Maurocalcine and Peptide A Stabilize Distinct Subconductance States of Ryanodine Receptor Type 1, Revealing a Proportional Gating Mechanism*

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Maurocalcine (MCA) isolated from Scorpio maurus palnus venom shares 82% sequence identity with imperatotoxin A. Both scorpion toxins are putative mimics of the II-III loop peptide (termed peptide A (pA)) of α1,-dihydropyridine receptor and are thought to act at a common site on ryano-dine receptor type 1 (RyR1) important for skeletal muscle EC coupling. The relationship between the actions of synthetic MCA (sMCA) and pA on RyR1 were examined. sMCA released Ca²⁺ from SR vesicles (EC₅₀ = 17.5 nM) in a manner inhibited by micromolar ryano-dine or ruthenium red. pA (0.5–40 μM) failed to induce SR Ca²⁺ release. Rather, pA enhanced Ca²⁺ loading into SR and fully inhibited Ca²⁺, caffeine-, and sMCA-induced Ca²⁺ release. The two peptides modified single channel gating behavior in distinct ways. With Ca²⁺-carrying current, 10 nM to 1 μM sMCA induced long lived subconductances having 48% of the characteristic full open state and occasional transitions to 29% at either positive or negative holding potentials. In contrast, pA stabilized long lived channel closures with occasional burst transitions to 65% (s1) and 86% (s2) of the full conductance. The actions of pA and sMCA were observed in tandem. sMCA stabilized additional subconductance states proportional to pA-induced subconductances (i.e. 43% of pA-modified s1 and s2 substates), revealing a proportional gating mechanism. [³H]Ryano-dine binding and surface plasmon resonance analyses indicated that the peptides did not interact by simple competition for a single class of mutually exclusive sites on RyR1 to produce proportional gating. The actions of sMCA were also observed with ryano-dine-modified channels and channels deficient in immunophilin 12-KDa FK506-binding protein. These results provide evidence that sMCA and pA stabilize distinct RyR1 channel states through distinct mechanisms that allosterically stabilize gating states having proportional conductance.

Excitation-contraction (EC)¹ coupling in muscle cells is the signaling process by which electrical stimuli arriving at the transverse tubule membrane transmit information to the sarcoplasmic reticulum (SR) to release intracellular Ca²⁺ necessary for muscle contraction. Skeletal type and cardiac type EC coupling differ in their dependence on extracellular Ca²⁺ entry. Functional interaction between dihydropyridine receptors (DHPRs) within the transverse tubule and Ca²⁺ release channels/ryanodine receptors (RyRs) within SR defines the type of EC coupling and its underlying mechanism. During cardiac EC coupling, a small influx of Ca²⁺ through cardiac DHPRs is required to open RyR2 (1–5), whereas skeletal type EC coupling does not require entry of external Ca²⁺. Instead, membrane depolarization triggers the opening of RyR1 through a mechanism involving conformational coupling of skeletal DHPRs (α₁₂,-subunit) and RyR1 (6–8).

Expression of cDNAs encoding cardiac/skeletal muscle chimeric α₁-DHPRs in dysgenic myotubes, which lack endogenous α₁₁-DHPR, identified the a site within the cytoplasmic loop between repeats II and III (the cytosolic II-III loop; amino acids 666–791) essential for the physical coupling with RyRs and skeletal EC coupling (9, 10). Experiments with the full-length skeletal II-III loop peptide showed specific activation of RyR1 but not RyR2 channels incorporated into planar lipid bilayers and in radioligand binding studies with [³H]Hryanodine (11, 12). The domain II-III loop peptide essential for RyR1 activity was further refined to the region between Arg⁶⁸¹ and Leu⁶⁹⁰ of termed peptide A (pA) (13, 14). Studies of how pA modifies single channel gating behavior revealed both activating and inhibitory properties on RyR1 depending on the concentration, the free cis Ca²⁺ concentration, and the holding potential (12, 15). These actions of pA were also found to extend to RyR2, suggesting a modulatory influence downstream of the skeletal α₁₁-DHPR/RyR1 interaction (12). However, expression of α₁₁-DHPR chimeras in dysgenic myotubes has shown that the pA region of the II-III loop was not essential for engaging skeletal type EC coupling (16), whereas a 46-amino acid fragment (Leu⁷¹⁰–Gln⁷⁶⁵) was essential for bidirectional signaling (9, 17–19). Although the action of pA and related peptides may not be directly involved in mediating bidirectional signaling in skeletal EC coupling, they are very useful in defining basic properties of RyR gating.

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§ The abbreviations used are: EC, excitation-contraction; SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; α₁,DHPR, skeletal α subunit of DHPR; FKBP12, FK506-binding protein of 12 kDa; IpTxa, imperatotoxin A; pA, peptide A; MCA, maurocalcine; RyR1, ryanodine receptor type 1; sMCA, synthetic maurocalcine; SPR, surface plasmon resonance; HPLC, high pressure liquid chromatography; SERCA, sarco-endoplasmic reticulum calcium transport ATPase; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TM, transmembrane.
The mechanism by which pA alters RyR1 channel function has not yet been fully defined. Imperatordye A (IpTx-R), a 3-amino acid peptide isolated from the scorpion Pandinus imperator, was shown to be a high affinity agonist of RyR1 channels (20, 21). Nanomolar IpTx-R increased high affinity H\textsuperscript{1}Hryanodine-binding and induced rapid Ca\textsuperscript{2+} release from SR vesicles. More recent studies have shown that IpTx-R prolonged the duration of Ca\textsuperscript{2+} sparks in frog skeletal muscle (22, 23) and significantly increased the amplitude and the rate of Ca\textsuperscript{2+} release in developing skeletal muscle (24). In measurements of single channel currents, IpTx-R stabilized long living subconductance states in skeletal and cardiac muscle ryanodine receptor channels having 43 and 28% of the native full transition at the holding potential of −40 and +40 mV, respectively (25). IpTx-R was proposed to mimic the actions of pA based on their similar RyR1-activating properties, possibly through a common effector site. In combination, the RyR1 channel complex that cannot be explained by simple distinct subconductance states through an interaction with the mechanism by which sMCa alters RyR1 function and its relationship to sMCa-modified RyR1 channel gates in competition at a common effector site. In combination, the RyR1 channel complex that cannot be explained by simple distinct subconductance states through an interaction with the system (25, 27).

Recently, maurocalcine (MCa), a novel peptide isolated from the venom of the scorpion Scorpion maurus palmaeus, has been found to possess 82% sequence identity with IpTx-R (27, 28). MCa shares the highly basic structural domain identified in peptide A and IpTx-R important for interactions with RyR1. Preliminary work has shown that synthetic MCa (sMCa) activates RyR1 channels by inducing long living subconductance states (27). Both IpTx-R- and sMCa-modified channels can reversibly transit between subconductance states and fast gating states. The actions of these peptides were also found to be additive with those of ryanodine, resulting in additional substates from the ryanodine-locked half-conducting state. However, the predominant subconductance induced by sMCa differs from that of IpTx-R. At a holding potential of +40 mV, the predominant substates induced by sMCa and IpTx-R are 48 and 28% of the full conductance, respectively, suggesting that the structural difference in these peptides may induce slightly different channel conformations that stabilize different unitary conductances (25, 27).

In the present investigation, we define in detail the mechanism by which sMCa alters RyR1 function and its relationship to the actions of pA. We found that sMCa and pA stabilize distinct subconductance states through an interaction with the RyR1 channel complex that cannot be explained by simple competition at a common effector site. In combination, the peptides reveal that the sMCa-modified RyR1 channel gates in stable proportional conductances. Gating in proportional conductances was also observed in the presence of sMCa and ryanodine and of sMCa and channels deficient in FKBP12. These results reveal that RyR1 channels gate in stable proportional conductances in the presence of allosteric modulators that mediate actions through distinct effector sites and are consistent with an iris model regulating a single permeation pore.

**EXPERIMENTAL PROCEDURES**

**Materials**—[H\textsuperscript{1}H]Ryanodine was obtained from PerkinElmer Life Sciences with specific activity of 57 Ci/mmol and purity of >90%. Unlabeled ryanodine was purchased from Calbiochem. Purified natural phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL) or Northern Lipids (Vancouver, Canada). Peptide A was a generous gift from Dr. Noriaki Ikemoto (Boston Biomedical Research Institute, Boston, MA). sMCa was made by the solid phase peptide synthesis method (27), purified with HPLC and ion exchange chromatography, and verified with HPLC, amino acid analysis, and mass spectrometry. The structural authenticity of pA utilized in the present investigation was confirmed by two methods, mass spectrometry and amino acid sequence analysis. The peptide was found to have a major peak at the anticipated molecular weight (M\textsubscript{r} = 2330) and have the amino acid sequence reported for the DHPB-II-III loop (Thr\textsuperscript{717}-Leu\textsuperscript{730}). All other chemicals were commercially obtained at the highest purity available.

**Preparation of Skeletal Muscle SR Membranes**—Membrane vesicles enriched in RyR1-FKBP12 complex and SERCA pumps were prepared from rabbit fast twitch skeletal muscle based on the method of Saito et al. (29). Briefly, freshly ground muscle was homogenized in ice-cold buffer containing 5 mm imidazole-HCl, pH 7.4, 300 mm sucrose, 10 μM leupeptin, and 100 μM phenylmethylsulfonyl fluoride. Differential centrifugation was performed to obtain a heavy SR fraction, and junctional SR was collected from the 38–45% (w/w) interface of a discontinuous sucrose gradient. The junctional SR was then resuspended to 10–15 mg/ml (30), frozen in liquid N\textsubscript{2}, and stored at −80 °C until needed.

**Fluorescent Ca\textsuperscript{2+} Transport Measurement**—Ca\textsuperscript{2+} transport across SR vesicles was measured with the membrane-impermeant Ca\textsuperscript{2+}-sensitive dye, antipyrilazo III, using a diode array spectrophotometer (model 8452; Hewlett Packard, Palo Alto, CA). Skeletal SR vesicles (50 μg/ml) were added to 1.15 ml of ATP-regenerating buffer consisting of 95 mm KCl, 20 mm potassium MOPS, 7.5 mm sodium pyrophosphate (31), 250 μM antipyrilazo III, 12 μg of creatine phosphokinase, 5 μM phosphocreatine, and 1 mM MgATP, pH 7.0 (final volume of 1.2 ml). Transport assays were performed at 37 °C in temperature-controlled cuvettes with constant stirring. SR vesicles were loaded with sequential additions of CaCl\textsubscript{2} that constituted ~80% of their loading capacity. Net Ca\textsuperscript{2+} fluxes across SR vesicles were measured by monitoring extravesicular changes in free [Ca\textsuperscript{2+}] by subtracting the absorbance of antipyrilazo III at 710 nm from absorbance at 790 nm for 2–4 s intervals. The loading capacity of the SR vesicles was determined by the sequential addition of 24 or 12 nmol of Ca\textsuperscript{2+} until the vesicles cannot uptake any Ca\textsuperscript{2+} or start calcium-induced calcium release. The maximum amount of Ca\textsuperscript{2+} accumulated by SR vesicles defines the loading capacity. At the end of each experiment, the total intravesicular Ca\textsuperscript{2+} was determined by the addition of 3 μl of the Ca\textsuperscript{2+} ionophore A23187 and the absorbance signals were calibrated by the addition of 12 or 24 nmol of CaCl\textsubscript{2} from a National Bureau of Standards stock solution. Test compounds were either added before loading (pretreatment) or after the last addition of Ca\textsuperscript{2+} was accumulated by the vesicles.

**The Specific Binding of [H\textsuperscript{1}H]Ryanodine to 12 μg of SR protein was performed with two distinct assay protocols, each with a slight modification of the original method (32, 33). Protocol A used a buffer composed of 3 nm [H\textsuperscript{1}H]ryanodine, 200 mM KCl, 10 mM HEPES, ~7 μM free Ca\textsuperscript{2+}, pH 7.2, and incubation was performed at 36 °C for 1.5 h. Protocol B used a buffer composed of 1 nm [H\textsuperscript{1}H]ryanodine, 140 mM KCl, 15 mM NaCl, 50 μM free Ca\textsuperscript{2+}, 240 mM PIPES, 10% sucrose, pH 7.4, and incubation was at 37 °C for 3 h. Additions of test compounds were made to the radiolabeled assay buffer, singly or in combination, prior to the addition of SR.

To test whether sMCa and pA interacted in a classic competitive manner to modulate RyR1 conformation, the complete dose response for the Ca\textsuperscript{2+} -induced enhancing specific [H\textsuperscript{1}H]ryanodine binding was assayed in the absence and presence of pA (15 or 30 μM) under the buffer conditions described above.

Separation of bound and free [H\textsuperscript{1}H]ryanodine was performed by filtration through a Whatman GF/B glass fiber filter using a Brandel (Gaithersburg, MD) cell harvester. Filters were washed twice with 3 ml of ice-cold buffer containing 20 mM Tris-HCl, 250 mM KCl, 15 mM NaCl, 50 μM Ca\textsuperscript{2+}, pH 7.1. Filters were then soaked overnight in 5 ml of scintillation mixture (Ready Safe; Beckman) and bound radioactivity was determined by scintillation spectrometry. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled ryanodine. Each experiment was performed in duplicate or triplicate and repeated at least two times.

**BiAcore Analysis of Peptide Interactions with RyR1**—Real-time surface plasmon resonance (SPR) experiments were performed on a BIAcore biosensor system 1000 at 25 °C. Biotinylated sMCa was immobilized on the sensor chip surface coated with streptavidin (sensor chip SA). Purified RyR1 was prepared from heavy SR vesicles solubilized in 1% CHAPS (34). sMCa was isolated by elution of the solubilized SR proteins as previously described (35). Before SPR experiments, purified RyR1 was dialyzed overnight at 4 °C in a buffer containing 150 mM NaCl, 10 mM HEPES, pH 7.4, 0.005% polysorbate in order to reduce the sucrose and NaCl concentration.

In these conditions, RyR1 has been characterized by an apparent sedimentation coefficient of 30 S that corresponds to a homotrimer of 565-kDa subunits (36, 37). sMCa-RyR1 interaction was measured by injection of purified RyR1 at 2 μg/ml (~1 nm in its tetrameric form) in a buffer containing 10 mM HEPES, 150 mM NaCl, 2 mM EGTA, 2 mM CaCl\textsubscript{2} (pCa 5), 0.005% polysorbate 20, pH 7.4. Nonspecific binding was

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measured by injecting RyR1 over a control surface saturated with biotin instead of biotinylated sMCA. To study the possible competition of sMCA and pA at a mutually exclusive site, RyR1 was preincubated for 30 min with 20 μM pA prior to injection onto the biosensor chip containing immobilized sMCA.

Single Channel Recording—Ca\(^{2+}\) current through single RyR1 channels incorporated into planar bilayer lipid membranes was measured in an asymmetric CsCl (10:1 or 1:10 cis/trans) solution. The bilayer lipid membrane was formed from a mixture of phosphatidylethanolamine and phosphatidylcholine (5:2, w/w) at 50 mg/ml in decane, across a 150–300-μm aperture in a 1.0-ml polystyrene cup. SR vesicles were added to the cis side of the chamber at a final concentration of 0.1–10 μg/ml. The cis solution contained 500 mM CsCl, 7 mM CaCl\(_2\), 20 mM HEPES, pH 7.4, and the trans solution contained 50 mM CsCl, 7 mM free Ca\(^{2+}\), 20 mM HEPES, pH 7.4. After a single fusion event, the vesicles are quickly removed by perfusion with 7 volumes of identical buffer without vesicles. Under conditions of a 10:1 (cis/trans) Cs\(^{+}\) gradient, a holding potential of +30 to +40 mV drives the current from the cis to the trans chamber. To drive the current from trans to cis, the CaCl in the cis solution was lowered to 50 mM by perfusion, and the CsCl in the trans side was raised to 500 mM by bolus addition from a 4 μl CsCl stock.

Measurements of trans to cis current were made at a holding potential of −40 mV. Single channel current was measured under voltage clamp using a Dagan 3890A integrating patch clamp (Dagan Instruments, Minneapolis, MN). Holding potentials were with respect to the trans (ground) chamber, and positive current was defined as current flowing from cis to trans. Current signals were captured at 10 kHz and filtered at 1 kHz using a four-pole Bessel filter. Data were digitized with a Digitida 1200 interface (Axon Instruments, Burlingame, CA) and stored on a computer for subsequent analysis. The experiments were performed at room temperature and were replicated at least three times. Unless otherwise stated, test chemicals were sequentially added to the cis solution after an initial period of recording control channel behavior.

Single channel activity was analyzed with pCLAMP 6.0 (Axon Instruments). Open events for full conductance and subconductances were defined as intervals at which the currents exceeded 50% of maximum open level and the defined subconductance level, respectively. Open probability (P\(_o\)) for unmodified channels (control channels) was calculated from 60–90 s of continuous record using the PSTat program. For peptide A-modified channels, the overall open probability is calculated from the whole recording time, whereas the within group open probability is calculated only from the groups of time points when channels are at rapid gating states. Current levels were analyzed by mean-variance analysis, and peaks in the all points amplitude histogram were fitted with Gaussian functions. Dwell open and closed times for relatively fast gating events (duration of <50 ms) were calculated from least-square fits of biexponential function using the PSTat software. Arithmetic average time is used for analyzing the long subconductance states induced by sMCA and the long closed time induced by pA, because there are not enough events to fit with a reasonable biexponential function.

RESULTS

Maurocalcine Induces Ca\(^{2+}\) Release from SR Vesicles by Activating RyR1—Fig. 1 compares the amino acid sequences of MCA, IpTX\(_a\), and pA, showing a common basic domain terminating with an amino acid possessing a hydroxyl-containing side chain that has been proposed to contribute essential structure for activating RyR1 (26). It is therefore conceivable that MCA shares many properties with IpTX\(_a\) and pA in the manner in which it modulates RyR1. The present work therefore elucidates the mechanism(s) by which sMCA and pA alter the function of SR Ca\(^{2+}\) transport and RyR1 channel function.

To assess how MCA alters Ca\(^{2+}\) fluxes across skeletal muscle SR vesicles, macroscopic Ca\(^{2+}\) transport measurements were performed under pyrophosphate-supported active loading conditions. After SR vesicles were sequentially loaded with Ca\(^{2+}\) to ~80% of their capacity and extravesicular Ca\(^{2+}\) level returned to the base line (~150 mM free Ca\(^{2+}\)), sMCA (20 mM) quickly induced Ca\(^{2+}\) release (Fig. 2A). The addition of ryanodine receptor inhibitors, ruthenium red (5 μM), or ryanodine (500 μM) at the plateau of Ca\(^{2+}\) release, blocked RyR1 channels, and led to Ca\(^{2+}\) reuptake into vesicles, suggesting that sMCA induced Ca\(^{2+}\) release by selective activation of RyR1. The actions of sMCA on SR Ca\(^{2+}\) release were concentration-dependent, with an EC\(_{50}\) of 17.5 mM (Fig. 2, B and C). The loading capacity of SR vesicles has been shown to be determined by a balance between Ca\(^{2+}\) uptake through ATP-dependent SERCA pumps and Ca\(^{2+}\) release through ruthenium red-sensitive and -insensitive efflux pathways (38). Fig. 2D shows that sMCA (20 μM) significantly decreased the loading capacity of SR vesicles, and this effect was abolished by ruthenium red at a concentration previously shown to completely block RyR1 in this assay (39). This provides additional evidence that sMCA selectively activates RyR1 with no measurable effects on either leak channels or SERCA pumps.

pA Inhibits Ca\(^{2+}\)–-, Caffeine–, and sMCA-induced Ca\(^{2+}\) Release from SR Vesicles—pA was initially reported to enhance the binding of \([^{3}H]\)ryanodine to RyR1 in the concentration range of 1–50 μM (13). Here, an unexpected result was that pA (2–40 μM) failed to induce Ca\(^{2+}\) release from actively loaded SR membrane vesicles, but instead ≥5 μM caused the base line of the dye signal to gradually decline (Fig. 3A, traces 3 and 4). This observation provided the impetus to examine the possible RyR1-inhibitory activity of pA. Indeed, pA (2–10 μM) introduced into the transport medium 2 min before bolus addition of 60 μM Ca\(^{2+}\) dose-dependently inhibited Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Fig. 3A). pA also inhibited caffeine-induced Ca\(^{2+}\) release in a concentration-dependent manner (IC\(_{50}\) = 1.5 μM; Fig. 3B). It is worth noting that 5 μM pA completely inhibited caffeine-induced Ca\(^{2+}\) release, in agreement with the recent report in which micromolar pA was shown to decrease P\(_o\) of single channels reconstituted in bilayer lipid membranes (12). However, unlike the reported activating effect of submicromolar pA on single channel P\(_o\), we did not observe that pA, from submicromolar to micromolar concentrations (0.1–40 μM), was able to induce Ca\(^{2+}\) efflux from actively loaded SR. Additional experiments with a combination of pA and thapsigargin were performed to determine whether pA enhances Ca\(^{2+}\) release.
under passive conditions (in the absence of SERCA-mediated reuptake of Ca^{2+}). After active Ca^{2+} loading of the SR vesicles was complete, pA (0.1–10 μM) failed to enhance thapsigargin-induced Ca^{2+} release (not shown). Consistent with an inhibitory action on RyR1, 5–20 μM pA increased the loading capacity of SR in a concentration-dependent manner (Fig. 3C), and in support of distinct mechanisms of action, pA (≥10 μM) was found to inhibit sMCa-induced Ca^{2+} release in a dose-dependent manner (Fig. 3D).

Since it is generally agreed that the amount of high affinity \(^{[3]H}\)ryanodine-binding measured in radioligand receptor analysis reflects the degree of channel activity, we examined how sMCa (0.01–300 nM) (Fig. 4A) or peptide A (0.03–50 μM) (Fig. 4B) influenced occupancy. In accordance with the results obtained from macroscopic Ca^{2+} transport experiments, sMCa dose-dependently increased \(^{[3]H}\)ryanodine binding with an EC\(_{50}\) of 1.2 nM. By comparison, the dose response exhibited by pA was bell-shaped, with an EC\(_{50}\) of 266 nM and an IC\(_{50}\) of 23.5 μM. Considering their sequence homology, the mechanism by which sMCa and pA interact with RyR1 to produce seemingly antagonistic effects on SR Ca^{2+} release was further investigated using two methodological approaches, \(^{[3]H}\)ryanodine binding analysis and real time SPR analysis. If sMCa and pA compete for one or more common, mutually exclusive effector sites (i.e. classic competition), then increasing the concentration of sMCa would be expected to overcome the inhibition of \(^{[3]H}\)ryanodine binding produced by a fixed concentration of pA. Fig. 5A shows that the presence of 15 or 20 μM pA reduced the ability of sMCa to enhance \(^{[3]H}\)ryanodine binding by 50 and 100%, respectively, even at a concentration of sMCa nearly 1,000-fold EC\(_{50}\) (1 μM). Thus, indirect analysis using \(^{[3]H}\)ryanodine-binding as an indicator of channel conformation indicated that, in combination, the peptides did not exhibit classic competitive interaction at RyR1. The SPR technique permitted more direct (without the use of \(^{[3]H}\)ryanodine) examination of whether sMCa and pA interact by competing for a common site on RyR1. SPR sensorgrams revealed that RyR1 failed to interact with biotin, whereas RyR1 interacted strongly with biotinsMCa (Fig 5B, traces a and b, respectively). In consonance with results using \(^{[3]H}\)ryanodine-binding analysis, 1 nM RyR1 oligomer that had been pretreated for 30 min with a great excess of pA (20 μM) maintained its ability to strongly interact with immobilized biotinsMCa (Fig 5B, trace c), and the amplitude of the interaction was not significantly lower than control (Fig. 5C, n = 3 separate SPR sensorgrams obtained with and without pretreatment of RyR1 with pA). Taken together, these results indicate that the antagonistic actions of sMCa and pA on RyR1 function were not mediated by simple competitive displacement at a common binding site.

sMCa and pA Modulate RyR1 Behavior by Stabilizing Distinct Conducting States—Macroscopic Ca^{2+} transport, radioligand binding, and SPR analyses suggested that sMCa and pA exert their effects on RyR1 through distinct mechanisms. We more directly explored whether the two peptides modify channel gating behavior by interacting at distinct effector sites or with multiple common effector sites. Based on sequence homology, the latter hypothesis was tested by determining whether the actions of the two peptides were additive by analyzing their influence singly and in combination on single RyR1 channels reconstituted into planar lipid bilayers. Fig. 6A shows 16 s of continuous record of a native RyR1 channel rapidly gating between closed and full open states. The
addition of sMCa (50 nM) to the cis (cytoplasmic) solution resulted in the enhancement of channel open probability (Po) from 0.03 to 0.79) by inducing a predominant long lived subconductance state at 48% of the channel's native full-open level. Micromolar ryanodine irreversibly locks RyR channels in a highly stable conformation approximating 50% of the full conductance that infrequently closes but never opens to the full state (40, 41). By contrast, sMCa modified the characteristic rapid gating transitions into a long lived 48% subconductance that frequently transitioned to periods of fast gating between full open and closed conformations (Fig. 6B). Reversible association and dissociation of sMCa from RyR1 probably accounted for transitions between modified and native gating modes. The amplitude of the subconductance gating is independent of the concentration of sMCa (5 nM to 1 μM, n = 15). The major subconductance of 45% was achieved with sMCa at both positive (Fig. 6A and B) and negative (Fig. 6C and D) holding potentials, and the IV relationship of the sMCa-modified channel was linear and did not exhibit rectification of Cs⁺ current (not shown). The major subconductance in sMCa-modified channels is not changed by allosteric modulators of channel gating that stabilize full conductance openings such as bostadin 10 (42) and ATP. sMCa stabilizes subconductances with amplitude of 45 and 46% of the full conducting level in the

**Fig. 3.** Peptide A inhibits Ca²⁺ release induced by ryanodine receptor activators. A, pA inhibits Ca²⁺-induced Ca²⁺ release (CICR). Different concentrations of pA (0, trace 1; 2 μM, trace 2; 5 μM, trace 3; 10 μM, trace 4) were added 2 min before adding 60 μM Ca²⁺. B, pretreatment with pA inhibits caffeine (4 mM)-induced Ca²⁺ release. The pA concentrations tested were 0 (trace 1), 1 μM (trace 2), 2 μM (trace 3), and 5 μM (trace 4). C, pA increased Ca²⁺ loading capacity in skeletal muscle SR vesicles in a concentration-dependent manner. The loading capacity without pA is considered as 100%. The p value for comparing the loading capacity in the presence and absence of 5, 10, and 20 μM of pA was 0.002, <0.001, and 0.004, respectively (Student's t test). D, pA inhibits the sMCa-induced Ca²⁺ release. pA (0, trace 1; 2 μM, trace 2; 5 μM, trace 3; 10 μM, trace 4) was added 2 min before adding sMCAs (20 nM).

**Fig. 4.** Maurocalcine and peptide A have different effects on [³H]ryanodine binding. The radioactive ryanodine binding assays were performed with protocol B as described under “Experimental Procedures.” The experiments were repeated in two different preparations each in triplicate. Data are presented as mean ± S.E.
nels. These results indicate that the action of sMCa on RyR1 is behavior characteristic of control channels, and reintroduction of sMCa from the cis chamber quickly restored the fast gating state of product SCorpion Peptide-induced Proportional Gating of RyR1

presence of bastadin 10 (n = 3) and ATP (n = 3), respectively. The average mean open time of the sMCa-modified 48% conductance state was >700 ms (n = 8 channels). On rare occasions, gating transitions to 29 and 16% subconductances were recorded with the sMCa-modified channels as previously reported (27). Because of their scarcity, subconductances below 48% were not analyzed in detail. To further address reversibility of the sMCa-modified channel, the cis chamber was perfused with 10 volumes of buffer lacking sMCa (Fig. 7). Removal of sMCa from the cis chamber quickly restored the fast gating behavior characteristic of control channels, and reintroduction of the scorpion peptide immediately induced the long living subconductance states characteristic of sMCa-modified channels. These results indicate that the action of sMCa on RyR1 is fully reversible.

pA modified the gating properties of RyR1 channels in a manner distinct from sMCa. pA (0.5–20 μM) induced long lived closed states interspersed with bursts of channel gating activity (Fig. 8). pA decreased the overall P_o of the RyR1 channels by stabilizing very long closed states (P_o from 0.26 to 0.060, n = 10 channels). The average closed time of events >10 ms was 45 ms for unmodified channels compared with 463 ms for pA-modified channels. The percentage of time accounted by long closures of control (10 ms) was greatly increased by pA, from 37 to 91%. However, the mean closed times for pA-modified channels during the burst of rapid gating activity were not significantly altered (control τ_c1 = 0.8 ms, 61%; τ_c2 = 4.9 ms, 39% versus peptide A-modified τ_c1 = 0.8 ms, 64%; τ_c2 = 3.8 ms, 35%; n = 10 channels). Distinct modes of channel gating were observed within a burst of activity, and the subconductance states were easily defined. pA induced prominent transitions to subconductances having 65% (s1) and 86% (s2) of the full conductance (Fig. 8B), and these events were summarized in the amplitude histogram shown in Fig. 10A (second panel). Within the active gating periods, the pA-modified channels exhibited ~3-fold higher P_o compared with the unmodified channel (Fig. 8, compare A and B). pA-modified channels exhibited longer mean open times (τ_o1 = 0.73 ms (66%) and τ_o2 = 3.9 ms (34%)) compared with unmodified channels (τ_o1 = 0.46 ms (83%) and τ_o2 = 2.7 ms (17%)).

sMCa and pA Modify RyR1 Channels in an Additive Manner—A possible molecular interaction between sMCa and pA on RyR1 was further investigated by sequentially adding both of the peptides to the cytoplasmic side of reconstituted channels. The addition of 5 μM pA modified the typical rapidly gating RyR1 channel (Fig. 9, first trace) to a burst gating mode (Fig. 9, second trace). In addition to occasional transitions to the full open state, two prominent subconductances within the gating episodes were 65% (s1 state) and 86% (s2 state) of full conductance level (Fig. 8, second trace). The subsequent addition of 50 nM sMCa induced virtually no observable long lived openings characteristic of sMCa-modified channels. (Fig. 9, third trace). Increasing the sMCa to 1 μM (in the presence of pA) induced long lived subconductance states, indicating that both sMCa and pA can co-occupy the RyR1 channel complex (Fig. 9, fourth trace). Most interestingly, the presence of pA and sMCa in combination stabilized subconductance states that were not seen with either sMCa or pA alone, indicating the simultaneous binding and additive effects of pA and sMCa. Specifically, in n = 8 channels, pA-modified channels demonstrated gating transitions to 65% (s1), 86% (s2), or 100% of the full chord conductance during a burst of gating events. Binding of sMCa further modified these transitions to 28, 37, or 43% of the full conductance, respectively, all of which were proportional to the pA-modified states with a factor of 0.43 (Figs. 9 and 10). The modulatory effect of sMCa reveals a fundamental property of how allosteric modulators influence RyR1 channel conductances. Instead of stabilizing a particular subconductance state, sMCa imposes proportional control of channel conductance relative to the existing gating transitions. The concept of proportional gating was further analyzed by defining the ratio between the conductances of the sMCa-modified states and the counterpart states without sMCa as the “proportional gating factor” (a). For any given gating state with conductance S, the binding of sMCa induces a subconducting state of product a × S (noted as aS; e.g. aS1, aS2, and afull in Fig. 10). In order to calculate a proportional gating factor, amplitude histograms were constructed from local transitions that included gating events in the presence of pA and in the presence or absence of sMCa. The examples of those transitions chosen were shown by the solid lines (a–c) at the bottom of the fourth panel in Fig. 9. Fig. 10A shows representative histograms derived from within bursts of gating activity in the same channel. The addition of sMCa to a pA-modified channel induced subconductance states that shifted proportionally to those produced by pA (traces 3–5). The proportional gating factors were calculated for every transition in sMCa/pA-modified channels (from n = 8 channels) and were grouped according to their respective current levels (Fig. 10B). Although there was a reasonable amount of variance in the calculation because the histograms were derived from a limited number of events taking place during bursts of activity, the calculated propor-
tional gating factors for all groups were closely centered at 0.43 for all of the current transitions.

Occasionally, native channels in the absence of pharmacological modifiers exhibit periods of gating transitioning from the closed state to a subconductance level (Fig. 11, first trace), probably due to the loss of FKBP12 from the channel complex during preparation. This provided an opportunity to test the hypothesis that sMCa also imposes a proportional gating factor on channels modified by means other than pA. Once sMCa was added to channels displaying subconductance behavior, additional subconductances proportional to the original current levels with a proportional gating factor of 0.45 were easily identified within the trace (Fig. 10, second and third panels). This mode of modulation was also demonstrable with ryano-

Fig. 6. Maurocalcine induces long lived substates at +40 and −40 mV holding potential. 16 s of continuous measurement of a single channel current without (A) and with 5 nM of sMCa (B) were recorded in the asymmetric solution using Cs⁺ as current carrier. The cis buffer contains 500 mM CsCl, 20 mM HEPES, 50 μM Ca²⁺, and the trans buffer contains 50 mM CsCl, 20 mM HEPES, ~7 μM free Ca²⁺. The holding potential is +35 mV. The closed and full conductance opening levels are labeled as C and O, respectively, and the subconductance levels are indicated by the dotted lines between closed and open states. In separate experiments, 16 s of continuous measurements of single channel current at a holding potential of −40 mV were recorded without (C) and with 5 nM of sMCa (D) in cis buffer containing ~7 μM free Ca²⁺. Traces a–d represent 2 s of recordings at indicated positions from A–D, respectively.
Once sMCa was introduced to channels locked in the characteristic ryanodine-modified half-state, it produced additional subconductances of the ryanodine-modified channel with proportional factors of 0.57 and 0.39 (27). These results demonstrated that sMCa imposed a proportional gating factor on the native channel conductance, subconductances stabilized by allosteric ligands, and subconductances induced by altered protein-protein interactions. Therefore, proportional gating appears to reflect a universal property of how sMCa modifies channel gating behavior.

**DISCUSSION**

**sMCa and pA Modulate RyR1 Channels through Different Mechanisms**—Using a macroscopic Ca\(^{2+}\) transport assay, \(^{3}H\)ryanodine binding analysis, SPR techniques, and measure-
ments of single channel currents, we have investigated the molecular mechanisms by which sMCa and pA modulate RyR1. sMCa is a high affinity activator of the channels. Like its closely related scorpion toxin, IpTx, sMCa rapidly induces Ca\(^{2+}\) release from SR vesicles through a ruthenium red and ryanodine-sensitive pathway, increases high affinity \(^{3}H\)ryanodine binding, and increases channel activity by stabilizing long lived subconductance states. However, their interactions with RyR1 are not identical, since the predominant subconductance induced by sMCa is different from that of IpTx. The holding potential of +40 mV, the predominant substates induced by sMCa and IpTx are 48 and 28\% of the full conductance, respectively. The rectifying effect of IpTx is not obvious in sMCa-modified channels, since the substates induced by sMCa at negative potential also hold 48\% of the native full conducting current. One possible explanation is that in the present experiments the influence of sMCa on channel behavior was tested with asymmetric Cs\(^{+}\) as current carrier, whereas comparable studies with IpTx were performed with symmetric K\(^{+}\) (25). However, the structural difference in these peptides may also account for this minor difference in their function.

pA was initially proposed to mimic the essential site for the physical coupling between DHP and RyR (13, 14). It has been shown to enhance the binding of \(^{3}H\)ryanodine, elicit Ca\(^{2+}\) release from SR vesicles, and induce long living subconductance of RyR channels in the concentration range of 1–50 \(\mu M\). The actual physiological role of this portion of the II-III loop in EC coupling, however, is challenged by in situ studies with skeletal/cardiac muscle DHPR chimeras expressed in dysgenic myotubes. These studies have identified a different region (amino acid residues 720–765) to be the critical site for DHPR and RyR interaction in skeletal muscle (9). Also, skeletal type EC coupling was unaffected when an \(\alpha_{1s}\)-DHPR with a scrambled sequence corresponding to pA (residues 681–690) was expressed by dysgenic myotubes (16). Since both active and inhibitory effects of peptide A on RyR channels have been reported, the mechanism of the interaction of peptide A with RyR1 must be complex (12, 15). In contrast to previous reports by El-Hayek et al. (13), we exclusively observed the inhibitory effect of pA. pA did not induce Ca\(^{2+}\) release from SR vesicles under active or passive assay conditions; rather, it inhibited Ca\(^{2+}\) release induced either by Ca\(^{2+}\), caffeine, or sMCa. pA also increased Ca\(^{2+}\) loading capacity in a concentration-dependent manner. Taken together, these observations indicate that a prominent action of pA is to inhibit RyR1-mediated SR Ca\(^{2+}\) release.

Moreover, pA modified single RyR1 channel gating behavior in a manner distinct from that of sMCa. pA stabilized long closed states of RyR1 and produced characteristic burst-gatings separated by very long closed intervals, consistent with the observations that micromolar pA inhibits channel activity (12, 15). pA stabilized openings to multiple subconductances, and the levels were distinct from those produced by sMCa. In our study, pA was inhibitory to channels with moderate activities (i.e., \(P_o = 0.05–0.4\), whereas pA-induced burst activity may become stimulatory in channels with very low activity (i.e., \(P_o = 0.001–0.006\)) as reported by other groups. Although the prominent inhibitory actions of pA raise doubt about its physiological role within \(\alpha_{1s}\)-DHPR in engaging EC coupling as originally suggested, its unique property of stabilizing multiple subconductance states makes it a valuable tool for studying the mechanism of action of sMCa. The behavior of pA-modified single channels provided a plausible explanation for the bell-shaped dose-response curve observed with \(^{3}H\)ryanodine binding analysis. pA increased mean open time within burst events, a state favorable for \(^{3}H\)ryanodine binding. Once bound to high affinity sites, \(^{3}H\)ryanodine has been shown to dissociate very slowly (\(t_{1/2} > 30\) min) (32). On the other hand, increasing con-

![Diagram](http://www.jbc.org/)

**Fig. 9.** **Maurocalcine and peptide A influence RyR1 channel gating in an additive manner.** Shown are four recordings of a representative (n = 8) experiment with the sequential addition of 5 \(\mu M\) peptide A, 50 \(nm\) sMCa, and 1 \(\mu M\) sMCa. Single channel currents were recorded at +35 mV as in Fig. 5 with 50 \(\mu M\) Ca\(^{2+}\) in the cis solutions. The additive nature of sMCa and pA on subconductance behavior indicates that they are binding to distinct effector sites. The subconductance states induced by further binding of sMCa are labeled as \(\alpha_{full}\) and \(\alpha_{S1}\), where \(\alpha\) is the proportional gating factor calculated as the ratio between the conductance of sMCa-modified and adjacent sMCa-unmodified states. See the legend to Fig. 10 for more details.
Concentrations of pA decrease the overall activity of ryanodine receptor channels and can account for the decrease of [3H]ryanodine binding. The first effect dominates at low micromolar concentrations of pA, and the inhibitory effect becomes increasingly dominant as concentration increases. The ability of pA to enhance gating activity of purified RyR1 was recently shown to

**Fig. 10.** The subconductance level induced by maurocalcine is dependent on the preexisting gating state. A, the amplitude histograms were constructed from selected fragments of single channel recordings to show the subconductances induced by peptide A only and together with sMCa (1 μM). The major conductances in peptide A-modified channels are 65% (S1 state), 86% (S2 state), and 100% (full) of the native full conductance. The subconductance states induced by further binding of sMCa are labeled as αS1, αS2, and αfull, accordingly. The proportional gating factor (α) is calculated as the ratio between the conductance of sMCa-modified and adjacent sMCa-unmodified states. B, summary plot of all the transitions and their proportional gating factors (α) grouped according to their conductance states (n = 8).

**Fig. 11.** Effects of maurocalcine in native channels with stable subconductionate gatings. A, the native ryanodine receptor channel has stable subconductance states at 80% (Sub1) and 60% (Sub2) of its full conductance. The addition of 5 nM sMCa (middle and bottom panels) induced subconductances (e.g. αSub1 and αSub2) proportional to their current conductance states with the proportional gating factor at 0.45 (n = 2). B, the amplitude histograms of the corresponding current traces in A.
be dependent on the presence of FKBP12 (43). These results suggest that the net effect of pA on RyR1 gating not only depends on the concentration of pA but also on the conformational state of RyR1 defined by the interaction of RyR1 with other SR proteins.

sMCa Imposes a Proportional Gating Factor on Channel Conductances—sMCa reversibly associates with RyR1 to stabilize long living subconductance states. In this paper, we identify that toxin-modified RyR1 exhibits proportional gating behavior that is additive to preexisting channel gating behavior, regardless of the predominating conductance or how it was achieved (e.g. dissociation of FKBP12 or pharmacological modification with ryanodine or pA). Ligand-induced subconductance gating of RyR1 has been proposed to be mediated either by conformational alterations or partial occlusions of the conduction pathway (44). For example, nanomolar ryanodine has been proposed to produce a long lived 50% conductance conformation and was interpreted as predominantly the result of partial occlusion, whereas higher concentrations fully occlude a single conductance pore. In the present study, we found that sMCa predominantly stabilizes a gating transition to 48% of the full conductance amplitude, and unlike ryanodine, the current level does not decrease with increasing sMCa concentration. The actions of sMCa can be best explained by a model that stabilizes RyR1 conformation by an allosteric mechanism that indirectly limits the conduction pore rather than by direct occlusion of the conduction pore by the scorpion peptide. In support of this interpretation, the relative amplitude of subconductance gating induced by sMCa is virtually unchanged under both positive and negative membrane potential. Considering the high sequence homology and functional similarity between IpTx3 and sMCa, it is highly likely that both peptides bind to the same effector site on RyR1. IpTx3 was shown by cryoelectron microscopy to bind to a cytoplasmic region far from the center of the conducting vestibule of RyR1, further supporting an allosteric mechanism for the toxins (46).

The three-dimensional structure of RyR1 determined by cryoelectron microscopy has revealed a mushroom-shaped structure consisting of a large square cytoplasmic domain with four peripheral clamp-shaped domains (domains 5–10 as defined by Radermacher et al. (47)) connected by “handle” domains (domain 3) (45, 47, 48). The transmembrane (TM) region composed of four subdomains (each from one subunit of RyR) forms the “stem” of the mushroom-shaped structure of the channel. Studies comparing open RyR1 modified by AMP-PCP/Ca2+ (46) or ryanodine/Ca2+ (48) with RyR1 in the closed state have revealed long range conformational changes in the TM domain and opening of a central pore induced by these activating reagents. The TM domain in the open state was found to be twisted counterclockwise by ~4°, possibly by shifting four TM subdomains in an iris-like manner (48). The proportional gating of RyR induced by sMCa is consistent with the central pore model and an iris-like mechanism. IpTx3 was shown to bind (one toxin molecule per channel subunit) to a cytoplasmic cavity between domain 3 and domain 7/8 (47), which is presumably also the binding site for sMCa. Domain 3 and domain 7/8 are connected to the central conducting vestibule through short “bridges.” Based on this structural information, occupation of the toxin sites may transmit long range conformational changes via a flexible bridging region that introduces a constriction onto the conduction pathway. In this manner, binding of sMCa to any one of the four RyR1 subunits can modify the conformational change of all four subunits.

The combined effect of sMCa and pA on channel conductance is the simple product of the individual effect of each peptide alone, suggesting that each peptide can independently occupy distinct class of effector sites on RyR1 and exert its effect additively. Based on the high sequence homology between MCa and IpTx3, it is likely that these scorpion peptides bind to overlapping sites within the clamp region. The exact locations of regions of RyR1 that recognize pA remain to be elucidated. Biochemical data from [3H]ryanodine-binding analysis and measurements of SPR presented here strongly suggest that sMCa and pA can occupy binding sites on RyR1 in tandem and that their interaction does not follow simple competition at a single class of mutually exclusive sites. Although the purified RyR1 utilized in SPR experiments is likely to be in its tetrameric form, the experiments aimed at assessing competition used a low concentration of RyR1 (1 nM) and a great excess of pA (20 μM) that is 150 times the previously reported IC50 for interaction of RyR1 and pA in solution (43). Under these assay conditions, it is therefore reasonable to assume that RyR1 is saturated with peptide regardless of its oligomeric form. Despite the saturating levels of pA, SPR indicated no diminution in the ability of RyR1 to interact with immobilized sMCa. However, we cannot totally discount possible allosterism between the sMCa and pA binding sites among the four symmetrical clamp regions of the oligomer, since higher concentrations of sMCa are needed to induce RyR1 long lived subconductance states in the presence of pA (Fig. 9). Moreover, the inhibitory actions of pA clearly dominate over the activating actions of sMCa. Most likely, this is the result of the long-lived channel closures induced by pA. The most plausible explanation is that sMCa and pA limit the counterclockwise twist of RyR1 (i.e. to less than the 4° for the full open state predicted by results from cryoreconstruction) (45) through their interactions with distinct effector sites, thereby limiting the final conductance of the channel (Fig. 12). Consistent with this interpretation, the proportional gating factor imposed by sMCa was constant in the presence and absence of diverse channel modulators that both do not change the unitary conductance of the channel such as bastadin 10 (42) and ATP and do promote subconductances (e.g.,
FKBP12-deficient channels). Even ryanodine-modified chan-
nels, which are thought to undergo profound conformational
changes within the conducting pathway, are subject to propor-
tional gating upon binding of sMCa. In conclusion, the propor-
tional gating imposed by sMCa-bound RyR1 appears to be
additive with the subconductances favored in the presence of
pA and reflects a common mechanism by which peptides inter-
acting with the clamp domains can allosterically limit channel
conductance, and it is consistent with an iris model of a single
conduction pore for RyR1.

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Maurocalcine and Peptide A Stabilize Distinct Subconductance States of Ryanodine Receptor Type 1, Revealing a Proportional Gating Mechanism
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