Capsaicin Stimulates Uncoupled ATP Hydrolysis by the Sarcoplasmic Reticulum Calcium Pump*

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In muscle cells the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA) couples the free energy of ATP hydrolysis to pump Ca\(^{2+}\) ions from the cytoplasm to the SR lumen. In addition, SERCA plays a key role in non-shivering thermogenesis through uncoupled reactions, where ATP hydrolysis takes place without active Ca\(^{2+}\) translocation. Capsaicin (CPS) is a naturally occurring vanilloid, the consumption of which is linked with increased metabolic rate and core body temperature. Here we document the stimulation by CPS of the Ca\(^{2+}\)-dependent ATP hydrolysis by SERCA without effects on Ca\(^{2+}\) accumulation. The stimulation by CPS was significantly dependent on the presence of a Ca\(^{2+}\) gradient across the SR membrane. ATP activation assays showed that the drug reduced the nucleotide affinity at the catalytic site, whereas the affinity at the regulatory site increased. Several biochemical analyses indicated that CPS stabilizes an ADP-insensitive E\(_{2}\)P-related conformation that dephosphorylates at a higher rate than the control enzyme. Under conditions where uncoupled SERCA was specifically inhibited by the treatment with fluoride, low temperatures, or dimethyl sulfoxide, CPS had no stimulatory effect on ATP hydrolysis by SERCA. It is concluded that CPS stabilizes a SERCA sub-conformation where Ca\(^{2+}\) is released from the phosphorylated intermediate to the cytoplasm instead of the SR lumen, increasing ATP hydrolysis not coupled with Ca\(^{2+}\) transport. To the best of our knowledge CPS is the first natural drug that augments uncoupled SERCA, presumably resulting in thermogenesis. The role of CPS as a SERCA modulator is discussed.

Transient increase in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) is a common mechanism in cellular signaling. In the working muscle, the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA) rapidly clears [Ca\(^{2+}\)]\(_{cyt}\) to ensure muscle relaxation. SERCA uses energy from ATP hydrolysis to build up a Ca\(^{2+}\) gradient across the SR membrane that can reach up to 4 orders of magnitude. SERCA is the best-characterized member of the P-type ATPase family (1). Our understanding of this ion pump was initially based on extensive biochemical and molecular biological studies (e.g., for review, see Refs. 2 and 3). Crystal structures of several SERCA isomers have provided essential structural information and largely exposed the molecular mechanism of this pump (Refs. 4–8; for review, see Refs. 9 and 10).

An interesting feature in the crystal structures of SERCA is that the cytoplasmic and the transmembrane (TM) domains, which are rigid structures, are interconnected with flexible links (4–10). Because of their importance in energy transduction, the functional importance of the flexible links in SERCA has been intensively investigated by mutational analyses (e.g., 11–13). As discussed previously (14), one of the functional roles of the flexible links could be to generate complex domain movements, possibly allowing the pump to isomerate among several distinct sub-conformations. Indeed, several SERCA isomerization steps have been added as branched reactions in the classical Post-Albers scheme (Scheme 1). Dominance of SERCA isomer(s) not participating in active Ca\(^{2+}\) transport \textit{in vivo} would likely lead to the modulation of SERCA function (e.g., Ref. 15 and see below).

Hypothetically, hydrolysis of one ATP molecule leads to transport of two Ca\(^{2+}\) ions from the cytoplasm to the SR lumen (17). However, substoichiometric efficiencies of SERCA are commonly observed in the presence of a Ca\(^{2+}\) gradient across the SR membrane. Uncoupling between hydrolysis and transport was shown to occur even in the absence of passive Ca\(^{2+}\) leak and was explained by hydrolytic cleavage of the inorganic phosphate from the phosphorylated intermediate before Ca\(^{2+}\) release to the lumen (18). This is associated with Ca\(^{2+}\) release to the cytoplasmic side, the so-called slippage (19). The energy from ATP hydrolysis not coupled to Ca\(^{2+}\) transport is presumably dissipated as heat. Definite information on whether SERCA hydrolyzes/synthesizes ATP, transports Ca\(^{2+}\), or generates heat has been obtained by measuring Ca\(^{2+}\) transport and ATP hydrolysis together with pump-mediated heat exchange (20). Subsequent investigations in this direction accentuated the presence of a regulatory mechanism(s) that would allow SERCA to generate heat at the disbursement of Ca\(^{2+}\) transport (15). This concept is indeed substantiated by studies showing that binding of sarcolin (a small regulatory protein that is abundant in the SR of skeletal muscle; for a recent review, see Ref. 21) results in uncoupling (22) and increased heat generation (23) by SERCA. Hence, interaction of sarcolin with SERCA shifts its conformational equilibrium toward isomers with an increased tendency to hydrolytic cleavage before trans-
location to the SR lumen (or Ca\textsuperscript{2+} release to the cytoplasm followed by hydrolytic cleavage).

CPS (Fig. 1A, inset) is the chemical compound that makes chili peppers taste hot. CPS is an agonist of the transient receptor potential vanilloid subfamily member 1 (TRPV1 or VR1), a non-selective cation channel abundantly expressed in sensory neurons (24). CPS intake has been linked with the suppression of several malignant transformations through a mechanism that is not fully understood. In particular, CPS treatment was reported to significantly slow down the proliferation of prostate cancer cells (25). In addition, many studies in the medical literature have linked the consumption of chili-containing meals with increased energy expenditure and fat oxidation (26). In this regard a seeming effect of chili consumption is the rapid increase in body core temperature and subsequent increased heat loss (27).

We have been interested in identifying molecular targets for naturally occurring pharmacologically active drugs to understand how these drugs exert their global effects (28, 29). Here we document that CPS activates ATP hydrolysis by SERCA with almost no effect on its Ca\textsuperscript{2+} transport function (under the conditions used in this study, see “Experimental Procedures”). Our data are consistent with a model in which CPS increases the steady state concentration of a SERCA isomer in which occluded Ca\textsuperscript{2+} is released from an E\textsubscript{P}-like form to the cytoplasmic side instead of the luminal side of the SR, resulting in uncoupling between ATP hydrolysis and Ca\textsuperscript{2+} transport. Hence, the increase in body metabolism and heat generation after CPS intake is likely a result of, at least in part, the direct effect of CPS on SERCA.

**Experimental Procedures**

Ca\textsuperscript{2+}-ATPase Preparation and Hydrolytic Activity—SR vesicles (a generous gift of J. V. Møller) were prepared as described previously (30). In other experiments unsided membrane fragments prepared by extraction with sodium deoxycholate were used. The hydrolytic activity of SERCA was determined by measuring the Ca\textsuperscript{2+}-dependent inorganic phosphate liberation from ATP after incubation at 37 °C in the presence of different substrate and ligand concentrations. The reaction mixture contained 20 mM MOPS (pH specified in separate figure legends), 130 mM KCl, 2 mM Tris-ATP, 0.5 mM EGTA, 5 mM MgCl\textsubscript{2}, and 3 μg of protein in intact SR vesicles or deoxycholate-extracted SR membranes. In some cases ATP hydrolysis in the presence of intact SR vesicles were performed in the presence of 2 μg ml\textsuperscript{-1} A23187 (unless otherwise indicated) to rapidly dissipate the Ca\textsuperscript{2+} gradient across the SR membrane. In control samples an excess of EGTA was added to the mixture, bringing the free Ca\textsuperscript{2+} concentration to about 5–10 nM (thus, SERCA activity in the presence of excess EGTA did not exceed 5% of the maximum ATP hydrolysis measured in the presence of Ca\textsuperscript{2+}). The free Ca\textsuperscript{2+} concentration was calculated using the program MaxChelator WinmaxC (freely available on the World Wide Web). Liberated inorganic phosphate was determined using a colorimetric method, as described previously (31). In other set of experiments the hydrolytic activity at low ATP concentrations was determined in the presence of 200 μM Tris ATP premixed with tracer amounts of \textsuperscript{32}P\textsubscript{ATP}, as described previously (32). Additional experiments were finally performed in which agonists and antagonists to the VR1 were employed to demonstrate the functional absence of passive Ca\textsuperscript{2+} fluxes through this particular channel during SERCA activity measurements.

Phosphoenzyme (EP) Measurements—Enzyme preincubated with either DMSO vehicle or CPS was rapidly mixed with an ice-cold phosphorylation medium producing 50 mM MOPS, pH 7.0 (unless otherwise indicated), 0.5 mM EGTA, 80 mM KCl, 2 mM MgCl\textsubscript{2}, 0.08 mg of SR protein/ml. The drug or solvent concentrations were maintained constant after mixing. The free Ca\textsuperscript{2+} concentrations are mentioned in separate figure legends. Phosphorylation was started by the addition of 10–50 μM Tris-ATP (containing 10–100 μCi [γ\textsuperscript{32}P]ATP) and quenched at various times with stopping solution consisting of 5% trichloroacetic acid and 1 mM sodium pyrophosphate. The acid stable EP was washed twice and collected by centrifugation, and its radioactivity was measured using scintillation counting. In other experiments dephosphorylation was investigated by diluting the phosphorylated enzyme in buffer containing ligands of interest for different time intervals, after which acid quenching, washing, and collection took place. Enzyme dephosphorylation from P\textsubscript{i} was measured at 24 °C in a reaction mixture containing 50 mM MOPS, pH 6.5, 8 mM MgCl\textsubscript{2}, 2 mM EGTA, various concentrations of [γ\textsuperscript{32}P]P\textsubscript{i}, and 0.4 mg of SR protein/ml in a total volume of 400 μl. The reaction was acid-quenched after 10 min of incubation by the addition of stopping solution, and the EP was processed as above. Hydrolysis of EP phosphorylated from P\textsubscript{i} was performed after 5 min of equilibration at 0 °C.

Cleavage with Proteinase K—Intact SR vesicles were incubated in a mixture containing 40 mM MOPS buffer (pH values in Fig. 7), 2 mM EGTA, 1 mM AMP-PCP, 5 mM MgCl\textsubscript{2}, 1.6 mg ml\textsuperscript{-1} SR protein, 0.028 mg ml\textsuperscript{-1} proteinase K (PK) in the presence or in the absence of 150 μM CPS. In some cases Ca\textsuperscript{2+} was added to produce a free Ca\textsuperscript{2+} concentration of ~130 μM. The reaction was started with the addition of PK, allowed to proceed for 25 min at 24 °C, and terminated with the addition of equal volume of SDS sample buffer containing 0.5% trichloroacetic acid.
Capsaicin Stimulates Uncoupled SERCA

FIGURE 1. CPS stimulation of ATP hydrolysis by SERCA. A, SERCA activity was measured at 37 °C in a reaction mixture containing 20 mM MOPS, pH 7.0, 130 mM KCl, 2 mM Tris-ATP, 0.5 mM EGTA, 5 mM MgCl₂, SR vesicles (○) or deoxycholate-extracted SR membrane fragments (●) containing 3 μg of protein, and the indicated CPS concentrations. The free Ca²⁺ concentration was 7.5 μM. Inorganic phosphate liberated from ATP was measured by a colorimetric method as described under “Experimental Procedures.” ATP hydrolysis measured in the presence of excess EGTA was subtracted from that in the presence of Ca²⁺, which did not exceed 2% of the Ca²⁺-dependent ATP hydrolysis. Data are expressed as a fraction of the activity measured in the absence of CPS. The inset shows the chemical structure of CPS. B, steady state ATP hydrolysis in the presence of intact SR vesicles was measured as in panel A but in the presence of the indicated A23187 concentrations, either in the absence (□) or in the presence (○) of 150 μM CPS. The free Ca²⁺ concentration was 6.6 μM. Data are expressed as mmol of liberated phosphate h⁻¹ mg⁻¹ protein.

acid to ensure complete suppression of the PK action (33). Protein fragments were analyzed by SDS-PAGE. Protein fragments in sample buffer were loaded onto 8% Tricine gels, and electrophoresis took place overnight at 150 V and 11 mA. After electrophoresis the gels were washed and stained with a Coomassie-based EZ blue solution (Sigma) according to the manufacturer’s instructions. Scanning and intensity determination of the dry gels was performed using ImageQuant TL image analysis software (Amer sham Biosciences) as previously applied (29).

Ca²⁺ Accumulation—Ca²⁺ accumulation was measured by incubating SR vesicles (10 μg/ml) in a reaction mixture (flux medium) containing 20 mM MOPS, pH 7.0, 80 mM KCl, 25% DMSO, 5 mM MgCl₂, 0.5 mM EGTA, 16 μM Tris-ATP in the absence or in the presence of 100 μM CPS (added to the vesicles before the assay), and 45CaCl₂ to produce a free Ca²⁺ concentrations of 2.2 μM. The reaction was started with the addition of SR vesicles to the flux medium, and 50 μl were taken at different time intervals and passed through Bio-Rex 70 columns (see Refs. 34 and 35). Ca²⁺ entrapped in vesicles was measured using scintillation counting. Control fluxes, which did not exceed 15% of maximum, were measured as above but in the presence of excess EGTA or in the absence of ATP.

Statistical Analysis—Mean values are shown, and differences between means were assessed with the unpaired t test. All procedures were taken from Graph Pad Prism.

RESULTS

CPS Activation of SERCA and the Effect of Substrates—Results presented in Fig. 1A illustrate the effect of different concentrations of CPS on the steady state ATP hydrolysis by SERCA, measured at neutral pH. Intact SR vesicles as well as deoxycholate-extracted SR membrane fragments were used. In both SERCA preparations CPS significantly stimulated the steady state Ca²⁺-dependent ATPase activity. The stimulation was more than 3-fold in the case of the intact SR vesicles and about 1.5-fold in the case of the fragmented membranes. After the addition of the necessary substrates to intact SR vesicles, a Ca²⁺ gradient across the vesicle membrane develops (e.g. Ref. 17). The establishment of a Ca²⁺ gradient across the vesicle membrane is likely responsible for the observed difference between the intact vesicles and the membrane fragments. Results shown in Fig. 1B confirmed this prediction and showed that the CPS stimulation of steady state ATP hydrolysis by SERCA significantly diminishes when the membranes of the intact SR vesicles were progressively made leaky by the addition of increasing concentrations of the Ca²⁺ ionophore A23187. Indeed, part of the stimulation of SERCA activity by CPS was found to be independent on the presence of a Ca²⁺ gradient, as indicated by the mild stimulation of activity at concentrations of A23187 more than 1.5 μg ml⁻¹ (a concentration that presumably produces maximum opening of the vesicles; Fig. 1B). This activation likely corresponds to the stimulation observed with the deoxycholate extracted SR membrane fragments (Fig. 1A, filled symbols). CPS, at concentrations up to 300 μM, did not affect the hydrolytic activity of the closely related Na,K-ATPase (data not shown).

pH Dependence—The effect of pH on the CPS stimulation of SERCA was investigated. As depicted in Fig. 2A, under conditions allowing Ca²⁺ accumulation in the SR (intact vesicles), CPS strongly activated ATP hydrolysis by SERCA at the acidic and neutral pH regimes, and the stimulation ceased strongly at alkaline pH. In contrast, in the absence of a Ca²⁺ gradient (leaky vesicles), CPS mildly stimulates ATP hydrolysis by SERCA in a way that is almost pH-independent (Fig. 2B).

ATP Dependence—The activation by various concentrations of ATP of the control and the CPS-treated SR vesicles is depicted in Fig. 3, showing more than 2-fold activation of ATP hydrolysis after CPS treatment. The ATP activation curves were analyzed by comparing two different fitting procedures, which revealed interesting information (see a similar analysis in
Capsaicin Stimulates Uncoupled SERCA

FIGURE 4. Ca\(^{2+}\) dependence of EP formation. SR vesicles were equilibrated at 0°C before dilution with the phosphorylation mixture, as described under “Experimental Procedures.” A, phosphorylation was performed in the presence of 20 mM MOPS, pH 7.4, 4 mM MgCl\(_2\), 0.5 mM EGTA, 80 mM KCl, 10 μM \([^{32}\text{P}]\text{ATP}\), and the indicated Ca\(^{2+}\) concentrations in the presence of either DMSO (□) or 150 μM CPS (○). A hyperbolic equation was used to fit the data, producing a \(K_d\) for Ca\(^{2+}\) activation of 1.2 ± 0.11 in control and 4.97 ± 0.31 μM in CPS-treated enzyme. B, time dependence of EP formation was measured as in A but in the reaction was instead performed at a fixed free Ca\(^{2+}\) concentration of 0.2 μM and was allowed to proceed for the indicated time intervals before acid quenching and collection. Symbols are identical to those in panel A. Fitting a single-phase exponential association to the data gave a rate constant \(k = 0.404 ± 0.03\) for control and 0.272 ± 0.07 for CPS-treated enzyme. Fitting a two-phase exponential association to the data gave \(k_1 = 0.045 ± 0.01\) and \(k_2 = 0.054 ± 0.01\) for control and \(k_1 = 0.269 ± 0.02\) and \(k_2 = 0.274 ± 0.01\) for CPS-treated enzyme. The inset shows the reciprocal of the EP level against time. C, in these experiments, SR membranes were first suspended in a mixture containing 30 mM MOPS, pH 7, 100 μM Ca\(^{2+}\), 4 mM ADP, and 8 mM dithiothreitol. After incubation for 90 min at 24°C, the vesicles were washed and suspended in MOPS buffer. This treatment was shown to completely block the \(E_1P\) to \(E_3P\) isomerization step without effect on the \(E_1\) to \(E_3P\) transition (see Sørensen et al. (38)). Phosphorylation was performed as in panel B. Symbols are identical to those in panels A and B.

for Ca\(^{2+}\) activation of the EP was 1.2 ± 0.11 for the control enzyme and 5.61 ± 0.31 μM for the CPS-treated enzyme.

FIG. 4B depicts the time dependence of EP formation measured at a free Ca\(^{2+}\) concentration of 0.2 μM. Fitting one phase exponential association to the data revealed an overall decrease in the rate of EP formation. However, fitting a two-phase exponential association to the data showed distinct effects of CPS on the rate of EP formation; CPS accelerated EP formation in the initial fast phase by about 6-fold, whereas the rate of EP formation in the slower phase slightly decreased (see the legend to Fig. 4B). This significant difference between the control and the CPS-treated vesicles was also demonstrated by blotting the reciprocal of the Y-variable against time, shown in the inset to Fig. 4B. Hence, the CPS-treated enzyme is phosphorylated at a lower final level, but the initial rate of phosphate transfer is faster than that of the control enzyme. That CPS does not interfere with the initial phosphate transfer was also indicated from experiments in which CPS was found to indeed enhance the rate of the \(E_1\) → \(E_3P\) transition. It has previously been shown that reduction of disulfide bonds in SERCA resulted in complete loss of the hydrolytic activity, with complete block of the \(E_1P\) to \(E_3P\) transition, without any effect on the \(E_1\) to \(E_3P\) transition (36). To this end, the effect of CPS on the time dependence of EP formation of enzyme incubated in the presence of Ca\(^{2+}\)/ADP plus 8 mM dithiothreitol (see details in Daiho, T., and Kanazawa (36)) was investigated. The results showed that CPS treatment resulted in an increase in both the rate and the magnitude of phosphorylation of the dithiothreitol-modified enzyme (Fig. 4C), indicating that the \(E_1\) to \(E_3P\) transition is enhanced after treatment with CPS.

The ATP affinity for EP formation was also measured and is depicted in Fig. 5, left panel. ATP stimulated the phosphorylation of the control enzyme with a high affinity (\(K_d = 0.358 ±

the supplemental material provided in Mahmmod (35). Fitting a Michaelis-Menten equation to the data showed that CPS treatment increases the ATP affinity. The analysis was satisfactorily in the case of the control enzyme but gave a bad fit in the case of the CPS-treated enzyme (see the legend to Fig. 3). On the other hand, fitting a two-site activation model to the data gave better fitting for both the control and the CPS-treated curves and revealed distinct effects of the ATP concentration. The nucleotide affinity at the high affinity site strongly decreased (the \(K_d\) increased from 7.2 ± 0.6 μM for the control enzyme to 27.3 ± 2.4 μM for the CPS-treated enzyme). In contrast, the nucleotide affinity at the low affinity site strongly increased (the \(K_d\) decreased from 3.70 ± 0.38 mm for the control enzyme to 1.70 ± 0.47 mm for the CPS-treated enzyme). Hence, in the presence of CPS, net ATP hydrolysis increases at higher ATP concentrations.

\(\text{Ca}^{2+}\) Uptake—ATP-powered \(\text{Ca}^{2+}\) transport was measured (see “Experimental Procedures”) in the presence or in the absence of CPS, showing that CPS had no effect on \(\text{Ca}^{2+}\) transport into the SR lumen (data not shown). We also determined that CPS does not significantly affect equilibrium \(\text{Ca}^{2+}\) binding to the SK membrane in the absence of ATP (data not shown).

EP Measurements—When ATP is added to enzyme preincubated with \(\text{Ca}^{2+}\), an EP intermediate is rapidly formed and reaches a steady state level, which is dependent on the rates of phospholipid transfer from ATP and subsequent hydrolytic cleavage of inorganic phosphate from the EP. The \(\text{Ca}^{2+}\) dependence of EP formation was measured in the absence or presence of CPS (Fig. 4A), showing that the maximum EP level decreased more than 3-fold after treatment with CPS. The \(K_d\)
Capsaicin Stimulates Uncoupled SERCA

FIGURE 5. Left panel, ATP dependence of EP formation. SR vesicles were equilibrated at 0 °C before dilution in a phosphorylation mixture as described under "Experimental Procedures." Phosphorylation was performed in the presence of 20 mM MOPS, pH 6.5, 0.2 mg ml⁻¹ SR protein, 80 mM KCl, 10 μM [³²P]ATP in the presence of either DMSO (□) or 150 μM CPS (○). The free Ca²⁺ concentration was 3.5 μM. The control data were best fit to a single hyperbolic equation, which gave a Kₘ for ATP activation of 0.358 ± 0.012 μM. In contrast, in the case of the CPS-treated enzyme the data were best fit to a two-phase hyperbolic equation, giving a Kₘ values of 1.24 ± 0.187 and 152.4 ± 35.6 μM. Right panel, phosphorylation from inorganic phosphate, was performed as described under "Experimental Conditions."

0.012 μM), and the maximum EP reached ~6 nmol·mg⁻¹. Consistent with data in Fig. 4, CPS was found to strongly decrease the steady state EP level (EPₘₐₓ, for the CPS-treated enzyme was ~2.3 ± 0.11 nmol·mg⁻¹). This decrease was associated with a significant drop in the ATP affinity for phosphorylation (see the legend to Fig. 5). In addition, CPS mildly enhanced the phosphorylation from inorganic phosphate at pH 6.5 (Fig. 5, right panel). No effect was observed at pH 7 (data not shown).

After phosphoryl transfer from ATP, an E₆(2Ca²⁺)−P intermediate is formed. This intermediate is unstable and reacts with ADP to form ATP. Measuring the EP decay after dilution in ADP-containing media can, thus, provide information on the conformational dynamics of the EP. Thus, the enzyme was first phosphorylated on ice for a short time period followed by chasing with 1 mM ADP for different time intervals, after which the EP was quenched with acid. As seen in Fig. 6, the control EP was rapidly dephosphorylated after the addition of ADP. Analysis of the data using a single exponential function gave a decay rate of 0.321 ± 0.023 s⁻¹ for the control enzyme. On the other hand, the EP decay rate was 0.149 ± 0.017 s⁻¹ in the case of the CPS-treated enzyme. This finding demonstrates a CPS-induced shift in the conformational equilibrium of SERCA to an ADP-insensitive isomer. Fitting a linear equation to the slow-decaying component, which provides information on the initial (zero time) level of ADP-insensitive form (28), showed that about 50% of the CPS-stabilized EP is ADP-insensitive. This should be compared with 27% for the control enzyme.

EP Decay—The decreased steady state EP level (Fig. 4, A and B, and Fig. 5) together with the increased rate of initial phosphate transfer (Fig. 4C) seen after CPS treatment suggests that the drug increases the rate of EP hydrolysis. Indeed, that CPS increases the rate of hydrolytic cleavage of SERCA was initially indicated from experiments in which the effect of CPS on the activation of SERCA by various concentrations of K⁺ (which enhances SERCA dephosphorylation; Refs. 37, 38) was studied. It was found that the drug increased the affinity of SERCA for K⁺ by about 2-fold (the Kᵥₐₛ for K⁺ activation was 50.1 ± 11.7 mM for the control and 26.5 ± 5.2 mM for the CPS-treated enzyme; data not shown). Hence, we investigated the effect of CPS on the hydrolysis of EP formed either from ATP or from inorganic phosphate. After rapid Ca²⁺ chelation with a high concentration of EGTA (to prevent further phosphorylation), the enzyme phosphorylated from ATP in the presence of CPS dephosphorylated at a faster rate compared with the control enzyme (Table 1).

Low affinity ATP interaction with the E₆P form, which is strongly modified by CPS (Fig. 3), is crucial for transition to E₃ and subsequent catalysis. Hence, the dephosphorylation of enzyme phosphorylated from inorganic phosphate (the “E₆P” form) was investigated in more detail. The enzyme was first phosphorylated from P₁ as described under "Experimental Procedures." The phosphorylated enzyme was transferred on ice,
and dephosphorylation was initiated by dilution in a pH 7.0 MOPS buffer containing the ligand of interest. As summarized in Table 1, ATP was as efficient as K$^+$ in initiating dephosphorylation of the control enzyme. On the other hand, ATP increased the rate of dephosphorylation of the CPS-treated enzyme by about 50%. ADP and AMP decreased the dephosphorylation rate of the control enzyme, and CPS treatment increased the rate of dephosphorylation in both cases by almost 3-fold, providing evidence that CPS enhances $E_2P$ hydrolysis by a mechanism that is independent of a specific adenine nucleotide.

**Cleavage with Proteinase K**—After selective cleavage of SERCA with PK at the link between A domain and TM3, the pump loses its ability to transport Ca$^{2+}$ and hydrolyze ATP but can be phosphorylated to form an $E_2(2Ca^{2+})$−P (39, 40). Subsequent investigations further showed that the $E_2$ form has a compact structure that is resistant to PK cleavage (41). Limited cleavage with PK has, therefore, been extensively used to provide useful information on the conformational dynamics of the pump (e.g. Refs. 42–45). Hence, we studied how CPS would affect the PK cleavage pattern of SERCA. As seen in Fig. 7, treatment with PK of SERCA pretreated with CPS induced profound differences in the cleavage pattern compared with samples not treated with CPS. At acidic pH and in the absence of Ca$^{2+}$, the p95C fragment appeared after treatment with PK of the CPS-treated SR vesicles (this occurs at both pH 6 and to a lesser extent at pH 6.5). In addition, the intensity of the p83C was also increased after PK cleavage of the CPS-treated enzyme compared with samples not treated with CPS (the p83C fragment was indeed about 70% more intense after PK treatment in the presence of CPS and in the absence of Ca$^{2+}$). The greater accumulation of the p83C fragment was undoubtedly obvious from the concurrent accumulation of the p28N fragment (see labels in Fig. 7, lower panel). The accumulation of the p95C fragment occurred only at acidic pH. On the other hand, the production of the p83C fragment increased in the CPS-treated vesicles both at acidic and neutral pH. Remarkably, CPS also induced accumulation of the p83C and p28N when cleavage was performed in the presence of Ca$^{2+}$. These findings demonstrate that CPS directly interacts with SERCA and specifically reduces the protection of Thr$^{242}$ against PK attack. It is noteworthy that treatment with CPS did not modify the PK cleavage pattern of the catalytic subunit of the pig kidney Na,K-ATPase (data not shown).

**Effect of CPS on the Ligand-induced Uncoupling of SERCA**

Several studies have indicated that the uncoupled SERCA reactions occur through definite isomerization steps in the reaction cycle (see branched pathways in Scheme 1). Indeed, several treatments were reported to dissect the uncoupled activity from the coupled activity of SERCA (to the best of our knowledge, all SERCA modifications reported so far were shown to produce pumps with inhibited uncoupled transport). Thus, treatment of SERCA with relatively high concentrations of fluoride (46), low temperatures, or dimethyl sulfoxide (DMSO) (47) have been shown to increase the coupling ratio of SERCA concurrently with inhibition of the uncoupled ATP hydrolysis (indicated from changes in the caloric yield). Accordingly, it was of interest to investigate how CPS would affect ATP hydrolysis by SERCA under conditions where the coupled SERCA were functionally distinct from uncoupled SERCA using the treatments mentioned above. Intriguingly, whereas CPS stimulated ATP hydrolysis by SERCA in the absence of fluoride (Fig. 8, left panel, circles; see also Figs. 1–3), an inhibition was apparent in the presence of 20 mM fluoride (Fig. 8, left panel, diamonds). Remarkably, the activation by CPS in the absence of fluoride was found to be more potent than the inhibition in the presence of fluoride. In addition, the activation by CPS increased at higher temperatures than at lower temperatures (Fig. 8, middle panel), indicating that the augmentation of ATP hydrolysis by CPS occurs through the uncoupled pathway(s), which are enhanced by increased temperatures (47). Finally, the stimulation of ATP hydrolysis was abolished when the assays were performed in the presence of 25% DMSO (Fig. 8, right panel).
Capsaicin Stimulates Uncoupled SERCA

**FIGURE 8. Effect of CPS on SERCA in the presence of treatments known to inhibit uncoupled transport.** The hydrolytic activity was measured in a reaction mixture containing 20 mM MOPS pH 7.0, 130 mM KCl, 2 mM Tris-ATP, 0.5 mM EGTA, 5 mM MgCl$_2$, 6 μg ml$^{-1}$ SR vesicles, and the indicated CPS concentrations. The free Ca$^{2+}$ concentration was 7.5 μM in all cases. Left panel, the reactions were performed in the absence (squares) or presence (diamonds) of 20 mM NaF. Fitting the data using a sigmoid dose-response function gave an EC$_{50}$ of 39.8 ± 1.07 μM for the stimulation and 122.8 ± 2.95 μM for the inhibition. Middle panel, the reactions were performed at 25 °C (circles), 30 °C (triangles), or 35 °C (squares). Right panel, the assays were performed in the absence (circles) or presence (squares) of 25% DMSO.

**DISCUSSION**

In this study we introduce CPS as a SERCA “stimulator.” Under conditions allowing formation of a Ca$^{2+}$ gradient across the SR membrane, CPS produced a substantial stimulation of ATP hydrolysis (Figs. 1–3). Indeed, stimulation by CPS was also observed in leaky SR vesicles in the presence of relatively high Ca$^{2+}$ concentrations (data not shown). This strongly indicates that the activation by CPS depends on Ca$^{2+}$ interaction with the low affinity luminal sites. The steady state EP level is reduced after CPS treatment, yet the CPS-treated enzyme is phosphorylated rapidly to form an $E_1P(2Ca^{2+})$ intermediate (Fig. 4C). This reduction in the EP level is likely a consequence of the rapid rate of dephosphorylation induced by the drug (Table 1). The pH dependence (Fig. 2) and the reduced sensitivity for ADP after CPS treatment (Fig. 6) indicated that CPS stabilizes an $E_2$-like SERCA isomer. PK cleavage experiments revealed that in the presence of CPS, the links between the A domain and TM domains 2 and 3 are more exposed (Fig. 7). An increase in ATP hydrolysis not coupled with active Ca$^{2+}$ transport presumably results in increased thermogenesis (15, 46, 47).

This study attempted to find how slippage is induced by CPS. According to the Albers-Post scheme, an $E_1P(2Ca^{2+})$ intermediate is formed after phosphoryl transfer from ATP. This form can react with ADP to form ATP, resulting in ATP synthesis. Nevertheless, ADP release from the $E_1P(2Ca^{2+})$ is probably necessary for Ca$^{2+}$ to transfer from the occlusion sites to the SR lumen in consonance with the $E_1P(2Ca^{2+})$ → $E_2P(2Ca^{2+})$ reaction (here Ca$^{2+}$ is bound with low affinity). Low affinity interaction of ATP is presumably important for proton release to the cytoplasm and isomerization to the $E_2$ form. Uncoupling is an inherent deviation from the normal physiological route and requires alternative isomerization steps (Scheme 1). Hence, uncoupling of SERCA requires ADP dissociation from the catalytic site, Ca$^{2+}$ slippage to the cytoplasm, and hydrolytic cleavage. According to our data, it does seem reasonable to consider an $E_1P(2Ca^{2+})$ → $E_2P(2Ca^{2+})$ transition, producing an isomer that releases Ca$^{2+}$ to the cytoplasm. We showed that CPS stabilizes a form with a decreased ADP affinity (Fig. 6) and increased regulatory interaction with ATP (Fig. 3). The uncoupled reaction is likely a consequence of conformational changes facilitated primarily by a high Ca$^{2+}$ concentration in the SR in addition to the nucleotide composition and possibly also the lipid composition (see Ref. 51 and below).

Digestion with PK of SERCA in the $E_2$ form facilitates cleavage between Thr$^{242}$ and Glu$^{243}$, producing a p83C and p28N fragments (39). An extra cleavage between Leu$^{119}$ and Lys$^{120}$ is observed in the $E_2$ conformation, producing p95C and p14N fragments (40). Recent studies have shown that SERCA undergoes significant conformational fluctuations in the native membrane milieu (45, 52). These fluctuations were reported to occur even in the presence of ligands that are expected to stabilize a given conformation; that is, isomerization between different conformers in a substrate-independent manner (45). We have shown that CPS treatment resulted in the exposure of the PK cleavage sites in SERCA (Fig. 7). Indeed, the specific exposure of the link between the A domain and TM3 is underestimated by the observation that in the presence of CPS, the p83C fragment

Evidence for the Functional Absence of VR1 in the Rabbit SR Vesicles—Previous studies have indicated the absence of passive Ca$^{2+}$ leak through inositol 1,4,5-trisphosphate-sensitive Ca$^{2+}$ channels in the SR membranes (48). Nevertheless, low expression of VR1 in the SR membranes of rat skeletal muscle was reported in a single report (49). Because CPS is a VR1 agonist (24), the presence of VR1 channels in the rabbit SR membranes would provide a pathway for passive Ca$^{2+}$ leak, and this would result in indirect SERCA stimulation through the dissipation of the Ca$^{2+}$ gradient across the SR membrane. Consequently, it was necessary to investigate whether or not VR1 functionally occurs in the SR membranes used in this study. Because VR1 is an unspecific cation channel (50), the addition of excess Na$^+$ would interfere with the passive Ca$^{2+}$ flux through the VR1 channel. It was found that the activation by CPS was the same in the absence or in the presence of 200 mM NaCl (data not shown). In addition, treatment with the VR1 antagonist capsazepine did not influence the stimulation produced by CPS (Fig. 9, left panel). Finally, treatment with the potent VR1 agonist resiniferatoxin did not elicit an increase in SERCA activity (Fig. 9, right panel). Thus, there is no evidence for the presence of functional VR1 in the SR vesicles used in this study.

**FIGURE 9. Evidence for the functional absence of VR1 in SR membranes.** The hydrolytic activity was measured in a reaction mixture containing 20 mM MOPS pH 7.0, 130 mM KCl, 2 mM Tris-ATP, 0.5 mM EGTA, 5 mM MgCl$_2$, 6 μg ml$^{-1}$ SR vesicles. Ca$^{2+}$ was added to produce a free Ca$^{2+}$ concentration of 10 μM. Left panel, the assays were performed in the presence of the indicated concentrations of capsazepine (circles), a potent VR1 antagonist. Diamonds show the activity obtained when excess EGTA was added to each reaction assay. Right panel, the assays were performed in the presence of the indicated CPS concentrations either in the absence (squares) or in the presence (triangles) of 20 μM resiniferatoxin, a potent VR1 agonist (24).
Capsaicin Stimulates Uncoupled SERCA

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REFERENCES

1. Kühbaurndt, W. (2004) Nat. Rev. 5, 282–295
2. Andersen, J. F. (1995) Biosci. Rep. 15, 243–261
3. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28815–28818
4. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
5. Toyoshima, C., Nomura, H., and Tsuda, T. (2004) Nature 432, 361–368
6. Olesen, C., Serensen, T. L.-M., Nielsen, R. C., Møller, J. V., and Nissen, P. (2004) Science 306, 2251–2255
7. Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14489–14496
8. Olesen, C., Picard, M., Winther, A. M., Gyrup, C., Morth, J. P., Oxvig, C., Møller, J. V., and Nissen, P. (2007) Nature 450, 1036–1042
9. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
10. Møller, J. V., Nissen, P., Serensen, T. L., and le Maire, M. (2005) Curr. Opin. Struct. Biol. 15, 387–393
11. Daiho, T., Yamasaki, K., Wang, G., Danko, S., Izuka, H., and Suzuki, H. (2003) J. Biol. Chem. 278, 39197–39204
12. Yamasaki, K., Daiho, T., Danko, S., and Suzuki, H. (2004) J. Biol. Chem. 279, 2202–2210
13. Daiho, T., Yamasaki, K., Danko, S., and Suzuki, H. (2007) J. Biol. Chem. 282, 34429–34447
14. Takahashi, M., Kondou, Y., and Toyoshima, C. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 5800–5805
15. de Meis, L. (2003) J. Biol. Chem. 278, 41856–41861
16. Clausen, J. D., McIntosh, D. B., Anthonisen, A. N., Woolley, D. G., Vilsen, B., and Anderssen, J. P. (2007) J. Biol. Chem. 282, 20686–20697
17. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. (1980) J. Biol. Chem. 255, 3025–3031
18. Yu, X., and Inesi, G. (1995) J. Biol. Chem. 270, 4361–4367
19. Fortea, M. I., Soler, F., and Fernandez-Belda, F. (2000) J. Biol. Chem. 275, 12521–12529
20. de Meis, L. (2001) J. Biol. Chem. 276, 25078–25087
21. Bhupathy, P., Babu, G. J., and Periasamy, M. (2007) J. Mol. Cell. Cardiol. 42, 903–911
22. Smith, W. S., Brodburng, R., East, J. M., and Lee, A. G. (2002) Biochem. J. 361, 277–286
23. Mall, S., Broadbridge, R., Harrison, S. L., Gore, M. G., Lee, A. G., and East, J. M. (2006) J. Biol. Chem. 281, 36597–36602
24. Szallasi, A., and Blumberg, P. M. (1999) Pharmacol. Rev. 51, 159–211
25. Møller, J. V., Dahl, I. M., and Nissen, P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14489–14496
26. Olesen, C., Picard, M., Winther, A. M., Gyrup, C., Morth, J. P., Oxvig, C., Møller, J. V., and Nissen, P. (2007) Nature 450, 1036–1042
27. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
28. Smith, W. S., Brodburng, R., East, J. M., and Lee, A. G. (2002) Biochem. J. 361, 277–286
29. Mall, S., Broadbridge, R., Harrison, S. L., Gore, M. G., Lee, A. G., and East, J. M. (2006) J. Biol. Chem. 281, 36597–36602
30. Szallasi, A., and Blumberg, P. M. (1999) Pharmacol. Rev. 51, 159–211
31. Møller, J. V., Dahl, I. M., and Nissen, P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14489–14496
32. Olesen, C., Picard, M., Winther, A. M., Gyrup, C., Morth, J. P., Oxvig, C., Møller, J. V., and Nissen, P. (2007) Nature 450, 1036–1042
33. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
34. Smith, W. S., Brodburng, R., East, J. M., and Lee, A. G. (2002) Biochem. J. 361, 277–286
35. Mahmmoud, Y. A. (2008) Nature 450, 1036–1042
36. Daiho, T., and Kanazawa, T. (1994) J. Biol. Chem. 269, 11060–11064
37. Shigekawa, M., and Pearl, L. J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 647–655
38. Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14489–14496
39. Daiho, T., Yamasaki, K., Wang, G., Danko, S., Izuka, H., and Suzuki, H. (2003) J. Biol. Chem. 278, 39197–39204
40. Lenoir, G., Picard, M., Gauron, C., Montigny, C., Maréchal, P. L., Falson, P., le Maire, M., Møller, J. V., and Champeil, P. (2004) J. Biol. Chem. 279, 9156–9166
41. Danko, S., Daiho, T., Yamasaki, K., Kamidochi, M., Suzuki, H., and Toyo-
Capsaicin Stimulates Uncoupled SERCA

shima, C. (2001) FEBS Lett. 489, 277–282
42. Inesi, G., Ma, H., Lewis, D., and Xu, C. (2004) J. Biol. Chem. 279, 31629–31637
43. Hua, S., Xu, C., Ma, H., and Inesi, G. (2005) J. Biol. Chem. 280, 17579–17583
44. Bartolommei, G., Tadini-Buoninsegni, F., Hua, S., Moncelli, M. R., Inesi, G., and Guidelli, R. (2006) J. Biol. Chem. 281, 9547–9551
45. Inesi, G., Lewis, D., Toyoshima, C., Hirata, A., and de Meis, L. (2008) J. Biol. Chem. 283, 1189–1196
46. Reis, M., Farage, M., de Souza, A. C., and de Meis, L. (2001) J. Biol. Chem. 276, 42793–42800
47. Barata, H., and de Meis, L. (2002) J. Biol. Chem. 277, 16868–16872
48. Logan-Smith, M. J., Lockyer, P. J., East, J. M., and Lee, A. G. (2001) J. Biol. Chem. 276, 46905–46911
49. Xin, H., Tanaka, H., Yamaguchi, M., Takemori, S., Nakamura, A., and Kohama, K. (2005) Biochem. Biophys. Res. Commun. 332, 756–762
50. Gunthorpe, M. J., Benham, C. D., Randall, A., and Davis, J. B. (2002) Trends Pharmacol. Sci. 23, 183–191
51. Starling, A. P., East, J. M., and Lee, A. G. (1995) J. Biol. Chem. 270, 14467–14470
52. Montigny, C., Picard, M., Lenoir, G., Gauron, C., Toyoshima, C., and Champeil, P. (2007) Biochemistry 46, 15162–15174
53. Hwang, S. W., Cho, H., Kwak, J., Lee, S.-Y., Kang, C.-J., Jung, J., Cho, S., Min, K. H., Suh, Y.-G., Kim, D., and Oh, U. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6155–6160
54. Smart, D., Gunthorpe, M. J., Jerman, J. C., Nasir, S., Gray, I., Muir, A. L., Chambers, J. K., Randall, A. D., and Davis, J. B. (2000) Br. J. Pharmacol. 129, 227–230