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Quinacrine inhibits the calcium-induced calcium release in heavy sarcoplasmic reticulum vesicles

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Quinacrine is a fluorescence probe useful for studying the effect of local anesthetics. The interaction of quinacrine and sarcoplasmic reticulum membranes measured by fluorescence spectroscopy indicates the presence of a saturable binding site. Typical local anesthetics are able to displace quinacrine bound to heavy sarcoplasmic reticulum membranes. The effectiveness of that displacement decreases in the order dibucaine > tetracaine > benzocaine > lidocaine > procaine > procainamide, indicating that the size and hydrophobicity of quinacrine are major determinants in the binding process. The use of radioactive tracer and a rapid filtration technique reveals that quinacrine interacts, at lower concentrations, with sarcoplasmic reticulum membranes by blocking the Ca^{2+}-induced Ca^{2+} release. Higher quinacrine concentrations also affect the Ca^{2+}-pump activity.

Introduction

The antimalarial drug quinacrine (atebrin) was first introduced in biochemical studies owing to its ability to interact with DNA. Thus, it inhibits DNA synthesis in *Escherichia coli* [1], it is a weak frameshift mutagen [2] but a potent antimutagen in bacteria [3] and stains adenine-thymine rich regions of DNA in chromosomes [4]. It was also used with energy transduction membranes from mitochondria and chloroplasts as a H^+ gradient indicator [5,6]. Moreover, quinacrine was proved to behave like a typical local anesthetic, since it blocks the depolarization caused by carbamylcholine on *Electrophorus electricus* electroplaque in vivo and enhances in vitro the affinity of the cholinergic receptor for acetylcholine in *Torpedo marmorata* membrane fragments [7]. This effect apparently takes place on specific saturable sites of membrane proteins being related with the pharmacological action of local anesthetics [8,9]. An additional advantage of quinacrine over conventional local anesthetics is that the quinacrine–membrane interaction can be monitored by fluorescence spectroscopy.

The present study was undertaken to characterize the interaction of quinacrine with the sarcoplasmic reticulum membrane isolated from skeletal muscle. The competition between quinacrine and local anesthetics, measured as a displacement of the quinacrine bound to SR terminal cisternae, allowed us to obtain some clues about the molecular features involved in the binding process. Indeed, we observed the functional characteristics of the quinacrine effect, by studying the Ca^{2+} movement across the SR membrane.

Materials and Methods

Microsomal membranes. Microsomal membranes derived from sarcoplasmic reticulum terminal cisternae (heavy SR) were obtained from hind leg of rabbit white muscle as described by Saito et al. [10]. A membrane fraction enriched in sarcoplasmic reticulum longitudinal tubules (referred to as light SR) was obtained by the procedure of Eletr and Inesi [11].

Free Ca^{2+} concentration. The free Ca^{2+} concentration was fixed by a Ca^{2+}-EGTA buffer according to a computer program [12] which accounts for the different ligands concentrations present in the reaction medium.

Protein concentration. The protein concentration was determined by the procedure of Lowry et al. [13] using bovine serum albumin as standard.

Abbreviations: SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; K_p, partition coefficient; IC_{50}, concentration of local anesthetic necessary to inhibit half of the quinacrine-induced fluorescence increase.

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Fluorescence measurements. Fluorescence measurements were carried out at 25°C with a Bio-Logic Optical System (Echirolles, France). The excitation wavelength (350 nm) was selected by a monochromator, whereas the fluorescence emission was detected at 90° through a GG475 cut-off filter (Ealing Electro-Optics, Holliston, MA, U.S.A.), 475 nm being the wavelength of 50% maximum transmission.

Samples were placed on standard quartz cuvettes (10×10 mm cross-section) being stirred from the top by a propeller. In the fluorimetric titrations, the fluorescence of the incubation medium 50 mM Mops (pH 6.8), 80 mM KCl and different quinacrine concentrations (up to 60 μM) was initially recorded (F). The increase in fluorescence (ΔF) was measured by adding SR membranes to give a final concentration of 0.1 mg protein/ml. A different cuvette was used for each quinacrine concentration tested. The inner filter effect was measured by adding the membranes to the incubation medium in the absence of quinacrine. This value was subtracted from each ΔF measured.

Displacement of quinacrine fluorescence by local anesthetics was measured as before, but including different concentrations of dibucaine, tetracaine, benzocaine, lidocaine, procaine or procainamide in the incubation medium of 50 mM Mops (pH 6.8), 80 mM KCl and 20 μM quinacrine. In some cases it was necessary to warm and stir the medium to dissolve the anesthetic. The chemical structures of the drugs used are shown in Fig. 1.

Partition coefficients. Partition coefficients were measured at room temperature. 1 ml of local anesthetic in 200 mM sodium phosphate (pH 6.8) and 1 ml of *n*-octanol were vortexed for 1 min and then centrifuged at low speed. Anesthetic concentrations in the aqueous phase were measured spectrophotometrically before and after the mixing at the following wavelengths: dibucaine, 325 nm; tetracaine, 310 nm; benzocaine, 285 nm; lidocaine, 265 nm; procaine, 290 nm; and procainamide, 290 nm. The concentration of anesthetic in the organic phase was calculated by difference. The initial anesthetic concentrations were chosen to obtain maximum accuracy in the spectrophotometric measurements. Previously, the millimolar absorption coefficients ($mM^{-1} \cdot cm^{-1}$) at pH 6.8 were calculated from the anesthetics absorbance spectra (dibucaine, 4.07; tetracaine, 19.57; benzocaine, 17.76; lidocaine, 0.43; procaine, 15.15; procainamide, 15.80). The partition coefficient, K_p, is defined as the ratio of the anesthetic concentration in the organic phase to the anesthetic concentration in the aqueous phase.

Ca^{2+} uptake. Ca^{2+} uptake was measured as following: heavy SR (0.1 mg protein/ml) was incubated at 25°C in 50 mM Mops (pH 6.8), 80 mM KCl, 10 or 0.2 mM $MgCl_2$, 50 μM $^{45}CaCl_2$ (approx. 4000 cpm/nmol). In some cases 50 μM quinacrine or 5 μM ruthenium red (freshly prepared according to the purity percentage given by Sigma Chemical Co.) was also added. The active loading was initiated by adding 10 mM acetylphosphate. At different time intervals, aliquots of 0.8 ml (0.08 mg protein) were filtered (Millipore HAWP 0.45 μm pore size) and the filters were rinsed with 2 ml of medium 50 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and then collected for determination of the radioactive Ca^{2+}.

ATPase activity. ATPase activity of leaky vesicles was measured by a spectrophotometric procedure [14] using an ATP-regenerating system. Heavy SR (0.04 mg pro-

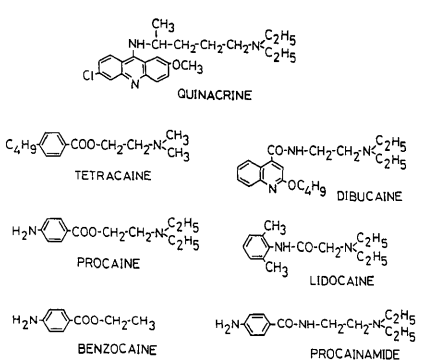

Fig. 1. Chemical structures in the free base form of the drugs used in this study.

tein/ml) was incubated at 22°C in 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 50 μM $CaCl_2$, 20 μg/ml pyruvate kinase, 20 μg/ml lactate dehydrogenase, 2 mM phospho*enol*pyruvate, 0.17 mg NADH/ml, 10 μM A23187 and different quinacrine concentrations (50–500 μM). The reaction was initiated by adding 1 mM ATP. The specific activity was calculated from the initial slope change of the absorbance value measured at 340 nm.

Ca^{2+}-induced Ca^{2+} release. The Ca^{2+}-induced Ca^{2+} release was measured by rapid filtration technique [15]. Heavy SR (3 mg protein/ml) was incubated for 2 h at room temperature in a medium consisting of 20 mM Mops (pH 6.8), 80 mM KCl, 4 mM $^{45}CaCl_2$ (approx. 4500 cpm/nmol). Samples of 0.03 ml (0.09 mg protein) were initially diluted in 0.87 ml of 20 mM Mops (pH 6.8), 80 mM KCl, 4 mM $CaCl_2$ and then 0.8 ml aliquots were layered on Millipore filters (DAWP 0.65 μm pore size), rinsed with 4 ml of dilution medium (20 mM Mops (pH 6.8), 80 mM KCl, 4 mM $CaCl_2$) and immediately flushed for 0.01–1 s with the corresponding medium as explained in the legend of Fig. 6. The filters were solubilized to measure the associated Ca^{2+} by a radiometric technique.

In other experiments, the flushing time was fixed at 0.5 s and the release obtained by addition of 20 mM Mops (pH 6.8), 80 mM KCl, 1 mM EGTA and 0.84 mM $CaCl_2$ (5 μM free Ca^{2}) was taken as 100%. To measure the inhibition of Ca^{2+} release, the medium was supplemented with different quinacrine concentrations. Measurements under conditions of no release i.e., with 20 mM Mops (pH 6.8), 80 mM KCl, 2 mM EGTA and 10 mM $MgCl_2$ were taken as a blank. A double reciprocal plot of the percentage of Ca^{2+} release inhibition as a function of the quinacrine concentration was used. The apparent inhibition constant (K_i) was calculated from the $-1/x$ intercept value as described by Fleischer et al. [16].

Results

Quinacrine is a convenient fluorescence probe in studies of SR membranes, since the interaction of quinacrine with these membranes leads to an increase in its fluorescence quantum yield. When sarcoplasmic reticulum vesicles were exposed to different quinacrine concentrations, an increase in the fluorescence intensity (ΔF) showing saturable properties can be measured. This effect was observed in microsomal preparations derived from different regions of the sarcoplasmic reticulum network 'in vivo'. The affinity of quinacrine for the heavy SR fraction with a $K_d \approx 8$ μM (Fig. 2B) was higher than that observed for a preparation enriched in longitudinal tubules ($K_d \approx 15$ μM) (Fig. 2A). In both cases the measurable increase in fluorescence corresponded to 5–6% of the quinacrine fluorescence in solution.

Since the quinacrine structure (Fig. 1) resembles that of a typical local anesthetic (aromatic ring connected to a tertiary amine group by a short alkyl chain), the competition of local anesthetics for quinacrine binding to these membranes was studied, in order to characterize the quinacrine binding site. The local anesthetics used were selected on the basis of contrasting physicochemical properties. As can be seen in Fig. 3, the fluorescence increase after addition of 20 μM quinacrine to heavy SR is displaced by different local anesthetics, the order of effectiveness being: dibucaine > tetracaine

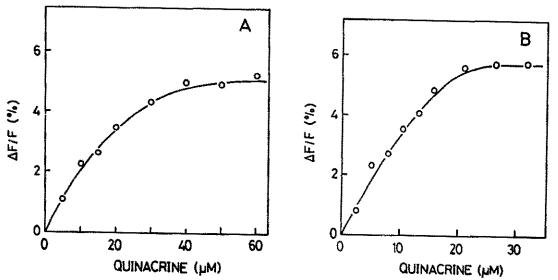

Fig. 2. Fluorimetric titration of the quinacrine binding site in light (A) and heavy (B) sarcoplasmic reticulum membranes. The fluorescence of the reaction medium containing 50 mM Mops (pH 6.8), 80 mM KCl and different quinacrine concentrations (up to 60 μM) was initially recorded (F). The fluorescence increase (ΔF) was measured for each quinacrine concentration after addition of SR membranes (0.1 mg protein/ml final concentration). The inner filter effect due to the turbidity of the membranes was evaluated by adding the microsomes to the reaction medium in the absence of quinacrine and was subtracted in each measurement.

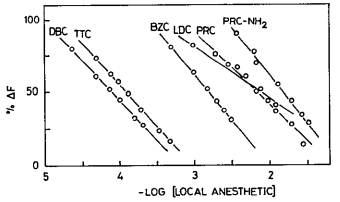

Fig. 3. Displacement of the quinacrine bound to SR membranes by different local anesthetics. The decrease in fluorescence, expressed as percentages, was measured by adding heavy SR (0.1 mg protein/ml final concentration) to the reaction medium 50 mM Mops (pH 6.8), 80 mM KCl, 20 μM quinacrine and different concentrations of the local anesthetic used. The inner filter effect was taken into consideration. DBC dibucaine; TTC, tetracaine; BZC, benzocaine; LDC, lidocaine; PRC, procaine; PRC-NH_2, procainamide.

> benzocaine > lidocaine > procaine > procainamide. The effectiveness of this competition can be adequately expressed by the IC_{50} value i.e., the concentration of local anesthetic necessary to inhibit half of the quinacrine-induced fluorescence increase. Lower IC_{50} values indicate higher effectiveness. In addition, the IC_{50} value represent the affinity of local anesthetics for binding to SR membranes.

In order to evaluate the structure-activity relationship of quinacrine interacting with the microsomal membranes, two basic physico-chemical properties of the local anesthetics that are often related to the potency of anesthesia, namely molecular weight and hydrophobicity [17], were considered. Table I reveals that the

TABLE I

Displacement of bound quinacrine (IC_{50}), molecular weight and partition coefficient of different local anesthetics

The IC_{50} values, expressed in millimolar concentrations, correspond to the concentration of each local anesthetic necessary to displace 50% of the quinacrine-dependent fluorescence increase. The partition coefficient was measured between *n*-octanol and buffer (pH 6.8).

Anesthetic	IC_{50} (mM)	Molecular weight (free base)	K_p (pH 6.8)
Dibucaine	0.08	344	200
Tetracaine	0.12	265	45
Benzocaine	1.58	165	71
Lidocaine	6.76	234	18
Procaine	7.59	236	0.3
Procainamide	14.50	235	> 0.02

molecular size of the local anesthetics and the hydrophobicity, as measured by the partition coefficient between *n*-octanol and buffer at pH 6.8, are involved in their interaction with the membrane. Thus, dibucaine and tetracaine, which are the most efficient local anesthetics (low IC_{50} values), are large in size and have a high K_p, whereas lidocaine, procaine and procainamide, with similar molecular weights, present lower effectiveness (high IC_{50}) related to their low hydrophobicities. Benzocaine, a neutral anesthetic lacking the terminal amine group (Fig. 1), with the lowest molecular size, presents an intermediate IC_{50} value, probably due to its high hydrophobicity.

We also studied the effect of quinacrine on the functional properties of the sarcoplasmic reticulum terminal cisternae. Thus Ca^{2+} uptake was measured under two critical experimental conditions. When the

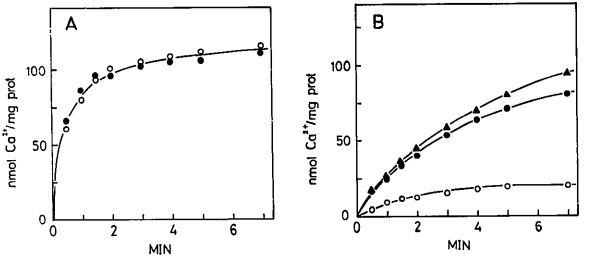

Fig. 4. Time course of Ca^{2+} uptake in heavy SR measured in the presence of 10 mM Mg^{2+} (A) or 0.2 mM Mg^{2+} (B). Heavy SR vesicles (0.1 mg protein/ml) were incubated at 25°C in a medium consisting of 50 mM Mops (pH 6.8), 80 mM KCl, $MgCl_2$ as indicated before, and 50 μM $^{45}CaCl_2$. After addition of 10 mM acetylphosphate, aliquots of 0.8 ml were filtered (Millipore HAWP 0.45 μm). The filters were subsequently rinsed with 2 ml of medium 50 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and solubilized for determination of the associated $^{45}Ca^{2+}$. Open circles represent control experiment, whereas closed circles correspond to the same experiment when 50 μM quinacrine was present in the incubation medium. In B the effect elicited by 5 μM Ruthenium red (closed triangles) is shown.

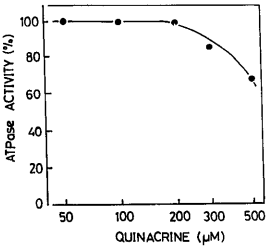

Fig. 5. Effect of quinacrine on the ATPase activity of heavy SR. Steady-state ATPase activity was determined by measuring the NADH oxidation at 340 nm in a medium containing 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 50 μM $CaCl_2$, 0.04 mg SR protein/ml, pyruvate kinase (20 μg/ml), lactate dehydrogenase (20 μg/ml), 2 mM phospho*enol*pyruvate, 0.17 mg NADH/ml, 10 μM A23187, 1 mM ATP and 50–500 μM quinacrine.

experiments were performed in the presence of 10 mM Mg^{2+} the time-course of the Ca^{2+} uptake, driven by acetylphosphate, was unaffected by the presence of low quinacrine concentrations (50 μM) in the reaction medium, as can be seen in Fig. 4A. In the other case, when the Mg^{2+} concentration was 0.2 mM (Fig. 4B), the Ca^{2+} accumulated by the vesicles was low, the addition, however, of the same quinacrine concentration gave rise to a remarkable increase in the accumulation of Ca^{2+}. The effect elicited by quinacrine was similar to that promoted by Ruthenium red.

Since the Ca^{2+} uptake is driven by the Ca^{2+} pump, we studied the effect of quinacrine on the ATPase turnover, measured in the presence of the Ca^{2+} ionophore A23187. Our data show (Fig. 5) that quinacrine only has an inhibitory effect on the ATPase activity at higher concentrations (above 200 μM).

To study the effect of quinacrine on the Ca^{2+} release, heavy SR vesicles were allowed to equilibrate with 4 mM radioactive Ca^{2+} added to the medium (passive loading). Under those conditions, the microsomal vesicles can store 90–100 nmol Ca^{2+}/mg protein. Then, when the extravesicular free Ca^{2+} concentration was reduced to 5 μM, a rapid Ca^{2+} release process was observed using a rapid filtration technique. The presence of low quinacrine concentrations in the external medium blocks the release of Ca^{2+} (Fig. 6A). By studying the extent of the Ca^{2+} release in the presence of different quinacrine concentrations (Fig. 6B), an apparent K_i of 35 μM can be calculated.

Discussion

The structure of quinacrine consists of an acridine ring where the nitrogen has a pK_a around 7.7 and a tail which contains a secondary and a tertiary amine group whose pK_a are 6.5 and 10.3, respectively [6]. Since the tertiary amine group (diethylamino) is completely protonated at neutral pH as well as 83% of the acridine amine group and 24% of the third basic group, a re-

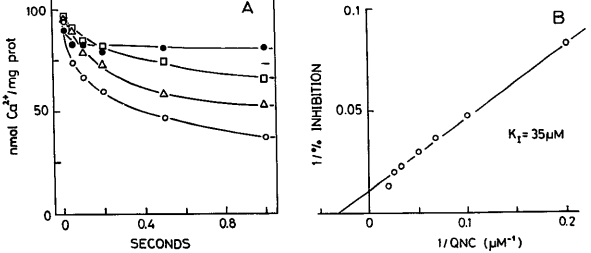

Fig. 6. Effect of quinacrine on the Ca^{2+}-induced Ca^{2+} release in heavy SR (A) and determination of the apparent inhibition constant (B). Passive loading of the vesicles (3 mg protein/ml) was carried out for 2 h at room temperature in a medium consisting of 20 mM Mops (pH 6.8), 80 mM KCl and 4 mM $^{45}CaCl_2$. For each experimental point, a sample of 0.03 ml was diluted in 0.87 ml of medium 20 mM Mops (pH 6.8), 80 mM KCl, 4 mM $CaCl_2$. 0.8 ml of the diluted vesicles were placed on Millipore filter (0.65 μm pore size) under vacuum in the rapid filtration apparatus and rinsed with 4 ml of the same medium containing nonradioactive Ca^{2+}. The release was studied by flushing the filters for various time intervals with the following media: 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA (●); 20 mM Mops (pH 6.8), 80 mM KCl, 1 mM EGTA, 0.84 mM $CaCl_2$ (5 μM free Ca^{2+}) (○). The latter medium was also supplemented with 15 μM (△) or 50 μM (□) quinacrine (QNC). In B, the inhibition of Ca^{2+} release was measured as described before. Samples on the filters (0.08 mg protein) were flushed for 0.5 s with 20 mM Mops (pH 6.8), 80 mM KCl, 1 mM EGTA, 0.84 mM $CaCl_2$ (5 μM free Ca^{2+}) and different quinacrine concentrations. See Materials and Methods for a more detailed description.

markable electrostatic effect for quinacrine binding along with hydrophobic forces due to the aromatic group is expected. The interaction of quinacrine with acidic phospholipid vesicles suggests that the drug is most probably localized with the acridine ring near the polar head of the phospholipid, while the alkyl chain moves freely in the aqueous phase [18].

Quinacrine mimics the local anesthetics action, since it has been shown to block the neuromuscular transmission in mammalian synapses by specific interaction with the acetylcholine receptor [19,20]. Studies in axonal membranes have shown [8,9]) that quinacrine appears to have a binding site as measured by fluorescence spectroscopy.

A qualitatively similar result was obtained for the interaction of quinacrine with SR membranes, since the addition of micromolar quinacrine concentrations gives rise to a saturable increase of the fluorescence intensity. The increase of quantum yield observed when quinacrine interacts with a membrane has been interpreted as a decrease in polarity of the environment and immobilization [18]. The similarity of the K_d values for quinacrine in light and heavy SR suggests that the saturable site may represent an interaction between the drug and some membrane component of a different nature present in each membrane fraction. We cannot rule out a nonspecific interaction with the lipidic matrix, although this possibility appears to be more feasible when high concentrations of quinacrine were used (effect on the Ca^{2+} pump at concentrations above 200 μM) as occurs with the presence of local anesthetics at millimolar concentrations on the Ca^{2+} uptake and release from SR [21–26].

The fact that increasing concentrations of local anesthetics decreases the equilibrium binding of quinacrine to membrane fragments provides further evidence for the proposal that the quinacrine binding site is the same as the local anesthetics binding site [9]. Thus, quinacrine can also provide information on the occupancy of the local anesthetic site by other clinically useful local anesthetics. In addition, these experiments reveal the structural characteristics involved in the binding of quinacrine. Firstly, we will discuss the significance of the partition coefficients used in this study. Although the K_p has often been taken as indicative of solubility in biological membranes [27,28], it is more likely that it is related to a hydrophobic parameter with no indication of the true anesthetic concentration in a membrane [29]. In fact, the octanol solubility could be indicative of an amphiphilic binding site with both polar and apolar domains, since the binding of small organic molecules to albumin or hemoglobin can also be correlated with the octanol/water partition coefficient [30,31]. The solubility in biological membranes could be more properly expressed by a hydrocarbon/water partition coefficient.

TABLE II

Percentage of local anesthetic, cationic form, at pH 6.8

The cationic form, at pH 6.8, was determined by the expression: $\log([AH^+]/[A]) = pK_a - pH$, where AH^+ and A are the cationic and neutral forms related to the dissociation constant K_a [36].

Anesthetic	% Cationic form
Dibucaine	98.05
Tetracaine	98.05
Benzocaine	0.00
Lidocaine	92.64
Procaine	99.22
Procainamide	99.61

Examining the structures of the local anesthetics used and bearing in mind the significance of the K_p it can be observed that the presence of a quinoline ring and butoxi group (dibucaine) or the addition of a butyl group to benzene (tetracaine) increases the effectiveness for the quinacrine–local anesthetic displacement. From these data the relevance of the quinacrine aromatic hydrophobicity is evident along with the molecular size for binding to the membranes. The nature of the alkyl substitutions (dimethyl or diethyl) in the terminal amine group as well as the hydrophilic bond (ester or amide) in the alkyl chain does not appear to play an important role in the competition process. For instance, benzocaine (with a structure similar to procaine, except that the terminal diethylamino group is absent) displaces quinacrine more easily than procaine. Also, tetracaine that is an ester-linked anesthetic is less efficient than dibucaine, but more than lidocaine even though dibucaine and lidocaine have an amide link. These examples also question the relevance of the charged and uncharged forms of the tertiary amine group since benzocaine (with no tertiary amine group) always remains in the uncharged form whereas the other local anesthetics are in a high proportion in the protonated form at pH 6.8 (Table II). Moreover, the meaning of the pK_a for a compound interacting with the electrostatic charges of a membrane is difficult to analyze [29].

In this report we have also shown the functional characteristics of the quinacrine–SR membranes interaction by taking advantage of the Mg^{2+} concentration dependence on the Ca^{2+} uptake and release [32]. We know that 10 mM Mg^{2+} blocks the Ca^{2+} release pathway in heavy SR [33], therefore, under these experimental conditions, we can observe selectively the effect of quinacrine on the Ca^{2+} uptake process. It is shown that 50 μM quinacrine does not modify the Ca^{2+} uptake profile nor the ATPase activity measured in the presence of the Ca^{2+} ionophore A23187. Furthermore, when the Mg^{2+} concentration was as low as 0.2 mM to favor the release over the uptake, quinacrine behaved like Ruthenium red [34,35] by blocking the Ca^{2+} release pathway, therefore the addition of 50 μM quinacrine

increases the Ca^{2+} accumulated inside the vesicles. These data were further confirmed by direct radiometric measurements. Under the experimental conditions selected, i.e., passive loading of the vesicles and absence of ATP, quinacrine prevents the Ca^{2+}-induced Ca^{2+} release. The apparent inhibition constant gives the drug potency of quinacrine on the heavy SR in blocking the Ca^{2+}-induced Ca^{2+} release.

It is concluded that the quinacrine effect is concentration dependent. Quinacrine, at lower concentrations, inhibits the Ca^{2+} release pathway, whereas higher concentrations also affect the Ca^{2+}-ATPase activity. Since quinacrine interacts with SR at lower concentrations than do local anesthetics, it can be used as a fluorescence local anesthetic probe.

Acknowledgments

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