Diethyl pyrocarbonate modification of the ryanodine receptor/Ca^{2+} channel from skeletal muscle

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Exposure of junctional sarcoplasmic reticulum (SR) membranes or purified ryanodine receptor to the histidine-specific reagent diethyl pyrocarbonate (DEPC) led to concentration- and time-dependent inactivation of ryanodine binding. The pH-dependence of the inactivation of ryanodine binding by DEPC and the reversal of this inactivation by hydroxylamine suggests the modification of histidine residue(s) by the reagent. Kinetic analysis of the time course of inactivation of ryanodine binding by DEPC suggests that the inactivation resulted from modification of a single class of histidine residue per ryanodine-binding site. The degree of inactivation of ryanodine binding by DEPC was decreased when high NaCl concentrations were present in the modification medium. The binding affinities for ryanodine and Ca^{2+} were not altered by DEPC modification. This modification decreased the total ryanodine-binding sites. DEPC modification increased the Ca^{2+}-permeability of the SR vesicles. A variety of bivalent cations prevented the DEPC inactivation of ryanodine binding in a series of decreasing efficiency: $Mn^{2+} > Ba^{2+} > Mg^{2+} > Ca^{2+}$, similar to their effectiveness in inhibiting ryanodine binding. It is suggested that a histidine residue(s) in the ryanodine receptor is involved, either in the binding of Ca^{2+}, or in a conformational change that may be required for Ca^{2+} binding to its binding site(s). This modification of the ryanodine receptor resulted in inactivation of ryanodine binding and activation of Ca^{2+} release.

INTRODUCTION

In muscle cells, Ca^{2+} release from the sarcoplasmic reticulum (SR) plays an important role in excitation–concentration coupling [1,2]. A protein involved in the release of Ca^{2+} to the myoplasm space has been identified as the target of the toxic alkaloid ryanodine [3,4]. This protein has been purified and shown to be a homotetrameric complex [5–7]. It is accepted that the ryanodine-sensitive Ca^{2+} channels are a central component of excitation/contraction coupling in heart and skeletal muscle [5,8].

The purified ryanodine receptor contains an intrinsic Ca^{2+} channel activity which is regulated by various modulators such as caffeine, ATP, calmodulin, Mg^{2+} and Ca^{2+} [5]. The dependence of Ca^{2+} release and ryanodine-binding activities on Ca^{2+} concentration suggests that the ryanodine receptor/Ca^{2+}-release channel possesses high-affinity, activating, and low-affinity, inhibitory, Ca^{2+}-binding sites [9–12]. Analysis of the amino acid sequence of the ryanodine receptor, deduced from the cDNA sequence, has led to predications of the location of high- and low-affinity Ca^{2+} binding sites [13–15].

Diethyl pyrocarbonate (DEPC) reacts with histidyl residues in proteins to yield the *N*-carbethoxyhistidyl derivative [16]. This modification can be reversed by the addition of hydroxylamine, which is specific for the *N*-carbethoxyhistidyl derivative [16]. DEPC at millimolar concentrations was shown to induce Ca^{2+} release from SR vesicles [17].

In this study we demonstrate that DEPC modifies a histidyl residue in the ryanodine receptor, probably at the Ca^{2+}-binding site(s), and this leads to inhibition of ryanodine binding.

MATERIALS AND METHODS

Materials

ATP, EGTA, EDTA, Tris, Mes, Mops, DEPC, histidine, $BaCl_2$, $MnCl_2$, $MgCl_2$, spermine–agarose and CHAPS were obtained from Sigma Chemical Co. [^{3}H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear. Unlabelled ryanodine was purchased from Calbiochem.

Membrane preparation

Junctional SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito et al. [18]. In most of the experiments the fraction R_4 was used. The membranes were suspended to a final concentration of about 25 mg of protein/ml in a buffer containing 0.3 M sucrose and 10 mM Tricine, pH 8.0, and stored at −70 °C.

Purification of the ryanodine receptor

Ryanodine receptor was purified by the spermine–agarose method [19]. The purified protein (14–50 μg/ml) was assayed for [^{3}H]ryanodine binding (in 0.1 ml) as described below for the membranes, except that soybean lecithin (0.5 mg/ml) was present in the assay medium. After 2 h at 30 °C, the bound ryanodine was assayed by poly(ethylene glycol) 600 precipitation in the presence of carrier protein (1.4 mg/ml BSA), followed by filtration through Whatmann GF/B filters and washes with 3 × 4 ml of 10 % poly(ethylene glycol) solution [19]. Protein concentration of SR membranes was determined as described by Lowry et al. [20], and that for the purified ryanodine receptor as described by Kaplan and Pedersen [21].

Modification by DEPC

SR membranes (1–2 mg/ml) or purified ryanodine receptor (14–50 μg/ml) in 50 mM Mes, pH 6.0, were incubated with the indicated concentration of DEPC at 25 °C for the indicated time. After quenching the DEPC that had not reacted with 20 mM histidine, pH 7.0, samples were assayed for ryanodine binding.

Abbreviations used: DEPC, diethyl pyrocarbonate; SR, sarcoplasmic reticulum.
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DEPC stock solutions were prepared by diluting DEPC into dry acetonitrile immediately before use. The concentration of acetonitrile in control and DEPC-containing samples was 2% or less.

[^{3}H]Ryanodine binding

Unless otherwise indicated, junctional SR membranes (final concn. 0.5 mg/ml) were incubated with 20 nM [^{3}H]ryanodine (sp. radioactivity 30 Ci/mmol) in a standard binding solution containing 1.0 M NaCl, 20 mM Mops, pH 7.4, and 50 μM $CaCl_2$, for 1–2 h at 37 °C. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of protein samples (50 μg) through Whatmann GF/C filters, followed by washing with 3 × 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 liquid-scintillation counting. Non-specific binding was determined in the presence of 25 μM unlabelled ryanodine.

Ca^{2+} efflux from passively loaded vesicles

SR vesicles were incubated with or without DEPC as described above, and then the membranes were collected by centrifugation (40000 *g*, 30 min). The pellets were resuspended to 3 mg/ml in a medium containing 0.3 M KCl, 20 mM Mops, pH 6.8, and 0.4 mM $CaCl_2$ (containing $^{45}CaCl_2$, 5×10^4 c.p.m./nmol) and incubated for 2 h at 24 °C. For Ca^{2+}-efflux assay, the loaded vesicles (20 μl) were placed on 0.45 μm-pore nitrocellulose filters and rinsed with different volumes of 0.3 M KCl/20 mM Mops (pH 6.8)/1 mM EGTA solution for the indicated time. The flow rate was about 1 ml/s.

RESULTS

Ryanodine-binding activity, of either SR membranes or purified ryanodine receptor, is lost when incubated with relatively low concentrations of DEPC at pH 6.0. For example, the binding of ryanodine was completely inhibited when SR membranes were incubated with 1.0 mM DEPC at 25 °C for 10 min (Figure 1a). Similar results were obtained with the purified ryanodine receptor (Figure 1b). However, as shown in Figure 1(b), the presence of phospholipids (5 mg/ml) in the modification medium strongly decreased inactivation of ryanodine binding by DEPC. This may result from an interaction of DEPC with phospholipids.

Figure 2 shows the time course of inactivation of ryanodine binding at different DEPC concentrations. The inactivation of ryanodine binding by DEPC appears to be pseudo-first-order, with a $t_{\frac{1}{2}}$ of 3 min in the presence of 1.2 mM DEPC. Since the inactivation is pseudo-first-order, either a single group or two or more exactly equivalent groups are probably involved in the inactivation of ryanodine binding by DEPC. This is also demonstrated in Figure 2(b), where the data were replotted as described by Levy et al. [22]. The plot of log $t_{\frac{1}{2}}$ (the time required for 50% inhibition of ryanodine binding) as a function of log [DEPC] yields a straight line with a slope of 0.99 (Figure 2b). These results are consistent with DEPC modification of a single class of site and that this modification eliminates ryanodine binding.

Figure 3 shows the effect of NaCl on the inactivation of ryanodine binding by DEPC when present in the modification medium. NaCl decreased the inhibition of ryanodine binding by the reagent. This protection by NaCl may suggest that the protein conformation(s) stabilized by high salt concentration is less sensitive to DEPC.

Inactivation of ryanodine binding by DEPC as a function of the pH of the modification medium is shown in Figure 4. As shown, the degree of inactivation by 0.3 or 0.6 mM DEPC is markedly decreased at alkaline pH, where the pK_a of the reactive group residue appears to be less than 6.2. These results suggest that inactivation of ryanodine binding by the reagent is due to modification of histidyl residue(s). It has been shown that in phosphate buffer, pH 6.5, DEPC reacts with histidine residues relatively specifically [16]. It has also been shown, however, that a thiol such as *N*-acetylcysteine also reacts with DEPC under slightly acidic conditions to yield a somewhat unstable product absorbing at 240 nm, but only in carboxylate buffers [23]. Therefore, the effect of preincubation of SR with DEPC in different buffers (Mes, Mops, phosphate) on ryanodine binding was tested. A similar degree of inhibition of ryanodine binding by DEPC was obtained when the modification was carried out in the presence of the different buffers at the same pH.

The specific modification of histidine by DEPC could be

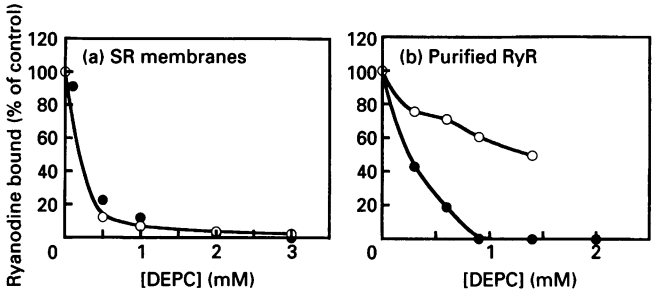

Figure 1 Inactivation of ryanodine binding to junctional SR membranes and purified ryanodine receptor by modification with DEPC

SR membranes (1 mg/ml) (**a**) or purified ryanodine receptor (14 μg/ml) (**b**) were incubated with the indicated concentration of DEPC in 50 mM Mes, pH 6.0. After 10 min incubation at 25 °C, histidine (pH 7.0) was added to a final concentration of 20 mM. Samples were assayed for ryanodine binding, as described in the Materials and methods section. In (**a**), ○ and ● indicate two different experiments of a total of 10 similar experiments, and in (**b**) ○ indicates the presence of phospholipids (5 mg/ml) during the incubation with DEPC. Control activities (100%) were (**a**) 6.5 (○) and 7.0 (●) and (**b**) 247 (●) and 395 (○) pmol/mg of protein for SR and purified ryanodine receptor (RyR) respectively.

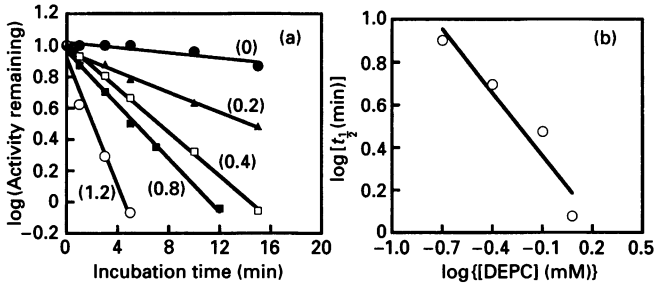

Figure 2 Time course of DEPC inhibition of ryanodine binding

SR membranes (1.0 mg/ml) were incubated without (●) or with (○, ▲, ■, □) different concentrations of DEPC as described in the Materials and methods section. The reaction was terminated at various times by addition of histidine, pH 7.0, to a final concentration of 20 mM. Samples (50 μl) were assayed for ryanodine binding as described in the Materials and methods section. The half-time for inactivation ($t_{\frac{1}{2}}$) was calculated for each DEPC concentration (**a**). A double-logarithmic plot of $t_{\frac{1}{2}}$ of inactivation against DEPC concentration is shown in (**b**). The slope value, $n = 0.99$ ($r = 0.96$). The DEPC concentrations used were: 0.2 (▲), 0.4 (□), 0.8 (■) and 1.2 mM (○). Control activity (100%) = 10 pmol of ryanodine bound/mg of protein.

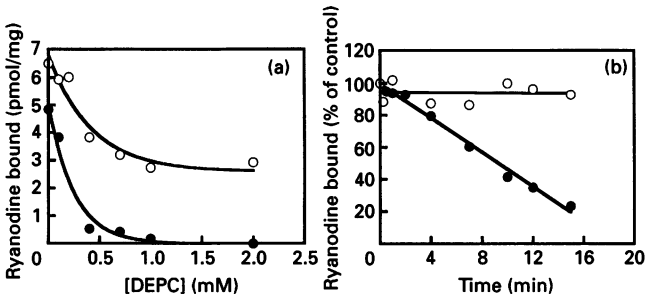

Figure 3 Effect of NaCl on the inactivation by DEPC of ryanodine binding

In (**a**), SR membranes (1 mg/ml) were incubated for 10 min in 50 mM Mes, pH 6.0, with the indicated concentration of DEPC in the absence (●) or the presence (○) of 0.5 M NaCl. In (**b**), SR membranes (1 mg/ml) were incubated for the indicated time with and without 0.4 mM DEPC in 50 mM Mes, pH 6.0, in the absence (●) or the presence (○) of 0.5 M NaCl. Control activity (100%) was 8.0 pmol/mg of protein. Ryanodine binding was assayed as described in the Materials and methods section.

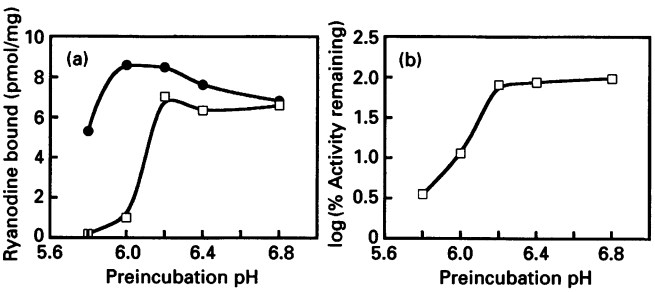

Figure 4 pH-dependence of ryanodine-binding inactivation by DEPC

SR membranes (1.0 mg/ml) were incubated without (●) or with DEPC (0.6 mM; □) at different pH values as described in the Materials and methods section. The buffers used were: 25 mM Mes for pH 5.8, 6.0, 6.2, 6.4 and 6.8, and 20 mM Mops for pH 6.8 and 7.5. The log of percentage of activity remaining versus incubation pH is presented in (**b**).

Table 1 Effect of hydroxylamine treatment on the inhibition of ryanodine binding by DEPC modification of SR membranes

SR membranes were incubated without or with 0.4 mM DEPC for 10 min (first preincubation), and then hydroxylamine from a 0.5 M stock solution, pH 7.0, was added to the indicated final concentrations. After 20 min at 22 °C (second incubation), the samples were centrifuged and the pellets were resuspended in 0.25 M sucrose/10 mM Tricine (pH 8.0)/1 mM histidine and assayed for ryanodine binding and protein concentration as described in the Materials and methods section. Control activity (100%) was 2.2 pmol of ryanodine bound/mg of protein. This activity is lower than in the other experiments, because of the long period of incubation at pH 6.0.

Preincubation conditions		Ryanodine bound
First	Second	(% of control)
1. Mes, pH 6.0	–	100
2. Mes, pH 6.0	NH_2OH (10 mM)	100
3. Mes, pH 6.0	NH_2OH (40 mM)	137
4. Mes, pH 6.0 + DEPC	–	27
5. Mes, pH 6.0 + DEPC	NH_2OH (10 mM)	101
6. Mes, pH 6.0 + DEPC	NH_2OH (40 mM)	126

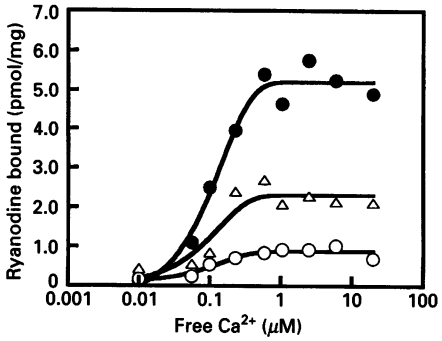

Figure 5 Ca^{2+}-dependency of ryanodine binding by unmodified and DEPC-modified membranes

Unmodified (●) and DEPC-modified (△, 0.3 mM; ○, 0.6 mM), membranes were assayed for ryanodine binding in the presence of 0.2 mM EGTA and the indicated free Ca^{2+} concentrations. Free Ca^{2+} concentration was based on the EGTA association constant reported by Fabiato [31].

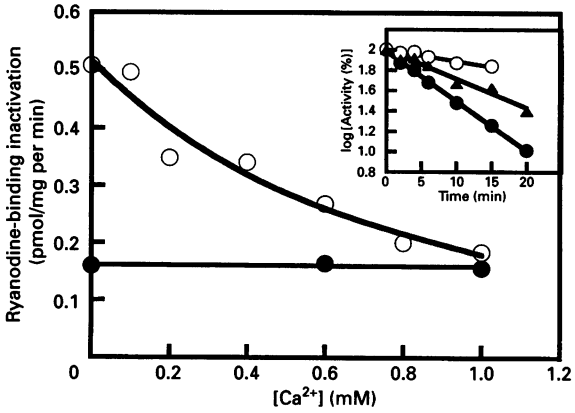

Figure 6 Effect of $CaCl_2$ on the inactivation by DEPC of ryanodine binding

SR membranes were incubated without or with DEPC (0.6 mM) in the absence and the presence of the indicated concentrations of $CaCl_2$ for different times as described in Figure 1. The apparent first-order inactivation rate of ryanodine binding in the absence (●) and the presence (○) of different concentrations of Ca^{2+} is shown. The Ca^{2+} concentrations used were 0.1, 0.2, 0.4, 0.6 and 1 mM. Inset shows representative time courses of ryanodine-binding inactivation with DEPC in the absence (●) and the presence of 0.2 mM (▲) and 1 mM (○) $CaCl_2$. Ryanodine binding was assayed as described in the Materials and methods section.

supported by reversal of the inhibition of ryanodine binding by hydroxylamine [16]. Since hydroxylamine by itself inhibited ryanodine binding (results not shown), the untreated and DEPC-treated membranes, incubated with hydroxylamine, were washed with sucrose buffer before the assay of ryanodine binding. Table 1 shows that hydroxylamine treatment of DEPC-modified membranes reverses the inhibition of ryanodine binding.

The following experiments demonstrate that the modification by DEPC did not change the Ca^{2+}-dependency of the ryanodine binding, nor the binding affinity of the receptor for ryanodine. As has been shown previously [5,9,10], ryanodine binding is Ca^{2+}-dependent (Figure 5). Under the conditions used (1.0 M NaCl and pH 7.4), the Ca^{2+}-dependency of binding by unmodified and DEPC-modified membranes was similar. The concentration of Ca^{2+} giving half-maximal stimulation (C_{50}) of ryanodine binding was 150–200 nM Ca^{2+} ($n = 2$) in the unmodified or DEPC-modified membranes. However, we found that DEPC modification decreased the total ryanodine-binding sites

Table 2 Effect of bivalent cations on the inactivation of ryanodine binding by DEPC

In Expt. I, SR membranes (1 mg/ml) were incubated at 25 °C in 50 mM Mes, pH 6.0, without (control) and with DEPC (0.4 mM) and in the presence or the absence of the indicated bivalent cations. After 10 min of incubation, EDTA and $CaCl_2$ were added to decrease the free metal concentration and to bring the Ca^{2+} concentration to about 50 μM, and then ryanodine binding was assayed. In Expt. II, ryanodine binding was assayed in the absence and the presence of different concentrations of the indicated bivalent cations, as described in the Materials and methods section, except that the NaCl concentration was 0.2 M. The results are of two different experiments from which the IC_{50} and Hill coefficient (h) were obtained. The enhancement of ryanodine binding in the control by preincubation with $CaCl_2$ or $MnCl_2$ is probably due to their protection against some inactivation of ryanodine binding caused by the incubation of SR at acidic pH.

	[^{3}H]Ryanodine bound		
	(pmol/mg of protein)		(% of control)
Bivalent cation present in:	Control	DEPC	
Expt. I: Preincubation			
None	8.7	1.2	15
$CaCl_2$ (0.5 mM)	12	11.2	93
$CaCl_2$ (1.0 mM)	8.4	7.9	94
$BaCl_2$ (0.5 mM)	8.4	4.1	49
$MgCl_2$ (0.5 mM)	7.8	5.3	68
$MgCl_2$ (1.0 mM)	4.8	5.2	108
$MnCl_2$ (0.5 mM)	12.2	5.2	43
$MnCl_2$ (1.0 mM)	8.5	4.4	52
$MnCl_2$ (3.0 mM)	7.0	4.9	70
Expt. II: Ryanodine binding		IC_{50} (mM)	h
$CaCl_2$		0.6	0.90
$MgCl_2$		0.5	1.03
$BaCl_2$		0.25	1.09
$MnCl_2$		0.1	0.95

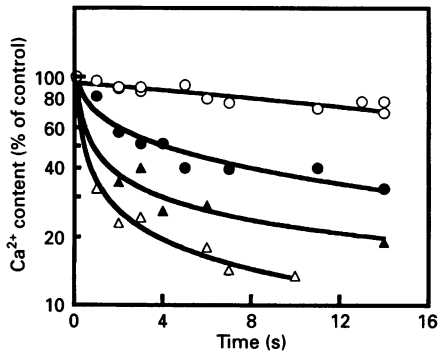

Figure 7 DEPC-induced Ca^{2+} efflux from passively loaded SR vesicles

SR membranes untreated (○) or treated with 0.4 mM (●) or 0.8 mM (▲) DEPC were loaded with $^{45}CaCl_2$ and assayed for Ca^{2+} efflux as described in the Materials and methods section. (△) indicates control membranes loaded with $^{45}CaCl_2$ for 90 min and then incubated with 0.5 μM ryanodine for 30 min, before the assay of Ca^{2+} efflux. Ryanodine-binding activities were 5.2, 2.9 and 2.1 pmol/mg of protein for controls and membranes treated with 0.4 mM or 0.8 mM DEPC respectively. Ca^{2+} content of the loaded vesicles (100%) was 8 nmol/mg of protein.

without directly affecting the binding affinity (results not shown). Thus the C_{50} for Ca^{2+} in both SR preparations is for the unmodified ryanodine receptor.

Figure 6 shows that the presence of $CaCl_2$ during the modification of SR membranes with DEPC protects against the inactivation of ryanodine binding by DEPC. Ca^{2+} decreases the rate of inactivation of ryanodine binding by DEPC (Figure 6). In Figure 6 a plot of the apparent first-order inactivation rate of ryanodine binding by DEPC in the absence and the presence of different Ca^{2+} concentrations is presented. Ca^{2+} affords complete protection, with half-maximal protection obtained at 0.4 mM.

Table 2 shows that not only Ca^{2+}, but also Mg^{2+}, Ba^{2+} and Mn^{2+}, all protected against the inactivation of ryanodine binding by DEPC. Table 2 shows that Ca^{2+} and other bivalent cations, when present in the assay medium, inhibited the binding of ryanodine (Expt. II). The results show a similar order of effectiveness of the bivalent cations in protection against inactivation of ryanodine binding by DEPC and in inhibition of ryanodine binding (Table 2). In these experiments we used 0.2 M NaCl, since high salt concentration (1 M) increases by about 2-fold the cation concentration required for 50% inhibition of ryanodine binding (results not shown).

The effect of DEPC modification on Ca^{2+}-permeability of the SR membranes is shown in Figure 7. As shown, DEPC treatment activates Ca^{2+} efflux from SR vesicles. SR modification by increased DEPC concentration enhances both the degree of Ca^{2+} efflux and the degree of inhibition of ryanodine binding. Similar results were reported previously with higher DEPC concentrations and actively loaded SR vesicles [17]. We have not tested, however, whether the membrane permeability for other ions has not been changed.

The number of histidyl residues reacting with DEPC per molecule of the purified ryanodine receptor could not be determined, because of the relatively low molar absorption coefficient of the *N*-carboethoxyhistidyl derivative (3200 $M^{-1} \cdot cm^{-1}$) [24], and the high molecular mass of the ryanodine receptor.

DISCUSSION

The inactivation of ryanodine binding by DEPC shown here amply substantiates the role of histidyl residues in the binding of ryanodine to its receptor. Our results indicate that modification of histidyl residues with DEPC results in a dose-dependent decrease in specific ryanodine binding to both the membrane-bound and the soluble, purified, ryanodine receptor (Figure 1). This decrease is due to a decrease in the number of ryanodine-binding sites, and may suggest that a histidyl residue modified by DEPC is closely associated with the ryanodine-binding sites. Studies on the relationship between the ryanodine-binding site and the DEPC-modification site are difficult, since ryanodine binds very tightly to its receptor, making meaningful competition experiments between ryanodine and DEPC impractical. However, since Ca^{2+} protects against the inactivation of ryanodine binding by DEPC, it is possible that the alkylated histidyl residue is involved either in Ca^{2+} binding to the high- or the low-affinity sites, or in conformational changes that may occur upon Ca^{2+} binding and which are involved in activation or inactivation of ryanodine binding. The suggestion that the covalent modification of the ryanodine receptor by DEPC occurs at, or affects, the Ca^{2+}-binding site(s) is discussed below.

DEPC reacts with many nucleophiles at pH > 7.0, but it is specific for the imidazole ring of histidine at pH 6.0 [16]. Although it has not been demonstrated that the effects of DEPC are due to modification of ryanodine-receptor histidyl residue(s), several indirect lines of evidence are consistent with the notion that DEPC alkylates a histidine moiety. (*a*) DEPC treatment at pH $\leq$ 6.0 specifically modifies histidyl residues in a number of soluble proteins [16]. The pH-dependence of DEPC inactivation

(Figure 4) suggests the involvement of reactive group(s) with a pK_a value of about 6.2, which may represent the pK_a of the reactive imidazole group. (*b*) Hydroxylamine displaces the ethoxycarbonyl moiety from the imidazole nitrogen of histidine [16], and regenerates the ryanodine-binding activity of the DEPC-modified membranes (Table 1). (*c*) It has been shown that exposure of the SR vesicles to light in the presence of the dye Rose Bengal, an operation that leads to photo-oxidation of histidyl residues [25], caused an activation of Ca^{2+} release and inhibition of ryanodine binding [17], as well as an activation of reconstituted Ca^{2+}-release channels [26]. It has been shown also that illumination of Ca^{2+}-release channels, isolated from sheep cardiac SR, in the presence of Rose Bengal resulted in the loss of ryanodine binding and the appearance of channels with sub-conductance states [27].

Of unique interest is the effect of Ca^{2+} and other bivalent cations on the action of DEPC on ryanodine receptor. Ca^{2+} diminished the inactivation of ryanodine binding by DEPC (Figure 6). Thus a role for histidine residue(s) in the binding of Ca^{2+} might be expected, in view of the protection against the DEPC inactivation of ryanodine binding by Ca^{2+} and several other bivalent cations. Two types of Ca^{2+} regulatory sites have been defined: a lower-affinity inhibitory site, and a higher-affinity binding site, which activates Ca^{2+} release [2,11,12] and ryanodine binding [5,9,10]. The effect of Ca^{2+} in both the protection against inactivation of ryanodine binding with DEPC and inhibition of ryanodine binding can be mimicked by other bivalent cations such as Ba^{2+}, Mg^{2+} and Mn^{2+}. These cations do not activate Ca^{2+} release or ryanodine binding [28]. The activation by Ca^{2+} is proposed as being due to the occupation of the high-affinity binding sites, with a Hill coefficient of about 2.0. The inhibition of ryanodine binding by these cations, together with their Hill coefficient of about 1.0 obtained for the inhibitory effect (Table 2), suggest that these cations probably occupied the low-affinity binding site(s) responsible for inactivation of Ca^{2+}-release-channel activity. Thus we suggest that the protection afforded by the bivalent cations against the inactivation of ryanodine binding by DEPC is due to the occupation of the low-affinity binding site by these cations. Protection against DEPC modification by Ca^{2+} or other bivalent cations suggests that the binding of Ca^{2+} results in a change in the environment of the histidyl residue attacked by DEPC. The protection is either directly due to steric hindrance, i.e., Ca^{2+} and DEPC bind at the same site in the ryanodine receptor, or indirectly due to a Ca^{2+}-induced change in protein conformation, which alters the reactivity of the histidyl residue. The observation that Ca^{2+} efflux from Rose-Bengal-modified membranes was not inhibited by millimolar Mg^{2+}[17], assuming that it modifies the same histidyl residue, supports our suggestion that the modified histidyl residue is located in the inhibitory, low-affinity, bivalent-cation-binding site(s).

In several cation-binding proteins the histidyl residues are directly involved in the ligation of cations to form important hydrogen bonds with carboxylic groups. For example, Zn in the active site of human carbonic anhydrase is tetrahedrally co-ordinated by three histidine side chains and a H_2O molecule [29], and Cu ions in cytochrome oxidase are bonded to a histidine side chain [30].

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In conclusion, our results show that DEPC modification of SR membranes results in diminished ryanodine binding and opening of the Ca^{2+}-release channel. It seems possible that this modification stabilizes an open protein conformational state that does not bind ryanodine. If, as suggested, DEPC modifies the low-affinity Ca^{2+}-binding site(s) on the ryanodine receptor, it may serve as a useful probe to localize these sites, and to help to elucidate their involvement in the control of channel gating.

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