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Modulation of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase by pentobarbital

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The dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity of sarcoplasmic reticulum vesicles upon the concentration of pentobarbital shows a biphasic pattern. Concentrations of pentobarbital ranging from 2 to 8 mM produce a slight stimulation, approximately 20–30%, of the ATPase activity of sarcoplasmic reticulum vesicles made leaky to Ca^{2+}, whereas pentobarbital concentrations above 10 mM strongly inhibit the activity. The purified ATPase shows a higher sensitivity to pentobarbital, namely 3–4-fold shift towards lower values of the $K_{0.5}$ value of inhibition by this drug. These effects of pentobarbital are observed over a wide range of ATP concentrations. In addition, this drug shifts the Ca^{2+} dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity towards higher values of free Ca^{2+} concentrations and increases several-fold the passive permeability to Ca^{2+} of the sarcoplasmic reticulum membranes. At the concentrations of pentobarbital that inhibit this enzyme in the sarcoplasmic reticulum membrane, pentobarbital does not significantly alter the order parameter of these membranes as monitored with diphenylhexatriene, whereas the temperature of denaturation of the (Ca^{2+} + Mg^{2+})-ATPase is decreased by 4–5 C°, thus, indicating that the conformation of the ATPase is altered. The effects of pentobarbital on the intensity of the fluorescence of fluorescein-labeled (Ca^{2+} + Mg^{2+})-ATPase in sarcoplasmic reticulum also support the hypothesis of a conformational change in the enzyme induced by millimolar concentrations of this drug. It is concluded that the inhibition of the sarcoplasmic reticulum ATPase by pentobarbital is a consequence of its binding to hydrophobic binding sites in this enzyme.

Introduction

It is now well-documented that local anesthetics, such as dibucaine and tetracaine [1–3] and alcohols [4,5], inhibit the (Ca^{2+} + Mg^{2+})-ATPase and the Ca^{2+} pump of sarcoplasmic reticulum both from skeletal and from cardiac muscle. Although these actions are exerted at concentrations of these drugs higher than those producing anesthesia in vivo, by several reasons a better knowledge of the molecular mechanism of inhibition of the sarcoplasmic reticulum Ca^{2+} pump by anesthetics is likely to be helpful to clarify the molecular basis of anesthesia. To cite only some of them: (i) the implication of an altered Ca^{2+} buffering in the presynaptic terminals of nerve cells in anesthesia has been suggested [6,7]; (ii) the Ca^{2+} fluxes across the sarcoplasmic reticulum membrane proceed at a rate similar to that reported for the plasma membrane of nerve cells [8–10]; (iii) the regulatory pattern of the Ca^{2+} channels of the sarcoplasmic reticulum membrane is similar to that of the plasma membrane of nerve cells, e.g., regulation by voltage, phosphorylation and active Ca^{2+} pump coupled to the hydrolysis of ATP [11,12] and (iv) the sarcoplasmic reticulum membrane is one of the best characterized membranes, both at a biochemical and at a biophysical level.

Due to these reasons we have carried out studies on the modulation of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase activity by pentobarbital, one of the most often used barbiturates in model studies of anesthesia [13,14]. In addition, pentobarbital has been

Abbreviations: ATPase, (Ca^{2+} + Mg^{2+})-ATPase; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IU, unit of enzyme producing 1 μmole product per min per mg protein; PEP, phospho*enol*pyruvate; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid.

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shown to inhibit the Ca^{2+} uptake in vascular smooth muscle [15] and, more recently, to alter Ca^{2+} fluxes across the plasma membrane of adrenal chromaffin cells at the concentrations that inhibit the stimulus-secretion coupling in these cells [16]. A close correlation between hypnotic and anticonvulsant properties of a barbiturate, 5-(2-cyclohexylideneethyl)-5-ethyl barbituric acid, and alterations of synaptosomal Ca^{2+} influx has also been shown [17]. These results further support the hypothesis that the mode of action of barbiturates involves alteration of the chemical transmission at a synaptic level rather than only disturbances of axonal impulse propagation [18–20].

It has already been shown by different groups of investigators that the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum is modulated by a relatively large variety of hydrophobic drugs, some anesthetics among them [1–5,21–23]. We have recently suggested that the inhibition of this $(Ca^{2+} + Mg^{2+})$-ATPase by local anesthetics dibucaine and tetracaine may be related to the disruption of the lipid annulus around the protein [24]. A similar explanation has been proposed to account for the inhibition of this enzyme by another hydrophobic compounds, such as pyrethroids or insecticides [23,25]. This possibility is of relevance to the mechanism of action of barbiturates, for Lee [26] has suggested that these drugs can act through this basic mechanism in nerve cells plasma membranes, then perturbing the function of proteins playing a major role on neuronal excitability. Elsewhere, we have suggested that this is likely to be the basic mechanism of inhibition of the plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase of synaptosomes by the local anesthetics dibucaine and lidocaine [27], an inhibition of especial relevance to the phenomenon of anesthesia because this is likely to be the most important transport system involved in maintaining low Ca^{2+} cytosolic levels [28] and also because this enzyme system is inhibited by local anesthetic concentrations close to those that block the axonal conduction [6]. The results presented in this paper extend our previous studies to pentobarbital and fully supports the hypothesis that the inhibition of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase activity is related to the interaction of this anesthetic with specific hydrophobic domains in this enzyme.

Materials and Methods

Sarcoplasmic reticulum has been purified from rabbit (New Zealand White) hind leg muscle as indicated elsewhere [29]. $(Ca^{2+} + Mg^{2+})$-ATPase was purified from sarcoplasmic reticulum by affinity chromatography through Affi-Gel blue as described by Gafni and Boyer [30]. The purity of the $(Ca^{2+} + Mg^{2+})$-ATPase was checked by SDS-gel electrophoresis (7.5% acrylamide) and found to be more than 95% pure from the absorbance at 550 nm of Coomassie blue stained gels. Protein concentration was measured following the method of Lowry et al. [31], using bovine serum albumin as standard. Lipids were determined as total phosphorus following the method of Barlett [32]. The $(Ca^{2+} + Mg^{2+})$-ATPase activity has been measured using the coupled enzyme system [33] with the following assay mixture: 0.1 M Tes, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2.5 mM ATP, 0.2 mM NADH, 0.42 mM PEP, 7.5 IU pyruvate kinase, 18 IU lactate dehydrogenase (pH 7.45). The effects of pentobarbital on the $(Ca^{2+} + Mg^{2+})$-ATPase activity were further assessed by direct measurements of the released inorganic phosphate following the method of Fiske and SubbaRow [34]. The Ca^{2+}-independent ATPase activity was measured in the presence of 3.4 mM EGTA, and only those preparations showing a value of this activity lower than 10% of total uncoupled $(Ca^{2+} + Mg^{2+})$-ATPase activity were used in this study. 'Leakiness' of sarcoplasmic reticulum vesicles was assessed by measuring the $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence and in the absence of A23187 (1–2 μg/ml). Only those preparations showing a 3–4-fold stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity at 20–22°C upon addition of A23187 have been used in this study. The specific activity of purified $(Ca^{2+} + Mg^{2+})$-ATPase ranged typically between 5 and 7 IU at 25°C and contained 35–40 mol lipid per mol of protein monomer. Calcium uptake was measured using arsenazo III as the metallochromic dye [35] from the difference of absorbance between 650 and 700 nm, obtained by simultaneous readings at these wavelengths with a Hewlett-Packard 8451A diode array spectrophotometer. The conversion of absorbance change into Ca^{2+} concentration change was carried out upon calibration with EGTA · Ca^{2+} solutions. All the kinetic results presented in this paper have been confirmed in, at least, duplicate experiments carried out with three different preparations of sarcoplasmic reticulum vesicles.

The labeling of the sarcoplasmic reticulum membranes with fluorescein isothiocyanate has been done as described elsewhere [29,36]. Fluorescence measurements were carried out with a spectrofluorimeter Hitachi-Perkin Elmer, Model 650-40, using excitation and emission wavelengths of 475 and 515 nm, respectively, at room temperature.

Scanning calorimetry measurements have been carried out using a differential scanning calorimeter Microcal MC-2 operating at a scanning rate of 60 C°/h under approx. 1.5 kg/cm^2 pressure during the scan. The buffer used in scanning calorimetry experiments has been: 50 mM Tes, 0.1 M KCl, 0.25 M sucrose, 2 mM dithiothreitol (pH 7.4). The samples were carefully degassed before loading the calorimeter. The pH was carefully readjusted to 7.4 when necessary after addition of pentobarbital.

The measurements of the polarization of fluorescence of DPH have been carried out at 25 °C as previously described [24].

Calcium efflux from sarcoplasmic reticulum preloaded with 5 to 20 mM $CaCl_2$, by overnight incubation on ice, has been measured using the fluorescence of chlortetracycline as in [37,38]. The rate of chlortetracycline diffusion across these membranes has been calculated from the phase of increase of fluorescence after mixing of chlortetracycline solution with vesicles preloaded with $CaCl_2$. In our experimental conditions (pH 7.45 and 25 °C), fitting the data to a first-order exponential process we have obtained a value of 2.7 min^{-1} for the rate of chlortetracycline diffusion through the membrane of sarcoplasmic reticulum. These values agree satisfactorily with data reported by other investigators [37,38]. Because the passive outflow of Ca^{2+} proceeds at a rate of, at least, 10-fold slower [39] it follows that the rate of Ca^{2+} efflux can be accurately estimated by fitting the decay of chlortetracycline fluorescence to the exponential equation of a first order kinetic process. The buffer used for these experiments had the following composition: 50 mM Tes (pH 7.45), 0.1 M KCl, 1 mM EGTA, 10 μM chlortetracycline and the indicated concentration of pentobarbital. All the results of calcium efflux determined using the fluorescence of chlortetracycline have been confirmed with, at least, three different membrane preparations.

Chemicals

Bovine serum albumin, chlortetracycline, deoxycholate, DPH, ATP, phospho*enol*pyruvate, EGTA, fluorescein isothiocyanate, phenylmethylsulfonyl fluoride, β-mercaptoethanol, Sephadex G-50 and Tes were obtained from SIGMA. A-23187, pyruvate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim. All the other chemicals used in this study were obtained from Merck. Affi-Gel blue (100–200 mesh) was obtained from Bio-Rad. Triton X-100 is a trade mark of Rohm & Haas, Co. Pentobarbital was obtained from Servá, and was prepared immediately before use.

Results

1. Dependence of the ATPase activity upon the concentration of pentobarbital

Fig. 1 shows that pentobarbital inhibits both the ATPase activity of sarcoplasmic reticulum made leaky with A23187 and that of purified ATPase. The purified ATPase is about 3-fold more sensitive to inhibition by this drug. On the contrary, sealed sarcoplasmic reticulum appears to be rather insensitive to this drug, see also the Fig. 1. Similar results have been obtained at 37 °C. On the other hand we have confirmed these results measuring the ATPase activity by directly monitoring the release of inorganic phosphate by the method of Fiske and SubbaRow [34]. In these experimental conditions (< 10 μg protein per ml) the correction of aqueous pentobarbital concentration by adsorption to the membranes can be neglected, as determined from the amount of this compound adsorbed to the sarcoplasmic reticulum which is removed upon pelleting these membranes by centrifugation. Either with leaky sarcoplasmic reticulum vesicles or with purified ATPase the inhibition of the ATPase activity by pentobarbital shows two distinct phases, showing a slight activation (20–30%) at low pentobarbital concentrations (e.g., 2–4 mM) followed by an abrupt inhibition at higher pentobarbital concentrations (apparent Hill coefficient ≥ 6). Furthermore, in the presence of 2 and 3 mM pentobarbital the activity of purified ATPase continuously decays as a function of time, being this inactivation adequately fitted to a first-order kinetic process. The rate constant of the inactivation process of purified ATPase is 0.089 min^{-1} and 0.438 min^{-1} in the presence of 2 mM and 3 mM pentobarbital, respectively. This is also observed with leaky sarcoplasmic reticulum, but at higher pentobarbital concentrations, e.g., in the presence of 11 mM pentobarbital the rate constant of the inactivation process is 0.18 ± 0.02 min^{-1}. The values of the ATPase activity indicated in the Fig. 1 in the presence of different pentobarbital concentrations correspond to the rate of ATP hydrolysis per minute, averaged during 1–2 min after starting the reaction. Therefore, only in the case of the purified ATPase and for pentobarbital concentrations higher than 2 mM underestimation due to the above mentioned inactivation process is a significant problem and in these cases the ATPase activity has been calculated from the data obtained in a period of 1 min

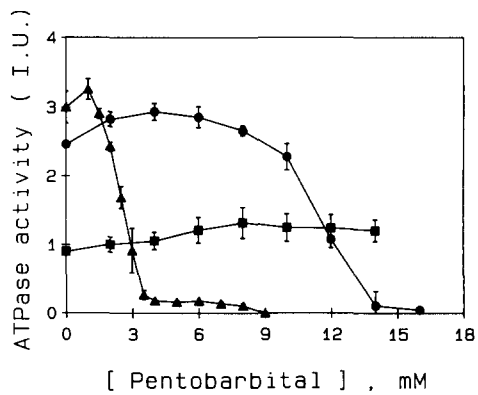

Fig. 1. Dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity upon the concentration of pentobarbital: sealed sarcoplasmic reticulum vesicles (■), sarcoplasmic reticulum vesicles made leaky with A23187 (1 μg per ml) (●) and $(Ca^{2+} + Mg^{2+})$-ATPase purified from sarcoplasmic reticulum membranes following Gafni and Boyer [30] (▲). The composition of the assay medium was as indicated in Materials and Methods. Other experimental conditions: 25 °C and 3–5 μg protein per ml, except for the case of the experimental series of sealed sarcoplasmic reticulum, in which case it was 15 μg protein per ml.

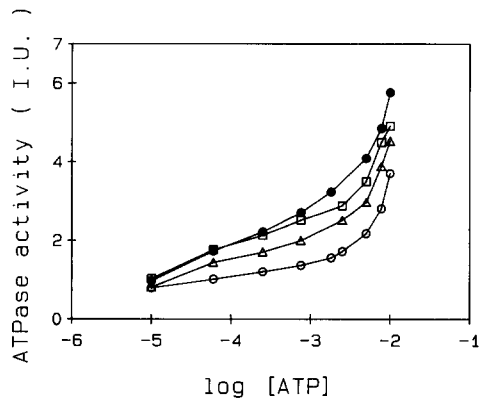

Fig. 2. Effect of pentobarbital on the dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity of leaky sarcoplasmic reticulum vesicles upon the concentration of ATP. The different symbols correspond to the following pentobarbital concentrations: none (○), 2 mM (●), 6 mM (□) and 10 mM (△). Temperature 25 °C; protein concentration: 3.1 μg per ml; A23187 concentration: 1 μg per ml. Other experimental conditions as indicated in Materials and Methods.

after starting the reaction. In addition, the activity of leaky sarcoplasmic reticulum incubated with 18–20 mM pentobarbital for 30 min only recovers to about 4% of the control value upon removal of pentobarbital by a 2-h dialysis step of the sarcoplasmic reticulum membranes, thus, strongly suggesting that the slow inactivation follows from denaturation of the ATPase.

2. Effect of pentobarbital on the ATP dependence of the ATPase activity

The dependence of the ATPase activity upon the concentration of ATP contain useful information regarding the catalytic mechanism of this enzyme [40].

Fig. 2 shows that the effect of pentobarbital on the (Ca^{2+} + Mg^{2+})-ATPase activity of leaky sarcoplasmic reticulum is seen over a wide range of ATP concentrations. In addition, it can be appreciated that the stimulation of the ATPase by lower concentrations of this drug is similar in the overall range of ATP · Mg^{2+} concentrations tested herein, implying an stimulatory effect of pentobarbital at concentrations of ATP · Mg^{2+} well below those required to produce the regulatory effect of the substrate. The effect of stimulatory concentrations of pentobarbital on the dependence of the ATPase activity upon ATP · Mg^{2+} closely mimic the effect of increasing concentrations of Mg^{2+} on this activity (Ref. 41 and results not shown). Similar results have been obtained using purified ATPase.

3. Effect of pentobarbital on the dependence of the ATPase activity upon the concentration of Ca^{2+}

The dependence of the ATPase activity upon the concentration of Ca^{2+} is biphasic [40]. As shown by Gould et al. [40] the analysis of this dependence provides important clues on the kinetic mechanism of action of the ATPase in sarcoplasmic reticulum membranes. At 37 °C the effect of various concentrations of pentobarbital on the dependence of the ATPase activity of leaky sarcoplasmic reticulum membranes upon free Ca^{2+} concentrations is presented in the Fig. 3. It can be readily observed that concentrations of pentobarbital effectively inhibiting the ATPase activity markedly decrease the affinity of Ca^{2+} to the ATPase as well, both to the high-affinity and to the low-affinity binding sites, from approximately 0.2 μM and 0.44 mM (control) to 1.13 μM and 1 mM (in the presence of 10 mM pentobarbital), respectively. These $K_{0.5}$ values have been obtained from Hill plots of the kinetic data presented in the Fig. 3 (not shown). Additionally we have obtained Hill coefficients of 1.3 ± 0.1 and 1.2 ± 0.1 for the activation and inhibition by Ca^{2+}, respectively, both in the absence and in the presence of pentobarbital. On the contrary, concentrations of pentobarbital that stimulates the ATPase of leaky sarcoplasmic reticulum membranes (e.g., 2 mM) leave essentially unaffected the affinity of Ca^{2+} towards both the high and low-affinity binding sites. Similar results have been obtained at 25 °C, except that in this latter case the observed shift of the $K_{0.5}$ of the high and of the low-affinity binding sites to Ca^{2+} has been 0.3 μM and 0.32 mM (control) to 0.7 μM and 1.5 mM (in the presence of 10 mM pentobarbital), respectively. It appears, thus, that the shift of the affinity of Ca^{2+} to higher values is readily seen only at inhibitory concentrations of pentobarbital.

4. Effect of pentobarbital on Ca^{2+} accumulation by sarcoplasmic reticulum vesicles

The steady-state level of Ca^{2+} accumulated within sarcoplasmic reticulum vesicles has been measured using the indicator Arsenazo III [35], as indicated in Methods. We have studied the effect of several millimolar pentobarbital concentrations on the level of Ca^{2+} accumula-

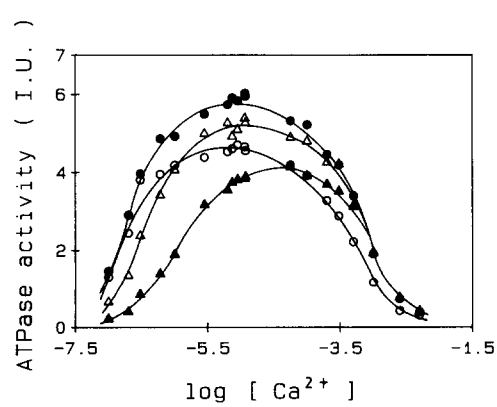

Fig. 3. Effect of pentobarbital on the dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity of leaky sarcoplasmic reticulum vesicles upon the free concentration of Ca^{2+}. The different symbols correspond to the following pentobarbital concentrations: none (○), 2 mM (●), 6 mM (△) and 10 mM (▲). Temperature 37 °C; protein concentration: 5.2 μg per ml; concentration of A23187: 1 μg per ml. Other experimental conditions as indicated in Materials and Methods.

tion. We have found that the steady-state level of Ca^{2+} accumulation by sarcoplasmic reticulum vesicles decreases in the presence of concentrations of pentobarbital higher than 4 mM. In the presence of 10 mM pentobarbital, the accumulation of calcium by sarcoplasmic reticulum vesicles is decreased to about 20–30% of that of sarcoplasmic reticulum vesicles in the absence of pentobarbital.

5. Effect of pentobarbital on the fluorescence of fluorescein bound to the ATPase

The fluorescence of fluorescein bound to the sarcoplasmic reticulum ATPase has been shown to monitor displacements between the major conformational states of the ATPase [42,43]. Because an altered kinetic behaviour can derive from an altered distribution of conformation states of the ATPase we have studied the effect of pentobarbital, and of Ca^{2+} and vanadate in the fluorescence of fluorescein (see Fig. 4). It has to be recalled that Ca^{2+} shifts the ATPase to the E_1 state and vanadate to the E_2 state, and that the E_2 state shows about 10% higher fluorescence intensity than the E_1 state, in good agreement with the results reported by other investigators [42,43]. It can be readily seen that pentobarbital shifts the enzyme to a conformational state of the ATPase showing higher fluorescence of fluorescein, e.g., to an E_2-like form. The effect of vanadate on the intensity of the fluorescence of fluorescein is significantly decreased in the presence of 4 to 8 mM pentobarbital, whereas that induced by Ca^{2+} remains unchanged or is increased, thus, supporting this hypothesis. From these results we have obtained the values listed of the E_2/E_1 equilibrium constant in the presence of several pentobarbital concentrations (Table I). The fact that increasing the temperature to 37 °C decreases the effect of pentobarbital on the fluorescence

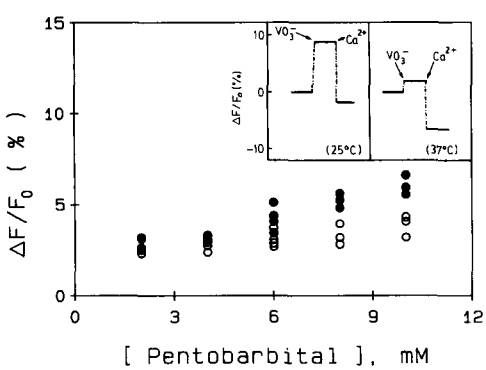

Fig. 4. Dependence of the fluorescence of fluorescein labelled sarcoplasmic reticulum ($\lambda_{exc} = 475$ nm; $\lambda_{em} = 515$ nm) upon the concentration of pentobarbital. Different symbols correspond to the titration carried out at 25 °C (●) and at 37 °C (○). Inset: effect of addition of 200 μM vanadate and of 400 μM Ca^{2+} indicated by arrows, both at 25 °C and at 37 °C. Protein concentration: 17.5 μg per ml. Labeling ratio 1 mol fluorescein per mol of ATPase. Other experimental conditions as indicated in Materials and Methods.

TABLE I

Effect of pentobarbital on the E_2/E_1 equilibrium constant of the ATPase in our sarcoplasmic reticulum preparations

Addition	E_2/E_1 equilibrium constant [a]	
	25 °C	37 °C
None (control) [b]	0.22	3.5–4.5
2 mM pentobarbital	0.65–0.90	(c)
4 mM pentobarbital	0.75–0.95	(c)
6 mM pentobarbital	1.0 –1.75	(c)
8 mM pentobarbital	1.60–2.30	(c)
10 mM pentobarbital	2.30–3.60	(c)

[a] The value of the equilibrium constant has been estimated by taking the fluorescence in the presence of 400 μM Ca^{2+} as that of the E_1 form and the fluorescence in the presence of 200 μM vanadate as that of the E_2 form [43].

[b] Buffer: 100 mM Tes, 100 mM KCl, 5 mM $MgCl_2$ and 100 μM EGTA, (pH 7.4).

[c] More than 95% of the ATPase in the E_2 form.

of fluorescein bound to the ATPase also supports this hypothesis, as it has been shown that the $E_1 \rightarrow E_2$ transition is endothermic [43].

6. Effect of pentobarbital on the order parameter of sarcoplasmic reticulum membranes

Because the activity of the sarcoplasmic reticulum ATPase is modulated by the lipid state [44,45], we have considered the possibility that the effects of pentobarbital on this enzyme could be correlated to changes on the order parameter of sarcoplasmic reticulum membranes. The order parameter has been calculated from DPH polarization measurements as indicated by Pottel et al. [46]. As a result we have found that up to 10 mM pentobarbital the alteration of the order parameter is negligible, e.g., from 0.62 ± 0.02 (control) to 0.58 ± 0.02 (in the presence of 10 mM pentobarbital). On the other hand, at this concentration of pentobarbital there is already an appreciable inhibition of the ATPase. These values agree with the value of the order parameter of the sarcoplasmic reticulum membrane determined by other investigators [46].

7. Effect of pentobarbital on the DSC profile of sarcoplasmic reticulum membranes

As shown elsewhere [24] sarcoplasmic reticulum membranes show an endothermic peak in DSC scans centered at approx. 53 °C, a peak that corresponds to the denaturation of the ($Ca^{2+} + Mg^{2+}$)-ATPase. Fig. 5 shows the effect of pentobarbital on the DSC scan of sarcoplasmic reticulum membranes. The decrease of the concentration of pentobarbital in the aqueous phase in these experimental conditions (0.5 mg protein per ml) has been found to be lower than 10% of total pentobarbital concentration, as determined from the amount of this anesthetic adsorbed to the sarcoplasmic reticu-

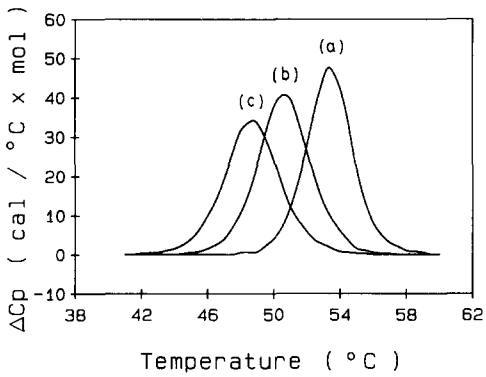

Fig. 5. Effect of pentobarbital on the DSC profile of sarcoplasmic reticulum membranes. The different scans correspond to the following pentobarbital concentrations: (a) none, (b) 9 mM and (c) 12 mM. The experimental conditions are indicated in Materials and Methods.

lum that is removed upon pelleting these membranes by centrifugation. It can be observed that pentobarbital shifts the endothermic peak several degrees towards lower temperatures at the concentrations that strongly inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase activity of leaky sarcoplasmic reticulum vesicles (see Fig. 1). It is to be recalled that other anesthetics, such as dibucaine and tetracaine, also lower the denaturation temperature of sarcoplasmic reticulum membranes, in the concentration range that inhibits the ATPase activity of leaky sarcoplasmic reticulum membranes [24]. However, the enthalpy of the denaturation process (i.e., the area below the peak) is only slightly altered, e.g., 10% decrease in the presence of 12 mM pentobarbital. The ΔH value of the denaturation of the $(Ca^{2+} + Mg^{2+})$-ATPase in the sarcoplasmic reticulum membrane is 500 ± 50 kcal/mol of protein monomer, calculated assuming that about 70% of the membrane protein is ATPase [47].

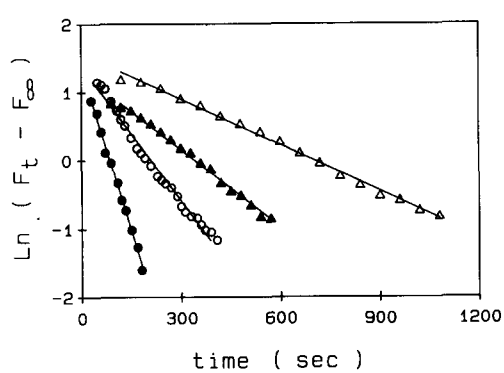

Fig. 6. Effect of pentobarbital on the efflux of Ca^{2+} from sarcoplasmic reticulum vesicles preloaded with 20 mM $CaCl_2$, monitored with the fluorescence of chlortetracycline. The different symbols correspond to the following concentrations of pentobarbital: none (△), 2 mM (▲), 6 mM (○) and 10 mM (●). Protein concentration: 150 μg per ml. Other experimental conditions as indicated in the Materials and Methods. The following rate constants of calcium efflux (s^{-1}) are obtained from these data: 0.0022 (no pentobarbital); 0.0039 (2 mM pentobarbital); 0.0066 (6 mM pentobarbital) and 0.016 (10 mM pentobarbital).

8. Effect of pentobarbital on the passive calcium efflux from preloaded sarcoplasmic reticulum membranes

We have used the fluorescence of chlortetracycline to monitor this process, as in Refs. 37 and 38. The sarcoplasmic reticulum vesicles were preloaded with Ca^{2+} by overnight incubation at 4°C with a buffered solution containing 5–20 mM $CaCl_2$. Fig. 6 shows the effect of pentobarbital on the decay of the fluorescence of chlortetracycline upon the time after dilution of sarcoplasmic reticulum vesicles in a solution depleted of Ca^{2+} by addition of EGTA. The results are presented in the form of a semilogarithmic plot, from where the rate constant of the process has been calculated. The values obtained are indicated in the legend of the figure. It follows from these results that pentobarbital increases several-fold the rate of Ca^{2+} efflux, at the concentrations that stimulate the ATPase activity of sealed sarcoplasmic reticulum vesicles.

Discussion

The modulation of the ATPase by pentobarbital shares several characteristics with the modulation of this enzyme system by anesthetics, such as the local anesthetics dibucaine and tetracaine [39] and alcohols [5]. In this regard, it is to be noted that in all the cases at the lower concentrations there is a phase of insensitivity or even slight activation, up to 20–30% stimulation, and then inhibition at higher concentrations of the drug. In addition, the dependence of the activity upon the concentration of pentobarbital in the inhibitory range is very steep, as it also occurs for the case of dibucaine and tetracaine [39]. This inhibition is not correlated to a significant alteration of the order parameter of the lipids of the sarcoplasmic reticulum membrane, thus, the possibility that it can be attributed to changes on this parameter seems unlikely. A similar inhibitory pattern has been observed with dibucaine, tetracaine and alcohols [5,39], and we have suggested that this inhibition may be a consequence of the disruption of the lipid annulus around the ATPase. Three additional observations reported in this paper are also consistent with this hypothesis: (i) the inhibition of the ATPase appears to be related to a slow irreversible process, likely protein denaturation, and delipidation enhances the rate of ATPase inactivation; (ii) the denaturation of the ATPase is shifted towards lower temperatures at the concentrations of pentobarbital that inhibit the ATPase activity, and delipidation strongly decreases the stability of this enzyme; (iii) the purified ATPase shows higher sensitivity to pentobarbital. Taking into account that the lipid to protein ratios of our preparations of purified ATPase and of sarcoplasmic reticulum are approximately 35–40 and 90–100 moles of lipids per mole of ATPase monomer, respectively, there is a good correlation between inhibition of the

ATPase activity and the molar ratio between pentobarbital and membrane lipids.

The conformation of the ATPase also appears to be altered by pentobarbital in native sarcoplasmic reticulum membranes, as indicated by the effects of this drug on the fluorescence of fluorescein covalently bound to the enzyme. The results suggest that pentobarbital shifts the ATPase towards an E_2-like state, see the Table I. In addition, these results support the hypothesis of a direct interaction of pentobarbital with the ATPase in the concentration range that activates this enzyme. The effects of pentobarbital on the $K_{0.5}$ value for Ca^{2+} binding to the high-affinity binding sites of the ATPase also agree with this hypothesis. Nevertheless, the effects of pentobarbital on the dependence of the ATPase upon free Ca^{2+} concentration is rather complex, as illustrated by the fact that the $K_{0.5}$ of both the high-affinity and the low-affinity Ca^{2+} binding sites is shifted towards higher values by pentobarbital. Because the low-affinity binding sites of Ca^{2+} are though to be the Ca^{2+} binding sites in the E_2 conformation whereas the high-affinity Ca^{2+} binding sites reflects Ca^{2+} binding to the E_1 conformation, these results suggest that besides displacement of the E_1/E_2 conformational equilibrium, other factors must be sought to account for the modulation by pentobarbital of the Ca^{2+} dependence of the ATPase activity. Among them, the putative implication of a significant surface potential change upon pentobarbital adsorption on the sarcoplasmic reticulum membrane deserves consideration.

The dependence of the saturation by Ca^{2+} of the ATPase activity upon the surface potential on sarcoplasmic reticulum membranes has been already pointed out [48,49]. This is especially relevant to the point addressed here because the Ca^{2+} binding sites seems to be relatively close to the lipid/water interphase, approx. 10–20 Å [49,50,51]. Therefore, we have determined the apparent adsorption coefficient of pentobarbital to the sarcoplasmic reticulum membranes at millimolar pentobarbital concentrations, using protein concentrations ranging from 2 to 10 mg per ml. We have found that in the presence of millimolar pentobarbital concentrations the sarcoplasmic reticulum lipid bilayer adsorbs pentobarbital at a molar ratio of 1 mol pentobarbital per 3–5 phospholipid molecules, showing this adsorption process saturation characteristics (results not shown). A similar behavior has already been shown for dibucaine adsorption to pure lipid bilayers [52] and in the case of pentobarbital it can be rationalized in terms of the positive free energy contribution of electrostatic interactions, i.e., the negative effect of adsorbed charges upon the adsorption of additional charged molecules. Although the pK value of pentobarbital in aqueous solution is 7.96 at an ionic strength of 0.16 [53], the apparent pK of protonation of hydrophobic compounds adsorbed to lipid bilayers has been shown to shift about -1.5 pH units due to the electric properties of the lipid/water interphase [21,54]. It follows that pentobarbital bound to the membrane will mostly be in its unprotonated monoanionic form, which is therefore the form likely involved in modulation of the ATPase activity. Under the experimental conditions of kinetic measurements, millimolar pentobarbital is already saturating the sarcoplasmic reticulum membrane, and the change of the surface density of charge upon adsorption of this drug has been calculated to range between $-(1/150)$ and $-(1/300)$ (electronic charge/Å^2). On these grounds, we have calculated the change of the zeta potential of the lipid bilayer in the presence of 5–10 mM $MgCl_2$ (to approach the salt concentration used in kinetic experiments) using Equation 41 of Ref. 55. In the presence of millimolar aqueous pentobarbital concentrations we have obtained a value of zeta potential change from -25 to -40 mV. This will, at most, shift the apparent $K_{0.5}$ value of saturation of the $(Ca^{2+} + Mg^{2+})$-ATPase by Ca^{2+} to lower values, for an increased negative charge density at the lipid/water interphase should increase the local concentration of cations nearby. On these grounds, it can be reasonably excluded that the changes of the surface potential induced by pentobarbital could in itself account for the experimentally observed shift of the $K_{0.5}$ value of Ca^{2+} dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase. Thus, this effect is likely related to the above referred conformational reorganization of the ATPase upon interaction with pentobarbital.

Considering previous studies on the modulation of the ATPase by hydrophobic compounds [5,21–23,39] the weak activation of the ATPase by pentobarbital can be rationalized in, at least, two different ways: (i) it can be related to the observed increase of the permeability to Ca^{2+} of sarcoplasmic reticulum membranes in the presence of pentobarbital, which will partially release the inhibition of the ATPase by accumulated Ca^{2+}, or (ii) it can be a consequence of the direct interaction of this drug with hydrophobic binding sites in the ATPase. The observed stimulation of the ATPase activity of leaky sarcoplasmic reticulum vesicles and of purified ATPase by pentobarbital, however, strongly support that the latter hypothesis can account for most of the observed stimulation.

The effects of pentobarbital on the dependence of the ATPase activity upon the ATP concentration reveals that low (activating) concentrations of this drug mimic the effect of lowering the concentration of Mg^{2+} (Fig. 2 and results not shown and see also Fig. 1 of Ref. 41). It has been recently shown that the inhibition of the ATPase activity by Mg^{2+} can be adequately simulated by kinetic competition between Mg^{2+} and $Mg \cdot ATP$ for binding to the nucleotide-binding site on the E_1 and E_2 conformations of the ATPase and on the phosphorylated forms of this enzyme, and in this latter case also

by competition between Mg^{2+} and Mg · ADP [41]. Moreover, the possibility that these effects of pentobarbital could be attributed to the surface potential change due to the adsorption of the drug on the sarcoplasmic reticulum membrane seems unlikely because the ATP binding site is located far away from the lipid/water interphase [56], at a distance where potential changes coming from a 25–30 mV surface potential change can be calculated to be lower than 1 mV [24]. Therefore, these results provide additional support to the hypothesis of binding of pentobarbital to specific domains in the ATPase and suggest that either these domains partially overlap with ATP binding sites or the conformation of the catalytic center is altered by an allosteric mechanism upon binding of pentobarbital to the $(Ca^{2+} + Mg^{2+})$-ATPase.

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