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Activation phosphorylation and dephosphorylation dynamics of Ca^{2+}/Calmodulin-dependent protein kinase Iα in HeLa cells

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), a multifunctional CaM-activated protein kinase, is involved in various Ca^{2+} signaling pathways including neuronal development. Here, we characterize the phosphorylation at Thr177 (an activation Thr residue) and subsequent dephosphorylation dynamics of CaMKIα in HeLa cells upon physiological stimulation that elevates intracellular Ca^{2+}. ATP induced CaMKIα phosphorylation within 5–10 min; phosphorylation was then blocked by CaMKK inhibitor TIM-063, or by the depletion of extracellular Ca^{2+}. This was followed by gradual dephosphorylation to basal levels within 30–60 min. Histamine induced CaMKIα phosphorylation, peaking within 3–4 min; this process was abolished by treatment with either TIM-063 or intracellular Ca^{2+} chelation using BAPTA-AM and thapsigargin; however, not by extracellular Ca^{2+} depletion. CaMKIα was then rapidly dephosphorylated to basal levels within 10 min. Consistently, histamine-induced (but not ATP-induced) CaMKIα phosphorylation was absent in triple IP_3 receptor-knockout HeLa cells. Dephosphorylation of CaMKIα after ATP-induced phosphorylation was unaffected by okadaic acid or CaMK phosphatase (CaMKP, known as PPM1F) inhibitors (ANS and ANDS). We found that HeLa cell extracts contained Mg^{2+}/Mn^{2+}-dependent CaMKIα dephosphorylation activity that was insensitive to ANS and ANDS. Furthermore, co-expression of PP2Cα fully abolished ATP-, histamine-, or ionomycin-stimulated CaMKIα phosphorylation, which is consistent with *in vitro* dephosphorylation of CaMKIα at Thr177 by recombinant PP2Cα. Taken together, these results reveal that agonist-induced Ca^{2+} influx from the extracellular space or release from intracellular stores transiently activates CaMKK-CaMKIα signaling in HeLa cells, which is shut off by dephosphorylation catalyzed by PP2Cα as a promising candidate for CaMKIα phosphatase.

1. Introduction

In Ca^{2+}/calmodulin-dependent protein kinase cascade (CaMK cascade), an upstream CaMK kinase (CaMKK) phosphorylates the downstream CaMKs, including CaMKI and CaMKIV at each activation-loop Thr residue (Thr177 in CaMKIα and Thr196 in CaMKIV, respectively), resulting in significant induction of the kinase activity [1–4]. CaMKI is a member of Ca^{2+}/CaM-dependent multifunctional monomeric kinases which consists of four isoforms (CaMKIα, β, γ, δ) derived from distinct genes [5–9]. Various modes of stimulation, including depolarization (40–60 mM KCl) in PC12 cells [10] or NG108 cells [11], bicuculline in hippocampal neurons [12], as well as lipopolysaccharide in RAW 264.7 cells (murine macrophage cell line) [13], increase the concentration of intracellular Ca^{2+} leading to the phosphorylation of

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activation-loop Thr in CaMKI by CaMKK, as both kinases are Ca^{2+}/CaM-dependent enzymes. CaMKK/CaMKI cascade plays an important role in various neuronal development including axonal outgrowth [14,15], enlargement of hippocampal dendritic spines [16], activity-dependent synaptogenesis [17], activity-dependent translational initiation in cultured hippocampal neurons [12]. Furthermore, CaMKK/CaMKI cascade contributes to the regulation of actin filament organization through phosphorylation of myosin II in HeLa cells [18] and controls cell cycle progression and proliferation, suggesting its potential involvement in cancer development. CaMKI has been shown to promote G1/S phase transition by regulating cyclin D1 expression in breast cancer cells [19]. The CaMKKβ/2-CaMKIα-CREB axis was recently shown to drive castration-resistant prostate cancer growth by regulating cholesterol metabolism [20]. In contrast to the activation of CaMKI by phosphorylation with CaMKK, the inactivation mechanism of CaMKI by dephosphorylation in cells is not fully understood. For example, it is unclear which phosphatase is responsible for reversibly inactivating CaMKIα *in vivo*, although PP2A (PPP2CA) [21] and PP2C family including CaMK phosphatase (CaMKP also known as POPX2 or PPM1F) [22,23] have been shown to dephosphorylate and inactivate CaMKIα *in vitro*. In the present study, we characterized the dynamics of CaMKK-mediated phosphorylation (activation) after physiological stimulation and subsequent dephosphorylation (inactivation) of CaMKIα in HeLa cells using pharmacological and biochemical approaches.

2. Materials and methods

2.1. Materials

An expression plasmid for HA-rat CaMKIα (pME-HA-CaMKIα) was constructed using the pME18s-HA vector. GST-CaMKIα 1–293, Lys49Glu (K49E) was expressed in *Escherichia coli* (JM109) and purified as previously described [24]. GST-rat CaMKKβ/2 were expressed in *E. coli* BL21 Star (DE3) and purified using glutathione-sepharose chromatography (Cytiva, Uppsala, Sweden) [25]. CaMKKβ/2 was prepared as follows: purified GST-CaMKKβ/2 (142 μg) was incubated overnight with 2 units of PreScission protease (Cytiva) at 4 °C to cleave GST followed by removing GST and uncleaved enzyme with glutathione-sepharose. Phospho-GST-CaMKIα 1–293, K49E was prepared as follows: GST-CaMKIα 1–293, K49E (1.4 mg) was phosphorylated with CaMKKβ/2 in a solution containing 100 mM HEPES (pH 7.5), 20 mM $Mg(Ac)_2$, 2 mM DTT, and 2 mM ATP overnight at 30 °C, followed by purification with glutathione-sepharose chromatography and a purified sample was dialyzed against 150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT, and 1 mM EDTA. Anti-phospho-CaMKIα at Thr177 monoclonal antibody (clone 9H8) was previously generated [26]. Recombinant human PP2Cα (NP_066283.1) was expressed in *E. coli* BL21 (DE3) and purified as recently described [27]. Expression plasmid for HA-tagged human PP2Cα (residues 2–382, pME-HA-PP2Cα) was constructed by PCR using pGEX-KG-PreS-PP2Cα [27] as a template and the primers 5′-GGAGCATTTTTAGACAAGCCAAAGATGG-3′ and 5′-TTACCACATATCATCTGTTGATGTAGAG-3′ with PrimeSTAR HS (TaKaRa Bio Inc., Kusatsu, Japan), followed by ligation into the EcoRV site in pME18s-HA vector. Anti-HA (12CA5) antibody and an anti-GST antibody (#27457701V) were purchased from Roche Applied Sciences (Indianapolis, IN, USA) and Invitrogen (Rockford, IL, USA), respectively. A CaMKK inhibitor TIM-063 (2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one) and an inactive analogue (TIM-062, 2-hydroxy-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one) were synthesized as previously described [28]. Okadaic acid and calyculin A were obtained from Wako Pure Chemical Industries (Osaka, Japan) and LC Laboratories (Woburn, MA, USA), respectively. ANS (1-amino-8-naphthol-4-sulfonic acid, #A0369) and ANDS (1-amino-8-naphthol-2,4-disulfonic acid, #A0363) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Histamine dihydrochloride (#081–03551) was purchased from FUJIFILM Wako Pure Chemical. BAPTA-AM (#126150–97–8) and thapsigargin (#67526–95–8) were obtained from Funakoshi (Tokyo, Japan) and FUJIFILM Wako Pure Chemical, respectively. All other reagents were obtained from commercial sources.

2.2. HeLa cell culture

Wild-type HeLa cells (Human cell line derived from cervical cancer, RRID: CVCL_0030) and IP_3R-null HeLa cell lines lacking all three IP_3R subtypes, IP_3R1, IP_3R2, and IP_3R3 (HeLa TKO cells) were generated by CRISPR/Cas9 genome editing using a high-fidelity Cas9 nuclease as previously described [29] and cultured in 6-well plates or 10-cm dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 in mycoplasma-free conditions.

2.3. Phosphorylation of HA-CaMKIα in HeLa cells

Expression plasmid for HA-rat CaMKIα (pME-HA-CaMKIα, 1 μg) was transfected into HeLa cells in 6-well plates using polyethylenimine "MAX" (Polysciences, Inc. Warrington, PA, USA), according to the manufacturer's protocol. After approximately 24 h of culture, cells were cultured in serum-free medium for 6 h with or without inhibitors (10 mM EGTA, 20 μM BAPTA-AM, 2 μM thapsigargin, 10 μM TIM-063, 10 μM TIM-062) and then stimulated with 1 μM ionomycin, 10 μM ATP or 10 μM histamine for the indicated time periods. Transfected cells were cultured in the absence or presence of 30 μM ANS or 30 μM ANDS for 24 h prior to stimulation. Before stimulation, the cells were cultured in serum-free medium for 6 h and then treated with 10 μM ATP for the indicated time periods. After serum depletion for 4 h, the transfected HeLa cells were cultured further in the absence or presence of 100 nM okadaic acid for 2 h without serum. The cells were then stimulated with 10 μM ATP for the indicated time periods. Stimulation was terminated by addition of 200 μL of 1× SDS-PAGE sample buffer, followed by immunoblot analysis with anti-pThr177 antibody or anti-HA antibody.

2.4. In vitro dephosphorylation of CaMKIα

HeLa cells cultured in a 10-cm dish were lysed with 500 μL of ice-cold lysis buffer containing (100 mM Tris–HCl pH7.5, 0.2 mM EGTA, 0.2 mM PMSF, 0.01% Tween 20, 2 mM DTT) and then centrifuged at 15,000 rpm for 10 min to obtain supernatant (HeLa cell extracts) which was stored at -80 °C until use for the dephosphorylation assay. Phospho-GST-CaMKIα 1–293, K49E (8.3 μg/mL) was incubated with either lysis buffer, HeLa cell extracts (0.3 mg/mL) or recombinant PP2Cα (0.3–191 nM) in a solution containing 100 mM Tris–HCl pH 7.5, 0.2 mM EGTA, 0.01% Tween 20, 2 mM DTT in the presence of 5 mM $MgCl_2$ and 2 mM $MnCl_2$ or 1 mM EDTA with or without protein phosphatase inhibitors at 30 °C for the indicated time periods. After terminating the reaction by addition of 2× SDS-PAGE buffer, the samples were subjected to SDS-10% PAGE, followed by immunoblot analysis with an anti-phospho-CaMKIα (at Thr177) antibody and an anti-GST antibody.

2.5. Phosphorylation of HA-CaMKIα in HeLa cells co-expressing with HA-PP2Cα

Expression plasmids encoding HA-tagged rat CaMKIα (pME-HA-CaMKIα, 0.5 μg) and HA-tagged PP2Cα (pME-HA-PP2Cα, 0.5 μg) were co-transfected into HeLa cells cultured in 6-well plates using polyethylenimine "MAX" (Polysciences, Inc., Warrington, PA), according to the manufacturer's instructions. At approximately 24 h post-transfection, the cells were incubated in serum-free medium for 6 h and subsequently stimulated with either 10 μM ATP or 1 μM ionomycin for the indicated time periods. Stimulation was terminated by the addition of 200 μL of 1× SDS-PAGE sample buffer. Cell lysates were then subjected to immunoblot analysis using an anti-phospho-CaMKIα (at Thr177) antibody or anti-HA antibody. The expression of HA-CaMKIα

and HA-PP2Cα was verified by their distinct electrophoretic mobilities.

2.6. Other methods

Immunoblot analysis was performed with the indicated primary antibodies and secondary horseradish peroxidase-conjugated anti-mouse IgG (#NA931V, GE Healthcare), anti-rabbit IgG (#NA934V, GE Healthcare) or anti-goat IgG (#6420–05, SouthernBiotech, Birmingham, AL, USA). A chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA, USA) was used for signal detection of the immunoblots. The phosphorylation level of CaMKIα at Thr177 was quantified by densitometric scanning of the immunoblot using ImageJ software [30] and expressed as phospho-CaMKIα/total CaMKIα. The protein concentration of the samples was estimated using Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as the standard. Student's *t* tests were used to evaluate the statistical significance of two-group comparisons. Probability (p) values < 0.05 were considered statistically significant. Data are representative of at least three independent experiments.

3. Results

3.1. Phosphorylation and dephosphorylation dynamics of CaMKIα at Thr177 in HeLa cells by ATP and histamine stimulation

The degree to which CaMKI is activated depends on the phosphorylation of its activation loop Thr (Thr177) in CaMKIα [1–4]. To investigate the activation phosphorylation and dephosphorylation dynamics of CaMKIα in HeLa cells, we performed assays with increasing concentration of Ca^{2+} and assessed the degree of phosphorylation at Thr177 in exogenously expressed HA-tagged CaMKIα (HA-CaMKIα). We chose two physiological ligands to raise intracellular Ca^{2+} levels: ATP and histamine. ATP stimulates purine receptors, P2X receptors (ATP-gated cation channels) and P2Y receptors (G-protein coupled receptors), which induces Ca^{2+} influx from the extracellular space as well as intracellular Ca^{2+} storage (e.g., in the endoplasmic reticulum) [31,32]. Histamine induces activation of $G\alpha_q$/11-proteins, thus stimulating the activity of phospholipase C (PLC), which generates inositol-1,4,5-trisphosphate (IP_3), which in turn releases Ca^{2+} from intracellular stores through IP_3 receptors [33]. Based on previous reports showing that an EC_{50} value of ATP for Ca^{2+} mobilization in HeLa cells is 5.1 μM [34] and that 10 μM histamine successfully mediates IP_3-induced Ca^{2+} release in HeLa cells [29], we stimulated HeLa cells with either 10 μM ATP or 10 μM histamine. We found that ATP stimulation at 10 μM rapidly induced CaMKIα phosphorylation with a peak reached within 5–10 min (Fig. 1A). The continued presence of the agonist for extended periods of time resulted in a gradual dephosphorylation to the basal level within 60 min. ATP-induced transient phosphorylation of CaMKIα is driven by rapid influx and subsequent efflux of Ca^{2+}; conversely, ionomycin stimulation results in sustained CaMKIα phosphorylation without its dephosphorylation up to 60 min post-stimulation (Fig. 1B). Histamine (10 μM) stimulation also induced phosphorylation of CaMKIα (with a peak reached within 3–4 min); however, rapidly reduced the phosphorylation back to the basal level within <10 min (Fig. 1C); thus, the action of histamine occurs in a much narrower timeframe than in the case with ATP stimulation (Fig. 1A).

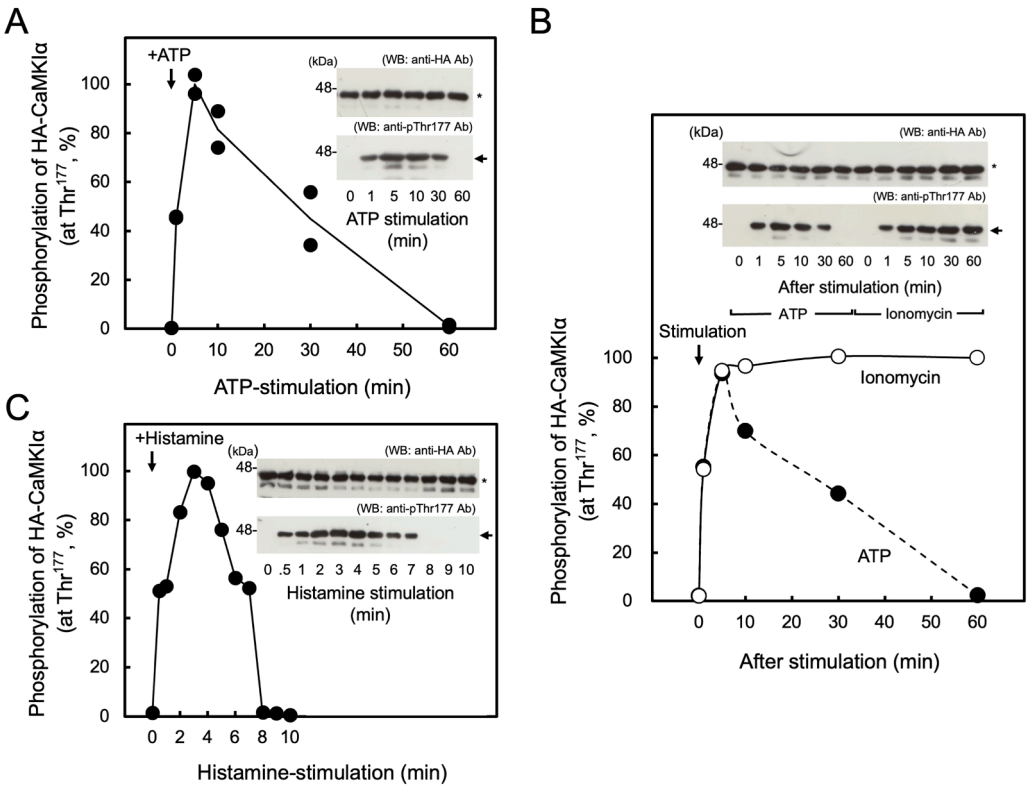

Fig. 1. Agonist-induced phosphorylation and subsequent dephosphorylation of CaMKIα in HeLa cells. HeLa cells were transfected with an expression plasmid for HA-CaMKIα and, after serum depletion for 6 h, cells were stimulated with 10 μM ATP (A and B, closed circle), 1 μM ionomycin (B, open circle) or 10 μM histamine (C, closed circle) for the indicated time periods as described in "Materials and methods." Cell lysates were subjected to immunoblot analysis using anti-HA antibody (*inset upper panels*) and anti-pCaMKIα (at Thr177) antibody (*inset lower panels*). The phosphorylation level of HA-CaMKIα at Thr177 was quantified based on immunoreactive signals on the blots (phospho-HA-CaMKIα/total HA-CaMKIα), expressed as a percentage of the average value of 5 min–ATP stimulation (A), as a percentage of the value of 30 min–ionomycin stimulation (B), or as a percentage of the value of 3 min–histamine stimulation (C) of HA-CaMKIα phosphorylation and plotted. Asterisks and arrows in *inset panels* indicate HA-CaMKIα and phospho-HA-CaMKIα at Thr177, respectively. The molecular masses in kilodaltons (kDa) are indicated on the left (*inset panels*).

3.2. Characterization of CaMKIa phosphorylation in HeLa cells

To characterize agonist-induced CaMKIα phosphorylation in HeLa cells, we performed a number of pharmacological assays (Fig. 2). We found that the induction of HA-CaMKIα phosphorylation in transfected HeLa cells by either ATP (Fig. 2A) or histamine (Fig. 2B) stimulation, was completely abolished by treatment with a CaMKK inhibitor, TIM-063 (10 μM) but not with an inactive analogue (TIM-062) [28]. These results indicates that CaMKK is responsible for the phosphorylation of CaMKIα in HeLa cells as a response to either ATP or histamine stimulation. ATP-induced CaMKIα phosphorylation in HeLa cells was completely abolished by depletion of extracellular Ca^{2+} with EGTA treatment but not by intracellular Ca^{2+} chelator, BAPTA-AM and thapsigargin (SERCA inhibitor) (Fig. 2A). This result indicates that ATP drives CaMKK activation by stimulating Ca^{2+} channels associated P2X receptors to induce the intracellular Ca^{2+} concentrations, rather than through P2Y receptors coupled with inositol turnover. In contrast, histamine-induced CaMKIα phosphorylation in HeLa cells was not affected by EGTA treatment but suppressed by treatment with BAPTA-AM and thapsigargin (Fig. 2B), confirming that histamine receptor-mediated inositol turnover increases the Ca^{2+} concentration from intracellular Ca^{2+}-stores (ER) to activate CaMKK. These results were confirmed by the fact that histamine-induced CaMKIα phosphorylation was not observed in HeLa cells lacking three IP_3R subtypes, IP_3R1, IP_3R2, and IP_3R3 (HeLa TKO cells [29]) (Fig. 2C). However, ATP-induced CaMKIα phosphorylation was not affected in TKO cells.

3.3. Pharmacological characterization of CaMKIα dephosphorylation in HeLa cells

As shown in Figs. 1A and 1C, CaMKIα was rapidly dephosphorylated to the basal level within 30–60 min and 10 min after ATP and histamine stimulation, respectively. To characterize the mechanism of dephosphorylation of CaMKIα in cells, we attempted to identify the protein phosphatase(s) responsible for CaMKIα dephosphorylation in HeLa cells. PP2A (PPP2CA) [21] and CaMK phosphatase (CaMKP, also known as POPX2 or PPM1F) [22] have been shown to dephosphorylate and inactivate CaMKI *in vitro*. Therefore, we treated ATP-stimulated HeLa cells in the absence or presence of 30 μM CaMKP inhibitors (ANS and ANDS [35], Figs. 3A and 3B) or 100 nM okadaic acid (OA, Fig. 3C), to test whether these phosphatase inhibitors can impair the CaMKIα dephosphorylation in HeLa cells. Treatment with any of the inhibitors (ANS, ANDS, and OA) did not appear to affect the level of CaMKIα phosphorylation in cells following stimulation with ATP. Using an anti-pThr antibody, we observed that OA-treatment increased global protein phosphorylation in transfected HeLa cells (see Figure S1), indicating that OA successfully suppressed target protein phosphatases in these cells.

3.4. CaMKIα phosphatase in HeLa cell extracts

To characterize the CaMKIα dephosphorylation activity in HeLa cells, we carried out an *in vitro* dephosphorylation assay using glutathione *S*-transferase (GST)-fused phospho-CaMKIα 1–293, K49E as a substrate (Fig. 4). Fig. 4A shows that HeLa cell extracts exhibit significant CaMKIα phosphatase activity in a reaction solution containing 5 mM $MgCl_2$ and 2 mM $MnCl_2$. As shown in Fig. 4B, CaMKIα phosphatase activity in HeLa cell extracts was not affected by addition of CaMKP (PPM1F) inhibitors (at concentrations: ANS 30 μM and ANDS 60 μM). This result is consistent with the findings of the cell-based assay (Figs. 3A and 3B). We confirmed that the dephosphorylation of phospho-GST-CaMKIα 1–293, K49E (at Thr177) by FLAG/GST-human CaMKP

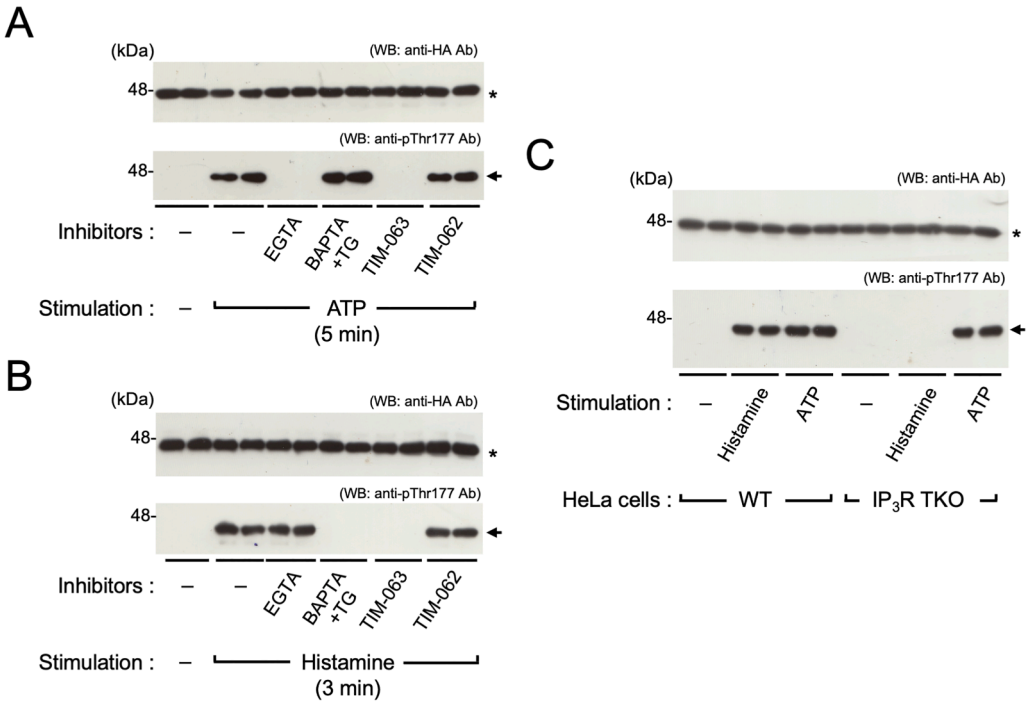

Fig. 2. Characterization of agonist-induced phosphorylation of CaMKIα in HeLa cells.
HeLa cells were transfected with an expression plasmid for HA-CaMKIα and after serum depletion in the absence (–) or presence of pharmacological reagents (10 mM EGTA, 20 μM BAPTA-AM /2 μM thapsigargin [BAPTA+TG], 10 μM TIM-063 or TIM-062) for 6 h, cells were stimulated without (–) or with 10 μM ATP for 5 min (A) or 10 μM histamine for 3 min (B) as described in "Materials and methods". (C) an expression plasmid for HA-CaMKIα was transfected into wild type (WT) or IP_3 receptor triple knockout HeLa cells (IP_3R TKO) and after serum depletion for 6 h, cells were stimulated without (–) or with 10 μM ATP for 5 min (ATP) or 10 μM histamine for 3 min (Histamine). Cell lysates were subjected to immunoblot analysis using anti-HA antibody (*upper panels*) and anti-pCaMKIα (at Thr177) antibody (*lower panels*). Asterisks and arrows indicate HA-CaMKIα and phospho-HA-CaMKIα at Thr177, respectively. The molecular masses in kilodaltons (kDa) are indicated on the left. Experiments were performed in duplicate.

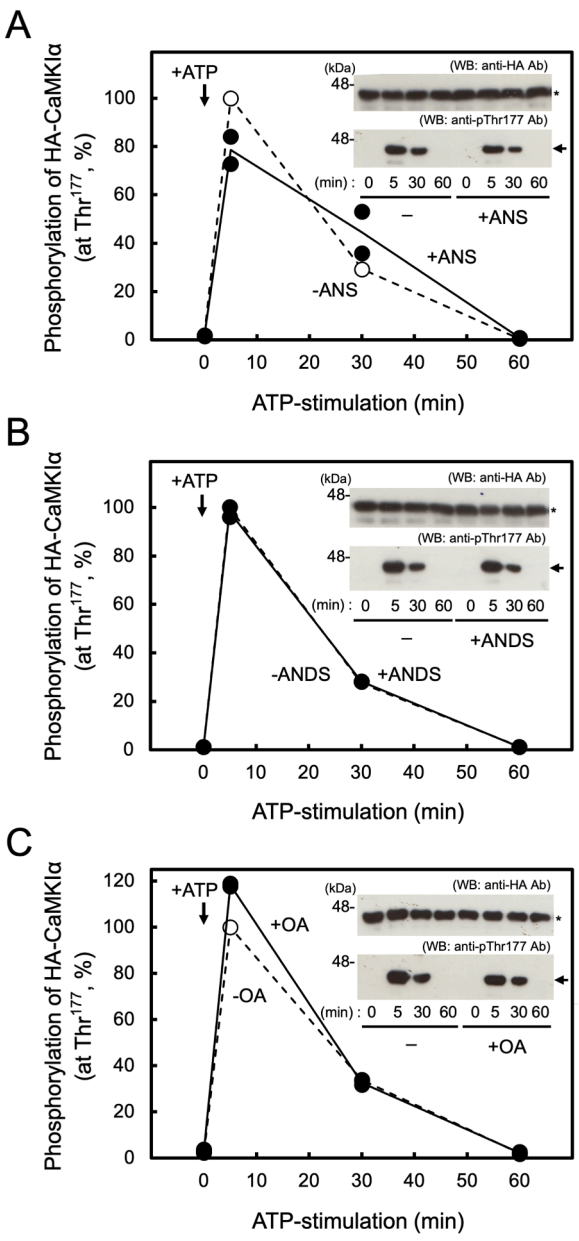

Fig. 3. Pharmacological characterization of CaMKIα dephosphorylation in HeLa cells.

HeLa cells were transfected with an expression plasmid for HA-CaMKIα and then cultured in the absence (A, B, –) or presence of 30 μM ANS (A, +ANS) or 30 μM ANDS (B, +ANDS) for 24 h prior to stimulation. The cells were cultured in serum-free medium for 6 h before stimulation and then treated with 10 μM ATP for the indicated time periods as described in "Materials and methods." After serum depletion for 4 h, the transfected HeLa cells was cultured further in the absence (C, –) or presence of 100 nM okadaic acid (C, +OA) for 2 h without serum. The cells were then stimulated with 10 μM ATP for the indicated time periods. Cell lysates were subjected to immunoblot analysis using anti-HA antibody (*inset upper panels*) and anti-pCaMKIα (at Thr177) antibody (*inset lower panels*). The phosphorylation level of HA-CaMKIα at Thr177 was quantified based on immunoreactive signals on the blots (phospho-HA-CaMKIα/total HA-CaMKIα), expressed as a percentage of the value of 5 min–stimulation in the absence of inhibitors. Asterisks and arrows in *inset panels* indicate HA-CaMKIα and phospho-HA-CaMKIα at Thr177, respectively. The molecular masses in kilodaltons (kDa) are indicated on the left (*inset panels*). Each experiment was performed in duplicate in the presence of phosphatase inhibitors, along with a single time-course experiment in the absence of inhibitors.

(PPM1F) was inhibited by the addition of either ANS (30 μM) or ANDS (60 μM) *in vitro* (see Figure S2). These results suggest that CaMKP does not exist and mediate CaMKIα dephosphorylation in HeLa cells, given that ANS and ANDS have been shown to inhibit CaMKP activity *in vitro* with IC_{50} values of 3 and 6 μM, respectively [35]. Despite the fact that the PP1/PP2A inhibitor (OA) had no effect on dephosphorylation of CaMKIα in HeLa cells after ATP stimulation (Fig. 3C), CaMKIα phosphatase activity in HeLa cell extracts was partially suppressed by relatively high concentrations of OA (200 nM) and calyculin A (CA) (20 nM) (Fig. 4C). This suggests that the HeLa cell extracts contain PP2A or PP1, which are able to dephosphorylate CaMKIα *in vitro* as shown previously [21]. However, these phosphatases are unlikely to dephosphorylate CaMKIα in the cells. Residual CaMKIα phosphatase activity (30%–50% of total activity) (Fig. 4C), which is insensitive to CA and OA, may contribute to the dephosphorylation of CaMKIα in the cells. To test whether other members of Mn^{2+}/Mg^{2+}-dependent protein phosphatases (PPM family) are involved in OA-insensitive phosphatase activity in HeLa cell extracts, we incubated phospho-GST-CaMKIα 1–293, K49E with HeLa cell extracts in the presence of 200 nM OA, together with either 1 mM EDTA or 5 mM $MnCl_2$/2 mM $MgCl_2$, for up to 120 min and examined its Thr177 phosphorylation level (Fig. 4D). In good agreement with the results shown in Fig. 4C, CaMKIα phosphatase activity in HeLa cell extracts was observed in the presence of OA and Mn^{2+}/Mg^{2+}, however it was impaired by the addition of OA and EDTA. These results suggest that OA- and CA-insensitive phosphatase is a *bona fide* CaMKIα phosphatase in HeLa cells, which is likely metal-dependent phosphatase (s) (PPM family), but not CaMKP (PPM1F).

3.5. Dephosphorylation of CaMKIα at Thr177 by PP2Cα in vitro and in HeLa cells

Next, we examined whether another member of PPM family, PP2Cα (known as PPM1A), which is insensitive to OA [36], CA [37], ANS and ANDS [35], dephosphorylates CaMKIα at Thr177. As evident from the *in vitro* assay (Fig. 5A), recombinant PP2Cα dephosphorylates CaMKIα (phospho-GST-CaMKIα 1–293, K49E) at Thr177, which is consistent with the findings of a previous study [23]. To test whether PP2Cα dephosphorylates CaMKIα in HeLa cells, we stimulated the HA-CaMKIα/HA-PP2Cα-coexpressing HeLa cells with either 10 μM ATP (Fig. 5B), 10 μM histamine (Fig. 5C) or 1 μM ionomycin (Fig. 5D) and analyzed the phosphorylation of HA-CaMKIα. In contrast to HA-CaMKIα-expressing cells, where ATP or histamine induced transient CaMKIα phosphorylation and subsequent dephosphorylation in a manner similar to Figs. 1 and 3, co-expression of HA-PP2Cα completely abolished ATP- and histamine-stimulated CaMKIα phosphorylation. In a similar manner to ATP or histamine stimulation, ionomycin treatment induced sustained phosphorylation of HA-CaMKIα and co-expression of HA-PP2Cα completely abolished ionomycin-induced CaMKIα phosphorylation (Fig. 5D). We confirmed that reducing the amount of HA-PP2Cα in HeLa cells restored CaMKIα phosphorylation induced by ATP stimulation (Figure S3). These results clearly indicate that PP2Cα is capable of dephosphorylating CaMKIα at Thr177 *in vitro* and in cells.

4. Discussion

The CaMKK/CaMKI activation cascade has been shown to contribute to a wide variety of calcium signaling pathways in many cell types, including the regulation of neuronal functions and cancer development [10–17,19,20]. Unlike the activation mechanism of CaMKI, which involves the phosphorylation of an activation Thr residue by CaMKK, the inactivation mechanism by dephosphorylation remains unclear. Here, we characterized the phosphorylation of CaMKIα at Thr177, which was induced by stimulation with the physiological agonists ATP and histamine, and the subsequent dephosphorylation of CaMKIα in HeLa cells. The dynamics of the CaMKK- and phosphatase-mediated phosphorylation and dephosphorylation of the activation loop Thr residue (Thr177)

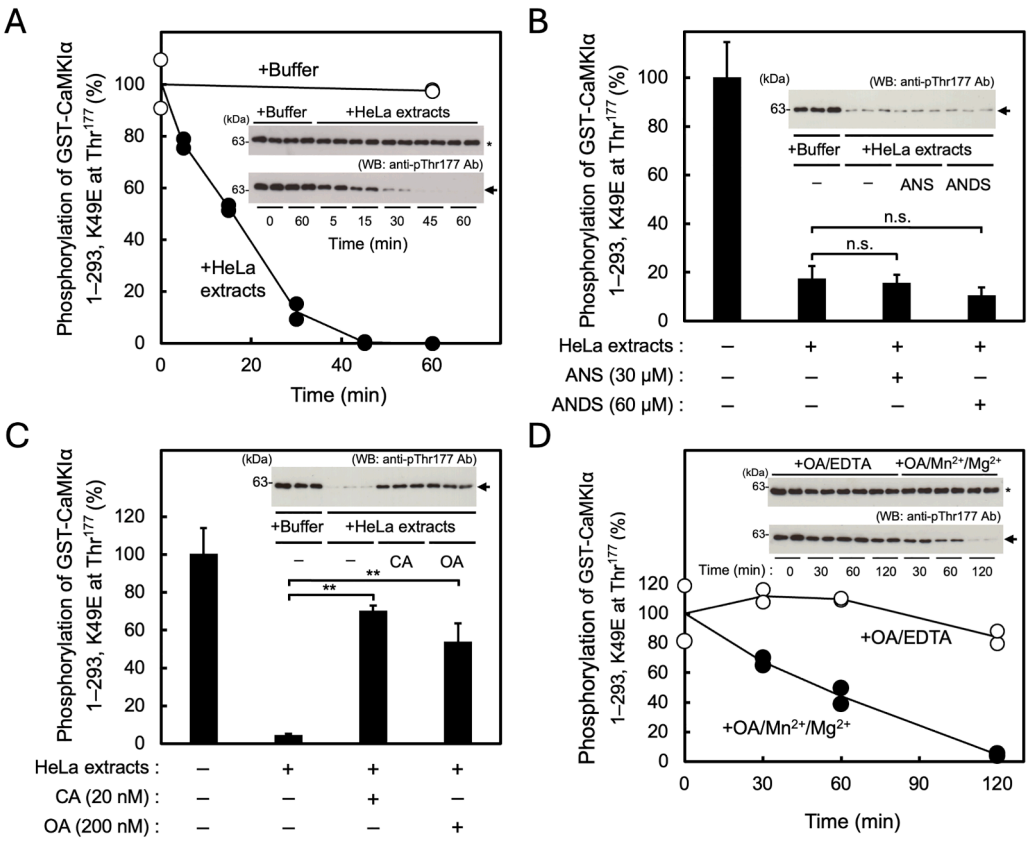

Fig. 4. CaMKIα-phosphatase activity in HeLa extracts.
(A) Phospho-GST-CaMKIα 1–293, K49E was incubated with HeLa cell extracts (0.3 mg/mL, closed circle, +HeLa extracts) or lysis buffer (open circle, +Buffer) at 30 °C for the indicated time periods as described in "Materials and methods." (B and C) Phospho-GST-CaMKIα 1–293, K49E was incubated with HeLa cell extracts (0.3 mg/mL, +, +HeLa extracts) or lysis buffer (–, +Buffer) for 60 min in the absence (–) or presence of ANS (B, 30 μM), ANDS (B, 60 μM), calyculin A (C, *CA*, 20 nM) or okadaic acid (C, OA, 200 nM). (D) Phospho-GST-CaMKIα 1–293, K49E was incubated with HeLa cell extracts (0.3 mg/mL) in the presence of 200 nM okadaic acid and 1 mM EDTA (open circle, +OA/EDTA) or 200 nM okadaic acid and 5 mM $MnCl_2$/2 mM $MgCl_2$ (closed circle, +OA/Mn^{2+}/Mg^{2+}) at 30 °C for the indicated time periods. After terminating the reaction, the samples were subjected to immunoblot analysis using anti-GST antibody (A and D, *inset upper panels*) and anti-pCaMKIα (at Thr177) antibody (*inset panels*). The phosphorylation level of GST-CaMKIα 1–293, K49E at Thr177 was quantified based on immunoreactive signals on the blots (phospho-GST-CaMKIα 1–293, K49E/total GST-CaMKIα 1–293, K49E), expressed as a percentage of the average value in the absence of HeLa cell extracts (A, B, C) or at the 0 min time point (D). Asterisks and arrows in *inset panels* indicate GST-CaMKIα 1–293, K49E and phospho-GST-CaMKIα 1–293, K49E at Thr177, respectively. The molecular masses in kilodaltons (kDa) are indicated on the left (*inset panels*). The assays (A and D) were performed in duplicate for each time point, and the results (B and C) represent the mean ± SD of triplicate assays. Statistical differences are marked with either n.s. (statistically not significant) (B) or double asterisks, $p < 0.05$ (C) *versus* phosphorylation of GST-CaMKIα 1–293, K49E treated with HeLa cell extracts without inhibitors.

in CaMKIα were demonstrated to be agonist-dependent in HeLa cells. ATP- and histamine-stimulation rapidly induced CaMKIα phosphorylation with a peak reached within 3–5 min through Ca^{2+} influx from extracellular space and intracellular Ca^{2+} store, respectively. In HeLa cells, the increase in CaMKIα phosphorylation induced by ATP appears to be caused primarily by Ca^{2+} influx mediated by P2X Ca^{2+} channels rather than via P2Y receptors (Fig. 6). It could be due to the fact that ATP-induced CaMKIα phosphorylation was completely abolished by depletion of extracellular Ca^{2+}, whereas it was observed in cells treated with thapsigargin/BAPTA-AM or in triple IP_3 receptor-knockout cells (Fig. 2). This finding is supported by a report indicating that HeLa cells express a functional $P2X_7$ receptor [38]. On the other hand, consistent with a previous report [39], our results also demonstrate that histamine-induced phosphorylation of CaMKIα is mediated by G-protein-coupled receptors via IP_3-mediated Ca^{2+} mobilization from intracellular Ca^{2+} stores such as ER (Figs. 2C and 6). Conversely, the dephosphorylation of CaMKIα proceeds more rapidly after histamine stimulation than after ATP-stimulation (Fig. 1). The distinct temporal profiles of CaMKIα phosphorylation at Thr177 and dephosphorylation in HeLa cells observed in this study—relatively prolonged Thr177 phosphorylation by ATP stimulation and transient phosphorylation via histamine—may reflect a sophisticated biological strategy. This system may allow epithelial cells to discern the nature and urgency of extracellular stimuli, thereby adjusting the duration of downstream cellular responses such as tissue repair or immune cell recruitment. Regarding the dephosphorylation mechanism of CaMKK/CaMKIα signaling, pharmacological approaches using phosphatase inhibitors suggested that CaMKP (PPM1F, [22]), PP2A (PPP2CA, [21]) known as CaMKIα phosphatases and PP1 (PPP1C) were not responsible for CaMKIα dephosphorylation in HeLa cells after agonist stimulation (Fig. 3). *In vitro* phosphatase assay and overexpression experiments in HeLa cells revealed that PP2Cα may contribute to the direct dephosphorylation of CaMKIα (Figs. 5 and 6). Abrogation of ATP- and histamine-induced phosphorylation of CaMKIα by PP2Cα co-expression in HeLa cells is unlikely due to the effect on the P2X receptor and histamine receptor, respectively, considering that ionomycin-induced sustained phosphorylation of CaMKIα was also completely abolished by co-expression of PP2Cα. However, given that the PP2C (metal-dependent protein phosphatase, PPM) family comprises 20 different isoforms [40], further experiments using gene silencing techniques are required to specify and identify *bona fide* endogenous CaMKIα-phosphatase or determine whether the phosphatases act redundantly to dephosphorylate CaMKIα in the cells, which may be depending on cell types. In this study, we used the overexpression method to analyze the precise

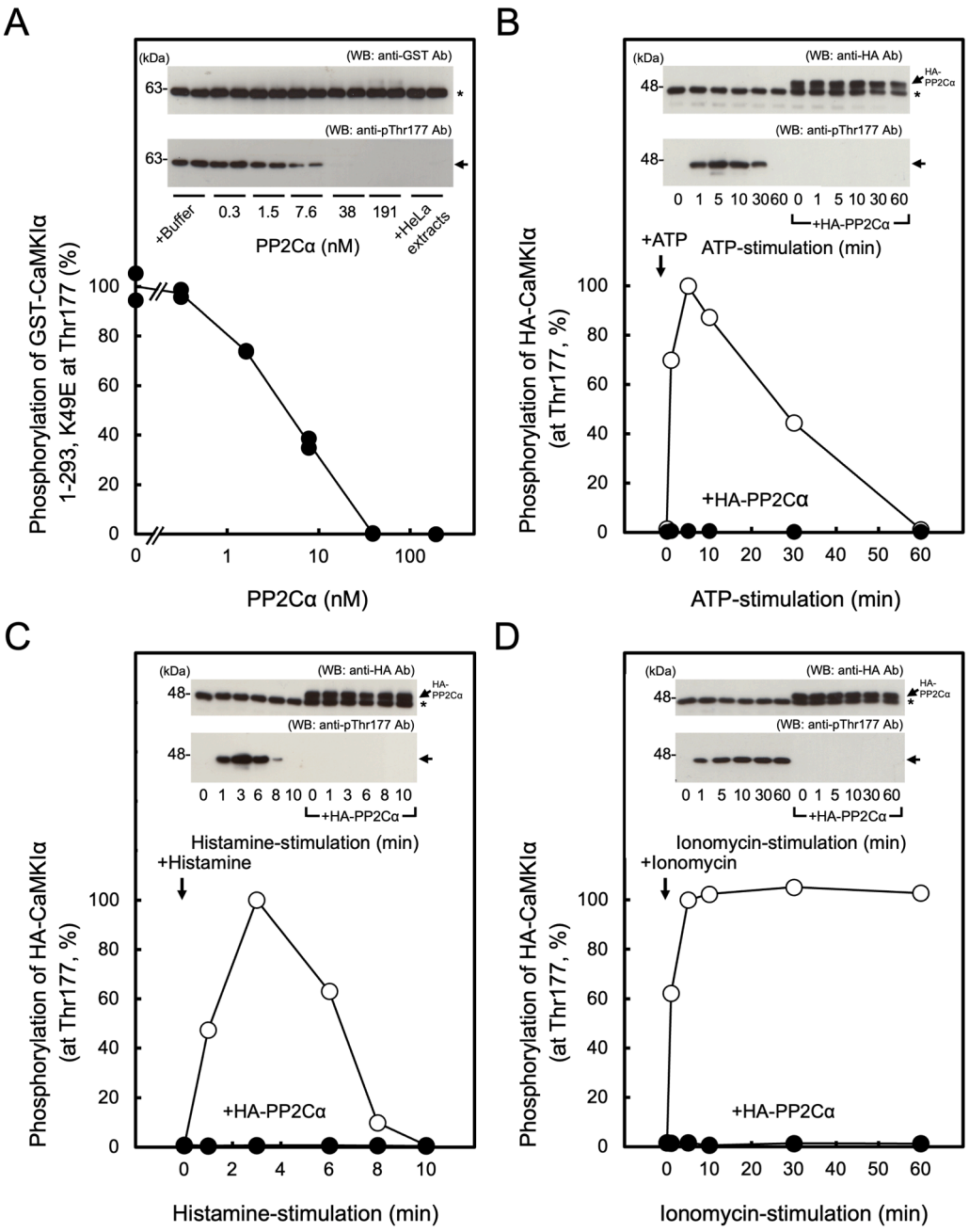

Fig. 5. PP2Cα dephosphorylates CaMKIα at Thr177 *in vitro* and in HeLa cells.
(A) Phospho-GST-CaMKIα 1–293, K49E was incubated with or without (+Buffer) the indicated concentrations (0.3–191 nM) of recombinant human PP2Cα for 60 min at 30 °C as described in "Materials and methods." After terminating the reaction, the samples were subjected to immunoblot analysis using anti-GST antibody (*inset upper panel*) and anti-pCaMKIα (at Thr177) antibody (*inset lower panel*). The phosphorylation level of GST-CaMKIα 1–293, K49E at Thr177 was quantified based on immunoreactive signals on the blots (phospho-GST-CaMKIα 1–293, K49E/total GST-CaMKIα 1–293, K49E), expressed as a percentage of the average value without PP2Cα. Experiments were performed in duplicate. (B, C, D) HeLa cells were transfected with an expression plasmid for HA-CaMKIα in the presence (closed circle) or absence (open circle) of an expression plasmid for HA-PP2Cα, and after serum depletion for 6 h, cells were stimulated with either 10 μM ATP (B), 10 μM histamine (C) or 1 μM ionomycin (D) for the indicated time periods. Cell lysates were subjected to immunoblot analysis using anti-HA antibody (*inset upper panels*) and anti-pCaMKIα (at Thr177) antibody (*inset lower panels*). The phosphorylation level of HA-CaMKIα at Thr177 was quantified based on immunoreactive signals on the blots (phospho-HA-CaMKIα/total HA-CaMKIα), expressed as a percentage of the value of 5 min– (B, D) or 3 min– (C) HA-CaMKIα phosphorylation in the absence of HA-PP2Cα and plotted. Asterisks in *inset upper panels* indicate GST-CaMKIα 1–293, K49E (A), HA-CaMKIα (B, C, D) and arrows in *inset lower panels* indicate phospho-Thr177. Arrows in *inset upper panels* (B, C, D) indicate HA-PP2Cα. The molecular masses in kilodaltons (kDa) are indicated on the left (*inset panels*).

phosphorylation/dephosphorylation kinetics of CaMKIα, which forces a cell to produce the kinase at artificially high levels. Therefore, we need to confirm the agonist-induced phosphorylation/dephosphorylation profile of the endogenous CaMKIs including their various isoforms [5–9].

5. Data statement

All relevant data are included in the article and supporting information. Information and reasonable requests for resources and reagents are available from the corresponding authors on reasonable request.

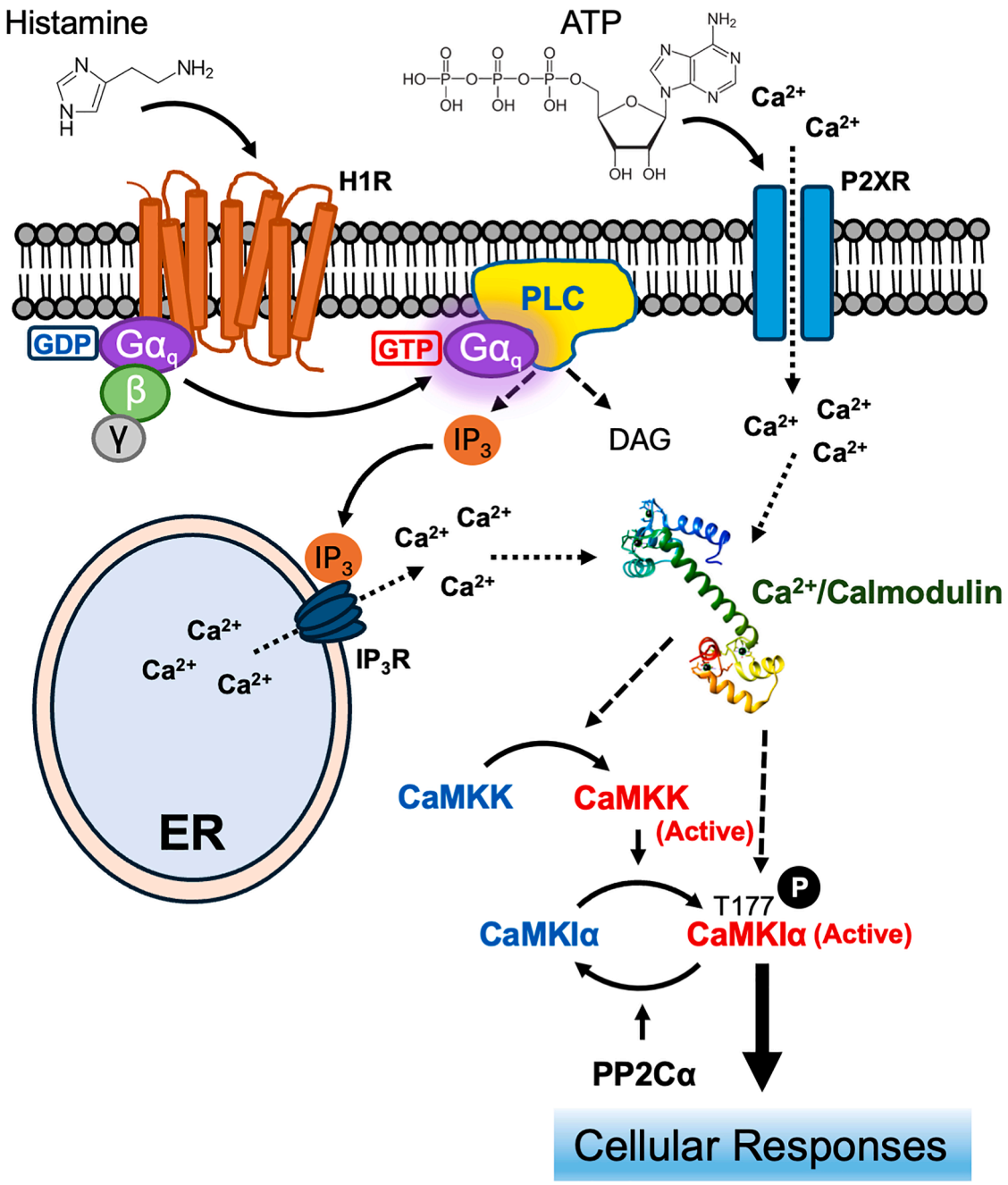

Fig. 6. Schematic representation of ATP and histamine-stimulated CaMKK/CaMKIα signaling cascade.
The molecular structure of CaM used in this model was obtained from the Protein Data Bank (PDB) entry 1UP5 [41] visualized using the UCSF Chimera [42]. H1R, histamine H1 receptor; P2XR, P2X purinoreceptor; $G\alpha_q/\beta/\gamma$, G_q-protein $\alpha/\beta/\gamma$ subunit; CaMKK, Ca^{2+}/CaM-dependent protein kinase; CaMKIα, Ca^{2+}/CaM-dependent protein kinase Iα; PP2Cα, protein phosphatase 2Cα; PLC, phospholipase C; ER, endoplasmic reticulum; IP_3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; T, Thr residue. P in a black circle indicates phosphorylation.

CRediT authorship contribution statement

Yerun Chen: Writing – original draft, Visualization, Investigation, Data curation. **Masao Miki:** Writing – review & editing, Investigation, Data curation. **Masaki Magari:** Writing – review & editing, Supervision. **Satomi Ohtsuka:** Supervision, Investigation, Funding acquisition, Data curation. **Masumi Eto:** Supervision, Resources. **Atsuhiko Ishida:** Supervision, Resources. **Futoshi Suizu:** Writing – review & editing, Supervision. **Akihiro Mizutani:** Supervision, Resources. **Hideaki Ando:** Resources. **Katsuhiko Mikoshiba:** Supervision, Resources. **Hiroshi Tokumitsu:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.ceca.2026.103183.

Data availability

Data will be made available on request.

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