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The interaction of spermine with the ryanodine receptor from skeletal muscle

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The effect of polyamines on ryanodine binding activity of junctional sarcoplasmic reticulum membranes is described. Spermine stimulated the binding of ryanodine to its receptor up to 5-fold, with half-maximal stimulation obtained with 3.5 mM. Spermidine and putrescine also stimulated ryanodine binding, but they were about 12-fold less potent. The degree of stimulation is dependent on the NaCl concentration present in the assay medium. Spermine has no effect on the Ca^{2+}-dependency of ryanodine binding but it increases the ryanodine binding affinity (K_d) by about 5.6-fold. Both the rate of ryanodine association with, and dissociation from, its binding site were affected by spermine. Spermine also stimulates the photoaffinity labelling by 3-*O*-(4-benzoyl)benzoyl[α-^{32}P]ATP ([α-^{32}P]BzATP) of the ryanodine receptor, increasing the BzATP binding affinity. We suggest that the stimulatory effect of spermine on ryanodine binding is due to its specific interaction with the ryanodine receptor. This spermine interaction enabled us to develop a new, one-step, fast and with high yield method for the purification of ryanodine receptor (Shoshan-Barmatz, V. and Zarka, A. (1992) Biochem. J. 284, in press).

Introduction

The factors that regulate Ca^{2+} release from sarcoplasmic reticulum (SR) *in vitro* have been extensively studied [1–3], but the actual mechanism that triggers the release *in vivo* remains unknown. It is now well accepted that the ryanodine receptor is a key component in the excitation-contraction coupling. Ryanodine binds specifically, and with high affinity, to a receptor in the junctional regions of the SR membrane network [4,5]. The ryanodine receptor protein has been isolated from rabbit skeletal and cardiac muscle SR by a number of groups using a range of solubilization and separation techniques [6–13]. Purification of the receptor protein has permitted detailed structural, functional and biochemical characterization [7–11,14,15]. The high-affinity ryanodine binding complex consists of a 30 S homotetramer and is equivalent to the 'feet' structures and it forms a channel following its incorporation into planar phospholipid bilayers.

Spermine is one of the polyamines, polycationic metabolites in prokaryotic and eukaryotic cells. Their function is not yet clear; it has been suggested that they are involved in cell growth, cell differentiation and in the regulation of biosynthetic pathways [16]. The interaction of polyamines with membranes has been suggested by several reporters [17–19]. Among these interactions are their ability to modify synaptic transmission [17] and the activity of Na, K-ATPase [18,19].

The functions of polyamines in the maintenance of Ca^{2+} homeostasis have also been reported [20–26]. Spermine was found to stimulate Ca^{2+} uptake by mitochondria [20,21] and both spermine and spermidine in the range of micromolar concentrations, were also found to block Ca^{2+} release from SR induced by various drugs [22]. Several other studies [23–26] suggest that polyamines serve as intracellular messengers which provoke an increase in cytoplasmic Ca^{2+} concentration.

In this communication we describe the interaction of spermine with the ryanodine receptor.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis (hydroxymethyl)-ethyl]glycine, Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; BzATP, 3'-*O*-(4 benzoyl)benzoyladenosine triphosphate.

Materials and Methods

Materials

ATP, EGTA, EDTA, Tris, Tricine, Mops, spermine, spermidine and putrescine were obtained from Sigma Chemical Co. [α-^{32}P]BzATP was synthesized and purified as described by Williams and Coleman [27]. [α-^{32}P]ATP was obtained from Amersham, and [^{3}H]ryanodine (60 Ci/mmol) was purchased from New England Nuclear. Unlabelled ryanodine was purchased from Calbiochem, and ruthenium red from Fluka.

Membrane preparations

Junctional SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito et al. [28]. In most of the experiments the fraction R_4 was used. The membranes were suspended to a final concentration of about 25 mg protein/ml in a buffer containing 0.25 M sucrose, 10 mM Tricine (pH 8.0) and 1 mM histidine and stored at −70°C. Protein concentration was determined by the method of Lowry et al. [29].

[^{3}H]Ryanodine binding

Unless otherwise indicated, junctional SR membranes (final concentration of 0.5 mg/ml) were incubated with 20 nM [^{3}H]ryanodine (spec. act. 30 Ci/mmol), in a standard binding solution containing 0.2 M NaCl, 25 mM Mops (pH 7.4) and 50 μM $CaCl_2$, for 1 to 2 h at 37°C. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of protein aliquots (50 μg) 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 retained radioactivity was determined by liquid scintillation counting techniques. Non specific binding was determined in the presence of 25 μM unlabelled ryanodine.

Photoaffinity labelling of ryanodine receptor by [α-^{32}P]BzATP

SR membranes (1 mg/ml) were irradiated in the presence of 2 μM of [α-^{32}P]BzATP ($4 \cdot 10^6$ cpm/nmol) in 50 μl of 25 mM Mops (pH 7.4) and 0.2 M NaCl and 1 mM EDTA. The radiation from 15W, UV lamp (G15T8) was for 4 min at a distance of 5 cm. The irradiated membranes were immediately diluted 1:1 with a sample buffer containing 125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol 4% (w/v) SDS and 2% (v/v) β-mercaptoethanol and were incubated for 3 min at 100°C. The samples were analyzed by SDS-PAGE as described below. Autoradiography of the dried gels was carried out using Kodak X-omat film. Quantitative analysis of the labelled protein bands was performed by densitometric scanning of the autoradiogram using a Hoefer GS 300 scanning densitometer.

Gel electrophoresis

The analysis of protein profile by SDS-polyacrylamide slab gel electrophoresis was performed according to the discontinuous buffer system of Laemmli [30] in 1.5 mm thick slab gels with 4–15% acrylamide. Gels were stained with Coomassie brilliant blue.

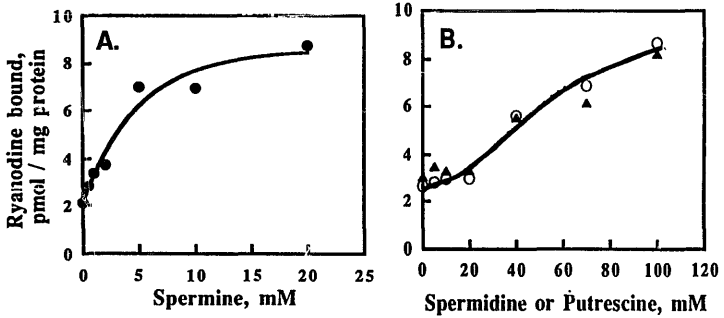

Fig. 1. Stimulation of ryanodine binding to the junctional SR membranes produced by polyamines. SR membranes (0.5 mg/ml) were assayed for ryanodine binding in the absence and the presence of the indicated concentration of spermine (●), spermidine (○) and putrescine (▲) as described in Materials and Methods. This is one of four identical experiments.

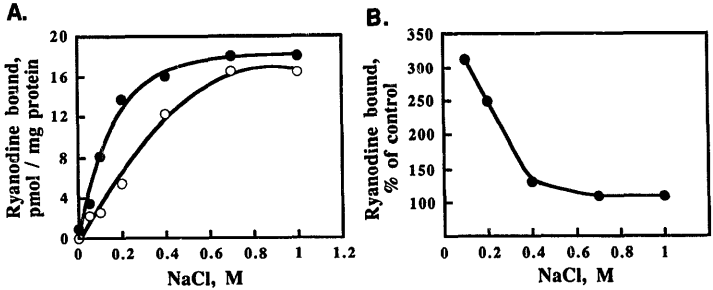

Fig. 2. Effect of NaCl concentration on ryanodine binding and on the degree of stimulation of ryanodine binding by spermine. SR membranes (0.5 mg/ml) were assayed for ryanodine binding in the absence (○) and the presence (●) of 10 mM spermine as described under Materials and Methods except that the NaCl concentration was varied. The data in A are presented as percentage of stimulation of ryanodine binding by spermine in B. This is one of two identical experiments.

Results

The effect of the aliphatic polyamines; spermine, spermidine and putrescine on [^{3}H]ryanodine binding to junctional SR membranes is shown in Fig. 1. A stimulation of about 4-fold was obtained with 15 mM of spermine and 100 mM of spermidine or putrescine. Furthermore, half-maximal stimulation was obtained with 3.5 mM of spermine compared to 40 mM of spermidine or putrescine. These results are listed in Table I along with the polyamine structure and their charge. As will be discussed below, it seems unlikely that the difference in the polyamine potency is attributable to differences in the respective number of charges.

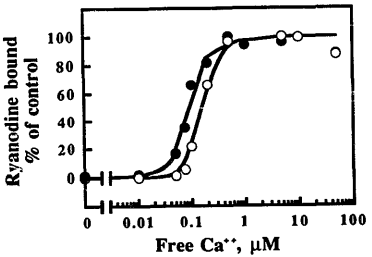

Fig. 3. Ca^{2+}-dependency of ryanodine binding in the absence and the presence of spermine. SR membrane (0.5 mg/ml) were assayed for ryanodine binding in the absence (○) and in the presence (●) of 10 mM spermine, and in the presence of 0.2 mM EGTA and the indicated free Ca^{2+} concentrations. Free Ca^{2+} concentration was calculated with a computer program using the apparent binding constant at pH 7.4 of $2.99 \cdot 10^7$ M^{-1}. Control activity (100%) = 1.86 and 5.6 pmol/mg protein in the absence and the presence of spermine, respectively.

The degree of stimulation of ryanodine binding by spermine is dependent on the NaCl concentration present in the ryanodine binding medium, the stimulation decreasing as the NaCl concentration increased (Fig. 2). This is not surprising since high NaCl concentrations stimulate ryanodine binding [7], thus in the presence of high NaCl concentration, the binding is close to its maximal level.

The effects of spermine on the [^{3}H]ryanodine binding properties of the receptor were determined by testing its effect on the Ca^{2+}-dependence of ryanodine binding (Fig. 3), on the affinity of ryanodine for its high-affinity site (Fig. 4), and on the rate of ryanodine association with and dissociation from its binding site (Fig. 5).

As it has been shown previously [31] ryanodine binding is Ca^{2+}-dependent (Fig. 3). Under the conditions adopted (0.2M NaCl and pH 7.4), the Ca^{2+}-dependency obtained in the absence or the presence of 10 mM spermine was similar. Half-maximal stimulation

TABLE I

Effect of different polyamines on ryanodine binding

Experimental conditions are as described in Fig. 1. C_{50}, the concentration required for half-maximal stimulation of ryanodine binding.

Polyamine	Formula	C_{50} (mM)	No. of charges
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	3.47	+4
Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	40.0	+3
Putrescine	$NH_2(CH_2)_3NH_2$	40.0	+2

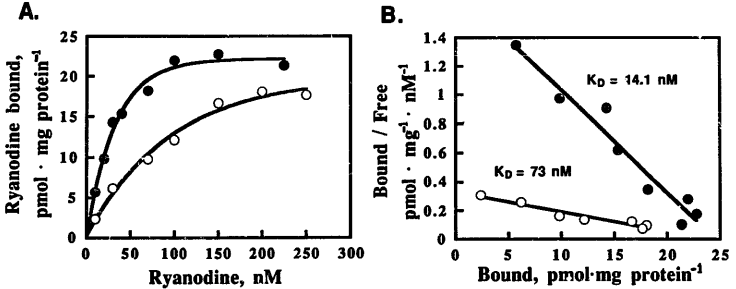

Fig. 4. The influence of spermine on the affinity of the ryanodine binding site. [^{3}H]ryanodine binding to SR membranes (1 mg/ml) was assayed as described in Materials and Methods in the presence of 0.2 M NaCl with (●) or (○) without 10 mM spermine. [^{3}H]ryanodine was diluted 1:1 or 1:4 with unlabelled ryanodine. Scatchard plots analysis are shown in B. The calculated B_{max} were 24.1 and 24.6 pmol/mg protein in the absence and the presence of spermine, respectively.

of ryanodine binding by Ca^{2+} was obtained with 100 to 200 nM ($n = 2$) in the absence or the presence of spermine.

Fig. 4 and Fig. 5 exemplify the effects of spermine on equilibrium binding parameters.

Fig. 4 shows the binding of [^{3}H]ryanodine as a function of its concentration in the absence and the presence of 10 mM spermine. Scatchard plots analysis of ryanodine binding (at 0.2 M NaCl) in the presence and the absence of 10 mM spermine indicates that spermine increased the apparent binding affinity (K_d) about 5.6-fold, from 81 ± 8.7 ($n = 5$) to 14.5 ± 0.6 nM ($n = 3$), similar to the K_d obtained in the presence of 1.0 M NaCl (data not shown). In contrast, similar B_{max} values were obtained with 0.2 M NaCl ± 10 mM spermine or high NaCl concentrations ($B_{max} = 21.7 \pm 4.2$

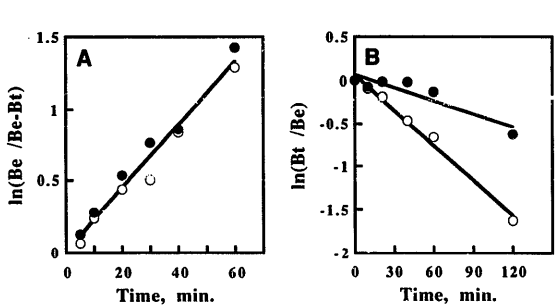

Fig. 5. Effect of spermine on equilibrium binding of [^{3}H]ryanodine and dissociation kinetics. In A, SR membranes (1 mg/ml) were incubated with 20 nM ryanodine as in Fig. 1, in the absence (○) and the presence (●) of 10 mM spermine. After the indicated time, aliquots were assayed for bound ryanodine (B_t) The maximal amount of ryanodine bound at the plateau (B_e) was 3.2 and 10.5 pmol/mg protein in the absence and the presence of spermine, respectively. In B, SR membranes were incubated with 20 nM ryanodine as in Fig. 1. After 2 h aliquots were assayed for bound ryanodine ($B_e = 3.2$ pmol/mg protein). Dissociation of bound ryanodine was initiated by 100-fold dilution with the binding medium (without ryanodine) with (●) or without (○) 10 mM spermine. The residual bound ryanodine at the indicated time (B_t) was determined. The calculated dissociation constant (k_{-1}) was 0.0136 and 0.005 min^{-1} in the absence and the presence of spermine, respectively. The rate constant for association (k_1) was calculated from the k_{obs} (0.022 min^{-1}) as described previously [46] using the following equation: $k_{obs} = k_1[L][R]/Be$), where [L] = ryanodine concentration, [R] = ryanodine receptor concentration = B_{max} (25.4 pmol/mg protein).

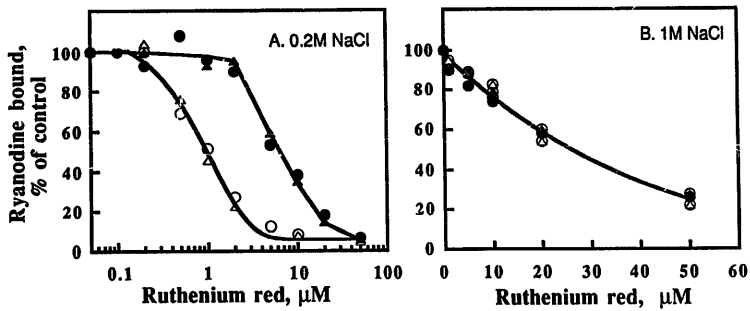

Fig. 6. The effect of spermine and of high NaCl concentration on the inhibition of ryanodine binding by RR. [^{3}H]Ryanodine binding was assayed in 0.2 M (A) or 1 M NaCl (B) in the presence (▲, ●) and the absence of 10 mM spermine (○, △) and/or the indicated concentration of RR. The triangle and circle symbols represent two different experiments. Control activities (100%) were in A: 2.3 and 8.0 and B: 12.5 and 13.5 pmol ryanodine bound/mg protein in the absence and the presence of spermine, respectively.

pmol/mg protein, ($n = 8$). These results suggest that the increase in ryanodine binding produced by spermine (at low NaCl concentrations) is attributable to increased receptor affinity for the ligand.

The effect of spermine on the association of ryanodine with its receptor is shown in Fig. 5A. Spermine increased ryanodine binding by 3.3-fold with no effect on the observed association rate constant (k_{obs}). [^{3}H]Ryanodine associates with its binding sites with a $k_{obs} = 0.0233 \pm 0.0063$ min^{-1} ($n = 2$). The calculated pesudo-first-order association rate constants (k_1) obtained in the absence and the presence of spermine were $1.5 \cdot 10^5 \pm 1.2 \cdot 10^4$ and $5.41 \cdot 10^5 \pm 8.4 \cdot 10^4$ M^{-1} min^{-1} ($n = 2$). When the dissociation of ryanodine receptor radioligand complex at equilibrium was initiated by 100-fold dilution, the dissociation was monophasic ($k_{-1} = 0.011 \pm 0.0024$ min^{-1}, $n = 3$). Spermine decreased the dissociation of ryanodine from its binding site by 2.5-fold ($k_{-1} = 0.00436 \pm 0.0006$ min^{-1}, $n = 3$). The K_d based on the calculated association and dissociation constants are 73 nM and 8.0 nM in the absence and the presence of spermine, respectively, which are close to the K_d calculated from saturation experiments (Fig. 4).

Ruthenium red (RR) is a polycationic dye which, inhibits ryanodine binding at micromolar concentrations [5,31] as well as Ca^{2+} release from isolated vesicles [32] and the activity of single Ca^{2+} release channels incorporated into lipid bilayers [33]. The relationship between the RR and spermine-binding sites is demonstrated in Fig. 6. At 0.2 M NaCl, RR inhibits ryanodine binding with half-maximal inhibition occurring at about 1 and 6 μM in the absence and the presence of 10 mM spermine, respectively. However, increasing NaCl concentration to 1 M, produced an increase in the concentration of RR required for half-maximal inhibition, either in the absence or the presence of 10 mM spermine to 26 μM (Fig. 6). These results do not reflect a simple competition between RR and spermine.

The effect of spermine on the ryanodine receptor properties was also examined by testing its effect on the regulatory nucleotide binding site. Fig. 7 shows the effect of spermine on the labelling of the receptor ATP binding site by the photoaffinity analog of ATP, 3′-*O*-(4-benzoyl)benzoyladenosine triphosphate (BzATP). Spermine stimulates the labelling by [α-^{32}P]BzATP of the ryanodine receptor. Quantitative analysis carried out by densitometric scanning of the autoradiogram and determination of the peak-area of the ryanodine receptor indicate that the stimulation of BzATP binding by spermine is a result of spermine increasing receptor affinity for BzATP (Fig. 7B).

Discussion

In this study, the effect of spermine on ryanodine binding activity of junctional SR is described. Spermine, stimulates the binding of ryanodine to its receptor up to 5-fold. Stimulation of ryanodine binding was also obtained with spermidine and putrescine but they are much less potent (Fig. 1 and Table I). This enhancement in ryanodine binding is dependent on the assay conditions (Fig. 2) and it is due to an increase in the receptor affinity for the ligand by affecting both the rate constants for association and dissociation. In the presence of 0.2 M NaCl, where the binding is not at the maximum level, spermine increases the binding

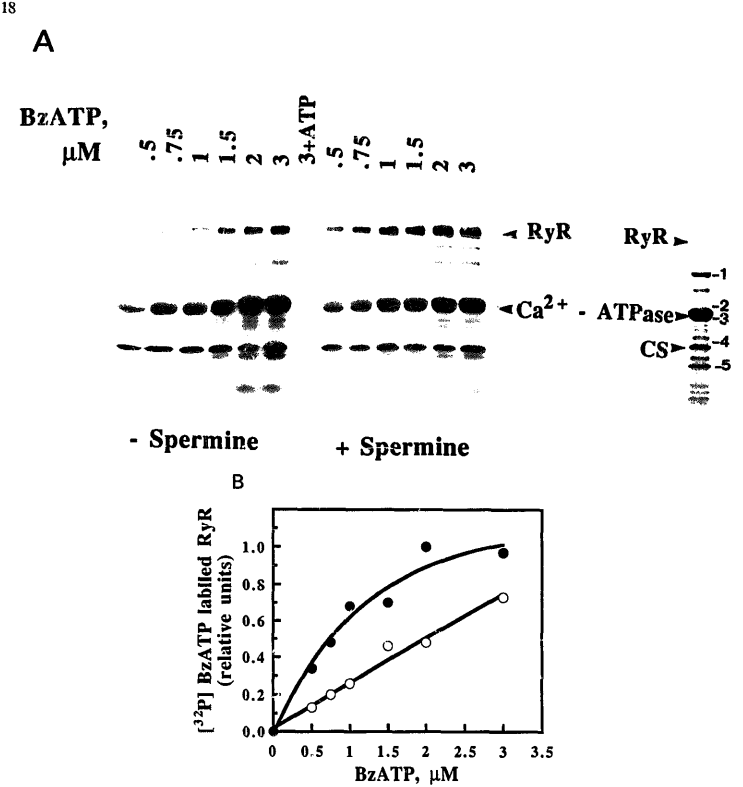

Fig. 7. Effect of spermine on the labeling of SR proteins by [α-^{32}P]BzATP. SR membranes were incubated with the indicated concentration of [α-^{32}P]BzATP in the absence (○) and the presence (●) of 0.5 mM spermine, and in the presence of 5 mM ATP (lane 7) The samples were irradiated for 4 min with UV light and prepared for SDS-PAGE as described in Materials and Methods. The autoradiogram and the Coomassie blue stained sample (the lane on the right) are shown in A and the quantitative analysis of the autoradiogram is shown in B. RyR, ryanodine receptor, CS, calsequestrin. Molecular weight standards, labeled on the right (1 to 5), were: myosin, 200000; β-galactosidase, 116000; phosphorylase b, 97400; bovine serum albumin, 66200 and ovalbumin 42700 (Bio-Rad).

affinity by over 5-fold, to the affinity observed in the presence of 1.0 M NaCl, suggesting that like high ionic strength [6], spermine maintains optimal receptor site conformation. The receptor conformation maintained by spermine is also optimal for the binding of ATP as followed by the binding of the photoreactive analog; [^{32}P]BzATP (Fig. 7).

Interestingly, ruthenium red, another polycationic species, in contrast to spermine, completely inhibits the binding of ryanodine both in the absence or the presence of spermine (Fig. 6).

The effect of the polyamines on ryanodine binding can be explained by considering them as only polyvalent cations. Although the effectiveness of the

polyamines is not defined exclusively by the number of positive charges (Table I), electrostatic interactions should be considered as one of the parameters in their mechanism of action. Because the charges are distributed at fixed distances along the flexible carbon chain, they are able to bridge critical distance (e.g. 1.6 and 1.1 nm for spermine and spermidine, respectively [16]). Thus, these features allow specific interactions which are not shared by inorganic cations such as Mg^{2+} and/or Ca^{2+}. Accordingly, a number of possible mechanisms may account for the effect of spermine on the ryanodine receptor activity. One of these relates directly to its polybasic nature; namely it has a particularly high affinity for polyanions. Since spermine will be in the protonated form at pH 7.4 with four positively charged moieties [34], there is an appreciable chance for bridging protein-protein carboxyl groups. Ryanodine receptor has a highly acidic nature [35,36] with isoelectric point of 3.7 [37]. Because of the abundance of negatively charged residues in the receptor protein, spermine presumably is able to associate non-specifically with these negatively charged groups. In other words, the modulation of ryanodine binding by spermine is due to electrostatic interactions provided by spermine. This possibility is consistent with the stimulatory effect of high salt concentration on ryanodine binding. In fact, 10 mM spermine produced a similar degree of enhancement of the ryanodine binding affinity of the receptor as 1.0 M NaCl. However, the observation that spermidine, which has three positive charges compared to four of spermine, is 12-fold less potent is contrary to the suggestion of non-specific electrostatic interactions.

Another possibility is that the pronounced effects of spermine on ryanodine and BzATP binding to the receptor could be due to spermine interaction with a specific site on the receptor. This possibility is supported by the data in Table I, and by the specific purification of ryanodine receptor on spermine-agarose column [39]. This specific site is not likely to be the high-affinity Ca^{2+} binding site since spermine had no significant effect on the Ca^{2+}-dependency of the ryanodine binding (Fig. 3). The interaction of spermine with the inhibitory low-affinity, Ca^{2+} binding site is ruled out because spermine exerts its stimulatory effect in the presence of low concentrations of Ca^{2+}, where the low-affinity Ca^{2+} binding site would not be occupied. It should be mentioned that the above possible mechanism for the stimulatory effect of spermine is consistent with the polyamine-Ca^{2+} exchange reaction hypothesis [23,24]. This hypothesis suggests that polyamines serve as intracellular messengers that increase free cytosolic [Ca^{2+}] by binding to anionic sites on the receptor and displace bound Ca^{2+} with consequent opening up of Ca^{2+} channels [23,24].

Another possible mechanism to account for the effect of spermine is its binding to phospholipids [38]. We have found, however, that spermine stimulated the binding of ryanodine to the purified receptor in the presence of a relatively high concentration (10 mg/ml) of phosphatidylcholine [39].

Since spermine stimulated ryanodine binding to the purified protein [39], it is unlikely that alterations in membrane surface charge, like those demonstrated for polyamines in other systems [38,40,41] are responsible for the stimulation of ryanodine and ATP binding to the receptor.

Thus, it seems that the stimulatory effect of spermine on ryanodine binding is due to its specific interaction with the ryanodine receptor, which led to stablization of a conformational state with higher binding affinity for ryanodine and for ATP.

The existence of a recognition site for polyamines on the NMDA receptor complex and the modulation of this receptor by polyamines have been demonstrated recently [42].

Spermine, spermidine and putrescine are endogenous constituents of muscle tissue [41] up to 0.8, 0.6 and 0.04 μmol/g wet weight of skeletal muscle, respectively [43,44]. These physiological levels might be capable of in situ modulation of ryanodine receptor activity. Palade [22] found that spermine at the range of micromolar concentrations inhibited the drug-induced Ca^{2+} release, from isolated SR vesicles, and suggested that spermine might be a physiological SR Ca^{2+} channel regulator. Spermine also antagonizes the halothane-induced contractures in muscle, although this effect was related to its interaction with phospholipase A_2 [45].

Polyamines including spermine are known to regulate a wide variety of physiological and biochemical processes (see Introduction), and it has been suggested that they may constitute a new class of hormones and second messenger [16,23,24]. Although the functions attributed to the aliphatic polyamines are numerous, their modes of action at a molecular level is not yet clear.

The interaction of spermine with the ryanodine receptor, enabled us to develop a new, fast and simple method for the purification of the ryanodine receptor from skeletal muscle [39].

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