



Xestospongine C, a selective and membrane-permeable inhibitor of IP₃ receptor, attenuates the positive inotropic effect of α -adrenergic stimulation in guinea-pig papillary muscle

¹Shigeki Miyamoto, ¹Masanori Izumi, ¹Masatoshi Hori, ²Motomasa Kobayashi, *¹Hiroshi Ozaki & ¹Hideaki Karaki

¹Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan and ²Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka, Suita, Osaka 569-0871, Japan

1 We evaluated the role of the inositol 1,4,5-triphosphate (IP₃) receptor-mediated Ca²⁺ release on the positive inotropic effects of α -adrenergic stimulation using a novel, potent, selective membrane-permeable blocker of IP₃ receptor, xestospongine C.

2 Guinea-pig papillary muscle permeabilized with saponin exhibited spontaneous oscillatory contractions in solution buffered with pCa²⁺ 6.5 by a low concentration of EGTA. The oscillatory activity was increased by adding 100 μ M IP₃ and abolished by 1 μ M ryanodine or 30 μ M cyclopiazonic acid. Xestospongine C (3 μ M) inhibited the IP₃-induced increase in the oscillatory contractions without affecting basal oscillations.

3 In intact papillary muscle, xestospongine C (3 μ M) inhibited the positive inotropic effects of phenylephrine, resulting in a rightward and downward shift of the concentration-response curve for phenylephrine. On the contrary, xestospongine C did not affect the concentration-response curve for phenylephrine obtained in the presence of ryanodine (1 μ M).

4 On the other hand, xestospongine C affected neither basal contractions nor the positive inotropic effects of a high extracellular Ca²⁺ concentration (3.2 mM) or that of isoprenaline (1 and 10 nM).

5 These results suggest that the IP₃-mediated increase in Ca²⁺ release is involved in the positive inotropic effects of α -adrenergic stimulation in the guinea-pig cardiac muscle.

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Abbreviations: IP₃, inositol 1,4,5-triphosphate; SR, sarcoplasmic reticulum; PSS, physiological salt solution; CICR, Ca²⁺-induced Ca²⁺-release

Introduction

It has been shown that inositol 1,4,5-triphosphate (IP₃), increased by the activation of a phosphoinositide signalling cascade, enhances Ca²⁺ efflux from the sarcoplasmic reticulum (SR) in cardiac muscle (Nosek *et al.*, 1986). Although the increase in Ca²⁺ release is expected to be involved in receptor-mediated positive inotropy, the role of IP₃ on the positive inotropic effects of α -adrenergic stimulation in cardiac muscle is still controversial (for review see Fedida *et al.*, 1993; De Jonge *et al.*, 1995). To determine the role of IP₃, it would be useful to have drugs that interfere with the IP₃-mediated Ca²⁺ release. Although heparin and decavanadate have been utilized for this purpose, conclusive evidence has not been obtained because these agents are nonselective (for review see Wilcox *et al.*, 1998). Xestospongine C, isolated from the sponge *Xestospongia* sp., has recently been shown to be a membrane-permeable blocker of IP₃-mediated Ca²⁺ release (Gafni *et al.*, 1997; Wilcox *et al.*, 1998). This compound is suggested to be a potent and highly sensitive inhibitor of IP₃ receptor with IC₅₀ of 350 nM, which is 30 times lower than the IC₅₀ for ryanodine-receptor (Gafni *et al.*, 1997). In the present experiments, we used xestospongine C to examine the role of IP₃ receptor on cardiac contraction.

Methods

Measurement of muscle force in papillary muscle

Male guinea-pigs (350–500 g) were anaesthetized with gaseous diethylether and the heart was rapidly removed and washed in ice-cold (4°C) normal physiological salt solution (PSS) (mM) NaCl 145, KCl 5, CaCl₂ 1.6, MgCl₂ 1, glucose 10 and HEPES 10. Papillary muscles with a diameter less than 1 mm were excised from the right ventricle. The muscle preparation was perfused continuously (12 ml min⁻¹) with a pre-warmed (30°C), pre-oxygenated physiological salt solution (PSS) solution. Muscle tension was measured isometrically and the resting tension applied to each preparation was adjusted to give 90% of the maximum tension development. During the equilibration period, the muscles were stimulated electrically by 5-ms square pulses at 0.5 Hz with a voltage 1.2 fold greater than the threshold intensity, using an electronic stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan) and a pair of platinum wires. Contractile force was digitized at the sampling rate of 110 points s⁻¹. The amplitudes of isometric contraction elicited by a stimulation frequency of 0.5 Hz in the presence of 1.6 mM Ca²⁺ were used as the control (100%).

The effects of phenylephrine and isoprenaline on contractions were examined in the presence of propranolol (1 μ M) and phentolamine (1 μ M), respectively, to avoid the interference

*Author for correspondence.

with the β - and α -adrenoceptor-mediated effects, respectively. Muscles were pretreated with xestospongine C ($3 \mu\text{M}$) for 30 min, and then phenylephrine was cumulatively applied to obtain a concentration-response curve.

Skinned muscle preparations

Small bundles of muscle tissue (120 – $150 \mu\text{m}$ in diameter and 1 – 2 mm in length) were obtained by teasing tissue from guinea-pig papillary muscle. The bundles were skinned with $50 \mu\text{g ml}^{-1}$ saponin for 30 min in relaxing solution (see below). Both ends of each bundle were tied with monofilament silk thread. One end of each bundle was connected to an isometric transducer, and the other end was fixed with a stainless steel pin to the siliconized floor of a chamber under a resting tension of 0.5 mN and equilibrated for 60–90 min. During this period, $10 \mu\text{M}$ Ca^{2+} was repeatedly applied until the peak force became reproducible. Experiments were carried out at room temperature ($\sim 22^\circ\text{C}$).

The relaxing solution contained magnesium (methanesulphonate) $_2$ (5.25 mM), potassium methanesulphonate (40 mM), ATP (5.3 mM), PIPES (20 mM), phosphocreatine (10 mM), creatine phosphokinase (20 unit ml^{-1}), FCCP ($1 \mu\text{M}$), E-64 (1 mg ml^{-1}), and EGTA (10 mM). The ionic strength was adjusted to 0.2 M , and the pH was adjusted to 7.1 at 20°C . Free Ca^{2+} concentration was changed by adding an appropriate amount of Ca^{2+} -(methanesulphonate) $_2$. Force oscillations were introduced by exposing the tissue to relaxing solution

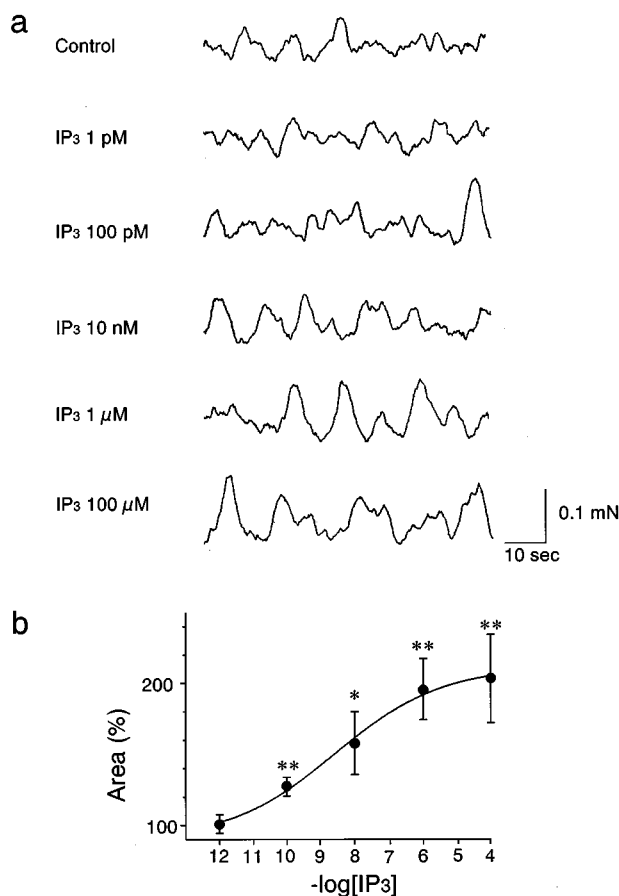


Figure 1 Effects of IP_3 (1 pM – $100 \mu\text{M}$) on the force oscillations in saponin-skinned muscles. Force oscillations were introduced by low-EGTA (0.05 mM) and $\text{pCa}^{2+} 6.5$ solution. (a) Typical trace of effect of IP_3 . (b) Summarized data of (a). The area of the force oscillations during 1 min was used for the quantitative assessment of effects of IP_3 . * $P < 0.05$, ** $P < 0.01$ vs the basal force oscillations.

containing 0.05 mM EGTA in which the free Ca^{2+} concentration was adjusted to $\text{pCa}^{2+} 6.5$ by adding Ca^{2+} (methanesulphonate) $_2$. The ionic compositions of these solutions were calculated by a computer program developed by Fabiato (1981) with slight modification. The area of the force oscillations during a 1-min period was used for the quantitative assessment of the effects of IP_3 and xestospongine C.

Chemicals

The chemicals used were phenylephrine, phentolamine, isoprenaline, propranolol, cyclopiazonic acid, saponin (Sigma, St. Louis, MO, U.S.A.), EGTA (Wako Pure Chemicals, Tokyo, Japan) and IP_3 (Dojindo Laboratories, Kumamoto, Japan). Xestospongine C was isolated and purified from an Okinawan marine sponge, *Xestospongia* sp. (Kobayashi *et al.*, 1998). Cyclopiazonic acid and xestospongine C were dissolved in dimethylsulphoxide and ethanol, respectively.

Statistics

The numerical data were expressed as mean \pm s.e.mean. Differences between mean values were evaluated by paired

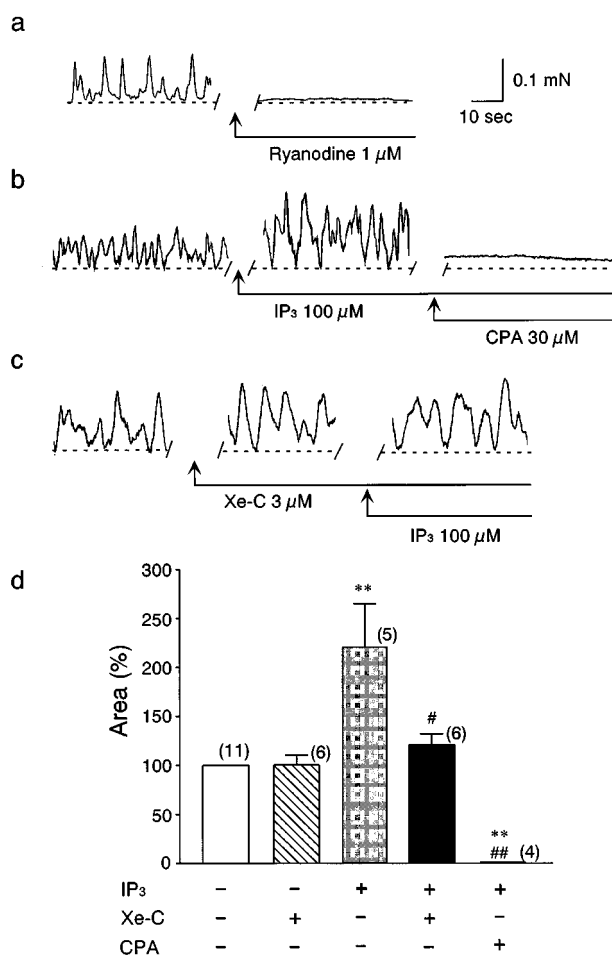


Figure 2 Effects of xestospongine C (Xe-C, $3 \mu\text{M}$) on the force oscillations in saponin-skinned muscles. (a) Typical trace of effects of $1 \mu\text{M}$ ryanodine on the force oscillations. (b) Typical trace of $100 \mu\text{M}$ IP_3 -induced potentiation of oscillations and the effects of addition of cyclopiazonic acid (CPA, $30 \mu\text{M}$). (c) Typical trace of the effect of $100 \mu\text{M}$ IP_3 in the presence of $3 \mu\text{M}$ xestospongine C. (d) Summarized data of (b) and (c). The numbers of the experiments is shown near each column. ** $P < 0.01$ vs the basal force oscillations. # $P < 0.05$, ## $P < 0.01$ vs the results obtained in the presence of IP_3 .

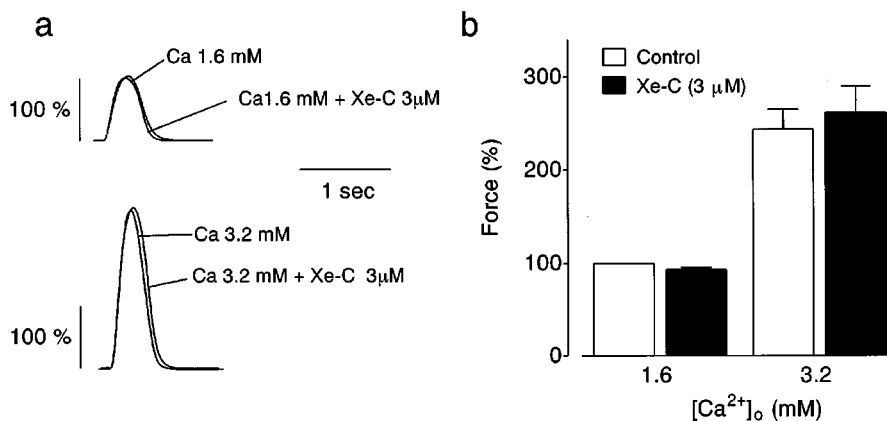


Figure 3 Effects of $3 \mu M$ xestospongins C (Xe-C) on the force of contractions in 1.6 and 3.2 mM $[Ca^{2+}]_o$. (a) Typical tracings of the force. (b) Summarized data ($n=4$). Each column represents the mean \pm s.e.mean. Values obtained in normal PSS at 0.5 Hz were taken as 100%.

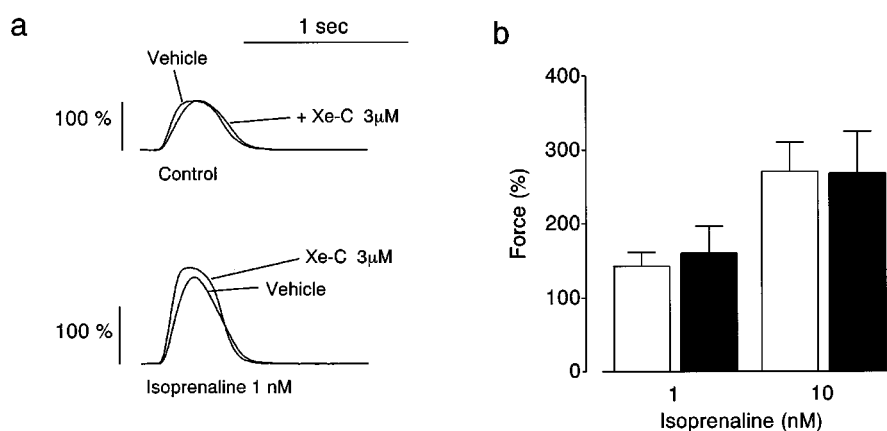


Figure 4 Effects of $3 \mu M$ xestospongins C (Xe-C) on the positive inotropic effects of isoprenaline (Iso). (a) Typical tracings of the force. (b) Summarized data ($n=5-10$). Each column represents the mean \pm s.e.mean. Values obtained in normal PSS at 0.5 Hz were taken as 100%.

Student's *t*-test and, where appropriate, analysis of variance (ANOVA) followed by the Bonferroni test. A probability of less than 0.05 was taken as a statistically significant difference.

Results

The effects of xestospongins C on force oscillations in saponin-skinned muscles

In saponin-skinned guinea-pig papillary muscle, force oscillations were observed in $pCa^{2+} 6.5$ solution (Figure 1a), and the addition of IP_3 (100 pM to 100 μM) augmented the force oscillation in dose-dependent manner (Figure 1a,b), as has been reported by Nosek *et al.* (1986). Using these force oscillations in saponin-skinned fibre, we examined the pharmacology of xestospongins C on cardiac SR functions.

The basal spontaneous force oscillations were completely inhibited by 1 μM ryanodine (Figure 2a) or by 30 μM cyclopiazonic acid, a SR Ca^{2+} -ATPase inhibitor (data not shown), suggesting that the force oscillations were induced by the rhythmic Ca^{2+} release from the SR. The force oscillations stimulated by 100 μM IP_3 were also abolished by 30 μM cyclopiazonic acid. On the other hand, the basal force oscillations were not changed by 3 μM xestospongins C. However, in the presence of xestospongins C, IP_3 failed to enhance the force oscillations (Figure 2c). These results are summarized in Figure 2d.

The effects of xestospongins C in intact muscle

The application of 3 μM xestospongins C had no effect on the peak force of contraction in normal PSS (Figure 3a). An increase in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from 1.6 to 3.2 mM increased the peak force. Xestospongins C (3 μM) did not affect the positive inotropic effect of the elevation in $[Ca^{2+}]_o$ (Figure 3a,b).

Positive inotropic effects were also elicited by 1 nM isoprenaline in the presence of 1 μM phentolamine (Figure 4a). Xestospongins C (3 μM) did not change the isoprenaline-induced inotropism (Figure 4a). The effects of xestospongins C on 1 and 10 nM isoprenaline-induced inotropic action are summarized in Figure 4b.

As shown in Figure 5a, phenylephrine (1 and 10 μM), in the presence of 1 μM propranolol, increased the twitch contractions in a concentration-dependent manner. Xestospongins C partially but significantly inhibited the positive inotropic effect of phenylephrine. Figure 5b shows the effects of various concentrations of phenylephrine on twitch contractions in the absence or presence of 3 μM xestospongins C. The results indicate that the concentration-response curve for phenylephrine was shifted to the lower right by xestospongins C.

Ryanodine (1 μM) decreased the twitch contraction in normal PSS, and xestospongins C (3 μM) alone did not change the contractions obtained in the presence of ryanodine (Figure 6). In the presence of ryanodine, the positive inotropy of phenylephrine was inhibited, resulting in the downward shift

of the dose-response curve for phenylephrine. As shown in Figure 6, xestospongins C had no effects on the dose-response curve for phenylephrine obtained in the presence of ryanodine.

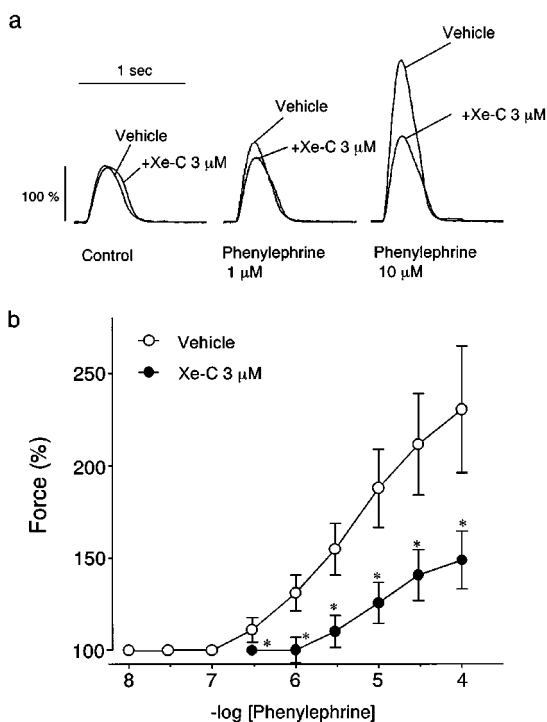


Figure 5 Effects of 3 μM xestospongins C (Xe-C) on the positive inotropic effects of phenylephrine. (a) Typical tracings of the xestospongins C on the contractions in the presence of 1 and 10 μM phenylephrine. (b) Effects of xestospongins C on the concentration-response curve of phenylephrine. Open and closed symbols represent the results in the absence and presence of xestospongins C, respectively. Each value represents the mean \pm s.e.mean. * $P < 0.05$ vs the vehicle. Vehicle, $n = 8$. Xe-C, $n = 10$.

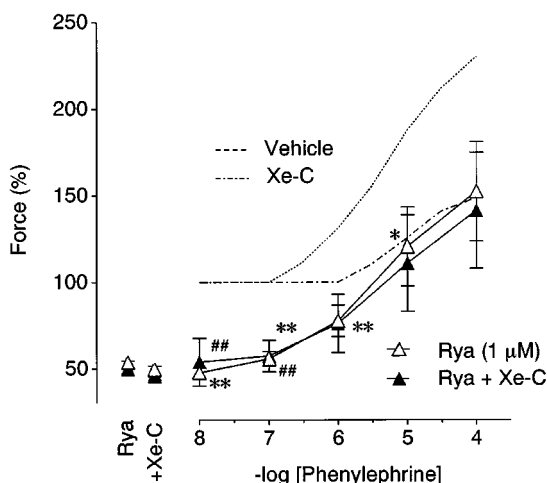


Figure 6 Effects of 3 μM xestospongins C (Xe-C) on the positive inotropic effects of phenylephrine in the presence of ryanodine. Open and closed symbols represent the results in the absence and presence of xestospongins C, respectively. Control responses in the absence of ryanodine (dashed lines) are adopted from Figure 5b. Each value represents the mean \pm s.e.mean ($n = 7$). * $P < 0.05$, ** $P < 0.01$ vs the vehicle data in Figure 5b. ## $P < 0.01$ vs the data obtained in the presence of xestospongins C in Figure 5b.

Discussion

In saponin-skinned papillary muscles, spontaneous force oscillations were observed in a low-EGTA/pCa²⁺ 6.5 solution and these oscillations were completely abolished by ryanodine or cyclopiazonic acid (Figure 2). These results suggest that the force oscillations are mediated by the spontaneous Ca²⁺ release from the SR through the ryanodine-receptor, as has been reported by Nosek *et al.* (1986) and Vites & Pappano (1990). In guinea-pig papillary muscle, IP₃ (100 pM–100 μM) augmented the force oscillation in a dose-dependent manner (Figure 1) and 3 μM xestospongins C inhibited the IP₃-induced potentiation in force oscillations without affecting the basal force oscillations (Figure 2). These results suggest that in guinea-pig papillary muscle skinned with saponin retains the IP₃-mediated pathway for Ca²⁺-release from the SR. The lack of the effect of xestospongins C on the steady state oscillations suggests the high selectivity of this inhibitor for IP₃-receptor over the ryanodine-receptor (Gafni *et al.*, 1997).

In the intact papillary muscle, 3 μM xestospongins C inhibited the phenylephrine-induced positive inotropic action. However, xestospongins C had no effects on the basal twitch contractions or the positive inotropic effects elicited by the increase in [Ca²⁺]_o. Furthermore, the positive inotropic action of isoprenaline, which is mediated by increasing the activities of voltage-dependent Ca²⁺ channels and SR Ca²⁺-ATPase, was also unaffected by xestospongins C (Figure 4). These results suggest that IP₃-mediated Ca²⁺ release is involved only in an α -adrenoceptor mediated positive inotropy.

Ryanodine inhibited not only the basal contraction, but also the positive inotropy of phenylephrine, suggesting that Ca²⁺ induced Ca²⁺ release (CICR) contributes to the positive inotropy of phenylephrine. Xestospongins C, however, did not inhibit the positive inotropic effect of phenylephrine in the presence of ryanodine (Figure 6). These results suggest that IP₃-mediated Ca²⁺ release and Ca²⁺-induced Ca²⁺ release are derived from the same intracellular Ca²⁺ source in cardiac muscle. In addition, these results support the notion that xestospongins C (up to 3 μM) has no effect on cellular functions in the absence of SR function. In this study, we examined the effects of 10 μM xestospongins C and found that this concentration of xestospongins C decreased the contraction in normal PSS to approximately 70% (our unpublished observation), suggesting that higher concentration of xestospongins C might interfere with other components of the contractile responses.

The present data also showed that 3 μM xestospongins C, which almost completely inhibits IP₃ induced Ca²⁺ release *in vitro*, only partially inhibited the inotropic action of α -adrenergic stimulation. This result suggests that other mechanisms, such as the activation of protein kinase C, contribute to the α -adrenoceptor mediated positive inotropy, since it has been shown that not only [Ca²⁺]_i (Woo & Lee, 1999) but also a myofilament Ca²⁺ sensitivity (Gambassi *et al.*, 1992; De Jonge *et al.*, 1995) is increased through the activation of protein kinase C. Alternatively, the membrane permeability is not high enough to access SR IP₃ receptors.

In conclusion, our results provide initial evidence of the role of the IP₃ receptor on the positive inotropy elicited by α -adrenergic stimulation.

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