The Cysteine-rich Secretory Protein Domain of Tpx-1 Is Related to Ion Channel Toxins and Regulates Ryanodine Receptor Ca$^{2+}$ Signaling*

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The cysteine-rich secretory proteins (Crisp) are predominantly found in the mammalian male reproductive tract as well as in the venom of reptiles. Crisps are two-domain proteins with a structurally similar yet evolutionary diverse N-terminal domain and a characteristic cysteine-rich C-terminal domain, which we refer to as the Crisp domain. We presented the NMR solution structure of the Crisp domain of mouse Tpx-1, and we showed that it contains two subdomains, one of which has a similar fold to the ion channel regulators BgK and Shk. Furthermore, we have demonstrated for the first time that the ion channel regulatory activity of Crisp proteins is attributed to the Crisp domain. Specifically, the Tpx-1 Crisp domain inhibited cardiac ryanodine receptor (RyR) 2 with an IC₅₀ between 0.5 and 1.0 μM and activated the skeletal RyR1 with an AC₅₀ between 1 and 10 μM when added to the cytoplasmic domain of the receptor. This activity was nonvoltage-dependent and weakly voltage-dependent, respectively. Furthermore, the Tpx-1 Crisp domain activated both RyR forms at negative bilayer potentials and showed no effect at positive bilayer potentials when added to the luminal domain of the receptor. These data show that the Tpx-1 Crisp domain on its own can regulate ion channel activity and provide compelling evidence for a role for Tpx-1 in the regulation of Ca$^{2+}$ fluxes observed during sperm capacitation.

Tpx-1 (testis specific protein-1) was originally identified in the mouse (1) and later found in the male reproductive tract of the human, guinea pig, rat, and horse (2–5). Tpx-1 is a member of the cysteine-rich secretory proteins (Crisp)² that are in turn a subgroup of the CAP protein superfamily (abbreviated from Crisp, Antigen 5, and Pr-1 (6)). The CAP proteins each contain a structurally related and unique domain, the CAP domain. Previously, there has been no biological activity attributed specifically to this domain.

In mammals, there are at least four Crisp proteins, Crisp-1, Tpx-1 (or Crisp-2), Crisp-3, and Crisp-4. Crisp-1 proteins are expressed predominantly in the epididymides where they coat the surface of sperm during epididymal maturation (12) and have been implicated in sperm oocyte binding and the regulation of capacitation (13–17). Tpx-1 is expressed only in the testes and localized to specific regions in the spermatozoa, notably the acrosome of the head, the outer dense fibers, and longitudinal columns of the fibrous sheath in the sperm tail and the connecting piece of the neck (3, 18). Transfection experiments have also suggested that Tpx-1 is involved in adhesion between germ cells and Sertoli cells within the seminiferous epithelium (19). Crisp-3 is expressed more widely, including the salivary gland, pancreas, prostate, and B-cells (12, 20–22). Crisp-4 proteins are expressed exclusively in the epididymal epithelium in an androgen-dependent manner (23).

No clear biological function has been attributed to any mammalian Crisp protein, and characterizations have historically focused on expression location and timing to infer function. Nonmammalian Crisp proteins have, however, provided preliminary biochemical data. XCrisp expression occurs exclusively in the hatching gland of Xenopus and is associated with cellular membranes (24). The XCrissp Crisp domain is indirectly required for the degradation of the vitelline envelope through activation of the degradation pathway. Helothermine, a Crisp protein from the venom of the Mexican beaded lizard, causes the reversible concentration-dependent blockage of voltage-gated Ca$^{2+}$ and K$^+$ channels and ryanodine receptors (RyR) (25–28). Helothermine is the only Crisp protein known to regulate RyRs. Crisp proteins have also been identified in the venom from a range of snakes (29–33), and many have been shown to have specific K$^+$ or Ca$^{2+}$ ion channel inhibition activities and to block depolarization-induced muscle contraction (reviewed in Ref. 34). PsTx is one of the more comprehensively characterized Crisp proteins and has been shown to block CNGA2 channels through interaction with regions in or near the channel pore (35). Investigations on venom Crisp proteins were performed using full-length native proteins containing both the CAP and Crisp domains. As such, it remains unclear whether the ion channel inhibition activity can be attributed to the CAP domain, the Crisp domain, or to both.

Recently the crystal structures of Stecrisp and Triflin, Crisp proteins

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² The abbreviations used are: Crisp, cysteine-rich secretory proteins; RyR, ryanodine receptor; r.m.s.d., root mean square deviations; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence; HPLC, high pressure liquid chromatography; ICR, ion channel regulator; TOCSY, total correlated spectroscopy.

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The atomic coordinates and structure factors (code 2A05) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://rcsb.org/).

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from the venom of the snake *Trimersurus stejnegeri* and *Trimersurus flavoviridis*, respectively, were determined (9, 36). They showed that the CAP domain and the cysteine-rich Crisp domain were present as two discrete domains. The CAP domains had the same α-β-α fold as other CAP proteins P14a, Ves V5, and GAPR-1 (7, 8, 37). The Crisp domains included a linker region, containing two crossed disulfide bridges, and a domain with structural homology to the BgK (38) and ShK (39) ion channel inhibitor toxins from sea anemones.

As a first step toward defining the in vivo function of Tpx-1, we have determined the structure of the Crisp domain of recombinant mouse Tpx-1 by using NMR, and based on its homology with several ion channel inhibitors and published reports of RyR in mammalian sperm (40–43), we tested its ability to regulate RyR activity. We show that the Tpx-1 Crisp domain can elicit the activation of RyR1 and the inhibition of RyR2 when added to the cytoplasmic domain of the receptor and the activation of both forms when added to the luminal domain of the receptor. These data show the first structural representation of a mammalian Crisp domain and its structural homology to vertebrate and invertebrate toxins. We also show, for the first time, direct evidence that the Crisp domain is responsible for the ion channel inhibition activity previously observed in full-length Crisp proteins of lizard and snake origin.

**EXPERIMENTAL PROCEDURES**

**Cloning Expression and Purification of **15**N-Labeled Tpx-1 Crisp Domain**—The eDNA fragment encoding the Crisp domain of mouse Tpx-1 (beginning at CASCP) was subcloned into pTriEx3 (Neo) (Novagen), and the recombinant protein containing an N-terminal His6 tag and a thrombin cleavage site was expressed in *ORIGAMI B(DE3) pLacI* (Novagen), which facilitated the formation of nickel-nitrilotriacetic acid-immobilized metal affinity chromatography (to remove the His6 tag) and semi-preparative reversed phase HPLC to separate folded and mis-folded forms. The Tpx-1 Crisp domain was at least 95% pure, and ~98% of the purified recombinant Tpx-1 Crisp domain contained 15N as determined by liquid chromatography-mass spectrometry. Unlabeled protein was prepared using the same method, except expression was carried out in LB medium.

**Generation of Denatured Tpx-1 Crisp Domain for RyR Channel Control Experiments**—Mis-folded Tpx-1 Crisp domain was reduced with 10 mM dithiothreitol (Sigma) overnight and subsequently alkylated with 30 mM iodoacetamide (Sigma) for 90 min in the dark. The completeness of the alkylation reaction was assessed by mass spectroscopy. Alkylated protein was purified by reversed phase HPLC and freeze-dried. Immediately prior to use, proteins were resuspended in the required buffer, denatured by heating at 50 °C for 10 min, followed by rapid cooling on ice.

**NMR Spectroscopy**—NMR spectra were acquired at 500 MHz and 25 °C on a Bruker DRX500 equipped with a triple resonance TXI cryoprobe. Three-dimensional 15N-TOCSY-HSQC and 15N-NOSY-HSQC (45) experiments were acquired on a sample of ~1 mM uniformly 15N-labeled Tpx-1 Crisp domain in 90% H2O, 10% D2O, pH 5.8. pH values were uncorrected meter readings at room temperature. The sample was lyophilized and resuspended in 2H2O prior to the acquisition of the following two-dimensional experiments: double quantum-filtered correlated spectroscopy (46), TOCSY (47), NOESY (48), and 13C-HSQC. Acquisition parameters for the various experiments are summarized in Table 1.

Spectra were processed using XWINNMR and were analyzed using the program SPARKY (49). Sequence specific resonance assignments were made using standard methods (50).

**Structure Calculations**—Initial rounds of structure calculation were performed using the CANDID module as implemented in CYANA 1.0.6 (51). The final round of structure calculations was performed using XPLOR-NIH (52) using experimental NOE-derived distance constraints and C-α and C-β chemical shifts supplemented with Ramachandran data base potentials that were turned on during the annealing protocol. Structures were calculated using a simulated annealing protocol in torsion angle space (53) and finally subjected to energy minimization.

**RyR Channel Activity Measurements**—All methods for RyR channel activity measurements, including the preparation of sarcoplasmic reticulum vesicles, single channel techniques, analysis of channel activity, and the statistical analysis were performed as described previously (54). Tpx-1 Crisp domain was added to the cis chamber containing the cytoplasmic domain of RyR at concentrations of 0.1, 1.0, 10, and 50 μM. Tpx-1 Crisp domain was added to the trans chamber containing the luminal domain of RyR at 10 μM. All channel recordings using denatured Tpx-1 Crisp domain were done using 10 μM protein.

**RESULTS**

**NMR Spectroscopy**—1H and 15N resonances were assigned from NOESY-HSQC and TOCSY-HSQC experiments using standard procedures (50). A single peak was obtained for each 1H,15N pair in the HSQC experiment (Fig. 1) consistent with a single set of conformations in solution.

**TABLE 1**

| Experiment Parameters for NMR Data | Points acquired | Mixing time | Solvent |
|------------------------------------|-----------------|-------------|---------|
| **F3**                             | **F2**          | **F1**      |         |
| 15N-TOCSY-HSQC                     | 2048            | 40          | 128     |
| 15N-NOSY-HSQC                      | 2048            | 40          | 200     |
| DQF-COSY                           | 2048            | 400         | NA      |
| TOCSY                              | 2048            | 400         | NA      |
| NOESY                              | 2048            | 600         | 80 ms   |
| 15C-HSQC                           | 2048            | 128         | 200 ms  |

*NA indicates not assessed.*
Assignment of Tpx-1 Crisp Domain Disulfide Bonds—The disulfide bond connectivity in the Tpx-1 Crisp domain was determined from analysis of the NOESY spectra, which were examined for the presence of CAH/H9251–CBH/H9252 and CAH/H9252–CBH/H9252 correlations (55, 56). The absence of such NOEs was used to exclude potential disulfide bonds. This analysis suggested the disulfide bonding arrangement in the recombinant Tpx-1 Crisp domain to be Cys189–Cys196, Cys192–Cys201, Cys205–Cys238, Cys214–Cys232, and Cys223–Cys236. No anti-diagnostic NOEs were observed between CAH/H9251 and CBHN, which indicated that all cysteines were disulfide-bonded (57). This analysis was supported by the recently reported crystal structure of a native Crisp domain from Stecrisp (9).

The Tpx-1 Crisp Domain Structure Determination—Final structure calculations using XPLOR-NIH generated 894 distance constraints, including 230 long range NOEs, 226 medium range NOEs, 251 short range NOEs, 187 intra-range NOEs, and 39 CBCA shifts. Following structural refinement, a family of 23 structures (from a total of 49) with the lowest energies and least residual violations of the experimental restraints were chosen to represent the structure of the Tpx-1 Crisp domain. A summary of the structural statistics for this family of structures is given in Table 2. The structures have no violations of distance or dihedral restraints greater than 0.2 Å or 5°, respectively. They have good covalent geometry and favorable nonbonded contacts. 75% of the backbone φ/ψ angles are in the most favored region of the Ramachandran plot.

The positional root mean square deviations (r.m.s.d.) of the backbone heavy atoms and angular order parameters are shown in Fig. 2. From these data it is apparent that the Tpx-1 Crisp domain contains two distinct and folded subdomains; one includes two short β-strands and the other contains three short α-helices (Fig. 3A). The subdomains are linked via a poorly ordered loop encompassing residues Asn203–Ser204, and Fig. 3B highlights the relative rotational freedom of each subdomain. Stereoviews of the family of structures superimposed over the heavy atoms of the individual subdomains are presented in Fig. 3, C and D. Mean pairwise r.m.s.d. for the two subdomains are 0.78 and 1.29 Å over the backbone and heavy atoms of Cys192–Thr202 and 0.31 and 0.74 Å over the backbone and heavy atoms of residues Asp206–Cys236. No NOEs are observed between the two domains, suggesting that there is little or no interaction between them in solution. Coordinates for the final family of structures of Tpx-1 Crisp domain structures have been deposited in the Protein Data Bank under the accession code 2A05.

Comparison of the solution structure of Tpx-1 Crisp with the crystal structure of Stecrisp revealed that the two proteins adopt a similar fold. The positional r.m.s.d. of backbone heavy atoms (N, C, C-α) of the Tpx-1 Crisp domain and Stecrisp is 1.4 Å over amino acids Cys192 to

### Table 2

**Structural statistics for the family of Tpx-1 Crisp domain structures**

| Statistics for the family of 23 Tpx-1 Crisp structures |
|-------------------------------------------------------|
| Mean r.m.s.d. from experimental restraints             |
| NOE (Å)                                               | 0.0154 ± 0.0024 |
| δCα (ppm)                                            | 1.051 ± 0.089   |
| δCα (ppm)                                            | 0.748 ± 0.117   |
| Mean r.m.s.d. from idealized covalent geometry         |
| Bonds (Å)                                            | 0.0019 ± 0.0002 |
| Angles (°)                                           | 0.313 ± 0.018   |
| Improper     (°)                                      | 0.298 ± 0.0191  |
| Mean energies (kJ mol⁻¹)                              |
| NOE                                                  | 6.5 ± 1.86      |
| δCα/Cα                                               | 21.39 ± 3.99    |
| Bond                                                 | 3.02 ± 0.68     |
| Angle                                                | 22.17 ± 2.57    |
| Improper (°)                                         | 5.39 ± 0.70     |
| L-J                                                   | −123.67 ± 12.53 |
| Total                                                | −65.19 ± 13.73  |

FIGURE 1. 1H-15N HSQC spectrum of the Tpx-1 Crisp domain. Labels represent assignments for the cross-peaks, with the numbering referring to the position of the corresponding residue in mature Tpx-1. The side chain NH2 amide protons of the asparagine and glutamine residues are indicated with horizontal straight lines.
The Crisp domain of Tpx-1 and other Crisp proteins were responsible for this activity. We undertook experiments to test this hypothesis. That the Crisp domain of Tpx-1 and other Crisp proteins were responsible for this activity. The Crisp domain of Tpx-1 and other Crisp proteins were responsible for this activity. We undertook experiments to test this hypothesis.

The Tpx-1 Crisp Domain Shows a Subtype-specific Regulation of the Cytoplasmic Domain of RyR—The Tpx-1 Crisp domain, at concentrations between 0.1 and 50 μM, was added to the cis solution bathing the cytoplasmic side of RyR channels in lipid bilayers. The activity of the channels changed rapidly in a RyR subtype-specific manner when the protein was added. Cardiac RyR2 channels were inhibited, whereas skeletal RyR1 channels were activated. The addition of 10 μM denatured Tpx-1 Crisp domain to the cytoplasmic domain of RyR1 and RyR2 had no effect on the mean current at positive or negative applied bilayer potentials (Table 3).

The data in Fig. 5A show the decline in activity of a single RyR2 channel after addition of 1 μM Crisp domain to the cytoplasmic domain of RyR2 and further inhibition when the concentration was increased to 10 μM. The inhibition, apparent in the 3-s segments of activity and in the all-points histograms obtained from 30-s recordings, occurred within the 15-s stirring period after the protein was added to the solution. The inhibition was rapidly reversible when the 10 μM protein was perfused from the chamber (Fig. 5A) but did not reverse when 50 μM protein was removed, presumably because washout of the protein was incomplete, and the remaining Crisp domain after perfusion was sufficient to inhibit the channels. Although occasional full conductance openings were observed, most openings in the presence of the Tpx-1 Crisp domain were brief and poorly resolved. The average data obtained at bilayer potentials of +40 and −40 mV (Fig. 5, B and C, respectively) show the following: (a) the effects of the Tpx-1 Crisp domain on RyR2 inhibition were not voltage-dependent, (b) the IC₅₀ was between 0.5 and 1 μM, and (c) incomplete recovery after removal of higher concentrations of the protein.

The effect of the Tpx-1 Crisp domain on RyR2 gating was to decrease the duration of channel openings and increase the closed periods. In 5 of 11 experiments, single channel recordings showed that the mean open time with 10 μM Crisp domain fell from 3.8 ± 1.2 to 2.3 ± 0.4 ms, whereas the mean closed time increased from 125.6 ± 33.48 to 1177 ± 607.8 ms (Fig. 5).

In marked contrast to the inhibitory effect on cardiac RyR2 channels, the Crisp domain enhanced the activity of skeletal RyR1 channels when added to the cytoplasmic domain (Fig. 6). The recordings in Fig. 6 were obtained from a bilayer containing two RyR1 channels. Long openings from one channel were apparent with additional brief openings of a second channel sometimes summing with the first channel. After adding the Tpx-1 Crisp domain at 1 and 10 μM, the duration of the openings increased, and more summed events were seen (Fig. 6A). The activation occurred without any increase in the single channel conductance. Changes in activity of the brief 1.5-s segments of activity shown in the...
Tpx-1 Crisp Domain Regulates RyR

**FIGURE 4. Sequence alignment of the Crisp domain of selected Crisp proteins.** 100% conserved amino acids are shaded. Tpx-1 structural elements and cysteine bonding architecture are indicated at the top of the alignment. Numbering is based on the translated Tpx-1 sequence. Also indicated are the structural elements observed in the Stecrisp crystal structure. Regions corresponding to the Hinge and ICR in the Crisp domain are indicated at the bottom of the alignment.

**TABLE 3**
Relative mean current after addition of 10 μM Tpx-1 Crisp domain to the luminal and cytoplasmic domains of RyR subtypes

| Applied potential | Folded Tpx-1 Crisp domain | Denatured Tpx-1 Crisp domain |
|-------------------|---------------------------|-----------------------------|
|                   | Luminal domain            | Cytoplasmic domain          | Luminal domain | Cytoplasmic domain |
|                   | +40 mV                     | −40 mV                      | +40 mV         | −40 mV             |
| RyR1              | 1.10 ± 0.27                | 1.81 ± 0.51                 | 1.46 ± 0.13    | 2.41 ± 0.26        |
|                   | n = 6                      | n = 6                       | n = 6          | n = 6              |
| RyR2              | 1.11 ± 0.34                | 2.72 ± 0.75                 | 0.36 ± 0.16    | 0.35 ± 0.14        |
|                   | n = 7                      | n = 7                       | n = 7          | n = 8              |

The effect of the Tpx-1 Crisp domain on RyR1 gating was measured in 2 of the 15 experiments in which the opening of only one channel was observed. The range of mean open times increased from 2.7 ± 3.5 to 4.2 ± 7.3 ms, and the mean closed times fell from 156 ± 670 to 16 ± 198 ms. Similar increases in open periods and decreases in closed durations could be observed in all multiple channel recording like those in Fig. 6.

The Tpx-1 Crisp Domain Caused an Increase in Mean Current through RyR1 and RyR2 at Negative Bilayer Potentials When Added to the Luminal Domain—Table 3 shows that addition of 10 μM Tpx-1 Crisp domain to the trans solution bathing the luminal domain of RyR1 and RyR2 caused an increase in the mean channel current when a bilayer potential of −40 mV was applied. A change in mean current was observed within the 15-s mixing period and over several subsequent 30-s recording periods. Mean current through RyR2 was significantly greater than control current (p = 0.032), and there was a nonsignificant trend for increased mean current for RyR1 (p = 0.09). No change in mean channel current was observed on either channel with an applied bilayer potential of +40 mV. Recordings returned to control levels following perfusion. It was not possible to determine changes in mean open and closed times as no single channel recordings were observed.

The addition of 10 μM denatured control Tpx-1 Crisp domain to the luminal domain of RyR1 and RyR2 had no effect at positive or negative applied bilayer potentials (Table 3).

**DISCUSSION**

Ion channels play a critical role in maintaining cellular homeostasis, and endogenous and exogenous regulators of ion channels are therefore of significant interest from a biological and pharmacological perspective. Crisp proteins appear unique as they contain a conserved domain that regulates ion channel activity and are introduced exogenously in the venom from a range of species and are present endogenously in the male reproductive tract. In both of these environments they are highly abundant proteins (29, 59, 60).

We have determined the first NMR solution structure of a mammalian Crisp domain and have shown the following: 1) It has structural homology to the Crisp domain of Stecrisp (9) and to the voltage-sensitive K⁺ ion channel regulators BgK (38) and ShK (39) from sea anemones. 2) It can activate RyR1 and inhibit RyR2 channel openings when applied to the cytoplasmic domain of the receptor. 3) It can activate both RyRs when added to the luminal domain of the receptor at negative applied potentials. 4) This activity is specifically attributed to the tertiary structure of the protein domain as denatured protein had no effect. This is the first time the ion channel regulatory activity of a Crisp protein has been shown specifically to be associated with the Crisp domain, and Tpx-1 is the first soluble sperm protein shown to regulate RyR Ca²⁺ ion channel activity directly.

**The Tpx-1 Crisp Domain Has Two Subdomains**—Tpx-1 has two subdomains (Figs. 2 and 3) that are structurally similar to Stecrisp (9) (Fig.
7). The Tpx-1 Hinge encompasses amino acids Cys189 to Asn203, and the cysteine-rich domain encompasses C-terminal amino acids Ser204 to His243 (Fig. 4). Rather than the cysteine-rich domain, as used by Guo et al. (9), we suggest the use of the more descriptive term for the C-terminal subdomain based upon the functional data showing regulation of RyRs. Therefore, we propose naming this the ion channel regulator (ICR) domain.

The Hinge contains two short \( \beta \)-strands that are stabilized by crossed disulfide bonds that form a \( \beta \)-hairpin. The Stecrisp crystal structure showed the conserved LCTN motif in the Hinge to be in close proximity to a conserved hydrophobic core of the CAP domain with extensive hydrogen bonding resulting in a stable interaction (9). The absence of NOEs and structural restraint between the subdomains of the Tpx-1 Crisp domain suggests that within the full-length Crisp protein the ICR will freely rotate in solution relative to the Hinge and the CAP domain and implies two discrete activities for the CAP domain and the ICR.

The Tpx-1 ICR Is Homologous to Venom Toxins—The Tpx-1 ICR forms an extremely stable and compact hydrophobic core. It is stabilized by three disulfide bonds whose conserved architecture and similar spacing with Stecrisp (9) and a family of voltage-sensitive K\(^+\) channel blockers, notably ShK (39) and BgK (38), results in a similar domain fold. The similarity in function of Tpx-1 to ShK and BgK is consistent with this structural similarity. The primary sequence homology between Crisp domains from human, mouse, snake, and lizard origin suggest that each of these domains will have a similar domain structure and function (Fig. 4). Indeed, similar activities have been observed for a range of native full-length Crisp proteins of reptile origin (34); however, it is now with absolute confidence that this activity can be attributed to the Crisp domain. Although this investigation has not conclusively demonstrated that the ion channel regulatory activity is restricted to the ICR, the structural relationship to the ShK and BgK toxins suggest this is the most likely function. The ICR is encoded by a distinct exon and present only in a subpopulation of the CAP superfamily, suggesting it has been acquired during the course of evolution and encodes for a separate and discrete activity, which is consistent with protein structural data.

**The Tpx-1 Crisp Domain Is a Conserved Structural Element Displaying a Complex Ion Channel Regulatory Activity toward the RyRs**—As Tpx-1 is localized to intracellular compartments in mature spermatozoa prior to the acrosome reaction (18), we were concerned in this investigation with understanding Tpx-1 function in relation to the release of Ca\(^{2+}\) from intracellular stores in sperm. In addition, given that helothermine has been shown to inhibit skeletal and cardiac RyRs (28), we thought it prudent to begin our investigation on this Ca\(^{2+}\) ion channel. Although snake venom Crisp proteins have complex pharmacological activities, opposing activities on ion channel subtypes by Crisp proteins have not been reported previously. Furthermore, despite testing a range of snake venom Crisps (reviewed in Ref. 34), only helothermine has been shown to regulate RyR. The RyR1 stimulatory and RyR2 inhib-
The Tpx-1 Crisp domain regulates RyR1 channels. A, recordings of 1.5 s of activity from a bilayer containing two RyR1 channels at a bilayer potential of +40 mV are shown to the left, and the mean current measured from 30 s of activity (at +40 and −40 mV) under each condition are to the right. As labeled, activity is shown under control conditions, after addition of 1 and then 10 μM Crisp and finally after perfusion of Crisp from the cis chamber. B and C, the bins show average relative open probability (P_o) ± SEM for activity at +40 and −40 mV, respectively, under control (con) conditions, in the presence of the indicated concentrations of Crisp and after perfusion of 10 or 50 μM Crisp from the cis chamber. Data were obtained from 15 experiments, with at least five observations at each concentration of Crisp. Asterisks indicate values significantly different from control. The dot over the perfusion data indicates a significant fall in relative P_o after perfusion.

The Tpx-1 Crisp domain regulates RyR1 channels. A, recordings of 1.5 s of activity from a bilayer containing two RyR1 channels at a bilayer potential of +40 mV are shown to the left, and the mean current measured from 30 s of activity (at +40 and −40 mV) under each condition are to the right. As labeled, activity is shown under control conditions, after addition of 1 and then 10 μM Crisp and finally after perfusion of Crisp from the cis chamber. B and C, the bins show average relative open probability (P_o) ± SEM for activity at +40 and −40 mV, respectively, under control (con) conditions, in the presence of the indicated concentrations of Crisp and after perfusion of 10 or 50 μM Crisp from the cis chamber. Data were obtained from 15 experiments, with at least five observations at each concentration of Crisp. Asterisks indicate values significantly different from control. The dot over the perfusion data indicates a significant fall in relative P_o after perfusion.

The data show that the affinity of the Tpx-1 Crisp domain for the inhibition site on the cytoplasmic domain of RyR2 is greater than that for the activation site on the cytoplasmic domain of RyR1. In particular, 1) the concentration of the Tpx-1 Crisp domain required for inhibition of RyR2 was half that required for the activation of RyR1 and; 2) RyR2 inhibition could not be reversed at higher Tpx-1 Crisp domain concentrations following perfusion. The 1000–10,000-fold dilution of protein following perfusion, which would have left 5–50 nM of the Tpx-1 Crisp domain, was sufficient to maintain inhibition. A greater affinity of the Tpx-1 Crisp domain for the inhibition site on RyR2 is in contrast with that reported for helothermine (28), which had a greater affinity for the activation site on RyR1. Although reasons for these different affinities are not currently clear, it reminds us that Crisp proteins have subtle and complex regulatory activities, as highlighted by the 30-fold difference in activity on CNGA2 channels for the 97% identical PsTx and pseudecin (30).

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