Short Communication

Transient Increase in Intracellular Calcium in Streptomyces alboniger Produced by Pamamycin-607, an Aerial Mycelium-inducing Substance

Masahiro Natsume,† Junko Tazawa, Hiroshi Abe, Yoshihisa Kudo,* Satoru Kondo,**†† and Shingo Marumo**

Department of Applied Biological Sciences, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan
*Laboratory of Neuropharmacology, Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan
**Department of Applied Biosciences, Nagoya University, Nagoya 464-01, Japan
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Effects of pamamycins on [Ca$^{2+}$], in Streptomyces alboniger were examined by microscopic fluorometry. Pamamycin-607, which has aerial mycelium-inducing activity, induced a transient increase in [Ca$^{2+}$], dose-dependently, but the inactive pamamycin-649 had no effect on [Ca$^{2+}$].

Aerial mycelium differentiation from substrate mycelia in actinomycetes is accompanied by morphological and physiological changes. We isolated pamamycin-607 (MW 607), an aerial mycelium-inducing substance, from Streptomyces alboniger IFO 12738.1,2 and found that Ca$^{2+}$ regulates the aerial mycelium formation of many actinomycetes, including S. alboniger.3 We also reported that Ca$^{2+}$ signal modulators inhibit aerial mycelium formation in S. alboniger and that for its activity pamamycin-607 requires Ca$^{2+}$.4 These results suggest that pamamycin-607 induces an increase in [Ca$^{2+}$], which becomes the trigger for aerial mycelium formation. We examined the effects of pamamycin-607 on [Ca$^{2+}$], in S. alboniger and compared them with those of pamamycin-649,5 an inactive homologue of MW 649.

[Ca$^{2+}$], was measured by microscopic fluorometry with Fura-2 as the indicator.6 Fura-2 was loaded into S. alboniger mycelia as follows: slant culture of S. alboniger IFO 12738 was inoculated into a 500-ml shaking flask containing 100 ml of S1 medium (glucose 1%, peptone 0.3%, yeast extract 0.4%, glycerin 0.05%, NaCl 0.4%, MgSO$_4$·7H$_2$O 0.05%, K$_2$HPO$_4$ 0.1%, pH 7.2), after which the flask was incubated at 28°C for 2 days on a reciprocal shaker. A 0.5-ml portion of this culture was transferred to an L-tube containing 2 ml of GPYP medium7 in which 10 μM of Fura-2-AM was dissolved. The inoculated L-tube was cultured with shaking for one day at 28°C. The media used were developed for the preparation of streptomycete protoplasts and are characterized by high concentrations of glycine. Mycelia of streptomycetes grown in these media are sensitive to lysozyme action because the cell wall structure becomes coarse.8 Mycelia cultured in Hickey and Tresner's medium9 (used for the bioassay of pamamycins and not containing glycine) were not stained by Fura-2-AM.

Fura-2-loaded mycelia of S. alboniger (about 0.5 mm diameter) were attached to a well coated with Cell-Tak (Collaborative Research, Bedford, MA) then perfused with MP3 medium (P3 medium10 without sucrose) at 1 ml/min. After 2 to 3 min of perfusion, the medium was changed to pamamycin-containing MP3 medium for 1 min, then returned to MP3 medium. The ratios of the fluorescent intensities at 340 nm/360 nm (F340/F360) were measured at 4 or 5 points in the field of view every 0.5 or 1.2 s. The [Ca$^{2+}$] was estimated by fitting the F340/F360 value to a calibration curve prepared beforehand from known concentrations of Ca$^{2+}$. A different mycelial specimen was used in each experiment.

The time course of [Ca$^{2+}$], in S. alboniger at 20 μM of pamamycin-607 is shown in Fig. 1 (line plot without symbol). [Ca$^{2+}$], increased immediately after treatment with pamamycin-607 was begun then gradually decreased. The peak [Ca$^{2+}$], value was 2.0 times the average [Ca$^{2+}$], before the pamamycin application. To clarify the results, we calculated the mean of the [Ca$^{2+}$], values every 30 sec using software data analysis and the values were plotted (Fig. 1, line plots with symbols). The magnitude of the [Ca$^{2+}$], increase became smaller as the concentration of pamamycin-607 was lowered from 20 to 10 then to 5 μM. The steady state [Ca$^{2+}$], value varied from 50 to 150 nm.

![Fig. 1. Dose-dependent Effects of Pamamycin-607 on [Ca$^{2+}$], Changes in S. alboniger IFO 12738. Pamamycin-607-containing medium was perfused during the period indicated by the bar. Changes in [Ca$^{2+}$], values at 20 μM of pamamycin-607 were measured at 5 points in the field of view every 0.5 s, and the mean values plotted (line plot without symbol). Symbols represent the 30 sec-averages of [Ca$^{2+}$], values measured every 0.5 s.](image-url)

* To whom correspondence should be addressed.
† Present address: Central Research Laboratories, Hokko Chemical Industry Co., Ltd., 2165 Toda, Atsugi, Kanagawa 243, Japan.

Abbreviation: [Ca$^{2+}$], concentration of intracellular calcium ion.
Effects of Pamamycin-607 on $[Ca^{2+}]_i$ in \textit{S. alboniger}

Fig. 2. Digitized Image of $[Ca^{2+}]_i$ Change in \textit{S. alboniger} IFO 12738 Produced by Pamamycin-607.

The fluorescence intensity of the Fura-2-loaded mycelium has been converted to the brightness seen in the image. Objective lens, 20 x; A, just before application of pamamycin-607; B, 40 s after application (20 $\mu$M); C, 80 s; D, 480 s.

Fig. 3. Aerial Mycelium Formation in \textit{S. alboniger} IFO 12738 after One-min Treatment with Pamamycin-607 or 649.

A, control; B, pamamycin-607 treated; C, pamamycin-649 treated. Mycelia were treated with pamamycin-607 or 649 for 1 min, spread on plates, and the plates incubated at 28°C for 4 days.

depending on the experiment, but the standard error was small. The scatter in the $[Ca^{2+}]_i$ values probably is due to the pellet-like shape of the mycelial specimen and to the extent of the uptake and hydrolysis of Fura-2-AM. These factors raise the background intensity of fluorescence.\textsuperscript{51}

The digitized image of $[Ca^{2+}]_i$ change at 20 $\mu$M pamamycin-607 is shown in Fig. 2. Before pamamycin-607 treatment (Fig. 2A), the entire mycelium shows low $[Ca^{2+}]_i$. Treatment with pamamycin-607 induced a $[Ca^{2+}]_i$ increase a little way in from the edge of the mycelium (Fig. 2B), the intense $[Ca^{2+}]_i$ area spreading outward to the edge with time (Fig. 2C and D).

Effects of pamamycin-649 on $[Ca^{2+}]_i$, were examined. Pamamycin-649, a 1:1 mixture of pamamycin-649A and -649B, has no aerial mycelium-inducing activity.\textsuperscript{51} No change in $[Ca^{2+}]_i$ occurred upon the treatment with pamamycin-649 (data not shown).

To clarify the relationship between the transient increase
in $[\text{Ca}^{2+}]_i$ and aerial mycelium-induction, we treated mycelia cultured as described in the section on $[\text{Ca}^{2+}]_i$ measurement with 50 $\mu$M of pamamycin-607 or -649 for 1 min then washed and spread them on Hickey and Tresner’s agar plates containing cerulenin $^{11}$ (60 $\mu$g/ml). After 4 days of incubation at 28°C, we observed whether aerial mycelia were formed. Under these conditions, *S. alboniger* IFO 12738 could not form aerial mycelia (Fig. 3A) because pamamycin production was inhibited by cerulenin, but substrate mycelia grew normally. Mycelia treated with pamamycin-607 formed aerial mycelia, but those treated with pamamycin-649 did not (Fig. 3B, C). These results suggest that the increase in $[\text{Ca}^{2+}]_i$ produced by pamamycin-607 causes aerial mycelium-induction in *S. alboniger*.

Ours is the first report of measurement of the $[\text{Ca}^{2+}]_i$ of an actinomycete showing a correlation with physiological functions. We now are investigating the $[\text{Ca}^{2+}]_i$ regulation mechanism that is affected by pamamycin-607 and the reaction that takes place between $[\text{Ca}^{2+}]_i$ and aerial mycelium formation.

**References**