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Effects of tetrandrine on calcium transport, protein fluorescences and membrane fluidity of sarcoplasmic reticulum

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1 To understand whether the molecular mechanism of Tetrandrine (Tet)'s pharmacological effects is concerned with sarcoplasmic reticulum calcium transport so as to be involved in myocardial contractility, we observed the effects of Tet on calcium transport and membrane structure of rabbit skeletal muscle sarcoplasmic reticulum vesicles (SR) and rat cardiac sarcoplasmic reticulum vesicles (CSR).

2 Calcium uptake was monitored with a dual-wavelength spectrophotometer. Protein conformation and fluorescence polarization were measured by fluospectrophotometric method and membrane lipids labelled with fluorescence probes for SR, respectively.

3 128 μmol l^{-1} Tet reduced the initial rate of calcium uptake to 59% of control 6 min after reaction. Tet un-competitively inhibited SR Ca^{2+},Mg^{2+}-ATPase activity, causing the stoichiometric ratio of SR Ca^{2+}/ATP to decrease to 1.43 from 2.0 of control.

4 Inhibitory rates on SR Ca^{2+},Mg^{2+}-ATPase by Tet were reduced from 60% in the absence of phosphate to 50% in the presence of phosphate and reduced from 92% in 1 mmol l^{-1} ATP to 60% in 5 mmol l^{-1} ATP.

5 Tet markedly reduced SR intrinsic protein fluorescence, while it slightly decreased the thiol(SH)-modified protein fluorescence of SR labelled with N-(3-pyrene)-maleimide.

6 Tet slightly increased fluorescence polarization in the middle and deep layers of SR membrane lipids labelled with 7- or 12-(9-anthroyloxy) stearic acid (AS) probes, whereas it did not change that of SR labelled with 1,6-diphenyl-1,3,5-hexatrine (DPH).

7 These results revealed that prevention of SR calcium uptake by Tet was due to inhibition of the SR calcium pump Ca^{2+},Mg^{2+}-ATPase, changes in spatial conformation of the pumps protein molecules and a decrease in the extent of motion of membrane lipid molecules, thus altering the regulation of $[Ca^{2+}]_i$ and myocardial contractility.
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Abbreviations: 2-,7-, and 12-AS, 2-,7- and 12-(9-anthroyloxy) stearic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; CSR, cardiac sarcoplasmic reticulum vesicles; DPH, 1, 6-diphenyl-1, 3, 5-hexatrine; EGTA, ethyleneglycol-bis-β-aminoethyl ether-N,N′; λ em, emission wavelength; λ ex, excitation wavelength; N-(3-P)-M, N-(3-pyrene) maleimide; Pi, inorganic phosphate; SR, skeletal muscle sarcoplasmic reticulum vesicles; Tet, tetrandrine

Introduction

Tetrandrine (Tet), isolated from the root of *Stephania tetrandra* (*S, Moore*) is a bisbenzylisoquinoline alkaloid. Tet decreases blood pressure (Kwan & Deng, 1991), is an anti-angina agent (Xia *et al*., 1994) and is a therapeutic for silicosis (Liu *et al*., 1995). Furthermore it was reported that Tet was a calcium-antagonist (Yan *et al*., 1997; Deng *et al*., 1993). Some physiological effects of Tet were observed on contraction of coronary arterial strips and uterus, but its pharmacological action at molecular level is still unclear. The sarcoplasmic reticulum calcium transport plays an important role in cardiac excitation-contraction coupling and regulation of myocardial contractility. To understand whether the molecular mechanism of Tet's pharmacological effects is concerned with sarcoplasmic reticulum calcium transport so as to be involved in myocardial contractility, effects of Tet, as an amphiphile, on sarcoplasmic reticulum calcium transport were investigated in our laboratory. In a previous study (Chen & Chen, 1990), a comparative study of Tet and stearamine, both amphiphiles with positive charge, was conducted showing an influence on Ca^{2+},Mg^{2+}-ATPase activities in skeletal muscle sarcoplasmic reticulum vesicles (SR) which have come to represent the 'gold standard' for work in this field. The inhibition of the enzyme by stearylamine was increased along with an increase in the SR membrane: amphiphile ratio, while inhibitory effect of Tet on the enzyme was not influenced by this parameter, indicating a variable mechanism of action of the amphiphiles effect on membranes. In this study, we wished to further determine the molecular basis of action of Tet as an amphiphile. We observed effects of Tet on the initial rate of SR calcium uptake, SR Ca^{2+},Mg^{2+}-ATPase activity, intrinsic protein fluorescence and thiol (SH)-modified protein fluorescence of the SR calcium pump, and SR membrane lipid fluidity, and analysed relationship of changes between structure and function of SR to further understand effects of Tet on SR calcium transport and the relation of the Tet action to the regulation of intracellular Ca^{2+} concentration and myocardial contractility.

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Methods

Preparation of rabbit skeletal muscle sarcoplasmic reticulum vesicles (SR)

For fractionation of muscle, all operation was carried out in the cold. The New Zealand rabbit was killed by a blow on the head and the white muscle in thigh was cut in small pieces and placed in 0.01 mol l^{-1} $NaHCO_3$ solution (muscle : solution = 1 : 5). The homogenate after homogenization in a Waring Blendor was spun at $9000 \times g$ for 10 min (4°C). The supernatant was passed through eight layers of cheesecloth to remove floating fat material and centrifuged at $9000 \times g$ for 20 min (4°C). The supernatant was passed through cheesecloth again and centrifuged at $30{,}000 \times g$ for 45 min (4°C). The pellet was resuspended in 0.6 mol l^{-1} KCl – 20 mmol l^{-1} MOPS solution, pH 6.8, homogenized and centrifuged at $30{,}000 \times g$ for 45 min (4°C). The pellet was rehomogenized in 10% sucrose (about 30 mg ml^{-1}). The cytochrome c oxidase activity and the ouabain-sensitive Na^+,K^+-ATPase activity demonstrated that there was almost no contamination by mitochondria or by sarcolemma.

Preparation of rat cardiac sarcoplasmic reticulum vesicles (CSR)

CSR preparation was described previously by our laboratory. Briefly, CSR was purified from hearts from Wistar rat (Grade II) by means of differential centrifugation. The molecular weight of CSR Ca^{2+}, Mg^{2+}-ATPase was 18 kDa, as determined by SDS – PAGE. The microstructure observed by electron microscope showed that the CSR vesicles were intact. The ouabain-sensitive Na^+,K^+-ATPase activity demonstrated that there was almost no contamination by sarcolemma.

Protein content

Protein concentration was determined according to the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Calcium uptake

Calcium uptake was measured with a Shimadzu UV 3000 spectrophotometer (Scarpa *et al.*, 1978). The spectrophotometer was zeroed using a cuvette containing a 2 ml solution of 50 mmol l^{-1} potassium phosphate, 100 μmol l^{-1} antipyrylazo III, 5 mmol l^{-1} Mg ATP, 0 – 128 μmol l^{-1} Tet and 40 mmol l^{-1} histidine-120 mmol l^{-1} KCl, pH 6.8 after equilibrium of the solution to 25°C. Forty μmol l^{-1} $CaCl_2$ as a standard was added separately four times. The reaction was initiated by addition of 12 μg of SR. The difference change of absorbance, due to SR taking up Ca^{2+} previously bound to antipyrylazo III, was monitored at 790 and 720 nm for calculation of SR calcium uptake. The rates of calcium uptake and calcium release were expressed as μmol mg^{-1} - min^{-1}.

Ca^{2+},Mg^{2+}-ATPase activity

Assays of Ca^{2+},Mg^{2+}-ATPase activities were performed with two different methods of kinetic analysis and colorimetric analysis. The kinetic method for determining ATPase activities was carried with a Hitachi UV 200-20 spectrophotometer by our laboratory. Briefly, for total Ca^{2+},Mg^{2+}-ATPase activities, the reaction mixture of 1.5 ml contained 5 mmol l^{-1} MgATP, 40 μmol l^{-1} $CaCl_2$, 50 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} phosphoenol-pyruvate, 0.2 mmol l^{-1} NADH, 0.15 units pyruvate kinase, 2.25 units lactate dehydrogenase, 12 mg l^{-1} SR protein, 40 mmol l^{-1} histidine-120 mmol l^{-1} KCl, pH 6.8 at 25°C. The reaction conditions for basal ATPase measurement was the same as for total ATPase, except that $CaCl_2$ was omitted from the reaction solution in the presence of 1 mmol l^{-1} EGTA. Ca^{2+},Mg^{2+}-ATPase activity was calculated as the difference between total ATPase and basal ATPase activities. For experiments with addition of Tet to the enzyme, SR was incubated with Tet for 5 min, at 25°C, following which the reaction was initiated by addition of ATP. Enzyme activity was expressed as μmol NADH mg^{-1} min^{-1} or Δ A 340 min^{-1}. The colorimetric method (blue colour at 820 nm) for determining ATPase activities was measured as inorganic phosphate (P_i) liberation (Chen *et al.*, 1956). Only if is there no inorganic phosphate, which develops colour, in the reaction solution, can the colorimetric method be selected. The colorimetric method of determining ATPase activity is described as follows. The reaction solution (2 ml) for total Ca^{2+},Mg^{2+}-ATPase activity contained 5 or 1 mmol l^{-1} MgATP, 12 μmol l^{-1} $CaCl_2$, 12 mg l^{-1} SR protein, 40 mmol l^{-1} histidine-120 mmol l^{-1} KCl, pH 6.8, at 25°C. The reaction condition for basal ATPase measurement was the same as that for total ATPase, except that $CaCl_2$ was omitted from the reaction solution in the presence of 1 mmol l^{-1} EGTA. Enzyme activity was expressed as μmol P_I mg^{-1} min^{-1}.

Spectra of intrinsic protein fluorescence

Intrinsic protein fluorescence spectra of the SR calcium pump protein were monitored with a Hitachi MPF-4 spectrofluorimeter at an emission wavelength (λ em) of 300 – 380 nm and an excitation wavelength (λ ex) of 290 nm, at 25°C in 3 ml of reaction solution of 5 mmol l^{-1} MgATP, 50 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 40 mmol l^{-1} histidine-120 mmol l^{-1} KCl, pH 6.8, 24 mg l^{-1} SR protein and Tet (incubated with SR in the reaction solution for 5 min before reaction). The fluorescence emission of the reagent solution including Tet with the omission of SR was used as control and subtracted automatically from each sample by machine.

Spectra of thiol (SH)-modified protein fluorescence

SH-modified protein fluorescence spectra of SR were measured at 25°C using a λ em of 360 – 420 nm and a λ ex of 347 nm in the reaction solution mentioned above except that N-(3-P)-M labelled SR (24 mg l^{-1}) was used instead of unlabelled SR. The fluorescence emission of reaction solution with Tet, in the absence of labelled SR, as control was also deducted from each sample. For labelling SR, N-(3-P)-M was dissolved in acetone and incubated with SR for 1.5 h at a SR (g l^{-1}) : N-(3-P)-M (μmol l^{-1}) ratio of 1 : 60 and then centrifuged at $30{,}000 \times g$ for 40 min, at 4°C. After washing with cold distilled water three times to remove free N-(3-P)-M, the pellet was suspended in 10% sucrose (protein concentration of around 7 g l^{-1}).

Fluorescence polarization measurements

SR membrane fluidity can be analysed with a Hitachi MPF-4 spectrofluorimeter by means of polarization measurements. For analysis of total membrane fluidity in the whole

membrane layer, the lipid in SR was labelled with 1,6-diphenyl-1,3,5-hexatrine (DPH). 1×10^{-3} mol l^{-1} DPH stock solution in tetrahydrofuran (C_4H_8O) was diluted to 2×10^{-5} mol l^{-1} before use. SR protein (2 mg) was mixed with DPH (1 ml of 2×10^{-5} mol l^{-1}) and incubated for 1 h, at 25°C. The solution was centrifuged at $30{,}000\times g$ for 40 min, at 4°C. The pellet was washed with cold distilled water three times to remove free DPH and then suspended in 10% cold sucrose. This preparation was then diluted to 2.4 g l^{-1} just before use. Fluorescence polarization (P) of SR labelled with DPH was measured at a λ em of 432 nm and a λ ex of 362 nm in the same reaction solution as in intrinsic protein fluorescence assay, at 25°C, except that SR labelled with DPH was used, instead of unlabelled SR. P value was calculated according to the data of I_{VV}, I_{VH}, I_{HV} and I_{HH}. For analysis of membrane lipid fluidity in each layer, the lipids in the surface, middle and deep layers of SR membrane were labelled with 2- or 7- or 12-(9-anthroyloxy) stearic acid [2-AS, 7-AS and 12-AS] probes, respectively. The three AS stock solutions of 1×10^{-3} mol l^{-1} dissolved in methanol were diluted to 2×10^{-4} mol l^{-1} with water, respectively before use. At an AS : SR ratio of 1 : 144 (mg : mg), SR was mixed with the 2-, 7- and 12-AS probes, respectively and incubated for 1 h, at 25°C, and then centrifuged at $30{,}000\times g$ for 40 min, at 4°C. The pellet was washed three times to remove free AS molecules and then resuspended in 10% of cold sucrose solution and diluted to 2.4 g l^{-1} prior to use. Fluorescence polarization for each layer was measured at a λ em of 460 nm and a λ ex of 390 nm in reaction solutions mentioned above, except that SR labelled with 2-,7- and 12-AS probes were used, respectively instead of DPH labelled SR.

Reagents

1,6-diphenyl-1,3,5-hexatrine (DPH), antipyrylazo III,3-[N-morpholino]propanesulphonic acid (MOPS), ethyleneglycol-bis-β-aminoethyl ether-N,N′(EGTA), histidine, KCl, bovine serum albumin and lactic dehydrogenase were obtained from Sigma Co. Pyruvate kinase, phosphoenol-pyruvate, NADH and ATP-Na_2 were obtained from Boehringher Mannheim. N-(3-pyrene)-maleimide [N-(3-P)-M], 2-(9-anthroyloxy) stearic acid [2-AS], 7-(9-anthroyloxy) stearic acid [7-AS] and 12-(9-anthroyloxy) stearic acid [12-AS] were obtained from Molecular Probes Co. $CaCl_2$, Dowex AG 50 W-X8 (200-400 mesh hydrogen form), sucrose (super-grade), 2,5-diphenyl oxazole (PPO) and Tris were obtained from MC/B (USA), Bio-Rad, Schevarz/Mann Inc, Carl-Roth and Merk, respectively. Tetrandrine was produced by Zhejiang Jinhua Farmacia-Factory in China and a stock solution of 16 mmol l^{-1} Tet in water was used in the experiments. The reagents from foreign countries were ordered by the Department of Equipment and Supplies, Chinese Academy of Medical Sciences, Beijing, China. All other reagents used were local products of analytical grade.

Results

Calcium uptake and release

In the presence of 50 mmol l^{-1} potassium phosphate, SR calcium content was maximal 5–6 min after the addition of SR and decreased gradually (Figure 1). When 128 μmol l^{-1} Tet in a range of 32–192 μmol l^{-1} was added before reaction, the calcium content, initial rate of calcium uptake

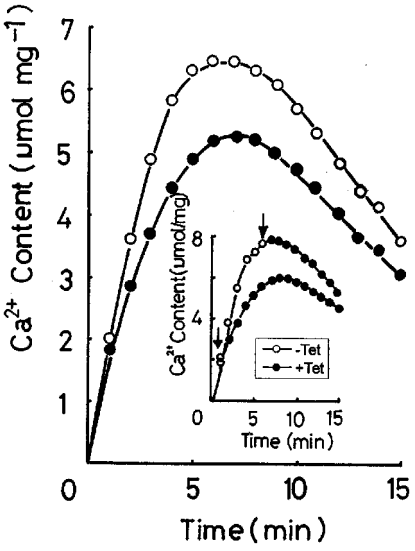

Figure 1 The time course of sarcoplasmic reticulum calcium uptake and release in the presence of 128 μmol l^{-1} tetrandrine. Calcium uptake of sarcoplasmic reticulum vesicles from rabbit skeletal muscle in the absence and presence of 128 μmol l^{-1} tetrandrine were performed as described under Methods. The inset shows effects of 128 μmol l^{-1} tetrandrine on sarcoplasmic reticulum calcium uptake with Tet added in the first and sixth minutes after the start of the reaction with the sarcoplasmic reticulum, respectively.

(μmol mg^{-1} min^{-1}) and rate of spontaneous calcium release (μmol mg^{-1} min^{-1}) 6 min after the reaction were reduced to 80, 59 and 74% of control, respectively (Figure 1). To further study the time course of the effect of Tet on rates of calcium uptake and release, Tet was added 1 min after the initiation of the reaction. Here the initial rate of calcium uptake was shown to decrease to 1.22 (58% of control) from 2.10 (control), while addition of Tet at the sixth minute after the reaction did not change the rate of spontaneous calcium release (the inset to Figure 1). In addition, inhibitory effect of Tet on the rates of SR calcium uptake and release was concentration-dependent, and enhanced with increasing Tet concentrations within the range of 32–192 μmol l^{-1}. These results indicate that Tet inhibited SR calcium uptake more than SR calcium release (Table 1).

Ca^{2+},Mg^{2+}-ATPase activities and ratio of Ca^{2+}/ATP

Phosphate influences Tet inhibition on SR Ca^{2+},Mg^{2+}-ATPase activities. In reaction solutions with addition of phosphate, to reaction solutions the same as those of calcium uptake, the K_M of SR Ca^{2+},Mg^{2+}-ATPase activities determined by kinetic assay was 64 μmol l^{-1} towards ATP. Indeed this value is similar to that (53 μmol l^{-1}) measured by colorimetric analysis (Chen & Chen, 1987). In the above reaction solution, 32–256 μmol l^{-1} Tet had an inhibitory effect on the enzyme activities in a concentration-dependent manner with maximal inhibition rates of 50%. The IC_{50} was around 80 μmol l^{-1} (Figure 2, curve 1). Tet showed uncompetitive kinetics with K_i of 225 μmol l^{-1} obtained from both Linewearer-Burk (Figure 3A) and Dixon (Figure 3B) plots, respectively. In reaction solutions containing 5 mmol l^{-1} Mg ATP without phosphate, the inhibitory effect of 50 μmol l^{-1} Tet on Ca^{2+},Mg^{2+}-ATPase activity was maximal by inhibition rate of 60% with an IC_{50} of 18.5 μmol l^{-1} by colorometric analysis (Figure 2, curve 2). Thus, it demonstrates that Tet, in the absence of phosphate,

Table 1 Effects of tetrandrine on the initial rate of calcium uptake, ATP hydrolysis and Ca^{2+}/ATP ratios from rabbit skeletal muscle sarcoplasmic reticulum vesicles (SR)

Tetrandrine (μmol l^{-1})	*Initial rate of calcium uptake* (μmol mg^{-1} min^{-1})	*Ca^{2+}, Mg^{2+}-ATPase activity* (μmol min^{-1} mg^{-1})	*Ca^{2+}/ ATP ratios***	*Calcium release* (μmol min^{-1} mg^{-1})
0*	1.83	0.919	1.99	0.584
32	1.66	0.831	1.99	
64	1.23	0.711	1.72	0.587
128	0.789	0.611	1.29	0.434
192	0.719	0.504	1.43	0.353
256		0.472		

Calcium uptake and the ATP hydrolysis with spectrophotometric assay were performed as described under Methods. *The results in the absence of Tet represent as 100%. **The ratios of rates of calcium uptake and of ATP hydrolysis by the Ca^{2+}, Mg^{2+}-ATPase.

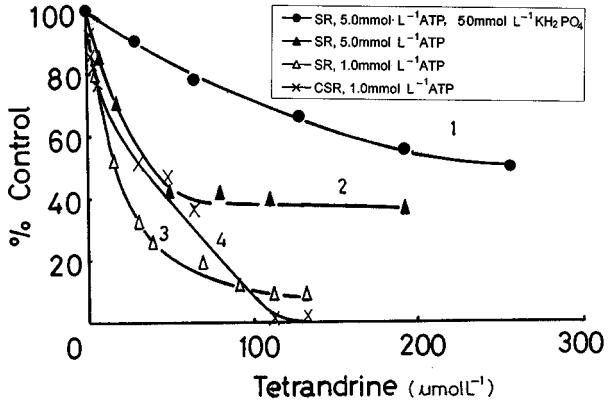

Figure 2 Comparisons of effects of phosphate and ATP concentrations, respectively, on sarcoplasmic reticulum Ca^{2+} +,Mg^{2+}-ATPase activities with tetrandrine solutions of increasing concentrations. Enzyme assays were performed as described under Methods in the presence of 12 mg l^{-1} rabbit skeletal muscle sarcoplasmic reticulum vesicles (SR) or rat cardiac sarcoplasmic reticulum vesicles (CSR). In the presence of 5 mmol l^{-1} ATP and SR, curves 1 and 2 were measured by UV spectrophotometric assay (using reaction solution in the presence of 50 mmol l^{-1} KH_2PO_4) and by colorimetric method (using that in the absence of KH_2PO_4), respectively. Curves 3 and 4 were determined by colorimetric method without KH_2PO_4 in the presence of 1 mmol l^{-1} ATP using SR and CSR, respectively.

inhibited the enzyme significantly more than in the presence of phosphate.

The ATP concentrations also influence inhibition of SR Ca^{2+},Mg^{2+}-ATPase activities by Tet. The extent of inhibition of the enzyme by Tet was related to ATP concentration. When the ATP concentration in the reaction solution without phosphate was 1 mmol l^{-1}, 112 μmol l^{-1} Tet was found to have maximal inhibitory effects on the Ca^{2+},Mg^{2+}-ATPase activity measured either from rabbit SR or from rat CSR with colorimetric analysis. The inhibition rates of the enzyme from either SR or CSR were changed by 92 and 100% with an IC_{50} of 15 μmol l^{-1} (Figure 2, curve 3) and 40 μmol l^{-1} (Figure 2, curve 4), respectively. The above results indicate that the Tet inhibitory effect on CSR as measured with the colorimetric method are comparable to those (data not shown) obtained with the kinetic analysis. These data indicate that an elevation in ATP concentration (to 5 from 1 mol l^{-1}) resulted in remarkable decreased inhibitory effect of Tet on the enzyme activity (reduction from 92 to 60%).

As shown in Table 1, hydrolysis of 1 mole ATP by the calcium pump protein transported 2 moles Ca^{2+} ions across the membrane, a Ca^{2+}/ATP ratio of 2, similar to that reported by literature (De Meis, 1981). Addition of Tet, with concentrations ranging from 64 to 192 μmol l^{-1}, to the reaction solution caused the stoichiometric ratio of SR Ca^{2+}/ATP to decrease from 2.0 (control) to 1.43. This indicates that the extent of Tet inhibitory effect on calcium transport was slightly more than that on calcium pump enzyme activities.

Intrinsic protein fluorescence and SH-modified protein fluorescence

Intrinsic protein fluorescence reflects the spatial conformation of protein molecules related to aromatic amino acids. Intrinsic protein fluorescence spectra of the SR calcium pump protein were monitored at 25°C, using a λ em of 300–380 nm and a λ ex of 290 nm, with an emission maximum observed at 330 nm (Figure 4). Following addition of 16, 32, 64 and 128 μmol l^{-1} Tet, this remarkable shift of the emission maximum was not exhibited, while relative fluorescences in emission intensity were reduced to 87, 81, 71 and 40% of control, respectively. These were due to the conformational changes by the transfer, during Tet action, of the enzymatic aromatic amino acids from an inner, nonpolarized environment to the outer, polarized environment. The fluorescence intensity of SR labelled with N-(3- P)-M (termed SH-modified protein fluorescence), which modified Cys^{344} and Cys^{364} of the Ca^{2+},Mg^{2+}-ATPase near to the active site, may indicate conformational changes in this domain. After incubation of 24 mg l^{-1} SR labelled with N-(3-P)-M in 16–256 μmol l^{-1} Tet solution; for 5 min, the Tet concentrations of 128 and 256 μmol l^{-1} slightly decreased the SH-modified protein fluorescence intensity to 92 and 84% of control, respectively, at a λ em of 360–420 nm and a λ ex of 347 nm (Figure 5). However the extents of decreases in SH-modified protein fluorescence intensity were far less than decreases in intrinsic protein fluorescence intensity.

Membrane fluidity

As shown in Table 2, Tet did not change fluorescence polarization of SR labelled with DPH at a λ em of 432 nm and a λ ex 362 nm after incubation of 24 mg l^{-1} SR labelled with DPH in 64 and 128 μmol l^{-1} Tet solution for 5 min, a Tet : SR ratio (μmol l^{-1} : mg l^{-1}); of 2.67 : 1 and 5.33 : 1, respectively. This can reflect the motion and fluidity of lipid molecules. 2-AS, 7-AS and 12-AS, of which carbon atoms combined with a luminous anthroic group at the different positions of aliphatic acid chain, can incorporate into the surface, middle and deep layers of SR membrane lipid, respectively so that the fluorescence polarization of SR labelled with 2-AS, 7-AS and 12-AS may reflect membrane

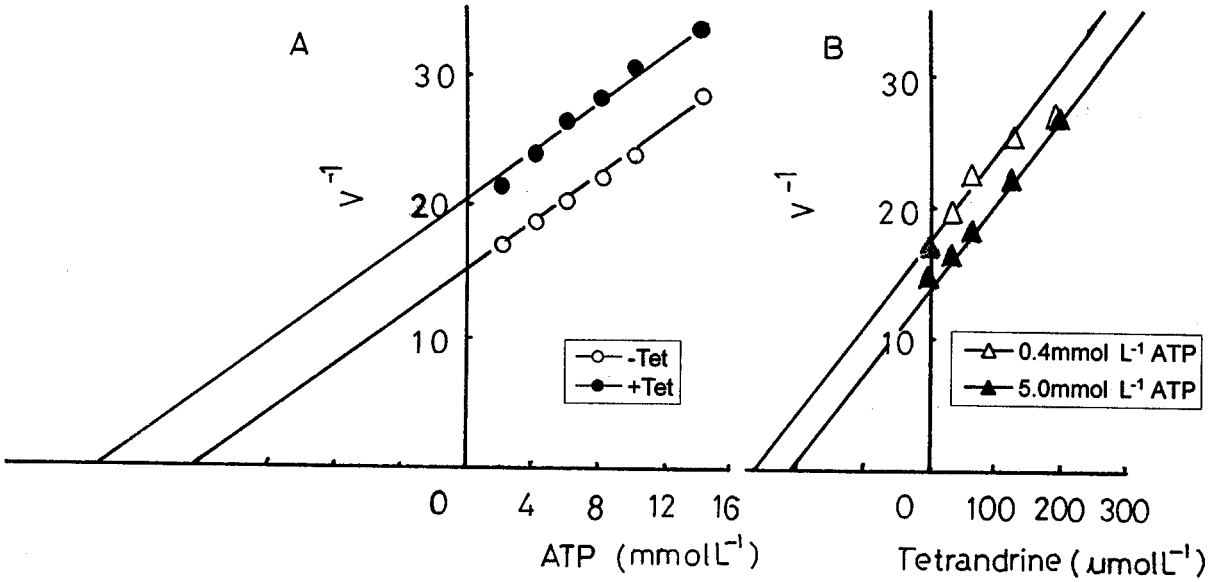

Figure 3 Uncompetitive inhibition of sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase by tetrandrine. Enzyme assays were measured by UV spectrophotometric method as described under Methods in the presence of 12 mg l^{-1} SR and 50 mmol l^{-1} KH_2PO_4. (A) Shows Linewearer-Burk plot in the absence and presence of 64 μmol l^{-1} tetrandrine with $K_M = 64$ μmol l^{-1}. (B) Shows Dixon plot in the presence of 0.4 and 5 mmol l^{-1} ATP with $K_i = 225$ μmol l^{-1}. V : A340 min^{-1}.

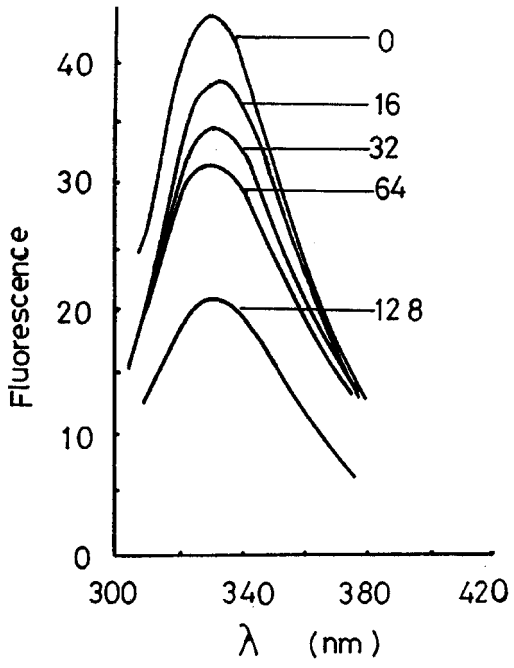

Figure 4 Fluorescence emission spectra of sarcoplasmic reticulum calcium pump protein in tetrandrine solutions of differing concentrations. Tetrandrine concentrations (μmol l^{-1}) are as indicated. Conditions were as described under Methods. λ ex = 290 nm, λ em = 330 nm.

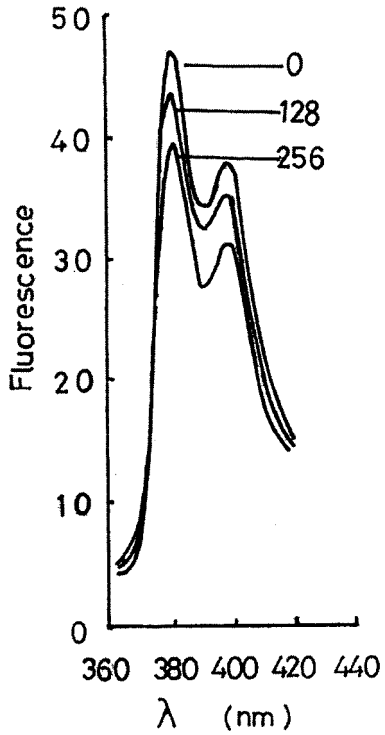

Figure 5 Fluorescence emission spectra of SH-modified protein of N-[3-P]-M labelled sarcoplasmic reticulum with tetrandrine. Tetrandrine concentrations (μmol l^{-1}) are as indicated. Conditions were as described under Methods with λ ex = 347 nm, λ em = 380 nm.

fluidity corresponding to the three layers of SR membrane. When 48 mg l^{-1} SR (labelled with 2-AS or 7-AS; or 12-AS) were incubated with 128 and 256 μmol l^{-1} of Tet respectively (a Tet : AS-SR ratio of 2.67 : 1 or 2.56 : 1) fluorescence polarization of 7 AS-SR and 12 AS-SR were distinctly increased with more of an increase in that of 2 AS-SR at a λ em of 460 nm and a λ ex of 390 nm. This suggests that Tet may lead to slightly decreased membrane fluidity in the middle and deep layers of SR (Table 2).

Discussion

Recently the mechanism of the effects of Tet on several systems was investigated including calcium and potassium channels (Wang *et al*., 1997; 1999; Wu *et al*., 2000), cell proliferation (Tian *et al*., 1977), platelet aggregation (Kim *et al*., 1999), cellular adhesion (anti-rheumatic medicines) (Chang *et al*., 1999), immunosuppressants (Lai *et al*., 1999),

Table 2 The effect of tetrandrine on fluorescence polarization of sarcoplasmic reticulum membrane lipid with DPH or AS as molecular probes

	Tet/SR Protein (μmol mg^{-1})		
Ratio	*0*	*2.67*	*5.33*
DPH-SR	0.164±0.007	0.167±0.005	0.164±0.006
2 AS-SR	0.194±0.008	0.205±0.004	0.203±0.004
7 AS-SR	0.182±0.006	0.198±0.007	0.207±0.006
12AS-SR	0.140±0.004	0.158±0.005	0.168±0.008

Determination of fluorescence polarization, and labelling of SR with DPH and AS were performed as described under Methods. Fluorescence polarization assay with labelled DPH-SR was carried out in the presence of 64 or 128 μmol l^{-1} tetrandrine using 24 mg l^{-1} DPH-SR. Fluorescence polarization assay with labelled AS-SR was carried out in the presence of 128 or 256 μmol l^{-1} tetrandrine using 48 mg l^{-1} of 2AS-SR (or 7AS-SR and 12AS-SR). ($n=3$).

but little has been reported on the action of Tet on SR calcium transport. In the present study, we demonstrated that Tet inhibited SR calcium uptake, and analysed the effects of Tet on SR Ca^{2+},Mg^{2+}-ATPase activity, protein fluorescence and membrane fluidity. *In vitro*, microsomal vesicle active transport of Ca^{2+} prepared from cardiac and skeletal muscle (mainly containing SR) utilizes energy liberated by the hydrolysis of the high-energy phosphate bonds of ATP, as demonstrated during the early 1960s (Katz, 1992). Prevention of high Ca^{2+} concentrations within the SR can be prevented by inhibition of the calcium pump. Our results showed that Tet inhibited SR calcium uptake and SR Ca^{2+},Mg^{2+}-ATPase activity in parallel. This indicated that transport of Ca^{2+} into SR was ATP-dependent and the inhibition of SR calcium uptake by Tet could be due to the ability of Tet to inhibit the SR Ca^{2+},Mg^{2+}-ATPase.

Tet and stearamine, both amphiphiles, were able to inhibit SR Ca^{2+},Mg^{2+}-ATPase activity. The inhibition of the enzyme by stearylamine was increased along with an increase in the SR membrane: amphiphile ratio, while Tet was not influenced by this parameter (Chen & Chen, 1990). In addition, stearlamine decreased intrinsic protein fluorescence of SR calcium pump protein in emission intensity (Chen & Chen, 1993) and enhanced the fluorescence polarization of SR labelled with DPH (Chen & Chen, 1992). In this study, Tet also decreased the relative fluorescence of SR in emission intensity, but did not change the fluorescence polarization of SR labelled with DPH. These results indicate that Tet's hydrophilic property is more than its hydrophobicity, while stearylamine has a stronger hydrophobicity. Tet's effect was probably through direct action on the SR calcium pump to cause an alteration of the active site of the enzyme and to lead to the decline in both the enzyme activities and calcium transport. Furthermore a prevention of SR calcium uptake by Tet was due to inhibition of the SR calcium pump Ca^{2+},Mg^{2+}-ATPase and the changes in spatial conformation of its protein molecules, which may involve in the regulation of cellular Ca^{2+} concentration and influence the heart to adjust its mechanical behaviour response to a variety of physiological stimuli.

SR membrane consists of a phospholipid bilayer containing a number of intrinsic membrane proteins and phospholipids (Katz, 1992). Intactness of SR membrane plays an important role in regulating its function-transportation of cytosolic Ca^{2+}. The SR calcium pump Ca^{2+},Mg^{2+}-ATPase protein molecule contains about 1000 amino acids including 13 tryptophanes (Brandl *et al*., 1986), among which most of them are located at the boundary between the membrane and water in membrane-lipid domain with the other tryptophan located at ATP binding site (the 522nd amino acid) in the cytosolic domain. In addition, this calcium pump molecule also contains 24 cysteines (Brandl *et al*., 1986; Inesi & Kurzmack, 1984), among which Cys^{344} and Cys^{364} are related to the decomposition of phosphorylated intermediate (EP) in a high-energy state and to calcium transport (Saito-Nakatsuka *et al*., 1987). In this study, Tet was found to have the kinetic characteristics of a un-competitive inhibitor on the SR Ca^{2+},Mg^{2+}-ATPase and a reduction in both intrinsic protein fluorescence and SH-modified protein fluorescence intensities of the SR calcium pump protein. On this basis, it was assumed that Tet may act at a domain out of the substrate binding site of the enzyme, through binding to the complex (ES) between enzyme and substrate to give arise to the changes in spatial conformation of the calcium pump protein. This would explain the larger extent of decrease in emission intensity resulting in transfer of most of the amino acids in the membrane and water boundary from an inner, non-polarized environment to an outer, polarized environment. However, Tet elicited slight SR protein conformational changes in the domain located at Cys^{344} and Cys^{364} as seen when these groups were labelled with N-(3-P)-M. Here the SH-modified protein fluorescence intensity was slightly decreased, indicating that conformational changes of the calcium pump proteins molecules related to aromatic amino acids might further contribute to the inhibitory effects of Tet on both SR calcium uptake and enzyme activities. These experiments revealed that effects of Tet on SR calcium pump were non-specific in both the kinetics of enzyme action and spatial conformation of protein, which was similar to non-specific action of Tet found in other physiological experiments (Yao *et al*., 1983; Wu *et al*., 1998) and to some pharmacological properties of nonselective calcium-antagonist (Fang *et al*., 1982).

In this study, it was shown that elevation in the ATP concentration or addition of phosphate can decrease the inhibitory effects of Tet on SR Ca^{2+},Mg^{2+}-ATPase. Comparing curves 2 and 3 in Figure 2, addition of 5 mmol l^{-1} ATP to reaction solution caused a reduction in inhibition of the enzyme by Tet, demonstrating that ATP as substrate for calcium transport was able to prevent inactivation of the SR Ca^{2+},Mg^{2+}-ATPase. Our data were quite similar to that of other studies where ATP at higher concentration stimulated the calcium pump and the presence of a low-affinity regulatory sites appear to stimulate Ca^{2+} transport by accelerating the conversion of E_1P to E_2P (Katz, 1992). Calcium-precipitating anions like oxalate and phosphate during calcium pump reaction *in vitro* greatly increase the amount of calcium taken up by the isolated vesicles. This is due to ability of these anions to form a precipitate with calcium transported into the vesicles. The modification of the effects of Tet on calcium pump ATPase protein by phosphate remains unclear. In a previous study (Yu & Chen, 1990), phosphate distinctly stimulated Ca^{2+},Mg^{2+}-ATPase activity (isolated from either SR or CSR), and this may partly contribute to the increase of Ca^{2+} uptake into the SR. In this present study, comparing curves 1 and 2 in Figure 2, addition of 50 mmol l^{-1} phosphate to the reaction solutions led to a reduction in inhibition of the enzyme activity by Tet. This might partly be due to the ability of the negative charges in phosphate to neutralize the positive charge in Tet, as well as by a similar mechanism as mentioned above. The mechanism of action of Tet inhibition of SR calcium transport, and the

modulatory effects of both ATP and phosphate on Tet inhibition of SR Ca^{2+},Mg^{2+}-ATPase will be further investigated in detail.

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