Imperatoxin A ($IpTx_a$) from *Pandinus imperator* stimulates [^{3}H]ryanodine binding to RyR3 channels

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Abstract The effect of imperatoxin A ($IpTx_a$) on the ryanodine receptor type 3 (RyR3) was studied. $IpTx_a$ stimulates [^{3}H]ryanodine binding to RyR3-containing microsomes, but this effect requires toxin concentrations higher than those required to stimulate RyR1 channels. The effect of $IpTx_a$ on RyR3 channels was observed at calcium concentrations in the range 0.1 μM to 10 mM. By contrast, RyR2 channels were not significantly affected by $IpTx_a$ in the same calcium ranges. Single channel current measurements indicated that $IpTx_a$ induced subconductance state in RyR3 channels that was similar to those observed with RyR1 and RyR2 channels. These results indicate that $IpTx_a$ is capable of inducing similar subconductance states in all three RyR isoforms, while stimulation of [^{3}H]ryanodine binding by this toxin results in isoform-specific responses, with RyR1 being the most sensitive channel, RyR3 displaying an intermediate response and RyR2 the least responsive ones.

Key words: Sarcoplasmic reticulum; Excitation contraction coupling; Ryanodine receptor; Calcium release channel; Toxin

1. Introduction

Ryanodine receptors, RyRs, are intracellular Ca^{2+} release channels mainly known for their role in regulating Ca^{2+} release from the sarcoplasmic reticulum of striated muscles [1]. Three different genes encoding distinct isoforms of RyRs are known [2–5]. Recent evidence indicates that RyRs, in addition to striated muscles, are co-expressed in several cell types and that specific combination of RyR isoforms may be important to generate localized Ca^{2+} release events [6–8] and to influence cellular activities such as muscle contraction in neonatal skeletal muscle [9]. In neurons, co-expression of RyR isoforms seems to be important to generate specific changes in hippocampal synaptic plasticity and spatial learning [10]. However, the mechanism(s) by which RyRs are activated in cells other than muscle cells is not known.

Identification of the specific regulatory properties of Ca^{2+} release channels is important to better understand the molecular basis of Ca^{2+} signals. The Ca^{2+} channel activity of the three RyR isoforms is known to be sensitive to the effects of several physiological compounds among which Ca^{2+} (at low concentrations) and ATP promote the activity of the channels, while Mg^{2+} and Ca^{2+} (at high concentrations) have an inhibitory effect on channel activity [11]. Other compounds, such as caffeine and ryanodine, although not physiological agonists of these channels, have been used to study and differentiate the regulatory properties of the three isoforms. Cyclic adenosine 5′-diphosphate-ribose, a NAD^+ derivative, is a strong candidate for a physiological activator of RyRs [12–16].

Imperatoxin A ($IpTx_a$), a 33-amino acid peptide from the venom of the African scorpion *Pandinus imperator*, has been reported to stimulate [^{3}H]ryanodine binding to RyR1 but not to RyR2 channels [17–19]. Indeed, a structural domain of $IpTx_a$ appears to share structural and functional homology with an active peptide segment of the dihydropyridine receptor (DHPR) α_{1S} subunit. The peptide segment corresponds to amino acids 666–690 of the II–III loop of the α_{1S} subunit of DHPR, which has been demonstrated to interact with RyR1 to trigger Ca^{2+} release [20,21]. The peptide derived from the II–III loop of the α_{1S} can bind RyR1 and enhance [^{3}H]ryanodine binding. The homology between the II–III loop of α_{1S} and $IpTx_a$ peptides has also been substantiated by competition experiments, where increasing concentrations of the II–III loop peptide affect the capacity of $IpTx_a$ to bind RyR1 [19]. Here we report the response of RyR3 channels to Ca^{2+} and $IpTx_a$. These results reveal an isoform selectivity of this peptide in regulating RyR activity.

2. Materials and methods

2.1. Cell culture and transfection

About 8×10^5 HEK293 cells were plated on a 100 mm tissue culture dish 24 h before transfection. One hour before DNA addition medium was changed. 5 μg of RyR3 expression vector and 5 μg of carrier DNA were mixed in a solution containing HBS (5 g/l HEPES, 8 g/l NaCl, pH 7.1), 0.7 mM Na_2HPO_4, 0.7 mM NaH_2PO_4, 120 mM $CaCl_2$ and incubated for 30 min. Calcium phosphate precipitates were added to cells and incubated for 6 h.

For stable transfections, cells were selected with Geneticin sulfate G418 (Boehringer) at 800 μg/ml.

2.2. Microsomal protein preparation

Bovine skeletal muscle and transfected cells were used to prepare microsomal fractions containing RyR1 and RyR3 channels, respectively. Microsomes were prepared as described previously [22,23].

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

[^{3}H]Ryanodine (50–70 Ci/mmol) was from Amersham Life Science (Amersham Place, UK). [^{3}H]Ryanodine (7 nM) was incubated for 90 min at 36°C with 30–50 μg of microsomes from bovine skeletal muscle or transfected cells in 0.1 ml of medium B containing 0.2 M KCl, EGTA and $CaCl_2$ necessary to set free Ca^{2+} in the range from 10 nM to 1 μM and 10 mM Na-HEPES (pH 7.2) in the absence or in the presence of $IpTx_a$. Saturation curve was obtained using different [^{3}H]ryanodine concentrations (0.5, 1, 3, 5, 10, 20 nM) in the same previous medium.

Samples were always run in duplicate, filtered through Whatman GF/B glass fiber filters, washed with 3×5 ml of medium B without $CaCl_2$ and 2×5 ml EtOH 10%. The filters were placed in scintillation vials, 5 ml of liquid scintillation added, and the radioactivity measured in a Tri-Carb 2100 TR Packard β-counter. The specific binding was defined as the difference between the binding in the absence (total binding) and in the presence (non-specific binding) of 20 μM unlabeled ryanodine (Calbiochem, La Jolla, CA, USA).

2.4. Reconstitution of RyRs in planar lipid bilayers

Single channel recordings of RyRs were carried out as described previously [19–21]. Channel fusion was facilitated by applying positive voltage and the addition of Ca^{2+} in the *cis* solution to a final concentration of 10 μM. Channel recordings were performed under symmetrical conditions of 200 mM Cs-methanesulfonate. The bilayer membrane was held at constant potentials (usually −30 mV) when recording channel activity and 'rested' at 0 mV during addition or perfusion of $IpTx_a$. Analysis of single channel data was performed using the pClamp7 program and Origin 6.0 graphing software.

3. Results

3.1. Expression and characterization of RyR3 expressed in HEK293 cells

To study the functional properties of RyR3, embryonic human kidney cells (HEK293) were transfected with the full-length RyR3 cDNA from mink lung epithelial cells (Mv1Lu) [24,25] and different stable clones were isolated. The levels of expression and the correct localization of the recombinant RyR3 protein in the different selected clones were tested by Western blot analysis and immunofluorescence experiments. For all experiments, a representative RyR3 expressing clone, 4-115, and non-transfected HEK293 cells were used. Fig. 1A shows the Western blot analysis, performed with a polyclonal antibody against RyR3, of 50 μg of microsomes from bovine diaphragm (lane 1), RyR3-transfected cells (lane 2), mock-transfected and non-transfected HEK293 cells (lanes 3 and 4). The native and the recombinant RyR3 proteins show a similar migration profile, attesting that the RyR3 cDNA encodes a protein with molecular size and immunoreactivity indistinguishable from the native RyR3 channel. Immunofluorescence staining of control and RyR3-transfected HEK293 cells revealed that the RyR3 protein was localized to the endoplasmic reticulum (Fig. 1C,D). In addition, dose-dependent [^{3}H]ryanodine binding to the microsomal fraction of transfected cells was carried out. Fig. 1B reports the saturation curve obtained by incubating 50 μg of RyR3 microsomes with concentrations of [^{3}H]ryanodine varying from 0.5 to 20 nM. RyR3 showed a dissociation constant (K_d) for ryanodine of 9.2 nM and a maximal receptor density (B_{max}) of 0.262 pmol/mg protein.

It has been shown that Ca^{2+} has a biphasic effect on the RyR channel activity depending on a different Ca^{2+} concentration that gives rise to a bell-shaped curve [11]. To investigate the Ca^{2+} sensitivity of RyR3 channels, [^{3}H]ryanodine

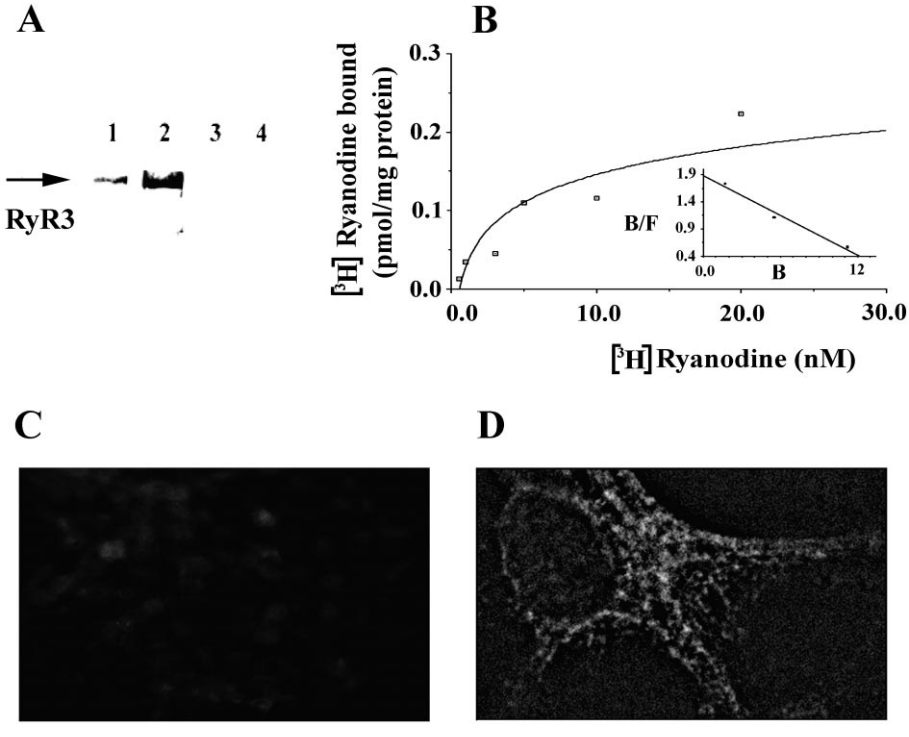

Fig. 1. Expression of RyR3 proteins. A: 50 μg of microsomes prepared from bovine diaphragm (lane 1), RyR3-transfected cells (4-115 clone) (lane 2), pcDNA3-transfected cells (lane 3) and untransfected HEK293 cells (lane 4) were tested for RyR3 protein by Western blot analysis. B: 50 μg of RyR3 microsomes (4-115 clone) were incubated for 90 min at 36°C with 0.5–20 nM [^{3}H]ryanodine in a solution containing 0.2 M KCl, 10 mM HEPES pH 7.4, 10 μM Ca^{2+}. Non-specific binding was defined as the binding of [^{3}H]ryanodine in the presence of 20 μM unlabeled ryanodine and has been subtracted in this and subsequent figures. The amount of [^{3}H]ryanodine bound was measured by membrane filtration on Whatman GF/B filters. Inset, Scatchard plot of saturation data of the specific binding. The K_d and B_{max} values were 9.8 nM and 0.262 pmol/mg of protein, respectively. C,D: Expressed RyR3 proteins were detected by immunofluorescence staining of untransfected (C) and transfected HEK293 (D) cells using anti-RyR3 antibody.

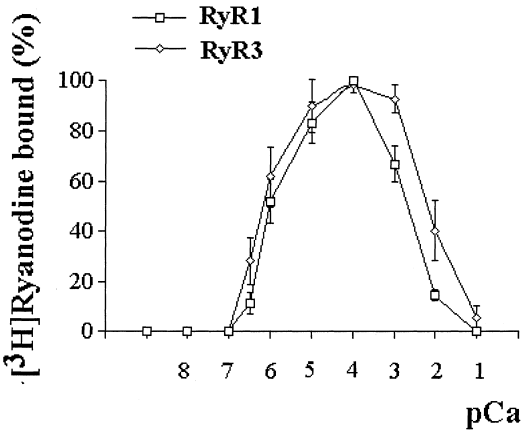

Fig. 2. Ca^{2+} dependence of [^{3}H]ryanodine binding. 50 μg of microsomes from bovine muscle, expressing RyR1, 50 μg of microsomes from RyR3-transfected cells were incubated with 7 nM [^{3}H]-ryanodine in a solution containing 0.2 M KCl, 10 mM HEPES pH 7.2, for 1.5 h at 36°C with various concentrations of free Ca^{2+} buffered with EGTA. Binding was normalized on the maximum binding value at 100 μM Ca^{2+}. Data are the mean ± S.D. of $n = 18$ (RyR1) and $n = 18$ (RyR3).

binding assays were performed in the presence of increasing concentrations of free Ca^{2+}. Fig. 2 shows the Ca^{2+}-dependent response of the RyR3 channel. Specific binding started to increase at 500 nM Ca^{2+} (pCa 6.5) and was maximal at 100 μM Ca^{2+} (pCa 4). At higher [Ca^{2+}], [^{3}H]ryanodine binding started to decrease. In addition to the Ca^{2+} sensitivity curve of RyR3 channels, Fig. 2 reports also similar experiments performed with RyR1 channels. Under these conditions, no major difference could be detected in the activating kinetics of the two channels. However, a small but significant difference between RyR1 and RyR3 was observed at concentrations between 1 and 10 mM Ca^{2+}, which corresponds to the inactivating part of the curve.

3.2. Dose–response effect of $IpTx_a$ on RyR3

To test whether RyR3 channels were affected by $IpTx_a$, [^{3}H]ryanodine binding assays were carried out with recombinant RyR3 protein expressed in HEK293 cells. Fig. 3A shows the dose-dependent effect of $IpTx_a$ at concentrations between 1 and 100 nM on [^{3}H]ryanodine binding to RyR3 microsomes in the presence of 10 μM Ca^{2+}. [^{3}H]Ryanodine binding in the absence of $IpTx_a$ was considered 100%. $IpTx_a$ was unable to enhance the [^{3}H]ryanodine binding to RyR3 microsomes at concentrations of 1 and 5 nM. At 10 and 100 nM $IpTx_a$ the [^{3}H]ryanodine binding reached a value of 109.0 ± 8.1% and 155.8 ± 12.8%, respectively. In contrast, $IpTx_a$ stimulated [^{3}H]ryanodine binding to RyR1 at a concentration of 5 nM (118.3 ± 10.0%) and stimulation increased to 150.6 ± 4.7% and to 241.2 ± 5.9% in the presence of 10 and 100 nM $IpTx_a$, respectively (Fig. 3B).

3.3. Differential sensitivity of RyR isoforms to $IpTx_a$: enhancement of [^{3}H]ryanodine binding

$IpTx_a$ has been found to preferentially stimulate [^{3}H]ryanodine binding to RyR1 and not to RyR2 channels [18,19]. The effect of $IpTx_a$ on RyR3 was therefore compared with those obtained on RyR1 and RyR2 channels (Fig. 4). In agreement with previous experiments, in the presence of 100 nM $IpTx_a$, the binding of [^{3}H]ryanodine to RyR3 microsomes was increased up to 151.0 ± 6.7%. The same $IpTx_a$ concentration stimulated [^{3}H]ryanodine binding to RyR1 to 219 ± 36.1%. No activation of [^{3}H]ryanodine binding was observed when RyR2 channels were used. These results confirmed that $IpTx_a$ can increase [^{3}H]ryanodine binding to RyR3 channels. This effect, although less pronounced than that obtained to RyR1, was significantly higher than that observed with RyR2 channels.

3.4. Ca^{2+} dependence of $IpTx_a$ effects on RyR3 channel

$IpTx_a$ has been reported to activate [^{3}H]ryanodine binding to RyR1 channels over a wide range of Ca^{2+} concentrations, producing a generalized increment of the bell-shaped binding curve. However, $IpTx_a$ displays erroneous effects on RyR2, activating binding moderately at low [Ca^{2+}] and inhibiting it also moderately at high [Ca^{2+}] [18,26]. This effect by $IpTx_a$ transforms the bell-shaped [^{3}H]ryanodine binding curve of RyR2 into a sigmoidal curve [18,26], suggesting that Ca^{2+} activation and inhibition sites are not affected equally by the peptide. Therefore, we analyzed the effect of $IpTx_a$ on the Ca^{2+} dependence of [^{3}H]ryanodine binding to RyR3 chan-

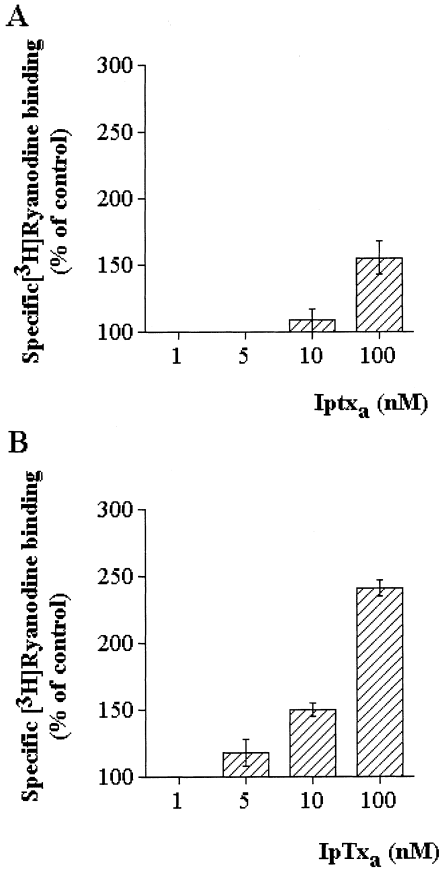

Fig. 3. Effect of $IpTx_a$ on [^{3}H]ryanodine binding. A: [^{3}H]Ryanodine (7 nM) was incubated with 50 μg of RyR3 microsomes in 0.2 M KCl, 10 μM $CaCl_2$ and 10 mM Na-HEPES (pH 7.2) for 90 min at 36°C in the presence of 1, 5, 10, 100 nM $IpTx_a$. Control binding (100%) was defined as the specific binding of [^{3}H]ryanodine in the absence of peptide toxin. Data are the mean (± S.E.M. of $n = 5$, 2, 7, 6 for 1, 5, 10 and 100 nM $IpTx_a$) of the percentage of binding increment induced by $IpTx_a$. B: [^{3}H]Ryanodine binding to RyR1 microsomes was carried out under the same conditions. Data are the mean ± S.E.M. of $n = 9$, 3, 10, 9 for 1, 5, 10 and 100 nM $IpTx_a$, respectively.

nels. Again, RyR1 was a pivotal comparison, both as a positive control for $IpTx_a$ and as an index of binding affinities of the Ca^{2+} activation and inhibition sites.

Fig. 5A shows that in the presence of $IpTx_a$ (filled circles), the binding curve maintains a bell shape and is increased about 1.5-fold compared to control. Moreover, $IpTx_a$ activates [^{3}H]ryanodine binding at all [Ca^{2+}], except for pCa 7 and pCa 2. The Ca^{2+} dependence of [^{3}H]ryanodine binding to RyR1 is shown in Fig. 5B. In the absence of $IpTx_a$ (control, open circles) [^{3}H]ryanodine binding reached the maximum value at pCa 4, falling sharply at pCa 3. In the presence of $IpTx_a$ (filled circles) [^{3}H]ryanodine binding increased up to 835.0 ± 6.9 fmol/mg protein at pCa 4, or about four-fold compared to control. At pCa 6, in the presence of $IpTx_a$, the [^{3}H]ryanodine binding to RyR3 was still increasing although the [^{3}H]ryanodine binding to RyR1 reached almost the plateau.

3.5. Single channel analysis of $IpTx_a$ effects on RyR3 channels

In a previous report, Tripathy et al. showed that RyR2, although insensitive to $IpTx_a$ in [^{3}H]ryanodine binding assays, was modified by the peptide in lipid bilayer recordings. A side-by-side comparison of effects of $IpTx_a$ on RyR1 and RyR2 revealed that the peptide increased [^{3}H]ryanodine binding to RyR1 only, even though $IpTx_a$ induced the appearance of kinetically indistinguishable subconductance states on RyR1 and RyR2 [26]. Thus, $IpTx_a$ presents itself as a modulator of RyRs with the rare property of producing divergent effects on [^{3}H]ryanodine binding and lipid bilayer assays. To test whether $IpTx_a$ was capable of modifying the single channel behavior of RyR3, we reconstituted microsomes from HEK293 cells expressing RyR3 and skeletal muscle microsomes (as source of RyR1) into lipid bilayers and recorded single channel activity in the absence and the presence of $IpTx_a$. As in previous studies [19,26], we recognized RyRs by their characteristic fast-flickering, high-conductance (~700 pS in monovalent cations) openings, their Ca^{2+} dependence, and their response to ryanodine (not shown).

Fig. 6 shows that $IpTx_a$ modified the activity of both RyR1 and RyR3 in a similar manner. Less than 1 min after addition of 300 nM $IpTx_a$ to the *cis* (cytosolic) side of the channel, RyR1 and RyR3 openings were transformed into long-lived

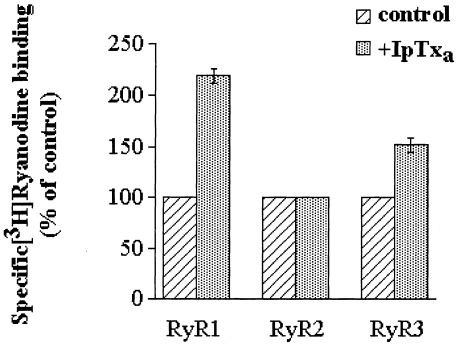

Fig. 4. Effect of $IpTx_a$ on [^{3}H]ryanodine binding to RyRs. 50 μg of RyR1 microsomes from bovine muscle, of RyR2 microsomes from bovine heart and microsomes from RyR3-transfected cells were incubated with 7 nM [^{3}H]ryanodine in 0.2 M KCl, 10 mM HEPES pH 7.4, 10 μM Ca^{2+} for 90 min at 36°C in the presence of 100 nM $IpTx_a$. The binding of [^{3}H]ryanodine in the absence of $IpTx_a$ corresponds to the control (100% specific binding). Data are the mean ± S.E.M. of $n = 27$ (RyR1), $n = 5$ (RyR2), and $n = 15$ (RyR3).

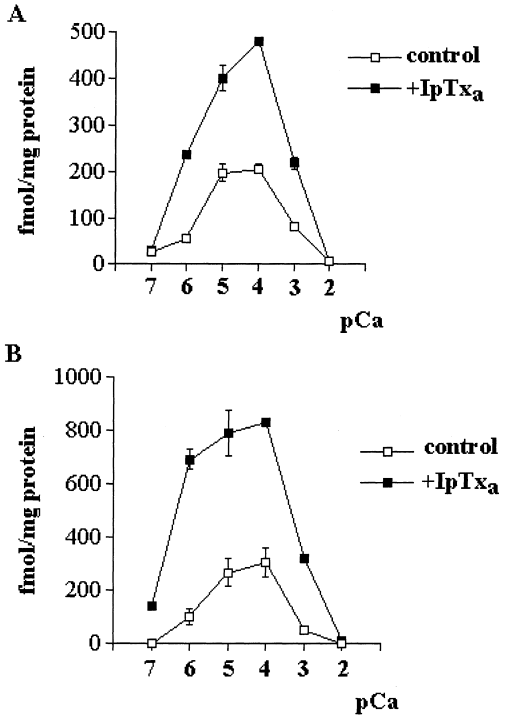

Fig. 5. Ca^{2+} dependence of $IpTx_a$ activation of [^{3}H]ryanodine binding. 50 μg of recombinant RyR3 (A) and 30 μg of bovine skeletal (B) microsomes were incubated for 90 min at 36°C with 7 nM [^{3}H]ryanodine in the absence (open squares) or in the presence (filled squares) of 100 nM $IpTx_a$. The incubation medium consisted of 0.2 M KCl, 10 mM Na-HEPES (pH 7.4) and $CaCl_2$ and EGTA concentrations to yield the indicated free [Ca^{2+}]. Ca^{2+}:EGTA ratios were calculated using the stability constants given by Fabiato [3]. Data points are the mean (± S.E.M.) of two independent determinations.

subconductance states of ~30% amplitude relative to the full conductance. Although of small amplitude, the $IpTx_a$-induced subconductance state displayed a mean open time that was > 100-fold longer than that of unmodified channels. Ion flow would therefore be expected to be greater for an $IpTx_a$-modified channel, despite its lower conductance. Thus, in the case of RyR1 and RyR3, the effects of $IpTx_a$ at the single channel level appear to be convergent with its effects on [^{3}H]ryanodine binding assays.

4. Discussion

A large number of physiological and pharmacological modulators of RyR channel activity have been tested to distinguish their capacity to differentiate the modality of activation and inactivation of the channels encoded by three RyR isoforms [11]. However, while a large number of data on regulation of RyR1 and RyR2 channels have been reported, far less is known about RyR3 channels. Most of the work performed on RyR3 channels has so far explored the sensitivity of these channels to Ca^{2+}. Although it is commonly accepted that Ca^{2+} has a biphasic effect on RyRs (activating at low and inhibitory at high concentrations), controversial results have been reported when comparing the activation and inactivation kinetics of RyR3 channels using different techniques. Single channel recordings of RyR3 channels reconstituted in lipid bilayer have revealed a 10-fold lower sensitivity to inactiva-

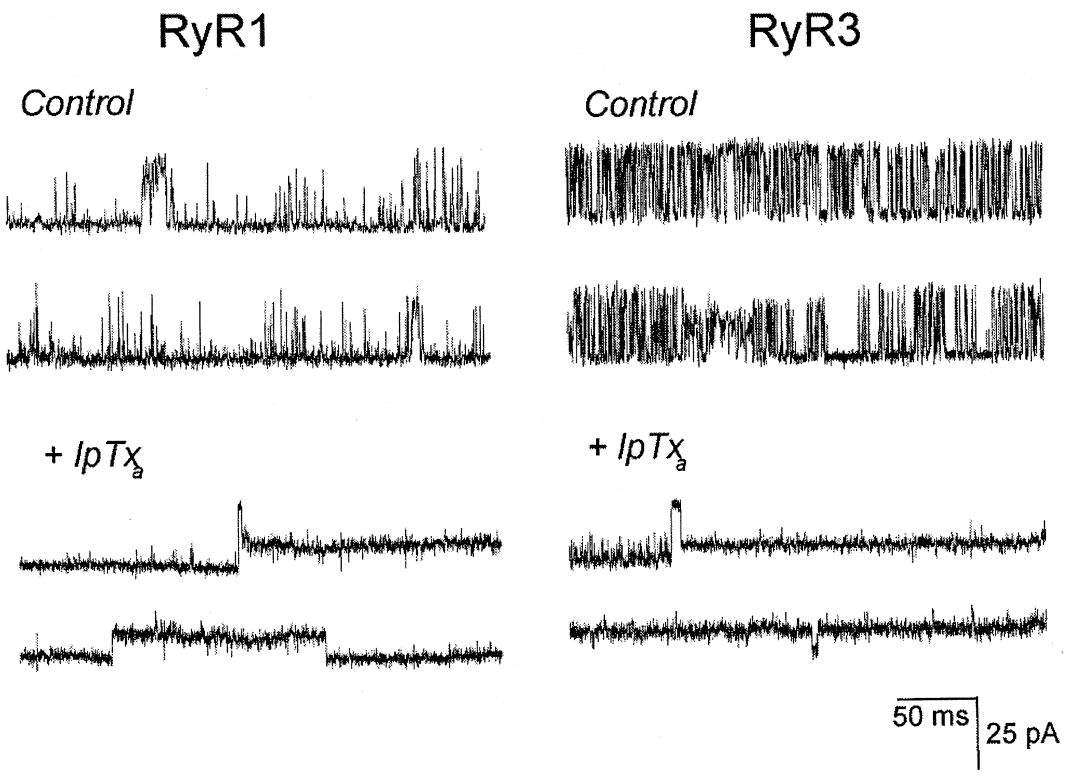

Fig. 6. Effect of $IpTx_a$ on single channel recording of RyRs. $IpTx_a$ induces a subconductance state in RyR1 and RyR3 channels. The top recordings were obtained under control conditions and the bottom recordings after addition of 300 nM $IpTx_a$ to the *cis* solution.

tion by high Ca^{2+} concentrations for RyR3 compared to RyR1 channels [15,16,27–29]. In contrast to single channel data, Murayama and Ogawa [30–31] reported that, in [^{3}H]ryanodine binding assays, a small but significant difference was observed only at low (activating) Ca^{2+} concentrations, while inactivation at higher Ca^{2+} concentrations was comparable between RyR1 and RyR3 channels. In the [^{3}H]ryanodine binding experiments described in the present report, a somewhat lower sensitivity to Ca^{2+} inactivation for RyR3 channels was observed compared to RyR1 channels, but this is certainly less evident than that reported in single channel analysis [15,16,27–29]. Whether these discrepancies reflect the regulatory effects of accessory proteins that are present in microsomal preparations used for [^{3}H]ryanodine binding and that are lost under experimental conditions for single channel analysis in lipid bilayers, similarly to what is reported for Calmodulin and $InsP_3$ receptors [32], is currently unknown.

A clearer distinction between the properties of the RyR3 channels and those of the two other RyR isoforms has been obtained by studying the effects of $IpTx_a$ on [^{3}H]ryanodine binding. In this study, we found that $IpTx_a$ stimulates [^{3}H]ryanodine binding to RyR3 channels. Although this response was smaller than that observed with RyR1 channels, which where activated at lower concentrations of $IpTx_a$, and reached higher levels of activation at saturating concentrations of $IpTx_a$, the effects of $IpTx_a$ on RyR3 channels differed from those observed with RyR2 channels. For example, the latter were found to be essentially insensitive to $IpTx_a$, in agreement with previous reports [18,19]. Similar to effects with RyR1 and again at variance with those on RyR2 channels [18,26], the effect of $IpTx_a$ on RyR3 channels was observed at all Ca^{2+} concentrations. Enhancement of [^{3}H]ryanodine binding was in fact observed both at Ca^{2+} concentrations that usually increase ryanodine binding to RyR3 channels as well as at Ca^{2+} concentrations that inhibit the binding.

An intriguing aspect, emerging from this and from previous studies on $IpTx_a$ effects on RyRs, is the apparent discrepancy between the distinctive responses in [^{3}H]ryanodine binding of the three RyR isoforms and the fact that, in single channel experiments, all three isoforms display a similar subconductance state following $IpTx_a$ treatment [19,26]. In this respect, it is interesting to note that $IpTx_a$ shares some resemblance with a region of the α_1 subunit of the skeletal muscle (α_{1S}) DHPR that has been implicated in binding to and activation of RyR1 channels [19]. However, in contrast to the effect on RyR1, the II–III loop of α_{1S} can neither bind nor activate RyR2 channels [20]. Actually, indirect evidence suggests that RyR3, like RyR2 channels, is not directly activated by the α_{1S} DHPR in skeletal muscle [33,34]. Therefore, the question is how can $IpTx_a$ (1) have distinctive effects on RyR isoforms as measured by [^{3}H]ryanodine binding (which is usually known to directly correlate with the open state of the channel); (2) be able to induce an almost identical subconductance in all three channels and (3) share an intriguing homology with the α_{1S} subunit that can activate and bind only RyR1 channels?

One way to rationalize these data is to divide the effects of $IpTx_a$ binding to RyRs in two events. The first event is an event common to all isoforms, which results in the induction of the subconductance state observed in single channel analysis in all three isoforms. Tripathy et al. [26] raised the question whether the subconductance state induced by $IpTx_a$ is due to occlusion by the toxin of the vestibule of the channels

or due to a conformational change. While this question is not definitively answered, the conformational change hypothesis is more favored. Here we propose that, in addition to this first event, which results in an equivalent change in all three isoforms, $IpTx_a$ can also induce an additional isoform-specific conformational change. This isoform-specific conformational change would then be responsible for the differences observed in [^{3}H]ryanodine binding experiments in the presence of $IpTx_a$ with the three RyR isoforms. Whether DHPR and $IpTx_a$ bind to the same site or to different sites on RyR1 channels is not known. Recent results indicate that there could be multiple sites of interaction between α_{1S} DHPR and RyR1 and that more than one region in both molecules are involved in RyR1/DHPR interaction [35,36]. It is therefore likely that $IpTx_a$ either binds to a smaller region of a larger interacting domain or to a site distinct from that of the α_{1S} DHPR. In both cases the determinant of $IpTx_a$ binding has to be conserved among the three isoforms, while those sequences that are necessary for α_{1S} DHPR binding in RyR1 are obviously not conserved in RyR2 and RyR3.

Recently images of $IpTx_a$ bound to RyR1 have been obtained by cryo-electronmicroscopy (EM) and three-dimensional reconstruction techniques [37]. Should the structure of the $IpTx_a$-bound RyR1 channels be distinguishable from those of $IpTx_a$-bound RyR2 and RyR3 channels, the hypothesized differences in conformation induced in RyR isoforms by $IpTx_a$ could be experimentally tested in the future by cryo-EM techniques.

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