INHIBITION OF THE ENERGY-LINKED FLUORESCENCE RESPONSE OF QUINACRINE WITH LOCAL ANESTHETICS

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1. Introduction

The fluorescence quenching of quinacrine (QA) which occurs upon association of the dye with energized membranes has been widely used as an indication of the development of the energy-pool [1–5]. The mechanism of the fluorescence quenching has generally included a direct role for the membrane [1,6–10], while simple diffusion of the diamine into the vesicle in response to the ΔpH cannot accurately account for the fluorescence quenching [11,12]. Furthermore, photoaffinity labeling studies [13] have suggested that QA interacts with specific hydrophobic sites on the energized membranes, possibly in a fashion similar to local anesthetics [14].

Quinacrine is structurally analogous to local anesthetics. As such, it exhibits several pharmacological properties in common with these anesthetic amines [15,16]. Like a typical anesthetic, in vivo QA blocks the response of *Electrophorous electricus*, electroplaque, in a non-competitive manner, in vitro QA enhances the affinity of the cholinergic receptor of *Torpedo mamorato* membrane fragments for acetyl choline. Quantitative studies on QA binding have indicated that there is a competition between QA and local anesthetics for the binding sites. Further, QA acts as a calcium antagonist displacing Ca^{2+} from its binding sites on calmodulin [17]. This report demonstrates that 2 local anesthetics can effectively inhibit the energy-linked fluorescence quenching of QA with submitochondrial membranes. This effect is seen most effectively with chlorpromazine (CP) and to a lesser extent, butacaine (BC), while other anesthetics tested had no effect. The effect is not, however, due to any uncoupling effect of the anesthetic.

Abbreviations: QA, quinacrine; 9AA, 9-aminoacridine; 9ACMA, 9-amino-3-chloro-7-methoxyacridine; BC, butacaine; CP, chlorpromazine; FCCP, *p*-trifluoromethoxycarbonyl cyanide–phenylhydrazone; NADH, nicotinamide adenine dinucleotide (reduced); SMP, submitochondrial particles

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2. Materials and methods

Submitochondrial particles (SMP) from heavy beef heart mitochondria were prepared as in [18,19]. The particles were treated with oligomycin (1 μg/mg protein), malonate, and suspended in 0.25 M sucrose to 25 mg protein/ml. NADH oxidase was measured with a Clark oxygen electrode in a medium consisting of 150 mM sucrose, 30 mM phosphate (pH 7.4) and 1.0 mM NADH at 25°C.

Fluorescence measurements were made with a SLM fluorescence spectrophotometer (model 4000) interfaced with a data processor (HP 9815A). The measurements were routinely obtained using an excitation slit of 4 nm and an emission slit of 8 nm.

Scatchard analysis of QA bound to the membrane indicates that CP competes with QA for the binding sites on the energized membrane. This competitive binding study (not shown) was done by a rapid filtration method using Millipore mixed cellulose–nitrate–acetate and cellulose–acetate (0.2 μm diam.) filters. However, 10–20% of the QA bound to these filters in the absence of the membranes, thereby making the determination of competitive-type inhibition difficult. Centrifugation was not considered an appropriate

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alternative. While it seems clear that QA and CP are competitive other methods for a more accurate determination will need to be developed.

Quinacrine · 2 HCl, chlorpromazine · HCl, tetracaine · HCl, procaine, lidocaine and butacaine hemisulfate were obtained from Sigma Chemical Co. All other chemicals were of the highest purity available commercially. Glass-redistilled water was used throughout.

3. Results and discussion

Fig.1 shows the inhibition of the energy-linked fluorescence response of QA upon the addition of CP. With increasing concentrations of CP, the extent of the response continually decreased until it eventually disappeared. This effect was independent of the order of addition; addition of CP while the membranes were energized had the same effect in releasing the fluorescence quenching (not shown). The equal cycle time of the fluorescence quenching induced upon the addition of 46 μM NADH in the absence and presence of CP indicates that CP is not uncoupling the membrane.

Butacaine (BC) also inhibits the energy-linked fluorescence response of QA but not as effectively as CP. Fig.2 shows the titration curve of the inhibition of the energy-linked fluorescence response of QA with both CP and BC. Clearly, an ~10-times greater concentration of BC was required to get the same

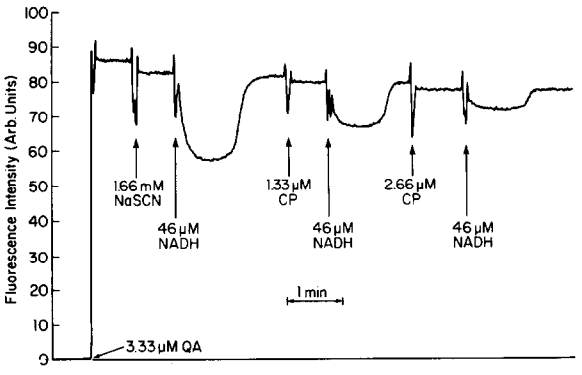

Fig.1. The effect of chlorpromazine on the energy-linked fluorescence decrease of QA. The reaction mixture consisted of 150 mM sucrose, 30 mM phosphate (pH 7.4) and 0.13 mg SMP protein/ml. Total vol. 3.0 ml. Other additions are as indicated. The fluorescence was measured with 420 nm excitation and 500 nm emission wavelength. $T = 25°C$.

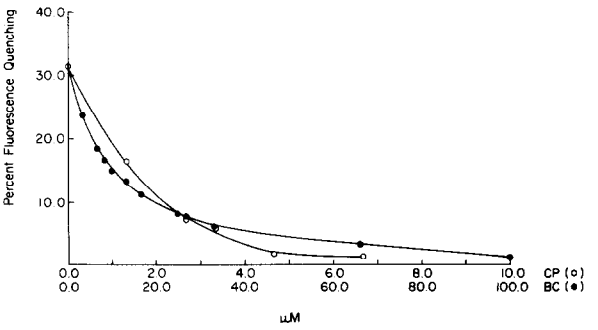

Fig.2. Titration of the energy-linked fluorescence response of QA with chlorpromazine (CP) and butacaine (BC). The conditions are as in fig.1. The app. K_i is the concentration of anesthetic which gives 50% inhibition of the energy-linked fluorescence response.

effect as with CP. The apparent K_i for CP is 1.5 μM, which is comparable to the K_d (2.2 μM) of QA [1]. The app. K_i of BC, however, is close to 10 μM. Thus, CP, but not BC, binds to the membrane with a higher affinity than QA.

Other local anesthetics tested, such as lidocaine, procaine and tetracaine, have little or no effect on the fluorescence response of QA. This indicates that the inhibition with CP and BC is not caused simply by charge repulsions with molecules absorbed to the surface of the membrane. The selectivity of the inhibition of the fluorescence response of QA indicated that there are stringent structural requirements for the binding sites. Indeed, there are considerable structural similarities between CP and QA, while the other anesthetics differ considerably.

Fig.3 shows the effect of CP and BC on NADH oxidase activity. Both CP and BC inhibited state 3 and, to a lesser extent, state 4 respiration. Under these conditions, however, neither anesthetic stimulated state 4 respiration. In the case of CP, in the concentration range which inhibited the energy-linked fluorescence response (<10 μM), there was no significant effect on the rate of either state 3 or 4 respiration. Although with BC there was considerable inhibition of state 3 respiration, state 4 respiration was not inhibited to an extent which would explain the release of the fluorescence quenching of QA. Thus, the inhibition of the energy-linked fluorescence response of QA by both BC and CP was not due to any uncoupling activity ascribable to the anesthetics.

The effect of CP on the energy-linked response

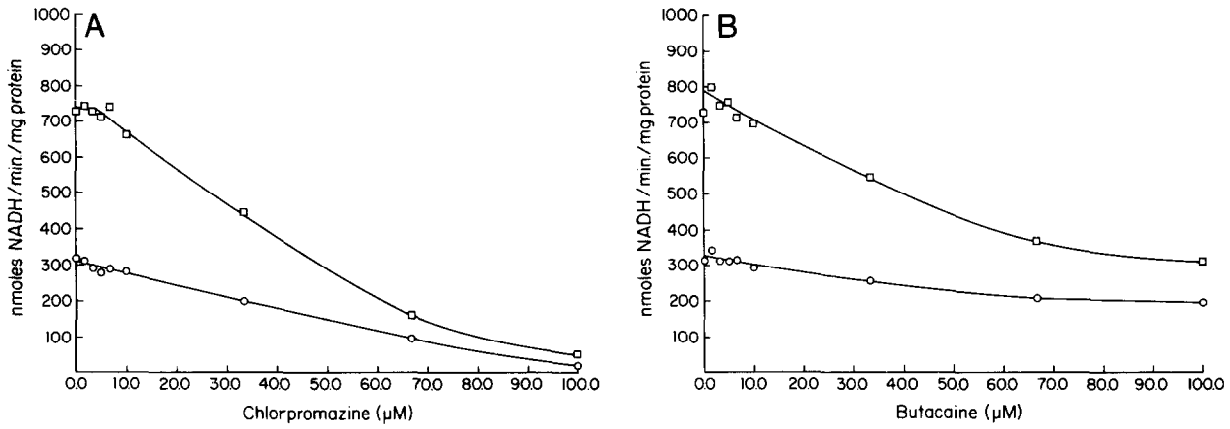

Fig.3. The inhibition of NADH oxidase by CP (A) and BC (B). The activities were measured in the presence (□) (state 3) and absence (○) (state 4) of FCCP (3.3 μM). The reaction mixture consisted of 150 mM sucrose, 30 mM phosphate (pH 7.4), 166 mM NaSCN and 0.13 mg SMP protein/ml. Total vol. 1.0 ml; $T = 25°C$.

presented in this paper is much greater than that with chloroplast membranes, as in [3]. With 22 μM chlorpromazine, 50% inhibition of the response was seen when using ~10 μM QA and less inhibition was seen with the lower concentrations of QA. In [3], however, the inhibition of the fluorescence response was interpreted to be due to an uncoupling effect of CP. This was not the cause of the inhibition of the fluorescence response with SMP (cf. below) and furthermore, does not explain the large difference seen in the effectiveness of CP with SMP relative to that with chloroplast. The cause for the smaller effect on the fluorescence response in chloroplast membranes is moot, but it may be due to differences in both the number and the type of binding sites. Since chloroplast membranes are composed of primarily neutral lipids [20] and much less protein the number of the binding sites may be expected to be much greater. Furthermore, with the absence of the charged phosphate groups of the lipids, the binding forces would be primarily hydrophobic and steric.

These results support the contention that there are specific binding sites for QA with the membrane. The question arises as to the significance of these sites and their relation to the energy-pool. Although only speculative, the sites may be a reflection of changes in the membrane which are required for ATP synthesis, such as an ordering and dehydration of the membrane lipids.

Our recent results indicate that the effect seen with these local anesthetics on the fluorescence response of QA is also seen with 9-aminoacridine (9AA) and 9-amino-6-chloro-2-methoxyacridine (9ACMA), though to different extents. These data are in line with [21] that there are specific binding sites for 9AA and 9ACMA with the membrane. If this were the case, then the ΔpH determinations made with these probes will be severely overestimated. These determinations have always assumed that there were no interactions between the membrane and the probe [22]. The validity of this assumption should, therefore, be re-evaluated. Indeed, such interactions may also occur between the membrane and the other amines, e.g. methylamine and ammonia.

Acknowledgements

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