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Original article

Three independent mechanisms contribute to tetracaine inhibition of cardiac calcium release channels

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ABSTRACT

Tetracaine is a tertiary amine local anaesthetic which inhibits ryanodine receptors (RyRs), the calcium release channels of the sarcoplasmic reticulum (SR). Tetracaine has been extensively used to study the role of the SR Ca^{2+} fluxes in muscle cells, yet a detailed understanding of tetracaine action on RyR channels is lacking. Here we investigate tetracaine effects in single channel recording of sheep cardiac RyRs in lipid bilayers. Tetracaine decreased channel conductance (block) and open probability (inhibition). The IC_{50} for inhibition had complex dependencies on membrane voltage and cytoplasmic [ATP], [Ca^{2+}] and pH. We identify three mechanisms underlying these actions. First, a voltage-dependent, slow inhibition in which luminal and cytoplasmic tetracaine compete for a common neutral/cation binding site within the trans-membrane RyR domain to induce long closed events (~100 ms). The apparent binding rate is proportional to the RyR closed probability, indicating that it only operates on closed channels. Second, a voltage-independent, pH sensitive fast inhibition in which cytoplasmic and luminal tetracaine compete for a site located on the cytoplasmic domain of the RyR to induce fast closed events (~2 ms). Its IC_{50} is not dependent on the open/closed conformation of RyR. Finally, a voltage-dependent block of the channel by cytoplasmic tetracaine reduced channel conductance. We develop a model for tetracaine inhibition which predicts that under diastolic conditions, *i.e.* when RyRs are mainly closed, the slow mechanism has the highest potency (IC_{50}~200 μM) of the three mechanisms and is therefore the dominant form of inhibition. However, during periods of Ca^{2+} release, *i.e.* when RyRs are open, the slow mechanism becomes ineffective, leaving the fast inhibition (IC_{50}~2 mM) as the dominant effect. Because of this closed state inhibition property, tetracaine loses its efficacy when RyRs open. This has the effect of increasing the feedback on SR Ca^{2+} release generated by cytoplasmic and luminal Ca^{2+}.

1. Introduction

In striated muscle, the action potential depolarises the voltage-dependent L-type Ca^{2+} channels in the sarcolemma and T-tubules, leading to a release of Ca^{2+} from the sarcoplasmic reticulum (SR, the main intracellular Ca^{2+} store) and a rise in cytoplasmic Ca^{2+} which stimulates and muscle contraction. Ryanodine receptors (RyRs) are the Ca^{2+} release channels in the SR membrane. Ryanodine and caffeine are commonly used experimentally to activate RyRs, stimulate Ca^{2+} release and deplete the SR whereas the local anaesthetic, tetracaine, is a classic RyR inhibitor. Together, these three pharmacological agents have been extensively used to study the role of the SR Ca^{2+} fluxes in Ca^{2+} handling by muscle cells. In this report we investigate the mechanisms for tetracaine inhibition of cardiac RyRs (the RyR2 isoform). The need to understand the mechanisms for pharmacological inhibition of RyRs is highlighted by the recent finding that certain class 1c drugs can repress Ca^{2+} waves and their associated cardiac arrhythmias by inhibiting RyR2 whereas RyR2 inhibition by tetracaine leads to increased store loading and stimulation of Ca^{2+} waves [1]. The markedly different therapeutic and pathological actions of these compounds are thought to stem from subtle differences in their inhibition kinetics [2]. Although the actions of tetracaine have been widely examined in muscle, the mechanism for tetracaine inhibition of RyRs is complex and poorly understood.

Tetracaine is a tertiary amine (pKa of 8.5 [3]) which induces closures in RyRs from cardiac [4–6] and skeletal muscle [3]. It inhibits them from both the luminal and cytoplasmic sides of the membrane in a reversible manner [3]. Hill coefficients of tetracaine concentration–responses (2.5 [3] and 1.5 [5]) indicate that channel closures result from cooperative binding of several tetracaine molecules but the specific multi-molecular mechanisms are unknown. The potency of tetracaine inhibition has been found to depend on the ionic strength [7] and adenine nucleotides [6,7] and 10-fold RyR to RyR variations in tetracaine potency have been reported for which there is still no explanation [3]. In the present study we use single channel recording to investigate these mechanisms in cardiac RyRs under a range of activating conditions and pH in order to develop a model for tetracaine inhibition. The model explains the

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dependence of tetracaine inhibition on the composition of intracellular milieu and provides a framework for understanding the action of tetracaine on cardiac muscle. The work follows on from a previous study where we showed that tetracaine causes both short-lived and long-lived channel closures in cardiac RyRs [1], indicating the presence of two inhibitory mechanisms (dubbed fast and slow mechanisms, respectively). Analysis of their voltage- and pH-dependencies, as presented here, allow us to predict the approximate locations for two distinct tetracaine binding sites and the specificity of these sites for charged and neutral forms of tetracaine. We find that the fast and slow mechanisms operate very differently on RyR gating. The slow mechanism utilises a binding site for neutral and cationic tetracaine which is strongly dependent on channel conformation, only operating whilst channels are in their closed state. Whereas the fast mechanism operates independently of the closed state of the channel and affinity of the tetracaine binding site decreases with increasing pH. These findings provide a new understanding of how tetracaine modulates Ca^{2+} release in muscle.

2. Materials and methods

2.1. Lipid bilayers, chemicals and solutions

SR vesicles were obtained from sheep hearts (RyR2) and these were reconstituted into artificial lipid bilayers as previously described [8]. Lipid bilayers were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt) in n-decane, (50 mg/ml). During vesicle fusion the *cis* (cytoplasmic) and *trans* (luminal) solutions contained 250 mM Cs^+ (230 mM $CsCH_3O_3S$, 20 mM CsCl) and 50 mM Cs^+ (30 mM $CsCH_3O_3S$, 20 mM CsCl), respectively. Due to the orientation of RyRs in the SR vesicles, RyRs added to the *cis* chamber incorporated into the bilayer with the cytoplasmic face of the channel orientated to the *cis* solution [9]. The caesium salts were obtained from Aldrich Chemical Company and Ca^{2+} was added to these solutions as $CaCl_2$ from BDH Chemicals. Prior to channel recording, the *trans* [Cs^+] was raised to 250 mM by addition of an aliquot of 4 M $CsCH_3O_3S$.

Unless otherwise stated, solutions were buffered to a redox potential of −232 mV (at pH 7.4) using a combination oxidised and reduced glutathione disulfide (GSSG (0.2 mM) and GSH (4 mM), respectively, calculated using the Nernst equation [10]). The rationale for redox buffering was to eliminate experimental variations in RyR activity arising from any redox sensitivity in the RyR2. It is known that both channel gating and tetracaine inhibition of the RyR1 isoform are sensitive to small changes in redox potential [11,12]. Since GSH and GSSG are the main intracellular redox buffers [13], combinations of GSH and GSSG were chosen to set the redox potential of the bathing solutions to cytoplasmic levels [10].

Tetracaine (tetracaine HCl>99%) was obtained from Sigma Chemicals. Adenosine 5′-triphosphate (ATP) was obtained as sodium salts from Sigma Chemicals. Solutions were pH buffered with 10 mM N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) for pH 7.4 and 10 mM Tris[hydroxymethyl]aminomethane (Trizma) for pH 9.5, both obtained from Sigma Chemicals. pH was titrated using CsOH (optical grade from ICN Biomedicals). Submicromolar free [Ca^{2+}] was buffered with 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, obtained as a tetra potassium salt from Molecular Probes) and titrated with $CaCl_2$. A Ca^{2+} electrode (Radiometer) was used to determine the purity of BAPTA, Ca^{2+} stock solutions and free [Ca^{2+}] in the experimental solutions against a 100 mM $CaCl_2$ standard (Fluka). During recordings the composition of the *cis* solution was altered either by addition of aliquots of stock solutions or by local perfusion of the bath. The local perfusion method allowed solution exchange within ~1 s between solutions in random sequence [14].

2.2. Acquisition and analysis of ion channel recordings

Recording and analysis of ion channel activity were carried out as described previously [15]. Electrical potentials are expressed using standard physiological convention (*i.e.* cytoplasmic side relative to the luminal side at virtual ground). Measurements were carried out at 23 ± 2 °C. During experiments the channel currents were recorded using a 50 kHz sampling rate and 5 kHz low pass filtering. Prior to analysis the current signal was digitally filtered at 1 or 2 kHz with a Gaussian filter and re-sampled at 5 or 10 kHz. Unitary current and time-averaged currents were measured using Channel2 software (P.W. Gage and M. Smith, Australian National University, Canberra). Mean open and closed durations were generally calculated from recordings of 100–1000 opening events. Dwell-time frequency histograms of channel open and closed events were obtained from single channel recordings and these were plotted using the "log-bin" method suggested by Sigworth and Sine [16]. The log-bin method displays exponentials as peaked distributions centred about their exponential time constant. Sampling bins were equally spaced on a log scale with 7 bins per decade.

3. Results

Initially we analysed tetracaine inhibition under conditions where the channels were maximally activated by cytoplasmic 1 mM $CaCl_2$ and 2 mM ATP at pH 7.4 (chelation of Ca^{2+} by ATP gives 0.45 mM free Ca^{2+}). Under these conditions RyRs had a mean open duration of ~100 ms and mean closed times of ~1 ms (*e.g.* Figs. 1A and B, 0 mM traces). The scarcity of native closed events allowed the tetracaine induced closures to be more clearly identified. Three forms of inhibition were evident in the single channel recordings shown in Figs. 1A and B. Firstly, tetracaine (mM in either cytoplasmic or luminal baths) caused brief channel closures that were partially resolved at 1 kHz recording bandwidth. Secondly, luminal and cytoplasmic tetracaine induced long channel closures of ~100 ms mean duration. Finally, tetracaine (cytoplasmic only) reduced the ionic conductance of the channel as seen by a decrease in the unitary current amplitudes (Fig. 1A, top four traces). We found that all these effects of tetracaine were completely reversible within the time frame of tetracaine washout (~2 s).

The fast and slow tetracaine induced closures caused a reduction in channel open probability (P_o; Figs. 1C and D) that was fitted with Hill curves using Eq. (1):

$$P_o = P_o control / \left(1 + \left([\text{tetracaine}] / IC_{50}\right)^H\right) \quad (1)$$

where IC_{50} is the half inhibitory concentration of tetracaine, $P_o control$ is the open probability of the channel in the absence of tetracaine and H is the Hill coefficient. Under the experimental conditions described above, the IC_{50}'s for cytoplasmic inhibition were lower than for luminal inhibition and the IC_{50}'s were lower at positive membrane potentials than at negative potentials (see Table 1 for Hill parameters).

Fig. 1E shows the effect of tetracaine on RyR unitary current. In the absence of tetracaine, the unitary current had a linear voltage-dependence with a conductance of 443 ± 5 pS. Cytoplasmic tetracaine (1 and 2 mM) caused a voltage-dependent reduction in the unitary current where the strongest effect was seen at positive membrane potentials. Luminal tetracaine caused no significant reduction in RyR2 conductance at concentrations up to 5 mM. The effect of cytoplasmic tetracaine on unitary current is analysed in Supplementary Fig. 1 which shows that the IC_{50} for current inhibition is voltage-dependent, increasing from 2 mM at −60 mV to 15 mM at +20 mV.

We investigated tetracaine inhibition of P_o and its dependence on cytoplasmic agonists such as ATP and Ca^{2+} by measuring [tetracaine]-responses in the absence and presence of cytoplasmic ATP and Ca^{2+} (Figs. 1C and D and Supplementary Fig. 2). We found 10-fold variations in the IC_{50}, depending on the concentrations of