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Neomycin: a novel potent blocker of communication between T-tubule and sarcoplasmic reticulum

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Abstract Ca^{2+} release from the sarcoplasmic reticulum (SR) was induced in isolated triads by direct stimulation of the SR moiety by polylysine, or stimulation via chemical depolarization of the transverse tubule (T-tubule) moiety. Polylysine-induced release was blocked by neomycin with an IC_{50} (the concentration for half-maximal inhibition) of 0.3 μM. However, the IC_{50} for neomycin block of depolarization-induced Ca^{2+} release sharply decreased in a voltage-dependent fashion, and it was 5.3 nM at a maximal extent of T-tubule depolarization. These results suggest that the high affinity binding of neomycin to the triad leads to the specific blocking of the signal transmission from T-tubule to SR.

Key words: Excitation–contraction coupling; Triad; Specific inhibitor; Neomycin

1. Introduction

Several types of blockers of Ca^{2+} release from the SR have been effectively used to characterize excitation–contraction (e–c) coupling properties of intact or skinned muscle fibers and isolated vesicles (reviews: [1–3]). One type, e.g. ruthenium red [4,5], directly blocks the ryanodine receptor (RyR)/Ca^{2+} channel [6–8]. The other type, e.g. dihydropyridines, blocks the voltage-dependent signal transmission from T-tubule to SR, as evidenced by the inhibition of charge movement [9,10] and depolarization-induced SR Ca^{2+} release with no effect on drug-induced Ca^{2+} release [11]. Neomycin, like ruthenium red, inhibits caffeine/polylysine- or Ca^{2+}-induced Ca^{2+} release at sub-μM to several μM [12–14] by specifically binding to the RyR [14], indicating that neomycin is a specific blocker of the RyR/Ca^{2+} channel. Here we report that neomycin works as a blocker of the RyR channel at sub- to several μM, while at much lower concentrations (1 nM–100 nM), it works as a specific blocker of the signal transmission from the T-tubule to the SR.

2. Experimental

2.1. Preparations

The triad-enriched microsomal fraction (triad) was prepared from rabbit leg and back muscle by differential centrifugation as described previously [15]. After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic enzyme inhibitors (0.1 mM PMSF, 10 μg/ml aprotinin, 0.8 μg/ml antipain, 2 μg/ml trypsin inhibitor), and 20 mM MES, pH 6.8 to a final protein concentration of 20–30 mg/ml. T-tubules and SR were separated by French press treatment of the triads, followed by sucrose density gradient centrifugation as described previously [16]; purified T-tubules on top of the 27% sucrose layer were sedimented and homogenized in the same solution as above. The fractions were frozen immediately in liquid nitrogen, and stored at −70°C.

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Abbreviations: MES, 2-(*N*)morpholino)ethanesulfonic acid; PMSF, phenylmethyl sufonyl fluoride; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system.

2.2. Ca^{2+} release assays

For polylysine-induced Ca^{2+} release, triads (1 mg/ml) were incubated in a solution containing 150 mM KCl, 1 mM Mg-ATP, an ATP-regenerating system (5.0 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase), 50 μM $CaCl_2$, 20 mM MES, pH 6.8. After completion of Ca^{2+} uptake into the SR, Ca^{2+} release was initiated by mixing one volume of the triad-containing solution with an equal volume of a solution containing 150 mM KCl, 6 μg/ml of 3.3 kDa polylysine, various concentrations of neomycin, and 20 mM MES, pH 6.8. The time course of polylysine-induced Ca^{2+} release was monitored with 10 μM arsenazo III or 2.5 μM fluo-3 as a Ca^{2+} indicator using a stopped-flow apparatus (BioLogic SFM-3).

To induce depolarization-induced Ca^{2+} release in the triads, we adopted the ionic replacement method (Na^+ replacement protocol: for details, see the legend to Fig. 1) originally used to induce T-tubule-mediated contraction in the frog skinned muscle fiber system [17,18]. In fact, this ionic replacement procedure generated T-tubule membrane depolarization in triads as shown by parallel measurements of T-tubule depolarization and SR Ca^{2+} release [19]. First, triads (1.6 mg/ml) were incubated in a solution containing 150 mM K gluconate, 5 mM Mg-ATP, an ATP-regenerating system, 15 mM NaCl, and 150 μM $CaCl_2$, 20 mM imidazole, pH 6.8. After incubation for at least 5 min to complete both T-tubule polarization (by mediation of the Na^+/K^+ pump) and Ca^{2+} loading of the SR (by the SR Ca^{2+} pump), one volume of the primed (polarized and Ca^{2+} loaded) triad-containing solution was mixed with nine volumes of depolarization solution whose compositions are shown in Table 1. Various concentrations of neomycin were added to the depolarization solution. The time course of induced Ca^{2+} release was monitored using the stopped-flow spectrophotometric system using either 2.5 μM fluo-3 or 10 μM arsenazo III as the Ca^{2+} indicator. The neomycin concentration-dependence of Ca^{2+} release inhibition was not affected by the type of Ca^{2+} indicator used. The initial rate of SR Ca^{2+} release was calculated by fitting exponential models to individual release curves (for details, see the legend to Table 2).

2.3. [^{3}H]Neomycin binding assay

To prepare the [^{3}H]neomycin probe, 125 μl stock solution of [^{3}H]*N*-succinimidyl propionate (0.125 mCi, 2.05 nmol; New England Nuclear/DuPont) was evaporated under nitrogen gas to remove the solvent, and 50 μl solution of 40 μM neomycin in 20 mM HEPES (pH 7.5) was added, then incubated at 22°C for 1 h. Unreacted succinimidyl propionate was quenched with 1 mM lysine. For the assays of [^{3}H]neomycin binding, 0.1 ml of T-tubule (1.0 mg/ml) or SR (3.2 mg/ml) vesicles, which had been primed in Solution A as described above, was mixed with 0.9 ml of depolarization solution (G10) containing 10 nM [^{3}H]neomycin. The mixture was immediately filtered through the Millipore filter (DA, 0.65 μm), air-dried and the radioactivity retained on the filter was scintillation-counted. Non-specific binding was determined by carrying out the same assays in the presence of 1.0 mM unlabeled neomycin added.

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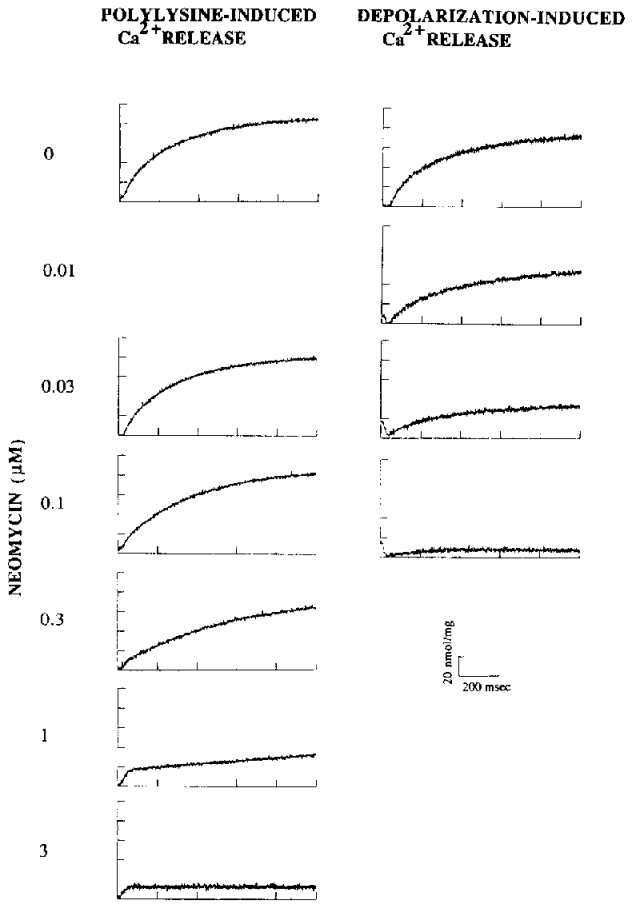

Fig. 1. Depolarization-induced Ca^{2+} release is much more sensitive to the release inhibitor neomycin than polylysine-induced Ca^{2+} release. T-tubule depolarization was produced by mixing one volume of the primed triads with nine volumes of depolarization solution containing 150 mM Na gluconate, 15 mM NaCl (which gave the maximum grade of K^+ to Na^+ replacement (G10), see section 2) and various concentrations of neomycin as indicated. The time course of induced Ca^{2+} release was monitored using fluo-3 in the stopped-flow system. For the assay of polylysine-induced release, Ca^{2+}-loaded triads were mixed with an equal volume of triggering solution containing 6 μg/ml of polylysine (M_r = 3,300), and the release time course was monitored using arsenazo III in the stopped-flow system. Each trace was obtained by signal-averaging a total of 105–140 traces originating from 3–4 different experiments.

3. Results

Addition of the maximally activating concentration of the RyR-specific trigger, polylysine (3 μg/ml ≈ 0.9 μM [20], or maximum extent of T-tubule depolarization (G10, see Table 1) yielded about the same magnitude of Ca^{2+} release, 70–80 nmol of Ca^{2+} release/mg protein at 1 s (Fig. 1). However, the sensitivity to the release blocker neomycin was found to be significantly different. Namely, polylysine-induced Ca^{2+} release was inhibited in the [neomycin] range of 0.3–3.0 μM, the same concentration range for the inhibition of caffeine- and Ca^{2+}-induced Ca^{2+} release [12,13]. In sharp contrast, depolarization-induced Ca^{2+} release was inhibited in a much lower concentration range (1–100 nM). Thus, 0.1 μM neomycin had virtually no effect on polylysine-induced Ca^{2+} release, while it produced almost complete inhibition of depolarization-induced Ca^{2+} release. The inhibition of Ca^{2+} release by neomycin occurred instantaneously, because neomycin was added only in the depolarization solution. These results suggest that there are two classes of neomycin binding sites: one, the known channel blocking site(s), and the other, a new site(s) with much higher affinity that is involved in blocking the T-tubule-to-SR signal transmission and is highly accessible to the neomycin binding.

The calculated values of the initial rate of Ca^{2+} release are shown as a function of the neomycin concentration present during Ca^{2+} release (Table 2, a and b; Fig. 2). The neomycin IC_{50} determined was 0.3 ± 0.18 μM (mean ± S.D., n = 3) for polylysine-induced Ca^{2+} release, and was as low as 5.3 ± 4.2 nM (n = 4) for depolarization (G10)-induced Ca^{2+} release (the P value between polylysine and G10 = 0.020). The neomycin IC_{50} for polylysine-induced Ca^{2+} release was about the same when lower polylysine dose (0.3 μg/ml) was used. Interestingly, however, the neomycin IC_{50} for depolarization-induced Ca^{2+} release varied significantly with the extent of T-tubule depolarization: 38 ± 9.6 nM (n = 3) at G3.25, and 16 ± 3.5 nM (n = 2) at G5.5, and 5.3 ± 4.2 nM (n = 4) at G10 as described above (the P value between G3.25 and G10 = 0.0016).

As shown in Fig. 3, 0.1 μM neomycin produced almost complete inhibition of depolarization-induced Ca^{2+} release (panel b). Nevertheless, polylysine induced a normal amount of Ca^{2+} release in the presence of 0.1 μM (panel c), which is comparable to the amount of Ca^{2+} release by simultaneous application of both T-tubule depolarization and polylysine (panel d). This indicates that 0.1 μM or lower concentrations of neomycin specifically blocked the pathway by which the Ca^{2+} channel is activated via T-tubule depolarization with no effect on the mechanism of direct stimulation by the RyR-specific ligand polylysine.

In order to examine the possibility that the high affinity neomycin binding site(s) might be in the T-tubule moiety, we determined the binding of [^{3}H]neomycin to the purified T-tubule, and compared with that to the triad. At 10 nM [^{3}H]neomycin (~ IC_{50} for neomycin block of depolarization-induced Ca^{2+} release), the specific activity of neomycin binding to the purified T-tubule fraction (2.8 ± 2.3 pmol/mg protein; n = 11 from 4 different preparations) was even smaller (P = 0.101) than that

Table 1
Composition of depolarization solution used to generate various extents of T-tubule depolarization

	Na gluconate (mM)	K gluconate (mM)	NaCl (mM)	fluo-3 (μM)	Imidazole (mM)
G10	150	0	15	2.5	20
G5.5	75	75	15	2.5	20
G3.25	37.5	112.5	15	2.5	20

The G values represent the ratios of ($[Na^+]_c$ after mixing the priming solution with the depolarization solution)/($[Na^+]_c$ of the priming solution), where $[Na^+]_c$ is the concentration of Na^+ in the reaction solution (c: cytoplasmic). The sum of $[K^+]_c$ and $[Na^+]_c$ was kept constant before and after mixing. Therefore, $[K^+]_c$ decreased upon increasing the G value. The pH of the solution: 6.8.

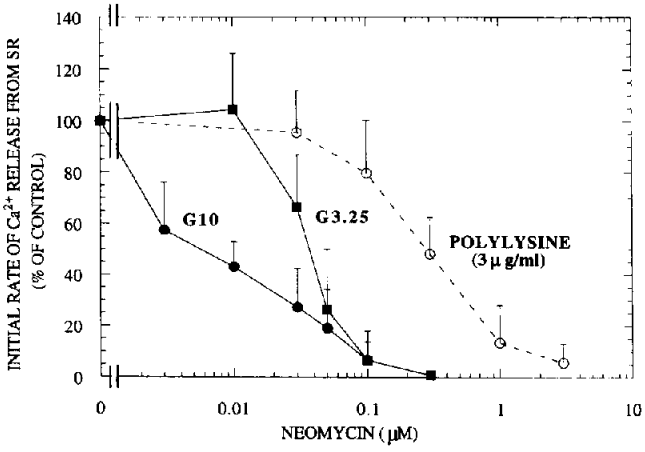

Fig. 2. The effect of neomycin on the initial rate of SR Ca^{2+} release induced by polylysine (○), maximal depolarization of T-tubule (●), and partial depolarization of T-tubule (■). The data shown in Table 2 were expressed as % control, and plotted as a function of neomycin concentration. Each datum point represents an average of 3–4 experiments ± S.D.

to the triad (4.4 ± 2.0 pmole/mg protein; n = 12 from 4 different preparations). Thus, the critical neomycin binding site(s) appears to be localized in the SR side, rather than the T-tubule side, of the signal transmission pathway (see section 4).

4. Discussion

Polylysine-induced release and depolarization-induced release described in this study are mediated via two distinctly different mechanisms. The former release is produced by direct stimulation of the SR moiety [14,20]. The latter is presumably controlled by the membrane potential changes in the T-tubule moiety of the triad [17–19]. However, both types of Ca^{2+} release are probably mediated by a common channel that resides in the RyR protein [8].

The neomycin IC_{50} value for the inhibition of polylysine-induced Ca^{2+} release reported here (0.3 μM) is essentially identical to the values reported in the literature for neomycin inhibition of SR Ca^{2+} release induced by caffeine (0.3 μM, [12]) or by Ca^{2+} (0.22–0.37 μM, [12,13]), suggesting that at these concentrations neomycin works as a blocker of a common release channel for all types of Ca^{2+} release. If neomycin works primarily as the RyR-specific common channel blocker, depolarization-induced Ca^{2+} release would have been inhibited in the same neomycin concentration range as for the polylysine-induced Ca^{2+} release. However, the neomycin IC_{50} for depolarization-induced Ca^{2+} release is much lower than that for polylysine-induced Ca^{2+} release. Furthermore, IC_{50} varied in a voltage-dependent manner. The inhibition is not due to the perturbation of T-tubule polarization by neomycin, because neomycin was added after completion of T-tubule polarization. We propose that there is an additional neomycin (site)s, which has much higher affinity for neomycin than the RyR channel blocking site(s) and is located in an area of the triad critical for the T-tubule-to-SR communication. The putative high affinity neomycin binding site(s) must be located in the cytoplasmic surface of the triad, because the inhibition occurred instantaneously upon mixing with neomycin.

Concerning the location of the high affinity neomycin bind-

Table 2a
The initial rates (nmol Ca^{2+} release/mg protein/s) of SR Ca^{2+} release induced by maximal depolarization (G10) and partial depolarization (G3.25) of T-tubule in the presence of various concentrations of neomycin

Type of Ca^{2+} release	Neomycin added (μM)						
	0	0.003	0.01	0.03	0.05	0.1	0.3
Depolarization-induced Ca^{2+} release (G10)	408 ± 200	212 ± 54	177 ± 108	89 ± 76	87 ± 94	32 ± 37	–
	(4)	(4)	(4)	(4)	(4)	(4)	
Depolarization-induced Ca^{2+} release (G3.25)	73 ± 2	80 ± 18	69 ± 13	49 ± 16	29 ± 6	5 ± 8	1 ± 1
	(3)	(2)	(3)	(3)	(2)	(3)	(3)

Table 2b
The initial rates (nmol Ca^{2+} release/mg protein/s) of SR Ca^{2+} release induced by polylysine (3 μg/ml) in the presence of various concentrations of neomycin

Type of Ca^{2+} release	Neomycin added (μM)					
	0	0.03	0.1	0.3	1.0	3.0
Polylysine-induced Ca^{2+} release	282 ± 119	274 ± 128	223 ± 96	134 ± 60	44 ± 40	22 ± 30
	(3)	(3)	(3)	(3)	(3)	(3)

Ca^{2+} release by polylysine and by maximal depolarization were produced and monitored as described in the legend to Fig. 1. For partial depolarization (G3.25), one volume of primed triads was mixed with nine volumes of depolarization solution (see Table 1) containing various concentrations of neomycin. Then, the time course of release was monitored in the same way as for Ca^{2+} release induced by maximal depolarization. The initial rates of Ca^{2+} release ($A \cdot k$) were calculated by fitting single $\{y = y_0 + A\,(1 - e^{-kt})\}$ or double $\{y = y_0 + A_1\,(1 - e^{-k_1 t}) + A_2\,(1 - e^{-k_2 t})\}$ exponential functions to the time course (obtained by signal averaging about 120 traces) of polylysine-induced and depolarization-induced Ca^{2+} release, respectively. The rapid descending phase during the initial 20 ms in the time courses of both polylysine-induced Ca^{2+} release and depolarization-induced Ca^{2+} release represents a mixing artifact, because this phase was present under non-depolarizing conditions, in the absence of added polylysine, and in the presence of the release blocker. Thus, the initial 20 ms portion was excluded from the curve fitting. Data represent the mean ± S.D. n: the number of experiments.

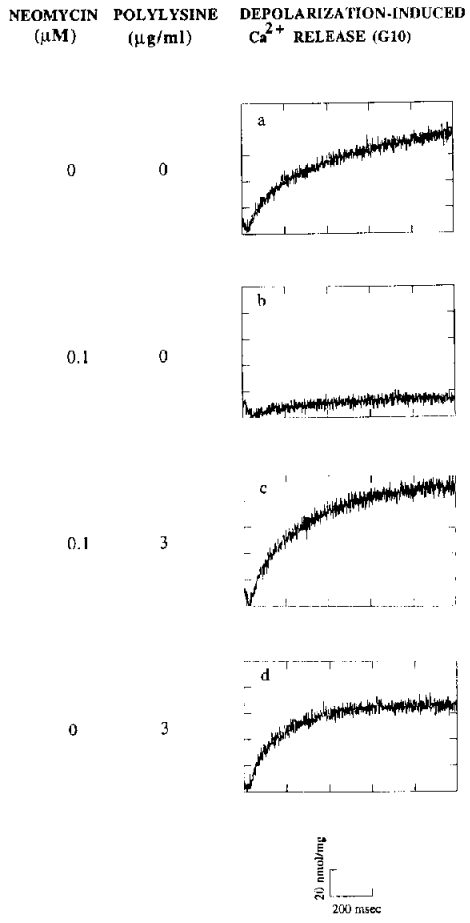

Fig. 3. Control experiments showing that polylysine is fully capable of inducing Ca^{2+} release (cf. panel c) even after complete inhibition of depolarization-induced Ca^{2+} release by 0.1 μM neomycin (cf. panel b). Ca^{2+} release was induced by the maximum extent of ionic replacement (G10) with various combinations of addition of neomycin and polylysine as indicated.

ing site(s), several possibilities remain to be investigated, such as the T-tubule membrane, the RyR, or a yet unidentified coupling protein located in the T-tubule/SR junction, etc. In view of the present finding that at 10 nM neomycin the specific activity of [^{3}H]neomycin binding to the purified T-tubule fraction is smaller than that to the total fraction of triad, its specific localization in the T-tubule appears to be unlikely. Together with our recent finding that at low concentrations (< several μM) neomycin binds almost exclusively to the RyR [14], we tentatively propose that the new neomycin site(s) described here is localized in the RyR, viz. in the putative signal-receiving domain of the RyR. In conclusion, neomycin would serve as a new tool, for both biochemical and physiological studies of e–c coupling, especially to identify and characterize key proteins or domains involved in the signal transmission from T-tubule to SR.

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