-34) and NKH477 to substantial  
392 inhibition of AC (Fig. 8) suggests that hyperactive cAMP signalling junctions regulate the  
393 changes in signalling to  $\text{IP}_3\text{R}$  that occur during sustained activation of AC. We conclude, and  
394 despite evidence that sustained stimulation with PTH(1-34) evokes internalization of  
395 functional AC signalling complexes (Ferrandon et al., 2009), that  $\text{PTH}_1\text{R}$  retains its ability to  
396 signal via hyperactive AC- $\text{IP}_3\text{R}$  signalling junctions during sustained stimulation. Sustained  
397 stimulation with PTH(1-34) in the presence of IBMX caused the global concentration of  
398 intracellular cAMP to increase to levels sufficient to sensitize  $\text{IP}_3\text{R}$  without the usual need for  
399 junctional delivery of cAMP. This was evident from the increased sensitivity to PTH(1-34)  
400 and NKH477 after sustained stimulation in the presence of IBMX, and its partial reversal by  
401 inhibition of AC with SQ/DDA (Fig. 8). Our demonstration that SQ/DDA can, under these  
402 experimental conditions, attenuate the effects of PTH(1-34) on carbachol-evoked  $\text{Ca}^{2+}$  signals  
403 reinforces our conclusion that hyperactive cAMP signalling junctions normally mediate the  
404 effects of PTH(1-34). Although the global increase in cAMP increased the sensitivity to  
405 PTH(1-34) and NKH477, it had no effect on the maximal response, which remained smaller  
406 than that evoked by acute stimulation. This demonstrates that ineffective delivery of cAMP to  
407  $\text{IP}_3\text{R}$  during sustained stimulation does not cause the diminished potentiation of carbachol-  
408 evoked  $\text{Ca}^{2+}$  signals. Instead, sustained increases in intracellular cAMP reduce the  
409 accumulation of cytosolic  $\text{IP}_3$  after carbachol stimulation (Fig. 3). We have not addressed  
410 whether this results from decreased production or enhanced degradation of  $\text{IP}_3$ . However, the  
411 diminished responses to carbachol during sustained stimulation with PTH(1-34) are

412 mediated by cAMP (Figs. 5-8), independent of PKA (Fig. 6), and dependent on delivery of  
413 cAMP within hyperactive signalling junctions (Fig. 8).

414 We conclude that PTH(1-34) via PTH<sub>1</sub>R stimulates AC and locally delivers cAMP at  
415 supersaturating concentrations to associated IP<sub>3</sub>R, thereby increasing their sensitivity to IP<sub>3</sub>  
416 and so potentiating the Ca<sup>2+</sup> signals evoked by carbachol (Fig. 8G). This junctional delivery  
417 of cAMP is maintained during sustained stimulation with PTH(1-34), but prolonged activity  
418 of the junctions leads to an inhibition of IP<sub>3</sub> accumulation. These cAMP junctions which  
419 behave as ‘on-off’, or digital, switches, allow fast and robust signalling from AC to its  
420 targets. The cAMP then mediates both the initial effects of PTH(1-34) on Ca<sup>2+</sup> signals and the  
421 longer term attenuation of the response without need for activation of PKA.

422

## 423 **Materials and Methods**

### 424 **Materials**

425 *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide  
426 dihydrochloride (H89) and 8-Br-cAMP were from R&D Systems (Minneapolis, MN, USA).  
427 2',5'-dideoxyadenosine (DDA), *N,N*-dimethyl-(3*R*,4*aR*,5*S*,6*aS*,10*S*,10*aR*,10*bS*)-5-(acetyloxy)-  
428 3-ethenyldodecahydro-10,10*b*-dihydroxy-3,4*a*,7,7,10*a*-pentamethyl-1-oxo-1*H*-naphtho[2,1-  
429 *b*]pyran-6-yl ester β-alanine hydrochloride (NKH477) and 9-(tetrahydro-2-furanyl)-9*H*-purin-  
430 6-amine (SQ22536) were from Merck Biosciences (Middlesex, UK). [2,8-<sup>3</sup>H]-adenine was  
431 from Perkin Elmer (Waltham, MA, USA). 1,2-*bis*(*o*-aminophenoxy)ethane-*N,N,N',N'*-  
432 tetraacetic acid (BAPTA) was from Molekula (Gillingham, UK). Carbamylcholine chloride  
433 (carbachol, CCh) and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich  
434 (Gillingham, UK). Ionomycin was from Apollo Scientific (Stockport, UK). Cell culture  
435 media, G-418, fluo 4AM and fura 2AM were from Life Technologies (Paisley, UK). 3-[5-  
436 (tert-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitril (ESI-09) was  
437 from Biolog Life Science Institute (Bremen, Germany). All PTH analogues were human  
438 forms and supplied by either Bachem (Bubendorf, Switzerland) or, for PTHrP(1-36), custom-  
439 synthesized by Selleckchem (Boston, MA, USA). Sequences of the analogues used are listed  
440 in supplementary material Table S1.

441

### 442 **Measurements of [Ca<sup>2+</sup>]<sub>c</sub>**

443 HEK-PR1 cells (~10<sup>5</sup> PTH<sub>1</sub>R/cell) were cultured as described (Tovey et al., 2008).  
444 Measurements of [Ca<sup>2+</sup>]<sub>c</sub> in cell populations were performed as previously described (Tovey  
445 et al., 2008). Briefly, confluent cultures of HEK-PR1 grown in 96-well plates were loaded

446 with fluo 4 by incubation with fluo 4AM (2  $\mu$ M, 20°C) in HEPES-buffered saline (HBS).  
 447 HBS had the following composition (mM): NaCl 135, KCl 5.9, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 1.5,  
 448 HEPES 11.6 and glucose 11.5, pH 7.3. After 1 h, loading medium was replaced with HBS,  
 449 and after 45 min cells were used at 20°C for measurements of [Ca<sup>2+</sup>]<sub>c</sub>. A fluorescence plate-  
 450 reader equipped to allow automated fluid additions (FlexStation 3, Molecular Devices,  
 451 Sunnyvale, CA, USA) was used to record fluorescence at intervals of 1.44 s (excitation at 485  
 452 nm; emission at >525 nm) (Tovey et al., 2006). Fluorescence (F) was calibrated to [Ca<sup>2+</sup>]<sub>c</sub>  
 453 from: [Ca<sup>2+</sup>]<sub>c</sub> = K<sub>D</sub>(F-F<sub>min</sub>)/(F<sub>max</sub>-F), where K<sub>D</sub> is the equilibrium dissociation constant of fluo  
 454 4 for Ca<sup>2+</sup> (345 nM); F<sub>min</sub> and F<sub>max</sub> were measured from cells treated with Triton X-100 (0.2  
 455 %, v/v) in the presence of BAPTA (10 mM) or CaCl<sub>2</sub> (10 mM).

456 For single-cell measurements of [Ca<sup>2+</sup>]<sub>c</sub>, near-confluent cultures of HEK-PR1 cells were  
 457 grown on poly-L-lysine-coated round coverslips (22-mm diameter) and loaded with fura 2 by  
 458 incubation with fura 2AM (2  $\mu$ M, 45 min, 20°C) in HBS. The medium was removed and  
 459 cells were incubated for a further 45 min in HBS at 20°C before single-cell imaging using an  
 460 Olympus IX71 inverted fluorescence microscope. Cells were alternately excited at 5-s  
 461 intervals with light (340 nm and 380 nm) from a Xe-arc lamp and monochromator, while  
 462 collecting emitted light at 510 nm using a Luca EMCCD camera (Andor Technology, Belfast,  
 463 UK) and MetaFluor software (Molecular Devices, Sunnyvale, CA). Autofluorescence was  
 464 determined at the end of each experiment by addition of ionomycin (1  $\mu$ M) and MnCl<sub>2</sub> (10  
 465 mM) and subtracted from measurements before computing fluorescence ratios (R =  
 466 F<sub>340</sub>/F<sub>380</sub>). These were calibrated to [Ca<sup>2+</sup>]<sub>c</sub> from:

$$[\text{Ca}^{2+}]_c = K_D \frac{(R - R_f)}{(R_b - R)} \cdot \frac{F_f}{F_b}$$

467 where the K<sub>D</sub> for fura 2 is 224 nM, R<sub>b</sub> and R<sub>f</sub> are the fluorescence ratios for fura 2 with and  
 468 without Ca<sup>2+</sup> bound, and F<sub>b</sub> and F<sub>f</sub> are the fluorescence recorded at 380 nm with and without  
 469 Ca<sup>2+</sup>.

470 Concentration-effect relationships were fitted to Hill equations using Prism version 5  
 471 (GraphPad, San Diego, CA, USA). Results are shown as means  $\pm$  s.e.m.. Statistical  
 472 comparisons of sensitivities used pEC<sub>50</sub> values (-log of the half-maximally effective  
 473 concentration, EC<sub>50</sub>). Because our experiments were performed over a prolonged period  
 474 using HEK-PR1 cells from different passages, there is some variability in the absolute  
 475 sensitivities to carbachol and PTH(1-34), and in the amplitudes of the Ca<sup>2+</sup> signals evoked.  
 476 All statistical comparisons are therefore between experiments performed in parallel and  
 477 analysed using paired Student's t-tests.

478

### 479 **Measurements of intracellular cAMP**

480 These assays were performed under conditions that replicate those used for measurements of  
481  $[Ca^{2+}]_c$ . HEK-PR1 cells were grown in 24-well plates until ~90% confluent,  $^3H$ -adenine (2  
482  $\mu Ci.well^{-1}$ ) was then added to the culture medium. After 2 h at 37°C in 5%  $CO_2$ , the medium  
483 was removed, cells were washed with HBS, and used for experiments in HBS at 20°C.  
484 Because many cells extrude cAMP into the extracellular medium (Copsel et al., 2011),  
485 reactions were terminated by first removing the medium and then adding ice-cold  
486 trichloroacetic acid (5% v/v, 1 mL). After 30 min on ice,  $^3H$ -cAMP was separated from other  
487  $^3H$ -adenine nucleotides by sequential column chromatography on Dowex cation exchange  
488 resin and alumina as previously described (Pantazaka et al., 2013). The activity of the eluates  
489 was determined by liquid scintillation counting and amounts of  $^3H$ -cAMP are expressed as  
490 percentages of the sum of the activities recovered in the  $^3H$ -cAMP,  $^3H$ -ADP and  $^3H$ -ATP  
491 fractions.

492

### 493 **Measurements of intracellular $IP_3$**

494 A Förster resonance energy transfer (FRET) sensor based on the  $IP_3$ -binding core of  $IP_3R1$   
495 (Tovey and Taylor, 2013) was used to measure cytosolic concentrations of  $IP_3$  in single  
496 HEK-PR1 cells under conditions similar to those used for measurements of  $[Ca^{2+}]_c$ . The  
497 plasmid and properties of the sensor were described previously (Tovey and Taylor, 2013).  
498 The sensor comprises the  $IP_3$ -binding core attached via short linkers to enhanced cyan  
499 fluorescent protein (CFP) at its N terminal and enhanced yellow fluorescent protein (YFP) at  
500 its C-terminal (see inset to Fig. 3A).  $IP_3$  binding causes a decrease in FRET. HEK-PR1 cells  
501 on poly-L-lysine-coated, 22-mm diameter, glass coverslips were grown for 48 h in 6-well  
502 plates to ~60% confluence. Cells were then transiently transfected with plasmid encoding the  
503  $IP_3$ -sensor (1  $\mu g$ ) using Lipofectamine LTX reagent with PLUS reagent, according to the  
504 manufacturer's instructions (Life Technologies, Paisley, UK). Cells were imaged after 48 h.  
505 An Olympus IX71 inverted fluorescence microscope with a 40x objective and a 440 nm/520  
506 nm dual band-pass dichroic mirror was used to record fluorescence from widefield images  
507 after excitation at 440 nm (to excite CFP). A Luca EMCCD camera (Andor Technology,  
508 Belfast, UK) was used to collect emitted fluorescence simultaneously at 1-s intervals from  
509 YFP (520–550 nm) and CFP (455–485 nm) using a Cairn Optosplit 2 image-splitter fitted  
510 with a 495-nm dichroic mirror. After correction for background fluorescence (determined  
511 from cytosolic areas of non-transfected cells), FRET ratios are presented as CFP



512 emission/YFP emission, so that the ratio increases (decreased FRET) after IP<sub>3</sub> binding. The  
513 transfection efficiency was ~65%, and 52 ± 2% (n = 17 coverslips) of transfected cells  
514 responded to carbachol (1 mM) with discernible FRET changes; only these responsive cells  
515 were included in analyses of the effects of PTH(1-34).

516

#### 517 **Supplementary materials available online**

518

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523

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622

623 **Fig. 1. Potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals by PTH(1-34) is mediated by**  
624 **cAMP. (A)** Local delivery of cAMP to  $\text{IP}_3\text{R}$  within ‘signalling junctions’ (red box) allows  
625 stimulation of  $\text{PTH}_1\text{R}$  to increase the sensitivity of  $\text{IP}_3\text{R}$  to  $\text{IP}_3$ . This potentiates the  $\text{Ca}^{2+}$   
626 release evoked by  $\text{IP}_3$  produced in response to activation of  $\text{M}_3$  muscarinic acetylcholine  
627 receptors ( $\text{M}_3\text{R}$ ). All-or-nothing activation of these signalling junctions is analogous to the  
628 behaviour of focally innervated skeletal muscle (lower panel), where release of acetylcholine  
629 at the neuromuscular junction (red box) evokes all-or-nothing contraction of individual  
630 myofibrils. Graded contraction of the muscle fibre then results from recruitment of  
631 contracting myofibrils (right panels). See text for further explanation. **(B)** Typical changes in  
632  $[\text{Ca}^{2+}]_c$  from a population of HEK-PR1 cells stimulated with a submaximal concentration of  
633 carbachol (CCh, 20  $\mu\text{M}$ ) alone (black) or with PTH(1-34) (100 nM, added 1 min before  
634 carbachol, red). BAPTA (2.5 mM) was added before carbachol to chelate extracellular  $\text{Ca}^{2+}$ .  
635 Results are means  $\pm$  s.d. from 2 wells in a single experiment. **(C)** Summary results show  
636 concentration-dependent effects of PTH analogues added 1 min before 20  $\mu\text{M}$  carbachol. **(D)**  
637 Effects of PTH analogues on intracellular cAMP measured after 1 min under conditions  
638 identical to those used for measurements of  $[\text{Ca}^{2+}]_c$ . Results show  $^3\text{H}$ -cAMP as a percentage  
639 of  $^3\text{H}$ -ATP,  $^3\text{H}$ -ADP and  $^3\text{H}$ -cAMP. Results (C-E) are means  $\pm$  s.e.m. from at least 3  
640 experiments. **(E)** Results from C and D were used to establish the relationship between  
641 cAMP and the potentiated carbachol-evoked increases in  $[\text{Ca}^{2+}]_c$  for cells stimulated with the  
642 indicated PTH analogues for 1 min. **(F)** Concentration-dependent effects of PTH analogues  
643 alone on the peak increases in  $[\text{Ca}^{2+}]_c$ . The reported abilities of the analogues to stimulate  
644 PLC and/or AC are shown.

645 **Fig. 2. Sustained stimulation with PTH(1-34) reduces potentiated carbachol-evoked**  
646 **Ca<sup>2+</sup> signals without affecting the Ca<sup>2+</sup> content of the intracellular stores. (A, B)**  
647 Populations of HEK-PR1 cells were incubated with the indicated concentrations of PTH(1-  
648 34) for 1-60 min in HBS before addition of BAPTA (2.5 mM) and either 20 μM (A) or 1 mM  
649 carbachol (CCh) (B). The code applies to both panels. Results (A and B) show means ±  
650 s.e.m., n = 4. **(C, D)** Single-cell analyses show the percentage of cells in which carbachol (1  
651 mM) evoked a detectable increase in [Ca<sup>2+</sup>]<sub>c</sub> in control cells or after stimulation with PTH(1-  
652 34) (100 nM) for 1 or 60 min (C), and the increase in [Ca<sup>2+</sup>]<sub>c</sub> evoked by carbachol under each  
653 condition (D). In these experiments, normal HBS was replaced by nominally Ca<sup>2+</sup>-free HBS  
654 5 min before addition of carbachol. Results (C and D) are from 3 coverslips each with ~65  
655 cells. **(E)** Effect of prolonged stimulation with PTH(1-34) on the Ca<sup>2+</sup> contents of the  
656 intracellular stores was assessed by incubating populations of cells with PTH(1-34) for 30  
657 min, before addition of BAPTA (2.5 mM) and then ionomycin (1 μM). Restoration of  
658 extracellular Ca<sup>2+</sup> (10 mM) at the end of the experiment confirmed that the indicator was not  
659 saturated by the Ca<sup>2+</sup> signals evoked by ionomycin. Results show a typical trace from 5 wells  
660 in 1 experiment. RFU, relative fluorescence units. **(F)** Similar experiments show the effects  
661 of treatment for 1 or 30 min with PTH(1-34) (100 nM) on the peak Ca<sup>2+</sup> signals evoked in  
662 Ca<sup>2+</sup>-free HBS by carbachol (20 μM, open bars) or ionomycin (1 μM, solid bars). Results are  
663 means ± s.e.m., n = 3.

664 **Fig. 3. Sustained stimulation with PTH(1-34) reduces carbachol-evoked increases in**  
665 **cytosolic IP<sub>3</sub> concentration.** (A) Cytosolic IP<sub>3</sub> was measured in single HEK-PR1 cells using  
666 a fluorescence resonance energy transfer (FRET) sensor during stimulation (3 min) with 1  
667 mM carbachol (CCh) (S1) and then, after washing, with 30 μM carbachol added 30 min later  
668 (S2). The trace shows typical results from a cell with no intervening PTH treatment. FRET is  
669 shown as CFP/YFP fluorescence, so that an increased signal (decreased FRET) corresponds  
670 to an increase in IP<sub>3</sub> concentration (see Methods). The inset shows the IP<sub>3</sub> sensor with  
671 excitation and emission (italics) wavelengths in nm. IBC, IP<sub>3</sub>-binding core. (B) Summary  
672 results (means ± s.e.m. for 62 cells from 6 coverslips) show ΔFRET (stimulated / basal  
673 signal) for cells stimulated with the indicated carbachol concentrations presented as either the  
674 first (S1) or second stimulus (S2, i.e. after 1 mM carbachol). (C, D) Typical results from  
675 single cells subject to similar treatments, but with PTH(1-34) (100 nM) added 1 min (C) or  
676 30 min (D) before, and then during, the second addition of carbachol. (E, F) Summary results  
677 show ΔFRET for the first and second carbachol stimulation (S1 and S2) as means ± s.e.m. for  
678 36 and 34 cells from 5 (E) and 7 (F) coverslips. (G) For each cell, ΔFRET measurements for  
679 the first (S1, 1 mM carbachol) and second stimulus (S2, 30 μM carbachol) were used to  
680 calculate S2/S1 for the indicated treatments. Results are means ± s.e.m. for 28-36 cells.  
681

682 **Fig. 4. Internalization of adenylyl cyclase signalling pathways does not contribute to**  
683 **diminished potentiation of Ca<sup>2+</sup> signals after sustained stimulation with PTH.** (A-D)  
684 Cells were stimulated for 1 or 30 min with PTH analogues before addition of carbachol (20  
685 μM) in Ca<sup>2+</sup>-free HBS. The peak increases in [Ca<sup>2+</sup>]<sub>c</sub> evoked by carbachol are shown. (E-H)  
686 Parallel measurements of intracellular cAMP measured under identical conditions. Results  
687 are means ± s.e.m., from at least 3 experiments. The code shown in panel A applies to all  
688 panels.

689 **Fig. 5. Diminished potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals after sustained**  
690 **increases in intracellular cAMP concentration. (A-C)** Peak increases in  $[\text{Ca}^{2+}]_c$  evoked by  
691 addition of carbachol (20  $\mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free HBS to cells preincubated with PTH(1-34) (A),  
692 NKH477 (B), or 8-Br-cAMP (C) for the indicated times.  $\Delta[\text{Ca}^{2+}]_c$  denotes the difference in  
693 the peak increase in  $[\text{Ca}^{2+}]_c$  evoked by carbachol alone and after each pretreatment. **(D)**  
694 Similar experiments show the effects of carbachol (20  $\mu\text{M}$ ) on the peak increase in  $[\text{Ca}^{2+}]_c$   
695 after the indicated combinations of treatments for 1 min (PTH(1-34)), 2 min (NKH477) or 5  
696 min (8-Br-cAMP) and 30 min. **(E)** Comparison of the relationship between cAMP and  
697  $\Delta[\text{Ca}^{2+}]_c$  for cells acutely stimulated with PTH(1-34) (1 min, n = 7), PTHrP(1-36) (1 min, n =  
698 3) or NKH477 (5 min, n = 8). **(F, G)** Relationships between cAMP and  $\Delta[\text{Ca}^{2+}]_c$  for cells  
699 stimulated with carbachol (20  $\mu\text{M}$ ) after acute or sustained stimulation with PTH(1-34) (F) or  
700 NKH477 (G). Results are means  $\pm$  s.e.m., n = 4 (A-D) or at least 3 (F, G). **(H)** Targets of the  
701 drugs used.

702

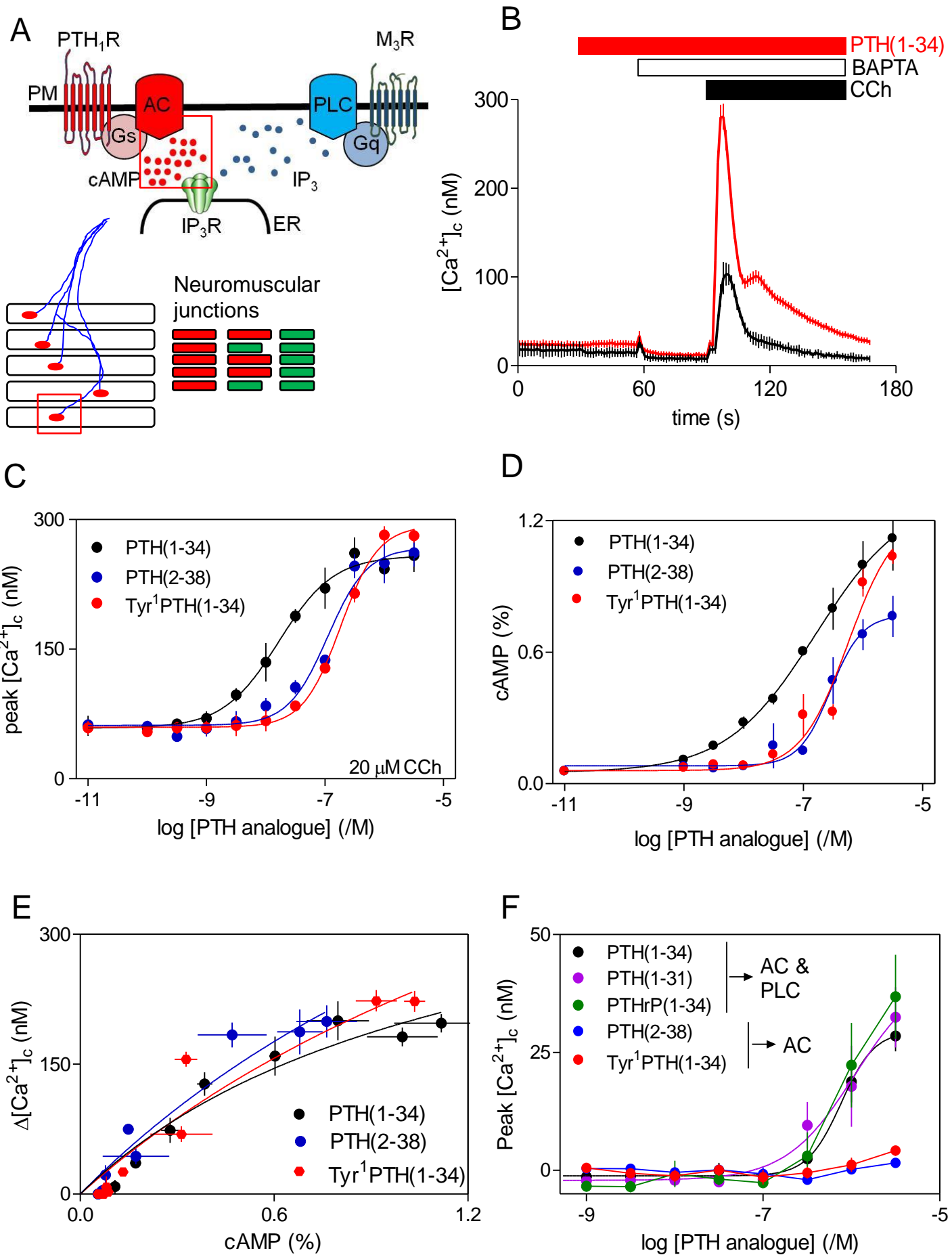
703 **Fig. 6. Neither acute nor sustained potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals**  
704 **requires activation of protein kinase A. (A-E)** Cells were incubated with H89 (10  $\mu\text{M}$ , 20  
705 min) to inhibit PKA before acute or sustained stimulation with PTH(1-34) (A, B), NKH477  
706 (C, D) or 8-Br-cAMP (10 mM) (E) followed by addition of carbachol (20  $\mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free  
707 HBS. Results show peak increases in  $[\text{Ca}^{2+}]_c$  evoked by carbachol as means  $\pm$  s.e.m., n = 3.  
708 **(F)** Targets of the drugs used.

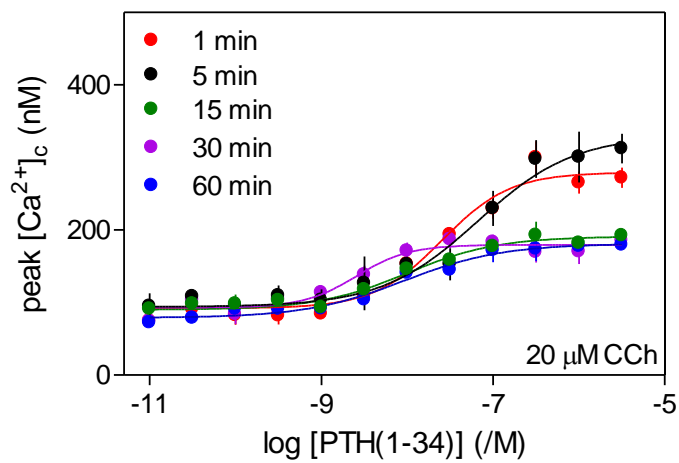
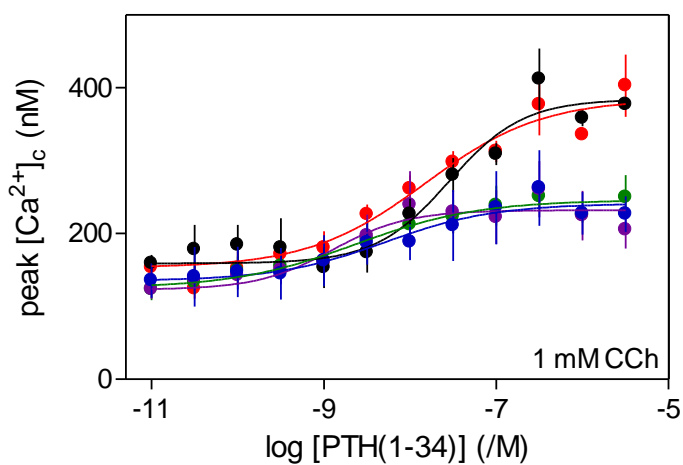
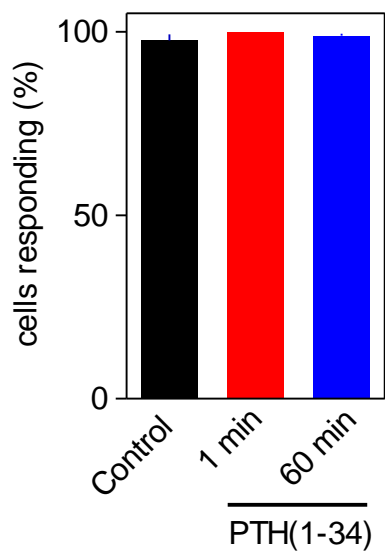
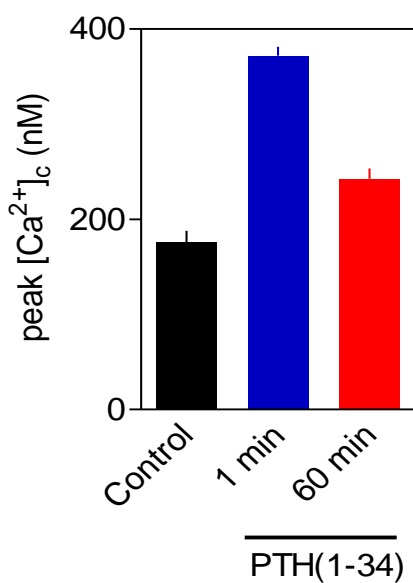
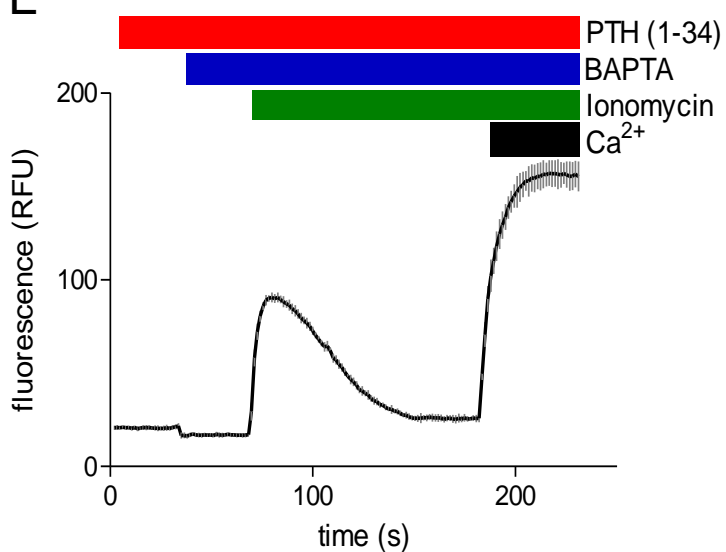
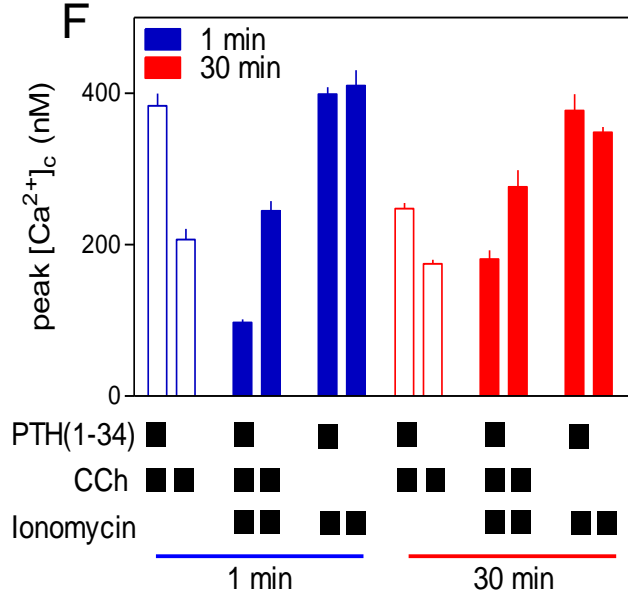


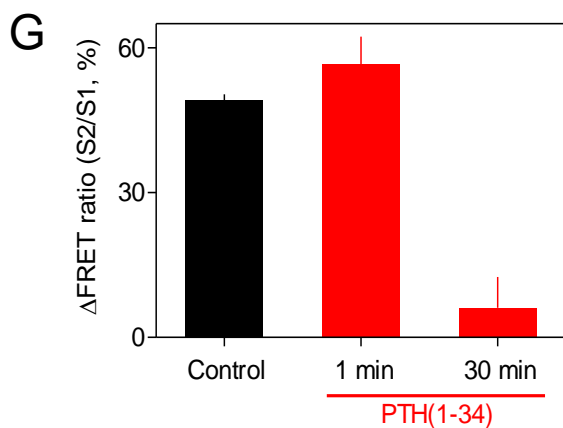
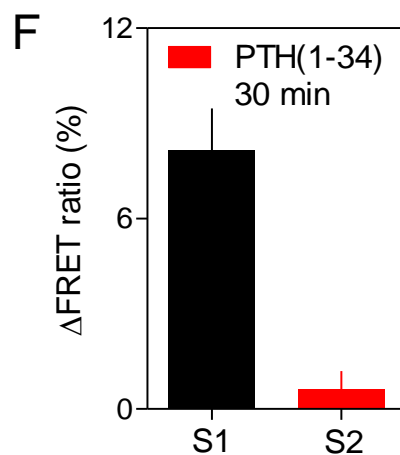
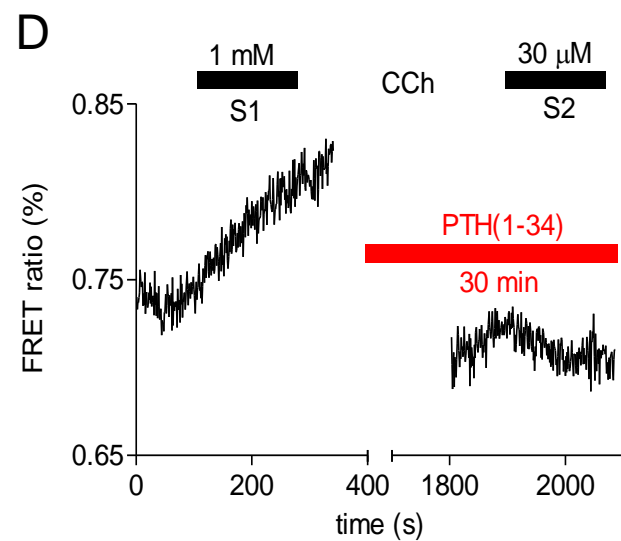
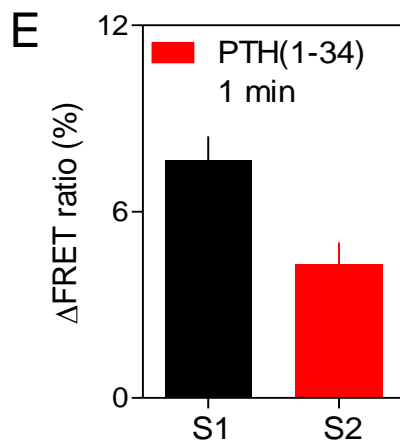
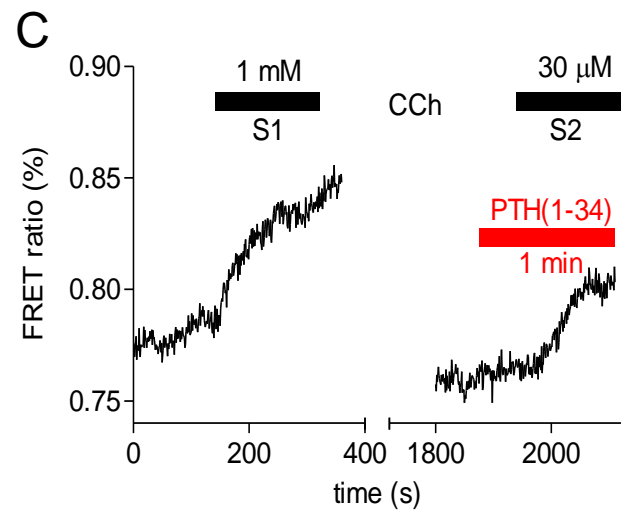
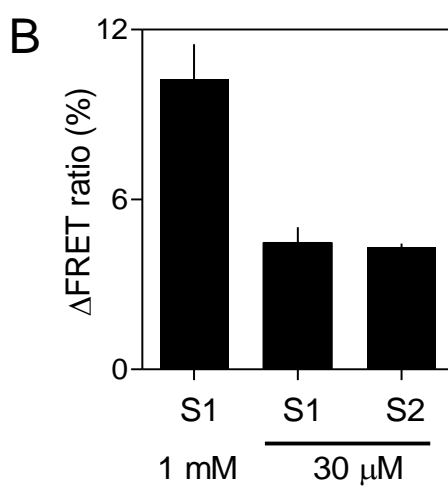
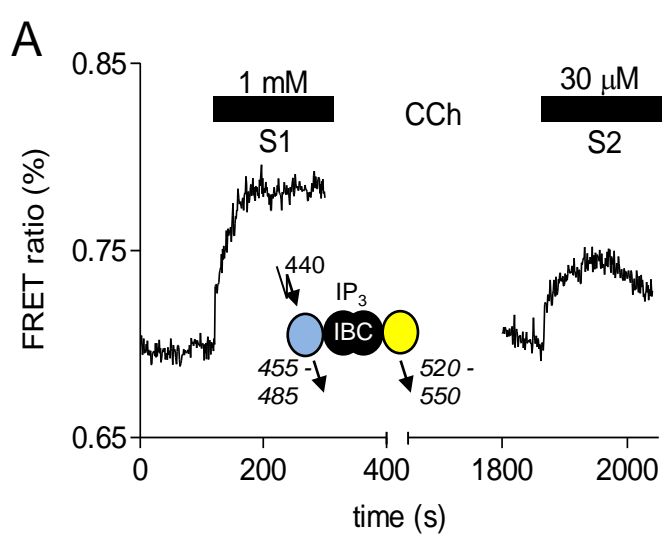
709 **Fig. 7. Acute potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals via cAMP signalling**  
710 **junctions. (A, B)** Cells were incubated with IBMX (1 mM, 5 min) before stimulation with  
711 PTH(1-34) for 1 min and then addition of carbachol (20  $\mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free HBS. Results show  
712 intracellular levels of cAMP (A) and the peak increases in  $[\text{Ca}^{2+}]_c$  evoked by carbachol (B).  
713 (C) Effects of IBMX (1 mM, 5 min) or SQ/DDA (1 mM SQ 22536 and 200  $\mu\text{M}$  DDA, 20  
714 min) on the increase in intracellular cAMP concentration evoked by NKH477 (300  $\mu\text{M}$ , 5  
715 min). (D) Targets of the drugs used. (E, F) Effects of similar treatments with IBMX of  
716 SQ/DDA on the peak  $\text{Ca}^{2+}$  signals evoked by carbachol (20  $\mu\text{M}$ ) after incubation with the  
717 indicated concentrations of NKH477 for 5 min. Results (A-E) are means  $\pm$  s.e.m., n = 3. (G)  
718 Communication between  $\text{PTH}_1\text{R}$  and  $\text{IP}_3\text{Rs}$  is proposed to be mediated by local delivery of  
719 supramaximal concentrations of cAMP from AC to  $\text{IP}_3\text{Rs}$  within junctional complexes. We  
720 suggest that the concentration-dependent effects of PTH are then mediated by recruitment of  
721 these all-or-nothing junctions, rather than from graded activity within each (Tovey et al.,  
722 2008).

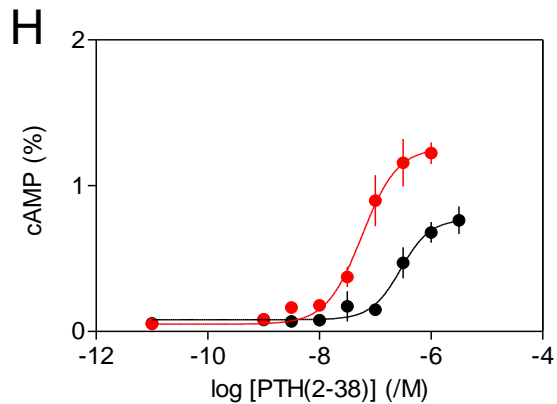
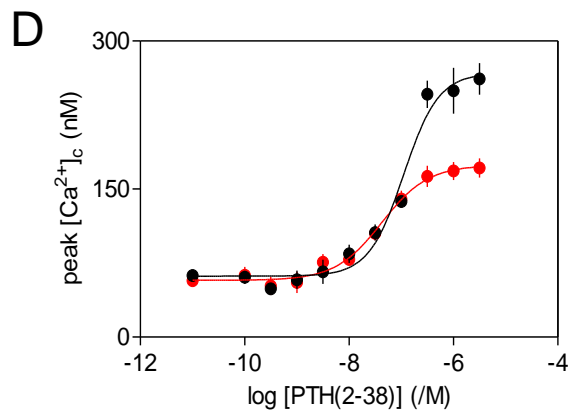
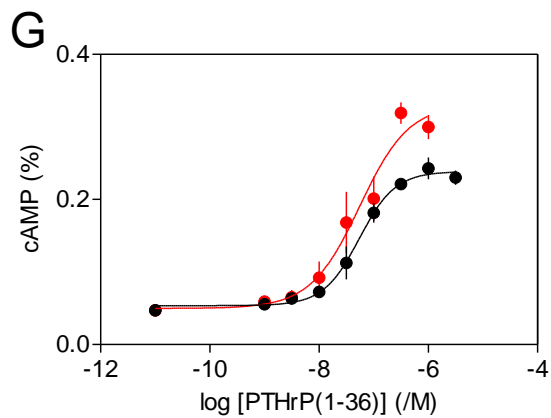
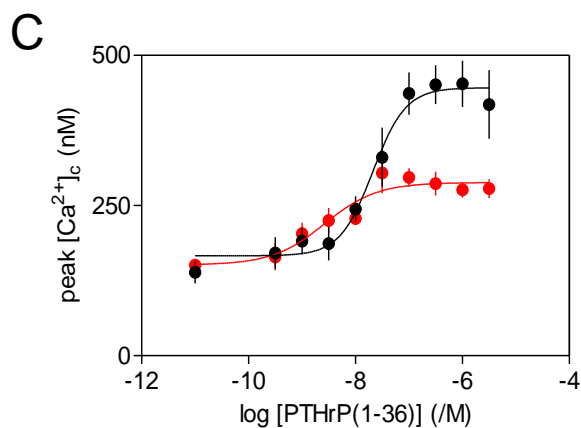
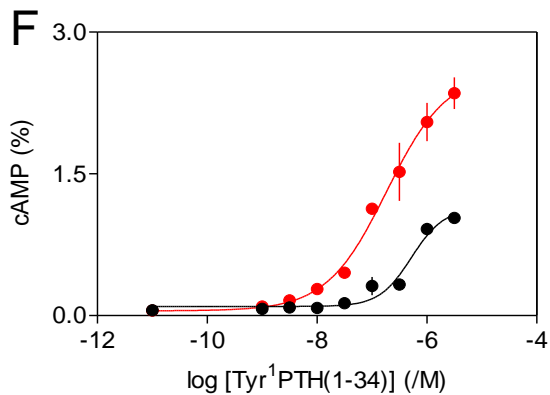
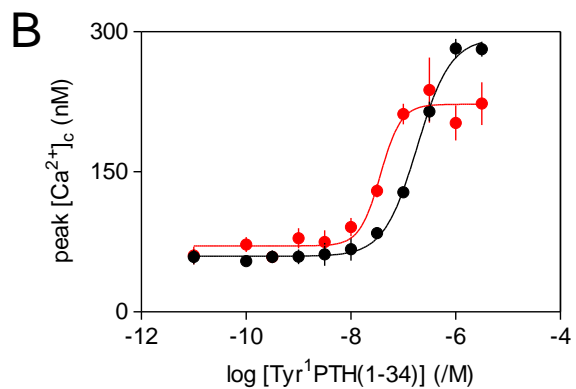
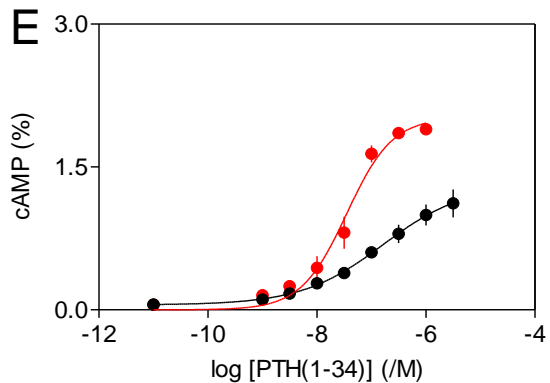
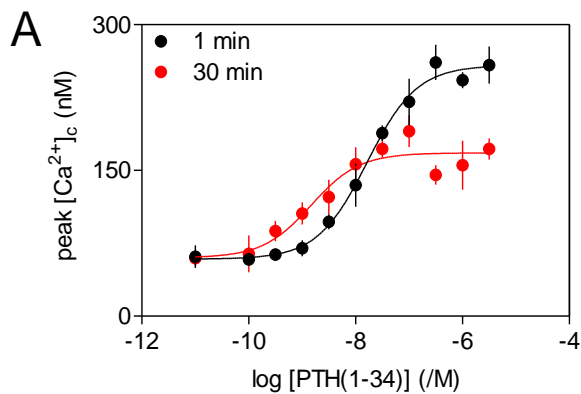
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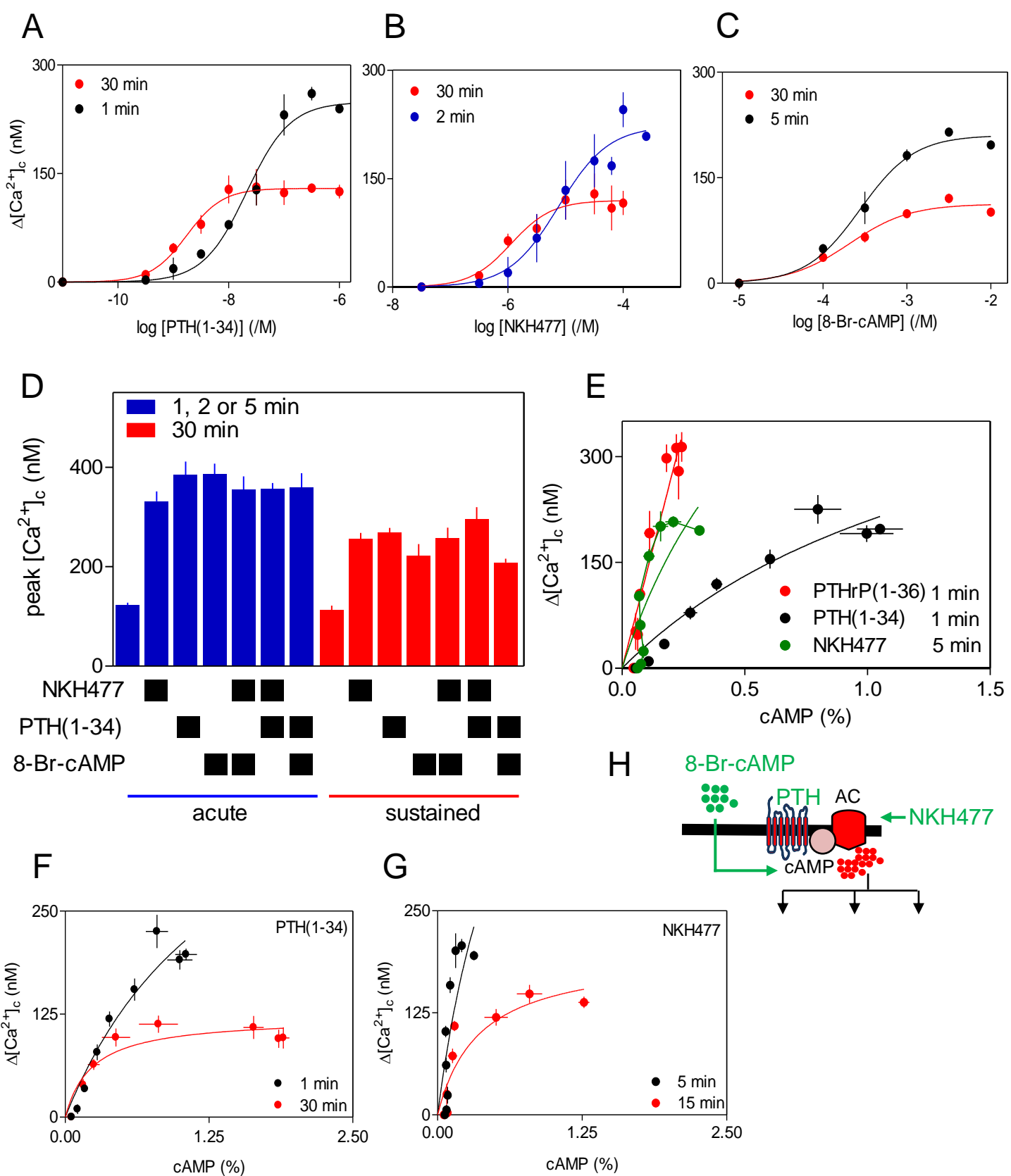
724 **Fig. 8. Sustained potentiation of carbachol-evoked  $\text{Ca}^{2+}$  signals is mediated by cAMP**  
725 **junctions. (A)** Effects of SQ/DDA and IBMX (concentrations as in Fig. 7C) on the increase  
726 in intracellular cAMP concentration evoked by NKH477 (300  $\mu\text{M}$ , 15 min). (B, C) Effects of  
727 the same treatments on the peak  $\text{Ca}^{2+}$  signals evoked by carbachol (20  $\mu\text{M}$ ) after incubation  
728 for 15 min with the indicated concentrations of NKH477. (D, E) Similar analyses of the  
729 effects of SQ/DDA and/or IBMX on the increase in intracellular cAMP concentration evoked  
730 by incubation with the indicated concentrations of PTH(1-34) for 60 min (D) or the peak  $\text{Ca}^{2+}$   
731 signals evoked by carbachol (20  $\mu\text{M}$ ) added 60 min after PTH(1-34) (E). Results (A-E) are  
732 means  $\pm$  s.e.m., n = 3. (F) Relationships between cAMP and  $\Delta[\text{Ca}^{2+}]_c$  for cells stimulated  
733 with PTH(1-34) for 60 min alone or after treatment with SQ/DDA or IBMX (G) Normally  
734 cAMP is delivered to  $\text{IP}_3\text{R}$  within signalling junctions (left panel), but massive accumulation  
735 of cAMP during sustained stimulation with PTH and IBMX (right panel) achieves global  
736 cytosolic cAMP concentrations sufficient to sensitize  $\text{IP}_3\text{R}$  beyond active junctions. (H)  
737 Targets of the drugs used.

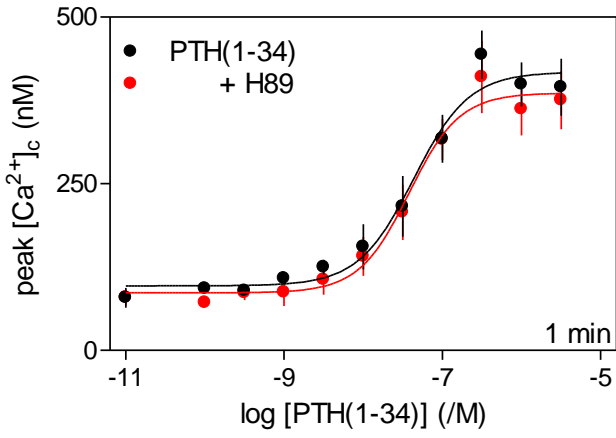
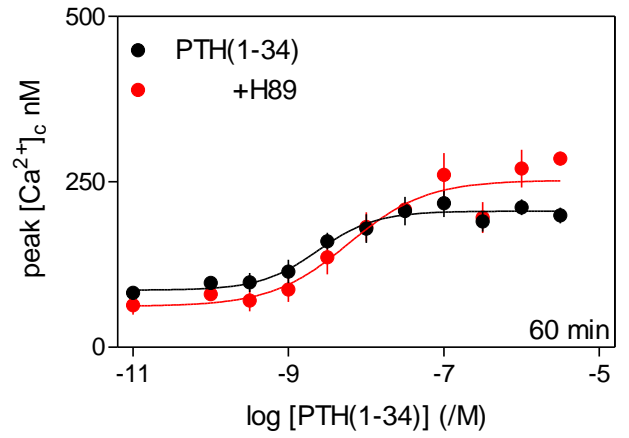
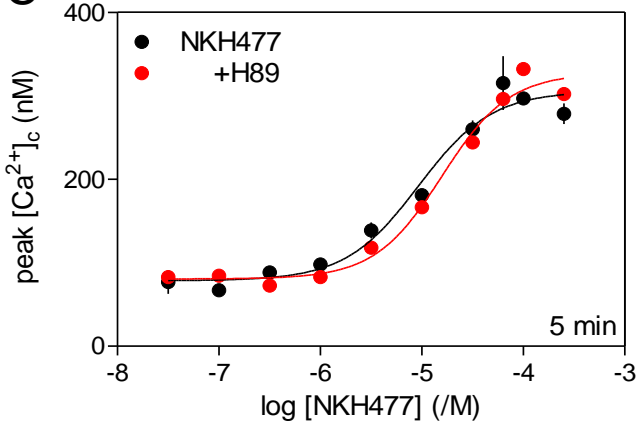
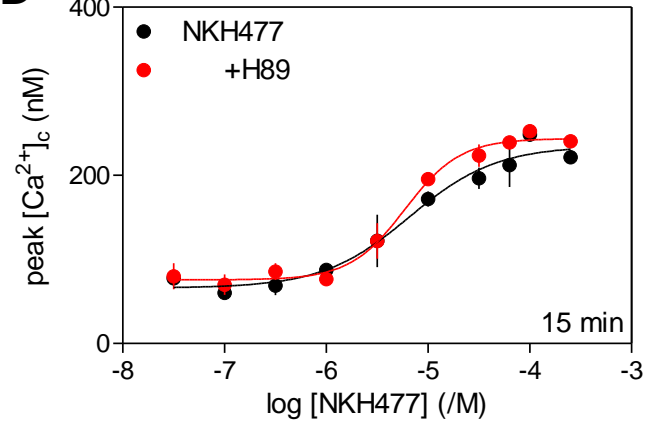
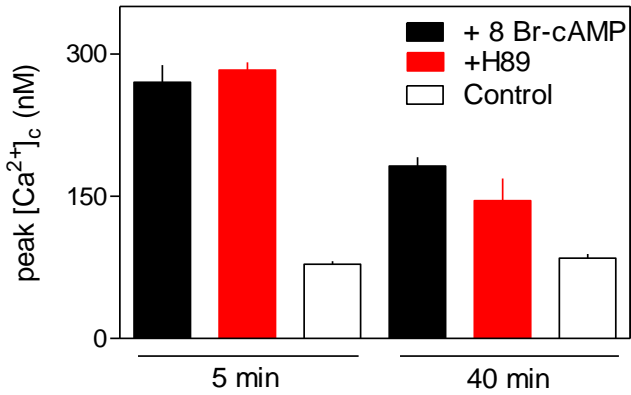


**A****B****C****D****E****F**







**A****B****C****D****E****F**