Amino- and Guanidinoacylryanodines: Basic Ryanodine Esters with Enhanced Affinity for the Sarcoplasmic Reticulum Ca^{2+}-Release Channel

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Amino- and guanidinoacyl esters of ryanodine were prepared to evaluate the effect of basicity on the binding affinity of these derivatives for the sarcoplasmic reticulum Ca^{2+}-release channel (SR CRC). In the presence of DCC and DMAP Cbz-β-alanine reacts with ryanodine in CH_2Cl_2 to give O_{10eq}-Cbz-β-alanylryanodine (**3a**), which on hydrogenolysis yields the β-alanyl ester (**4a**). *N,N'*-bis-Cbz-*S*-methylthiourea reacts with **4a** to yield β-*N,N'*-bis-Cbz-guanidinopropionylryanodine (**5a**). O_{10eq}-β-guanidinopropionylryanodine (**6a**) is obtained on hydrogenolytic deprotection of **5a**. The binding affinity of β-alanine ester (**4a**) and its glycyl congener (**4b**) is 2–3-fold greater, and that of the β-guanidinopropionyl ester (**6a**) and its acetyl congener (**6b**) 3–6-fold greater, than that of ryanodine. The effect of ryanodine on SR Ca^{2+} flux is of a biphasic nature: nanomolar levels open (activate) the channel, while micromolar levels close (deactivate) it. The base-substituted esters **4a** and **6a** both display a unidirectional effect: they only open the channel. An understanding of ryanodine's mode of action and the design of effective SR CRC activating and deactivating ryanoids for possible therapeutic application are major research objectives.

Chemical alteration of natural products[1–5] at times leads to *improvement*, only rarely—as in the case of Etoposide[5]—to *innovation* of therapeutic effectiveness. The latter achievement, indeed, presents a paradigm.

In recent years the two principal bioactive alkaloids, ryanodine (**1**)[6] and 9,21-dehydroryanodine (**2**),[7] isolated from the tropical shrub *Ryania speciosa* Vahl (Figure 1), have been studied extensively because of their characteristic role as modulators of the sarcoplasmic reticular Ca^{2+}-release channel (SR CRC, ryanodine receptor) of striated muscle;[8,9] their insecticidal activity and mammalian toxicity had previously been established.[9a,10]

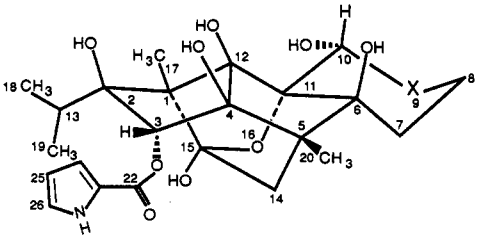
Evidence has very recently been presented for the presence of ryanodine receptors in brain[11] and other cells,[12] including neutrophils.[12c] It appears then a worthy challenge to attempt and translate the complex, high-potency in vitro actions and the in vivo toxicity[9a,10] of these ryanoids into therapeutic effectiveness for cardiac disease or perhaps alternate pathologies. Clearly, the *semi*synthesis of altered ryanodines[13] and evaluation of their in vitro pharmacological activity is an attractive starting point. Determining the relative binding affinity (RBA) of these ryanoids may assist in mapping the binding domain. In addition, some of these derivatives may serve as molecular probes,[14] for example in affinity chromatography[14a] and in photoaffinity labeling[14b] of the ryanodine receptor. Also, the search for putative endogenous effectors of this receptor in the mammalian system will be facilitated by the availability of suitable derivatives (vide infra).

To explore the effect of base substitution on ryanoid binding we availed ourselves of ryanodine's accessible, secondary C10-hydroxyl function[10] to prepare O_{10eq}-amino- and -guanidinoacyl esters.[14b,c] Here we report the *semi*-synthesis and preliminary pharmacological evaluation of O_{10eq}-β-alanyl- (**4a**), -glycyl- (**4b**), -β-guanidinopropionyl- (**6a**), -guanidinoacetylryanodine (**6b**), the Cbz-protected intermediates (**3a, 3b, 5a, 5b**) and the *anhydro*-ryanodine ester (**7**). Some of these esters have been shown to possess enhanced affinity for the receptor, relative to ryanodine (**1**), and to display a unidirectional effect on Ca^{2+}-release channels.

Chemistry

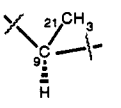
Basic esters (**4a, 4b, 6a, 6b**) of ryanodine (**1**) were prepared according to Scheme I. O_{10eq}-Cbz-β-alanylryanodine (**3a**)[13b,c] was synthesized from **1** and *N*-Cbz-β-alanine by the method of Neises and Steglich.[15] Hydrogenolysis of **3a** in the presence of Et_3N gives O_{10eq}-β-alanylryanodine (**4a**),[13b,c] which reacts with *N,N'*-bis-Cbz-*S*-methylthiourea[16] under mild conditions to yield O_{10eq}-β-*N,N'*-bis-Cbz-guanidinopropionylryanodine (**5a**). Hydrogenolysis (Et_3N) of **5a** affords O_{10eq}-β-guanidinopropionylryanodine (**6a**).

In the presence of aqueous HCl hydrogenolysis of **3a**, in addition to ester **4a**, yields a byproduct characterized



1

Ryanodine X =



2

9,21-Dehydroryanodine X =

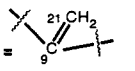


Figure 1

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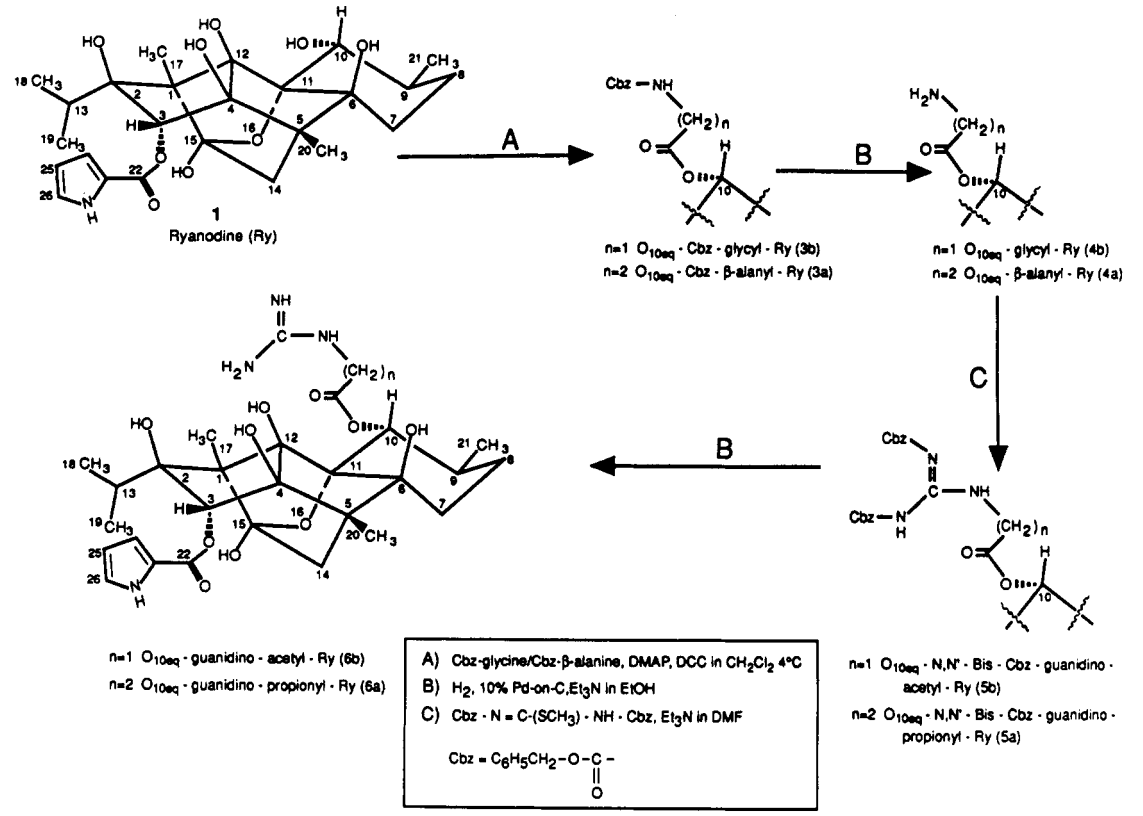
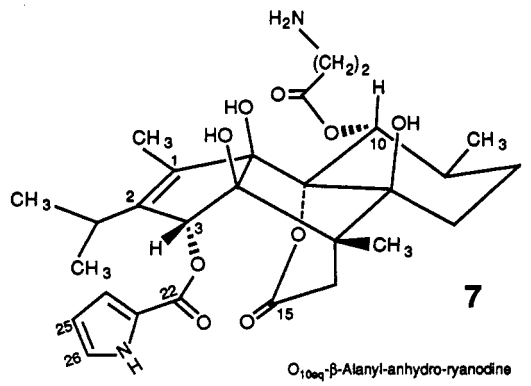
Scheme I. Semisynthesis of O_{10eq}-Guanidino- and -Aminoacyl Esters of Ryanodine



as β-alanyl-*anhydro*-ryanodine (**7**).

The preparation of the guanidinoacetyl ester (**6b**) similarly proceeds from **1** and Cbz-glycine via O_{10eq}-Cbz-glycyl- (**3b**), O_{10eq}-glycyl- (**4b**), and O_{10eq}-*N,N'*-bis-Cbz-guanidinoacetylryanodine (**5b**); hydrogenolysis (Et_3N) of **5b** affords the O_{10eq}-guanidinoacetate of **1** (**6b**).



The basic esters **4** and **6** of **1** are shown to be of pharmacological interest (vide infra); in view of the relatively greater abundance of dehydroryanodine (**2**),[8,14b] preparation of the corresponding basic esters of **2** is now underway.[17] Compared to **1** and **2**, other known natural ryanoids,[18] the less polar "ryania diterpene esters" A, B, C_1, C_2, and D,[18a] the more polar "Rania diterpene esters" E and F,[18b,c] ryanodyl 3-pyridine-3-carboxylate,[18d] and 18-hydroxyryanodine[10] are minor constituents and are not readily available for chemical alteration.

Pharmacological Results and Discussion

A cursory comparison of the relative binding affinity (RBA) of the esters 3–6 with that of **1** (Table I) reveals a 3–6-fold enhanced affinity of the basic esters **4** and **6**, a slightly enhanced affinity of the mono-Cbz protected ester **3**, and a 7–20-fold lesser affinity of the di-Cbz protected esters **5a** and **5b**.

Table I. Relative Binding Affinity of Ryanoids and O_{10eq}-Ryanodine Esters for the Sarcoplasmic Reticular Ca^{2+}-Release Channel[a]

		IC_{50} (nM) ± SD
1	ryanodine	6.2 ± 0.4
2	dehydroryanodine	8.9 ± 1.2
	O_{10eq}-Ryanodine esters	
3a	Cbz-β-alanyl[b]	5.9
3b	Cbz-glycyl	4.8 ± 0.6
4a	β-alanyl	2.6 ± 0.4
4b	glycyl	1.8 ± 0.4
5a	β-*N,N'*-bis-Cbz-guanidinopropionyl[b]	43.6
5b	*N,N'*-bis-Cbz-guanidinoacetyl[b]	135.6
6a	β-guanidinopropionyl	1.1 ± 0.1
6b	guanidinoacetyl	1.8 ± 0.2
	Ry-*hemi*-succinate ($^-OOCCH_2CH_2COO_{10}$-Ry[d])	>1000.0
	N-methyl Ry-succinamidate ($CH_3HNOCCH_2CH_2COO_{10}$-Ry)	49.4 ± 2.7
7	β-alanyl-*anhydro*-ryanodine[b]	149.0
	anhydro-ryanodine[c]	>1000.0

[a] IC_{50} is the concentration of the unlabeled ryanodine or ryanodine ester at which 50% of the high affinity binding sites on the skeletal SR CRC/Ry receptor are occupied. [b] Single experiment. [c] Reported for *anhydro*-Ryanodine: IC_{50} >10 μM. (Waterhouse, et al. *J. Med. Chem.* **1987**, *30*, 710–716. [d] Gerzon, et al. Reg. Meet. Am. Chem. Soc., Indianapolis, 1991, Abstr. MEDI 342.

The enhanced binding affinity of the positively charged esters **4** and **6** to the receptor indicates that an anionic function may be proximate to the ryanodine binding site. That a negative charge does reside adjacent to the C-10 region is supported by the minimal binding affinity (IC_{50} > 1.0 μM) noted for the negatively charged O_{10eq}-succinate of **1**, $^-OOCCH_2CH_2COO_{10}$-ryanodine.[13a,c,19] Furthermore, $CH_3HNOCCH_2CH_2COO_{10}$-ryanodine, the corresponding, noncharged *N*-methylamide, regains receptor affinity; this

amide exhibits 24% of the affinity of 1. While the enhanced binding of the base-substituted esters (**4a,b** and **6a,b**) implicates a negative locus adjacent to the binding site, adequate binding of the mono-Cbz esters (**3a,b**) reveals a degree of steric tolerance at the region of the C10-equatorial hydroxyl. A low RBA of the two bulky bis-Cbz-guanidino esters (**5a,b**) suggests this steric tolerance to be of limited scope. Clearly, the RBA relationships among the ryanodines (**1** and **2**) and esters **3–6** reflects, among others, the effect of basicity and/or dimension (amino vs guanidino, $n = 1$ vs $n = 2$) of the substituent. To be able to assign a relative contribution factor to the one or the other parameter a larger series of derivatives would be necessary.

Ryanodine exhibits two opposing effects on the sarcoplasmic reticular Ca^{2+}-release channel (SR CRC).[8,9] Measuring the passive flux of $^{45}Ca^{2+}$ across the SR CRC (see the Experimental Section) allows quantitation of Ca^{2+} efflux from skeletal SR vesicles as a means of assessing both opening (activating) and closing (deactivating) effects of **1** and its esters in a single assay.[8,9,18c] In this assay **1** activates the SR CRC at low micromolar concentrations, activation reaching its maximum at about 30 μM; concentrations higher than 30 μM induce deactivation of the channel. An altered–unidirectional–pharmacological profile, differing from the biphasic profile of **1**, was observed for esters **4a** and **6a**. The activation curve for the β-alanyl ester (**4a**) is shifted to the left—toward lower concentrations—of that of **1**, maximal activation being reached at a concentration of 10 μM. This 3-fold-lowered shift parallels the 2–3-fold greater RBA for this ester (**4a**).

A perhaps more significant aspect, however, of the interaction of the β-alanyl ester (**4a**) with the receptor involves its overall effect on the channel.[20] Whereas **4a**, like **1**, induces full opening of the SR CRC, higher concentrations of **4a**, up to 1 mM, exhibit none of the closing action seen with high concentrations of **1**. In preliminary experiments the guanidino ester (**6a**), which has an RBA for the receptor about 6 times greater than that of **1**, also appears to be purely an activator of the SR CRC.[20] Thus, while the base-substituted esters (**4a**, **6a**) retain the ability to open this channel, they entirely lack the ability of **1** to close the SR CRC.

Whether the biphasic pharmacological activity profile of **1** can be attributed (a) to **1** interacting at two different, independent binding sites,[8,9f] (b) to different conformations of the ryanodine molecule interacting at a single binding site,[14b] or (c) to a combination of these (and perhaps other) factors[18c] are questions remaining to be answered in fiture studies. The selective channel activator action of the base-substituted esters **4a** and **6a** at the SR CRC may help to elucidate the mechanism of ryanodine's action.

Work in our and other laboratories pursues the chemical alteration of the ryanodines. In addition to the present C10-hydroxyl esters, the C9–C21 double bond in dehydroryanodine,[18b] and the pyrrolecarboxylic acid moiety[18d] are among the sites at which chemical alteration possibly may yield pharmacologic information. An understanding of ryanodine's mode of action and the design of effective SR CRC activating and deactivating agents for possible therapeutic application remain major research objectives.

Experimental Section

Melting points (uncorrected) were determined in open capillary tubes with a Mel-Temp apparatus, Laboratory Devices. Elemental analysis was performed by Midwest Micro Lab, Indianapolis, IN. Proton magnetic resonance (^{1}H NMR) spectra were obtained in CD_3OD or $CDCl_3$ solution on a Bruker AM 250 instrument; the proton chemical shift values are reported in ppm (δ) relative to internal tetramethylsilane. Mass spectra were determined on Vacuum Generators' VG CAB CSE instruments using glycerol with lithium iodide, giving ions ($M^+ + 7$), except for the guanidino ester derivatives (**6a,b**) which give $M^+ + 1$ ions. Analytical TLC was performed on 0.25-mm silica gel plates (Merck, Kieselgel 60, 230–400 mesh) using system CMaM ($CHCl_3$/CH_3OH/aqueous 40% CH_3NH_2, 98/2/0.2) or, for the guanidino esters, system BAWP (1-butanol/acetic acid/water/pyridine, 4/1/2/1, by volume).[16b]

***O_{10eq}*-Cbz-β-alanylryanodine (3a)** was prepared by a modification of the method of Neises and Steglich.[15] A stirred CH_2Cl_2 solution containing equimolar amounts of ryanodine (**1**), Cbz-β-alanine, and DCC with catalytic amounts of DMAP was allowed to react for 8 h at 4 °C. Termination of the reaction with H_2O and column chromatography (system **CMaM**) gave a 75% yield of the crystalline, protected ester (**3a**), mp 178–180 °C. TLC: R_f (CMaM) = 0.5. MS (FAB, LiI): $M^+ + 7 = 705$. Calcd ($C_{36}H_{46}N_2O_{12}$) mol. wt. = 698. ^{1}H NMR (CD_3OD): δ 7.32 (m, 5 H, phenyl aromatic protons), 7.06, 6.87, 6.23 (three double doublets, pyrrole hydrogens), 5.58 (s, 1 H, *H*-C3), 5.31 (d, 1 H, H_{ax}-C10), 5.07 (s, 2 H, PhCH_2O), 3.45 (t, 2 H, NHCH_2), 2.59 (m, 1 H, *H*-C13), 1.40 (s, CH_3-C1), 1.11 (d, 3 H, CH_3-C13), 0.90 (s, 3 H, CH_3-C5), 0.82 (d, 3 H, CH_3-C9), and 0.74 (d, 3 H, CH_3-C13). The designation of the product (**3a**) as the O_{10eq}-ryanodine ester (**3a**) is supported by the observation that the doublet for the C_{10ax}-hydrogen, present in the spectrum of **1** at 3.94 ppm, in the above spectrum of **3a** is shifted downfield to 5.31 ppm (cf. ref 10).

***O_{10eq}*-β-Alanylryanodine (4a).** Ester **3a** was hydrogenolyzed under hydrogen (40 lb/in.^{2}) with Pd–C (10%) in ethanolic solution containing equimolar Et_3N. Filtration of the catalyst, evaporation of the solvents under reduced pressure, and trituration of the solid residue with a pentane/ethyl ether mixture (9/1) yields crystalline β-alanylryanodine (**4a**), mp 182–184 °C. TLC (CMaM): R_f = 0.22. MS (FAB, LiI): $M^+ + 7 = 571$. Calcd ($C_{28}H_{40}N_2O_{10}$) mol. wt. = 564. ^{1}H NMR (CD_3OD): δ 7.03, 6.87, and 6.23 (three double doublets; pyrrole hydrogens), 5.58 (s, 1 H, *H*C3), 5.4 (d, 1 H, *H*C10), 3.0 (t, 2 H, H_2NCH_2), 2.60 (t, 2 H, CH_2CO), 2.56 (d, H_b) and 1.94 (d, H_a) (AB pattern, H_2C14), 2.26 (m, 1 H, *H*C13), 2.10 (m, 1 H, *H*C9), 1.40 (s, 3 H, CH_3-C1), 1.03 (d, 3 H, CH_3-C13), 0.89 (s, CH_3-C5), 0.85 (d, 3 H, CH_3-C9), and 0.74 (d, 3 H, CH_3-C13).

***O_{10eq}*-β-*N,N'*-Bis-Cbz-guanidinopropionylryanodine (5a).** The β-alanyl ester (**4a**, 100 mg, 0.17 mmol) in a stirred DMF solution (1 mL) containing Et_3N (0.2 mmol) was allowed to react with N,N'-bis-(Cbz)-*S*-methylisothiourea[16] (200 mg, 0.55 mmol) at room temperature for 18 h. Removal of DMF under reduced pressure (0.1 mmHg) at 35 °C and column chromatography of the oily residue on SILICAR gel (system CMaM) gave the ester **5a** (100 mg). TLC (CMaM): R_f = 0.72. MS (FAB, LiI) $M^+ + 7 = 881$. Calcd ($C_{45}H_{54}N_4O_{14}$) mol. wt. = 874. ^{1}H NMR (CD_3OD) relevant peaks: δ 7.5–7.3 (m, 10 H, 2 Ph groups), 5.6 (s, 1 H, *H*-C3), 5.35 (d, 1 H, *H*-C10), 5.26 (s, 2 H, ArCH_2O), 5.12 (s, 2 H, PhCH_2O), 3.72 (m, 2 H, CH_2NH), 2.70 (m, 2 H, CH_2CO).

***O_{10eq}*-β-Guanidinopropionylryanodine (6a).** Hydrogenolysis of **5a** (75 mg) with Pd–C (10%) in ethanol containing Et_3N, as described above for the hydrogenolysis of **3a**, followed by column chromatography (System CCaM), gave the crystalline guanidino ester (**6a**, 35 mg). TLC (BAWP): R_f = 0.73. MS (FAB, LiI): $M^+ + 1 = 607$. Calcd ($C_{29}H_{42}N_4O_{10}$) mol. wt. = 606. Anal. Calcd C, 57.43; H, 6.93; N, 9.24. Found; C, 51.78; H, 7.05; N, 8.29.

***O_{10eq}*-β-Alanyl-*anhydro*-ryanodine (7).** Cbz-β-alanylryanodine (**3a**, 0.1 mmol) was hydrogenolyzed using Pd–C (10%) in EtOH containing HCl (1 mL, 0.1 N). Filtration of the catalyst and evaporation of solvents gave a mixture of β-alanyl-*anhydro*-ryanodine (**7**) and β-alanylryanodine (**4a**) as the HCl salts. Conversion to the free bases (aqueous Na_2CO_3, $CHCl_3$) and column chromatography on SILICAR using $CHCl_3$ and then $CHCl_3$/CH_3OH/aqueous 40% CH_3NH_2 mixtures (92/2/0.2, 96/4/0.4, and 94/6/0.6), gave β-alanyl-*anhydro*-ryanodine (**7**) and ester **4a**. TLC (CMaM): R_f = 0.4. MS (FAB, LiI): $M^+ + 7 = 553$. Calcd ($C_{28}H_{38}N_2O_9$) mol. wt. = 546. ^{1}H NMR (CD_3OD): δ 7.06, 6.87, 6.26 (three doublets for pyrrole hydrogens), 6.17 (q, 1 H, *H*C3), 5.63 (d, 1 H, *H*C10), 3.42 (d, H_b) and 2.59 (d, H_a) (AB

pattern, H_2C14), 2.71 (m, 1 H, HC13), 3.07 (t, 2 H, H_2NCH_2), 2.61 (t, 2 H, CH_2CO), 2.05 (m, 1 H, HC9), 1.82 (d, 3 H, CH_3C1), 1.1 (d, 3 H, CH_3-C13), 0.99 (d, 3 H, CH_3-C9), 0.96 (s, 3 H, CH_3-C5), 0.91 (d, 3 H, CH_3-C13).

Designation of this byproduct as O_{10eq}-β-alanyl-*anhydro*-ryanodine (7) is based on the observation that the C3 proton, present as a singlet in the spectrum of the β-alanyl ester (**4a**) at 5.58 ppm, in that of the β-alanine *anhydro*-ester (7) appears as a quartet at 6.17 ppm. This C3 proton appears in the spectrum of 1 as a singlet at 5.63 ppm and as a quartet at 6.17 ppm in that of *anhydro*-ryanodine;[6b] a similar C3 proton shift obtains between ryanodol (singlet at 4.11 ppm) and *anhydro*-ryanodol (quartet at 4.70).[6d]

Cbz-glycylryanodine (3b). This ester was prepared from Cbz-glycine and **1**, analogously to **3a**. TLC (CMaM): R_f = 0.46. MS (FAB, glycerol): $M^+ + 23 = 707.3$. Calcd ($C_{35}H_{44}N_2O_{12}$) mol. wt. = 684. ^{1}H NMR ($CDCl_3$): δ 5.40 (d, 1 H, C10-*H*), 5.14 (s, 2 H, PhCH_2O).

Glycylryanodine (4b). Ester **4b** was prepared by hydrogenolysis of **3b** in the presence of Et_3N. TLC (CMaM): R_f = 0.23. Calcd for $C_{27}H_{38}N_2O_{10}$ mol. wt. = 550. No physicochemical data available because of small sample size.

Di-Cbz-guanidino acetylryanodine (5b). Ester **5b**, analogously to **5a**, was obtained from the reaction of **4b** and *N,N'*-bis-Cbz-*S*-methylisothiourea.[16] TLC (CMaM): R_f = 0.6. Calcd ($C_{44}H_{52}N_4O_{14}$) mol. wt. 860. No physicochemical data due to small sample size.

O_{10eq}-Guanidinoacetylryanodine (6b). Hydrogenolysis of **5b** in the presence of Et_3N, as in the preparation of **6a**, gave **6b**. TLC (BAWP): R_f = 0.8. MS (FAB, LiI): $M^+ + 1 = 593$. Calcd ($C_{28}H_{40}N_4O_{10}$) mol. wt. = 592. While the amino esters (**4a,b**) peak with lithium iodide in the mass spectrum at $M^+ + 7$, the more basic guanidino esters (**6a** and **6b**) peak at $M^+ + 1$.

Pharmacological Methods. The relative binding affinity (RBA) of ryanodine (**1**), dehydroryanodine (**2**) and esters **3–7** listed in Table I were determined using a competition assay, as described.[8,9b,c,18c] Briefly, rabbit skeletal SR membrane vesicles[9e–f] were incubated in the presence of 6.7 nM tritiated ryanodine and increasing concentrations of the unlabeled esters, to competitively displace the [^{3}H]ryanodine. The IC_{50} value of each of the esters was determined from the respective displacement curve using the computer binding analysis program EBDA/ligand.[21]

Passive flux of $^{45}Ca^{2+}$ through the SR Ca^{2+}-release channel[9e,f] was used to evaluate the ability of the basic esters **4a** and **6a** to open (activate) and close (deactivate) the channel. Briefly, junctional SR vesicles from rabbit skeletal muscle were passively loaded with $^{45}Ca^{2+}$ in the presence and in the absence of the esters. Ca^{2+} efflux was then initiated by diluting the vesicles 100-fold into a low Ca^{2+} concentration. $^{45}Ca^{2+}$ remaining in the vesicles was determined after a 3-s efflux period by termination and filtration of the vesicles followed by scintillation counting.

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