Sustained signalling by PTH modulates IP_3 accumulation and IP_3 receptors via cyclic AMP junctions

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Summary

Parathyroid hormone (PTH) stimulates adenylyl cyclase (AC) via type 1 PTH receptors (PTH_1R) and potentiates the Ca^{2+} signals evoked by carbachol, which stimulates formation of IP_3. We confirmed that in HEK cells expressing PTH_1R, acute stimulation with PTH(1-34) potentiated carbachol-evoked Ca^{2+} release. This was mediated by locally delivered cyclic AMP (cAMP), but unaffected by inhibition of protein kinase A (PKA), exchange proteins activated by cAMP, cAMP phosphodiesterases (PDE) or substantial inhibition of AC. Sustained stimulation with PTH(1-34) causes internalization of PTH_1R-AC signalling complexes, but the consequences for delivery of cAMP to IP_3R within cAMP signalling junctions are unknown. Here we show that sustained stimulation with PTH(1-34) or with PTH analogues that do not evoke receptor internalization reduced the potentiated Ca^{2+} signals and attenuated carbachol-evoked increases in cytosolic IP_3. Similar results were obtained after sustained stimulation with NKH477 to directly activate AC, or with the membrane-permeant analogue of cAMP, 8-Br-cAMP. These responses were independent of PKA and unaffected by substantial inhibition of AC. During prolonged stimulation with PTH(1-34), hyperactive cAMP signalling junctions, within which cAMP is delivered directly and at saturating concentrations to its targets, mediate sensitization of IP_3R and a more slowly developing inhibition of IP_3 accumulation.

Introduction

Parathyroid hormone (PTH) is the major endocrine regulator of plasma Ca^{2+} and phosphate concentrations and, with PTH-related peptide (PTHrP), it regulates bone remodelling (Potts and Gardella, 2007). Many effects of PTH and PTHrP are mediated by type 1 PTH receptors (PTH_1R), which are G-protein-coupled receptors (GPCR) (Mahon, 2012; Mannstadt et al., 1999). PTH receptors, along with other class II GPCRs, stimulate both adenylyl cyclase (AC) activity and an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) (Taylor and Tovey, 2012). The N-terminal fragments of PTH and PTHrP, PTH(1-34) and PTHrP(1-36), are sufficient for activation of PTH_1R (Mahon, 2012). However, PTH analogues differ in whether they favour PTH_1R coupling to G proteins or other signalling proteins, notably GPCR kinases and β-arrestins (Dean et al., 2008; Gesty-Palmer and Luttrell, 2011; Okazaki et al., 2008). Binding of β-arrestin to PTH_1R contributes to desensitization (Feinstein et al., 2011), but it also recruits additional signalling pathways (Gesty-Palmer et al., 2006) and initiates internalization of active $PTH_1R{\cdot}Gs{\cdot}AC$ signalling complexes via β-arrestin- and dynamin-dependent endocytosis (Ferrandon et al., 2009; Gidon et al., 2014). These complexes then continue to generate cAMP from early endosomal compartments (Feinstein et al., 2011; Ferrandon et al., 2009; Wehbi et al., 2013). Similar agonist-evoked internalization of functional signalling pathways occurs for some other GPCRs (Calebiro et al., 2010; Irannejad et al., 2013). The significance for the present work is that internalized PTH_1R signalling complexes and those at the plasma membrane may deliver cAMP to different intracellular compartments.

The links between cAMP and Ca^{2+} signalling by PTH_1R are complex (Taylor and Tovey, 2012). In most, though not all, cells (Mahon, 2012), PTH_1R activates Gs, stimulation of AC and so formation of cAMP. When PTH_1R or Gq is expressed at high levels, PTH_1R can also stimulate phospholipase C (PLC) (Taylor and Tovey, 2012), which catalyses formation of inositol 1,4,5-trisphosphate (IP_3), and so Ca^{2+} release from intracellular stores. Typically, such Ca^{2+} signals are evoked by higher concentrations of PTH than are required for stimulation of AC (Cupp et al., 2013; Okazaki et al., 2008; Takasu et al., 1999; Taylor and Tovey, 2012; van der Lee et al., 2013). Furthermore, some analogues of PTH favour coupling of PTH_1R to AC via Gs, while others favour PLC coupling (Cupp et al., 2013; Fujimori et al., 1991; Gesty-Palmer and Luttrell, 2011; Takasu et al., 1999) (supplementary material Table S1). Association of PTH_1R with the scaffold proteins, Na^+/H^+ exchange regulatory factors-1 and 2 (NHERF-1 and 2), both of which are expressed in HEK cells (Wang et al., 2010), favours coupling, via Gq or Gi/o, to PLCβ (Wang et al., 2007). Cyclic AMP can also

stimulate IP_3 formation because binding of cAMP to an exchange protein-activated by cAMP (EPAC-1) allows it to activate the small G protein, rap 2B, which then stimulates PLCε (Schmidt et al., 2001).

We have shown that in HEK cells stably expressing human PTH_1R (HEK-PR1 cells), PTH(1-34) stimulates AC. The cAMP produced directly sensitizes IP_3R to the IP_3 produced when receptors, like endogenous M_3 muscarinic receptors, stimulate PLC. Hence, concentrations of PTH(1-34) that do not alone evoke increases in $[Ca^{2+}]_c$ potentiate the Ca^{2+} signals evoked by carbachol, which activates muscarinic receptors (Short and Taylor, 2000; Tovey et al., 2008; Tovey et al., 2003; Tovey and Taylor, 2013). This potentiation is mediated by cAMP, but it requires the cAMP to be delivered at high concentrations from AC to IP_3R within a signalling complex that includes AC6 and IP_3R2. Furthermore, from evidence that even substantial inhibition of AC failed to attenuate signalling from PTH_1R to IP_3R, we proposed that within each signalling complex, cAMP is presented at concentrations more than sufficient to maximally sensitize associated IP_3Rs (Tovey et al., 2008). We describe the AC-IP_3R complex as a 'signalling junction' to capture an analogy with the neuromuscular junction of focally innervated skeletal muscle (Fig. 1A), where release of acetylcholine from presynaptic terminals saturates postsynaptic receptors and leads to all-or-nothing contraction of the myofibril. Graded contractions of the muscle then result from graded recruitment of these all-or-nothing fibrillar responses. Because this mode of signalling to IP_3R requires its close association with AC, we assessed whether the association is maintained during sustained stimulation with PTH(1-34) when PTH_1R signalling pathways may be reconfigured. We show that sustained stimulation with PTH leads to diminished potentiation of carbachol-evoked Ca^{2+} signals. This does not require internalization of PTH_1R. We provide evidence that the hyperactive cAMP signalling junctions that mediate sensitization of IP_3R by PTH also cause inhibition of IP_3 formation during sustained stimulation. Our results suggest that delivery of cAMP to its targets within signalling junctions allows rapid potentiation of IP_3R activity followed by a more slowly developing inhibition of IP_3 accumulation.

Results

PTH potentiates carbachol-evoked Ca^{2+} release via cAMP-mediated sensitization of IP_3 receptors

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

PTH(1-34), added 1 min before addition of a submaximal concentration of carbachol (20 μM), were concentration-dependent (Fig. 1C). Similar results, and with similar sensitivity to PTH(1-34), were obtained using a maximally effective concentration of carbachol (supplementary material Table S2).

At the highest concentrations used (>300 nM), PTH(1-34) alone evoked small (< 40 nM) increases in $[Ca^{2+}]_c$ (Short and Taylor, 2000) (Fig. 1F) that were unaffected by inhibition of AC, cyclic nucleotide phosphodiesterases (PDEs), protein kinase A (PKA) or EPACs (supplementary material Fig. S1, which also illustrates the targets of the inhibitors used). We do not detect stimulation of PLC by PTH(1-34) in HEK-PR1 cells (Short and Taylor, 2000; Tovey et al., 2008; Tovey and Taylor, 2013), but in some settings PTH_1R can activate Gq and PLC (see Introduction). We showed previously that an analogue of PTH, PTH(1-31), that stimulates AC but was thought not to stimulate PLC, mimicked PTH(1-34) by potentiating carbachol-evoked Ca^{2+} signals (Tovey et al., 2008). Conversely, PTH(3-34), which was thought to selectively activate Gq (Fujimori et al., 1991; but see Takasu et al., 1999), was ineffective (Tovey et al., 2008). A recent study challenges the utility of both analogues (Cupp et al., 2013). In CHO cells expressing PTH_1R, PTH(1-31) was indistinguishable from PTH(1-34) in stimulating AC and PLC (Takasu et al., 1999); while PTH(3-34) stimulated AC (with very low potency), but not PLC (Cupp et al., 2013). In the same study, PTH(2-38) and Tyr^1PTH(1-34) were as effective as PTH(1-34) in stimulating AC, but they failed to activate PLC (Cupp et al., 2013) (supplementary material Table S1). Selective activation of AC by PTH(2-38) and Tyr^1PTH(1-34) is consistent with evidence that N-terminal modifications of PTH attenuate coupling to PLC (Cupp et al., 2013; Takasu et al., 1999).

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

analogues, potentiation of carbachol-evoked Ca^{2+} signals was invariably evoked by lower concentrations of PTH (higher pEC_{50}, where pEC_{50} is the negative log of the half-maximally effective concentration) than was cAMP accumulation (Fig. 1C,D, supplementary material Table S3). The Ca^{2+} signals evoked by very high concentrations of PTH(1-34) probably result from stimulation of PLC. Our inability to detect IP_3 formation under these conditions (Tovey et al., 2008; Tovey and Taylor, 2013) is unsurprising when the Ca^{2+} signals evoked by PTH are small and they are detected only under conditions when the IP_3-evoked Ca^{2+} release is also maximally potentiated via the cAMP produced in response to PTH.

Potentiation of carbachol-evoked Ca^{2+} release by PTH requires neither protein kinase A nor EPACs

We have provided evidence that the effects of PTH(1-34) on carbachol-evoked Ca^{2+} signals require neither PKA nor EPACs (Tovey et al., 2008). The latter conclusion came from experiments in which a membrane-permeant analogue of cAMP that selectively activates EPACs (8-Br-2'-*O*-Me-cAMP) did not mimic the effects of PTH(1-34) or 8-Br-cAMP on carbachol-evoked Ca^{2+} signals. That conclusion is strengthened by results with a new membrane-permeant antagonist of EPAC1/2 (ESI-09) (Almahariq et al., 2013). ESI-09 (10 μM, 5 min) had no significant effect on the Ca^{2+} signals evoked by carbachol alone, the concentration-dependent potentiation by PTH(1-34) on carbachol-evoked Ca^{2+} signals, or the small Ca^{2+} signals directly evoked by high concentrations of PTH(1-34) (supplementary material Figs. S1E, S2A,B). It was impracticable to use higher concentrations of ESI-09 or more prolonged treatments because they directly inhibited carbachol-evoked Ca^{2+} release (supplementary material Fig. S2A,C). Others have also recently reported non-specific effects of ESI-09 (Rehmann, 2013). A competitive antagonist of EPACs like ESI-09 might be ineffective if high concentrations of cAMP are locally delivered to IP_3Rs from AC (Tovey et al., 2008). However, potentiation of carbachol-evoked Ca^{2+} signals by 8-Br-cAMP, which is uniformly distributed in the cytosol, was also unaffected by ESI-09 (supplementary material Fig. S2D).

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

HEK-PR1 cells the effects of PTH(1-34) on carbachol-evoked Ca^{2+} release are mediated by cAMP, which sensitizes IP_3Rs to IP_3 without need for activation of PKA or EPACs (Fig. 1A).

Sustained stimulation with PTH reduces potentiation of carbachol-evoked Ca^{2+} signals

PTH(1-34) stimulates delivery of cAMP to IP_3Rs within signalling junctions (Tovey et al., 2008; Tovey and Taylor, 2013). This, together with evidence that stimulation of AC at the plasma membrane is followed by internalization of functional $PTH_1R{\cdot}AC$ signalling complexes (see Introduction), prompted us to examine responses of HEK-PR1 cells to carbachol after sustained stimulation with PTH(1-34).

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

maximal potentiation of carbachol-evoked Ca^{2+} signals without affecting the Ca^{2+} content of the stores.

Sustained stimulation with PTH reduces intracellular concentrations of IP_3

The effects of acute and sustained stimulation with PTH(1-34) on the changes in cytosolic IP_3 concentration evoked by a submaximal concentration of carbachol (30 μM) were measured in single HEK-PR1 cells using a FRET-based IP_3 sensor. Cells were first stimulated with 1 mM carbachol (3 min, S1) to identify responsive cells (Fig. 3A). After washing and a 30-min recovery interval, cells were then stimulated with 30 μM carbachol (3 min, S2). The dual-stimulation protocol, with PTH(1-34) (100 nM) added 1 or 30 min before the second carbachol stimulus, allowed paired single-cell comparisons of treatments (S2/S1). This analysis reduced the variability arising from the limited dynamic range of the sensor. The control response shows that the FRET signal evoked by 30 μM carbachol was less than that with 1 mM carbachol (Fig. 3A), and it was unaffected by prior exposure to 1 mM carbachol (Fig. 3B). These results confirm that the sensor was not saturated by the experimental stimulus. Addition of PTH(1-34) 1 min before the second challenge had no effect on the response to carbachol (Fig. 3C,E). This is consistent with evidence that acute stimulation with PTH(1-34) does not stimulate PLC in HEK-PR1 cells (Tovey et al., 2008; Tovey and Taylor, 2013). However, a 30-min pretreatment with PTH(1-34) significantly reduced the increase in cytosolic IP_3 evoked by carbachol (Fig. 3D,F,G). We conclude that sustained stimulation with PTH(1-34) reduces the stimulatory effect of carbachol on the cytosolic levels of IP_3.

Internalization of AC signalling pathways does not mediate sustained effects of PTH on carbachol-evoked Ca^{2+} signals

We used PTH analogues that differ in their abilities to evoke internalization of PTH_1R to assess whether endocytosis of functional AC-signalling pathways contributes to the sustained effects of PTH(1-34) on carbachol-evoked Ca^{2+} signals. PTH(1-34) evokes receptor internalization and sustained signalling from endosomal AC, PTH(2-38) does not evoke receptor internalization, Tyr^1PTH(1-34) is a weak partial agonist for receptor internalization, and PTHrP(1-36) evokes receptor internalization but no persistent AC signalling (see supplementary material Table S1). The acute and sustained effects of each analogue on carbachol-evoked Ca^{2+} signals were similar to those evoked by PTH(1-34) (Fig. 4A-D, supplementary material Table S3). For each PTH analogue, the maximal amplitude of the Ca^{2+} signal evoked by carbachol was smaller after sustained stimulation, despite each causing

intracellular levels of cAMP to be greater after stimulation for 30 min relative to 1 min (Fig. 4E-H, supplementary material Table S3). Although PTHrP(1-36) mimicked the effects of PTH(1-34) in potentiating carbachol-evoked Ca^{2+} signals, it stimulated lesser cAMP accumulation. This is unexpected because others suggest that PTHrP(1-36) (Dean et al., 2008), PTHrP(1-34) and PTHrP(1-37) (Cupp et al., 2013) are as efficacious as PTH(1-34) in stimulating accumulation of cAMP, albeit in cells with 10-fold greater levels of PTH_1R expression (Dean et al., 2008). We have not further explored this issue. For most PTH analogues, the sensitivity to PTH of both cAMP accumulation and Ca^{2+} signalling increased during sustained stimulation (ΔpEC_{50} values in supplementary material Table S3). This suggests that a component of the increased sensitivity of the Ca^{2+} signals is probably due to the increased sensitivity of AC activation to PTH during prolonged stimulation. The more important point for the present work is that for all the PTH analogues, sustained stimulation causes greater accumulation of cAMP, but lesser potentiation of carbachol-evoked Ca^{2+} signals. Collectively, these results suggest that internalization of functional PTH_1R signalling complexes is unlikely to be responsible for the sustained effects of PTH on carbachol-evoked Ca^{2+} signals. We therefore assessed the effects of more directly evoking sustained elevations in intracellular cAMP concentration on carbachol-evoked Ca^{2+} signals.

Brief stimulation (1-5 min) with 8-Br-cAMP, PTH(1-34) or NKH477, a soluble analogue of forskolin that directly activates AC (Ito et al., 1993), caused similar potentiation of carbachol-evoked Ca^{2+} signals (Fig. 5A-C) and their maximal effects were non-additive (Fig. 5D). Because the three stimuli take different times to reach their targets, incubation periods (1-5 min) were optimized for each to achieve maximal potentiation of carbachol-evoked Ca^{2+} signals. The results extend previous work (Tovey et al., 2008) by confirming that cAMP alone mediates potentiation of carbachol-evoked Ca^{2+} signals by PTH(1-34). However, the relationship between intracellular cAMP and $\Delta[Ca^{2+}]_c$ is different for PTH(1-34), PTHrP(1-36) and NKH477 (Fig. 5E): the effects of PTH(1-34) on Ca^{2+} signals are associated with much larger accumulations of cAMP than are comparably potentiated Ca^{2+} signals evoked by PTHrP(1-36) or NKH477. This indicates that IP_3R cannot be responding to a uniformly delivered global increase in cytosolic cAMP.

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

sustained effects of PTH(1-34), NKH477 or 8-Br-cAMP were affected by inhibition of PKA, because treatment with H89 had no effect, under conditions where H89 inhibits PKA-mediated phosphorylation of proteins, (Tovey et al., 2008) (Fig. 6 and supplementary material Fig. S3A). There was also no effect of H89 on the amount of cAMP produced after stimulation with PTH(1-34) for 1 or 60 min. For cells treated with H89, amounts of intracellular cAMP detected after stimulation with 3 μM PTH(1-34) for 1 or 60 min were 95 ± 0.1 and 98 ± 0.2% of those detected in matched control cells (n = 3) (Supplementary material Fig. S3B) . We conclude that sustained elevations of intracellular cAMP, whether evoked by activation of PTH_1R or directly, attenuate the potentiation of carbachol-evoked Ca^{2+} signals. Neither the potentiation of Ca^{2+} signals by cAMP nor the diminished response after sustained elevation of cAMP requires activation of PKA.

Brief and sustained stimulation with PTH potentiate carbachol-evoked Ca^{2+} signals via cAMP junctions

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

Acute (1 min) potentiation of carbachol-evoked Ca^{2+} signals by PTH(1-34) was unaffected by substantially inhibiting cAMP formation (by inhibiting AC with SQ/DDA) or degradation (by inhibiting cyclic nucleotide phosphodiesterases with IBMX), although both treatments had the expected effects on intracellular cAMP (Fig. 7A,B supplementary material Table S6, Fig. S3C,D). Figure S3E demonstrates that if the cAMP that regulates IP_3R were uniformly distributed, the observed 60-70% inhibition of AC by SQ/DDA would cause a detectable inhibition of the effects of PTH(1-34) on carbachol-evoked Ca^{2+} signals. The lack of effect of SQ/DDA on Ca^{2+} responses is not, therefore, a limitation of our methods. Similar results were obtained when NKH477 was used to acutely stimulate AC. SQ/DDA and IBMX had the expected effects on intracellular concentrations of cAMP (Fig. 7C,D), but they had no effect on the potentiation of carbachol-evoked Ca^{2+} signals (Fig. 7E,F). These results confirm previous work, where we argued that the inability of SQ/DDA or IBMX to affect potentiation of carbachol-evoked Ca^{2+} 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_3R (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^{2+} 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^{2+} signals (Fig. 8A,B). These results suggest that the sustained effects of
322 PTH or direct activation of AC on carbachol-evoked Ca^{2+} 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^{2+} signals to PTH(1-34) and NKH477 without affecting the maximal
327 amplitude of the increase in $[Ca^{2+}]_c$ (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^{2+} 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^{2+}
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^{2+} signals.
334 Whereas SQ/DDA had no effect on the acute potentiation of Ca^{2+} 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^{2+} 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

massively increased, it achieves levels that can sensitize the Ca^{2+} signals evoked by carbachol without need for cAMP signalling junctions. Under these conditions, cAMP will sensitize both junctional IP_3R and extra-junctional IP_3R. Recruitment of the latter would be expected to be attenuated by inhibition of AC, while junctional signalling would be unaffected (Fig. 8G).

Discussion

Signalling from PTH_1R to Ca^{2+} signals via AC-IP_3R junctions

PTH(1-34) potentiates carbachol-evoked Ca^{2+} release by increasing the sensitivity of IP_3R (Fig. 1A). The potentiated response is mediated by cAMP, it requires neither protein kinase A nor EPACs, and probably results from cAMP binding directly to IP_3R or closely associated proteins (Tovey et al., 2010; Tovey et al., 2008). Despite cAMP being the essential link between PTH_1R and Ca^{2+} signalling, acute responses to all concentrations of PTH(1-34) or to direct stimulation of AC (with NKH477) were insensitive to inhibition of either AC (with SQ/DDA) or cyclic nucleotide phosphodiesterases (with IBMX), although each inhibitor had the expected effect on global concentrations of intracellular cAMP (Fig. 7, supplementary material Table S6, Fig. S3). This, together with the inconsistent relationship between intracellular cAMP and Ca^{2+} signals for different analogues of PTH and direct stimulation of AC (Fig. 5E), establish that the responses are not mediated by global cAMP signals uniformly delivered to the cytosol. Instead, we suggest that cAMP is delivered to IP_3R within signalling junctions at concentrations more than sufficient to fully sensitize associated IP_3R. We propose that the concentration-dependent effects of PTH(1-34) then result from recruitment of these digital junctions, rather than from graded activity within individual junctions (Fig. 7G) (Tovey et al., 2008). This evidence that potentiation of carbachol-evoked Ca^{2+} signals by PTH(1-34) requires local communication between AC and IP_3R motivated our analysis of sustained responses to PTH(1-34) during which functional AC signalling pathways are internalized (see Introduction).

Sustained signalling from PTH_1R via AC-IP_3R junctions

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

to fewer cells responding or to loss of Ca^{2+} from intracellular stores (Fig. 2), and they were unaffected by inhibition of PKA (Fig. 6). Acute and sustained Ca^{2+} responses to PTH analogues that differ in whether they evoke internalization of functional AC signalling complexes were similar to those evoked by PTH(1-34) (Fig. 4). Furthermore, acute and sustained responses to 8-Br-cAMP or direct activation of AC mimicked the responses evoked by PTH(1-34), and the maximal effects of sustained exposure to each stimulus were non-additive (Fig. 5A-D). Collectively, these results suggest that additional effects of active PTH_1R, like stimulation of phosphatidylinositol 3-kinase and Akt (Yamamoto et al., 2007), are unlikely to contribute to the sustained effects of PTH on CCh-evoked Ca^{2+} signals. Instead, we conclude that attenuated potentiation of carbachol-evoked Ca^{2+} signals during sustained exposure to PTH(1-34) is mediated by a sustained increase in cytosolic cAMP that does not require PKA or internalization of PTH_1R signalling complexes.

The insensitivity of the sustained responses to PTH(1-34) and NKH477 to substantial inhibition of AC (Fig. 8) suggests that hyperactive cAMP signalling junctions regulate the changes in signalling to IP_3R that occur during sustained activation of AC. We conclude, and despite evidence that sustained stimulation with PTH(1-34) evokes internalization of functional AC signalling complexes (Ferrandon et al., 2009), that PTH_1R retains its ability to signal via hyperactive AC-IP_3R signalling junctions during sustained stimulation. Sustained stimulation with PTH(1-34) in the presence of IBMX caused the global concentration of intracellular cAMP to increase to levels sufficient to sensitize IP_3R without the usual need for junctional delivery of cAMP. This was evident from the increased sensitivity to PTH(1-34) and NKH477 after sustained stimulation in the presence of IBMX, and its partial reversal by inhibition of AC with SQ/DDA (Fig. 8). Our demonstration that SQ/DDA can, under these experimental conditions, attenuate the effects of PTH(1-34) on carbachol-evoked Ca^{2+} signals reinforces our conclusion that hyperactive cAMP signalling junctions normally mediate the effects of PTH(1-34). Although the global increase in cAMP increased the sensitivity to PTH(1-34) and NKH477, it had no effect on the maximal response, which remained smaller than that evoked by acute stimulation. This demonstrates that ineffective delivery of cAMP to IP_3R during sustained stimulation does not cause the diminished potentiation of carbachol-evoked Ca^{2+} signals. Instead, sustained increases in intracellular cAMP reduce the accumulation of cytosolic IP_3 after carbachol stimulation (Fig. 3). We have not addressed whether this results from decreased production or enhanced degradation of IP_3. However, the diminished responses to carbachol during sustained stimulation with PTH((1-34) are

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

We conclude that PTH(1-34) via PTH_1R stimulates AC and locally delivers cAMP at supersaturating concentrations to associated IP_3R, thereby increasing their sensitivity to IP_3 and so potentiating the Ca^{2+} signals evoked by carbachol (Fig. 8G). This junctional delivery of cAMP is maintained during sustained stimulation with PTH(1-34), but prolonged activity of the junctions leads to an inhibition of IP_3 accumulation. These cAMP junctions which behave as 'on-off', or digital, switches, allow fast and robust signalling from AC to its targets. The cAMP then mediates both the initial effects of PTH(1-34) on Ca^{2+} signals and the longer term attenuation of the response without need for activation of PKA.

Materials and Methods

Materials

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

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

HEK-PR1 cells (~10^5 PTH_1R/cell) were cultured as described (Tovey et al., 2008). Measurements of $[Ca^{2+}]_c$ in cell populations were performed as previously described (Tovey et al., 2008). Briefly, confluent cultures of HEK-PR1 grown in 96-well plates were loaded

with fluo 4 by incubation with fluo 4AM (2 µM, 20°C) in HEPES-buffered saline (HBS). 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. After 1 h, loading medium was replaced with HBS, and after 45 min cells were used at 20°C for measurements of $[Ca^{2+}]_c$. A fluorescence plate-reader equipped to allow automated fluid additions (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA) was used to record fluorescence at intervals of 1.44 s (excitation at 485 nm; emission at >525 nm) (Tovey et al., 2006). Fluorescence (F) was calibrated to $[Ca^{2+}]_c$ from: $[Ca^{2+}]_c = K_D(F-F_{min})/(F_{max}-F)$, where K_D is the equilibrium dissociation constant of fluo 4 for Ca^{2+} (345 nM); F_{min} and F_{max} were measured from cells treated with Triton X-100 (0.2 %, v/v) in the presence of BAPTA (10 mM) or $CaCl_2$ (10 mM).

For single-cell measurements of $[Ca^{2+}]_c$, near-confluent cultures of HEK-PR1 cells were grown on poly-L-lysine-coated round coverslips (22-mm diameter) and loaded with fura 2 by incubation with fura 2AM (2 µM, 45 min, 20°C) in HBS. The medium was removed and cells were incubated for a further 45 min in HBS at 20°C before single-cell imaging using an Olympus IX71 inverted fluorescence microscope. Cells were alternately excited at 5-s intervals with light (340 nm and 380 nm) from a Xe-arc lamp and monochromator, while collecting emitted light at 510 nm using a Luca EMCCD camera (Andor Technology, Belfast, UK) and MetaFluor software (Molecular Devices, Sunnyvale, CA). Autofluorescence was determined at the end of each experiment by addition of ionomycin (1 µM) and $MnCl_2$ (10 mM) and subtracted from measurements before computing fluorescence ratios ($R = F_{340}/F_{380}$). These were calibrated to $[Ca^{2+}]_c$ from:

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

where the K_D for fura 2 is 224 nM, R_b and R_f are the fluorescence ratios for fura 2 with and without Ca^{2+} bound, and F_b and F_f are the fluorescence recorded at 380 nM with and without Ca^{2+}.

Concentration-effect relationships were fitted to Hill equations using Prism version 5 (GraphPad, San Diego, CA, USA). Results are shown as means ± s.e.m.. Statistical comparisons of sensitivities used pEC_{50} values (-log of the half-maximally effective concentration, EC_{50}). Because our experiments were performed over a prolonged period using HEK-PR1 cells from different passages, there is some variability in the absolute sensitivities to carbachol and PTH(1-34), and in the amplitudes of the Ca^{2+} signals evoked. All statistical comparisons are therefore between experiments performed in parallel and analysed using paired Student's t-tests.

Measurements of intracellular cAMP

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

Measurements of intracellular IP_3

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

emission/YFP emission, so that the ratio increases (decreased FRET) after IP_3 binding. The transfection efficiency was ~65%, and 52 ± 2% (n = 17 coverslips) of transfected cells responded to carbachol (1 mM) with discernible FRET changes; only these responsive cells were included in analyses of the effects of PTH(1-34).

Supplementary materials available online

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Fig. 1. Potentiation of carbachol-evoked Ca^{2+} signals by PTH(1-34) is mediated by cAMP. (**A**) Local delivery of cAMP to IP_3R within 'signalling junctions' (red box) allows stimulation of PTH_1R to increase the sensitivity of IP_3R to IP_3. This potentiates the Ca^{2+} release evoked by IP_3 produced in response to activation of M_3 muscarinic acetylcholine receptors (M_3R). All-or-nothing activation of these signalling junctions is analogous to the behaviour of focally innervated skeletal muscle (lower panel), where release of acetylcholine at the neuromuscular junction (red box) evokes all-or-nothing contraction of individual myofibrils. Graded contraction of the muscle fibre then results from recruitment of contracting myofibrils (right panels). See text for further explanation. (**B**) Typical changes in $[Ca^{2+}]_c$ from a population of HEK-PR1 cells stimulated with a submaximal concentration of carbachol (CCh, 20 μM) alone (black) or with PTH(1-34) (100 nM, added 1 min before carbachol, red). BAPTA (2.5 mM) was added before carbachol to chelate extracellular Ca^{2+}. Results are means ± s.d. from 2 wells in a single experiment. (**C**) Summary results show concentration-dependent effects of PTH analogues added 1 min before 20 μM carbachol. (**D**) Effects of PTH analogues on intracellular cAMP measured after 1 min under conditions identical to those used for measurements of $[Ca^{2+}]_c$. Results show ^{3}H-cAMP as a percentage of ^{3}H-ATP, ^{3}H-ADP and ^{3}H-cAMP. Results (C-E) are means ± s.e.m. from at least 3 experiments. (**E**) Results from C and D were used to establish the relationship between cAMP and the potentiated carbachol-evoked increases in $[Ca^{2+}]_c$ for cells stimulated with the indicated PTH analogues for 1 min. (**F**) Concentration-dependent effects of PTH analogues alone on the peak increases in $[Ca^{2+}]_c$. The reported abilities of the analogues to stimulate PLC and/or AC are shown.

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

Fig. 3. Sustained stimulation with PTH(1-34) reduces carbachol-evoked increases in cytosolic IP_3 concentration. (**A**) Cytosolic IP_3 was measured in single HEK-PR1 cells using a fluorescence resonance energy transfer (FRET) sensor during stimulation (3 min) with 1 mM carbachol (CCh) (S1) and then, after washing, with 30 μM carbachol added 30 min later (S2). The trace shows typical results from a cell with no intervening PTH treatment. FRET is shown as CFP/YFP fluorescence, so that an increased signal (decreased FRET) corresponds to an increase in IP_3 concentration (see Methods). The inset shows the IP_3 sensor with excitation and emission (italics) wavelengths in nm. IBC, IP_3-binding core. (**B**) Summary results (means ± s.e.m. for 62 cells from 6 coverslips) show ΔFRET (stimulated / basal signal) for cells stimulated with the indicated carbachol concentrations presented as either the first (S1) or second stimulus (S2, i.e. after 1 mM carbachol). (**C, D**) Typical results from single cells subject to similar treatments, but with PTH(1-34) (100 nM) added 1 min (C) or 30 min (D) before, and then during, the second addition of carbachol. (**E, F**) Summary results show ΔFRET for the first and second carbachol stimulation (S1 and S2) as means ± s.e.m. for 36 and 34 cells from 5 (E) and 7 (F) coverslips. (**G**) For each cell, ΔFRET measurements for the first (S1, 1 mM carbachol) and second stimulus (S2, 30 μM carbachol) were used to calculate S2/S1 for the indicated treatments. Results are means ± s.e.m. for 28-36 cells.

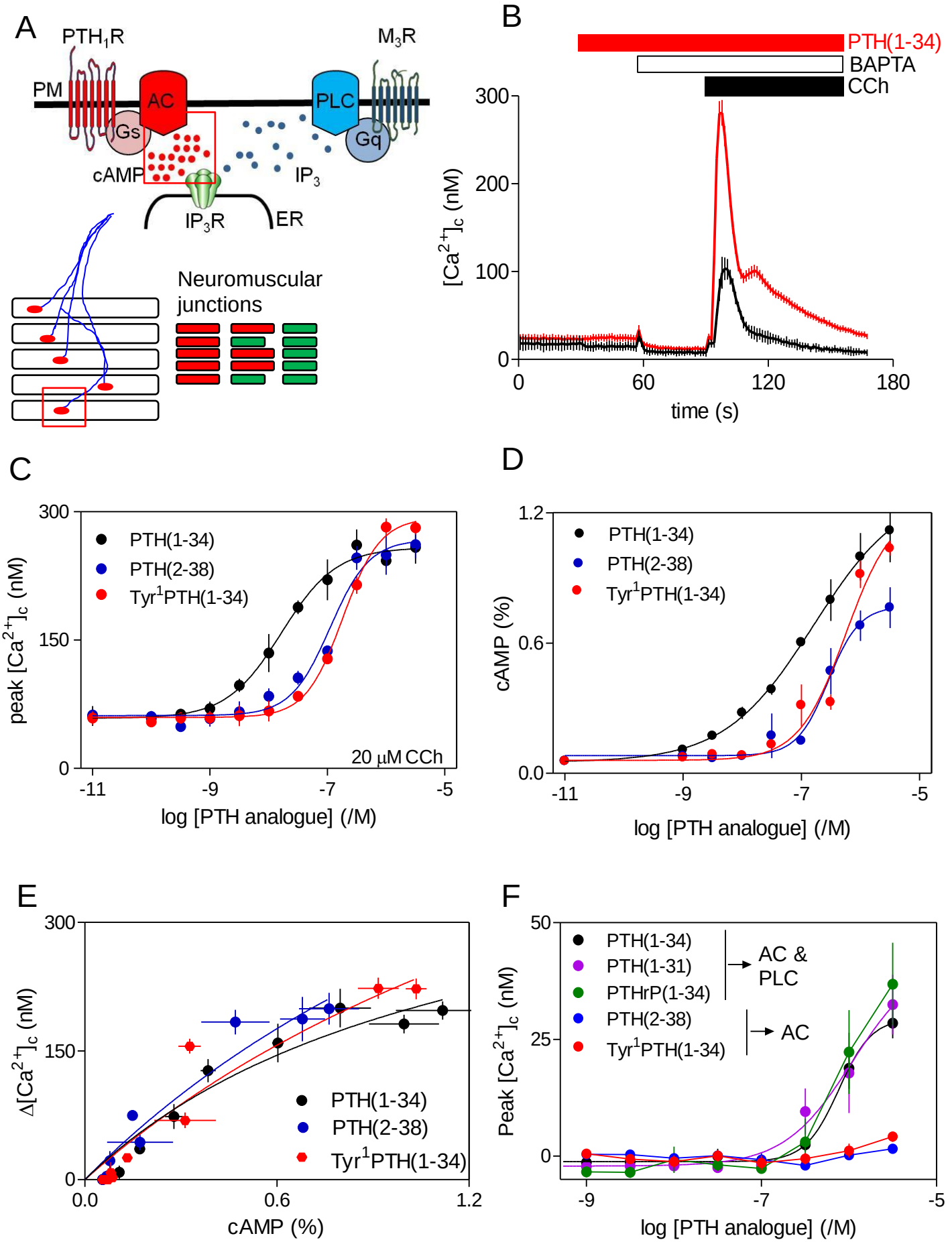
Fig. 4. Internalization of adenylyl cyclase signalling pathways does not contribute to diminished potentiation of Ca^{2+} signals after sustained stimulation with PTH. (**A-D**) Cells were stimulated for 1 or 30 min with PTH analogues before addition of carbachol (20 μM) in Ca^{2+}-free HBS. The peak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

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

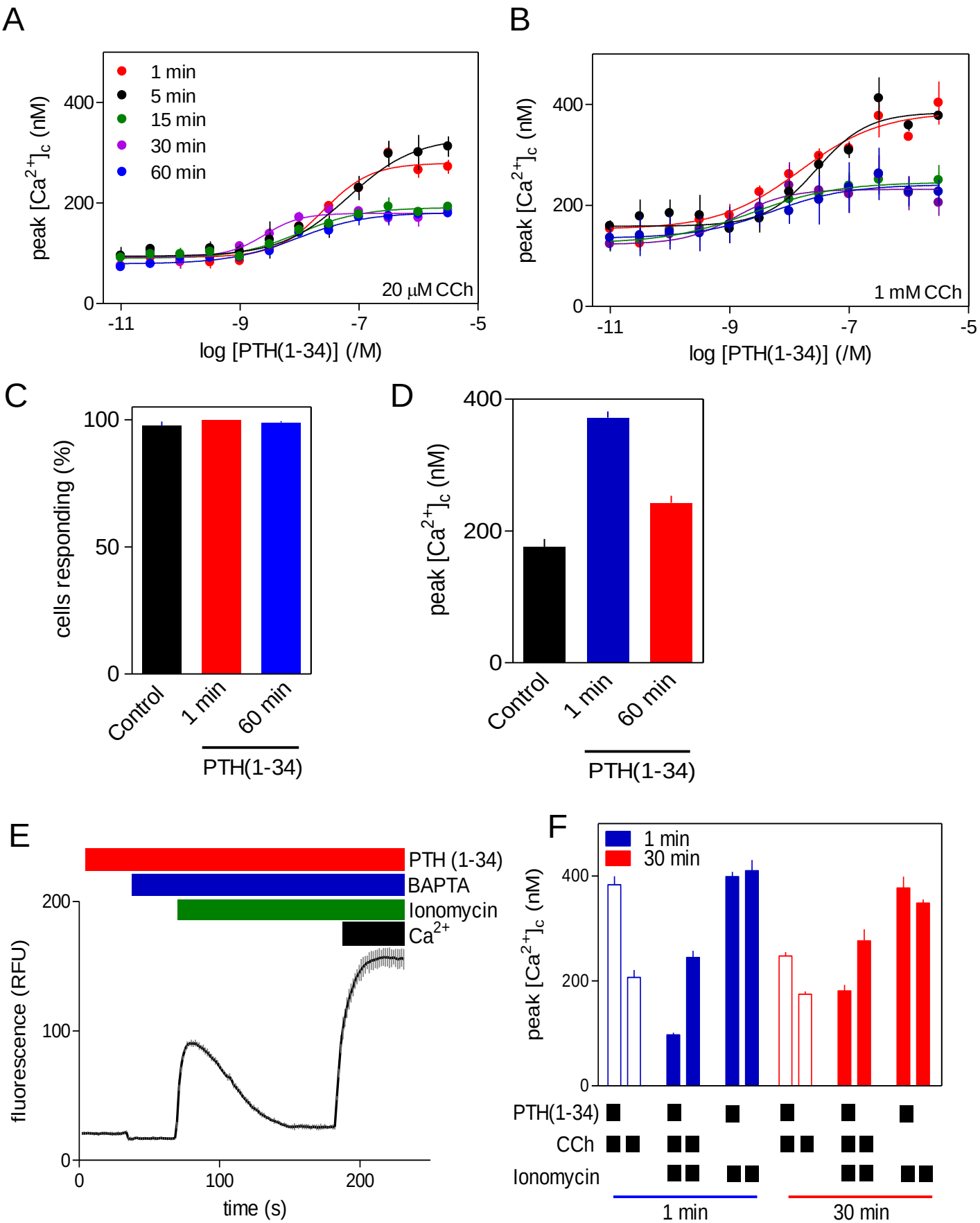
Fig. 6. Neither acute nor sustained potentiation of carbachol-evoked Ca^{2+} signals requires activation of protein kinase A. (A-E) Cells were incubated with H89 (10 μM, 20 min) to inhibit PKA before acute or sustained stimulation with PTH(1-34) (A, B), NKH477 (C, D) or 8-Br-cAMP (10 mM) (E) followed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

Fig. 7. Acute potentiation of carbachol-evoked Ca^{2+} signals via cAMP signalling junctions. (**A, B**) Cells were incubated with IBMX (1 mM, 5 min) before stimulation with PTH(1-34) for 1 min and then addition of carbachol (20 μM) in Ca^{2+}-free HBS. Results show intracellular levels of cAMP (A) and the peak increases in $[Ca^{2+}]_c$ evoked by carbachol (B). (**C**) Effects of IBMX (1 mM, 5 min) or SQ/DDA (1 mM SQ 22536 and 200 μM DDA, 20 min) on the increase in intracellular cAMP concentration evoked by NKH477 (300 μM, 5 min). (**D**) Targets of the drugs used. (**E, F**) Effects of similar treatments with IBMX of SQ/DDA on the peak Ca^{2+} signals evoked by carbachol (20 μM) after incubation with the indicated concentrations of NKH477 for 5 min. Results (A-E) are means ± s.e.m., n = 3. (**G**) Communication between PTH_1R and IP_3Rs is proposed to be mediated by local delivery of supramaximal concentrations of cAMP from AC to IP_3Rs within junctional complexes. We suggest that the concentration-dependent effects of PTH are then mediated by recruitment of these all-or-nothing junctions, rather than from graded activity within each (Tovey et al., 2008).

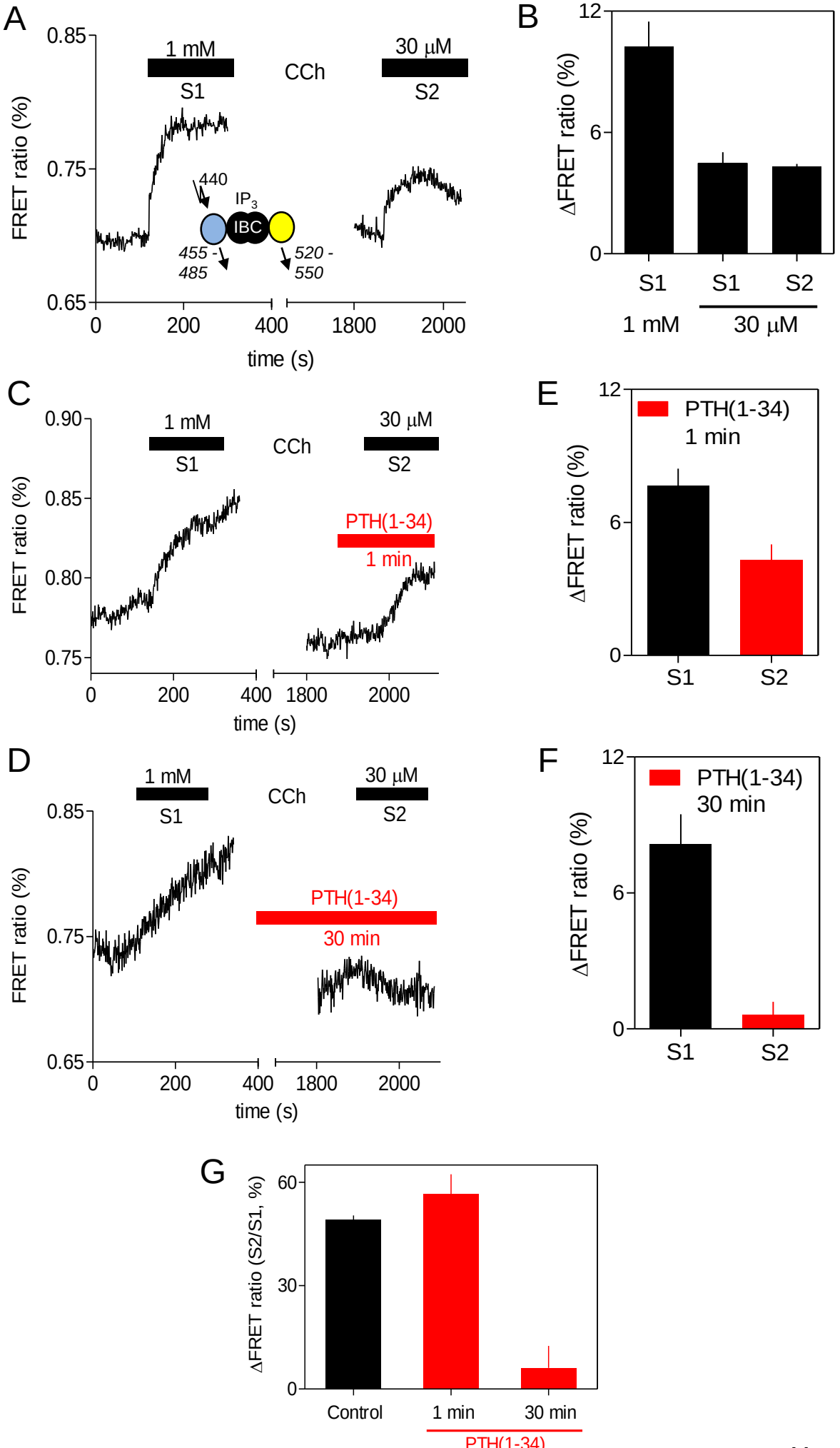
Fig. 8. Sustained potentiation of carbachol-evoked Ca^{2+} signals is mediated by cAMP junctions. (**A**) Effects of SQ/DDA and IBMX (concentrations as in Fig. 7C) on the increase in intracellular cAMP concentration evoked by NKH477 (300 μM, 15 min). (**B, C**) Effects of the same treatments on the peak Ca^{2+} signals evoked by carbachol (20 μM) after incubation for 15 min with the indicated concentrations of NKH477. (**D, E**) Similar analyses of the effects of SQ/DDA and/or IBMX on the increase in intracellular cAMP concentration evoked by incubation with the indicated concentrations of PTH(1-34) for 60 min (D) or the peak Ca^{2+} signals evoked by carbachol (20 μM) added 60 min after PTH(1-34) (E). Results (A-E) are means ± s.e.m., n = 3. (**F**) Relationships between cAMP and $\Delta[Ca^{2+}]_c$ for cells stimulated with PTH(1-34) for 60 min alone or after treatment with SQ/DDA or IBMX (**G**) Normally cAMP is delivered to IP_3R within signalling junctions (left panel), but massive accumulation of cAMP during sustained stimulation with PTH and IBMX (right panel) achieves global cytosolic cAMP concentrations sufficient to sensitize IP_3R beyond active junctions. (**H**) Targets of the drugs used.



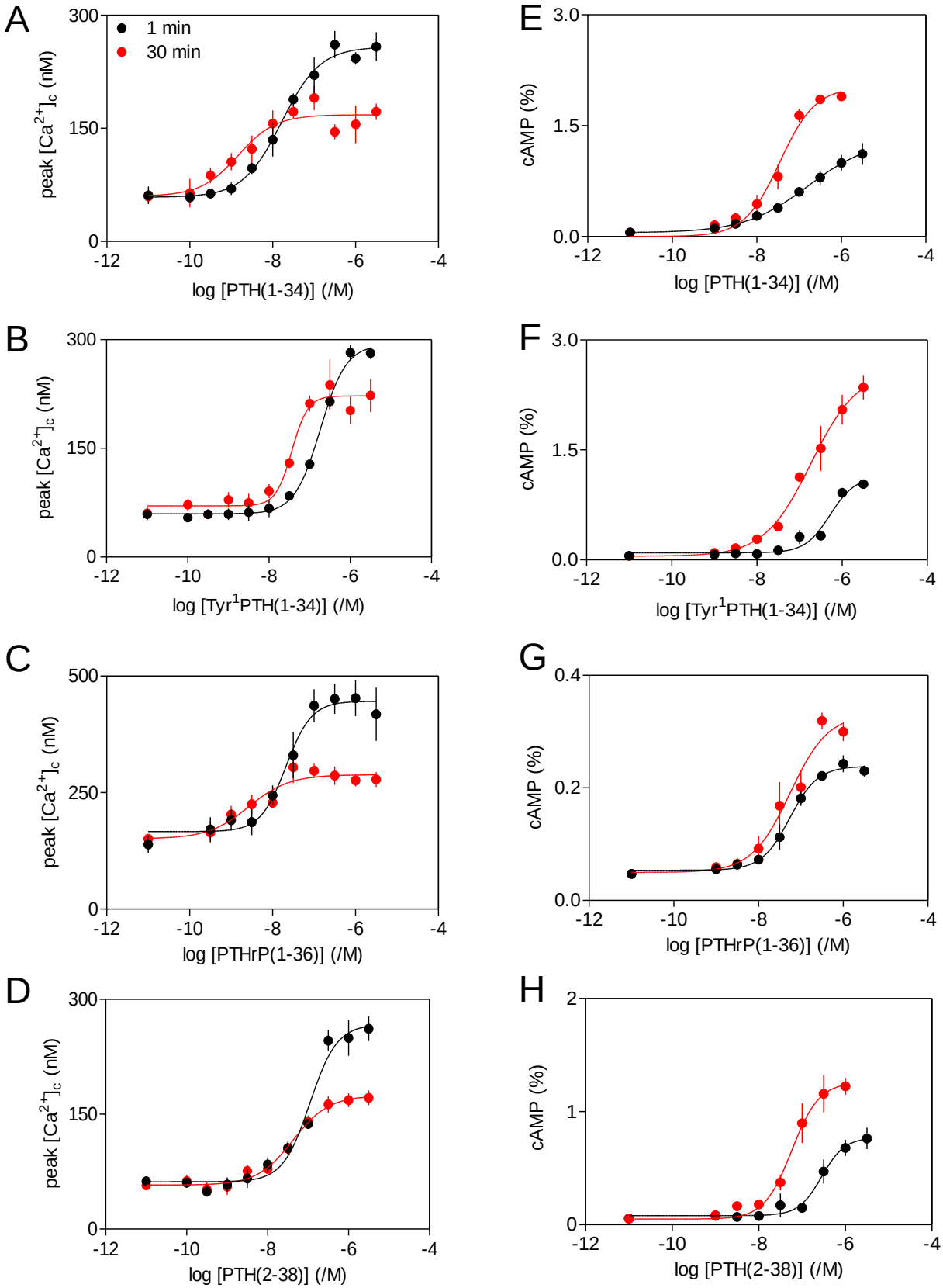
Meena *et al.*, Figure 1



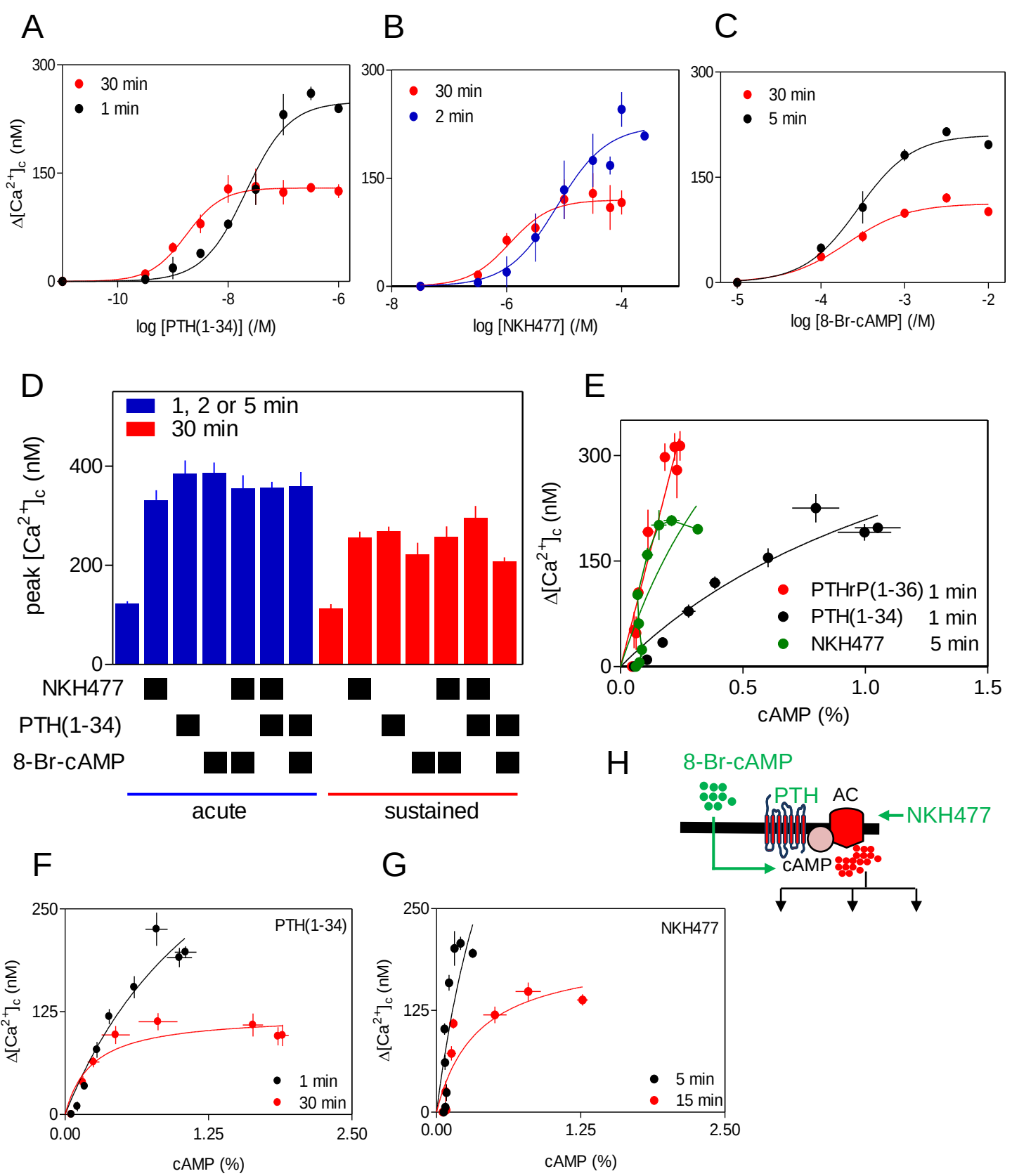
Meena *et al.*, Figure 2



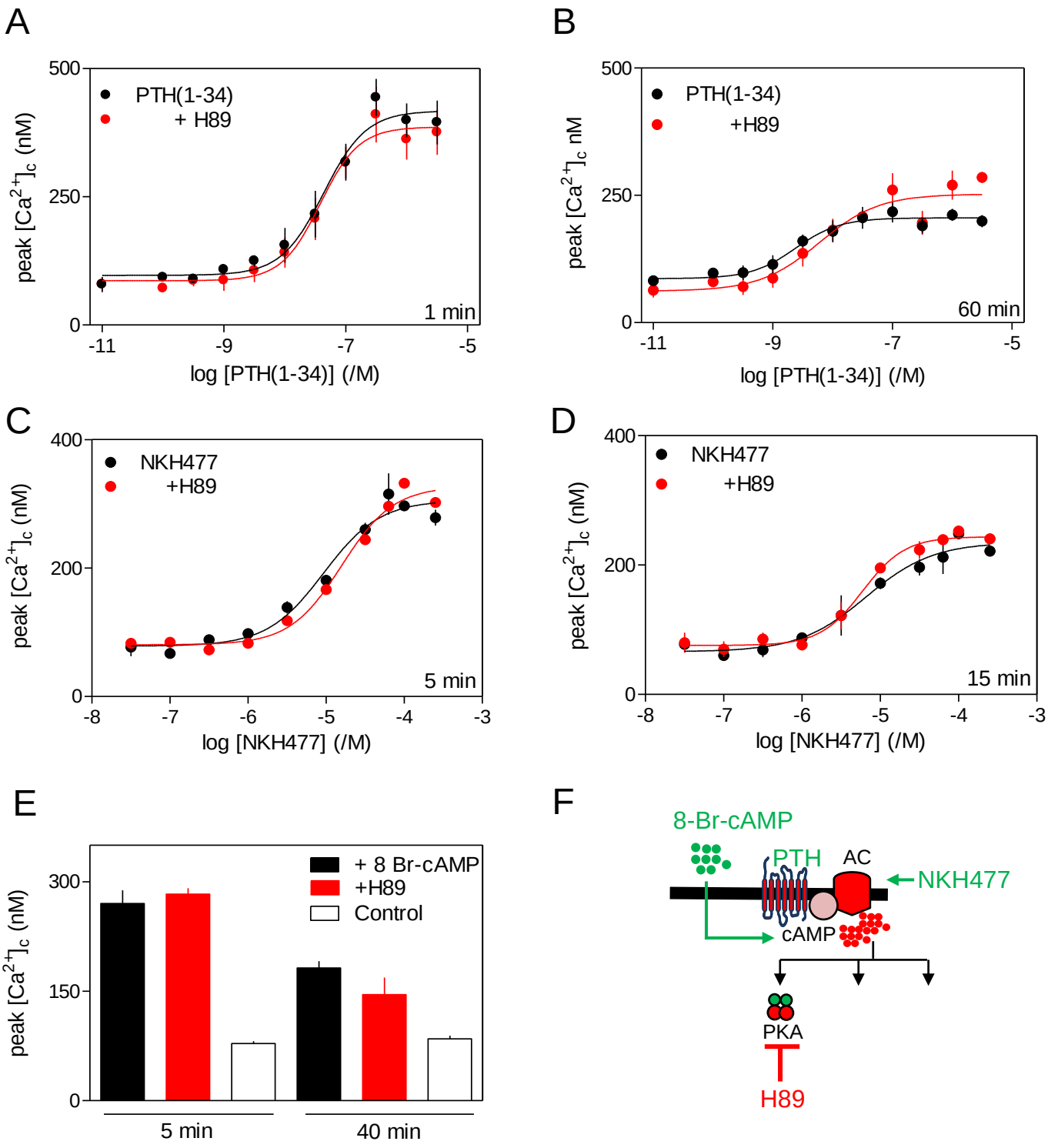
Meena *et al.* Figure 3



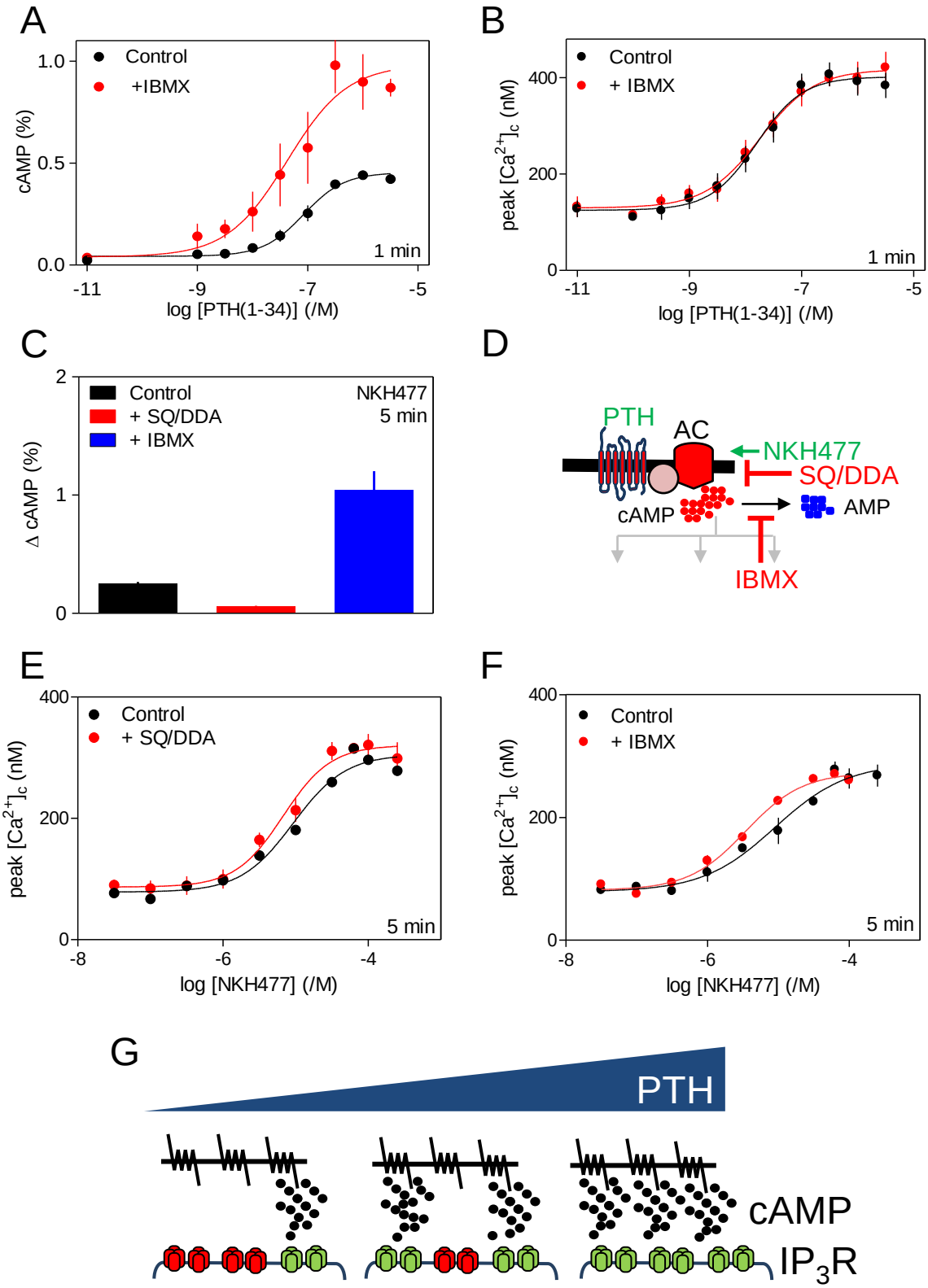
Meena *et al.*, Figure 4



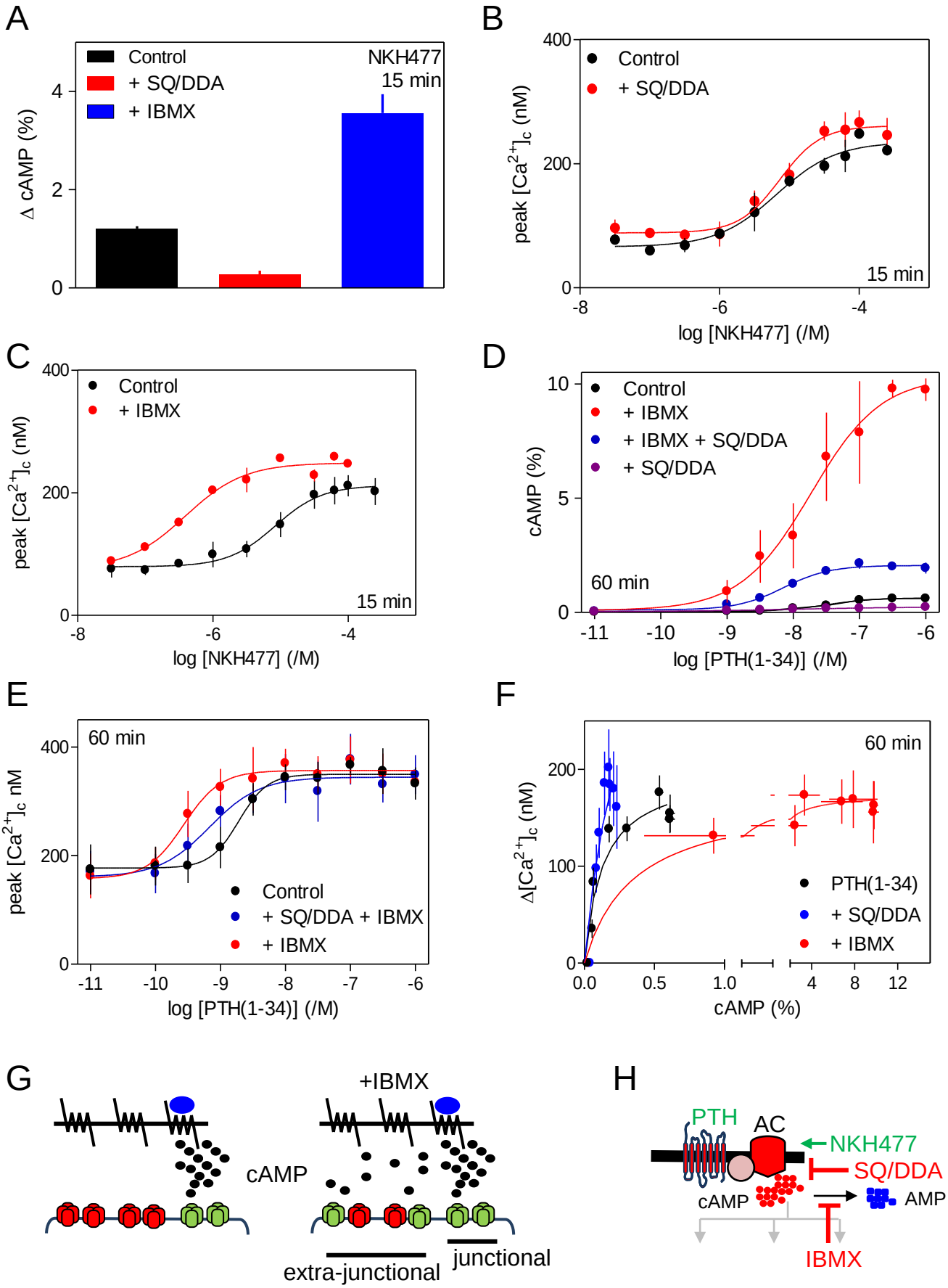
Meena *et al.*, Figure 5



Meena *et al.*, Figure 6



Meena *et al.*, Figure 7



Meena *et al.* Figure 8