The life cycle of *Dictyostelium discoideum* is accelerated via MAP kinase cascade by a culture extract produced by a synthetic microbial consortium

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Abstract

A cellular slime mold, *Dictyostelium discoideum,* is an amoeboid organism that has unique life cycle consisting of distinctly separated vegetative and developmental phases. Thus, this organism presents a rare opportunity in which to examine the effects of bioactive substances on separate cellular activities. In this research, we investigated the effect of a culture extract, termed EMXG, produced by a synthetic microbial consortium. EMXG promoted proliferative response of amoeba cells. It further accelerated the developmental phase, leading to the preferred fruiting body formation from fewer cells. Furthermore, EMXG modulated biological rhythm of this organism, that is, interval of oscillation of cAMP level observed in suspension starvation was significantly shortened. Concomitantly, the level of ERKB, a MAP kinase, was found to oscillate in a similar fashion to that of cAMP. Additionally, ErkB deficient mutant amoeboid cells did not respond to proliferative stimulation by EMXG. These lines of evidence point to a likelihood that MAP kinase cascade is involved, and further that ErkB could be the molecular target of EMXG.

Introduction

In recent years, more and more attentions have been directed towards the world of microorganisms. Metagenomic analysis that handles the collection of microorganisms as a whole revealed a huge number of novel genes with unknown functions [Proctor *et al.*, 2019; Barko *et al.*, 2018; Nishijima *et al.*, 2016]. Of particular interest resides in the gradual and steady flows of finding that a combination of microorganisms, or microbial consortium, exhibits novel effects that could not be expected from monocultures [Tsoi *et al.*, 2019; Tanoue *et al.*, 2019; Atarashi *et al.*, 2013; Netzker *et al.*, 2018]. A mixture of selected 17 Clostridia strains is a prerequisite for maximum Treg induction, with its subset being less active [Atarashi et al., 2013]. A similar situation with a combination of 11 bacterial strains has been reported in IFNγ + CD8 cell induction [Tanoue et al., 2019]. In these instances, synergistic cooperation among constituent bacteria has been postulated to exert its inducing activity in full. The exact molecular properties produced by these consortia are yet to be identified. These trends, however, have created renewed interests in microbial consortium, naturally occurring and synthetic as well, and opened up a novel avenue of applied microbiology.

EM, a short form of Effective Microorganisms, is a synthetic microbial consortium consisting, as main members, of photosynthetic bacteria, lactic acid bacteria and yeast. EM was serendipitously discovered by Teruo Higa in 1970s [Higa and Parr, 1994]. Since its discovery, EM itself or its culture extract has exerted numerous beneficial effects in such fields as agriculture, bioremediation, environmental cleanup and so forth [Olle and Willimas, 2015; Alagukannan and Ashokkumar, 2015;Lananan et al., 2014; Ekpeghere et al., 2012]. More recently, EM has proved to be effective in human health as well. Functional genomics and metabolome analyses have shown that ECEM, one of EM products, has anti-inflammatory and immunostimulatory effects in macrophage [Shintani *et al.*, 2015]. Furthermore, EM・XGOLD (abbreviated as EMXG in this communication), another EM product brought to the market as a health drink, has been reported to be effective in

maintaining human immunity function [Najima *et al.*, 2015]. However, its molecular mechanism on those effects is still unknown, so far.

Inspired by these findings, we initiated a more in-depth examination of the effect of EM products on cellular activity with the use of cellular slime mold *Dictyostelium discoideum* as a model. The advantage of this organism for the present research resides in its distinctly separated basic biological phases, i.e. growth and development, genomic homology to human counterparts, yet its simple lifecycle as well as its easy handling.

We report here that EMXG modulates cAMP oscillation rhythm in *Dictyostelium discoideum.* Since no factors no substances that affect the robust rhythm of this organism have been reported, the present result represents a unique and novel finding. Further studies revealed the level of ERKB, a MAP kinase, oscillates in a similar fashion to that of cAMP. Additionally, ErkB deficient mutant cells did not respond to proliferative stimulation by EMXG while wild-type cells did. These lines of evidence suggest the involvement of MAP kinase cascade in the modulation of cAMP rhythm by EMXG and may shed light on the still enigmatic aspects of biological oscillation.

Results

EMXG stimulates proliferation of *D. discoideum* amoeboid cells

To see the effect of EMXG on cell proliferation, AX2 cells, an axenic strain of *D. discoideum*, were cultivated in a synthetic medium containing various concentrations of EMXG, and cell numbers were monitored at a fixed time interval. As seen in Fig. 1, EMXG stimulated the growth of *D. discoideum* amoeboid cells in a dose dependent manner. Since EMXG without HL5 medium did not increase the number of the cells, the observed effect was solely due to non-nutrient component of EMXG (Fig.1b). The growth stimulation was also revealed by an alternative method. Lawn of *Klebsiella aerogenes* was spread over agar impregnated with or without EMXG. A drop of amoeba cell suspension (ca. 10^4 cells) was dropped onto the center of the agar plate. With this set up, the size of the spot becomes enlarged with time in a concentric manner as amoeba cells eat up the surrounding bacteria. The diameter of the circle could thus be regarded as a parameter of amoeba growth. Fig. 2 shows how one of such experiments looks. Accelerated expansion of the circle provided further evidence for the growth stimulation by EMXG.

EMXG promotes fruiting body formation

Amoeboid cells, placed on non-nutrient agar, start to aggregate and proceed through several morphogenetic stages, and finally lead to the formation of fruiting bodies. These processes are regarded as a developmental phase of *D. discoideum* life cycle. To see whether EMXG stimulates cells in this phase, 10 drops containing a fixed number of amoeboid cells were dropped onto agar plate containing various concentrations of EMXG or, as a control, no EMXG (Fig. 3). Approximately 48 hours later, the number of fruiting body, in terms of number of spore, was counted under dissecting microscope. As seen from Fig. 3, EMXG stimulated, dose dependently but not linearly, the fruiting body formation. Presently, it is not clear at which stage during development, i.e. from aggregation through migrating slug to fruiting body formation, EMXG exerts its influence. A possible inference could be that EMXG enhances the cellular cAMP level

which facilitates aggregation process leading to preferred fruiting body formation. This inference turned out to be the case later in our experiment shown in Table 1.

EMXG influences cAMP level and oscillation rhythm of amoeboid cells in suspension

Oscillation is one of the key but unresolved biological phenomena [Maroto and Monk, 2008]. It has been observed in different cell types and with a wide range of frequencies; neurons with periodicities in milliseconds, circadian clocks with about a day and mammalian cells with a range of a few seconds to hours [Wilson *et al.*, 2018; Somers *et al.*, 2018]. Although the underlying mechanisms of these phenomena differ depending on the cell type and its intrinsic periodicities, similarities have also been observed in oscillatory systems with similar periodicities. It has been shown that, a few hours after initiation of suspension culture of *D. discoideum* amoeboid cells, under starvation conditions, exhibit spontaneous oscillation of light-scattering as well as levels of cAMP with a periodicity of 6-7 min [Gerisch and Wick, 1975; Roos *et al.*, 1977; Tomchik and Devreotes, 1981]. This property is caused by contraction of cells, which seems to directly related to the level of extracellular cAMP level. Extraneous addition of cAMP to the suspension was shown to shift the cAMP oscillation phase. The observed stimulatory effect by EMXG of fruiting body formation suggests that EMXG may affect the level of cAMP. We also have detected EMXG promoted aggregate stream formation (Fig. 4) Prompted by these findings, it was of interest to see whether EMXG has an effect on the level of cAMP and further on the oscillation of cAMP level. Fig. 5 shows the result of one of such experiments, and, as summarized in Table 1, the interval of cAMP oscillation is significantly shortened. Since the periodicity of this cAMP oscillation has been known to be robust, its shortening by EMXG is a novel finding to our knowledge. No factors or substances that affect the oscillation interval have so far been reported. Therefore, it is of interest as well as of importance to identify what in EMXG exerts this capability. The basal level of cAMP with EMXG was also higher than that of control (Table 1), which may support the

previously mentioned inference that efficient fruiting body formation by EMXG could be due to enhanced production of cAMP.

The ERK pathway is involved in EMXG effect

Regarding the oscillatory behavior of cAMP level, genetic and biochemical investigations with large number of mutants demonstrated the interacting network consisting of a dozen proteins, which are essential for spontaneous oscillation [Michael *et al.*, 1998; Knetsch, *et al.*, 1996; Aubry *et al* 1997]. Computer simulation of a molecular circuit based on these results showed that it is able to account for the temporal and quantitative aspects of the oscillatory system [Maeda *et al.*, 2004]. The ERK protein is one of the central members of such a circuit and has been well known as molecular mediator of extracellular signals. We examined how *Dictyostelium* ERK (ERKB) proteins behave during amoeba cell suspension culture in the presence of EMXG. In Fig. 6, western blot analysis shows the shortening of peak interval of phosphorylated ERKB. This finding is further corroborated by an experiment shown in Fig.7. No growth stimulation was detected in *erk*B null mutant in the presence of EMXG. These two lines of evidence clearly point to the involvement of ERKB protein in cAMP oscillation control of this organism. Based on the proposed regulatory circuit of producing cAMP oscillation, it remains of interest how ERKB affects the activities of other members of the circuit. More importantly, the implication how the shortening of cAMP periodicity relates to the action of EMXG awaits further investigation.

Discussion

The life cycle of *Dictyostelium discoideum* is of particular interest (Loomis, 2014). The amoeboid cells released from spores of fruiting body by an appropriate shock grow as long as the food, such as bacteria, is available. Once the food is depleted, they stop growing and start to aggregate by a chemotactic mechanism using cAMP as a chemoattractant. The attraction to the centre of the aggregation is propagated in the fieled with a period of 6 to 7 min [Gerisch and Wick, 1975; Tomchik and Devreotes, 1981], although the period decreases down to 8 min as cells develop [Gerisch *et al.*, 1979]. The aggregates wander around as a form of slug, and through formation of two types of cells, namely, pre-spore and pre-stalk cells, finally form the fruiting body. Accordingly, all cells are in growth phase in the presence of food, and in its absence they are totally in the developmental phase. These two phases, i.e. growth and development, represent basic facets in biological development. In most cases, they proceed simultaneously to form a mass of cells mixed with various phases, making it hardly possible to examine them individually. Furthermore, many of its genes are homologous to human counterparts. These features make *D. discoideum* a model organism of choice for experimentally analyzing the effect of novel factors or substances unpredictably produced by microbial consortium.

The proliferative stimulation and preferred formation of fruiting bodies by EMXG could both be ascribed to the elevated cellular cAMP level as evident from Table 1. Close correlation between cell proliferation and cAMP has been reported [Schwebs *et al.*, 2018]. Still questions remain, for example, as to the exact action site of EMXG during *D. disoideum* cell cycle, its mode of action other than chemoattractant or how EMXG facilitates the enhanced formation of aggregating foci. Promoted proliferation was also observed in cultured *Tetrahymena*, a ciliated protozoa (our unpublished observation).

The modulation by EMXG of oscillatory rhythm of cAMP level warrants special mention. To evoke the spontaneous oscillation in this species, endogenous interactions of molecules involving protein kinase, ERKB, has been proved to be necessary [Knetsch, *et al*, 1996; Aubry *et al.*,

1997]. Further, this oscillatory regulation of cAMP level was predicted by assuming only six components, namely, cell surface cAMP receptor 1, CAR1, the MAP kinase, ERKB, intracellular phosphodiesterase, REGA, protein kinase A, PKA, and extracellular cAMP phosphodiesterase, PDE [Maeda *et al*, 2004]. The computational simulation with these six components predicted the oscillatory behavior of cAMP level. One other point worth mentioning is the fact the periodicity of this cAMP oscillation has been known to be robust. The model well explains many facets of the system like the periodicity of robustness and the amplitude variation caused by partial loss of some of the enzymes. It is, however, unable to predict any substantial effect on the cycle time. Therefore, our report here provides evidence that there is possible factors or substances that affect the oscillation interval. Our result that EMXG is effective in shortening cAMP oscillation interval represents an unexpected novel finding to our knowledge, and opens up a new avenue for uncovering the regulatory mechanism of cAMP oscillation in *D. discoideum*. It is of interest as well as of importance to identify what component in EMXG exerts this capability. Furthermore, the knowledge regarding how this shortening of periodicity is brought about could contribute to the understanding of yet enigmatic biological rhythm in general.

Experimental Procedures

Cell culture and growth assay

Axenic strain of *Dictyostelium discoideum*, AX2, was cultured in HL5 medium (15.4 g glucose, 7.15 g yeast extract, 14.3 g proteose peptone, 0.485g KH_2PO_4 and 1.28g $Na_2HPO_4 \cdot 12H_2O$) at 21˚C. Cells harvested from early logarithmic phase were used in most experiments unless otherwise stated. For measuring the effect of EMXG on vegetative phase, cells were inoculated at a density of 1.0 x 10^5 cells/ml in 20 ml 1/3 HL5 with various concentrations of EMXG and shaken at 120 rpm at 21°C. Cell density was monitored at a fixed time interval. Alternatively, a drop of cell suspension was spotted at the center of 5LP agar plate (0.5% lactose and 0.5 % bactopeptone and 1.5% agar) which is impregnated with or without EMXG and coated with a lawn of *Klebsiella aerogenes*. The size of the concentric circle produced by phagocytosis of amoeba was monitored as proliferative activity.

EMXG

EMXG is a health drink containing secondary metabolite produced by a synthetic microbial consortium called effective microorganism, termed EM. EM constitutes, as main members, of photosynthetic bacteria (*Rhodopseudomonas palustris*), two species of lactic acid bacteria (*Lactobacillus casei* and *Lactobacillus farranginis*) and two species of yeasts (*Saccharomyces cerevisiae* and *Candida ethanolica*). Component analysis showed that EMXG contains no detectable nutrient [Najima et al. 2015].

Assay for fruiting body formation

For observing the development, cells at a density of 1.0 x 10^6 cells/ml were harvested and resuspended in Bonner's standard solution [Bonner, 1947]. The droplets of 5 µl with various cell densities were spotted onto a 1.5 % non-nutrient agar plate. The plates were incubated at 21°C. Approximately 48 hours later, the number of soruses of fruiting bodies was counted under a dissection microscope (SZX12; Olympus, Tokyo, Japan).

Oscillation of cAMP level in suspension starvation of amoeba cells

The axenically grown cells were washed twice with 10 mM Na_2HPO_4/KH_2PO_4, pH 6.5 (PB) and shaken at a density of 1 x 10^7 cells/ml with or without EMXG. After starvation for 6 hr, 100 µl of the suspension were taken and added to 100 µl of 3.5% perchloric acid at the indicated times. After neutralization of the lysate by adding 50 µl of 50% saturated $KHCO_3$, the cAMP concentration was determined using cAMP Biotrak EIA System (Amersham Bioscience, U.K.).

Western blot analysis

One hundred µl aliquot of the cell suspension from the starvation culture, as in cAMP assay, were mixed with 2 x sample buffer and electrophoresed. The blotted membrane was probed with primary and secondly antibodies, and analyzed with an image analyzer (LAS1000; Fujifilm, Tokyo, Japan). Signal intensity was converted to arbitrary unit of density by using ImageJ software. Anti-phospho-p44/p42 MAP kinase antibody (#9101, Cell Signaling Technology) was used as the primary antibody for detecting phosphorylated *D. discoideum* ErkB [Maeda *et al.* 2004].

References

Alagukannan G, Ashokkumar M: Role of Effective Microorganisms (EM) in Sustainable Agriculture A Review and Recommendations, International Journal of Scientific Research, 2015; 4:577-578.

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T. Narushima S, Hase K, *et al*. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota, Nature, 2013;500: 232-236.

Aubry L, Maeda M, Insall R, Devreotes PN, Firtel RA: The *Dictyostelium* mitogen-activated protein kinase ERK2 is regulated by Ras and cAMP-dependent protein kinase (PKA) and mediates PKA function. Journal of Biological Chemistry, 1997;272: 3883-3886.

Barko PC, McMichael MA, Swanson KS, Williams DA: The Gastrointestinal Microbiome. Journal of Veterinary Internal Medicine, 2018;32: 9-25.

Bonner, JT, Savage LJ: Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 1947;106: 1-26.

Ekpeghere KI, Kim BH, Son HS, Whang KS, Kim HS, and Koh S.C: Functions of effective microorganisms in bioremediation of the contaminated harbor sediments, Journal of Environ. Science and Health, Part A, 2012;47,1: 44-53.

Gerisch G, Malchow D, Roos W, Wick U: Oscillations of cyclic nucleotide concentrations in relation to the excitability of *Dictyostelium cells*. Journal of Experimental Biology. 1979;81: 33-47.

Gerisch G, Wick U; Intracellular oscillations and release of cyclic AMP from *Dictyostelium* cells. Biochemical and Biophysical Research Communications, 1975;65: 364-370.

Higa T. Parr, JF: Beneficial and Effective Microorganisms for a Sustainable Agriculture and Environment. International Nature Farming Research Center, Atami, Japan. 1994.

Knetsch ML, Epskamp SJ, Schenk PW, Wang Y. Segall J.E, Snaar-Jagalska, BE: Dual role of cAMP and involvement of both G-proteins and ras in regulation of ERK2 in *Dictyostelium discoideum*. EMBO Journal, 1996;15: 3361-3368.

Lananan F, Hamid SHA, Din WNS, Ali N, Khatoon H, Jusoh A, Endut A: Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing Effective Microorganism (EM-1) and microalgae (Chlorella sp.), International Biodeterioration and Biodegradation, 2014;95: 127e134.

Loomis WF: Cell signaling during development of *Dictyostelium*, Developmental Biology. 2014;39: 1–16.

Maeda M, Lu S, Shaulsky G, Miyazaki Y, Kuwayama H, Tanaka Y, Kuspa A, Loomis WF: Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA. 2004;Science, 304: 875-878.

Maroto M, Monk NAM: Cellular Oscillatory Mechanisms, Advances in Experimaental Medicine and Biology, Springer Science + Business Media, LLC., Landes Bioscience, 2008;641

Michael T, Laub MT: A molecular network that produces spontaneous oscillation in excitable cells of *Dictyostelium*, Molecular Biology of the Cell, 1998;9: 3521-3532.

Najima M, Shintani M, Shimabukuro S, Munekata M: Improvement of immune function by EM･XGOLD, a health drink containing extract from culture of Effective Microorganisms (ECEM), Shinryo to Shinyaku, 2015;52: 78-84.

Netzker T, Flak M, Krespach MK, Stroe MC, Weber J, Schroeckh V, Brakhage, AA: Microbial interactions trigger the production of antibiotics. Current Opinion in Microbiology, 2018;45:117-123.

Nishijima S, Suda W, Oshima K, Kim SW, Hirose Y, Morita H, Hattori, M: The gut microbiome of healthy Japanese and its microbial and functional uniqueness. DNA Research, 2016;23: 125-133.

Olle M, Williams, I: The Influence of Effective Microorganisms on the Growth and Nitrate Content of Vegetable Transplants, Journal of Advanced Agricultural Technologies, 2015;2: 25-28.

Proctor LM, Sechi S, DiGiacomo ND, Fettweis JM, Jefferson KK, Strauss, JF 3rd, Rubens CE, Brooks JP, Girerd PP, Huang B, *et al.*: The Integrative HMP (iHMP) Research Network Consortium, The Integrative Human Microbiome Project. Nature, 2019;569: 641-648.

Roos W, Scheidegger C, and Gerisch G: Adenylate cyclase activity oscillation as signals for cell aggregation in *Dictyostelium discoideum*, Nature, 1977;266: 259-261.

Schwebs DJ, Pan M, Adhikari N, Kuburich NA, Jin T, Hadwiger JA: *Dictyostelium* Erk2 is an atypical MAPK required for chemotaxis. Cell Signalling, 2018:46: 154-165, 2018.

Shintani M, Mitsunaga F, Shimabukuro S, Nakamura S: Anti-inflammatory and immunostimulatory effects of extract from culture of Effective Microorganisms (ECEM) revealed by functional genomics and metabolome analyses, Food and Nutrition Sciences, 2015;6: 1115-1125.

Somers J, Harper REF, Albert JT: How Many Times? On the Sensory Basis and Computational Challenges of Circadian Systems. Frontiers in Behavioral Neuroscience, 2018;12, 211: eCollection.

Tanoue T, Morita S, Plichta DR, Skelly AN, Suda W, Sugiura Y, Narushima S, Vlamakis H, Motoo I, Sugita K, *et al.*: A defined commensal consortium elicits CD8 T cells and anti-cancer immunity. Nature, 2019;565: 600-605.

Tomchik K, Devreotes PN: Adenosine 3',5'-monophosphate waves in *Dictyostelium discoideum*: a demonstration by isotope dilution-fluorography. Science, 1981;212: 443-446.

Tsoi R, Dai Z, You L: Emerging strategies for engineering microbial communities. Biotechnology Advances, 2019;pii: S0734-9750(19)30047-3.

Wilson CJ, Higgs MH, Simmons DV, Morales, JC: Oscillations and Spike Entrainment. F1000Res. 2018 2018: 7.

411 Legends for Figures

412

413 Figure 1. (a) Stimulation of growth of *D. discoideum* amoeba cells by EMXG.
414 EMXG concentrations were: open circle, 0%; closed circle, 10%; open square,
415 50%; closed square, 100%. (b) EMXG does not increase the number of the
416 cells. Open circle, with PB; closed circle, with EMXG.

417

418 Figure 2. Stimulation of phagocytosis of *D. discoideum* amoeba cells by
419 EMXG. In this particular experiment, 10 μl of cell suspension containing
420 5,300 cells were dropped onto the center of agar plate covered by overnight
421 culture of *Klebsiella aerogenes.* Plates were photographed after 5 days of
422 incubation at 21 ℃. A: Control without EMXG, B: Agar contained 40%
423 EMXG.

424

425 Figure 3. Stimulation of fruiting body formation by EMXG. EMXG
426 concentrations: C, Control (no EMXG); E5 (5% EMXG), E10 (10% EMXG),
427 E20 (20% EMXG). **: Significant difference in comparison to the Control by
428 Steel test ($p<0.01$), a,b: different letters show significant difference by
429 Steel-Dwass test($p<0.01$)

430

431 Figure 4 Aggregation of wild-type cells under submerged condition with or
432 without EMXG. Cells were starved at 21 °C for 12hr in PB with or without
433 EMXG. Scale bar, 20 μm.

434

435 Figure 5. Shortening of cAMP oscillation interval by EMXG. Open circle,
436 without EMXG; closed circle, with EMXG.

437

438 Figure 6. Shortening of oscillation interval of phosphorylated ERKB protein.
439 Open circle, without EMXG; closed circle, with EMXG.

440

441 Figure 7. ErkB-deficient mutant is not responsive to the proliferative

442 stimulation by EMXG. EMXG concentrations were: open circle, 0%; closed
443 circle, 10%; open square, 50%; closed square, 100%.

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Author contributions

H. K. and T. H. performed the experiments and drafted the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Statement of Ethics

The study protocol was approved by the in committee of University of Tsukuba on research.

Table 1. The effect of EMXG on cAMP content with or without EMXG*

	Average concentration of cAMP (pmol / 10^7 cells)	**Cycle length (min)**
PB	1.319 ± 0.913	6 - 7
EMXG in PB	3.916 ± 1.637	4 - 6

* Cell suspensions were shaken in 10 mM PB (pH=6.5) for 2hr at the density of 1.0 x 10^7 cells/mL and added 6hr with cAMP pulse (30 nM at every 6 min). And then cells were washed with PB for 3 times and resuspended in PB at the density of 10^7 cells/mL and shaken for sampling.

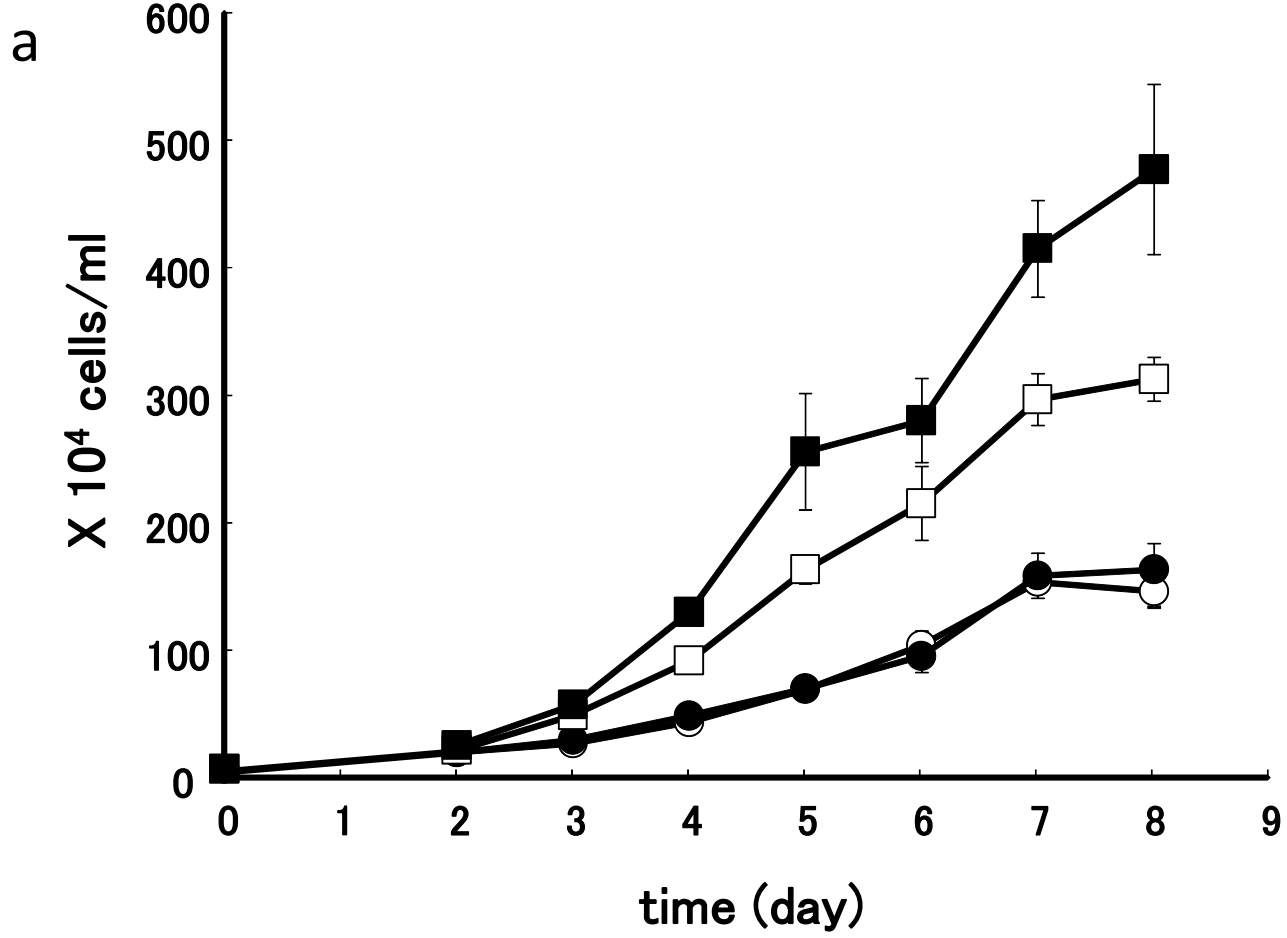


Fig. 1 (a). Stimulation of growth of *D. discoideum* amoeba cells by EMXG. EMXG concentrations were: open circle, 0% (control); closed circle, 10%; open square, 50%; closed square, 100%.

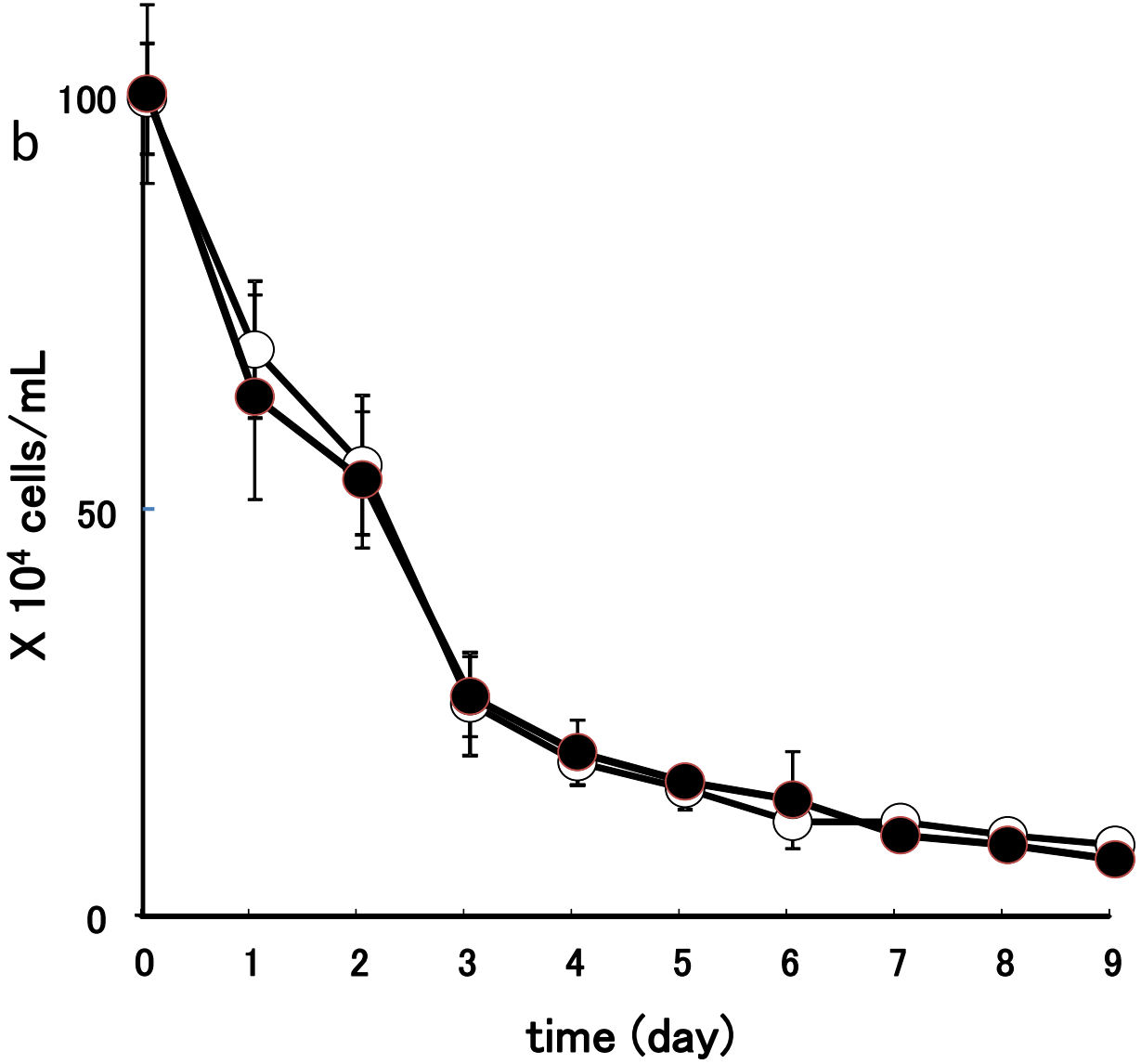
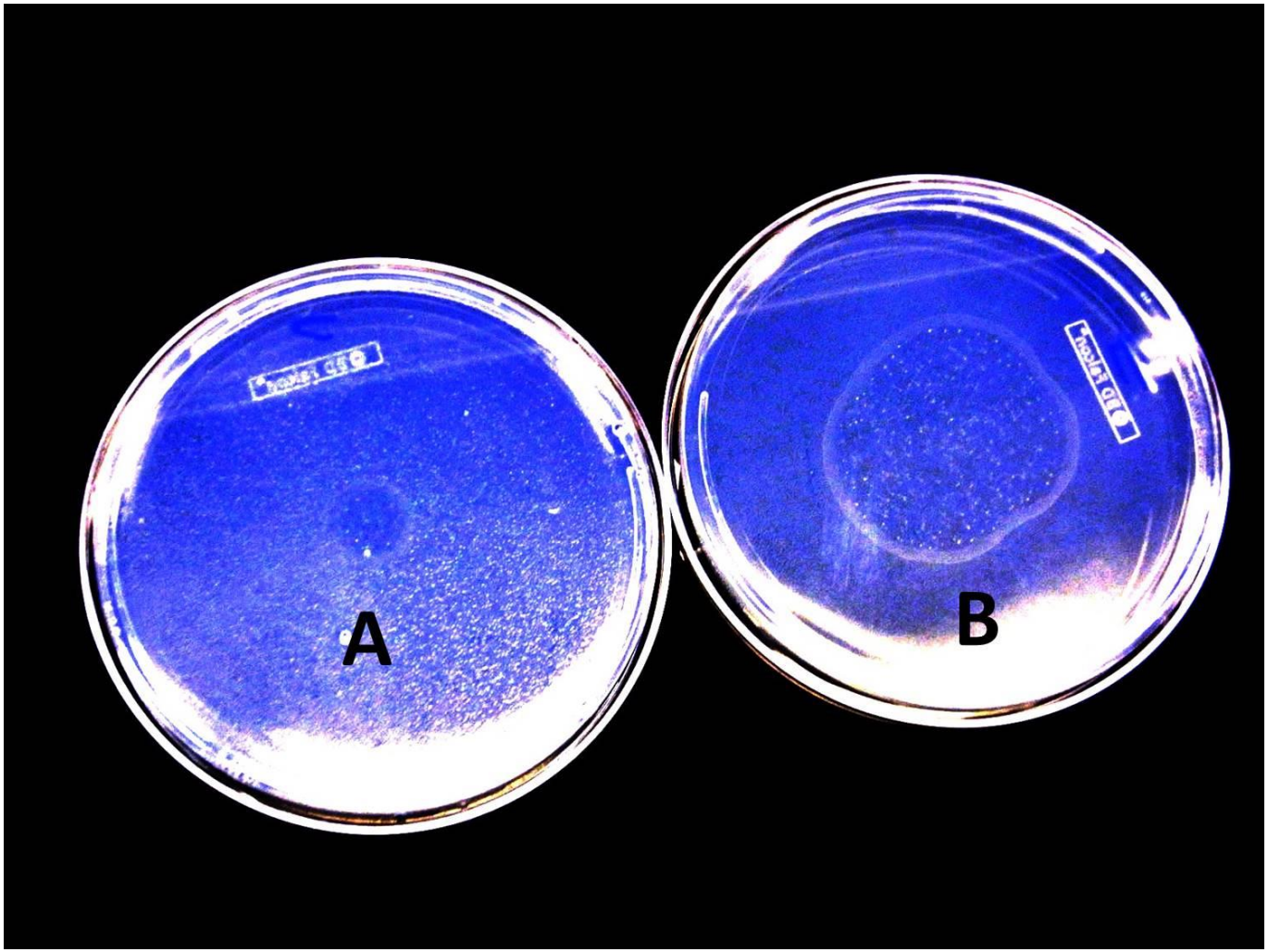


Fig. 1 (b). EMXG does not increase the number of the cells. Open circle, without EMXG; closed circle, with EMXG.



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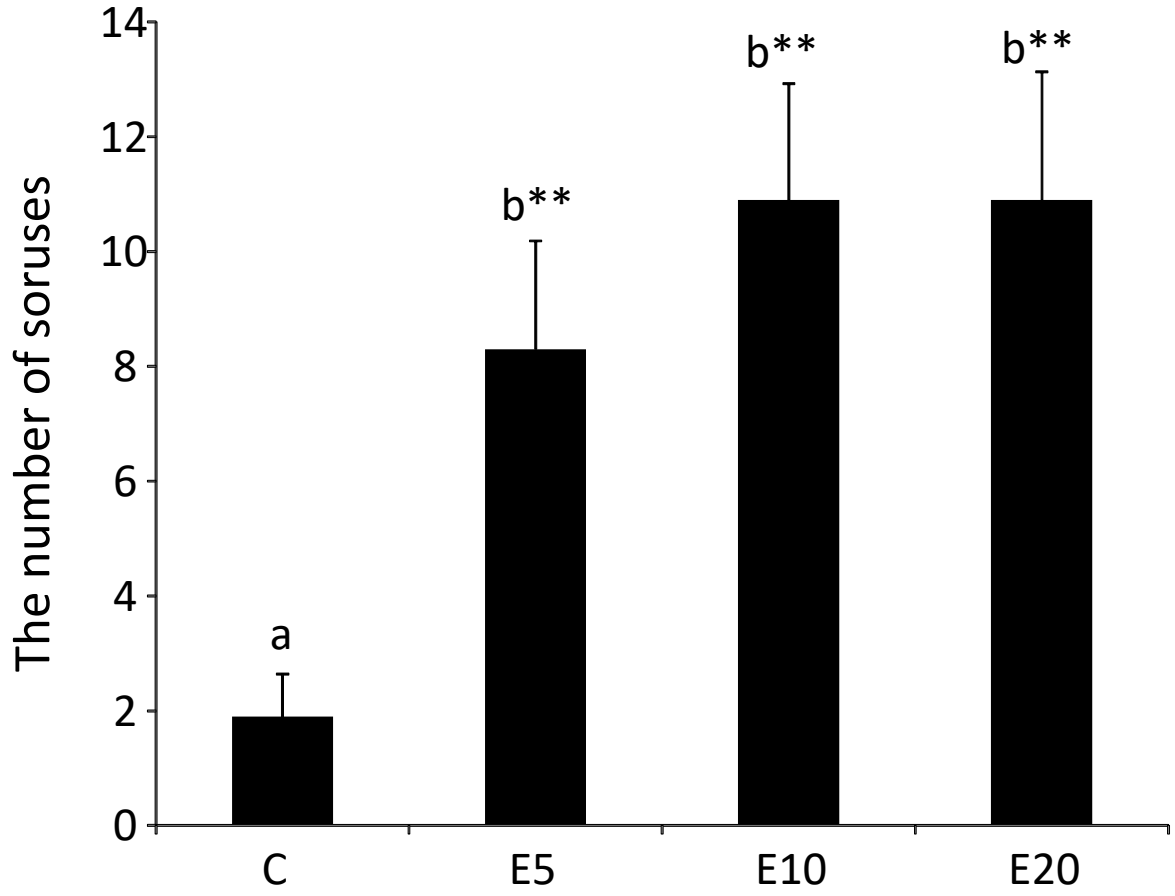


Fig. 3. Stimulation of fruiting body formation by EMXG. Drops of amoeba cell suspension were dropped onto non-nutritious agar plate. After 48 hours of incubation, fruiting body formation was monitored as numbers of fruiting bodies. EMXG concentrations: C, Control (no EMXG); E5 (5% EMXG), E10 (10% EMXG), E20 (20% EMXG). **: Significant difference in comparison to the Control by Steel test ($p<0.01$)
a,b: different letters show significant difference by Steel-Dwass ($p<0.01$)

PB

EMXG in PB

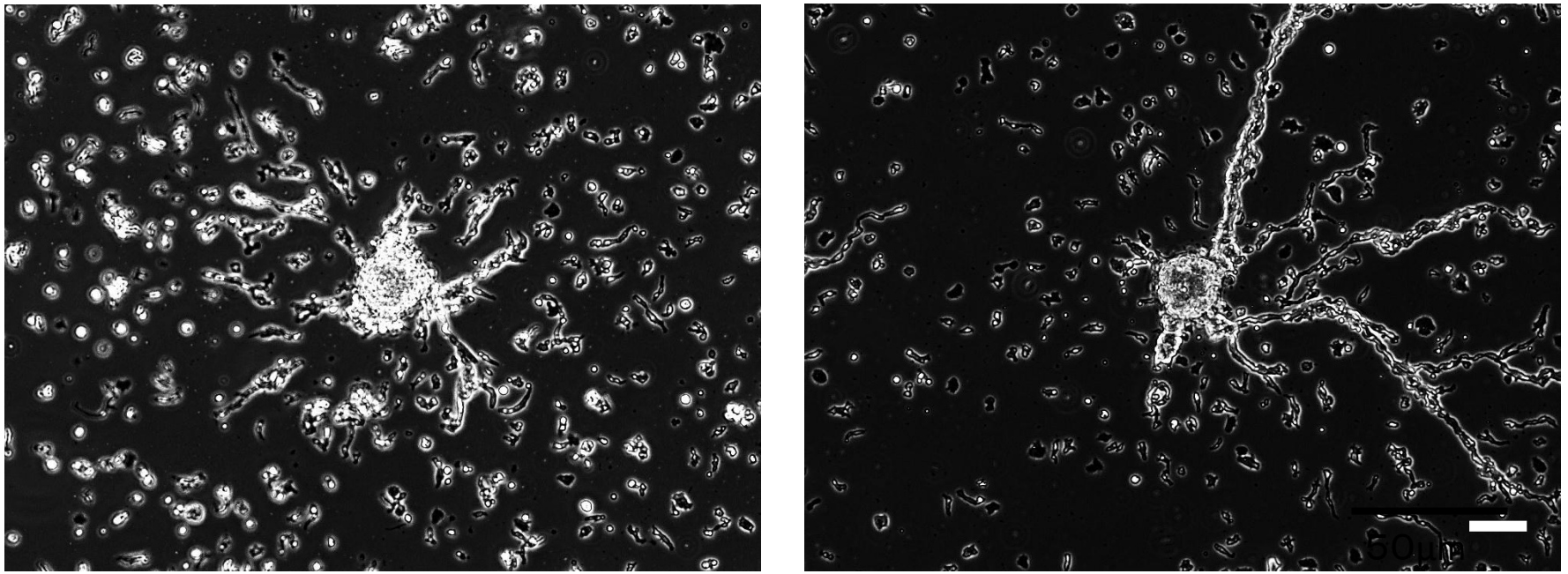


Fig. 4. Aggregation of wild-type cells under submerged condition with or without EMXG. Scale bar, 20 µm.

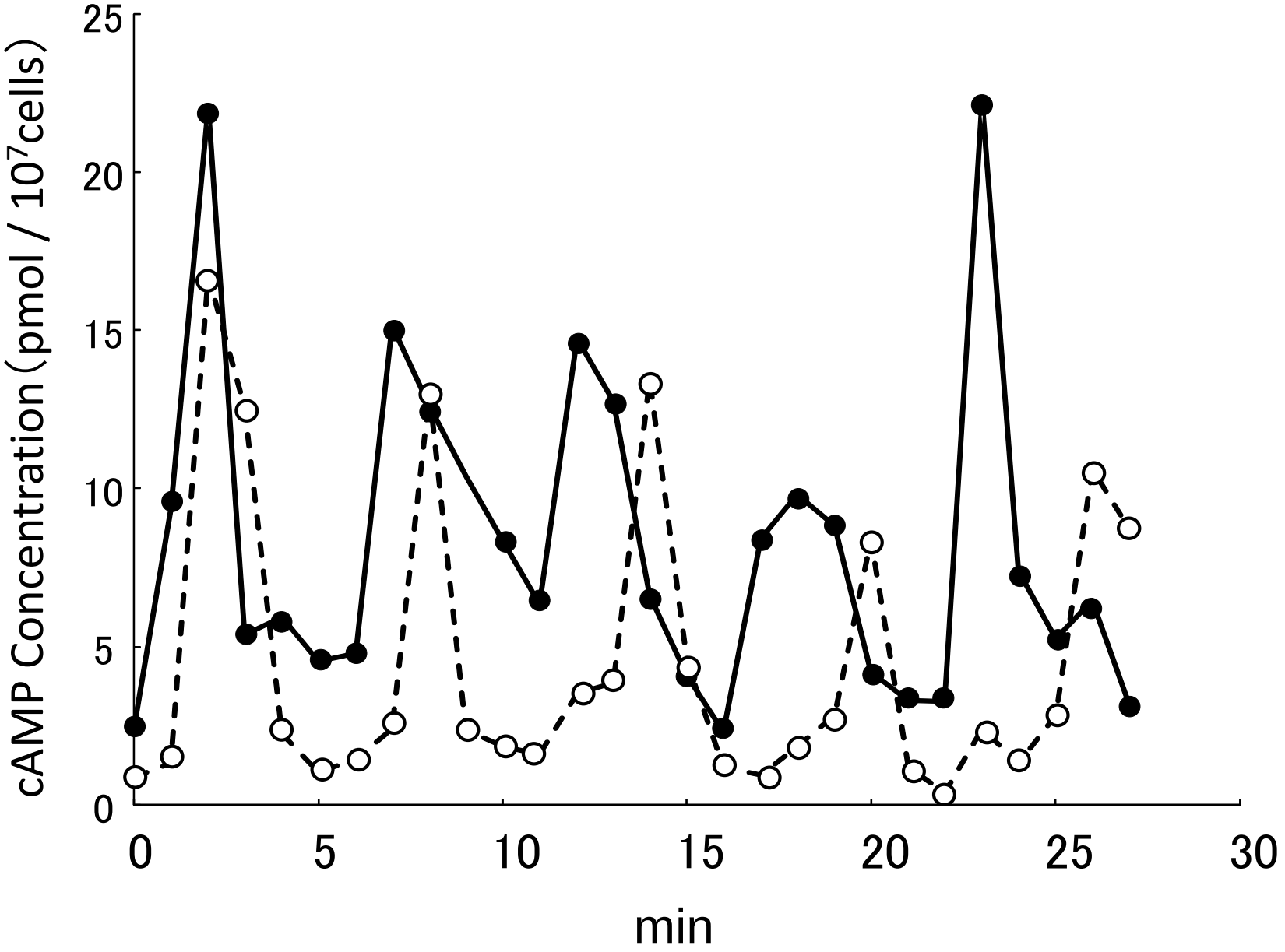


Fig. 5. Shortening of cAMP oscillation periodicity by EMXG. Open circle, control without EMXG; closed circle, in the presence of EMXG.

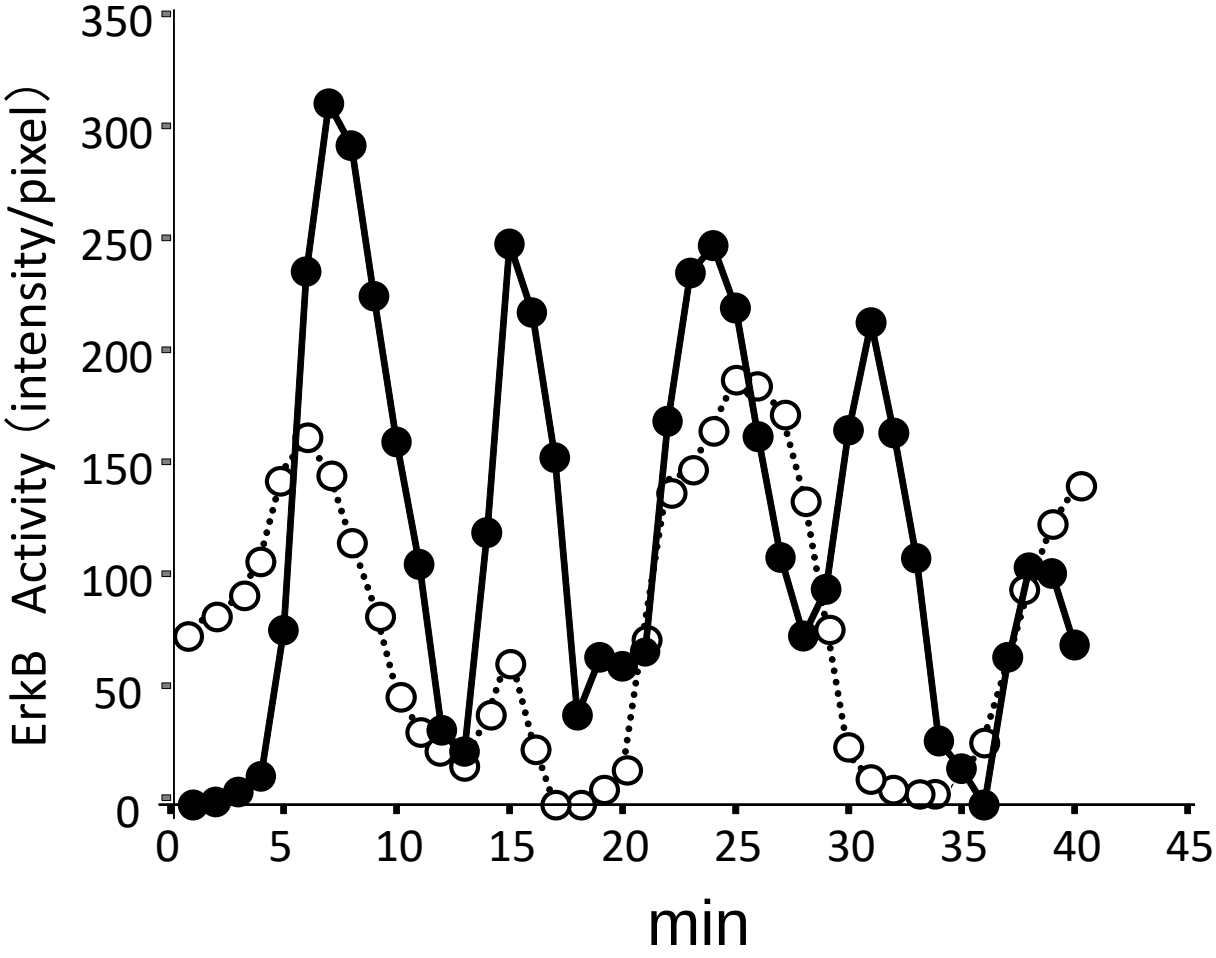
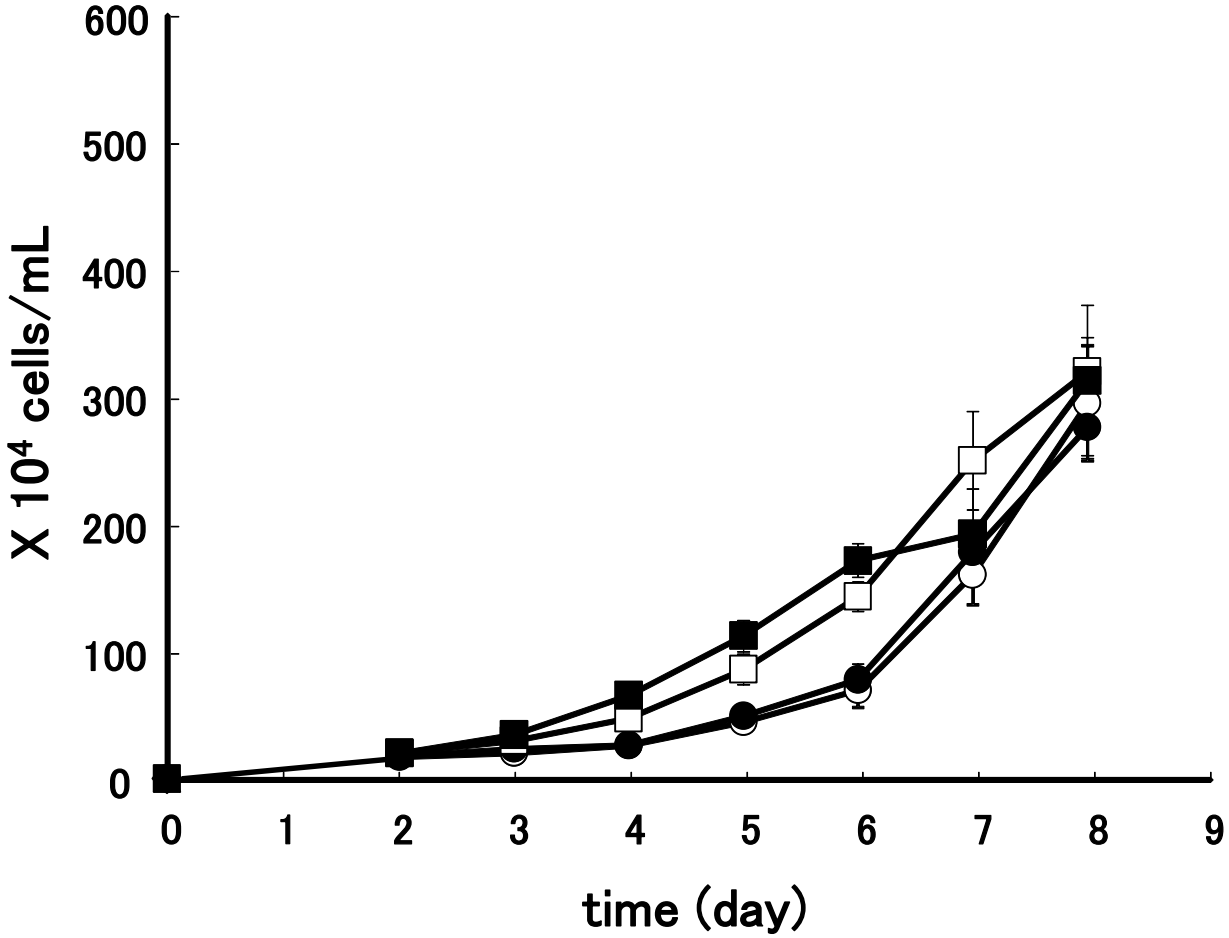


Fig. 6. Shortening of oscillation interval of phosphorylated ERKB protein. Open circle, control without EMXG; closed circle, in the presence of EMXG.



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