Anisodamine Causes the Changes of Structure and Function in the Transmembrane Domain of the Ca\(^{2+}\)-ATPase from Sarcoplasmic Reticulum

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The effects of anisodamine on the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum (SR) were investigated by using differential scanning calorimetry to measure the ability of anisodamine to denature the transmembrane domain and the cytoplasmic domain. Anisodamine significantly altered the thermotropic phase behaviors of the transmembrane domain of purified Ca\(^{2+}\)-ATPase. Specifically, the melting temperature of the transmembrane domain moved toward lower temperatures with the concentrations of anisodamine increasing and the thermotropic phase peak was abolished at 10 mM, indicating that the stabilized structure of the transmembrane domain in the presence of Ca\(^{2+}\) could be destabilized by anisodamine. Decreases of the intrinsic fluorescence and increases of the extrinsic fluorescence of ANS, a fluorescent probe, showed the exposure of tryptophan and hydrophobic region, respectively, suggesting again that anisodamine caused a less compact conformation in the transmembrane domain. A marked inhibition of the Ca\(^{2+}\) uptake activity of SR Ca\(^{2+}\)-ATPase was observed when the addition of anisodamine. The drug did not affect the cytoplasmic domain of the enzyme and only slightly decreased the ATPase activity of the enzyme at concentrations up to 10 mM. This was likely due to the destabilized protein transmembrane domain. To sum up, our results revealed that anisodamine interacted specifically with the transmembrane domain of SR Ca\(^{2+}\)-ATPase and inhibited the Ca\(^{2+}\) uptake activity of the enzyme.

Key words: anisodamine; Ca\(^{2+}\)-ATPase; differential scanning calorimetry (DSC); intrinsic fluorescence; 1-anilino-8-naphthalenesulfonate (ANS) fluorescence

Hyoscyamus niger L., a medicinal herb recorded in the famous ancient Chinese medical book, *Compendium of Materia Medica*, is widely dispersed throughout China. Anisodamine is isolated from these medicinal herbs and was first synthesized by Chinese scientists.1) Drugs in this family also include scopolamine and atropine. These drugs, showing an inhibitory effect on the cholinergic nerve function, as well as an improvement of the microcirculation, are extensively used in clinics, especially in cases of toxic shock and organophosphorus intoxication. The studies of the interaction between anisodamine and lipids have suggested that the primary target sites for anisodamine are most likely the lipid matrices of biological membranes. It has been observed that anisodamine increases the fluidity of membranes2) and induces the hexagonal phase of cardiolipin and dioleoylphosphatidylcholine (DOPC) liposomes.3) Recently we show that anticholinergic drugs including anisodamine and atropine can cause an interdigitated gel phase in dipalmitoylphosphatidylglycerol (DPPG) vesicles.4–6) Anisodamine also inhibits the activity of some enzymes,7,8) indicating that anisodamine may interact with functional membrane proteins either directly or indirectly. However, the detailed molecular mechanism has not been clarified. In particular, the drug binding sites in proteins have not been established yet.

As shown in Fig. 1, the anisodamine molecule has one monovalent trialkylamine cation, two hydrophilic hydroxyl groups, and one hydrophobic benzene ring. Raman spectroscopy reveals that an electrostatic interaction exists between the trivalkylamine group of the drug and the headgroup of acid lipids, and then the benzene ring of the drug is inserted into the phospholipid bilayer.9) However, no direct evidence has been provided to demonstrate anisodamine binding to discrete sites on membrane proteins. SR Ca\(^{2+}\)-ATPase is a suitable model protein to study the interaction between anisodamine and membrane proteins because it is a well characterized ion-transporting protein. SR Ca\(^{2+}\)-ATPase has high-affinity Ca\(^{2+}\) binding sites located in the transmembrane domain,10) therefore the thermotropic phase behaviors of the Ca\(^{2+}\)-ATPase in EGTA are different from that obtained in the presence of Ca\(^{2+}\) in differential scanning calorimetry (DSC) profiles. The

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Abbreviations: DSC, differential scanning calorimetry; SR, sarcoplasmic reticulum; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; Gm\(_1\), monosialo ganglioside-Gm\(_1\); Gm\(_3\), monosialoganglioside-Gm\(_3\); ANS, 1-anilino-8-naphthalenesulfonate; T\(_m\), transition temperature; \(\Delta H\), transition enthalpy
cytoplasmic domain containing the ATP binding subdomain denatures or unfolds at about 50 °C, while the transmembrane domain which is stabilized by Ca$^{2+}$ denatures at about 59 °C in the presence of 1 mM Ca$^{2+}$.

In this study, we investigated the thermotropic behaviors and conformational changes of purified SR Ca$^{2+}$-ATPase induced by anisodamine using three assays: DSC, intrinsic fluorescence, and ANS-binding extrinsic fluorescence. These data characterized the features of the interaction between anisodamine and SR Ca$^{2+}$-ATPase and would help to clarify the relationship between the conformational change and the activity of SR Ca$^{2+}$-ATPase.

Materials and Methods

Pyruvate kinase (PK), l-Lactic dehydrogenase (LDH), Phospho-enol pyruvate (PEP), 1-anilino-8-naphthalenesulfonate (ANS), and EGTA were obtained from Sigma. β-Nicotinamide adenine dinucleotide, reduced disodium salt (NADH) was obtained from Boehringer. ATP was purchased from Fluka. Dithiothreitol (DTT) was purchased from Bebco. Anisodamine was the product of the Chengdu Medicinal Factory. All other reagents were of analytical grade.

Sarcoplasmic reticulum isolation and the Ca$^{2+}$-ATPase purification. Sarcoplasmic reticulum (SR) was isolated from back and hind leg white muscles of New Zealand white rabbits and the Ca$^{2+}$-ATPase was purified from SR as described by Michelangeli et al.

The purity of SR Ca$^{2+}$-ATPase was checked by SDS-PAGE and found to be more than 95% pure. Protein concentration was measured by a modification of the Lowry method, using bovine serum albumin as the standard.

Differential scanning calorimetry (DSC). SR Ca$^{2+}$-ATPase was dialyzed overnight against buffer containing 20 mM Tris-HCl, 150 mM KCl (pH 7.2) in order to remove all the sucrose and dithiothreitol, then the samples were suspended in the same buffer at a protein concentration of 4 mg/ml. Anisodamine of 100 mM was prepared for stock solution by adding the buffer used for DSC and pH was carefully readjusted to 7.2. The free calcium concentration was buffered by a CaCl$_2$/EGTA solution and calculated with the aid of a computer program using previously reported association constants. All samples containing certain concentrations of Ca$^{2+}$ and anisodamine were incubated at 37°C for 30 min and the samples were degassed under vacuum for 10 min before the cells were loaded. The samples were run on a high sensitivity MC-2 differential scanning calorimeter interfaced to an IBM microcomputer (Microcal, Northampton, MA) and the transition temperatures (Tm) of the Ca$^{2+}$-ATPase were measured. The scan rate for all scans was 60 °C/h.

Intrinsic fluorescence measurement. SR Ca$^{2+}$-ATPase samples were treated with anisodamine as described above. The intrinsic fluorescence was measured with a Hitachi F4010 spectrofluorimeter at 30 °C with an excitation wavelength of 285 nm. The fluorescence emission spectra were recorded in the 300–400 nm ranges. Both excitation and emission slits were set at 5 nm.

ANS-binding fluorescence. ANS-binding fluorescence was used to study the changes in the surface hydrophobicity of proteins. The 18 μM protein of SR Ca$^{2+}$-ATPase was incubated with 1 mM Ca$^{2+}$ and a variety of concentrations of anisodamine at 37°C for 30 min in 50 mM Tris-HCl buffer, pH 7.4, then 20-fold excess ANS was added to every protein sample, incubated again at 25°C for 15 min. ANS-binding fluorescence spectra were obtained by using a Hitachi F4010 spectrofluorimeter at 25°C. The excitation wavelength was 378 nm, and the fluorescence emission spectra were recorded in the 400–600 nm ranges with a slit width of 5 nm.

ATPase activity assay. SR Ca$^{2+}$-ATPase at 4 mg/ml was incubated with 1 mM Ca$^{2+}$ and various concentrations of anisodamine for 30 min at 37°C. For ATP hydrolysis assays of the enzyme, the absorbance of NADH at 340 nm was monitored by using AR-30 m and the absorbance of arsenazo III at 675–685 nm by using ATP-regeneration in a solution of 40 mM Hepes, 100 mM KCl, 5 mM MgSO$_4$, 70 μM arsenazo III, 50 μM Ca$^{2+}$, 25 mM ATP (pH 7.2), and SR 75 μg protein/ml.

Calcium uptake assays. SR at 4 mg/ml was treated with anisodamine as described above, then calcium uptake was measured with a Hitachi 557 dual wavelength spectrophotometer at 30°C which monitored the absorbance of arsenazo III at 675–685 nm by using ATP-regeneration in a solution of 40 mM Hepes, 100 mM KCl, 5 mM MgSO$_4$, 70 μM arsenazo III, 50 μM Ca$^{2+}$, 25 mM ATP (pH 7.2), and SR 75 μg protein/ml.
Results

Conformational changes in the transmembrane domain of SR Ca\(^{2+}\)-ATPase by calcium

Figure 2 showed the original DSC thermograms of purified SR Ca\(^{2+}\)-ATPase in the presence of 1 mM EGTA (a), 1 mM Ca\(^{2+}\) (b), 1 mM Mg\(^{2+}\) (c) and 1 mM Sr\(^{2+}\) (d). The experimental conditions are described in Materials and Methods. The results were representative of two other DSC profiles.

![DSC Profiles of Purified Ca\(^{2+}\)-ATPase of SR in the Presence of 1 mM EGTA (a), 1 mM Ca\(^{2+}\) (b), 1 mM Mg\(^{2+}\) (c) and 1 mM Sr\(^{2+}\) (d).](image)

Destabilization of the transmembrane domain by anisodamine

The interaction between anisodamine and SR Ca\(^{2+}\)-ATPase in 1 mM Ca\(^{2+}\) clearly altered the thermotropic phase behaviors of the transmembrane domain (peak 2) but not those of the cytoplasmic domain (peak 1) (Fig. 3). The transition temperature of peak 2 increased slightly at 1 mM anisodamine, and then the temperature moved toward lower temperatures with the concentrations of anisodamine increasing. Simultaneously, the area of peak 2 abruptly decreased with increased anisodamine content. Finally, peak 2 was abolished at 10 mM anisodamine. It should be pointed out that the interaction between a local anaesthetic drug dibucaine with SR Ca\(^{2+}\)-ATPase induced only a decrease of transition temperature of peak 2. It is reasonable to believe that the interaction mechanism of anisodamine is different from that of dibucaine, although it’s not feasible to identify them in this experiment. All \(T_m\) values were listed in table 1 which showed that the stabilized structure of the transmembrane domain (peak

![DSC Profiles of Purified Ca\(^{2+}\)-ATPase of SR in the Presence of 1 mM Ca\(^{2+}\) Containing Various Anisodamine Concentrations. The final anisodamine concentrations were 0, 1, 3, 5, 7, 10 mM for the curves from a to f. Protein concentration, 4 mg/ml. Scanning rate, 60 °C/h. The experiments were done at least three times with the same results.](image)

Table 1. Effects of Various Concentrations of Anisodamine on Thermotropic Parameters of Cytoplasmic Domain (\(T_m\)) and Transmembrane Domain (\(T_{m2}\)).

<table>
<thead>
<tr>
<th>Anisodamine (mM)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T_m) (°C)</td>
<td>50.9</td>
<td>51.6</td>
<td>51.8</td>
<td>51.4</td>
<td>51.6</td>
<td>51.0</td>
</tr>
<tr>
<td>(T_{m2}) (°C)</td>
<td>60.0</td>
<td>60.7</td>
<td>59.4</td>
<td>59.0</td>
<td>57.4</td>
<td>/</td>
</tr>
</tbody>
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2: $T_m$ in 1 mM Ca$^{2+}$ began disruption due to the addition of anisodamine while the cytoplasmic domain (peak 1: $T_m$) had no great changes with increasing of anisodamine contents.

Quenching of intrinsic tryptophan fluorescence

Figure 4 showed the intrinsic fluorescence emission spectra of SR Ca$^{2+}$-ATPase in the presence of anisodamine. It could be seen that the intrinsic fluorescence intensity of the Ca$^{2+}$-ATPase at 334 nm decreased with the concentration of the drug increasing. Although the fluorescence emission intensity of the enzyme is decreased, the emission maximum is not shifted, indicating that the microenvironments of the tryptophan residues are similar with and without anisodamine. The difference in the emission intensity may be due to different microenvironments of the packing of side chains.21

ANS-binding fluorescence spectra

ANS was used as a hydrophobic fluorescence probe to measure the changes in surface hydrophobicity of SR Ca$^{2+}$-ATPase at various anisodamine concentrations. The difference in hydrophobicity reflects the change in exposure of hydrophobic regions of the enzyme. The fluorescence emission spectra for the Ca$^{2+}$-ATPase in the presence of anisodamine were shown in Fig. 5. Compared with the fluorescence emission spectrum without anisodamine (Fig. 5c), the spectra of the enzyme at various concentrations of anisodamine showed an apparent increase in fluorescence intensity at 470 nm except at 1 mM anisodamine (the emission maximum of ANS itself was at 520 nm and little change happened with the addition of anisodamine up to 10 mM, Fig. 5a, b). This indicates that the transmembrane domains of the Ca$^{2+}$-ATPase undergo a conformational change and increase the exposure of hydrophobic regions, which is consistent with the result of intrinsic fluorescence.

The effects of anisodamine on the activity of SR Ca$^{2+}$-ATPase

The changes in the ATPase activity of purified SR Ca$^{2+}$-ATPase and in the Ca$^{2+}$ uptake activity of the Ca$^{2+}$-ATPase in natural SR were monitored at different concentrations of anisodamine. As shown in Fig. 6, anisodamine had a slight effect on the ATPase activity of the enzyme at concentration up to 10 mM. In contrast, the drug markedly inhibited the Ca$^{2+}$ uptake activity with half-inhibition at approximately 4 mM (Fig. 7). The inhibition effect approached the maximum of 80% at 10 mM, although the uptake activity was slightly increased at 1 mM concentration. These results indicate that anisodamine has a weak interaction with the cytoplasmic domain containing the site of ATP for ATP hydrolysis and a significant interaction with the transmembrane domain that contains Ca$^{2+}$ binding sites.

Discussion

The SR Ca$^{2+}$-ATPase molecule consists of a large cytoplasmic domain, connected by a narrow stalk to the transmembrane helics with calcium binding sites which are expected to be located in the middle of the membrane (≈12 Å from the cytoplasmic/lipid bilayer junction). Evidence has been reported that Ca$^{2+}$ transport is associated with a conformational change induced in the enzyme by hydrolysis of ATP.22 The transmembrane domain, which denatured at 50°C (the same $T_m$ as the cytoplasmic domain) in 1 mM EGTA,
underwent a conformational change when calcium binding to the domain, and a new transition appeared at 60°C/C14. This compact structure of the subdomain region containing calcium sites may be necessary to form a stabilized calcium access from the calcium-binding sites to the lumen.

Anisodamine has a monovalent trialkylamino cation and a hydrophobic benzene ring (Fig. 1). Thus, the drug can be regarded as an amphiphilic molecule with one positive charge and one short non-polar moiety. The trialkylamino group interacts with the acid lipid first, then the non-polar benzene ring is incorporated into biological membrane.6) It is reasonable to believe that anisodamine may interact directly with SR Ca^{2+}-ATPase which results in a decline of the Ca^{2+} uptake activity of the enzyme. DSC results indicate that the drug interacts with the transmembrane domain (peak 2) in a much stronger way than with the aqueous regions of the enzyme.

The stabilized structure of the transmembrane domain can be destabilized by some anaesthetics, and induces a decrease of Ca^{2+} transport.18) It should be emphasized that anisodamine inhibits only the Ca^{2+} uptake activity rather than the ATPase activity of the Ca^{2+}-ATPase. In other words, the energy obtained from the hydrolysis of ATP can not induce Ca^{2+} transport through Ca^{2+} access because anisodamine attacks only the transmembrane domain of the Ca^{2+}-ATPase, and results in destabilization of the domain, indicating that this stabilized structure of calcium access by calcium is necessary for Ca^{2+} transport. This supports the DSC evidence that anisodamine interacts at the transmembrane level, either directly with the Ca^{2+}-ATPase or with annular lipids surrounding the protein.

In order to gain further insight into the conformational change of SR Ca^{2+}-ATPase, we used two complementary approaches to define the physical properties of the enzyme. Decreases of the intrinsic fluorescence and increases of the extrinsic fluorescence of ANS reflected the exposure of tryptophan residues and hydrophobic regions, indicating that anisodamine caused the conformation of the transmembrane domain to be less compact and this conformational change leaded to lower enzyme activity. These results are consistent with those from DSC. In addition, it is very interesting to note that the Ca^{2+}-ATPase is activated at 1 mM anisodamine (Fig. 7) which is in agreement with the conformational change of the enzyme, that is, transition temperature increases (Fig. 3), intrinsic fluorescence increases (Fig. 4) and extrinsic fluorescence of ANS decreases (Fig. 5), respectively. This means anisodamine at 1 mM can make the hydrophobic domain more compact. Similar relationship of structure and function, such as an antagonistic effect of Gm1 and Gm3 on the activity and conformation of the Ca^{2+}-ATPase, has been reported.23) Gm1 causes less compact conformation and lowers the activity of the enzyme, while Gm3 induces both the hydrophobic domain and the hydrophilic domain to be more compact and increases the enzymatic activity, indicating that compact structure is necessary for increased activities.

Different from soluble proteins, SR Ca^{2+}-ATPase, a membrane protein, is embedded in lipid bilayer. Any factor that affects the physical state of phospholipids may cause changes in the Ca^{2+}-ATPase in terms of conformation and function. Therefore, we could not exclude the possibility that the interaction between anisodamine and SR Ca^{2+}-ATPase might be indirect, i.e. through membrane lipids since even purified Ca^{2+}-ATPase contains annular lipids surrounding the protein.

Fig. 6. Effect of Anisodamine on the ATPase Activity of Purified Ca^{2+}-ATPase of SR.

The experimental conditions are described in Materials and Methods. The data were averages (± S.E.) of three independent experiments, each with three duplicates.

Fig. 7. Effect of Anisodamine on the Ca^{2+} Uptake Activity of the Ca^{2+}-ATPase in Natural SR.

A, The plot of Ca^{2+} uptake vs time. The final concentrations of anisodamine were 0, 1, 3, 5, 7, 10 mM for the curves from a to f. B, the plot of specific activity vs anisodamine concentrations. The data were calculated from A at 1 minute point. The experimental conditions as described under materials and methods. The data were representative of three independent experiments.
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References


