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Correspondence and requests for materials should be addressed to H.K. (hidekuwayama@biol.tsukuba.ac.jp)

Arachidonic Acid Enhances Caffeine-Induced Cell Death via Caspase-Independent Cell Death

Hidekazu Kuwayama

Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Tennodai, 1-1-1, Ibaraki 305-8572, Japan.

Caffeine is a globally consumed psychostimulant but can be fatal to cells at overdose exposures. Although caspase-dependent apoptosis plays a role in caffeine-induced cell death, the responsible intracellular signalling cascade remains incompletely understood. The cellular slime mould, *Dictyostelium discoideum*, does not possess caspase-dependent apoptotic machinery. Here, we observed that ablation of *D. discoideum plaA*, which encodes a phospholipase A2 (PLA_2) homolog, leads to a decreased rate of cell death under high caffeine concentrations and to enhanced cell death with the addition of arachidonic acid. Moreover, the inhibition of PLA_2 activity lead to a recovery of the survival rate in caspase-inhibited Hela cervical carcinoma cells under high caffeine concentrations, indicating that caffeine-induced cell death is enhanced via PLA_2-dependent signalling. Our results indicate that arachidonic acid may be a general second messenger that negatively regulates caffeine tolerance via a caspase-independent cell death cascade, which leads to multiple effects in eukaryotic cells.

The moderate ingestion of caffeine has a psychostimulant effect on the body. The molecular mechanism has been shown to involve caffeine binding as an antagonist[1] to cell surface adenosine receptors and/or blocking the binding site of cyclic AMP (cAMP) phosphodiesterase (PDE) and thereby decreasing its activity[2]. Further, excessive levels of caffeine, or caffeine overdose, can result in various systemic symptoms known as caffeine intoxication. At the cellular level, a high dose of caffeine results in various responses, including cell death, delays in the cell cycle, the impairment of DNA repair and recombination, and perturbed intracellular calcium homeostasis[3]. In the case of cell death, caffeine has been shown to exert its fatal effect by evoking an apoptosis cascade that involves PI3K/Akt/mTOR signalling[4,5]. However, all of the potential mechanisms by which a caffeine overdose results in cell death remain to be clarified.

Dictyostelium discoideum, which is known as the cellular slime mould, is a social amoeba that feeds on bacteria and grows by division until the bacteria are consumed. Upon starvation, the amoebae start to develop and form a fruiting body consisting of a mass of dormant spore cells suspended on dead stalk cells. This eukaryote has been utilized as a model organism to study the cellular function of genes. In this report, we show that caffeine triggers the activation of phospholipase A2 (PLA_2) and that arachidonic acid (AA), the product of this enzyme, acts as a negative regulator of cell tolerance in this microorganism, as well as in mammalian culture cells.

Results

Caffeine-induced PLA_2 activation enhances cell death via a caspase-independent cell death pathway. High caffeine concentrations are cytotoxic to *D. discoideum* (Fig. 1a). However, we found that a mutant strain lacking *plaA*, which encodes a calcium-independent patatin-like phospholipase A_2 γ, showed less sensitivity to high caffeine concentrations (Fig. 1b). Since AA is a primary product of PLA_2, we tested the effect of AA on caffeine tolerance in *plaA* mutants and observed that the addition of 20 μM AA significantly reduced caffeine tolerance in the null mutant, as well as in wild-type cells (Fig. 1c, d). Furthermore, AA production could be measured in wild-type cells upon stimulation with 20 mM caffeine, whereas no measurable AA was observed in *plaA* mutant cells (Fig. 1e), indicating that *plaA* is the gene responsible for AA production by caffeine stimulation. These observations indicate that caffeine activates *D. discoideum* PLA_2 and that the resultant AA leads to the suppression of survival under high caffeine concentrations.

Next, we tested the tolerance to caffeine in Hela cervical carcinoma cells, a mammalian cell line culture. Hela cells were found to be more tolerant of caffeine than *D. discoideum* cells. Nonetheless, the survival rate of Hela cells gradually decreased with 25–50 mM caffeine treatment over the course of 24 h (Fig. 2a). Bromoenol lactone

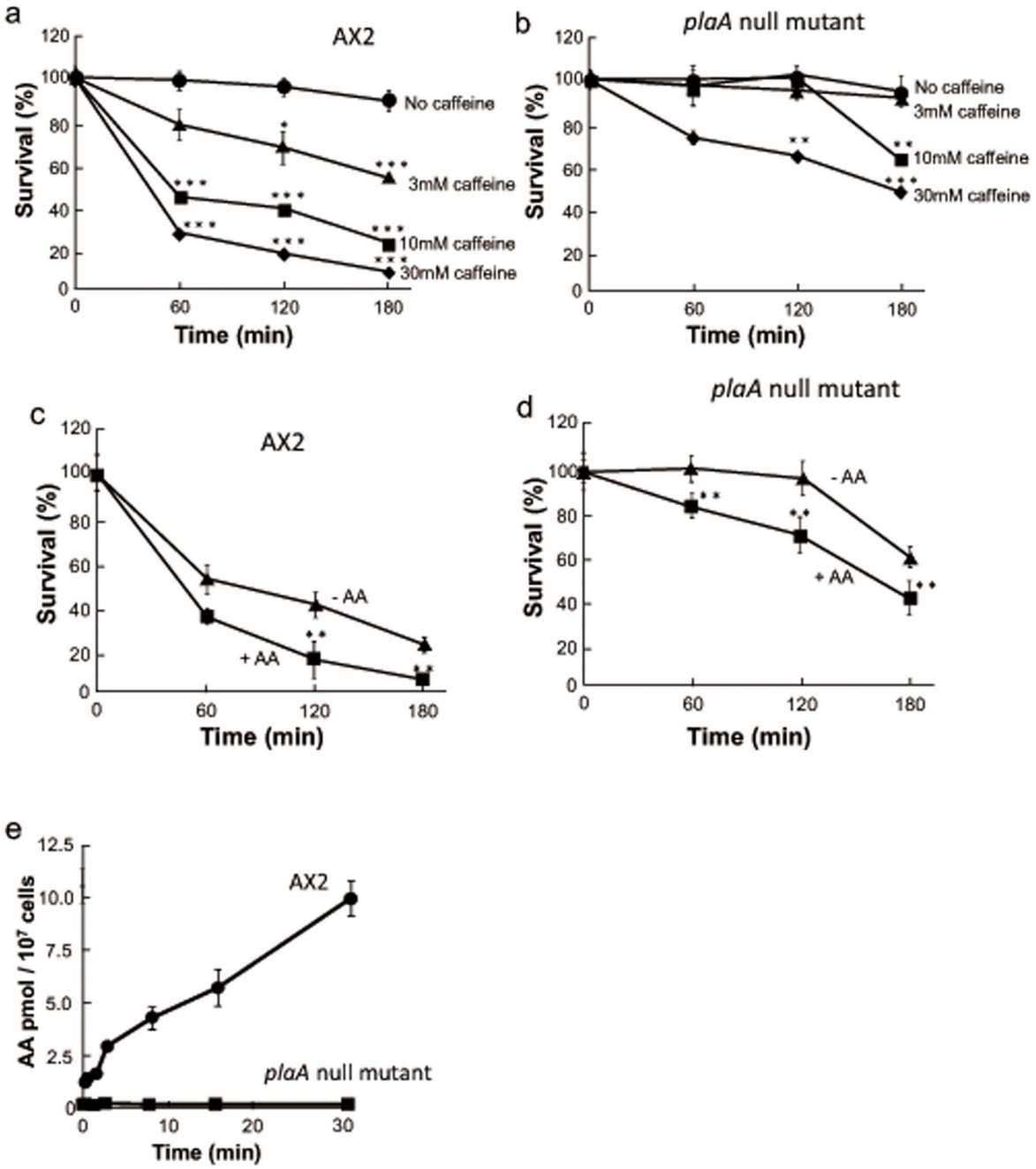

Figure 1 | The survival rate of *D. discoideum* cells in the presence of high caffeine concentrations. (a, b) Survival rate in various concentrations of caffeine (3 mM, 10 mM, and 30 mM) in wild-type AX2 (**a**) and *plaA* null cells (**b**). (c, d) Effect of 20 μM AA on the survival rate of wild type AX2 and *plaA* null mutant cells in 10 mM caffeine. (e) Arachidonic acid (AA) production following the addition of 10 mM caffeine to AX2 (filled circle) and *plaA* null mutant (open circle) cells. All error bars represent s.d. All results are presented as means ± s.d. of triplicated determinations of a representative experiment in 3 independent experiments. *$P < 0.01$, **$P < 0.005$, ***$P < 0.001$ in comparison with the data of no caffeine (**a**, **b**) and minus AA (**d**) at each time point (*t*-test) and between plus and minus AA (*t*-test) (**c**).

(BEL) is a widely used, general PLA_2 inhibitor[6]. We tested the survival of Hela cells in the presence of 50 mM caffeine with or without BEL. The addition of BEL significantly suppressed the caffeine-induced reduction in cell survival, indicating that the activation of PLA_2 reduces cell viability under high caffeine concentrations (Fig. 2b). A genomic study has predicted that no caspase-dependent apoptosis cascade is present in *D. discoideum*[7]. Hence, the caffeine-dependent intracellular signalling pathway in this organism is assumed to be independent of the caspase-dependent apoptotic signalling found in mammalian cells. Furthermore, stimulation with high concentrations of caffeine has been reported to cause a greater than 4-fold increase in AA levels in a mammalian culture cell-line[8]. Therefore, we measured the survival rate in Hela cells in the presence of the cell-permeable general caspase inhibitor, Q-VD-OPh[9] (Fig. 2b). After 24-h incubation in a medium containing 50 mM caffeine, the survival rate was reduced to 21.8 % ± 6.2 % in the absence of this inhibitor but recovered to 75.9 % ± 5.1 % in its presence, indicating that caffeine-induced cell death involves a caspase-dependent apoptosis cascade, as previously described[10]. Further, in the presence of Q-VD-OPh, a significant increase in the survival rate was observed by co-incubation with BEL, suggesting that PLA_2 activity also negatively contributes to survival under high

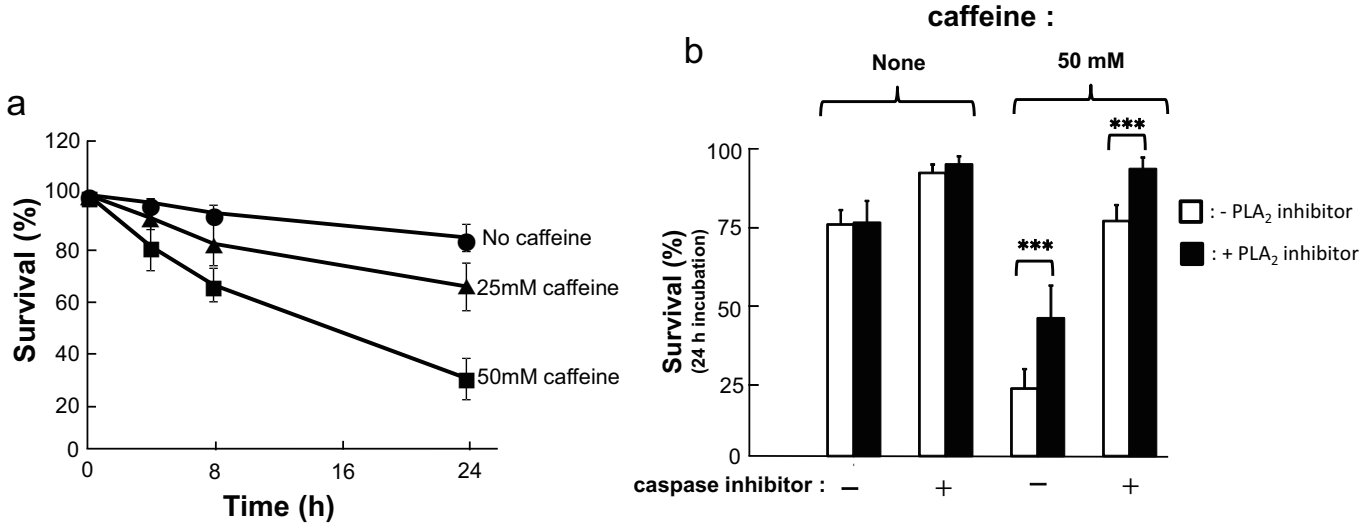

Figure 2 | The survival rate of Hela cells in the presence of high caffeine concentrations. (a) Survival rate in various concentrations of caffeine (25 mM and 50 mM) in Hela cells. (b) Survival rate in 50 mM caffeine with or without 10 μM Q-VD-OPh, a caspase inhibitor in the presence (filled bar) or absence (blank bar) of 5 μM bromoenol lactone (BEL), a phospholipase A_2 inhibitor. All data are taken from 15 images from 3 independent experiments and are presented as the mean ± s.d. (n > 658). ***P < 0.01 between plus and minus BEL (*t*-test).

caffeine concentrations in mammalian cells, independently of caspase-dependent apoptosis.

***plaA* is involved in the intracellular signalling pathway for cAMP degradation in *D. discoideum*.** In *D. discoideum*, caffeine has been used as a specific inhibitor of adenylyl cyclase[11]. cAMP is an autonomic, oscillatory, intercellular signalling compound mediating cell-cell communication and governing the development of this organism under starvation conditions[12]. At 3 to 5 mM concentrations, caffeine completely blocks adenylyl cyclase activity in *D. discoideum* cells and results in the failure of this organism to aggregate and make fruiting bodies[10]. In mutant *plaA* cells, even under high concentrations of caffeine, the cells were able to initiate the developmental process and form fruiting bodies (Fig. 3a). This indicates that PLA_2 is involved not only in caffeine-induced cell death but also in *D. discoideum* development. Spontaneous oscillation of cAMP production was measured in mutant and wild-type cells. In our assay conditions, the wild-type cells showed a transient accumulation of cAMP every 6 min, whereas mutant cells showed a prolonged interval of about 9 min between peaks (Fig. 3b). With the addition of caffeine, the oscillatory production of cAMP was completely blocked in both the wild-type and the null mutant cells, resulting in only a basal level of cAMP production. However, the basal cAMP concentrations were significantly higher in the null cells (Fig. 3c). This difference disappeared after co-incubation with 20 μM AA (Fig. 3d). These observations suggest 2 possibilities: 1) cAMP production is less inhibited by caffeine in the null mutant or 2) cAMP PDE is less active in the null mutant cells. To distinguish between these 2 possibilities, we monitored cAMP production in response to 2′-deoxy-cAMP (dcAMP). This compound is an extracellular stimulus that results in a pattern of cAMP production mimicking the spontaneous oscillatory production of cAMP. The kinetics of cAMP response upon dcAMP stimulation in both the mutant and wild-type showed a transient increase, with a peak at 2 min after cAMP stimulation. However, the reduction to a basal level was prolonged in the null mutant (Fig. 3e). Co-incubation with the extracellular PDE inhibitor, dithiothreitol, did not affect the prolonged cAMP production in the mutant (Fig. 3f). Moreover, incubation with the intracellular cAMP PDE inhibitor, IBMX, significantly reduced cAMP degradation in the wild-type but had no effect on the cAMP response in the plaA null mutant (Fig. 3g). This observation indicates that *plaA* is involved in the intracellular signalling pathway for cAMP degradation.

cAMP is not involved in the caffeine-resistant signalling pathway. *RegA* encodes a cAMP PDE that is responsible for cytosolic cAMP concentrations. The deletion mutant shows elevated intracellular cAMP and, thus, precocious development[13]. When *regA* null cells were allowed to develop on caffeine-containing, non-nutrient agar plates, smaller but normal fruiting bodies were formed (Fig. 4a). This indicates that *regA* is also a suppressor gene that can rescue the caffeine-dependent inhibition of development in *D. discoideum*, although the extent of the suppression was weaker than that of the *plaA* null mutant. Next, to investigate whether *regA* is involved in survival tolerance under high caffeine concentrations, we measured the survival rate of the *regA* null mutant in the presence of 30 mM caffeine. Unlike the *plaA* null mutant, the *regA* null mutant did not show a survival rate that was significantly different from that of wild-type (Fig. 4b). Furthermore, co-incubation with the cell-permeable cAMP analogue, 8-Br-cAMP, which can potentially behave like cAMP in intracellular spaces, did not improve the caffeine-induced cell death in wild-type (Fig. 4c), suggesting that the elevated intracellular cAMP in the *plaA* null mutant is not the cause of the increased caffeine tolerance under high caffeine concentrations. Therefore, PLA_2-AA was potentially participating in another molecular pathway to reduce the caffeine-induced cell death, in addition to its role in inhibiting intracellular cAMP PDE.

Discussion

Caffeine has been shown to induce apoptosis via the PI3K/Akt/mTOR/p70S6K signalling pathway[13]. Moreover, caffeine triggers autophagy through the inhibition of the PI3K/Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathway. Recently, AA was reported to effectively activate both mTOR complex 1 (mTORC1) and mTORC2 in cultured breast cancer cells[14]. Interestingly, AA-stimulated mTORC1 activation is independent of PI3-K, indicating that the AA produced upon caffeine stimulation causes mTORC1 activation. These observations suggest that complex cross-talk signalling occurs between AA and the PI3K/Akt/mTOR/p70S6K signalling pathways. Further investigations of the possible relationships between the two pathways are required to unravel the signalling cascade evoked by caffeine.

In this study, we show a novel function for PLA_2-AA in caffeine-induced cell death via a caspase-independent cell death signalling pathway. Because caffeine is known as a potent enhancer of

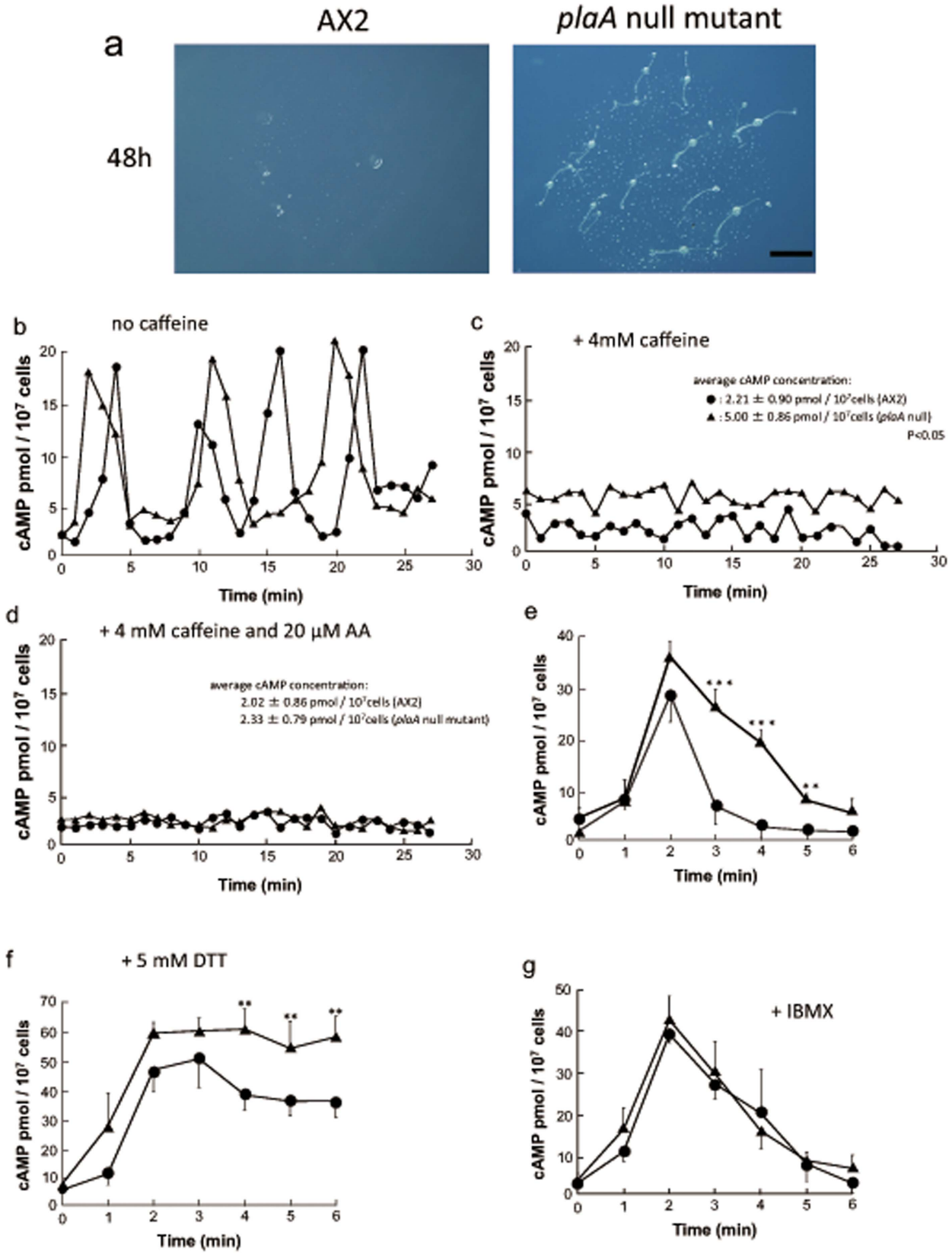

Figure 3 | Effect of caffeine on the development of *plaA* null cells. (a) Development of wild type AX2 and *plaA* null mutants on 4 mM caffeine-containing, non-nutrient agar plates. Bar, 1 mm. (b, c, d) Spontaneous production of cAMP in wild type AX2 (filled circle) and *plaA* null mutant (filled triangle) cells without caffeine (b), with 4 mM caffeine (c), and with 4 mM caffeine and 20 µM AA (d). (e, f, g) cAMP production by 5.0 µM 2′-deoxy-cAMP stimulation with no cAMP PDE inhibitors (**e**), with 5 mM dithiothreitol (DTT), an extracellular cAMP PDE inhibitor (**f**), and with 3 mM 3-isobutyl-1-methylxanthine (IBMX), an intracellular cAMP PDE inhibitor (**g**) in wild type AX2 (filled circle) and *plaA* null mutant (filled triangle) cells. Data in **b**, **c**, and **d** are presented as a representative experiment in 3 independent experiments. Data in **e**, **f**, and **g** are presented as the mean ± s.d. of triplicated determinations of a representative experiment in 3 independent experiments. **P < 0.05, ***P < 0.001 (*t*-test).

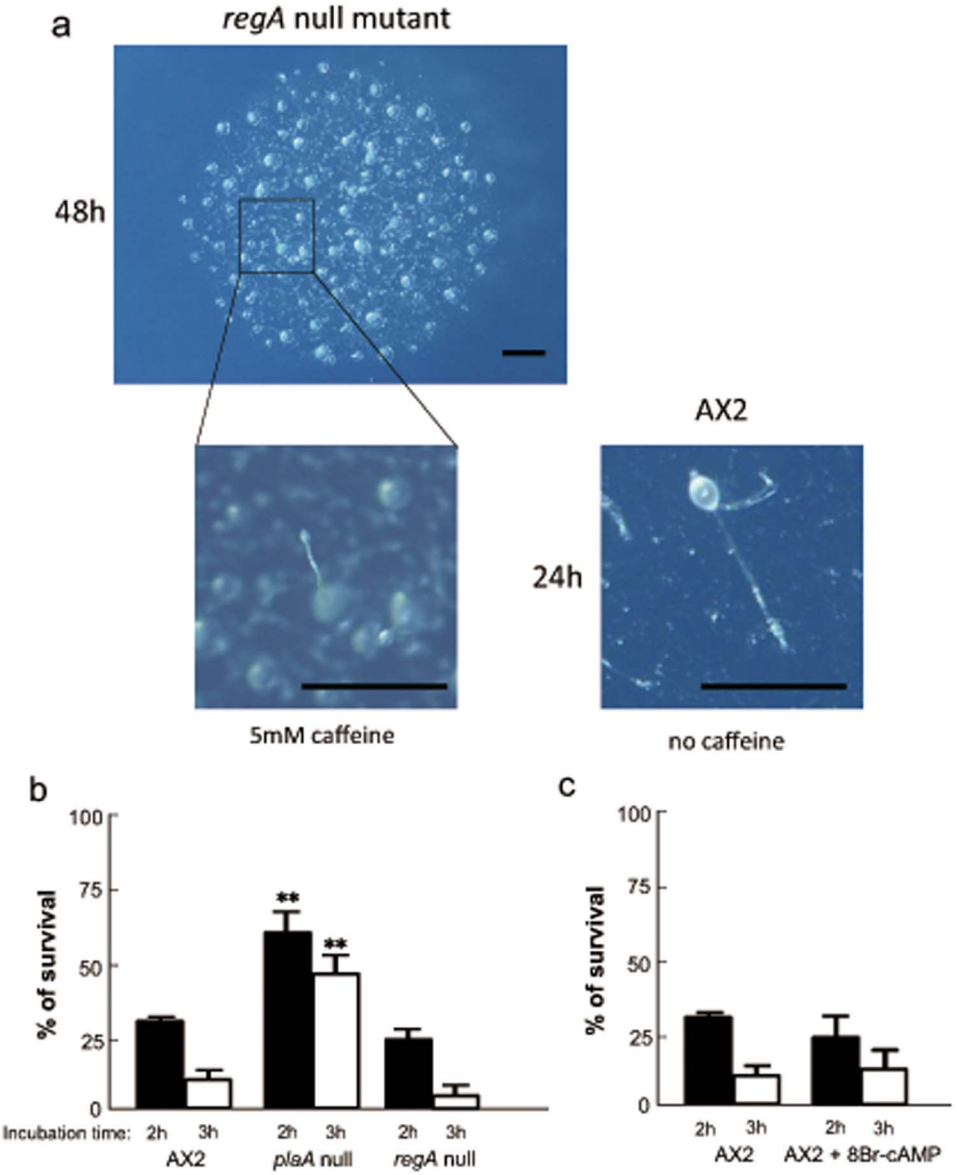

Figure 4 | Independence of intracellular cAMP signalling cascade on caffeine tolerance. (a) Development of *regA* null mutant on 4 mM caffeine-containing, non-nutrient agar plates. Bar, 1 mm. (b) The survival of *regA* null cells in 30 mM caffeine. **P < 0.01 versus AX2 (two-way ANOVA). (c) The survival of wild-type cells in 30 mM caffeine with 10 mM 8-Br-cAMP, a cell-permeable cAMP analogue. All data are presented as the mean ± s.d. of triplicated determinations of a representative experiment in 3 independent experiments.

anticancer drugs[15], it is important to note that strengthening the PLA_2-AA cascade might lead to the enhancement of antitumor potency, without inducing the caspase-dependent apoptosis cascade. The molecular mechanism of how caffeine inhibits *D. discoideum* development has been unravelled[4]. In this study, we also show that the PLA_2-AA cascade is at least partly involved in the inhibition of development by caffeine through elevating intracellular cAMP PDE activity, which did not appear to play a role in PLA_2-AA enhanced cell death. A number of caffeine-related genes has been isolated in *Saccharomyces pombe* in a genome-wide survey, but most of the resistant mutants were revealed to be constitutively activated for the oxidative stress-dependent *pap1* pathway. No PLA_2 gene was isolated in the screen[16]. AA is a well-known precursor of eicosanoids such as prostaglandin E and F, which are potent mediators of inflammation and immunity and which function as second messengers in the central nervous system[17,18]. However, in *D. discoideum*, no cyclooxygenase (COX) gene is present in the genome. This suggests that in *D. discoideum*, AA itself may play a role as a potent second messenger in the caffeine-induced signalling pathway, while in a mammalian system, both activation of PLA_2 and inhibition of COX genes may augment the production of AA, which mediates caffeine-induced cell death. It will be important to investigate the role of the AA downstream pathway to resolve the above issue.

As shown in Fig. 1c and d, treatment with AA was insufficient to restore caffeine sensitivity in the *plaA* null mutant to the level observed in wild type. This indicates that the PLA_2 enzyme may have another function in caffeine tolerance, in addition to its production of AA. Furthermore, as shown in Fig. 2b, PLA_2 inhibitor does not completely block the caffeine-induced cell death, indicating that the PLA_2-independent pathway is likely involved in this process, probably through autophagy via the PI3K/Akt/mTOR/p70S6K signalling pathway.

Interestingly, extracellular AA acts as a chemoattractant in *D. discoideum*[19]. Furthermore, *plaA* was originally isolated as a gene involved in chemotaxis, since *plaA* mutants result in the loss of chemotaxis in the PI3K/PTEN null background[20]. How PLA_2-AA

functions in intracellular chemotaxis signalling pathway is not unravelled yet, but studying the mode of the action by caffeine may enlighten its molecular mechanism. Considering that chemotaxis is a composite biological phenomenon comprising cell migration, cell polarity, and gradient sensing, caffeine may be also effective in inflammation and cancer invasion because chemotaxis plays a critical role in those cases[21].

Thus, in this study, we show that AA increases the sensitivity to caffeine-induced cell death. Since caffeine has been found to enhance the effect of anticancer agents[15], our discovery underscores that, in the chemotherapeutic treatment on cancer, caffeine increases its potential effect as the enhancer of anticancer agents in combination with the compulsory activation of AA production.

Methods

Wild-type *Dictyostelium discoideum* AX2 and *plaA* and *regA* null mutant cells were cultivated using a standard method[22]. The survival rate of D. discoideum cells was measured by shaking the cells in phosphate buffer (PB; 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 [pH 6.5]) at 21°C with the indicated caffeine concentrations and time. The survival rate was determined as described before[23]. The AA assay was performed using an AA assay reagent (Cayman) following the manufacturer's protocol. For the experiments involving *D. discoideum* development, cells were incubated on 1.5% agar PB plates with or without 4 mM caffeine at a density of 5.0×10^5 cells/cm^2. The cAMP assay was performed as described previously[24]. Hela cervical carcinoma cells were cultivated in DMEM (Sigma) supplemented with 10% foetal bovine serum (Sigma) at 37°C and 5% CO_2. The survival rate of Hela cells was measured by exchanging the medium with DMEM containing the indicated concentrations of caffeine and 5 μM propidium iodide (Sigma) with or without 10 μM Q-VD-OPh (R&D Systems, Inc.) and 5 μM bromoenol lactone (CAYMAN). Cell death was calculated by the ratio of the number of cells with fluorescent-stained nuclei to that of the total cells deduced from 15 DIC and fluorescent images, each of which contained 658–1,955 cells for one estimation. Images of *D. discoideum* development were collected using OLYMPUS SZX12 with DP Controller software. Images of Hela cells were collected using OLYMPUS IX71 with IPLab software.

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Author contribution

HK designed and performed all the experiments and drafted the manuscript.

Additional information

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