Multiple Actions of Imperatoxin A on Ryanodine Receptors

INTERACTIONS WITH THE II–III LOOP "A" FRAGMENT*

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Imperatoxin A is a high affinity activator of ryanodine receptors. The toxin contains a positively charged surface structure similar to that of the A fragment of skeletal dihydropyridine receptors (peptide A), suggesting that the toxin and peptide could bind to a common site on the ryanodine receptor. However, the question of a common binding site has not been resolved, and the concentration dependence of the actions of the toxin has not been fully explored. We characterize two novel high affinity actions of the toxin on the transient gating of cardiac and skeletal channels, in addition to the well-documented lower affinity induction of prolonged substates. Transient activity was (a) enhanced with 0.2–10 nM toxin and (b) depressed by >50 nM toxin. The toxin at ≥1 nM enhanced Ca2+ release from SR in a manner consistent with two independent activation processes. The effects of the toxin on transient activity, as well as the toxicity of the native state, were independent of cytoplasmic Ca2+ or Mg2+ concentrations or the presence of adenine nucleotide and were seen in diisothiocyanostilbene-2,2’-disulfonic acid-modified channels. Peptide A activated skeletal and cardiac channels with 100 nM cytoplasmic Ca2+ and competed with Imperatoxin A in the high affinity enhancement of transient channel activity and Ca2+ release from SR. In contrast to transient activity, prolonged substate openings induced by the toxin were not altered in the presence of peptide A. The results suggest that Imperatoxin A has three independent actions on ryanodine receptor channels and competes with peptide A for at least one action.

Excitation-contraction (EC)1 coupling is the process that facilitates Ca2+ release from the sarcoplasmic reticulum (SR) of muscle fibers following depolarization of the surface/transverse (t)-tubule membrane. A protein-protein interaction between the dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) underlies EC coupling in skeletal muscle. The DHPR L-type Ca2+ channel in the t-tubule membrane detects surface depolarization and transmits a signal to the RyR channel in the SR via an interaction between the cytoplasmic domains of the two proteins. The interacting region of the DHPR is located between the second and third transmembrane repeats in the α1 subunit (II–III loop) (1). The interacting regions of the RyR are less clearly defined but are likely to involve residues 1076–1112 (2) and residues 1837–2168 (3).

The recombinant DHPR II–III loop activates skeletal RyRs (4, 5). The loop has been arbitrarily divided into four segments, A, B, C, and D (6). The C region (residues 720–765) is strongly implicated in EC coupling (7–9), and a random coil peptide corresponding to this region modifies the activity of the skeletal RyR (10, 11). A second region of the II–III loop, the A region (residues 671–690), is of interest because its corresponding fragment induces Ca2+ release from the SR and enhances current flow through RyR channels with high affinity (5, 6, 10, 12–18). Although the A region is not essential for skeletal EC coupling in myocytes (7, 9), it may play a role in the DHPR-RyR interaction (8), and it is a useful probe for assessing RyR function (11, 17, 19).

The ability of peptide A to activate RyRs is highly correlated with its capacity to adopt an α-helical structure (15, 16, 18) and with the orientation of positively charged residues along one surface of the molecule (18). Curiously, two scorpion toxins, Imperatoxin A and Maurocalcine (having 82% sequence identity), have structural features in common with peptide A. Although the intrinsically disulfide-stabilized β-sheet structure of the toxins is vastly different from the α-helical structure of the A peptide, the toxins and peptide A share a similar surface orientation of positively charged residues (18, 20). Because of this structural similarity, several studies have examined the possibility that the scorpion toxins and peptide A bind to the same site on RyR1. Two studies have concluded that they bind to the same, or overlapping sites (13, 18), whereas a third study concluded that Maurocalcine/Imperatoxin A and peptide A bind to independent sites (19). These different conclusions could have arisen if more than one binding site exists for either the toxins and for peptide A, and not all sites are common to the two compounds. At least two binding sites for peptide A have been defined, one within the channel pore, which leads to voltage-dependent channel block (12), and a site (or sites) on the cytoplasmic domain of the channel, which support its voltage-independent actions (10, 12). Here we investigate the possibility that Imperatoxin A also has multiple actions on RyR activity and examine interactions of between the toxin and peptide A in modifying RyR channel gating.

We find that there are at least three separate actions of Imperatoxin A on cardiac and skeletal RyR channels that can be
distinguished by their affinity and reversibility and by their ability to compete with peptide A in its native and a modified form. The results show that peptide A competes with Imperatoxin A for the high affinity activation of transient channel openings. On the other hand, peptide A does not prevent the characteristic toxin-induced prolonged substate activity. The results suggest that there is at least one overlapping binding site for Imperatoxin A and peptide B as well as independent binding sites. These observations raise the question of which of the Imperatoxin A binding sites has been identified on the RyR (21) and whether this site is the site that also interacts with peptide A.

MATERIALS AND METHODS

Peptides—Peptides A and A1(D-R18) were synthesized as in Green et al. (18). Imperatoxin A was synthesized by Auspep Australia and folded using procedures outlined by Fajloun et al. (22).

Vesicle Preparation—Preparation of SR vesicles, Ca2+ release from SR, and single channel techniques have been described previously. Heavy skeletal SR vesicles were prepared from rabbit back leg and muscle (11, 12), whereas cardiac SR vesicles were prepared from sheep heart (23).

Single Channel Techniques—Blayers of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:5:2 w/w) (Avanti Polar Lipids, AlaBaster, AL) were formed across an aperture of ~200-μm diameter in the wall of a 1.0-ml Delrin cup (Cadillac Plastics, Australia). Terminal cisternae vesicles (10 μg/ml) were added to the cis chamber. The cytoplasmic side of channels incorporated into the bilayer faced the cis solution. Bilayer potential was controlled, and single channel currents were recorded, using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Bilayer potential is expressed as \( V_c \sim V_{\text{trans}} \) (cytoplasmic) – \( V_{\text{trans}} \) (membrane).

Bilayers were formed and vesicles incorporated using cis solutions containing (in millimolar): 230 CsCH\(_3\)O\(_3\), 20 CsCl, 5.0 CsCl, 10 TES (pH 7.4). Following incorporation, (a) the cis solution was replaced with an identical solution, except that the [Ca\(^{2+}\)] was between 0.1 and 100 μM and (b) 200 mM CsCl added to the trans chamber for symmetry. Drugs were added to the cis chamber and removed by perfusion with 10 μl of cis solution.

Analysis of Channel Activity—Channel activity was analyzed over one to two 30-s periods of continuous activity at +40 mV and then at –40 mV. Slow fluctuations in the baseline were corrected using an in-house baseline correction program (written by Dr. D. R. Laver). Channel activity was measured either as “mean current” (the average of all data points in a record) or as open probability (\( P_o \)), using a threshold analysis with the program Channel 2, (developed by P. W. Gage and M. Smith, John Curtin School of Medical Research). Measurements of mean current, performed on records from experiments containing 1–4 channels, included all channel activity from the smallest subconduction level to maximum openings. On the other hand, open probability (\( P_o \)), mean open time (\( T_o \)), and mean closed time (\( T_c \)) measurements are restricted to records in which the opening of a single channel only could be detected and exclude openings that fall within the baseline noise. In this case threshold levels for channel opening and closing were set to exclude baseline noise (a) at ~20% of the maximum single channel conductance when examining transient openings in the absence of prolonged substates or (b) at 50% of the maximum conductance or 50% of the steady state conductance, whichever was greater, when substates were present.

Channel activity is expressed as relative \( P_o \) to include data in which activity varied from ~0.001 to ~0.1 and data from bilayers containing more than one channel. Relative \( P_o \) was calculated either (a) from \( I/V_{\text{c}} \) at \( I/T \) is the mean current under test conditions and \( V_c \) is the control mean current, or (b) from \( I/\text{Pmax} \), where \( P \) is the open probability under test conditions and \( \text{Pmax} \) the control open probability. Because the mean current divided by the maximum current approximates open probability, \( I/\text{Pmax} \approx P_o \).

DIDS Modification—Channels modified by the disulfonic stilbene derivative, diisothiocyanostilbene-2,2-disulfonic acid (DIDS), were used in some cases with a cytoplasmic [Ca\(^{2+}\)] of 100 nM to enhance channel activity under control conditions. DIDS modification does not alter the regulation of RyRs by Mg\(^{2+}\), Ryanodine, or ruthenium red (24), although it can interact with other properties of RyRs (25–27). Channels were exposed to 100 or 300 μM DIDS in the cis chamber for 4–6 min, and then DIDS was removed by perfusion. Activity increased in the presence of DIDS and then fell with removal of the reversible component of activation. However, activity remained higher than before exposure to DIDS because of covalent bonds formed between isothiocyanate groups and NH\(_2\) OH, and aromatic groups on a variety of amino acid residues (28).

Ca\(^{2+}\) Release from SR—Extravesicular Ca\(^{2+}\) was monitored at 710 nm using a Cary 3 spectrophotometer (12). The cuvette solution was stirred continuously, and temperature was controlled at 25 °C. Skeletal SR (100 μg of protein) was added to the cuvette solution (final volume of 2 ml), containing (in millimolar): 100 KH\(_2\)PO\(_4\), (pH 7); 4 MgCl\(_2\); 1 NaN\(_3\), ATP; 0.5 antipyrindilavolo III. Ca\(^{2+}\)-ATPase activity was suppressed with thapsigargin (200 nM (29)). The same solutions were used with cardiac SR except that an ATP-regenerating system (phosphoenolpyruvate (5 mM) and pyruvate kinase (25 μM) was added, and Ca\(^{2+}\)-induced Ca\(^{2+}\) release was triggered by addition of 20 μM Ca\(^{2+}\) to the cuvette solution.

When toxin was examined alone, it was added 2 min after thapsigargin. When peptide was added before toxin, either peptide or an equivalent volume of water (vehicle) was added 2 min after thapsigargin, and then toxin or an equivalent volume of water (vehicle) was added after a further 2 min. Release rates in each experiment were measured 10–20 s after toxin addition. Ca\(^{2+}\) release rate, \( R \), as a function of [toxin] was fitted by a Hill equation, \( R = R_b + \frac{R_{max}}{1 + (\text{Tx}/\text{Tx}^\text{50})^n} \), where \( R_b \) is the baseline Ca\(^{2+}\) leak or Ca\(^{2+}\)-induced Ca\(^{2+}\) release in thapsigargin, \( R_{max} \) is the maximum toxin-induced release rate, \( \text{Tx} \) is [toxin], \( \text{Tx}^\text{50} \), the [toxin] for activation to 50% maximum, and \( n \) is the Hill coefficient.

Statistics—Average data are given as mean ± S.E. The significance of the difference between control and test values was tested using either (a) a Student’s t test, either one or two sided and either for independent or paired data, as appropriate or (b) using the non-parametric “sign” test (30). Differences were considered to be significant when \( p \leq 0.05 \).

RESULTS

RyR activity demonstrated two distinct gating modes, a “transient” mode and a maintained “substate” mode. The transient mode comprised all control channel activity and was observed in toxin-modified channels. In this mode, the duration of channel openings varied from ~0.5 to ~1000 ms with brief submaximal openings and longer openings to the maximum conductance. The maintained substate mode was seen only after exposure to Imperatoxin A and was characterized by openings lasting from ~1 to ~20 s. The number of transient openings increased with toxin concentrations between 200 μM and 20 μM, then declined with >20 μM toxin. Prolonged substate openings appeared with >100 μM toxin.

Imperatoxin A Alters the Probability of Transient RyR Channel Openings

Increased Transient Activity—Imperatoxin A applied at picomolar concentrations to the cis (cytoplasm) side of RyR channels caused an increase in the frequency of transient openings. The increase occurred rapidly, within the 30-s period of toxin addition and stirring. Prolonged substate openings were not induced with these low toxin concentrations. The activating effect on transient openings was only slowly reversible when the toxin was perfused from the cis chamber (Fig. 1, A–C).

Activity increased in cardiac (n = 20 experiments) and skeletal (n = 19) channels that were either Ca\(^{2+}\)-activated (cis Ca\(^{2+}\) of 10 or 100 μM; n = 18) or at sub-activating cis Ca\(^{2+}\) (100–300 nM) (n = 20), in the absence (n = 10) or presence of ATP (n = 17) (with 2 mM Mg\(^{2+}\), n = 4) or AMP-PNP (n = 5) and in DIDS-modified channels (n = 5) (Fig. 1, D and E). All channels were activated by toxin at concentrations up to 10 μM. At 50–300 nM, some channels were activated while others were inhibited. Channels recorded with low cis Ca\(^{2+}\) showed average activation at higher toxin concentrations than those recorded with higher activating cis Ca\(^{2+}\) (Fig. 1, D and E).

The changes in channel gating associated with increased activity were measured in a subset of data from skeletal and...
cardiac RyR channels (Table I). The increased activity of skeletal RyRs with 1 and 10 nM toxin was due to an increase in channel open time, and a reduction in closed time. The increase in open time was also seen in cardiac channels, but in contrast to skeletal channels, there was no significant change in closed durations at lower toxin concentrations.

**Inhibition of Transient Activity**—Transient channel openings decreased when the toxin was increased to between 50 and 500 nM (see Fig. 1C). Some channels were activated for several minutes after exposure to higher [toxin] and were then inhibited (Fig. 1A). Activity was rapidly restored to an activated (greater than control) level upon removal of the toxin, indicating rapid reversibility of the inhibitory action.

The inhibition of transient activity was independent of prolonged substate openings. Although all channels exhibiting prolonged substates also showed fewer transient openings, the number of transient openings was often substantially reduced in the absence of substate activity (100 nM toxin (Fig. 1A)). Inhibition reduced $P_o$ to less than control when the activity was initially high (in channels with activating cis Ca$^{2+}$ and/or ATP (Fig. 2A)). When activity was low (in the presence of 100–300 nM Ca$^{2+}$ and/or MgATP), $P_o$ fell to less than the toxin-activated level, but often remained higher than control (e.g. the skeletal channels in Table I).

The decline in transient activity was caused by an increase in closed time (Table I). Curiously, the open times in skeletal RyRs continued to increase as toxin concentration increased (Table I), perhaps indicating that the activating effect of the toxin increased with [toxin], but was overwhelmed by an independent inhibitory effect. Persistent inhibition was also seen following perfusion of inhibiting [toxin] from the cis chamber, when removal of inhibition revealed a strong slowly reversible activation (e.g. Fig. 2A). On average, cardiac RyRs showed a significant decline in open times and increase in closed dura-
Three discrete actions of Imperatoxin A on RyRs

The skeletal data were obtained either with 2 mM ATP and 10 μM Ca^{2+} in the cis chamber (n = 4) or with 300 nM Ca^{2+} and 2 mM MgATP (n = 3). The cardiac data were obtained with either 10 μM Ca^{2+} (n = 8) or with 10 μM Ca^{2+} plus 2 mM ATP (n = 4). The relative changes in channel parameters were similar under all conditions and similar at +40 mV and −40 mV. Therefore data under each condition at each potential are combined and expressed as relative open probability (P_o), relative open time (T_o), and relative closed time (T_c).

| Skeletal RyR | Toxin concentration |
|--------------|---------------------|
| Relative P_o| 1 nm                |
| Relative T_o| 4.30 ± 1.08*       |
| Relative T_c| 1.36 ± 0.45        |

| Cardiac RyR | Toxin concentration |
|-------------|---------------------|
| Relative P_o| 0.43 ± 0.07*       |

* A significant change in the open probability.

**Table I**

**Fig. 2. Inhibition of transient RyR activity by Imperatoxin A at ≥100 nM.** A, 5-s recordings at +40 mV (left) and 30-s all-points histograms (right) with 10 μM cis Ca^{2+}. Transient activity was initially activated (trace 2) and then inhibited (trace 3) by 100 nM toxin, with further inhibition by 500 nM toxin (trace 4) and some openings to the toxin-induced state (C). Activity was recovered to an activated level (relative to control) after toxin removal (trace 5). Average data in B were obtained at +40 and −40 mV. Open symbols indicate skeletal RyRs, and filled symbols indicate cardiac RyRs. ▲, 100 nM cis Ca^{2+}; 100 μM DIDS-modified (n = 5); ▼, 10 μM cis Ca^{2+}; 2 mM cis ATP (n = 4); ○, 10 μM cis Ca^{2+} and 2 mM cis ATP or AMP-PNP (n = 4). C, effects of Imperatoxin A on channel activity by Ca^{2+} and adenine nucleotides −40 and +40 mV. 200 nM toxin was added for 3–4 min before the cis [Ca^{2+}] was increased to 100 μM for 4 min and then 2 mM ATP or AMP-PNP added. Channels were either activated (left, n = 6) or inhibited (right, n = 5) by the toxin. Relative P_o is compared under control conditions (e, with 300 nM cis Ca^{2+}), with then toxin (T), cis [Ca^{2+}] of 100 μM (Ca) and then 100 μM plus 2 mM ATP or AMP-PNP (Ad, adenine nucleotide). The y axis is split to display the range of relative P_o values.

In DIDS-modified channels (n = 5) (Fig. 2B).

Inhibition of transient RyR activity by Imperatoxin A at ≥100 nM. A, 5-s recordings at +40 mV (left) and 30-s all-points histograms (right) with 10 μM cis Ca^{2+}. Transient activity was initially activated (trace 2) and then inhibited (trace 3) by 100 nM toxin, with further inhibition by 500 nM toxin (trace 4) and some openings to the toxin-induced state (C). Activity was recovered to an activated level (relative to control) after toxin removal (trace 5). Average data in B were obtained at +40 and −40 mV. Open symbols indicate skeletal RyRs, and filled symbols indicate cardiac RyRs. ▲, 100 nM cis Ca^{2+}; 100 μM DIDS-modified (n = 5); ▼, 10 μM cis Ca^{2+}; 2 mM cis ATP (n = 4); ○, 10 μM cis Ca^{2+} and 2 mM cis ATP or AMP-PNP (n = 4). C, effects of Imperatoxin A on channel activity by Ca^{2+} and adenine nucleotides −40 and +40 mV. 200 nM toxin was added for 3–4 min before the cis [Ca^{2+}] was increased to 100 μM for 4 min and then 2 mM ATP or AMP-PNP added. Channels were either activated (left, n = 6) or inhibited (right, n = 5) by the toxin. Relative P_o is compared under control conditions (e, with 300 nM cis Ca^{2+}), with then toxin (T), cis [Ca^{2+}] of 100 μM (Ca) and then 100 μM plus 2 mM ATP or AMP-PNP (Ad, adenine nucleotide). The y axis is split to display the range of relative P_o values.

Toxin-activated channels were further activated by Ca^{2+}, ATP, or AMP-PNP, whereas inhibited channels could not be activated to the same extent by these ligands (Fig. 2C). In this experiment, with 100 nM cis Ca^{2+}, cardiac RyR channels were either activated or inhibited by 200 nM toxin. The toxin-acti-
Peptide A Competes with Imperatoxin A for Activation of Transient Opening—It has been suggested that peptide A and Imperatoxin A compete for a single site on the RyR. Cardiac channels were activated by 1 nM toxin and by subsequent additions of peptide A up to 500 nM (Fig. 3A). Activity declined when the peptide was increased to 1 μM. However, the plateau of activation with peptide plus toxin was no greater than that with peptide alone, suggesting that there was no summation of the effects of the two compounds and supporting the concept of the same or overlapping binding sites. Similar results were obtained when channels were exposed to 10 nM toxin, although there was a significant reduction in activity when the [peptide] reached 500 nM. In a similar experiment (Fig. 3B), channels were first exposed to 50 nM peptide A and then to increasing concentrations of toxin. Activity tended to increase with peptide plus toxin up to 600 μM, but activation was significantly less than expected from the summation of two independent processes (Fig. 3B). Channel activity declined when higher concentrations of toxin were added with peptide. The decline in activity with higher concentrations of toxin plus peptide A (or modified peptide A (A1-R18D) (18)) was particularly apparent in skeletal RyR channels (Table II and Fig. 3, C and D). Transient openings declined with 1 μM toxin in the presence of 1 μM peptide A (Fig. 3), even though there was no inhibition of RyRs by either 1 nM toxin or 1 μM peptide A alone.

Prolonged Substate Openings Induced by Imperatoxin A

Imperatoxin A induces prolonged channel openings to substate levels in cardiac and skeletal RyRs (31). In the present experiments, toxin concentrations of 1–10 μM were required to consistently observe substate activity within 2–10 min of exposure to the toxin, although substate activity could occasionally be seen after prolonged exposure to 10–100 nM toxin. Channels demonstrated abrupt transitions between the substate and transient gating modes (Fig. 4A). In the substate mode, the channel was almost continually open with \( P_o \) close to 1.0 and average open times of \( -1-20 \) s. Substate gating was interrupted by bursts of transient activity with frequent channel openings from the closed to the maximum conductance levels (Fig. 4A).

The currents from the DIDS-modified channel in Fig. 4B, recorded with 100 nM Ca\(^{2+}\), illustrate three important features: 1) the fall in transient activity in the presence of 1 μM toxin; 2)
The similarity of the toxin's action (and substate levels) at \(+40\) and \(-40\) mV; and 3) rapid recovery of transient openings after removal of the toxin. The substate activity remained for several minutes after removal of the toxin and restoration of transient activity. Two or three different conductance levels are apparent in the toxin-induced substate activity (Fig. 4). The highest conductance substate dominated and was a similar fraction of the maximum conductance at \(+40\) and \(-40\) mV (Table III). The

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**TABLE II**

Combined actions of Imperatoxin A (IpTx) and either the native peptide A or modified peptide A (A1-R18D) on the relative probability of transient skeletal RyR channel activity.

|                | 1 nM IpTx | 10 nM IpTx | 1 \(\mu\)M peptide A |
|----------------|-----------|------------|----------------------|
| **n = 4**      | 3.01 ± 0.70° | 4.34 ± 1.12° | 3.53 ± 0.67°         |
| **n = 5**      | 2.70 ± 0.85° | 1.64 ± 0.29° | 0.71 ± 0.16°         |
| **n = 6**      | 6.71 ± 1.84° | 5.84 ± 1.95° | 2.37 ± 0.70°         |

*° A significant change in the open probability.

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**Fig. 4.** Imperatoxin A induces prolonged substate openings with transitions between the substate and transient gating. A, activity recorded at \(-40\) mV from a cardiac RyR with 10 \(\mu\)M cis Ca\(^{2+}\) and 500 nM Imperatoxin A (left), and all-points histograms (right). The upper trace is a 30-s recording with transitions (i) between the closed state, the dominant toxin-induced substate at \(\sim 25\%\) of the maximum conductance, and a lower substate at \(\sim 12\%\) and (ii) between the substate and transient gating modes. The record was filtered at 100 Hz to show the different substate levels. The lower trace is an expansion of the transitions between substate and transient gating. B, Imperatoxin A-induced substates and gating transitions in a DIDS-modified cardiac RyR channel at \(-40\) and \(+40\) mV, with 100 nM cis Ca\(^{2+}\). In each panel, 5-s current records are to the left, and 30-s all-points histograms are to the right. The first trace in each panel shows DIDS-modified activity, the second and third traces show activity after adding 1 \(\mu\)M toxin then after removal of the toxin. Note (a) the similar dominant substate level, and smaller substate levels (dotted line next to the closed level), at both potentials, (b) the strong inhibition of transient gating in the presence of the toxin, and (c) the rapid recovery of transient activity following removal of the toxin, but maintained substate activity.
Three Discrete Actions of Imperatoxin A on RyRs

Properties of the prolonged toxin-induced subconductance state in skeletal and cardiac RyR channels

Data shown are averages of that obtained with 500 nM and 1 μM toxin for six cardiac channels or for 100 nM and 1 μM toxin for six skeletal channels.

| Substrate | % I_max | Substate probability in 30-s record | P_o within substrate |
|-----------|---------|-----------------------------------|----------------------|
| CARDIAC   |         |                                   |                      |
| +40 mV    | 0.275 ± 0.022 | 0.900 ± 0.082  | 3040 ± 2063        |
| -40 mV    | 0.291 ± 0.020  | 0.973 ± 0.011  | 7295 ± 5225        |
| SKELETAL  |         |                                   |                      |
| +40 mV    | 0.331 ± 0.010  | 0.916 ± 0.033  | 2083 ± 930         |
| -40 mV    | 0.336 ± 0.009  | 0.924 ± 0.022  | 1380 ± 899         |

* A significant change in the open probability.

Amplitude of substate

DISCUSSION

Activation of transient gating of skeletal and cardiac RyR channels by picomolar concentrations of Imperatoxin A is described for the first time. Novel observations with DIDS-modified channels illustrate the robust nature of (a) high affinity activation and lower affinity inhibition of transient activity by Imperatoxin A, (b) toxin-induced substate gating, and (c) activation by peptide A. We show that peptide A competes with Imperatoxin A only for actions on transient channel gating. The toxin-induced substate persists in the presence of peptide A, with additional transient openings to conductance levels peculiar to the peptide. The results raise questions about the functional effects of binding to the site identified for Imperatoxin A (21) and whether peptide A also binds to the site. Finally the multiple actions of the toxin provide an explanation for divergent reports of the existence of a common binding site with peptide A (13, 18, 19).

Note that the rates of Ca^{2+} release from the crude cardiac SR preparation are significantly lower than those from the heavy skeletal SR preparation, largely because of an ~10-fold greater contamination with longitudinal SR vesicles that do not contain Ca^{2+} release channels.
Multiple Independent Actions of Imperatoxin A—The effects of Imperatoxin A are separated by concentration dependence, reversibility, gating, and interactions with peptide A. The increase in transient activity, with as little as 200 pM toxin, occurred with an increase in open time and decrease in closed times, and was irreversible in the short term. Activated channels were further activated by Ca\(^{2+}\) and ATP (or AMP-PNP). Peptide A competes with the toxin for transient activation. Toxin at >10 nM inhibited transient openings and prolonged closed times. Inhibited channels could not be fully activated by ATP or AMP-PNP. Inhibition was (a) rapidly reversed when the toxin was removed and (b) enhanced by peptide A. The third action of the toxin was the induction of prolonged substates that persisted after recovery of transient gating when the toxin was removed and persisted in the presence of peptide A. The enhancement of transient RyR activity by picomolar concentrations of Imperatoxin A has not been reported, possibly because previous studies have not systematically examined of the concentration dependence of the toxin, but focused on the toxin-induced substate (31) and compared it with peptide A-induced substates (13).

Ca\(^{2+}\) Release from SR—Impertoxin A at \(\geq 1\) nM released Ca\(^{2+}\) from SR. A dual activating effect of the toxin was suggested by the need for two Hill functions fit to the data. The fits suggested a small high affinity effect added to a large low affinity effect, corresponding, respectively, to the high affinity activation of transient gating and the lower affinity substate induction. The effects on Ca\(^{2+}\) release were at \(\sim 10\)-fold higher concentrations than effects on channels in bilayers. The reason for this shift to higher [toxin] in Ca\(^{2+}\) release experiments is unclear but is also seen with the DHPR II–III loop and loop peptides (4, 12). Because each of the four RyR subunits are likely to contain toxin binding sites, it is curious that the Hill coefficients were around 1. This could mean that binding of one...
The inhibition of transient activity was not reflected in Ca²⁺ release from SR, possibly because (a) of variations between individual channels in the toxin concentration at which inhibition was apparent, (b) activity may have remained greater than control in some channels, even though it was less than the maximum toxin-activated level, and (c) any inhibitory effect on transient activity would have been swamped by the current flowing through substate openings at higher [toxin]. Under the bilayer conditions, the Pₜ for transient openings with low cis [Ca²⁺] in the presence of inhibiting toxin concentrations was between 0.001 and 0.0001, and thus the average current flowing through one channel would be between 0.01 and 0.001 pA, assuming there was a maximum single channel current of 10 pA. In contrast, when the channel was open at the substate level at 30% of the maximum conductance for 80% of the time, the average current through the channel would be ~2.5 pA.

**Actions of Peptide A**—Peptide A also has multiple independent effects. First, it increases transient activity in skeletal RyRs with high affinity (≥10 nM) and releases Ca²⁺ from skeletal SR (5, 6, 10, 12–18). Ca²⁺-induced Ca²⁺ release from cardiac SR is enhanced by the peptide and cardiac RyRs activated at sub-activating cis [Ca²⁺]. The second action is a voltage-independent inhibition, seen specifically with the modified A1-R18D at ≥10 nM in skeletal and cardiac RyRs with 100 μM cis Ca²⁺. The native peptide A is less active with 100 μM cis Ca²⁺ than with lower [Ca²⁺] (12, 17). Because peptide structure is not Ca²⁺-dependent, this inhibition raises the possibility either that there is a separate inhibitory site or the activation site can become inhibitory. The third action is a voltage-dependent block of the skeletal and cardiac RyR pore with ≥5 μM peptide A, leading to transient substate activity, particularly when the current pulls the peptide into the pore (at +40 mV) (10, 12, 32). The substates have conductances of <10–80% of the maximum conductance, with lower levels dominating at positive potentials.

**Interacts between Imperatoxin A and Peptide A**—Common and Independent Binding Sites—The non-additive nature of activation of transient gating by Imperatoxin A and peptide A is indicative of identical or overlapping sites so that binding of either peptide or toxin precludes binding of the other compound. In contrast, the toxin-induced substate activity persisted in the presence of peptide A suggesting independent binding sites (Fig. 6). The results with inhibition are less easily explained. Addition of peptide plus toxin at activating concentrations for the individual compounds, resulted in a decline in activity at +40 and −40 mV and shift of inhibition to lower [toxin]. Peptide A alone does not cause a voltage-independent inhibition with 100 nM cis Ca²⁺ (previous section), so that the results could suggest that peptide A binds to a site near the toxin inhibition site to increase the affinity for the toxin. However, an additive effect cannot be ruled out, because the modified A1-R18D can cause voltage-independent inhibition under appropriate conditions. Thus the shift in inhibition to lower toxin concentrations could be explained by the compounds binding to the same or different inhibitory sites. Additive effects could not be determined because inhibition by either compound reduced transient channel activity to very low levels. Overall, these data suggest that there is (a) a common transient activation site for Imperatoxin A and peptide A, (b) independent substate induction sites for the toxin and peptide, and (c) a toxin inhibition site whose relationship to the peptide A site is unclear (Fig. 6).

**Similar Actions of the Toxin and Peptide on Skeletal and Cardiac RyR Channels**—Imperatoxin A induces similar substate activity in skeletal and cardiac RyR channels (Table III and Ref. 31). Similarly, both skeletal and cardiac II–III loop and II–III loop peptides can interact with the either the skeletal or cardiac RyR (33, 34). These results imply that a physical interaction can occur between either skeletal or cardiac RyRs and DHPRs, if the proteins were appropriately targeted (32). This is difficult to test, because DHPRs do not form tetramers and are not aligned with cardiac RyRs (35).

**The Imperatoxin A-induced Substate**—The toxin-induced substate was ~30% of the maximum conductance at both +40 and −40 mV in cardiac and skeletal RyRs. This differs from reported Imperatoxin A-induced substates of 43% at ~40 mV and 28% at ~40 mV (31), perhaps because we used Ca²⁺ as the current carrier (as opposed to K⁺), or native rather than CHAPS-solubilized RyRs. Maurocalcine-induced substates are also voltage-independent (19).

**Previous Studies of Imperatoxin A and Peptide A Binding**—Findings that peptide A and Imperatoxin A do, or do not, bind to one site are consistent with multiple actions of the compounds. Gurrola et al. (13) showed competition between the toxin and peptide A in Ca²⁺ release from SR, [³H]ryanodine binding and ¹²⁵I-Imperatoxin A binding. Because these parameters do not reveal channel gating, the competition could have been for any of the functional sites and was most likely for the transient activation site. Indeed Green et al. (18) found interactions between peptide A and effects of the toxin on transient gating. Chen et al. (19) report independent peptide A and Maurocalcine-induced substates. Thus previous reports are consistent with the present finding that the toxin and peptide A compete for transient activation, but not for substate induction. In addition, the observation that peptide A inhibits the increase in [³H]ryanodine binding induced by the Imperatoxin A (13), even though toxin and peptide individually activate Ca²⁺ release, is consistent with the fact that transient channel gating is inhibited when toxin and peptide A are added together.

A binding site for Imperatoxin A has been identified on the
cytoplasmic domain of the RyR, close to the membrane (21). Because the site is likely to be a high affinity site, toxin binding to it may activate transient openings and thus also be the peptide A binding site. This site is remote from RyR domain 6, which is closest to the t-tubule membrane and the DHPR (36, 37). If the A region of the II–III loop does interact with the RyR under any conditions in vitro, it is likely that the interaction is with a part of the protein that is closer to domain 6, than the toxin binding site. It is thus possible that the inhibitory action of the peptide A and the toxin are caused by binding to a site that differs from the activation site and is located closer to the T-tubule membrane (Fig. 6).

In conclusion, we find that Imperatoxin A has three independent functional effects on cardiac and skeletal RyR channels. The skeletal DHPR II–III loop-derived peptide (peptide A) competes with the toxin for its high affinity activation of transient channel openings. Peptide A modifies a lower affinity toxin-induced inhibition of transient activity and has an independent action on the lowest affinity effect of the toxin in inducing prolonged substrate activity. We suggest that there are three functional binding sites for the toxin and that at least one site is shared by peptide A.

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Multiple Actions of Imperatoxin A on Ryanodine Receptors: INTERACTIONS WITH THE II-III LOOP "A" FRAGMENT
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