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The effect of local anaesthetics on the ryanodine receptor/Ca^{2+} release channel of brain microsomal membranes

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The effects of various local anaesthetics (LAs) on ryanodine binding of the sheep brain ryanodine receptor were tested. Tetracaine and dibucaine inhibit the binding with half-maximal inhibition (CI_{50}) of 0.12 mM and 0.7 mM, respectively. Lidocaine and its analog QX-314, on the other hand, stimulate the binding up to 3-fold with half-maximal stimulation occurring with about 2 mM of the drugs. Lidocaine increases both the receptor affinity for ryanodine by about 5-fold and the rate of ryanodine association with its binding site by about 6-fold. Tetracaine and lidocaine also interact with the purified brain ryanodine receptor and produce inhibitory and stimulatory effects similar to those obtained with the membrane-bound receptor. The interaction of the LAs with the brain ryanodine receptor, as well as with the skeletal muscle receptor [J. Memb. Biol. 133 (1993) 171–182], suggest that ryanodine receptor possesses intrinsic binding site(s) for LAs.

Local anaesthetic; Ryanodine receptor; Brain microsome; Ca^{2+} release channel

1. INTRODUCTION

It is accepted that the primary mode of membrane conduction blockade by local anaesthetics (LAs) occurs through the inhibition of voltage-gated sodium channels [1]. However, other voltage-gated channels [1–3] and ligand-gated channels [4,5] are also sensitive to local anaesthetics.

Local anaesthetics also have diverse effects on other cell activities such as Ca^{2+} transport across the sarcoplasmic reticulum [6–8], inositol phospholipid hydrolysis [9], protein kinase C activity [10], and on the binding of the neutral alkaloid ryanodine by the skeletal [11] or the hepatic [12] receptors.

Recently [13–18] the presence of ryanodine binding protein in brain microsomes was demonstrated. The brain ryanodine receptor shares many of the characteristics of the muscle receptor: (a) possesses high- and low-affinity ryanodine binding sites [13,15,18]; (b) the protein exists as a tetrameric complex of about 420 kDa subunits [15,18]; (c) its incorporation into planar lipid bilayers results in the appearance of ryanodine-sensitive Ca^{2+} channels [13,18]; (d) both the ryanodine binding and channel activities are affected by various modulators such as ATP and Ca^{2+} [13,15,18,19].

In this study we demonstrate the interaction of several local anaesthetics with the membrane-bound and purified brain-ryanodine receptor.

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Abbreviations. EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis-(thyroxymethyl)-ethyl]-glycine; MOPS, 3-(*N*-morpholino)-propensulfonic acid; LAs, local anaesthetics.

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, EGTA, Tris, Tricine, MOPS, caffeine, tetracaine, dibucaine, and lidocaine were obtained from Sigma, and QX-314 was obtained from Alomone Labs, Jerusalem. [^{3}H]Ryanodine was purchased from New England Nuclear, and unlabelled ryanodine was obtained from Calbiochem. Dibucaine and lidocaine were prepared in ethanol and then diluted to 25% ethanol while stirring, and the pH was adjusted to about 7.0. The final ethanol concentration in control and LAs-containing samples never exceeded 2%.

2.2. *Membrane preparation*

Microsomal membranes were prepared from whole sheep brain essentially as described previously [20]. The brain was homogenized in ice-cold buffer A (0.32 M sucrose, 5 mM NaH_2PO_4, pH 7.4, 0.3 mM PMSF, 0.8 mM benzamidine, 0.5 μg/ml leupeptin and 0.5 μg/ml aprotenine) (8 ml per g wet weight) with 10 strokes of a motor-driven teflon–glass homogenizer. Homogenate was centrifuged for 10 min at $1,000 \times g$. The supernatant was retained, and the pellet was homogenized and centrifuged as before. The supernatants were combined and re-centrifuged for 60 min at $17,000 \times g$. The pellets were resuspended in buffer A (approximately 2.5 ml per g wet weight of brain) with a tight-fitting homogenizer. This material (6 ml) was layered on sucrose gradients of 7.5 ml each of 1.2, 1.0 and 0.8 M sucrose (containing 5 mM NaH_2PO_4 pH 7.4 and the protease inhibitors), centrifuged for 90 min at $100,000 \times g$ (Beckman SW27). The membranes within the 1 M sucrose and at the 1.0–1.2 M sucrose interface were collected, diluted slowly with 3 vols. of 5 mM NaH_2PO_4, pH 7.4 and pelleted at $100,000 \times g$ for 45 min. The pellets were resuspended in buffer A (without antiproteases), frozen in liquid nitrogen, and stored at

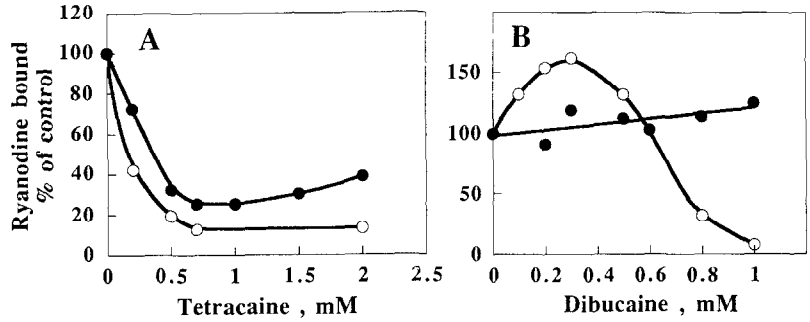

Fig. 1. Effects of tetracaine and dibucaine on ryanodine binding to brain microsomes. Brain microsomal membranes (2.5 mg/ml) were assayed for ryanodine binding in 0.5 M (○) or 1 M (●) of NaCl in the absence and in the presence of the indicated LAs as described in section 2. Control activities (100%) were: 35 and 84 fmol ryanodine bound/mg protein in the presence of 0.5 M NaCl and 1 M NaCl, respectively.

−70°C. Junctional SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito et al. [21].

2.3. *[^{3}H]Ryanodine binding*

Unless otherwise specified, 250 μg of brain microsomal membranes in 100 μl of a standard binding solution containing 0.5 M or 1 M NaCl, 20 mM MOPS, pH 7.4 and 50 μM $CaCl_2$ were incubated for 1–2 h at 37°C with 5 to 20 nM [^{3}H]ryanodine. Unbound ryanodine was separated from protein-bound ryanodine by filtration of the samples through Whatman GF/C filters, followed by washing three times with 5 ml of ice-cold buffer containing 0.2 M NaCl, 5 mM MOPS, pH 7.4 and 50 μM $CaCl_2$. The filters were dried, and the retained radioactivity was determined by standard liquid scintillation counting techniques. Specific binding of [^{3}H]ryanodine is defined as the difference between the binding in the absence and in the presence of 100 μM unlabelled ryanodine.

3. RESULTS

The effects of various concentrations of tetracaine and dibucaine on ryanodine binding by brain microsomal membranes are illustrated in Fig. 1. Tetracaine and dibucaine inhibited ryanodine binding with half-maximal inhibition (CI_{50}) occurring at about 0.12 mM and 0.7 mM for tetracaine and dibucaine, respectively. Dibucaine altered ryanodine binding in a biphasic manner; slight stimulation at low concentrations (< 300 μM) and inhibition at higher concentrations. This is similar to the effects of dibucaine on the skeletal muscle ryanodine receptor [11]. Ryanodine binding by the skeletal, cardiac and brain ryanodine receptor is strongly enhanced by increasing the ionic strength. Fig. 1 also shows that the degree of inhibition of ryanodine binding by tetracaine and dibucaine is dependent on the NaCl concentration present in the ryanodine binding medium. Increasing NaCl concentration from 0.5 M to 1 M decreases the inhibition by 0.5 mM of tetracaine from 81% to 65% (Fig. 1A). For dibucaine, however, increasing NaCl from 0.5 to 1 M prevented the inhibition of ryanodine binding by dibucaine (Fig. 1B).

The local anaesthetic lidocaine and its analog QX-314, on the other hand, stimulated the binding of ryanodine up to 2-fold (Fig. 2). However, the stimulation of ryanodine binding by QX-314 or lidocaine is decreased with increasing NaCl concentration, from stimulation of about 2-fold at 0.5 M NaCl to no stimulation in the presence of 1.0 M NaCl. This effect of NaCl is expected, since in the presence of 1 M NaCl ryanodine binding is close to its maximal level. The results in Figs. 1 and 2 suggest that the site of LA interaction is either influenced directly by ionic strength or indirectly due to NaCl stabilization of a protein conformation with a modified LA binding site.

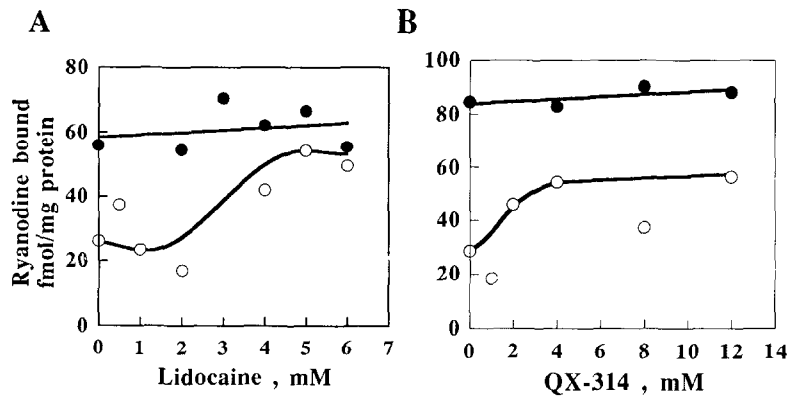

Fig. 2. Effects of lidocaine and QX-314 on ryanodine binding to the brain microsomes. Experimental conditions for ryanodine binding were as in Fig. 1. (○) and (●) indicate the presence of 0.5 M NaCl or 1 M NaCl, respectively.

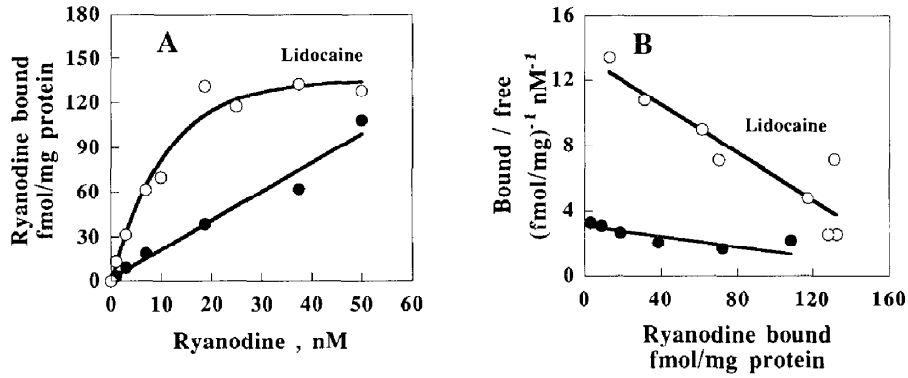

Fig. 3. The influence of lidocaine on the affinity of the ryanodine binding site. [^{3}H]Ryanodine was assayed as in Fig. 1 in the presence of 0.5 M NaCl in the absence (●) and in the presence (○) of 6 mM lidocaine, except that the [^{3}H]ryanodine concentration was varied. Saturation isotherms are shown in A and Scatchard plots in B. The calculated B_{max} values were: 190 and 185 fmol/mg protein, and K_d of 61 nM and 13.7 nM in the absence and in the presence of lidocaine, respectively.

Fig. 3 shows the binding of ryanodine as a function of its concentration in the absence and in the presence of lidocaine. Scatchard plots analysis of ryanodine binding in the absence and in the presence of 6 mM lidocaine indicates that lidocaine increased the apparent binding affinity (K_d) about 4.4-fold from 61 to 13.7 nM. In contrast, similar B_{max} values were obtained with 0.5 M NaCl ± 6 mM lidocaine (B_{max} = 190 and 184 fmol/mg protein). Thus the stimulation of ryanodine binding produced by lidocaine (at low NaCl concentration) is attributable to increased receptor affinity for the ligand.

Scatchard plot analysis of ryanodine binding in the absence and in the presence of 0.4 mM tetracaine indicates that tetracaine decreases the maximum binding sites (B_{max}) from 204 fmol/mg of protein to 116 fmol/mg of protein (n = 3) with no modification of the apparent binding affinity (K_d) (results not shown).

The effect of lidocaine on the ryanodine association with, and dissociation from, its binding site are shown in Fig. 4. Lidocaine increased ryanodine binding by 3- to 4-fold during the period of time tested (5 to 120 min, Fig. 4A, inset). Kinetic analysis of the data (Fig. 4A) indicates that lidocaine increased the observed association rate constant (K_{obs}). Ryanodine associates with its binding site with K_{obs} of 0.021 ± 0.0075 (n = 2) and 0.0521 ± 0.0013 (n = 2) min^{-1} in the absence and in the presence of lidocaine, respectively. The calculated pseudo-first-order association rate constants (K_1) obtained in the absence and in the presence of lidocaine were: $2.57 \times 10^{-4} \pm 0.29 \times 10^{-4}$ (n = 2) and $13.11 \times 10^{-4} \pm 0.74 \pm 10^{-4}$ (n = 2) M^{-1} · min^{-1} for the control and for lidocaine, respectively. Dissociation of bound ryanodine at equilibrium was initiated by 80-fold dilution (Fig. 4B). A similar monophasic dissociation was obtained in the absence and in the presence of 6 mM lidocaine (K_{-1} = 0.0147 min^{-1}). The K_d calculated from association and dissociation constants are 64 and 11.7 nM in the absence and in the presence of lidocaine, respectively, which are close to the K_d calculated from saturation experiments (Fig. 3).

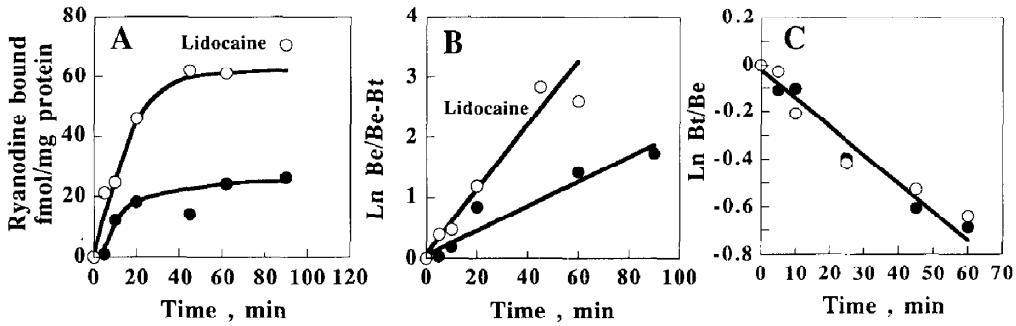

Fig. 4. Effect of lidocaine on equilibrium binding of [^{3}H]ryanodine and dissociation kinetics. In A, the membranes (2.5 mg/ml) were incubated with 10 nM ryanodine at 37°C, as in Fig. 1, in the absence (●) and in the presence (○) of 6 mM lidocaine. After the indicated incubation time, aliquots were assayed for bound ryanodine (Bt). The maximal amounts of ryanodine bound at the plateau (Be) were 32 and 66 fmol/mg protein, in the absence and in the presence of lidocaine, respectively. In B, the membranes were incubated with 10 nM ryanodine as in A. After 2 h, aliquots were assayed for bound ryanodine (Be = 33.0 fmol/mg protein). Dissociation of bound ryanodine was initiated by 80-fold dilution with the binding medium (without ryanodine) with (○) and without (●) 6 mM lidocaine, and determination of the residual ryanodine bound at the indicated time (Bt). The calculated K_{-1} was (0.0147) min^{-1} in the absence and in the presence of lidocaine. K_1 was calculated from the K_{obs}, as described previously [24], using the following equation: $K_{obs} = K_1[L][R]/Be$, where [L] = ryanodine concentration, [R] = ryanodine receptor = B_{max} (190 fmol/mg protein), $K_1 = 2.28 \times 10^{-4}$ and 12.37×10^{-4} M^{-1} · min^{-1} in the absence and in the presence of lidocaine, respectively.

The effects of different modulators of ryanodine binding to their receptor on the inhibition of the ryanodine binding by tetracaine are shown in Table I. ATP and caffeine, as shown previously [13,16] stimulated the binding of ryanodine to its receptor. The inhibition of ryanodine binding by tetracaine was not affected by caffeine, but it increased in the presence of ATP (Table I). The binding of ryanodine is absolutely dependent on μM concentrations of Ca^{2+} [16]. Ca^{2+} at the indicated concentrations has no significant effect on ryanodine binding, but it increases the binding inhibition by tetracaine from 70% to 100% (Table I).

The effects of different LAs on ryanodine binding to the purified ryanodine receptor are shown in Table II. As with the membrane-bound receptor tetracaine and dibucaine inhibited the binding, and lidocaine stimulated the binding to the purified receptor at the same range of concentrations. Thus, the experiments in Table II indicate that the drugs tested interact directly with the ryanodine receptor and that their binding sites are preserved in the purified receptor.

4. DISCUSSION

In this study, the effects of local anaesthetics on ryanodine binding to the brain membrane-bound and purified ryanodine receptor are presented. We found that dibucaine and tetracaine inhibited ryanodine binding to both membrane bound and purified ryanodine receptor, while lidocaine and QX-314 stimulated up to 3-fold ryanodine binding. These results indicate a specific interaction of LAs with the brain ryanodine receptor. The interaction of LAs with the purified ryanodine receptor suggests that the receptor possesses intrinsic binding site or sites for LAs. Similar results have been reported recently for the skeletal muscle ryanodine receptor [11]. Based on a structure–activity relationship, we proposed a model for the LAs site of interaction in the skeletal muscle ryanodine receptor [11]. Although in this study we have used only four different LAs with inhibitory and stimulatory effects on ryanodine binding, the results are very similar to those obtained with the skeletal muscle and, therefore, it is most probable that the nature of the LAs site of interaction is similar in both proteins.

Tetracaine and QX-314 (and also procaine) have recently been applied to single channels incorporated into planar lipid bilayers [25]. Tetracaine (150 μM) halved channel open probability (cf our reported CI50 of 120 μM for ryanodine binding, [11] and Fig. 1), while QX-314 (which stimulated binding, [11] and Fig. 2) did not reduce channel open probability.

The mechanism by which LAs exert their effects on nerves and muscles has been discussed extensively [1,6,22]. It is accepted, however, that there are specific interactions of LAs with Na^+ channel [22,23], acetylcholine receptor [5], Ca^{2+} channels [2,3] and K^+ channels [3]. The findings that LAs modify the activities of various channels may suggest the presence of a common binding site for LAs in the different ionic channels.

Since the brain ryanodine receptor is also a Ca^{2+} release channel [13,18], it is expected that, as with the SR membranes (see Table IV in ref. [11]), LAs would affect its activity as a Ca^{2+} release channel.

Table I

Effect of ATP, Ca^{2+} and caffeine on the inhibition of ryanodine binding by tetracaine

Additions	[^{3}H]Ryanodine bound (fmol/mg protein)	
	− Tetracaine	+ Tetracaine
None	48.5	14 (70)
ATP, 1 mM	69	0
$CaCl_2$, 0.2 mM	41	0
$CaCl_2$, 1.0 mM	54	0
Caffeine, 10 mM	96 5	26 (73)
Caffeine, 20 mM	99	30 (70)

[^{3}H]Ryanodine binding was assayed as in Fig. 1 in the absence or in the presence of tetracaine (0.4 mM) and of the indicated compounds. Free Ca^{2+} concentration was 50 μM in the absence and in the presence of ATP. The numbers in parentheses indicate the % of inhibition.

Table II

Comparison of the effects of local anaesthetics on ryanodine binding to the membrane-bound and the purified ryanodine receptor

Additions	Concentration (mM)	[^{3}H]Ryanodine bound, % of control (fmol/mg protein)	
		Membranes	Purified
None	–	35	7,974
Tetracaine	0.8	0	95
Dibucaine	1.6	9 4	0
Lidocaine	3	39	10,399
Lidocaine	6	60	13,065

The binding of [^{3}H]ryanodine (10 nM) to the brain microsomal membranes (2.5 mg/ml) and to the purified receptor (15.2 μg/ml) was determined as described in section 2, except that the assay temperature was 30°C, NaCl concentration was 0.5 M, and the indicated compounds were added to the assay medium. Brain ryanodine receptor was purified by the spermine-agarose method developed for the skeletal muscle ryanodine receptor [23] and its ryanodine binding activity was assayed also, as described in [23].

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