Mechanism of Stimulation of the Calcium Adenosinetriphosphatase by Jasmone†

A. P. Starling, G. Hughes, J. M. East, and A. G. Lee*

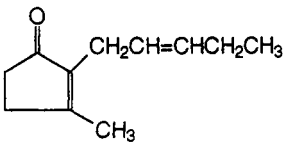
Department of Biochemistry and SERC Centre for Molecular Recognition, University of Southampton, Southampton SO9 3TU, U.K.

Received October 4, 1993; Revised Manuscript Received December 28, 1993®

ABSTRACT: The ATPase activity of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is increased ca. 3-fold at 25 °C and pH 7.2 by jasmone at a concentration of 100 μM, concentrations above 10 mM resulting in reduced stimulation. Stimulation by methyl jasmonate, menthol, or menthone requires much higher concentrations. Effects of jasmone are much less marked at 37 °C than at 25 °C, and much higher concentrations of jasmone are required to stimulate ATPase activity at pH 6.0 than at pH 7.2. The effects of jasmone on the ATPase are highly specific. Jasmone has no effect on the E1 ⇄ E2 equilibrium constant for the ATPase or on Ca^{2+} binding. The rate of phosphorylation by ATP is unaffected by jasmone, and only small effects are seen on the reaction of the phosphorylated ATPase with ADP. Jasmone does, however, increase the rate of dephosphorylation by a factor of 2 and the rate of dissociation of Ca^{2+} from the phosphorylated ATPase by a factor of 3. Jasmone decreases the level of phosphorylation of the ATPase by P_i in the absence of Ca^{2+} consistent with a decrease in the equilibrium constant $E2P_iMg \rightleftarrows E2PMg$. Reconstitution of the ATPase with dimyristoleoylphosphatidylcholine decreases the stoichiometry of Ca^{2+} binding from the usual 2:1 to 1:1. Unlike other hydrophobic molecules, jasmone failed to reverse this effect. Further, jasmone had very similar effects on the activity of the ATPase reconstituted with either dimyristoleoylphosphatidylcholine or dioleoylphosphatidylcholine, whereas other hydrophobic molecules caused a much greater stimulation of activity for the ATPase reconstituted with the short-chain lipid.

A number of inhibitors of the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR)[1] are known, including the sesquiterpene lactones thapsigargin (Brune & Ullrich, 1991; Sagara & Inesi, 1991; Kijima et al., 1991; Lytton et al., 1991; Wictome et al., 1992a,b; Sagara et al., 1992) trilobolide, and thapsivillosin A (Henderson et al., 1994b); nonylphenol (Michelangeli et al., 1990b); and the hydroquinone 2,5-di-*tert*-butyl-1,4-benzohydroquinone (Llopis et al., 1991; Wictome et al., 1992b; Lytton et al., 1991). Less is known, however, about molecules stimulating ATPase activity. It has been reported that addition of diethyl ether to the ATPase results in a 2-fold stimulation of ATPase activity at 22 °C (Salama & Scarpa, 1980; Bigelow & Thomas, 1987). Diethyl ether has also been reported to cause a decrease in order and an increase in fluidity in phospholipid bilayers and to increase the rate of rotation of the ATPase in the plane of the membrane (Bigelow & Thomas, 1987; Birmachu & Thomas, 1990); as a consequence, it has been suggested that the effects of diethyl ether on the activity of the ATPase could follow from the increased fluidity of the membrane. Short-chain alcohols have also been reported to increase the rates of motion within phospholipid bilayers and to increase ATPase activity, although at high concentrations alcohols inhibit the ATPase (Melgunov et al., 1988; Almeida et al., 1986; Kondo & Kasai, 1973). 3,3′,4′,5-Tetrachlorosalicylanilide, a lipophilic weak acid, has also been reported to stimulate the ATPase, possibly via effects on the lipid bilayer (Wakabayashi et al., 1988). Effects of phospholipids on the activity of the ATPase, however, have been suggested to follow not indirectly from changes in the properties of the phospholipid bilayer but directly from changes in the phospholipid–protein interaction leading to conformational changes in the ATPase (East et al., 1984; Michelangeli et al., 1990c, 1991; Lee, 1991).

We were interested to find molecules which might stimulate ATPase activity while being unlikely to have any effect on the properties of the phospholipid bilayer. Peppermint oil has been shown to cause relaxation of gastrointestinal muscle, with the most active ingredient of the oil being jasmone [3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one; I], a minor component of the oil (Hills et al., 1990). Here we show that jasmone causes a large increase in ATPase activity for the Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum and define the steps in the reaction sequence of the ATPase that are sensitive to binding of jasmone.



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MATERIALS AND METHODS

Jasmone, menthone, and menthol were obtained from Aldrich. Methyl jasmonate was a gift from Firmenich. Dimyristoleoylphosphatidylcholine [di(C14:1)PC], dioleoylphosphatidylcholine [di(C18:1)PC] and dinervonylphosphatidylcholine [(di(C24:1)PC)] were obtained from Avanti. Sarcoplasmic reticulum from rabbit skeletal muscle and purified Ca^{2+}-ATPase were prepared as described in Michelangeli et al. (1991). ATPase activity was determined at 25 °C as described (Michelangeli et al., 1991). Reconstitutions were performed as described in Michelangeli et al. (1990c). Ca^{2+} uptake by sealed SR vesicles (0.3 mg of protein/mL) was monitored spectrophotometrically by using the dye murexide in 40 mM Mes/Tris and 20 mM Mg^{2+}, pH 6.3. Spectra were

† We thank the SERC and the Wessex Medical Trust for financial support.

® Abstract published in *Advance ACS Abstracts,* February 15, 1994.

[1] Abbreviations: DPH, diphenylhexatriene; NBD, nitrobenzo-2,1,3-oxadiazole; SR, sarcoplasmic reticulum; di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C24:1)PC, dinervonylphosphatidylcholine.

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run on an Aminco DW2000 dual-wavelength spectrometer with a wavelength pair of 507 and 542 nm, as described in McWhirter et al. (1987).

Steady-state measurements of phosphorylation by $^{32}P_i$ were carried out in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA, 10 mM $MgSO_4$, the required concentration of P_i at 25 °C, and protein at a concentration of 0.2 mg/mL. After 15 s the reaction was quenched by addition of 10 volumes of quenching solution (25% trichloroacetic acid and 0.13 M phosphoric acid). The sample was placed on ice for 15 min, and then the precipitate was collected by filtration through Whatman GF/B glass fiber filters and finally counted in OptiPhase HighSafe 3. Steady-state measurements of phosphorylation by [γ-^{32}P]ATP were carried out at 25 °C in a medium containing 40 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, and the given concentrations of ATP and Ca^{2+}. Samples were quenched as above and collected by filtration on Whatman GF/C filters.

Steady-state measurements of Ca^{2+} release from the ATPase on addition of ATP were made using Antipyralazo III to monitor Ca^{2+} concentrations. The change in absorbance at 720–790 nm was monitored using an Aminco DW2000 dual-wavelength spectrophotometer. ATPase (0.3 mg/mL) was added to buffer (40 mM Mes/Tris, pH 6.0, and 20 mM $MgSO_4$) containing 100 μM Antipyralazo III. Aliquots of a concentrated solution of $CaCl_2$ were added to a final concentration of 50 μM. An aliquot of a concentrated solution of ATP was then added to give a final ATP concentration of 40 μM.

The time dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined using a Biologic rapid filtration device, at room temperature (typically 20 °C). Unless otherwise stated, a suspension of the ATPase in buffer (20 mM Hepes/Tris, 100 mM KCl, 20 mM $MgSO_4$, 100 μM $^{45}CaCl_2$, and 500 μM [^{3}H]sucrose), corresponding to 200 μg of ATPase, was loaded onto a Millipore HAWP 0.45-μm filter and then rapidly perfused with the same buffer but containing 2 mM ATP and 100 μM $^{40}CaCl_2$. The filter was then counted in Optiphase HiSafe 3. The amount of Ca^{2+} bound to the ATPase was calculated as previously described (Starling et al., 1993).

The time dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP at 25 °C was determined using a Biologic QFM-5 system. ATPase (0.2 mg/mL) was incubated in 20 mM Hepes/Tris, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl, and 100 μM $CaCl_2$. This was mixed with an equal volume of the same buffer containing 50 μM [γ-^{32}P]ATP, followed by quenching with 25% trichloroacetic acid and 0.13 M phosphoric acid. The precipitated protein was filtered, washed, and counted as described above.

The time dependence of dephosphorylation of the ATPase phosphorylated with $^{32}P_i$ was determined as described by Henao et al. (1991). The ATPase (4 mg/mL) was incubated in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM $^{32}P_i$, 20 mM $MgSO_4$, and 14% (v/v) dimetheyl sulfoxide. One volume of this suspension was mixed with 16 volumes of 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM $MgSO_4$, 5.3 mM ATP, and 200 μM jasmone, followed by quenching as described above. The time dependence of dephosphorylation of the ATPase phosphorylated with [γ-^{32}P]-ATP was determined using the triple-mixing capability of the Biologic QFM-5 system. The ATPase (0.2 mg/mL) was incubated in 20 mM Mes/Tris, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl, and 100 μM $CaCl_2$. This was mixed with an equal volume of the same buffer containing 50 μM [γ-^{32}P]ATP. After 100 ms, the mixture was mixed with an equal volume of 2.5 mM unlabeled ATP and 200 μM jasmone, in the same buffer, followed by quenching as described above.

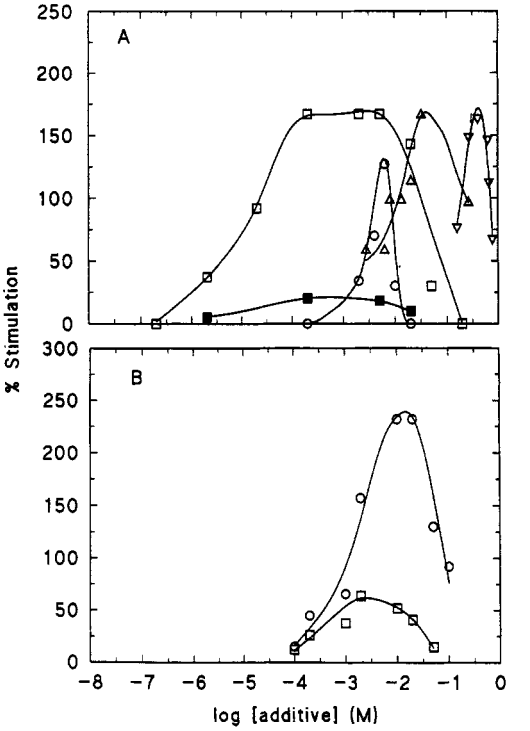


FIGURE 1: (A) Effects of jasmone (□), methyl jasmonate (○), menthone (△), and menthol (▽) on the ATPase activity of the purified ATPase (0.07 μM) measured at 2.1 mM ATP, pH 7.2, and 10 μM Ca^{2+} at 25 °C and of jasmone at 37 °C (■). (B) Effects of jasmone at pH 6.0 in 170 μM Ca^{2+} at 25 °C (○) and 37 °C (□). Results are expressed as percent stimulation of activity measured in the absence of jasmone.

The time dependence of ADP-induced dephosphorylation was determined by first incubating SR vesicles (0.2 mg/mL) in 50 mM MOPS, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl, 50 μM $CaCl_2$, and 50 μM [γ-^{32}P]ATP for ca. 1 min. This was then mixed in a 1:1 ratio with the same buffer containing 5 mM $MgSO_4$, 100 mM KCl, 10.08 mM $CaCl_2$, 10 mM EGTA, 3 mM ADP, and 400 μM jasmone (giving a final Ca^{2+} concentration of 44 μM), followed by quenching as described above.

The ATPase was labeled with NBD as described (Wictome et al., 1992a). Fluorescence measurements were performed at 25 °C using an SLM Aminco 8000C fluorimeter with excitation and emission wavelengths of 430 and 520 nm, respectively. To reduce the signal due to scattered light, the excitation beam was passed through a 450-nm long-wavelength cutoff filter (450FLO T-50, Andover Corporation) and the emission was passed through a Hoya Y50 500-nm short-wavelength cutoff filter. Tryptophan fluorescence was recorded with excitation and emission wavelengths of 290 and 340 nm, respectively. Fluorescence polarizations of DPH were measured at a DPH:di(C18:1)PC molar ratio of 1:100, with excitation and emission wavelengths of 348 and 426 nm, respectively, and corrected for instrumental polarization.

RESULTS

Effects of Jasmone on Steady-State Activity of ATPase. As shown in Figure 1A, addition of jasmone to the purified Ca^{2+}-ATPase at 25 °C at concentrations up to 10 mM results in an increase in ATPase activity by up to factor of ca. 3, followed by inhibition at higher concentrations. At 37 °C,

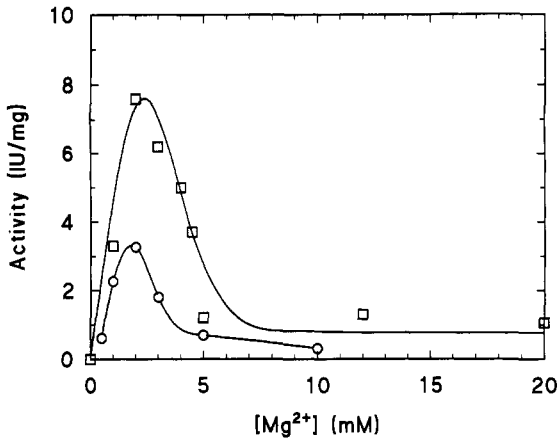


FIGURE 2: Effects of jasmone on the Mg^{2+} dependence of the activity (IU/mg of protein) of the purified ATPase, measured at pH 8.0 and 25 °C in the presence of 2.1 mM ATP and 0.07 μM ATPase: ATPase alone (○) and in the presence of 2 mM jasmone (□). All solutions contained 40 mM Tris/Hepes and 100 mM KCl.

effects on activity are very small (Figure 1A). A similar pattern is observed at pH 6.0 (Figure 1B) except that a much higher concentration of jasmone is required to produce maximal stimulation of activity. The major components of peppermint oil, menthol and menthone (Hills et al., 1990), only affected ATPase activity at very high concentrations, and concentrations resulting in activation and inhibition were much closer than for jasmone (Figure 1A); comparable effects were obtained over the same concentration range with hexanol (data not shown). Methyl jasmonate was ca. 100-fold less effective in stimulating the ATPase, and again concentrations resulting in activation and inhibition were much closer than for jasmone (Figure 1A). Effects of jasmone on activity were fully reversible.

At high pH values Mg^{2+} has been shown to inhibit ATPase activity, probably by binding to Ca^{2+} sites on the phosphorylated ATPase (Bishop & Al-Shawi, 1988; Michelangeli et al., 1990a). At pH 8.0 in the presence of 2.1 mM ATP, maximal ATPase activity was observed at 2 mM Mg^{2+}, with higher concentrations of Mg^{2+} causing inhibition; in the presence of 2 mM jasmone, activity was increased by a factor of ca. 2, again with concentrations of Mg^{2+} greater than 2 mM being inhibitory (Figure 2).

Ca^{2+} uptake by sealed SR vesicles can be followed spectrophotometrically by using the dye murexide to monitor the external Ca^{2+} concentration (McWhirter et al., 1987). At 25 °C, pH 6.3, and 50 μM ATP, maximal Ca^{2+} uptake was 139 and 123 nmol/mg of protein in the absence and presence of 20 μM jasmone, respectively. Following uptake of Ca^{2+} by SR vesicles, spontaneous release of some of the accumulated Ca^{2+} is observed (McWhirter et al., 1987); the rate of this spontaneous release was unaffected by jasmone (data not shown).

Effects of Jasmone on Partial Reactions of ATPase. Table 1 shows the maximal level of phosphorylation of the ATPase observed after 15 s of incubation of the ATPase with [γ-^{32}P]-ATP at 0.1 mM Ca^{2+} and at 1 mM Ca^{2+}. At 0.1 mM Ca^{2+} in the absence of jasmone, lower levels of phosphoenzyme formation are observed at 20 μM ATP than at 100 μM ATP, suggesting significant hydrolysis of the ATP after 15 s; in the presence of 2 mM jasmone, levels of phosphoenzyme formation are considerably reduced. In the presence of 1 mM Ca^{2+}, levels of phosphoenzyme formation are higher, and effects of jasmone are much less marked, particularly at 100 μM ATP.

Measurement of the time course of phosphorylation of the ATPase when the ATPase incubated in the presence of Ca^{2+}

Table 1: Phosphorylation of ATPase with [γ-^{32}P]ATP[a]

concn of ATP (μM)	concn of Ca^{2+} (mM)	level of phosphorylation (nmol of EP/mg of protein) ATPase	ATPase + 2 mM jasmone
20	0.1	1.24	0.12
100	0.1	3.6	0.29
20	1	2.6	1.1
100	1	3.3	3.3

[a] The ATPase (0.9 μM) was incubated with the given concentrations of [γ-^{32}P]ATP and Ca^{2+} for 15 s at pH 7.2, 25 °C, before the reaction was quenched.

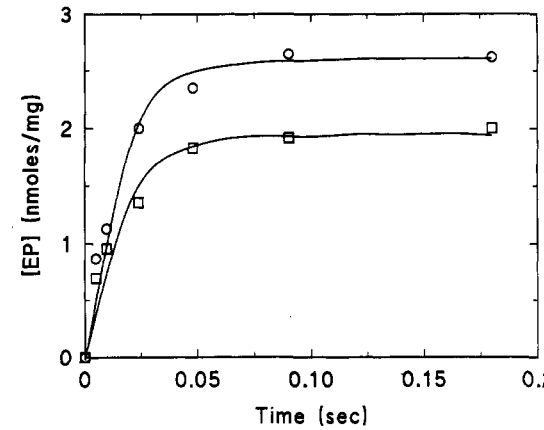


FIGURE 3: Rate of phosphorylation of the ATPase by [γ-^{32}P]ATP in the absence (○) or presence (□) of 200 μM jasmone. The ATPase (0.2 mg/mL) incubated in buffer (pH 7.2, 20 mM Hepes/Tris, 5 mM Mg^{2+}, 100 mM KCl and 100 μM Ca^{2+}) was mixed in a 1:1 ratio with the same buffer containing 50 μM [γ-^{32}P]ATP to give a final ATP concentration of 25 μM. The lines represent fits to single-exponential decays with the parameters given in the text.

was mixed with ATP showed that 200 μM jasmone had no significant effect on the rate of phosphorylation, which was determined to be 65 ± 9 s^{-1} and 66 ± 10 s^{-1} in the absence and presence of jasmone, respectively (Figure 3). However, the maximal level of phosphorylation was reduced by the presence of jasmone, from 2.6 nmol of EP/mg of protein to 1.9 nmol of EP/mg of protein (Figure 3).

Phosphorylation of the ATPase with ATP in uncoupled preparations leads to the release of Ca^{2+} into the medium which can be monitored by using Antipyralazo III. In the absence of jasmone, addition of 40 μM ATP to the ATPase led to a release of 6.5 nmol of Ca^{2+}/mg of protein, which reversed after hydrolysis of the ATP; in the presence of 200 μM jasmone, no release of Ca^{2+} could be detected (data not shown). A similar observation was made using $^{45}Ca^{2+}$. The ATPase was incubated with 100 μM $^{45}Ca^{2+}$ in buffer containing 20 mM Mg^{2+} and 100 mM KCl, at pH 7.2 and then perfused with the same medium containing 100 μM $^{45}Ca^{2+}$ and 2 mM ATP. Steady-state releases of Ca^{2+} of 4.1 and 1.6 nmol/mg of protein were observed in the absence and presence of 2 mM jasmone, respectively.

Phosphorylation of the ATPase by ATP in the presence of Ca^{2+} has been shown to cause a small transient decrease in tryptophan fluorescence intensity which is reversed when the added ATP is hydrolyzed (Figure 4; Champeil et al., 1986). In the presence of the inhibitor nonylphenol, the decrease in tryptophan fluorescence has been observed to be of greater magnitude and longer lived (see Figure 4), attributed to an increase in the proportion of the ATPase in the low-fluorescent E2 state in the presence of nonylphenol (Michelangeli et al., 1990b). Jasmone had little effect on the magnitude of the

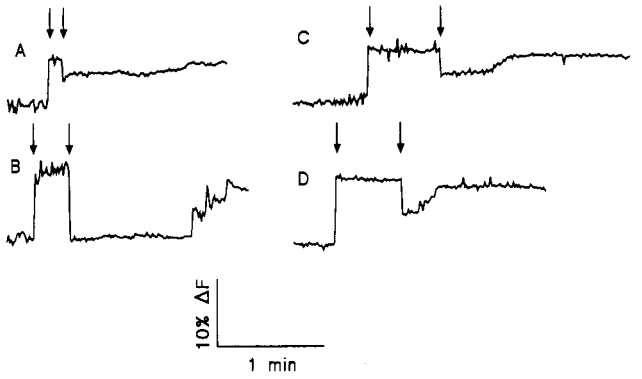


FIGURE 4: Effect of addition of Ca^{2+} (left arrow in each trace; final concentration, 250 μM) and ATP (right arrow in each trace; final concentration, 20 μM) on the tryptophan fluorescence intensity of the ATPase (1 μM) in 20 mM Hepes/Tris, pH 7.2, containing 25 μM EGTA, 100 mM KCl, and 5 mM Mg^{2+}: ATPase alone (A) or in the presence of 80 μM nonylphenol (B), 20 μM jasmone (C), or 2 mM jasmone (D).

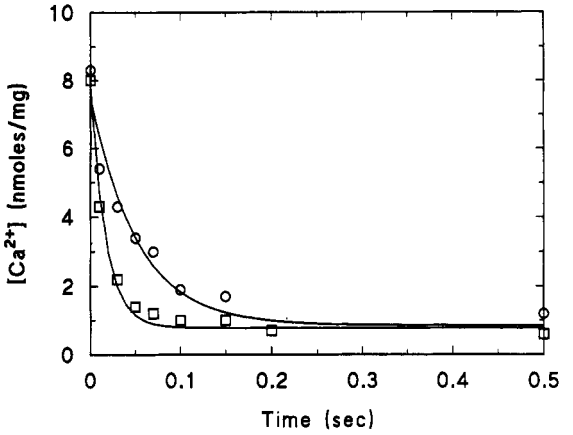


FIGURE 5: ATP-induced release of $^{45}Ca^{2+}$ from the ATPase. ATPase (0.4 mg/mL) was first equilibrated in pH 7.2 buffer (20 mM Hepes/Tris, 100 mM KCl, and 20 mM Mg^{2+}) containing 100 μM $^{45}Ca^{2+}$ and 0.5 mM [^{3}H]sucrose, and then 0.2 mg ATPase was adsorbed onto Millipore filters. The loaded filter was perfused for the given times with the same buffer containing 100 μM unlabeled Ca^{2+}, 0.5 mM [^{3}H]sucrose, and 2 mM ATP, in the absence (○) or presence (□) of 2 mM jasmone. The lines represent fits to single-exponential decays with the parameters given in the text.

fluorescence change seen following addition of ATP although the reversal of the change becomes faster, attributable to the increased rate of hydrolysis of ATP in the presence of jasmone (Figure 4).

The time dependence of Ca^{2+} release from the ATPase on phosphorylation can be followed using the technique of rapid filtration, in which the ATPase is adsorbed onto Millipore filters and perfused with specified media for defined periods of time. When the ATPase was incubated with 100 μM $^{45}Ca^{2+}$ in buffer containing 20 mM Mg^{2+} and 100 mM KCl, at pH 7.2, and then perfused with the same medium containing 100 μM unlabeled Ca^{2+} and 2 mM ATP, the rate of loss of $^{45}Ca^{2+}$ fitted to a single exponential process with rate constants of 20.0 ± 4.9 and 58.8 ± 8.3 s^{-1} in the absence and presence of 2 mM jasmone, respectively (Figure 5).

The effect of jasmone on the rate of dephosphorylation of E2P (Scheme 1) was investigated by first phosphorylating the ATPase with $^{32}P_i$ at pH 6.0 in the absence of Ca^{2+} and the presence of 14% dimethyl sulfoxide and then mixing it with an excess of a pH 7.5 medium containing KCl and ATP (Figure 6A). In the presence of dimethyl sulfoxide at pH 6.0, high levels of phosphorylation of the ATPase by P_i are obtained (de Meis, 1981), whereas at pH 7.5 in the presence of 100

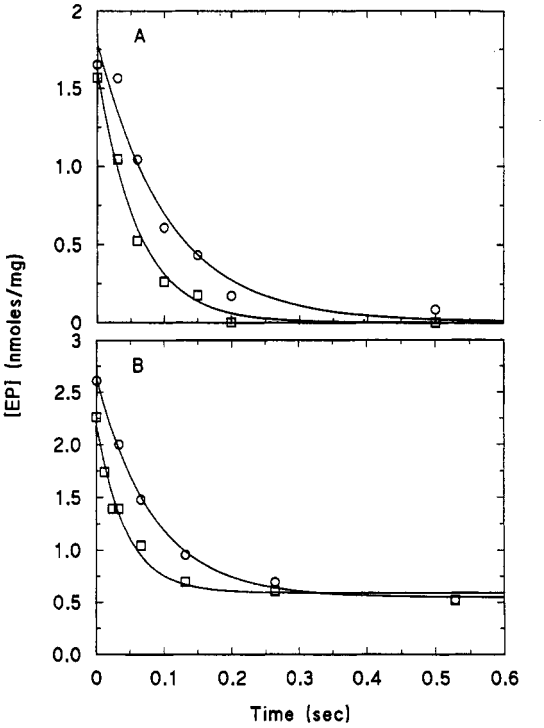
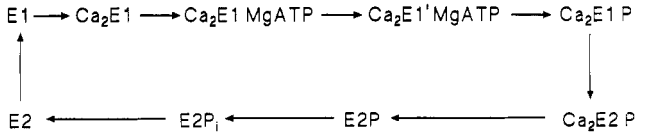


FIGURE 6: Effect of jasmone on the rate of dephosphorylation of the ATPase in the absence (A) or presence (B) of Ca^{2+}. (A) The enzyme syringe contained ATPase (4.0 mg/mL) in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM $^{32}P_i$, 20 mM Mg^{2+}, and 14% (v/v) dimethyl sulfoxide. The second syringe contained 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM Mg^{2+}, and 5.3 mM ATP and either no jasmone (○) or 200 μM jasmone (□). The contents of the enzyme syringe were mixed in a 1:16 volume ratio with the dephosphorylation mixture, and the reaction was quenched at the given times with 25% trichloroacetic acid. The lines represent fits to single exponentials with the parameters given in the text. (B) The enzyme syringe contained ATPase (0.2 mg/mL) in 20 mM Mes/Tris, pH 7.2, 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+}. This was mixed in a 1:1 ratio with a solution containing 50 μM [γ-^{32}P]-ATP in the same buffer. The mixture was incubated for 200 ms, and then mixed in a 1:1 ratio with the same buffer containing 2.5 mM unlabeled ATP and either no jasmone (○) or 400 μM jasmone (□). The reaction was quenched at the given times with 25% trichloroacetic acid. The lines represent fits to single exponentials with the parameters given in the text.

Scheme 1: Simplified Reaction Scheme for ATPase



mM KCl and the absence of dimethyl sulfoxide, levels of phosphorylation by P_i are very low (de Meis, 1981; Henao et al., 1991). As shown in Figure 6A, an initial level of phosphorylation of ca. 1.8 nmol of EP/mg of protein was observed under these conditions, with dephosphorylation fitting to single-exponential processes with rate constants of 9.5 ± 2.3 and 16.5 ± 1.8 s^{-1} in the absence and presence of 200 μM jasmone, respectively. Jasmone causes a large reduction in the level of phosphoenzyme formed from $^{32}P_i$ (Figure 7).

The effect of jasmone on the rate of dephosphorylation of the ATPase phosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+} was determined in a triple-mixing experiment. ATPase in the presence of Ca^{2+} was first incubated with 25 μM [γ-^{32}P]ATP for 200 ms to phosphorylate the ATPase, which was then mixed in a 1:1 ratio with 2.5 mM cold ATP in the presence or absence of 200 μM jasmone (Figure 6B). Dephosphorylation under these conditions fits to a single

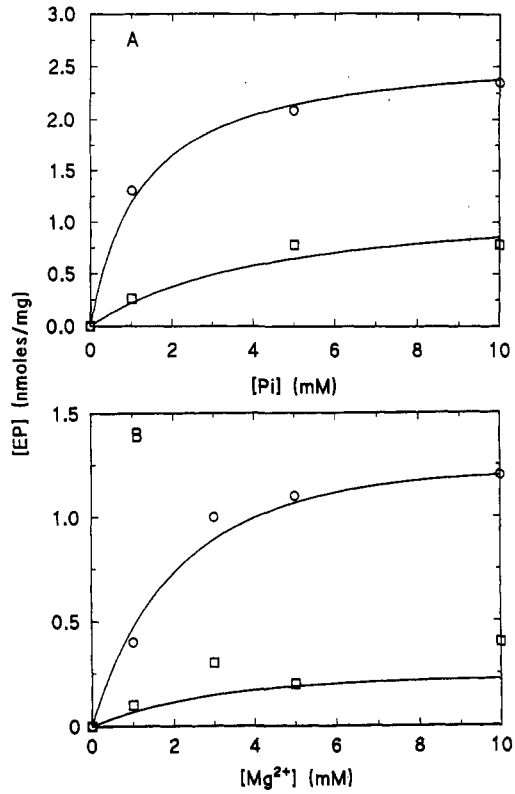


FIGURE 7: Effect of jasmone on the level of phosphorylation of the ATPase by phosphate. The ATPase (0.2 mg/mL) was incubated in 150 mM Mes/Tris, pH 6.2, 5 mM EGTA, and either (A) 10 mM Mg^{2+} in the presence of the given concentration of phosphate or (B) 1 mM P_i and the given concentration of Mg^{2+} in the absence (○) or presence (□) of 200 μM jasmone. The lines are simulations calculated as described in the text assuming a maximal level of phosphorylation of 3.2 nmol/mg of protein, with effective equilibrium constants (pH 6.2; no K^+) for phosphorylation of 15.0 in the absence of jasmone and 1.9 in its presence.

exponential, with rate constants of 11.8 ± 0.8 and 22.6 ± 3.2 s^{-1} in the absence and presence of 200 μM jasmone, respectively.

Finally, we studied dephosphorylation of the ATPase with ADP (Figure 8). The ATPase in sealed SR vesicles was phosphorylated with 50 μM [γ-^{32}P]ATP for ca. 1 min in the presence of Ca^{2+} and then mixed in a 1:1 ratio with buffer containing ADP and jasmone, to give final concentrations of ADP and jasmone of 1.5 and 0.2 mM, respectively, and a final Ca^{2+} concentration of 44 μM. A rapid initial decrease in [EP] was followed by a slower phase which fitted to a single-exponential process over the time period 20–100 ms; the rates of the slow phase were 12.9 ± 5.9 and 22.3 ± 3.2 in the absence and presence of jasmone, respectively, with the fraction of [EP] reacting in the fast phase increasing from 0.56 in the absence of jasmone to 0.69 in its presence.

Possible effects of jasmone on the E2–E1 equilibrium of the ATPase can be studied by making use of the conformational sensitivity of the fluorescence of NBD-labeled ATPase (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994a). It has been suggested that E2, E1, and Ca_2E1 are states of low, high, and intermediate fluorescence intensities, respectively, and that the E2 ⇄ E1 transition is pH sensitive, low pH favoring the E2 state (Wakabayashi et al., 1990; Henderson et al., 1994a). The presence of 200 μM jasmone had no effect on the fluorescence intensity of NBD-labeled ATPase or on the pH dependence of fluorescence intensity, suggesting no effect on the E2–E1 equilibrium (Figure 9).

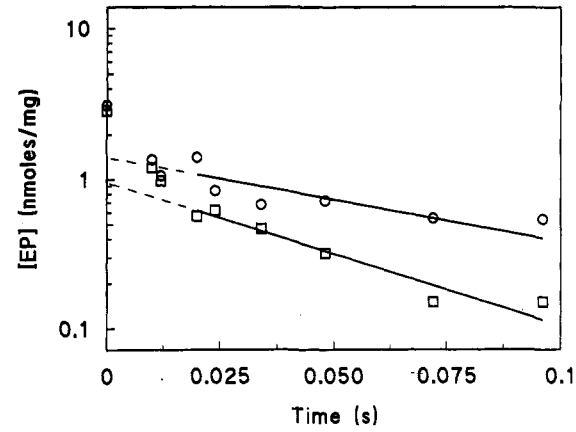


FIGURE 8: Effect of jasmone on the rate of dephosphorylation of the ATPase caused by addition of ADP. SR vesicles (0.2 mg/mL) were incubated in 50 mM MOPS, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl, 50 μM $CaCl_2$, and 50 μM [γ-^{32}P]ATP for ca. 1 min and then mixed in a 1:1 ratio with the same buffer containing 5 mM $MgSO_4$, 100 mM KCl, 10.08 mM $CaCl_2$, 10.0 mM EGTA, 3 mM ADP, and either no jasmone (○) or 400 μM jasmone (□) followed by quenching. The solid lines represent fits to single exponentials with the parameters given in the text, and the dashed lines show extrapolations to time zero giving the rapidly reacting fractions.

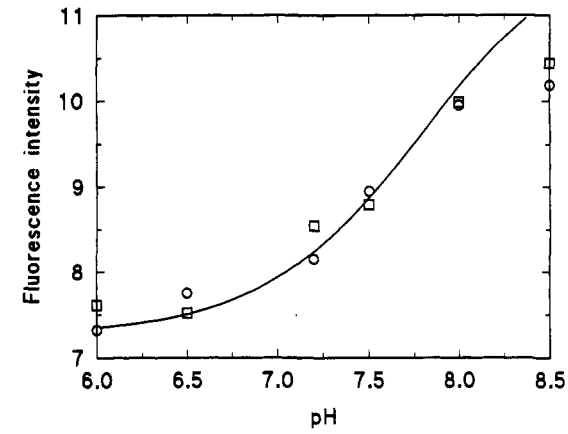


FIGURE 9: Effect of jasmone on the fluorescence intensity of NBD-labeled ATPase. Shown are the fluorescence intensities of NBD-labeled ATPase in the absence (○) or presence (□) of 200 μM jasmone. The solid line is a simulation of the data calculated using the parameters in Henderson et al. (1994a).

Table 2: Effect of Jasmone on Fluorescence Polarization of DPH in Bilayers of di(C18:1)PC[a]

jasmone (μM)	polarization (*P*)
0	0.144
20	0.138
200	0.145
2000	0.141

[a] di(C18:1)PC (128 μM) in 40 mM Hepes/Tris, pH 7.2, 25 °C.

Effects of Jasmone on the Lipid Bilayer. Possible effects of jasmone on the lipid bilayer component of the membrane were monitored using the fluorescence polarization of DPH incorporated into bilayers of di(C18:1)PC. Jasmone up to a concentration of 2 mM had no effect on fluidity as detected by DPH polarization (Table 2).

Effects of Jasmone on ATPase in Reconstituted Systems. Activities for the ATPase reconstituted into bilayers of di(C14:1)PC and di(C24:1)PC are lower than for the ATPase reconstituted with di(C18:1)PC (Michelangeli et al., 1991; Lee et al., 1991; Starling et al., 1993). Effects of 2 mM jasmone on the ATPase reconstituted with di(C18:1)PC at a phospholipid:ATPase molar ratio of 900:1 are comparable to

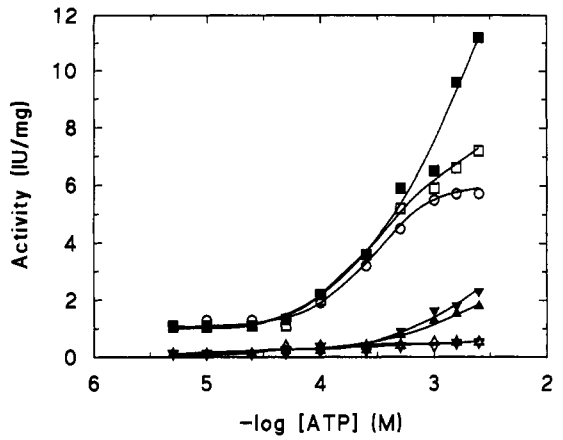


FIGURE 10: Effect of jasmone on the activity (IU/mg of protein) of the ATPase reconstituted with di(C18:1)PC, (○, □, ■), di(C14:1)-PC, (△, ▲) and di(C24:1)PC (▽, ▼) in the absence of jasmone (○, △, ▽) or in the presence of 20 μM (□) or 2 mM jasmone (■, ▲, ▼). [MgATP] = 2.1 mM; pH 7.2, 25 °C.

Table 3: Effect of Jasmone on Ca^{2+} Binding to Reconstituted ATPase[a]

system	Ca^{2+} bound (nmol/mg of protein)	
	-jasmone	+200 μM jasmone
ATPase[b]	8.9	9.4
di(C14:1)PC	4.9	3.8
di(C18:1)PC	9.0	7.9
di(C24:1)PC	4.2	3.4

[a] Ca^{2+} binding was measured in 20 mM Hepes/Tris, pH 7.4, 100 mM KCl, and 5 mM $MgSO_4$ (Starling et al., 1993). [b] Unreconstituted ATPase.

those seen with the native ATPase (Figure 10), although the effect of 20 μM jasmone is less, possibly due to partitioning of jasmone into the phospholipid. Effects of jasmone on the ATPase reconstituted with di(C14:1)PC and di(C24:1)PC are very similar.

It has been shown that on reconstitution with di(C14:1)PC or di(C24:1)PC the stoichiometry of Ca^{2+} binding to the ATPase changes from the usual 2:1 to 1:1 and that for the ATPase reconstituted with di(C14:1)PC the effect can be reversed by a variety of hydrophobic molecules (Starling et al., 1993). Addition of jasmone to the ATPase reconstituted with di(C14:1)PC had no effect on the level of Ca^{2+} binding (Table 3).

DISCUSSION

The activity of the Ca^{2+}-ATPase is dependent on the structure of the phospholipids surrounding it in the membrane, the phospholipid supporting the highest ATPase activity being di(C18:1)PC (Michelangeli et al., 1991; Starling et al., 1993). Thus hydrophobic molecules could, in principle, affect the activity of the ATPase by changing the properties of the phospholipid bilayer. However, direct effects on the ATPase appear equally likely (Lee et al., 1991). Some hydrophobic molecules have been shown to be able to bind to the lipid–protein interface (annular sites) of the ATPase, and it has been suggested that such binding leads to a decrease in ATPase activity due to displacement of phospholipid molecules from around the ATPase. Other hydrophobic molecules have been suggested to bind to sites on the ATPase (nonannular sites) either at protein–protein interfaces in ATPase oligomers or between transmembrane α-helices (Lee et al., 1991).

Recent studies have identified a series of highly specific, high-affinity inhibitors of the ATPase, the sesquiterpene lactones thapsigargin, trilobolide, and thapsivillosin A and 2,5-di-*tert*-butyl-1,4-benzohydroquinone (Brune & Ullrich, 1991; Sagara & Inesi, 1991; Kijima et al., 1991; Lytton et al., 1991; Wictome et al., 1992a,b; Sagara et al., 1992). Less is known about molecules that might lead to an increase in activity of the ATPase. Diethyl ether and short-chain alcohols have been shown to increase the activity of the ATPase (Kondo & Kasai, 1973; Salama & Scarpa, 1980; Almeida et al., 1986; Bigelow & Thomas, 1987; Birmachu & Thomas, 1990; Melgunov et al., 1988), but the effects of these molecules are likely to be nonspecific; they have been shown, for example, to affect rates of motion of phospholipids and of the ATPase in the membrane (Almeida et al., 1986; Bigelow & Thomas, 1987; Melgunov et al., 1988). The lipophilic weak acid 3,3′,4′,5-tetrachlorosalicylanilide has also been shown to stimulate the ATPase, and its effects are likely to be more specific since, although it binds to lipid bilayers, it has been shown to have no effect on bilayer fluidity (Barratt & Weaver, 1979; Wakabayashi et al., 1988).

Peppermint oil has been shown to cause relaxation of gastrointestinal smooth muscle, the most potent component of the oil being jasmone (Hills et al., 1990). As shown here (Figure 1A), jasmone at 25 °C causes a marked stimulation of activity of the Ca^{2+}-ATPase purified from rabbit skeletal muscle sarcoplasmic reticulum. As also shown in Figure 1A, the effect is markedly dependent on temperature, with little stimulation being observed at 37 °C. The temperature dependence can be compared to that seen with 3,3′,4′,5-tetrachlorosalicylanilide where marked stimulation was observed at 6 °C but not at 25 or 37 °C (Wakabayashi et al., 1988). Breaks are seen in Arrhenius plots of ATPase activity at about 25 °C (Lee et al., 1974) which would be consistent with different rate-controlling steps at high and low temperatures, the steps being modified by jasmone then being those that are rate controlling at low temperatures. Concentrations of jasmone causing relaxation of gastrointestinal muscle at 37 °C (Hills et al., 1990) are comparable to those affecting the skeletal muscle Ca^{2+}-ATPase (Figure 1). However, for the effect of jasmone on gastrointestinal muscle to follow from an effect on the ATPase, it would be necessary for the rate-controlling steps for smooth muscle and skeletal muscle ATPases to be different.

The concentration of jasmone resulting in stimulation of ATPase activity at pH 7.2 and 25 °C is less than that of menthol by a factor of ca. 10^4 (Figure 1). Octanol–water partition coefficients estimated using the parameters of Leo et al. (1971), however, are rather similar for jasmone and menthol (log *P* values of 2.9 and 3.3, respectively). The estimated partition coefficient for jasmone is similar to that for octanol, and octanol causes only slight stimulation of ATPase activity (data not shown). The effects of jasmone are therefore unlikely to follow from nonspecific partition into the phospholipid bilayer component of the membrane. We used the steady-state fluorescence polarization of DPH to monitor changes in the properties of the lipid bilayer. The steady-state polarization of DPH is sensitive to both order and rates of motion in the bilayer (Best et al., 1987). As shown in Table 2, jasmone has little effect on the fluorescence polarization, consistent with only small effects on the motional properties of the lipid bilayer; the change in fluorescence polarization of DPH observed on addition of 20 μM jasmone is equivalent, for example, to the change seen with an increase in temperature of ca. 2 °C [calculated from the data in Jones and Lee (1986)]. Effects of jasmone on the activity of the ATPase are likely therefore to follow from direct interaction with the ATPase.

We have interpreted effects on the kinetics of the ATPase in terms of the two-conformation, E2–E1 scheme (Scheme 1; de Meis, 1981). Jasmone had no significant effect on the E1–E2 equilibrium constant at any pH, as detected by the fluorescence intensity of NBD-labeled ATPase (Figure 9). Jasmone also had no significant effect on the high-affinity Ca^{2+} binding sites on the ATPase, as shown by measurements of the dependence of tryptophan fluorescence intensity or ATPase activity on Ca^{2+} concentration (data not shown). The observed effect of jasmone on the ATP dependence of ATPase activity suggests no significant effect on the affinity of the ATPase for ATP (data not shown).

Although jasmone had no effect on the rate of phosphorylation of the ATPase by ATP in the presence of Ca^{2+} (Figure 3), it did cause a significant reduction in the steady-state level of phosphorylation, consistent with an increased rate of dephosphorylation. As shown in Table 1, the effect of jasmone on steady-state levels of phosphorylation was reduced at high concentrations of Ca^{2+} where the rate of dephosphorylation is expected to be reduced. Direct measurement of the effects of jasmone on the rate of dephosphorylation of the ATPase phosphorylated by P_i showed that 200 μM jasmone increased the rate of dephosphorylation by a factor of ca. 2 (Figure 6A). Jasmone also decreased the steady-state level of phosphorylation of the ATPase by P_i (Figure 7). Phosphorylation of the ATPase by P_i requires the presence of Mg^{2+}, and it has been shown that inhibition of phosphorylation by spermidine follows from a reduction in affinity for Mg^{2+} (de Meis, 1991; de Meis et al., 1991). As shown in Figure 7B, inhibition by jasmone is independent of Mg^{2+} concentration. The data presented in Figure 7 could be fitted to the model for phosphorylation presented elsewhere (Froud & Lee, 1986; Michelangeli et al., 1991), assuming a decrease in the equilibrium constant for the phosphorylation step ($E2P_iMg \rightleftarrows E2PMg$) from 15.0 in the absence of jasmone to 1.9 in the presence of 200 μM jasmone with no effect on the affinity of the ATPase for P_i or Mg^{2+} (Figure 7); given the 2-fold increase in the rate of dephosphorylation of the ATPase, this would imply a marked decrease in the rate of the forward reaction in the presence of jasmone.

Jasmone also increases the rate of the $Ca_2E1P \rightarrow E2P$ step. In the experiment shown in Figure 5, the ATPase was incubated with 100 μM $^{45}Ca^{2+}$ and then perfused with 100 μM $^{40}Ca^{2+}$ and 2 mM ATP. Under the conditions of the experiment, the rate of phosphorylation of the ATPase was much faster than the observed rate of dissociation of $^{45}Ca^{2+}$ (see Figure 3 at 25 μM ATP) and the rate of dissociation of $^{45}Ca^{2+}$ from the unphosphorylated ATPase was slower than the rate of phosphorylation (Orlowski & Champeil, 1991; Henderson et al., 1994b). Thus the rate of dissociation of $^{45}Ca^{2+}$ from the ATPase reflects the rate of dissociation from the phosphorylated ATPase. As shown, the rate increased by a factor of 3 in the presence of 2 mM jasmone (Figure 5). The rate constant measured for dissociation of $^{45}Ca^{2+}$ in the absence of jasmone at pH 7.2 in 100 mM KCl (20.0 ± 4.9 s^{-1}) was significantly greater than that measured for the rate of dephosphorylation at pH 7.5 in 100 mM KCl (9.5 ± 2.2 s^{-1}; Figure 6), suggesting that in the overall process $E1Ca_2P \rightarrow E2$ the slowest step was dephosphorylation. This was confirmed by the experiment shown in Figure 6B in which the ATPase, first phosphorylated with [γ-^{32}P]ATP in the presence of Ca^{2+}, was dephosphorylated by mixing with excess unlabeled ATP; the rate of dephosphorylation under these conditions (pH 7.2, 100 mM KCl) was 11.8 ± 0.8 s^{-1} in the absence of jasmone and 22.6 ± 3.2 s^{-1} in the presence of 200 μM jasmone, respectively (Figure 6B). Thus at pH 7.2 in the presence of KCl the observed increase in rate of hydrolysis of ATP caused by jasmone is likely to follow largely from the increase in the rate of dephosphorylation. Kawashima et al. (1990) have shown that A23187 decreases the rate of dephosphorylation of the ATPase. At pH 7.2, 50% inhibition of the ATPase activity of the purified ATPase was observed at 17 μM A23187; 200 μM jasmone was found to stimulate ATPase activity in the presence of A23187, the concentration of A23187 required to inhibit activity by 50% increasing to 35 μM (data not shown). These results are also consistent with stimulation by jasmone following from an increase in the rate of dephosphorylation.

At pH 6.0, stimulation of ATPase activity by jasmone requires millimolar concentrations, and effects are comparable to those seen with methyl jasmonate at pH 7.2 (Figure 1). This would be consistent with the primary effect of jasmone being on the $Ca_2E1P \rightarrow E2$ change since, at pH 6.0, the $E2 \rightarrow Ca_2E1$ change is slow and rate determining in ATP hydrolysis (Henderson et al., 1994b).

Addition of ADP to the phosphorylated ATPase in Ca^{2+}-loaded SR vesicles leads to biphasic dephosphorylation, which has been interpreted (Scheme 1) in terms of rapid reaction of Ca_2E1P with ADP, Ca_2E2P being unreactive and having to undergo a slow transition to Ca_2E1P before reaction with ADP (de Meis, 1981). However, Stahl and Jencks (1987) have shown that the biphasic decay is consistent with formation of enzyme-bound ATP in a rapid burst followed by a slow dissociation of the bound ATP:

$$Ca_2EP + ADP \rightleftarrows Ca_2EPADP \rightleftarrows Ca_2EATP \rightleftarrows Ca_2E + ATP$$

Applying the quasi-equilibrium approach (Pickart & Jencks, 1982), this model predicts a burst size α given by

$$\alpha = [1 + K_{int}(1 + K_d^{ADP}/[ADP])]^{-1}$$

and a rate constant k for the slow phase given by

$$k = \alpha k_{off}$$

where K_d^{ADP} is the dissociation constant for ADP, K_{int} is the equilibrium constant for phosphorylation on the ATPase, and k_{off} is the rate constant for dissociation of ATP. As shown in Figure 8, jasmone has little effect on the burst size, so that neither K_{int} nor K_d^{ADP} is likely to be affected by jasmone. In terms of the above scheme the observed increase in the rate of the slow step (Figure 8) would imply an increase in the rate of dissociation of ATP. However, Fernandez-Belda and Inesi (1986) have shown that during the slow phase significant dephosphorylation to P_i can occur. The increase in rate of the slow step in Figure 8 (from 12.9 to 22.3 s^{-1}) is comparable to the increase in the rate of dephosphorylation seen on addition of jasmone and could therefore indicate that, in the presence of jasmone, the slow step contains a major contribution from the $E2P \rightarrow E2 + P_i$ step.

The increased rate of dissociation of Ca^{2+} from the phosphorylated ATPase appears not to involve any major change in the nature of the Ca^{2+} binding sites on E2P. Inhibition of ATPase activity at high concentrations of Ca^{2+} can be attributed partly to binding of Ca^{2+} to E2P and partly to the formation of CaATP, which is known to phosphorylate the ATPase with a rate slower than that of MgATP (Shigekawa et al., 1983; Yamada et al., 1986; Michelangeli et al., 1990a). The observed similarity of the Ca^{2+} concentrations causing inhibition of ATPase activity in the absence

and presence of jasmone (data not shown) suggests that jasmone does not change the affinity of the sites on E2P for Ca^{2+}. The inhibition of ATPase activity observed at high concentrations of Mg^{2+} at pH 8.0 have been attributed to binding of Mg^{2+} to the Ca^{2+} binding sites on E2P with subsequent inhibition of dephosphorylation (Bishop & Al-Shawi, 1988; Michelangeli et al., 1990a). As shown in Figure 2, the Mg^{2+} dependence of inhibition is also unaffected by addition of jasmone, again arguing against any large change in affinity of the Ca^{2+} binding sites on E2P.

Addition of jasmone results in a decreased release of Ca^{2+} from the ATPase at steady state in the presence of ATP (data not shown). Since the majority of the ATPase molecules will be phosphorylated in the presence of Ca^{2+} and ATP (Figure 3; Table 2), this implies an increase in the proportion of the ATPase in the Ca_2E1P state compared to that in the E2P state at steady state. Studies of the changes in tryptophan fluorescence intensity on addition of ATP are also consistent with the ATPase being present predominantly as Ca_2E1P in the steady state in the presence of jasmone. Nonylphenol, an inhibitor of the ATPase, has been shown to increase the rate of dephosphorylation of E2P but also to decrease the rate of the E2 → E1 transition, leading to a buildup of E2 at steady state (Michelangeli et al., 1990b). This has been detected, for example, by a decrease in tryptophan fluorescence for the ATPase in the presence of ATP and nonylphenol, since the tryptophan fluorescence intensity is lower for E2 than for Ca_2E1 or Ca_2E1P (Michelangeli et al., 1990b; see Figure 4). Unlike nonylphenol, jasmone has little effect on the change in tryptophan fluorescence intensity seen on addition of ATP (Figure 4). These experiments are therefore consistent with an increased rate of dephosphorylation of E2P with no effect on the rate of the E2 → E1 transition.

Effects of jasmone on the ATPase are distinct from those of other more hydrophobic molecules. Thus long-chain alcohols, sterols, and alkanes have all been shown to increase the activity for the ATPase reconstituted with the short-chain phospholipid di(C14:1)PC, often giving activities higher than those observed with di(C18:1)PC, the phospholipid which, on its own, supports highest activity (Lee et al., 1991; Froud et al., 1986; Starling et al., 1993). This is not observed with jasmone, where effects are similar for the ATPase reconstituted with di(C14:1)PC, di(C18:1)PC, or di(C24:1)PC (Figure 10). Reconstitution of the ATPase with di(C14:1)PC or di(C24:1)PC has been shown to reduce the Ca^{2+} binding stoichiometry from the usual 2:1 to 1:1, this effect being reversed for the ATPase reconstituted with di(C14:1)PC by oleic acid, methyl oleate, or oleyl alcohol (Starling et al., 1993). As shown in Table 3, jasmone has no effect on Ca^{2+} binding stoichiometry for the native ATPase or for the ATPase reconstituted with phospholipids with either short (C14) or long (C24) fatty acyl chains.

The nonpolar character of jasmone appears to be important for its effectiveness. Thus the concentration of methyl jasmonate required for stimulation is much greater than that of jasmone (Figure 1), suggesting that a nonpolar group at the 3 position in the cyclopentenone ring is important. Effects of cyclopentenol, cyclopentene, and cyclopentenone on the activity of the ATPase are very small (data not shown), suggesting an important role for the alkyl chain at the 2 position.

Jasmone increases the rates of dephosphorylation and of the Ca_2E1P → E2P step and decreases the equilibrium constant for phosphorylation by P_i but has no effect on the E2 ⇄ E1 equilibrium constant. The sesquiterpene lactone inhibitors, 2,5-di-*tert*-butyl-1,4-benzohydroquinone, and nonylphenol also decrease the equilibrium constant for phosphorylation by P_i but decrease the E1 ⇄ E2 equilibrium constant (Michelangeli et al., 1990b; Wictome et al., 1992a,b). The inhibitors all contain -OH groups which appear to be important for activity: 2,5-di-*tert*-butyl-1,4-benzoquinone is not an inhibitor of the ATPase, and similarly desoxytrilobolide has no effect on the ATPase, whereas trilobolide is a strong inhibitor (Wictome et al., 1993). Distinct strictural features therefore seem to be involved in stimulation and inhibition of the ATPase.

ACKNOWLEDGMENT

We thank Firmenich UK Ltd. for a gift of methyl jasmonate.

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