Calcium-independent activation of skinned cardiac muscle by secophalloidin

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Abstract Thin filament regulation of muscle contraction is believed to be mediated by both Ca^{2+} and strongly bound myosin cross-bridges. We found that secophalloidin (SPH, 5–8 mM) activates cross-bridge cycling without Ca^{2+} causing isometric force comparable to that induced by Ca^{2+}. At saturated [SPH], Ca^{2+} further increased force by 20%. SPH-induced force was reversible upon washing with a relaxing solution. However, there was more than 30% irreversible loss in subsequent Ca^{2+}-activated force. We hypothesize that SPH activates muscle via strongly bound cross-bridges. SPH-activated contraction provides a new model for studying the role of Ca^{2+} and cross-bridges in muscle regulation.

Key words: Secophalloidin; Cardiac muscle; Activation; Contraction; Regulation

1. Introduction

In resting striated muscles, interaction of myosin cross-bridges with actin is suppressed by regulatory troponin–tropomyosin complex on the thin filament (for recent reviews see [1–3]). Binding Ca^{2+} to troponin C (TnC) results in tropomyosin shift over the actin surface and exposure of myosin-binding sites on the actin previously blocked by tropomyosin. This switching of the thin filament from the 'off' to the 'on' state allows formation of force-generating myosin cross-bridges strongly bound to actin. The strongly bound cross-bridges stabilize the 'on' position of tropomyosin, thereby promoting further actomyosin interaction. Under conditions that favor strong actin–myosin binding (e.g. low MgATP concentrations) muscle can be activated by cross-bridges and contracts without Ca^{2+} [4]. At physiological MgATP concentrations, the strongly bound cross-bridges formed as a result of the Ca^{2+}-induced changes play an important role in muscle activation. A number of studies suggest that Ca^{2+} alone is unable to completely activate the thin filament, but rather allows the initial cross-bridge binding [5,6]. Thus, maximal activation requires both Ca^{2+} binding to TnC and cross-bridge binding to actin. Great progress in understanding the synergistic actions of Ca^{2+} and strongly binding cross-bridges has been gained by recent studies [7–12]. However, the specific details of the regulatory mechanism remain to be clarified.

Secophalloidin (SPH), a phalloidin derivative that binds weakly to actin (for review see [13]), is known to increase Ca^{2+} responsiveness of striated muscles [14]. As compared to other phallotoxins [15–17], SPH has a more pronounced effect acting at higher concentrations ($\sim$25% force increase in cardiac muscle at full Ca^{2+} activation with a half-maximal effect at 0.05–0.07 mM). SPH is unique in that its effect arises from binding to a site distinct from that of phalloidin. Such SPH properties arise from hydrolysis of the peptide bond between the first and seventh amino acid residues in phalloidin, resulting in the opening of the right cycle and appearance of the C- and N-terminals (Fig. 1).

Here, we report, for the first time, the ability of SPH (5–8 mM) to activate force in skinned cardiac muscle without Ca^{2+} to a level comparable to that activated by Ca^{2+}. This effect also arises from the binding of SPH to a site distinct from that for phalloidin. The structure–function relationship of the SPH molecule indicates a critical role of the C-terminal structure for SPH activity. A strong, Ca^{2+}-independent activation may be useful for further studies of the role of Ca^{2+} and cross-bridges in muscle regulation.

2. Material and methods

2.1. Preparations of SPH

SPH used in initial experiments was generously provided by Dr. Th. Wieland (Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany). Subsequently, different types of SPH (Fig. 1) were prepared. SPH lactone (SPH-L) was prepared from phalloidin by incubation with 50% aqueous trifluoroacetic acid [18]. In preparing SPH-hydroxy acid (SPH-H-A), lactone was opened by incubation a water solution of SPH-L (7–8 mg/ml, pH 8.5–8.7 adjusted with NH_4OH) for 70 h at room temperature. Each preparation was HPLC-purified. Analytical HPLC of each product on a Beckman Ultrasphere ODS (C-18) analytical HPLC column (5 μm; 80 Å; 4.6×250 mm) resulted in elution of the product as a single peak. Mass spectroscopy (electrospray ionization; ion trap detector) of products confirmed the expected molecular mass for each preparation.

2.2. Muscle experiments

Left ventricle muscle bundles from fresh bovine heart were obtained from a local abattoir and skinned with 1% Triton X-100 in rigor [17] or relaxing solution [19], then stored at −20°C in solutions containing 50% glycerol.

The experimental set-up and experimental protocols for studying isometric contraction were previously described [17]. Briefly, skinned

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Abbreviations: SPH, secophalloidin; SPH-L, secophalloidin lactone; SPH-H-A, secophalloidin hydroxy acid; TnC, troponin C; BDM, 2,3-butanedione monoxime; CDTA, *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid

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muscle bundles (maximal diameter 0.1–0.25 mm and sarcomere length of 2.1–2.3 μm) were mounted between a force transducer (AE 801) and a post, and then placed in a relaxing solution (75 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 100 mM MOPS (pH 7.0), 2 mM EGTA, 10 mM creatine phosphate, 250 U/ml creatine phosphokinase) at 20°C. To adjust pCa, 0.1 M $CaCl_2$ was used. Before testing SPH responses, several contraction–relaxation cycles were performed to evaluate the muscle preparation and to ensure its stability. Bovine cardiac muscle preparations are very robust and, after several initial cycles, can withstand many contraction–relaxation cycles without considerable loss of Ca^{2+}-activated force [17]. Therefore, stable force before SPH application was used as the control value.

To study the TnC involvement in SPH effects, TnC was exchanged by the method of Morimoto and colleagues [20] with some modifications. Briefly, muscles were incubated in 2 ml of a continuously agitated solution containing 5 mM *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), 40 mM Tris, 15 mM 2-mercaptoethanol, 0.6 mM NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin A (pH 8.4) for 80 min, the solution being renewed every 10 min. For reconstitution, muscles were incubated for 3–5 min in the relaxing solution with 0.1 mM dithiothreitol and 0.3 mg/ml bovine cardiac TnC kindly provided by Drs. H.M. Rarick and R.J. Solaro.

Results are presented as means ± S.E.M.

3. Results

3.1. Ca^{2+}-independent SPH-induced muscle activation

SPH-H-A added to a skinned muscle in a relaxing solution induced a large force (Fig. 2) comparable to maximal Ca^{2}-activated force (100 ± 9%, $n = 5$). At full SPH-H-A activation, addition of Ca^{2+} further increased force by 20 ± 5% ($n = 4$). 2,3-Butanedione monoxime (BDM) and phalloidin, which modulate force generated by cycling cross-bridges, were used to test the origin of the SPH-H-A-induced force. Exposure to 30 mM BDM caused a rapid and pronounced (~75%) loss of SPH-induced force (two experiments). Exposing muscle that was partially activated by SPH-H-A (1 mM) to phalloidin (80 μM) further increased force by ~30% (two experiments). We conclude that SPH-H-A activates cross-bridge cycling.

To further assess the role of Ca^{2+} in the SPH effect, muscles were partially activated by SPH-H-A in the relaxing solutions at different pCa (made by changing EGTA concentration with compensatory change in KCl concentration to keep the ionic strength constant). In two such experiments, the force activated by SPH-H-A at 10 mM EGTA was 94% and 106% of that generated by the same muscle at 2 mM EGTA. Thus, SPH-induced muscle activation is Ca^{2+}-independent.

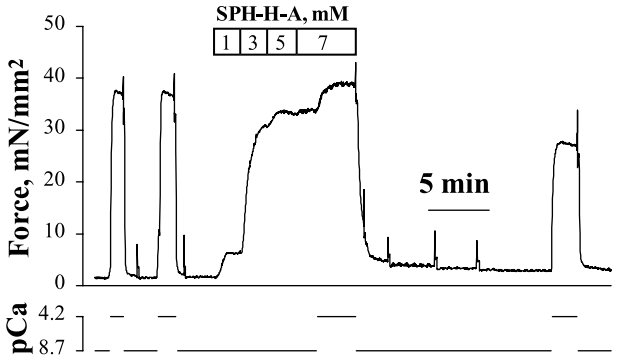
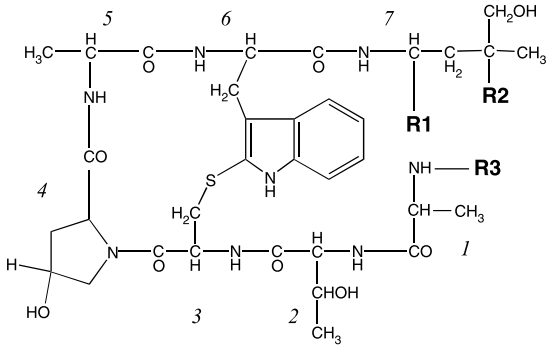


Fig. 2. Representative experiment comparing SPH-H-A- and Ca^{2+}-activated contraction. First two contractions are controls.

SPH-induced force was practically reversed by washing, but subsequent Ca^{2+}-activated force was considerably reduced (Fig. 2, 67 ± 3% of initial force, $n = 4$). This reduction in Ca^{2+}-activated force could not be restored by repeated washing with relaxing solution, by cycling between relaxing and activating solutions, by incubation with dithiothreitol, or by incubation with exogenous cardiac bovine TnC. Furthermore, force was not restored by TnC extraction and reconstitution with exogenous TnC (Fig. 3). Thus, force reduction was apparently irreversible and not mediated via an effect on TnC.

3.2. Role of the phalloidin site in SPH effects

SPH retains about 3% of phalloidin affinity to the specific actin site [21]. To probe a role of the phalloidin site in the observed SPH-H-A effects on force, SPH-H-A responses were assessed in muscle pretreated with phalloidin. Pretreatment with phalloidin, which binds practically irreversibly to actin, prevented the response of Ca^{2+}-activated muscle to all phallotoxins, except SPH [14–17]. In the present study, pretreatment with phalloidin did not prevent the Ca^{2+}-independent



Compound	R1	R2	R3	R1 & R2
SPH-H-A	– COOH	– OH	– H	
SPH-L			– H	– CO – O –
N-acetyl-SPH			– CO – CH_3	

Fig. 1. Chemical structures of secophalloidins. Figures in italics indicate numbers of amino acid residues.

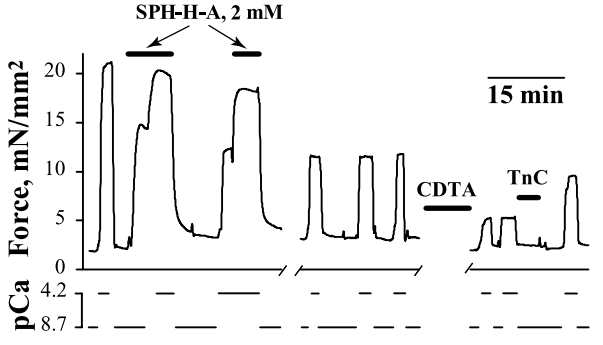


Fig. 3. Effect of TnC extraction followed by reconstitution on the force generated by muscle in which force had been preliminarily suppressed by SPH-H-A. During the first break in the record, the muscle was washed with relaxing solution for ~20 min. During the second break in the record, TnC was extracted with CDTA-containing solution for 80 min (see Section 2.2 for details). First contraction is control. During the next two contractions, force was suppressed by SPH-H-A. The following three contractions demonstrate stability of suppressed force. After reconstitution with TnC (the last contraction), force did not exceed that before TnC extraction.

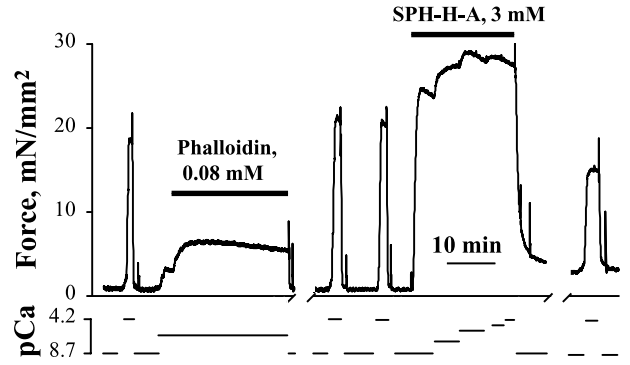


Fig. 4. Pretreatment with phalloidin does not prevent either the SPH-H-A-induced force activation or subsequent force suppression. During each break in the record, the muscle was washed with relaxing solution for ~20 min.

SPH-H-A-induced force response or the subsequent depression of Ca^{2+}-activated force (Fig. 4). Thus, we conclude that all SPH-H-A effects originate from binding sites distinct from the phalloidin site.

3.3. Structure–function relationship in SPH

The transformation of phalloidin to SPH results in the appearance of C- and N-terminals, which may influence force activation. The roles of these C- and N-terminals were systematically assessed (Fig. 5). In each of these experiments, the first and the last activation were induced by the same compound to evaluate stability of the response. The results demonstrated that the C-terminal structure is critical for SPH muscle activation. At the same concentration, SPH-L-induced force was less than 10% of that induced by SPH-H-A. Since the force response increased with SPH-L concentration and did not appear to saturate (upper panel of Fig. 5), it is possible that the hydroxy acid configuration at the C-terminal is important for affinity rather than for the effect of bound compound. On the other hand, the N-terminal is likely to have no role in the mechanism of SPH action since acetylation of the terminal amino group had no significant effect.

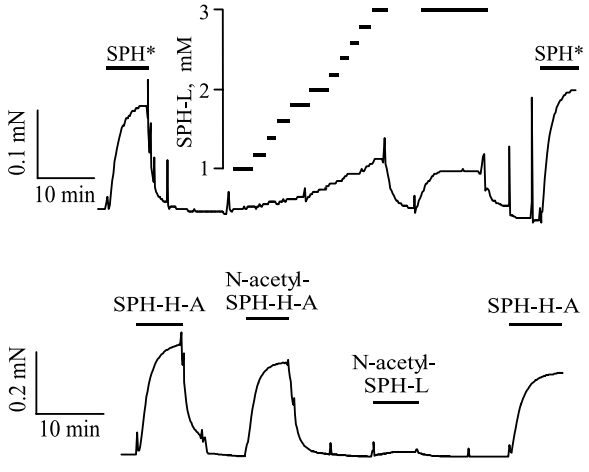


Fig. 5. Effect of N- and C-terminal structure on SPH ability to activate muscle without Ca^{2+}. If not indicated, concentrations of all compounds were 1 mM. Upper panel: Effect of lactone closure on the SPH effect. SPH*, Dr. Wieland's preparation, was a 1:1 mixture of SPH-L and SPH-H-A. Lower panel: Effects of N-terminal acetylation and of lactone closure in *N*-acetyl-SPH.

4. Discussion

The main finding of this study is Ca^{2+}-independent activation of cardiac muscle by SPH. Striated muscle can be activated by both Ca^{2+} and cross-bridges strongly bound to actin [1–3]. Therefore, two distinct mechanisms might exist for the Ca^{2+}-independent activation of cardiac muscle by SPH. One possibility is that SPH acts on the Ca^{2+} route directly by changing actin–tropomyosin–troponin interactions and thereby promoting, for example, a transition from the 'off' to the 'on' actin states without Ca^{2+}. A second possibility is a SPH-induced strengthening of actomyosin interaction at a given state of thin filament activation thereby promoting strongly bound cross-bridges which in turn can further activate the thin filament.

In the present study, we observed that maximal SPH-H-A-induced force was further increased (~20%) by Ca^{2+}. Previously, we found that maximal Ca^{2+}-activated force was further increased (~25%) by SPH [14]. Taken together these results prove different, non-competitive mechanisms of muscle activation by Ca^{2+} and SPH. Half-maximal force without Ca^{2+} was reached at ~2 mM SPH (Fig. 2), instead of 50–70 μM SPH at full Ca^{2+} activation [14]. Earlier we found ~0.7 pCa units leftward shift of the force–pCa curve induced by 1 mM SPH [22]. Thus, there is a strong positive reciprocal interaction between SPH and Ca^{2+}, probably involving changes in inter-protein interactions.

Non-competitive relationship between Ca^{2+} and SPH is consistent with a direct strengthening of the actomyosin interaction by SPH. The mutual increase in sensitivity also might account for the direct action of SPH on the actomyosin bond, since there is a pronounced feedback between strongly bound cross-bridges and Ca^{2+} activation in cardiac muscle [23]. In support of this idea, our SPH-H-A preparation was found to reduce ATPase activity of acto-S1 by 30–50% (S.S. Lehrer, personal communication), indicating SPH sensitivity of actomyosin itself.

To summarize, SPH activates cardiac muscle without Ca^{2+}. This property of SPH may make it a valuable tool to study the regulation of muscle contraction. Although the mechanism of SPH activation remains to be determined, SPH might activate muscle directly by changing actomyosin interaction.

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