Salicylic Acid Induces a Cytosolic Ca\(^{2+}\) Elevation in Yeast

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Cytosolic free calcium ion concentration ([Ca\(^{2+}\)]\(_{i}\)) after a salicylic acid (SA)-stimulus was monitored in cells of the yeast Saccharomyces cerevisiae expressing apaoequorin, which constitutes a Ca\(^{2+}\)-sensitive luminescent protein, aequorin, when combined with coelenterazine. SA induced a transient [Ca\(^{2+}\)]\(_{i}\) elevation that was dependent on the concentration of SA and pH of the SA solution. The SA-induced [Ca\(^{2+}\)]\(_{i}\) elevation was not reduced in Ca\(^{2+}\)-deficient medium, suggesting that Ca\(^{2+}\) was mobilized from an intracellular Ca\(^{2+}\) store(s). Benzoic acid, butyric acid and sorbic acid did not induce a [Ca\(^{2+}\)]\(_{i}\) elevation.

Key words: aequorin; cytosolic Ca\(^{2+}\); salicylic acid; Saccharomyces cerevisiae

Signal transduction pathways in eukaryotic cells have been investigated using yeast cells as a model system. Ca\(^{2+}\) is widely accepted as an important second messenger in intracellular signal transduction in eukaryotic cells. Stimulus-coupled change in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in animal cells have been studied using fluorescence dyes. Plant cells expressing apaoequorin which constitutes a Ca\(^{2+}\)-dependent luminescent protein, aequorin, with the chromophore coelenterazine, were successfully used for investigating stimulus-induced Ca\(^{2+}\) transients. However, studies on Ca\(^{2+}\) mobilization in yeast are limited to a few reports. For instance, Iida et al. reported a [Ca\(^{2+}\)]\(_{i}\) elevation induced by the mating pheromone (α factor) in a-mating type cells using a Ca\(^{2+}\)-indicator (fura-2). Nakajima-Shimada et al. transformed yeast cells with apaoequorin and monitored [Ca\(^{2+}\)]\(_{i}\) changes in response to mating pheromone and glucose in glucose-starved yeast cells. Cell cycle control by calcium and calmodulin in yeast has been well documented. An immediate and transient [Ca\(^{2+}\)]\(_{i}\) elevation in response to a hypoosmotic shock has been reported in apaoequorin transgenic yeast cells. However, the Ca\(^{2+}\) mobilizing mechanism into cytosol has remained unclear in yeast cells.

Nonsteroidal anti-inflammatory agents, such as salicylic acid (SA) or acetyl SA(aspirin), have been reported to induce cell cycle arrest and/or apoptosis in cancer cells in animals and heat shock response in human cells. In plant cells, SA functions as an intracellular and/or an intracellular signal relating to systemic acquired resistance, a disease response induced by pathogen infection. In yeast, it has been reported that SA decreased the viability of the cells. Recently, Giardina and Lis reported that sodium salicylate stimulates the binding of a heat shock transcription factor to the promoter region of a heat shock gene and represses the expression of a HSP gene. However, the upstream in the signal transduction pathway involved in SA-signaling has not been described well in any organisms.

In this study, we focused on a [Ca\(^{2+}\)]\(_{i}\) change in response to SA treatment in yeast cells expressing apaoequorin.

Materials and Methods

Culture of cells and constitution of aequorin. Yeast cells (S. cerevisiae H208-3B carrying pGPAQ19) were grown in a synthetic medium (SD medium) minus tryptophan at 25°C up to 5 × 10\(^{6}\) cells/ml. The cells were collected by centrifugation at 700 × g for 10 min, washed twice, and resuspended at 1 × 10\(^{6}\) cells/ml in a glucose-deficient SD (SD-glucose) medium to suppress the growth of the cells. The resuspended cells were incubated with 20 µM coelenterazine at 25°C for 3 h with shaking (130 rpm) to constitute aequorin. Cells were washed twice with SD-glucose medium to remove the remaining coelenterazine and resuspended in SD-glucose medium at 2.5 × 10\(^{6}\) cells/ml. This cell-suspension was used for the monitoring of aequorin luminescence within 2 h.

Chemicals. Coelenterazine, 2-(p-hydroxybenzoyl)-6-(hydroxypyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, were chemically synthesized as reported. SA was dissolved in 400 mM Mes-KOH (pH 4.0, unless otherwise indicated) containing 20% ethanol and added to the cell suspension at 1/20 volume (10 µl). The final concentration of SA was 5 mM, unless otherwise indicated.

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Abbreviations: BA, benzoic acid; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N, N, N′, N′-tetracetoic acid; [Ca\(^{2+}\)]\(_{i}\), cytosolic free calcium ion concentration; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid; IP\(_{3}\), inositol 1,4,5-trisphosphate; SA, salicylic acid; rlu, relative luminescence unit
The other chemicals were the highest grade commercially available.

Monitoring of aequorin luminescence. Monitoring of aequorin luminescence was done essentially as reported. The coelenterazine-loaded cell-suspension (190 μl) was transferred to a glass tube set in a luminometer (Chem-Glow photometer, American Instrument Co., Silver Spring, MD). Aequorin luminescence induced by the addition of SA or other compounds was recorded with a pen recorder (Rikadenki Co. Tokyo), and was expressed as relative luminescence units (rlu).

Results

SA-induced [Ca^{2+}]_{so} elevation
The aequorin luminescence indicating [Ca^{2+}]_{so} elevation was observed following the addition of SA to the aequorin-containing yeast cells (Fig. 1). When treated with 2 mM SA, a gradual luminescence increase was observed after a lag period (Fig. 1A, left middle trace). The higher concentrations of SA induced stronger luminescence with a shorter lag period. When treated with 5 mM SA, luminescence started at 5 s and peaked at 30 s, then returned quickly to the original level within 5 min (Fig. 1A, right bottom trace). No increase in luminescence was observed below 1 mM SA. The peak-height of aequorin luminescence was dependent on the concentration of SA (Fig. 1B). The peak-time also shifted depending on SA concentration.

The SA-induced [Ca^{2+}]_{so} elevation was dependent on the pH of SA solution (Fig. 2). The lower pH was more effective within the pH range examined (pH 4–5.5). No aequorin luminescence was observed when the buffer without SA was put on the cell suspension at any pH.

Effects of the other organic acids on [Ca^{2+}]_{so}
Several organic acids were tried on the aequorin-containing cells. SA induced a marked [Ca^{2+}]_{so} elevation with a lag period as observed above. Sorbic acid, butyric acid, or benzoic acid (BA) induced an immediate, small, and transient [Ca^{2+}]_{so} elevation (Fig. 3). In spite of the structural similarity between SA and BA, BA did not induce a marked [Ca^{2+}]_{so} elevation, suggesting that SA is highly specific in the induction of [Ca^{2+}]_{so} elevation.

Effects of extracellular Ca^{2+} concentration on the SA-induced [Ca^{2+}]_{so} elevation
To examine whether an influx of the extracellular Ca^{2+} is required for the SA-induced [Ca^{2+}]_{so} elevation or not, a Ca^{2+}-deficient synthetic (SD-Ca) medium was prepared. SD medium contained approximately 250 μM free Ca^{2+} when estimated with a Ca^{2+}-electrode (IS 561-Ca^{2+}, Philips). The free Ca^{2+} concentration of SD-Ca medium was below the detection limit of the Ca^{2+}-electrode (below 10 μM). This data coincides the previous data that SD-Ca medium contained 0.24 μM Ca^{2+}. The cell suspension was washed with SD-Ca medium containing 1 mM EGTA, and resuspended in the standard or

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**Fig. 1.** Effects of Various Concentration of SA on the [Ca^{2+}]_{so} in Yeast Cells.
SA was added to the coelenterazine-loaded cells as described in "Materials and Methods". A, Various concentration of SA (0–5 mM) was added to the cell suspension where indicated by the arrowheads. B, The peak height of the luminescence was plotted as a function of SA concentration.

**Fig. 2.** Effects of pH on the SA-Induced [Ca^{2+}]_{so} Elevation.
SA dissolved in Mes-KOH with indicated pH was added to the cell suspension. The peak height of the aequorin luminescence after SA addition was plotted as a function of pH of SA solution.
SD-Ca medium. There is no significant difference between the profiles of the SA-induced luminescence in the cells suspended in SD-Ca medium and those in SD medium (Fig. 4A). This indicates that Ca\(^{2+}\) was not derived from the extracellular Ca\(^{2+}\) in the medium.

When cells were incubated for 90 min in SD-Ca medium, the SA-induced luminescence decreased by 60% (Fig. 4B, center trace). The intensity of the luminescence did not reach to that in the high Ca\(^{2+}\) medium, even when 680 \(\mu\)M CaCl\(_2\) (comparable to the total Ca\(^{2+}\) in SD medium) was added to SD-Ca medium before the addition of SA (Fig. 4B, right trace). This result indicates that the intracellular Ca\(^{2+}\)-store was starved during the incubation in SD-Ca medium and was not refilled within a short period (1.5 min) by the addition of CaCl\(_2\). Inclusion of BAPTA (5 mM), a Ca\(^{2+}\) chelator, in the medium did not reduce the SA-induced [Ca\(^{2+}\)]\(_{30s}\) elevation (data not shown). These results suggest that Ca\(^{2+}\) required for the SA-induced [Ca\(^{2+}\)]\(_{30s}\) elevation is mobilized from an intracellular store(s) rather than from the extracellular medium.

**Discussion**

The signal transduction pathway following an SA stimulus has not been clear in any organism. In plants, the SA-signal transduction pathway has been extensively studied,\(^{10}\) and the initial event in SA recognition has been proposed to be the direct inhibition of catalase and the subsequent generation of H\(_2\)O\(_2\).\(^{10}\) However, this model is questioned in recent reports.\(^{17}\) Requirement of extracellular Ca\(^{2+}\) in SA-induced defense gene accumulation was reported in tobacco leaves\(^{18}\) and carrot suspension culture,\(^{10}\) suggesting that Ca\(^{2+}\) functions as a second messenger of SA-signaling in these plants. In the yeast cells, the involvement of Ca\(^{2+}\) in SA-signaling has not been reported. In this study, we monitored the [Ca\(^{2+}\)]\(_{30s}\) elevation after SA treatment in yeast cells by an apoaequorin-expressing system.

The SA-induced [Ca\(^{2+}\)]\(_{30s}\) elevation in yeast cells was dose- and pH-dependent. The pH dependence may be explained by a pH-dependent membrane permeability of SA.\(^{10}\) As the pKa value of SA is 2.98, SA dissociates at neutral pH, but approximately 10% of SA is protonated and discharged at pH 4.0. The discharged SA (SAH) might diffuse across the plasma membrane, stimulate a Ca\(^{2+}\) influx machinery (ies), and cause the [Ca\(^{2+}\)]\(_{30s}\) elevation. In this study, it is suggested that the [Ca\(^{2+}\)]\(_{30s}\) elevation is not induced by the simple acidification of the
cytosol caused by the diffusion of SAH into the cytosol, because both SA and BA (pKa = 4.19) can reach the cytosol by simple diffusion and acidify it.\textsuperscript{20}

Ca\textsuperscript{2+} depletion in the medium did not affect the SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation in a short period, suggesting that Ca\textsuperscript{2+} was not from the extracellular medium nor the cell-wall bound Ca\textsuperscript{2+}, but might be released from an intracellular store(s). If Ca\textsuperscript{2+} was from the extracellular medium or the cell-wall bound Ca\textsuperscript{2+}, the [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation would be reduced in a Ca\textsuperscript{2+}-deficient medium. A prolonged incubation of cells in the Ca\textsuperscript{2+}-deficient medium caused a decrease in the SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation and this decrease was not reversed by the addition of Ca\textsuperscript{2+} to the medium. The intracellular Ca\textsuperscript{2+} store might be depleted during the long-term incubation in Ca\textsuperscript{2+}-deficient medium and result in the reduction of the [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation. Refilling of Ca\textsuperscript{2+} in the intracellular store might be a relatively slow process, as the [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation was not fully restored within 1.5 min after Ca\textsuperscript{2+} addition. If Ca\textsuperscript{2+} came from the medium or the cell wall-bound Ca\textsuperscript{2+}, the reduced [Ca\textsuperscript{2+}]\textsubscript{cyt} would be replaced by the Ca\textsuperscript{2+} addition.

A H\textsuperscript{+}/Ca\textsuperscript{2+} antiporter (VCT)\textsuperscript{20} and a Ca\textsuperscript{2+}-pump (PMCI)\textsuperscript{20} located in the tonoplast, and a Ca\textsuperscript{2+}-pump (PMRI) located in the Golgi apparatus.\textsuperscript{20} Intracellular localization of these Ca\textsuperscript{2+} transporters suggests that Ca\textsuperscript{2+} is stored in the vacuole and the Golgi apparatus in yeast cells. However, the SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation was not inhibited by incubation with batrachomycin A (100 nm), a V-type ATPase inhibitor (data not shown).

A gene that is responsible for pheromone (α-factor)-induced Ca\textsuperscript{2+} uptake (MID1) was isolated by Iida et al.\textsuperscript{20} The gene product was located in the plasma membrane, thus Ca\textsuperscript{2+} was mobilized extracellularly through the plasma membrane in response to pheromone stimulus. We monitored the SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation in mid1 mutant cells expressing apoaequorin, but no difference was observed as compared with wild type cells (data not shown). This result suggests that Mid1 is not involved in the SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation.

When hypotonic shock was applied to yeast cells, a biphasic [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation occurred.\textsuperscript{23} Since only the second phase of [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation was canceled by BAPTA, Ca\textsuperscript{2+} for the second phase elevation might be from the medium, and Ca\textsuperscript{2+} for the first phase from the intracellular store. The first phase Ca\textsuperscript{2+} was considered to come through a stretch-activated channel, because it was blocked by gadolinium. However, the reported effects of gadolinium should be carefully evaluated, since it caused light scattering when it was added to the culture medium as indicated in this study.

The detailed mechanism of SA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation remained unclear, but it is different from that of the pheromone- or the hypotonic shock-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation. It is evident that Ca\textsuperscript{2+} is mobilized via different mechanisms in response to different stimulus in yeast cells.

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References