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FULL PAPER

Exploiting peptidomimetics to synthesise compounds that activate ryanodine receptor calcium release channels

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Abstract: Ryanodine receptor (RyR) Ca^{2+} release channels are essential for contraction in skeletal and cardiac muscle and are prime targets for modification of contraction in disorders affecting either the skeletal or heart musculature. We have designed and synthesized a number of compounds with structures based on a naturally occurring peptide (A peptides) that modifies the activity of RyRs. In total 34 compounds belonging to 8 different classes were prepared. The compounds were screened for their ability to enhance Ca^{2+} release from isolated cardiac SR vesicles, with 25 displaying enhanced Ca^{2+} release. Competition studies with the parent peptides indicated that the synthetic compounds were acting at a competing site. The activity of the most effective of the compounds BIT180 was further explored using Ca^{2+} release from skeletal SR vesicles and contraction in intact skeletal muscle fibres. The compounds did not alter tension in intact fibres, indicating that (as expected) they were not membrane permeable but importantly that they were not toxic to the intact cells. Proof in principal that the compounds would be effective in intact muscle fibres if rendered membrane permeable was obtained with a structurally related membrane permeable scorpion toxin (imperatoxin A) which did enhance contraction.

Introduction

Muscle contraction, underlying movement, respiration and heart beat, depends on the activity of ryanodine receptor (RyR) calcium ion channels which release Ca^{2+} from the internal sarcoplasmic reticulum (SR) calcium stores within skeletal and cardiac muscle fibres. Defects in Ca^{2+} signalling lead to atrial and ventricular arrhythmia and heart failure, as well as debilitating skeletal myopathies [1]. Since the RyR ion channel is essential for Ca^{2+} release and contraction, it is an ideal molecular target for manipulating contraction [2]. Drugs that modulate RyR activity can alleviate the effects of these cardiac and skeletal myopthies. For example dantrolene (or azumolene) is used to depress skeletal RyR (RyR1) activity during episodes of malignant hyperthermia [3], while JTV519 (S107) is a cardio-protective agent that stabilises RyR1 and cardiac RyR (RyR2) activity [4, 5]. While these two agents are generally effective, there are few other drugs that interact specifically with RyRs and have the potential for therapeutic applications.

We have previously described novel peptides corresponding to parts of the skeletal muscle dihydropyridine receptor (DHPR) which interact with the RyR [6] and have explored the possibility of using the peptides to modify RyR activity *in vivo* [7]. The cytoplasmic II-III loop between the 2nd and 3rd membrane repeats in the DHPR α1 subunit is essential for skeletal muscle contraction [8]. A 20 residue peptide corresponding to a sequence in the loop (A peptide) is active on skeletal and cardiac RyRs [6, 9]. We have used NMR techniques to determine the structural requirements for critical residues to either activate or inhibit RyRs [10-13].

We find that the ***A*** peptides activate the RyR with high affinity at low Ca^{2+} concentrations (100 nM to 1.0 μM) or inhibit with high affinity at higher Ca^{2+} concentrations (10 μM to 100 μM) [6, 9].The structural information that we used to design the synthetic compounds described in this manuscript comes from our NMR studies of peptides having a skeletal DHPR ***A*** sequence (***A1*** and ***A2DR18***), and the scorpion toxins Imperatoxin A (IPTXA), Maurocalcine and the phi-LITX-Lw1a toxin (LITX) [10-13]. The ***A*** peptides and scorpion toxins are thought to act at a similar site on the RyR [14-16]. The structure of the disulphide-linked toxins is more stable than the ***A1*** helix and the toxins activate RyR1 with a > 10-fold higher affinity than the ***A*** peptide. Strategic substitutions that increase structural stability also increase ***A*** peptide activity [11]. The ***A1*** peptide sequence is ^{671}TSAQKAKAEERKRRKMSRGL^{690} and by substituting Ser for Ala 17 and the D for L isomer of Arg 18 (***A2DR18***) we found the structural stability of this peptide had increased. The rate of Ca^{2+} release from SR vesicles is greater with the more structured ***A2DR18*** than with wild-type ***A1***. Our first approach to obtaining membrane permeable ***A*** peptides was to conjugate lipid tails to the peptides. This did not reduce peptide's activity on Ca^{2+} release [7], but they were toxic on whole cells and thus have not been explored further.

In this study we describe the synthesis of several classes of compounds with structural aspects based on common structural features of the DHPR II-III loop ***A1*** peptides and the RyR1-active scorpion toxins. We have tested the actions of these compounds on Ca^{2+} release from cardiac and skeletal SR vesicles and have examined the actions of the most active compound on intact skeletal muscle fibres. The synthetic compounds are effective activators of RyR channels in SR vesicles and appear to compete with the ***A*** peptides in these functions.

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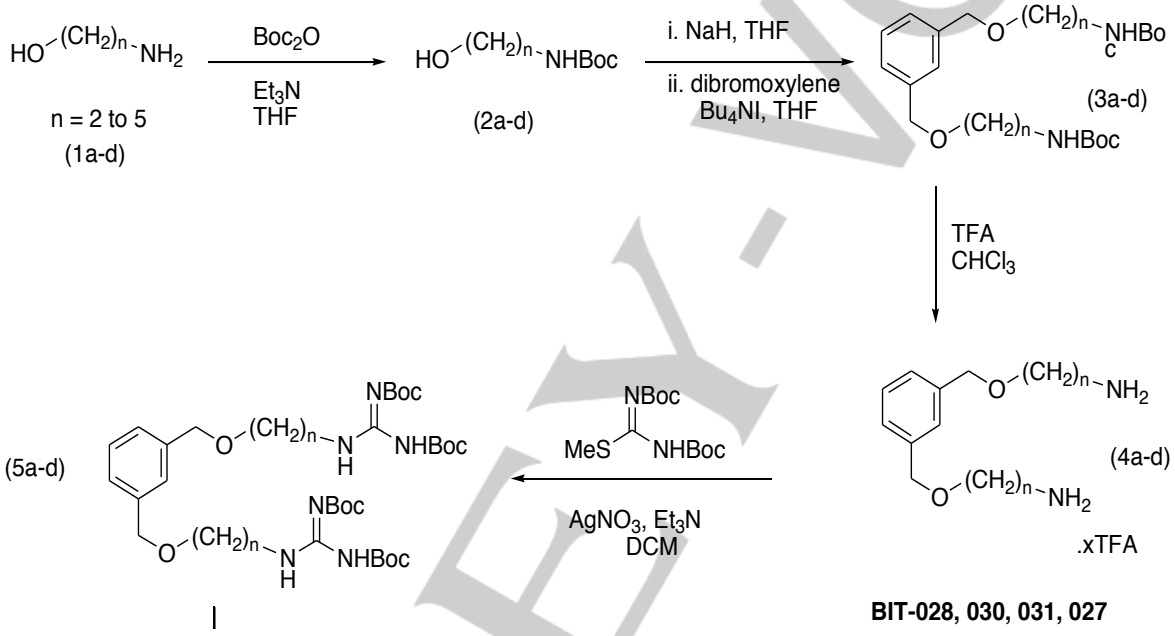
Results and Discussion

Synthesis of novel compounds.

As described in the introduction, we previously noted that a 20 amino acid α-helical peptide derived from the II-III loop region of the dihydropyridine receptor was capable of activating both the skeletal and cardiac RyRs and that the alignment of basic residues along the helix surface was responsible for this function. Moreover, we observed that enhancement of the α-helical nature of the peptide through modification of the amino acid sequence, promoted an increase in the activation of these peptides. In order to mimic the functional properties of these peptides, we synthesised a series of compounds whose key interactive features were retained. The compounds were made up of non-peptide frameworks comprising of cyclic and aromatics moieties onto which positively charged moieties were attached. These backbone frameworks were selected as suitable anchoring points for the attachment of flexible, positively-charged sidechains of variable length. In total, 35 compounds were prepared and tested. These compounds fell into 8 different structural classes, the methods for the development of each are shown in Schemes 1-8.



Scheme 1 Synthesis of BIT - 028, 027, 030, 031, compounds

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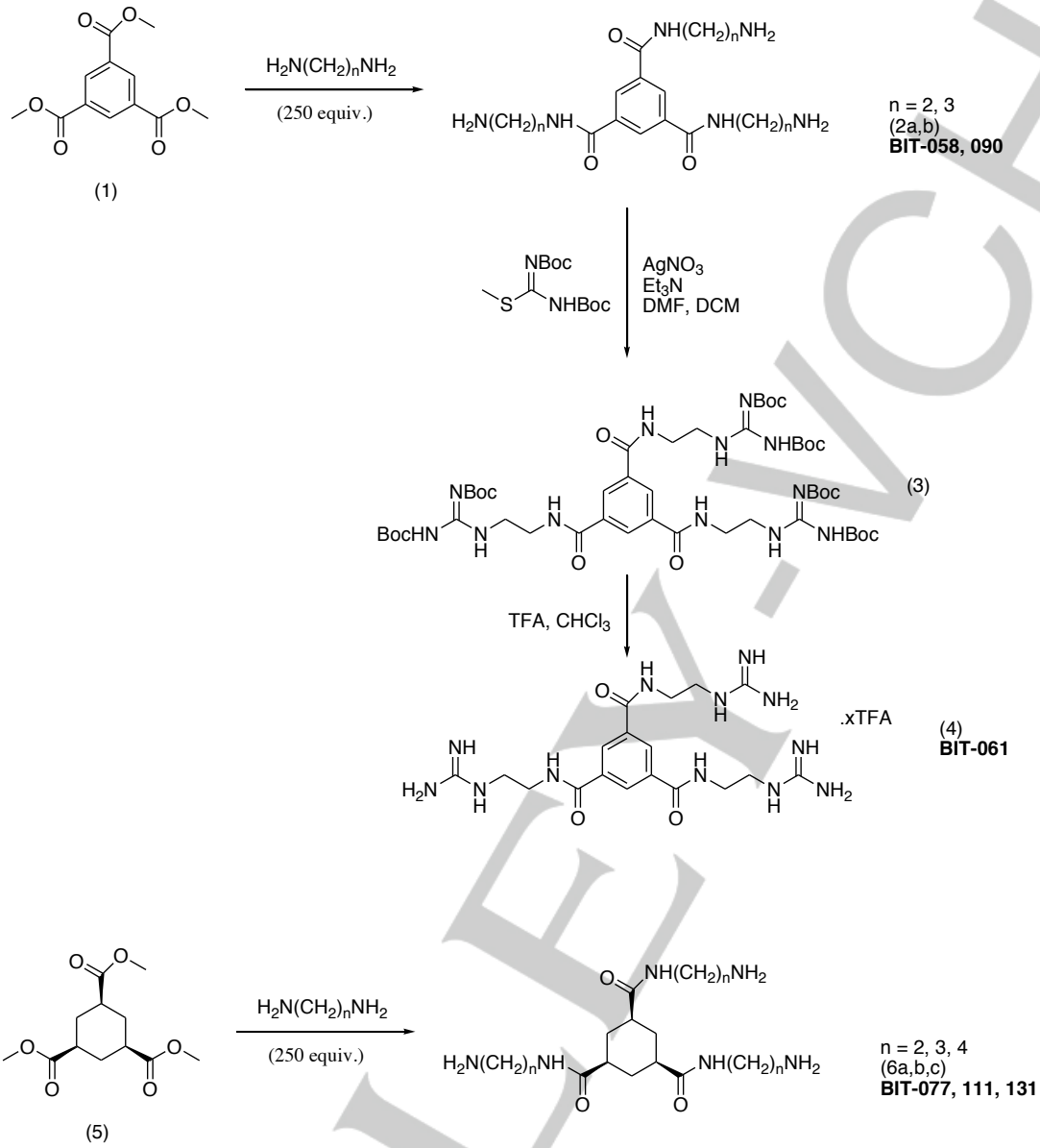

Scheme 2 Synthesis of BIT - 058, 061, 077, 090, 111, 131 compounds

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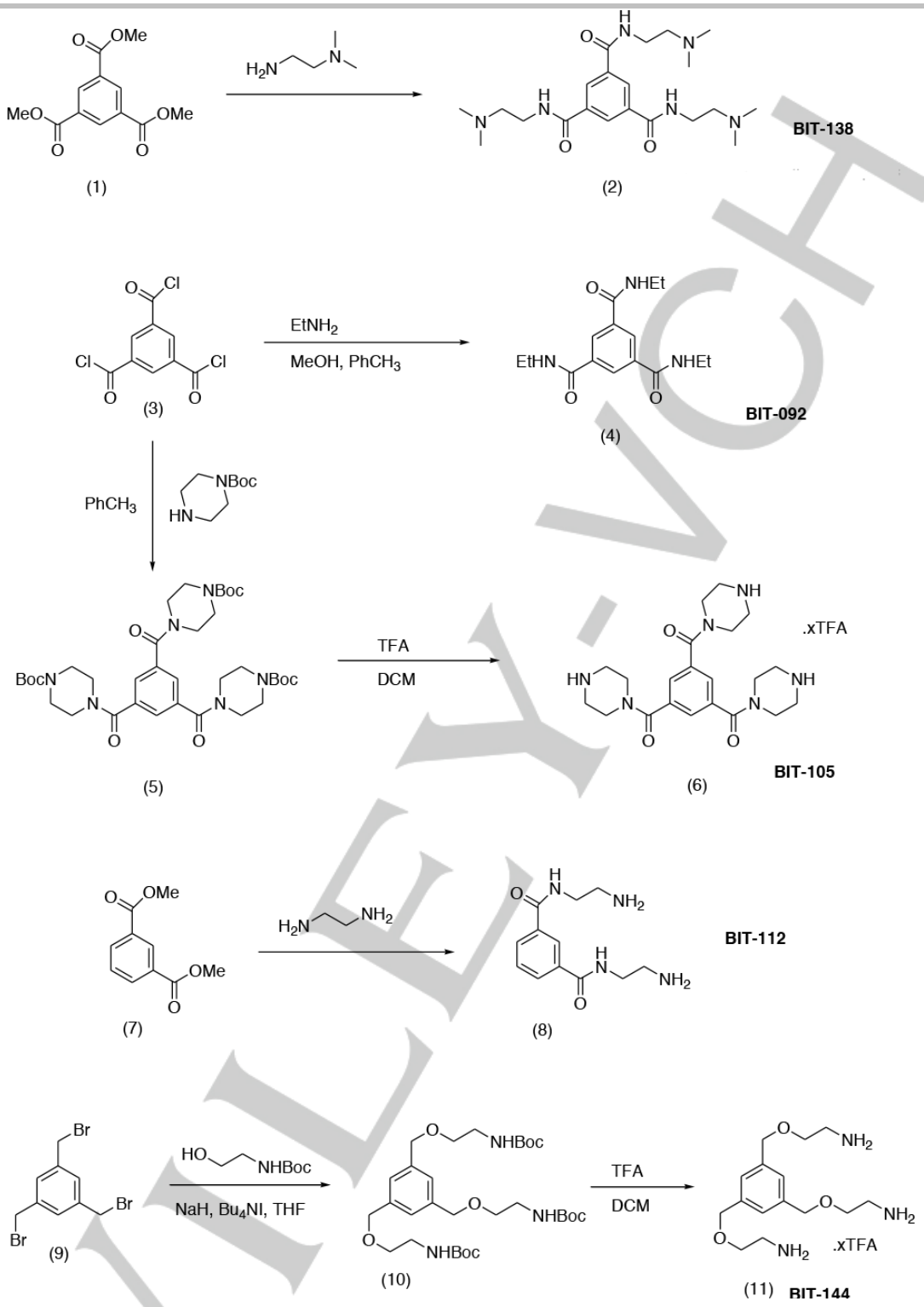

Scheme 3 Synthesis of BIT – 092, 105, 112, 138, 144 compounds

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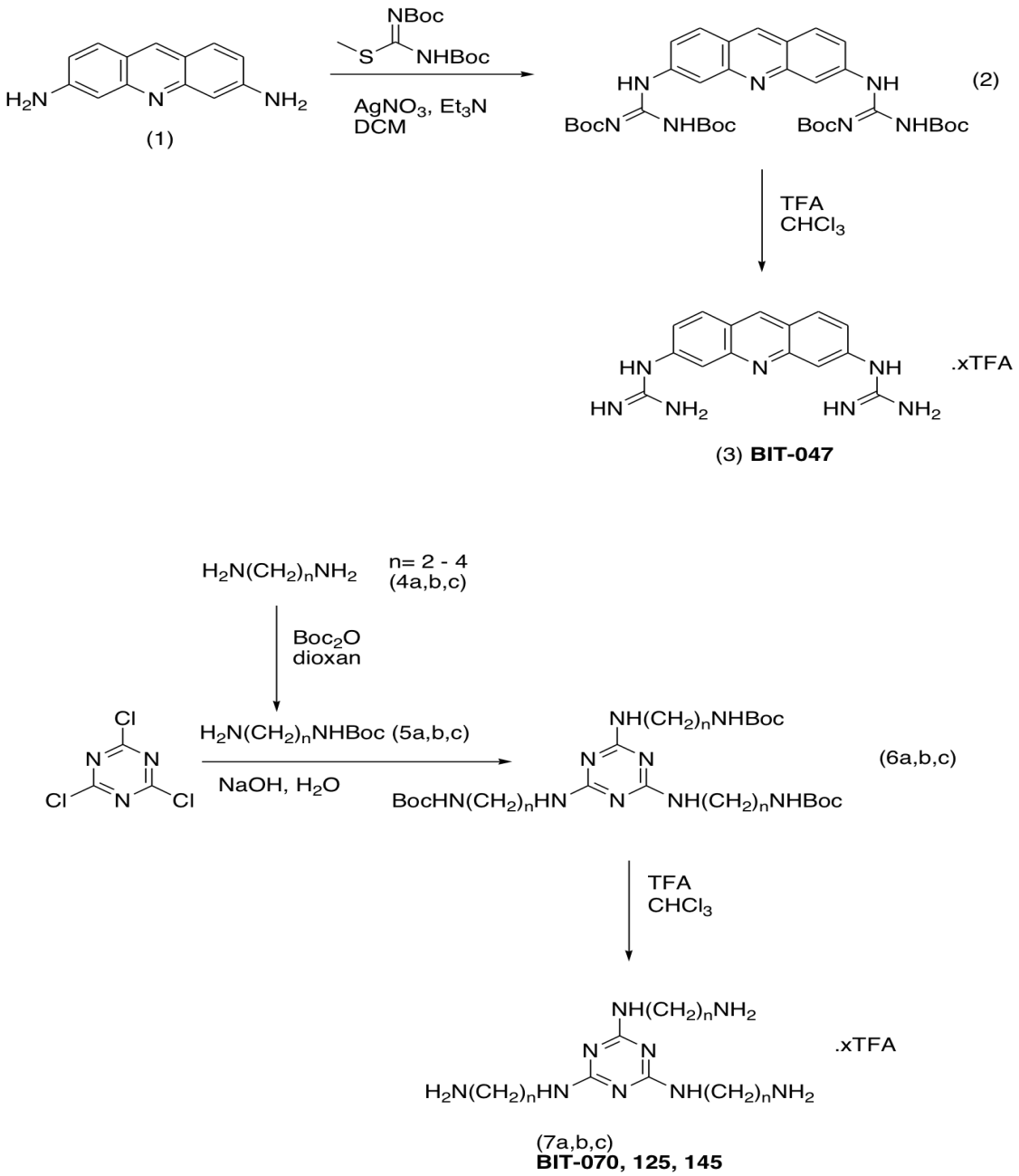

Scheme 4 Synthesis of BIT – 047, 070, 125, 145 compounds

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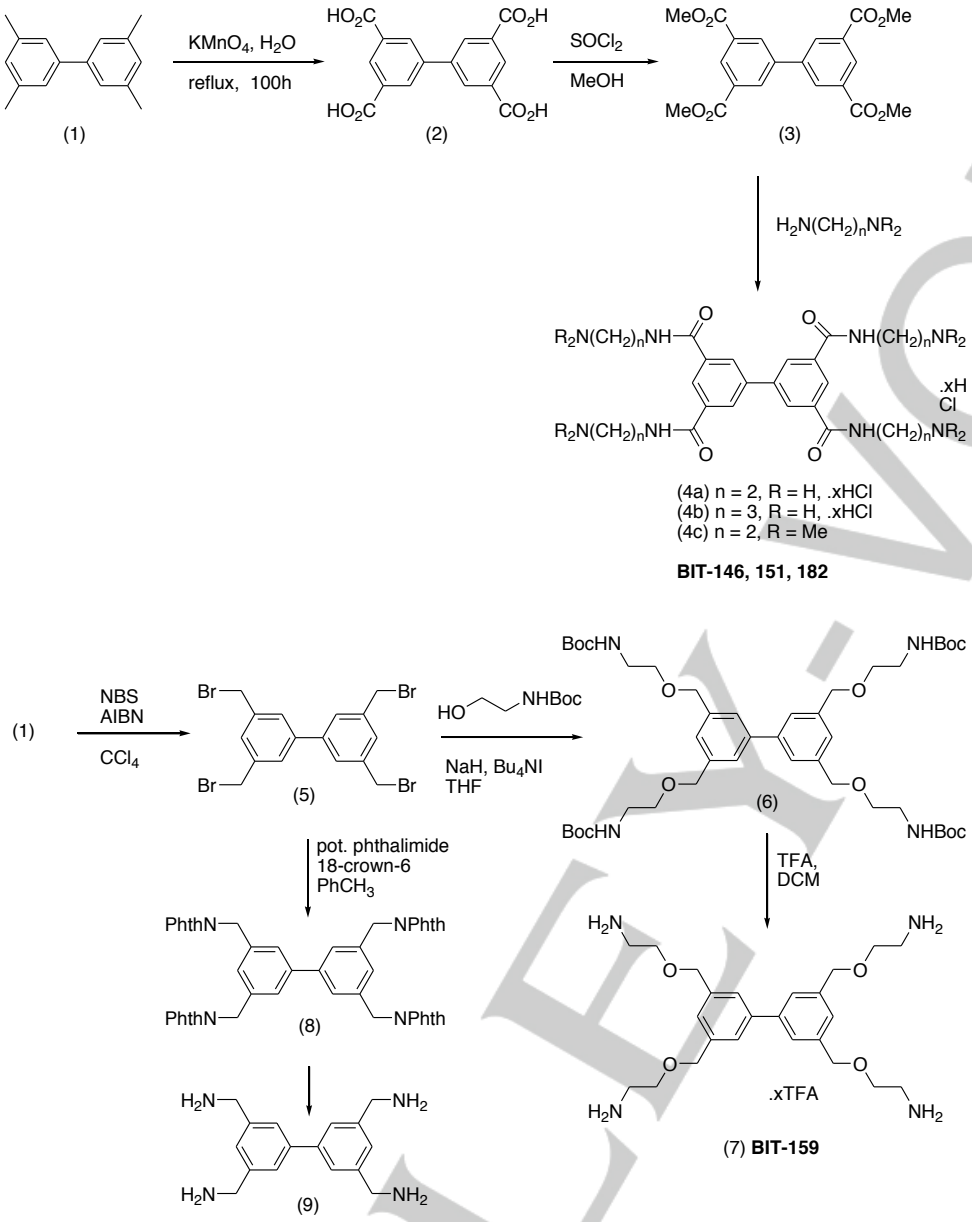

Scheme 5 Synthesis of BIT – 146, 151, 159, 182 compounds

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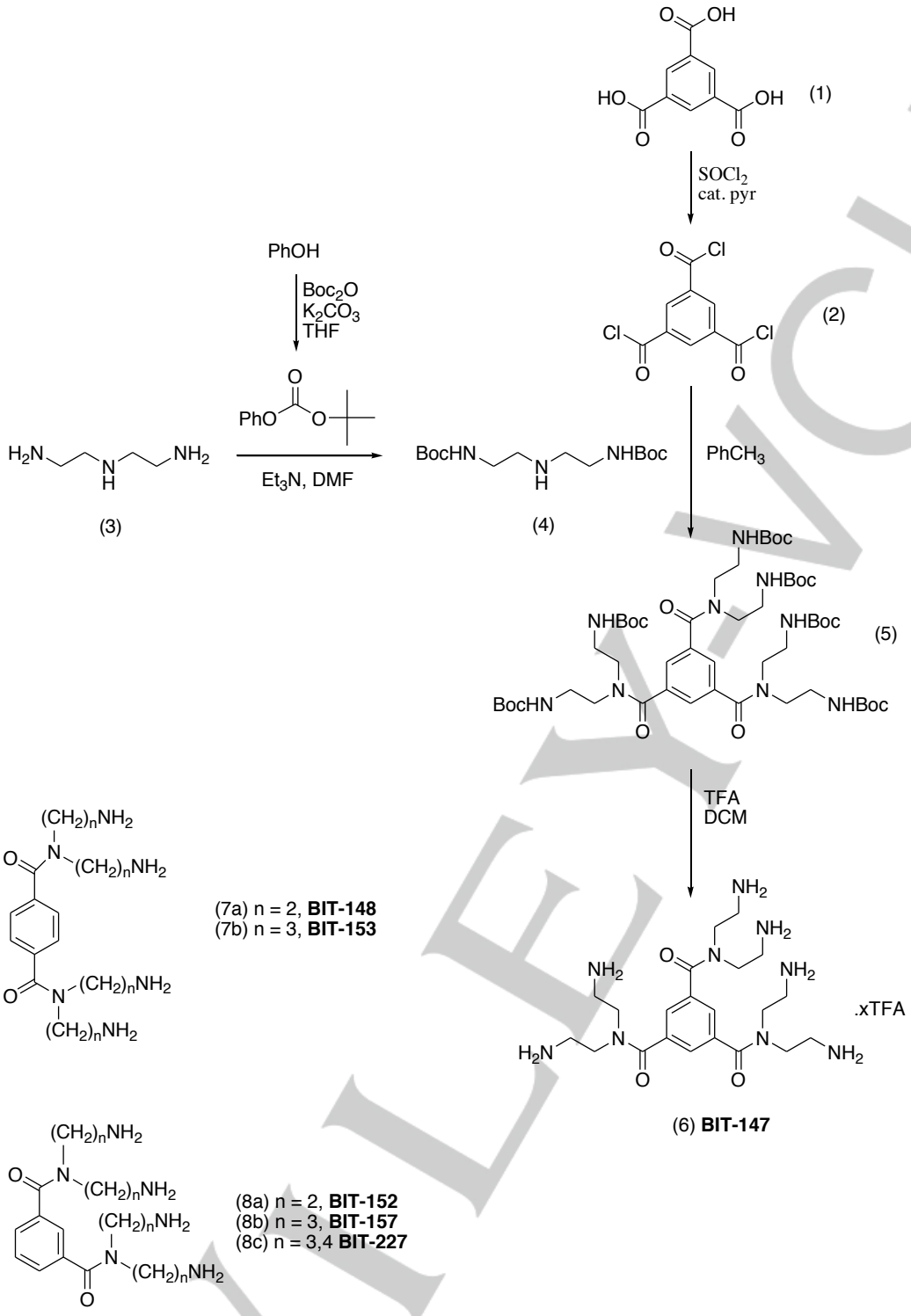

Scheme 6 Synthesis of BIT – 147, 148, 152, 153, 157, 227 compounds

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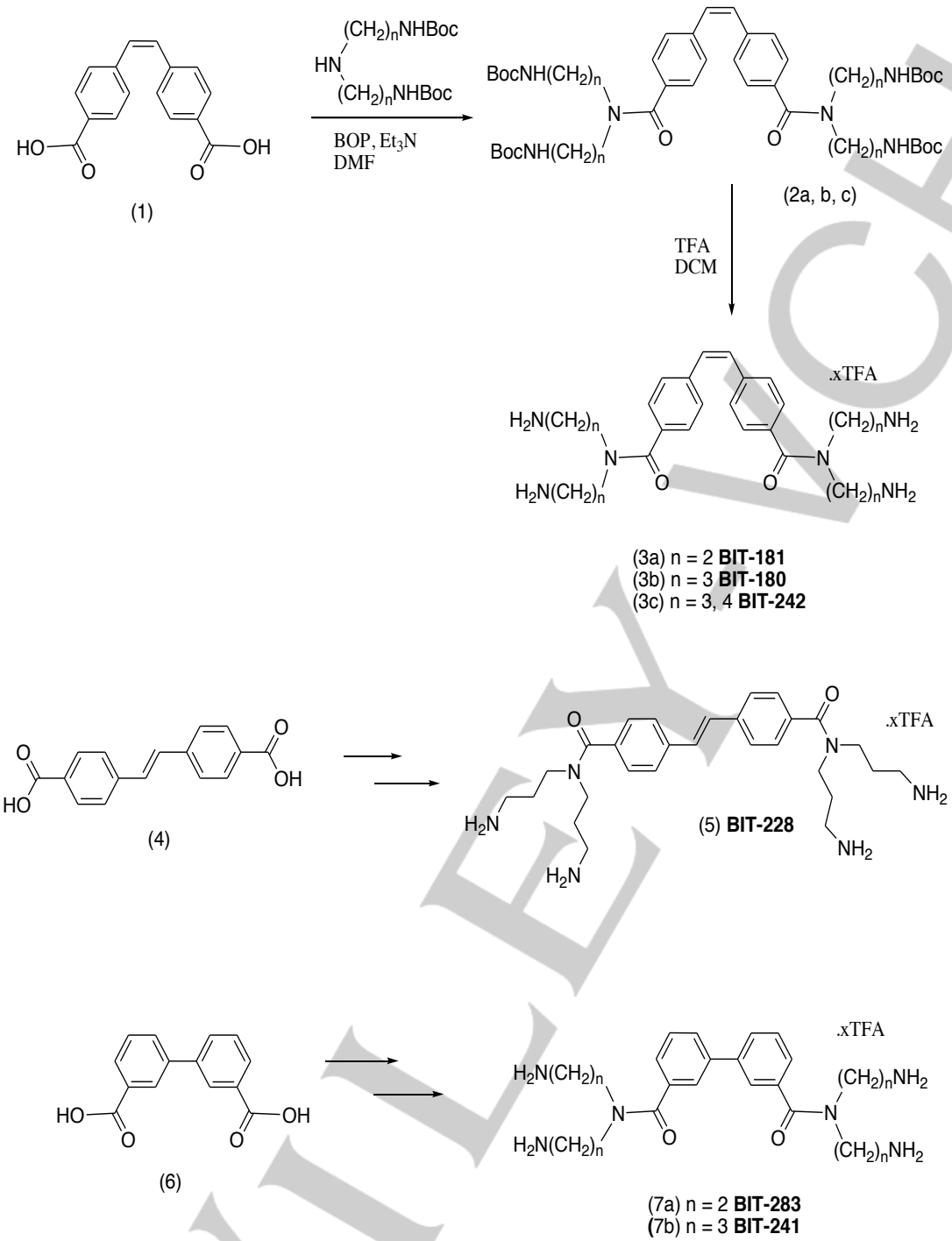

Scheme 7 Synthesis of BIT – 180, 181, 228, 241, 242, 283 compounds

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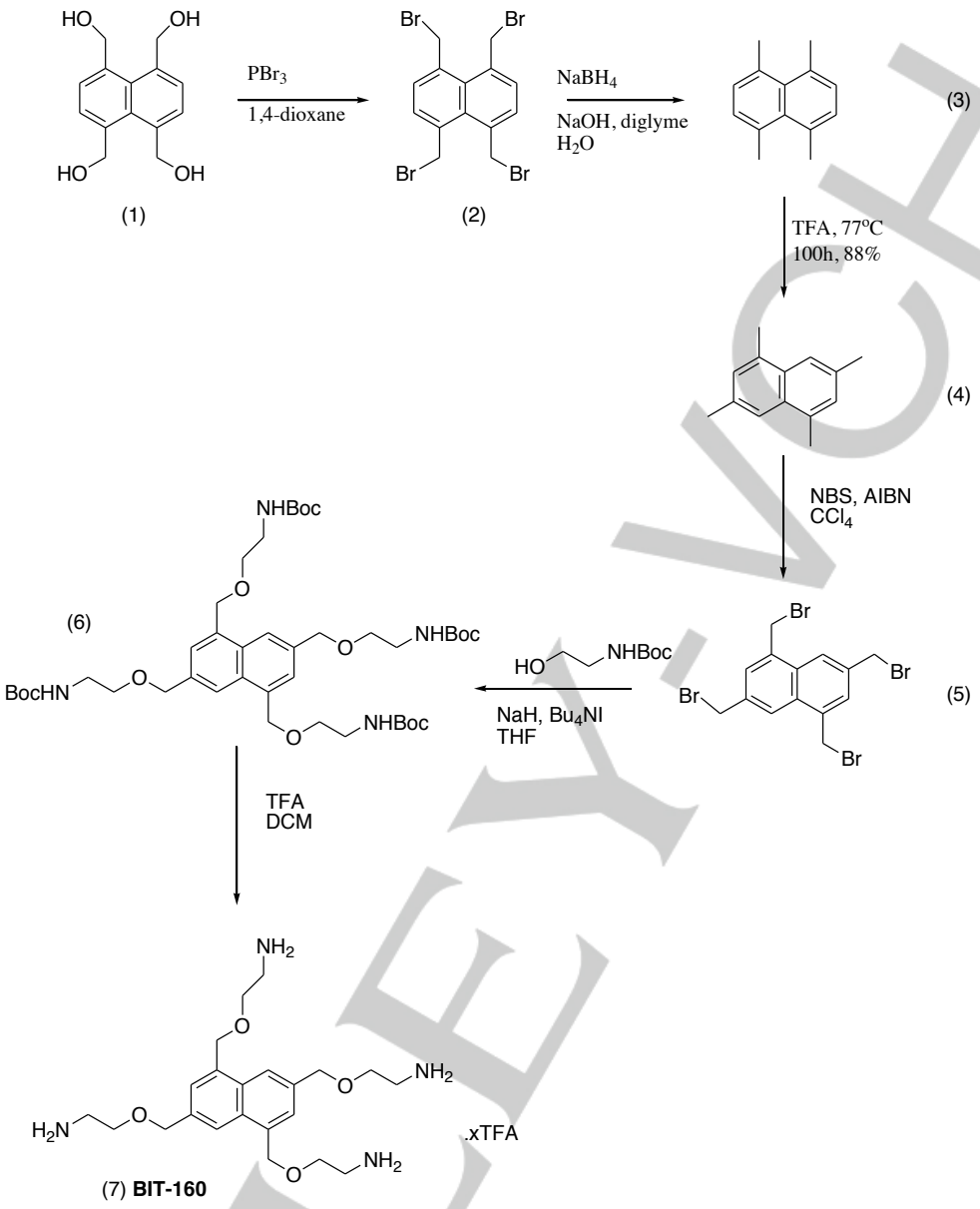

Scheme 8 Synthesis of BIT - 160

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2.2 Biological activity of compounds.

2.2.1 Activity assessment. The activity of the compounds was assessed using a Ca^{2+} release assay (Fig. 1A), in which Ca^{2+} levels were monitored using the Ca^{2+} sensitive dye, antipyralazo III. Cardiac or skeletal SR vesicles were first loaded with Ca^{2+} by adding 4 aliquots of $CaCl_2$, with ~3 min between the addition of each aliquot. Each addition increased the extravesicular [Ca^{2+}] by 7.5 μM, and then the [Ca^{2+}] declined over the ~3 min period, as Ca^{2+} was pumped into vesicles by the sarcoplasmic reticulum, endoplasmic reticulum Ca^{2+} ATPase (SERCA). Thapsigargin (200 nM) was then added to block the SERCA pump and the rate Ca^{2+} release in the presence of thapsigargin measured to evaluate resting (unstimulated) Ca^{2+} "leak" through the RyRs (slope of the green line in Fig. 1B). Ca^{2+} release was activated ~1 min after the introduction of thapsigargin by adding 4 mM caffeine to cardiac SR vesicles (Fig. 1) or 0.25 mM caffeine to skeletal SR. The rate of resting Ca^{2+} release, after thapsigargin addition and immediately before Ca^{2+} or caffeine addition was subtracted from the initial rates of Ca^{2+}- or caffeine-induced Ca^{2+} release (slope of the red line in Fig. 1B), to evaluate the degree of activation by Ca^{2+} or caffeine. The primary role of RyRs in both resting and activated Ca^{2+} release was confirmed when Ca^{2+} release was terminated by addition of 5.0 μM ruthenium red (RyR inhibitor). We ascertained that Ca^{2+} had been successfully loaded into the SR from the decline in Ca^{2+} concentration following each Ca^{2+} addition and also by the total amount of Ca^{2+} released after thapsigargin block. This is by the sum of resting Ca^{2+} release, activated Ca^{2+} release and Ca^{2+} remaining in non-releasable stores assessed from the increase in fluorescence at the end of the experiment following addition of the ionophore, A22187 (1.5 μg/ml). Experiments were performed with either vehicle (usually water) alone (as a control) or vehicle plus compound, added with caffeine or Ca^{2+}. The rate of Ca^{2+} release in the presence of drug was expressed relative to the rate of release with vehicle alone.

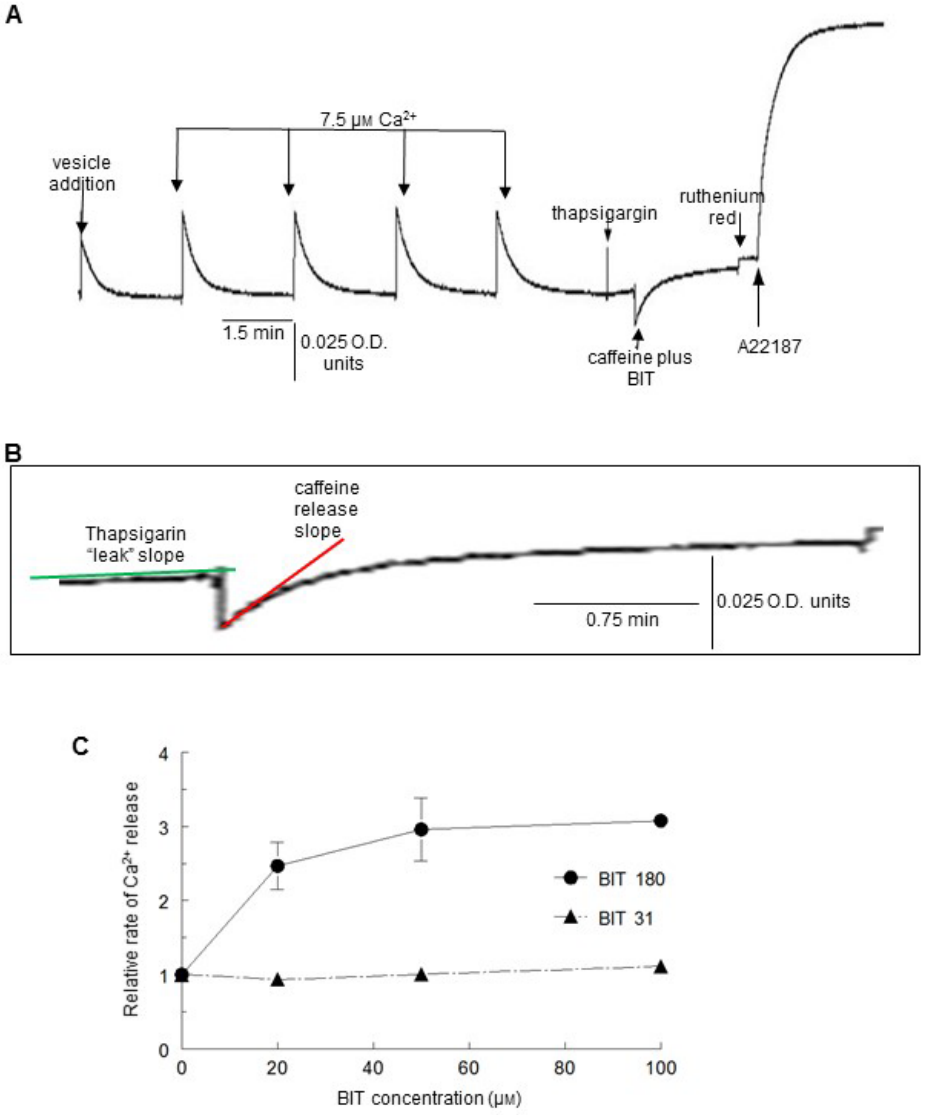

Figure 1. Effects of BIT compounds on caffeine-activated Ca^{2+} release from cardiac SR vesicles. ***A****) Recording of a typical Ca^{2+} release experiment. Cardiac SR vesicles were loaded into the standard buffer solution (see Experimental section) containing the Ca^{2+}*

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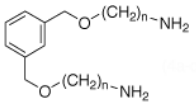
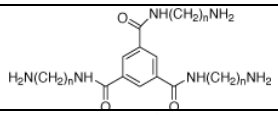
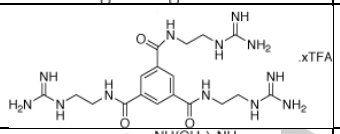
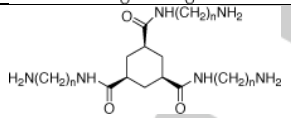
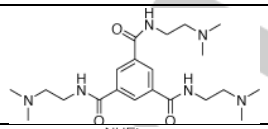
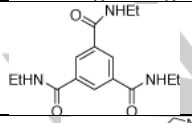
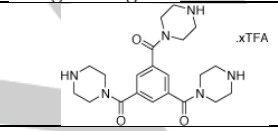
sensitive dye arsenazo III and appropriate concentrations of ATP and Mg^{2+} and ATP regenerating system to maintain activation of the Ca^{2+} ATPase to accumulate Ca^{2+} from the extravesicular solution. The recording shows absorbance at 710 nm as a function of time. SR vesicles were added initially and then four additions of 7.5 μM Ca^{2+}, with 3 min allowed for Ca^{2+} uptake between each addition. Thapsigargin (200 nM) was then added to inhibit the Ca^{2+} ATPase, then 4 mM caffeine added to activate Ca^{2+} release. After a further 3 min, 5 μM ruthenium red was added and finally 3 μg/ml of the Ca^{2+} ionophore A22187 to release all Ca^{2+} that had been taken up by the vesicles and not released by exposure to caffeine. ***B****) An expanded view of the sections of the record used to measure the effects of the BIT compounds. The green line shows the slope of release in thapsigargin which shows the baseline Ca^{2+} leak (i.e. resting Ca^{2+} release) before addition of caffeine. The red line indicates the initial rate of caffeine-induced Ca^{2+} release which was measured with caffeine addition alone (control) and with caffeine plus BIT compound.* ***C****) Examples of the concentration dependence of effects of the BIT compounds on 4 mM caffeine-activated Ca^{2+}release from isolated cardiac muscle SR. An activator (BIT 180, n = 3, filled circles) and an inactive compound (BIT 31, n = 4, filled triangles) are shown. The rate of caffeine-induced Ca^{2+} release in the presence of the BIT compounds is expressed relative to the rate of release in the absence of the compounds. Data is this and subsequent figures shown as mean ± SEM. Where error bars are not visible, they are contained within the symbols.*

All compounds were initially screened with cardiac SR vesicles and their level of activity in increasing the rate of Ca^{2+} release evaluated and ranked as strong, intermediate or weak (Table 1). Activity was first evaluated by the compounds ability to enhance Ca^{2+}-activated Ca^{2+} release (column 4, Table 1) and then the effects of compounds of interest on caffeine-activated Ca^{2+} release re-examined (column 5, Table 1). It was assumed in this and later experiments that effects on the rate of release occurred via actions on the RyR as there is no other release pathway present and because release was terminated by addition of ruthenium red. Fig. 1C shows examples of the average relative rates of Ca^{2+} release plotted as a function of drug concentration for two compounds. One of these compounds (BIT 180) enhanced Ca^{2+} release, while the other (BIT 31) was inactive. Similar graphs were constructed for each compound. A value of ~1 indicated no functional response, while values >1 indicated that RyRs had been activated (Table 1).

Table 1

BIT No:	Structure	n R	Classification of Ca^{2+} induced activation	Ca^{2+} induced increase -fold	Caffeine induced increase - fold
028 030 031 027		2 3 4 5	None Weak None None	1 1.1 1 1	
058 090		2 3	Intermediate None	1.4 1	2.0
061			N/A	N/A	
077 111 131		2 3 4	Weak Weak Weak	1.12 1.25 1.25	
138			None	1	1
092			None	1	1
105			None	1	

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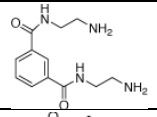
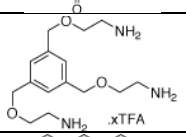
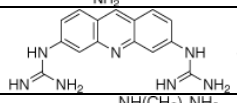
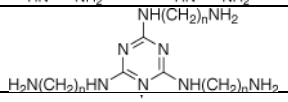
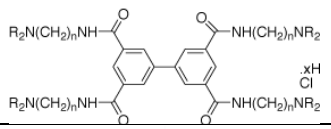
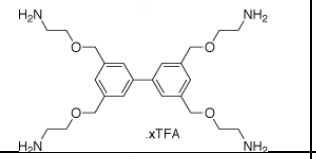
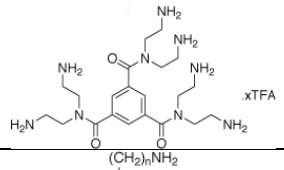
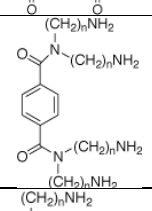
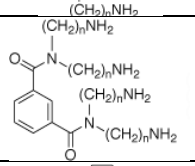
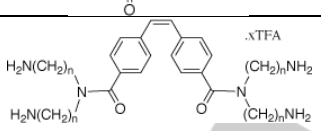
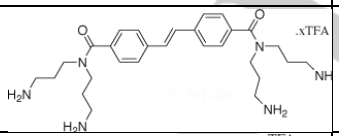
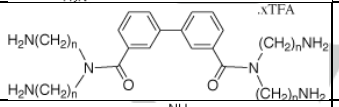
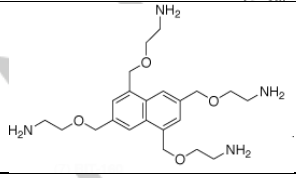
112			None	1	
144			None	1	
047			None	1	
125 145		3 4	Weak Weak	1.13 1.16	
146 151 182		2 H 3 H 2 Me	Weak None	1.3 1	
159			Intermediate	1.4	1.4
147			Slight inhibition	0.55	
148 153		2 3	Intermediate Weak	1.4 1.2	
152 157 227		2 3 3,4	Weak Weak Strong	1.1 1.2 2.0	 1.6 2.0
181 180 242		2 3 3,4	Weak Strong Intermediate	1.3 2.0 1.6	 4.0
228			Strong	2.4	2.4
283 241		2 3	Strong Strong	3.0 2.4	3.0 2.4
160			Weak	1.35	

n - number of CH_2 groups, R – chemical moieties

Twenty-one of the compounds increased the rate of Ca^{2+} release, with 5 compounds (BIT 180, 227, 241, 283, 228) producing strong activation to rates >2-fold greater than control. BIT 180 was the strongest activators increasing the average relative rate of release ~3-fold (filled and open circles respectively, Fig. 1C). A further four compounds (BIT 159, 58, 148, 242) produced intermediate activation to rates that 1.3 and 2 times control. Weak activation (<1.3-fold increase) was

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seen with 12 compounds (BIT 30, 125, 145, 146, 152, 77, 111, 131, 153, 157, 160, 181).

2.2.2 Further effects of BIT 180 on Ca^{2+} release from cardiac and skeletal SR.

As shown in Fig. 1C and Table 1, several of the compounds caused significant activation of Ca^{2+} release from cardiac SR vesicles. A comparison of the effects of one of the strongest activator, BIT 180, on Ca^{2+} release from cardiac skeletal SR vesicles is shown in Fig. 2A. BIT 180 produced similar increases in the relative rates of 4.0 mM caffeine-induced Ca^{2+} release from cardiac vesicles (open circles) and 0.25 mM caffeine-induced Ca^{2+} release in skeletal SR vesicles (filled circles). However, BIT 180 also enhanced resting Ca^{2+} leak from skeletal vesicles when added in the absence of caffeine (Fig. 2A filled triangles), although the increase in activity was stronger in the presence of caffeine, when RyR channels were more active (Fig. 2A filled circles). Notably, BIT 180 did not alter the rate of resting Ca^{2+} leak from cardiac vesicles if added alone in the absence of caffeine-activated release in 3 of 3 experiments (data not shown).

BIT 180 was more effective in enhancing caffeine-induced Ca^{2+} release from cardiac SR than the parent peptides, ***A1*** and ***A2DR18*** (Fig. 2B). As reported previously, activation by the mutant peptide ***A2DR18*** was greater than that of the native ***A1*** peptide at lower peptide concentrations <20 μM [7]. The ~2-fold increase in .the rate of Ca^{2+} release caused by ***A2DR18*** at 10 μM was similar to that caused by BIT 180 at the same concentration. However, while increasing concentrations of BIT .180 caused further increases in the rate of Ca^{2+} release to ~4-fold between 50 and 100 μM, the activity of the ***A1*** peptide decreased at peptide concentrations >40 μM, while that of ***A2DR18*** declined concentrations >20 μM. This previously reported low affinity inhibitory action of both ***A1*** and ***A2DR18*** [6] may have limited the degree of activation that could be observed with the peptides (if activation was masked by the inhibition). Therefore, the maximum activation of the peptides with that of BIT 180 could not be directly compared. Never the less, it is apparent the action of the mimetic is simpler than that of the parent peptide compound and a preferable template for an RyR activator.

It should be noted that higher concentrations of peptides and of the BIT compounds are required to see an immediate action of the peptide in Ca^{2+} release experiments, in contrast to bilayer experiments where activity can be followed for many minutes and slowly developing effects can be observed at lower concentrations. Therefore, we predict that lower concentrations of both peptides and the peptide mimetics would also be effective in the long term in future *in vivo* studies.

2.2.3 Competition between BIT 180 and the A peptides in enhancing caffeine-activated Ca^{2+} release.

To determine whether BIT 180 was acting at the same site as the parent compound we examined the ability of BIT 180 to enhance caffeine-activated Ca^{2+} release in the presence of peptide ***A1***. BIT 180 was added to the cuvette containing cardiac SR vesicles either alone or with the maximally activating concentration of 30 μM peptide ***A1.*** The compounds were added at the same time as caffeine and ~1 min after addition of thapsigargin (as in the protocol shown in Fig. 1A above). As before, the Ca^{2+} leak after thapsigargin addition was subtracted from the initial rate of caffeine-induced Ca^{2+} release, then the resultant rate expressed relative to the rate measured for addition of vehicle alone. The average data is shown in Fig 3A. Peptide ***A1*** significantly enhanced the rate of caffeine-induced Ca^{2+} in the absence of BIT 180 (zero [BIT 180]) as expected, but then prevented the activation by BIT 180 when it was added at concentrations of 20 to 100 μM (Fig. 3A). This suggested that 30 μM of peptide ***A1*** occupied the BIT 180 binding site and prevented BIT 180 from binding and causing its usual activation.

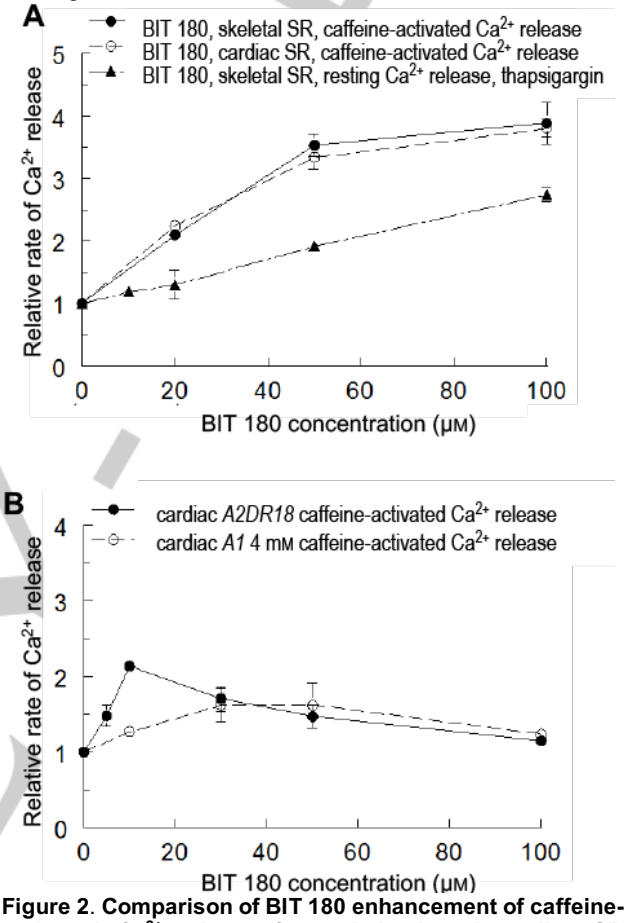

Figure 2. **Comparison of BIT 180 enhancement of caffeine-activated Ca^{2+} release from skeletal and cardiac SR vesicles and BIT 180 potentiation of resting Ca^{2+} release from skeletal SR.** *These experiments were performed using the protocol shown in Fig. 1.* ***A****) the rate of caffeine-induced Ca^{2+} release in the presence of BIT 180 is shown relative to that in the absence of BIT 180 (0 μM [BIT 180]. Data is shown for cardiac SR vesicles exposed to 4 mM caffeine plus indicated concentrations of BIT 180 (open circles, n = 3) and skeletal SR vesicles exposed to 0.25 mM caffeine plus indicated concentrations of BIT 180 (filled circles, n = 3). Data is also shown for the effect of BIT 180 on resting (non-activated) Ca^{2+} relase from skeletal SR vesicles with BIT 180 added after thapsigargin, without caffeine (filled triangles, n = 3).* ***B****) - effects of peptides* ***A1*** *(open circles, n = 2) and* ***A2DR18*** *(filled circles, n = 2), on 4 mM caffeine-induced Ca^{2+} release A2DR18 were performed, since the results were the same as those in a larger data set published previously [7].*

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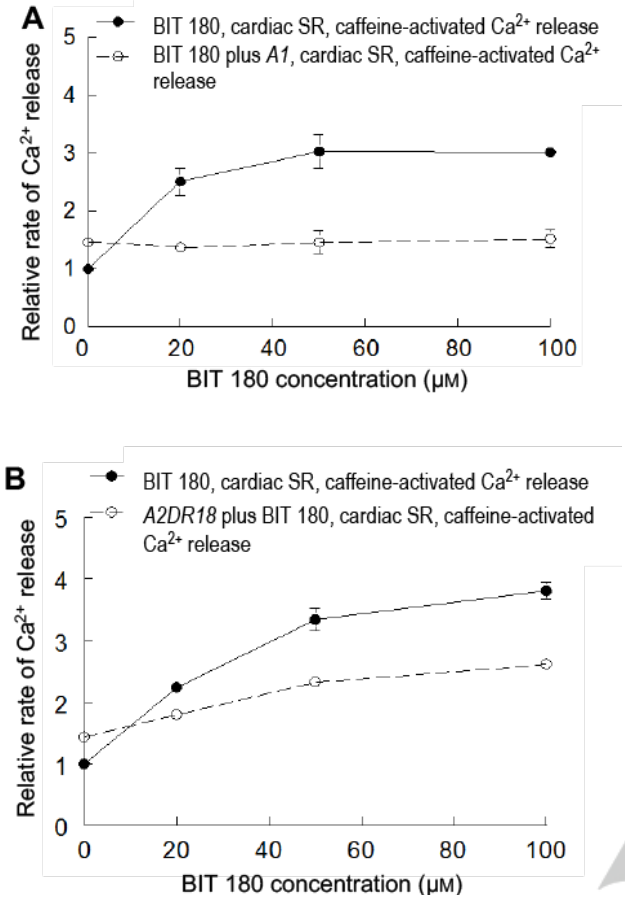

Figure 3. The action of BIT 180 on cardiac SR is inhibited by peptide *A1* and peptide *A2DR18*. *In **A** and **B**, 30 μM peptides **A1** or 20 μM **A2DR18** were added in the control (0 [BIT 180]) experiment and with each concentration of BIT 180. Rates of Ca^{2+} release are shown as a function BIT 180 concentration and expressed relative to the rate of Ca^{2+} release with caffeine alone, in the absence of BIT 180 (zero concentration on the x axis). Rates of Ca^{2+} release in the presence of peptides **A1** or **A2DR18** are also expressed relative to the rate of Ca^{2+} release in the absence BIT 180 or peptide (i.e. 0 [BIT 180] without peptide). **A**) - cardiac SR vesicles exposed to 4 mM caffeine plus BIT 180 at indicated concentrations in the absence (filled circles, n = 3) and presence (open circles, n = 3) of 30 μM peptide **A1**. **B**) - cardiac SR vesicles exposed to 4 mM caffeine plus BIT 180 at indicated concentrations in the absence (filled circles, n = 3) and presence (open circles, n = 3) of 20 μM peptide **A2DR18**.*

Competition between BIT 180 and ***A2DR18*** was also examined, using the maximally activating concentration of 20 μM peptide ***A2DR18***. ***A2DR18*** caused the expected significant increase in the rate of caffeine-induced Ca^{2+} release when added without BIT 180 ((zero [BIT 180, Fig. 3B). When BIT 180 was added with ***A2DR18***, there was a small additional increase in the rate of Ca^{2+} release, but the rate remained substantially less that with BIT 180 alone. An essentially similar result was obtained with caffeine-induced Ca^{2+} release from skeletal SR, although the increase in the relative rate of release from skeletal SR with ***A2DR18*** was significantly greater than that seen in cardiac SR (Fig. 4A).

2.2.4 Competition between BIT 180 and the A peptides in stimulating Ca^{2+} release in the absence of caffeine-activation.

Competition between BIT 180 and peptide ***A2DR18*** in enhancing the rate of resting Ca^{2+} leak from skeletal SR vesicles was measured. This experiment examined the ability of BIT 180 and peptide ***A2DR18*** to activate Ca^{2+} release themselves, in the absence of caffeine. The peptides were added following addition of thapsigargin, without caffeine. The record in Fig. 4B shows that addition of BIT 180 plus ***A2DR18*** produced a significant increase in the rate of Ca^{2+} release. As with caffeine-activated Ca^{2+} release, the slope immediately before addition of BIT 180 or peptide ***A2DR18*** plus BIT 180 was measured and subtracted from the initial slope after addition of the compounds. This was then expressed relative to the control measurement obtained when vehicle was added alone (Fig. 4C). The average data in Fig. 4C shows that BIT 180 alone produced a significant concentration-dependent activation of Ca^{2+} release from the skeletal SR vesicles in the absence of caffeine (this is also seen in the data in Fig. 2A above). (Fig 4C solid line). Peptide ***A2DR18*** alone enhanced the rate of Ca^{2+} release (i.e. open symbol at zero BIT 180 concentration in Fig 4C) by an amount that was similar to that with 20 μM BIT 180 (filled symbol at 20 μM BIT 180), but there were only marginal further increases when BIT 180 was added with ***A2DR18*** (Fig 4C, broken line).

In marked contrast to the substantial activation of Ca^{2+} release from skeletal SR vesicles by BIT 180 and BIT 180 plus ***A2DR18*** in the absence of caffeine-activation, neither BIT 180 nor BIT 180 plus ***A2DR18*** had any effect on resting Ca^{2+} release from cardiac SR vesicles in 3 experiments. These results indicate that both the activating BIT compound and the ***A2DR18*** peptide are substantially more effective in activating Ca^{2+} release from skeletal SR through RyR1 than activating Ca^{2+} release from cardiac SR through RyR2.

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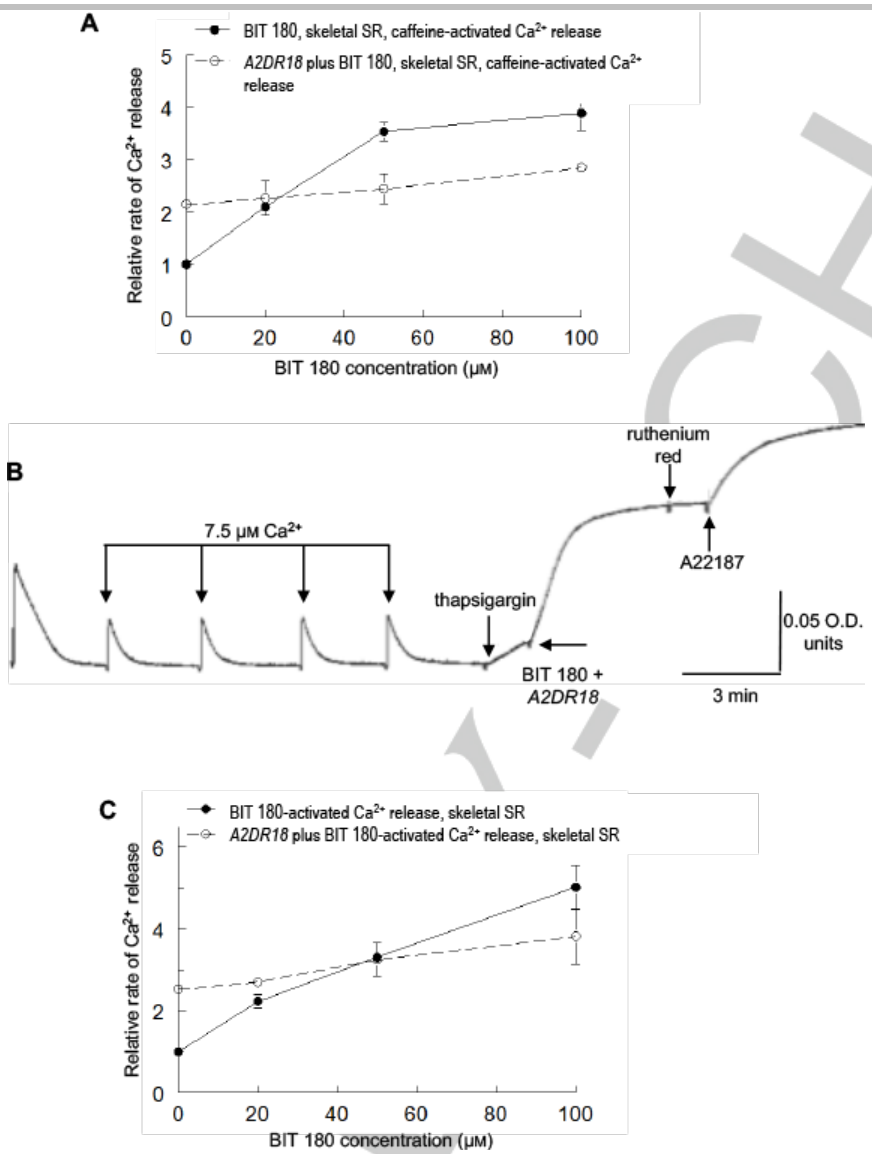

Figure 4. **The action of BIT 180 on activated and resting Ca^{2+} release from skeletal SR is inhibited by peptide *A1* and peptide *A2DR18*.** ***A****) – Average effects of BIT 180 alone, or added with* ***A2DR18,*** *on caffeine-induced Ca^{2+} release from skeletal SR. The skeletal SR vesicles were exposed to 0.25 mM caffeine plus BIT 180 in the absence (filled circles, n = 3) and presence (open circles, n = 3) of 20 μM peptide* ***A2DR18****. The protocol for the experiment and the normalization of the rates of Ca^{2+} release are the same as those described for cardiac SR in the legend to Figure 3.* ***B and C****) – the actions of BIT 180 alone, or added with* ***A2DR18****, on resting Ca^{2+} "leak" from skeletal SR following addition of thapsigargin.* ***B****) - a recording from a typical experiment used to obtain the data shown in* ***C.*** *Skeletal SR vesicles were loaded with Ca^{2+}, then exposed to thapsigargin for ~1 min before BIT 180 alone or with* ***A2DR18*** *were added, as described in the legend to Figure 1, except that in this case caffeine was not added to activate Ca2+ release.* ***C****) Average data for the effect of BIT 180 plus 20 μM A2DR18 on the rate of Ca^{2+} release. BIT 180 was added in the absence (filled circles, n = 3) and presence (open circles, n = 3) of 20 μM peptide A2DR18.*

Together, the results in Figs 3 and 4 suggest that, as with peptides ***A1*** and ***A2DR18*** occupied the BIT 180 binding site and prevented BIT 180 from binding and causing its usual activation. This result further suggests that the activating BIT compounds act at a common site that is also the ***A*** peptide binding site. Finally, the results suggest that, under resting conditions, skeletal SR is significantly more sensitive to activation by BIT 180 than cardiac SR, although both appear equally sensitive to BIT 180 during caffeine-induced Ca^{2+} release. It is worth noting that the open state of the RyR2 during caffeine-induced Ca^{2+} release simulates the state of RyR2 during cardiac excitation-contraction coupling, since caffeine-induced Ca^{2+} release mimics the physiological Ca^{2+}-induced Ca^{2+} release process [17]. The correlation between the state of the skeletal channel during caffeine-induced Ca^{2+} release and during excitation-contraction coupling, where RyR1 opening is governed by the voltage sensor is less clear.

Overall the results in this section indicate that the compounds may be more suited to boosting contraction in skeletal muscle under conditions in which Ca^{2+} release from the SR is depressed as it is in some debilitating genetic disorders of RyR1 [18, 19]. The fact that the compounds also act on the cardiac RyR2 may mean that a drug derivative will have to be targeted to skeletal muscle if they enhance resting Ca^{2+} release, induce arrhythmia and/or deplete SR Ca^{2+} stores [2]. However, this may not be a problem as BIT 180 did not appear to alter resting Ca^{2+} release from cardiac SR. It is also possible that enhanced Ca^{2+} release during systole in the intact

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heart may be avoided as there is an auto-correction mechanism that operates during systole to maintain the peak Ca^{2+} release at a pre-set level [20].

2.4 Effect of activators on skeletal muscle tetanic tension.

The effects of the BIT 180 on tetanic and twitch contractions in intact skeletal muscle was examined to determine whether the compound was membrane permeable and to assess potential effects on the surface membrane of muscle fibres. The protocol followed in this intact fibre experiment is illustrated in Fig. 5A. Twitch and tetanic tension were recorded over a control period, then following drug or vehicle addition for a period of 25 mins. During this time, the solution flow was ceased (to limit amounts of compound used). A subsequent 20 min perfusion to remove the drug and accumulated metabolites. There was a progressive run-down of tension during the period that perfusion was ceased in the absence of any drug addition, which was attributed to metabolite accumulation. However, the changes in tetanic tension were identical when either the vehicle alone (H_2O) or vehicle plus BIT 180 (Fig. 5B) were added. This lack of an effect on tension suggested that BIT 180 was unable to enter the muscle fibres. Most importantly, the lack of an effect indicated that BIT 180 did not have any toxic action on the electrical properties of external surface membrane of the muscle fibres.

To show that a structurally related membrane permeable compound would alter muscle tension in this system, we next examined the actions of Imperatoxin A (IPTXA) on intact skeletal muscle fibre tension. It has been shown that Maurocalcine and the structurally related IPTXA are membrane permeable and can enter cells, including intact cardiomyocytes [21, 22]. Thus, we expected that IPTXA would penetrate intact adult skeletal muscle fibres. The results show that the run-down of tetanic and twitch tension was much reduced in the presence of 100 or 500 nM IPTXA (Fig. 5C and D respectively) consistent with a significant activation by the toxin that had entered the cells. Tetanic tension was significantly greater than with water alone after 5 min and 15 min in100 or 500 nM IPTXA.

The duration of exposure to toxin was extended to 85 min to evaluate the extent of activation after more substantial fibre penetration by the toxin. The degree of potentiation was the same after 75 and 85 min, indicating the toxin had equilibrated within the cells by 75 min. The tension remained greater than control during washout, even though there was notably less recovery of tension after longer periods of incubation without solution flow. The degree of potentiation of the tetanic tension by the toxin was substantially greater than twitch potentiation (Figure 5D), perhaps indicating that the amplitude of the twitch was to some extent limited by Ca^{2+} pump activity which would be potentiated as soon as cytoplasmic Ca^{2+} was released by the single action potential that triggered the twitch. It is possible that the enhanced pump activity was overcome by the summation of Ca^{2+} release events during the tetanic stimulation.

The data in Fig. 5B-D has been replotted in Fig. 6, where the average twitch or tetanic tension in the presence of IPTXA is normalised to the average tension in control experiments at each time point during the experiment. The activating effect of the toxin on twitch and tetanus is clearly seen in this graph, as is the stronger action of the toxin on the tetanus. Also apparent is the fact that there was little difference between results obtained with 100 nM and 500 nM IPTXA. The data in Figs. 5 and 6 show that the potentiating effects of the toxin were maintained after resumption of perfusion, indicating that the toxin was not easily dissociated from RyR1 or washed

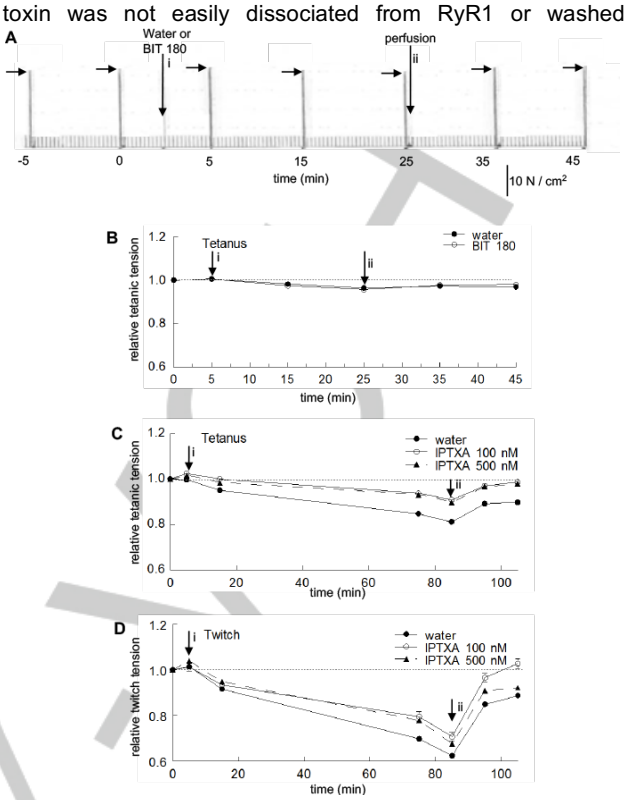

Figure 5. Effects of BIT 180 and IPTXA on twitch and tetanic contractions recorded from bundles of intact soleus muscle fibres from rat. ***A and B) –*** *effect of BIT 180 on contraction.* ***A) –*** *typical continuous tension recording showing the experimental protocol used. Stable control twitch (small vertical deflections elicited every ~25 s) and tetanic (larger vertical deflections elicited every ~10 min – amplitude indicated by horizontal arrows) contractions were established and recorded at 0 and -5 min time points. Perfusion was stopped and then BIT 180 (or vehicle, i.e. water alone) added 2.5 min after the second control tetanus, as indicated by the vertical arrow labelled i. Tetanii were then elicited at 5 min, 15 min and 25 min after adding BIT 180. Perfusion to remove BIT 180 commenced after the 25 min tetanus (as indicated by the vertical arrow labelled ii) and washout tetanii elicited after a further 10 and 20 min, as the compound was washed out of the bathing solution.* ***B****) – Average values were obtained for the amplitude of each tetanus, relative to the 2nd control tetanus. Data is shown for 100 µM BIT 180 in water (open circles, n = 7) or for the same volume of water lacking BIT 180 (filled circles n = 12).* ***C*** *and* ***D****) – average effects of IPTXA on tetanic and twitch tension respectively. In this experiment, the recording time with IPTXA was extended to the 85 min time point to observe the maximum effects of the toxin. Tetanic contractions were not elicited between the 15 and 75 min time points to avoid excess rundown. Average relative tetanic (****C****) or twitch (****D****) tension is shown for either the appropriate volume of water (filled circles, n = 9), or 100 nM IPTXA (open circles, n = 6) or 500 nM IPTXA (filled triangles, n = 8) in water, added after the control tetanus. Tetanic contractions were evoked at 5, 15, 75 and 85 min time points. Perfusion was commenced after the 85min tetanus and then two tetanic contractions evoked during the recovery period. The two vertical arrows, labelled (i) and (ii) in (****B****), (****C****) and (****D****) indicate the time of application of the vehicle or experimental compound (i) and then the start of perfusion and washout (ii) of the vehicle or experimental compound.*

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out of the muscle fibre. This is consistent with the known high affinity binding of the toxin to RyR1 [15]. The results are also consistent with the concentrations of toxin used exerting only the high affinity action in increasing the open probability of the RyR channel. Had the toxin had its lower affinity action of locking the RyR into a subconductance state [14, 16], we would expect that the chronic release of Ca^{2+} from the SR would induce a contracture and that the consequent depletion of Ca^{2+} in the SR would lead to a reduction in twitch and tetanic tension. Since neither a contracture, nor tension reduction were seen, we conclude that this low affinity action of the toxin did not occur. The ability of IPTXA to potentiate the twitch and tetanic tension provides proof in principal that the membrane permeable compounds with structures based on the structure of the peptides or toxin will target RyR channels in vivo and will modulate muscle fibre contraction.

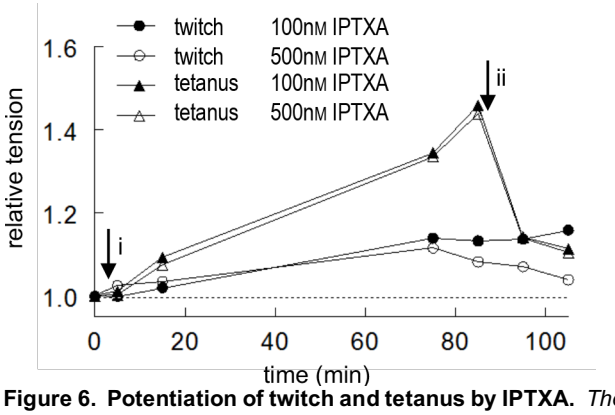

Figure 6. Potentiation of twitch and tetanus by IPTXA. *The potentiation of twitch and tetanic tension by IPTXA can be seen more clearly when the tension during exposure to the toxin is expressed relative to tension recorded after addition of the vehicle alone. The effective increase in twitch (circles) and tetanic (triangles) contractions during an 85min exposure to 100 nM (filled symbols) or 500 nM (open symbols) IPTXA. Error bars have been excluded for clarity. The vertical arrows labelled (i) and (ii) have the same meaning as that described in the legend to Figure 5.*

3 Conclusions

We have synthesized a number of positively charged compounds designed to mimic the functional effect of the α-helix structure of peptide ***A*** and its analogues derived from the skeletal muscle DHPR and have shown these compounds activate both the skeletal and cardiac RyRs. Furthermore, through competition experiments, these compounds have been shown to act upon similar binding sites to peptide ***A***. The compounds have been tested against intact skeletal muscle fibres and found to be incapable of altering the contractile response thus indicating that they are membrane impermeable. However, the fact that IPTXA, a structurally related membrane-penetrating peptide can activate intact muscle fibres raises the possibility that modified cell-penetrating analogues of these compounds may have a future role as probes for muscle function or as therapeutic compounds in skeletal muscle weakness.

Experimental Section

4.1 Chemistry

4.1.1 Scheme 1 (BIT 027, 028, 030, 031):

General : α,α'-dibromo-*m*-xylene was prepared from *m*-xylene using the procedure described by Rensing *et al*.[23] and gave identical NMR data to that of commercial material (Aldrich). N-Boc-amino alcohols (2a-d) were prepared using the conditions described by Caputo *et al*.[24] to prepare analogous compounds and are also commercially available (Sigma, Aldrich, Fluka). *N*,*N*'-bis-t-Boc-2-methyl-2-thiopseudourea was prepared by the method of Han *et al*[25].

***Compounds 4a-d*:** Sodium hydride (60% in mineral oil, 1.1 equiv.) was added to a cooled (ice bath) solution of N-Boc-amino alcohol (2a-d) (1 equiv.) in dry THF under nitrogen. The mixture was stirred for 25 min before addition of tetrabutylammonium iodide (0.1 equiv.) and α,α'-dibromo-*m*-xylylene (0.47 equiv.). Stirring was continued overnight at room temperature. The mixture was filtered through celite, the solvent removed *in vacuo* and 3a-d were purified by flash chromatography on silica gel (EtOAc / PE 3:7) to give colourless oils (yields 21-60%), which were suspended in $CHCl_3$. TFA (approx. 55 equiv.) was added and the mixture stirred for 6 h at room temperature. The solvent was removed *in vacuo* to give the products 4a-d in quantitative yield if x = 2.

(4a): δH (300 MHz, CD_3OD) 3.15 (4H, t, *J* = 5.0 Hz), 3.69 (4H, t, *J* = 5.0 Hz), 4.60 (4H, s), 7.35 (3H, s), 7.39 (1H, s); δC (75.5 MHz, CD_3OD) 139.3, 129.7, 128.6, 74.1, 66.9, 40.6; m/z (ES^+) 225 MH^+ (100%); HRMS $C_{12}H_{21}N_2O_2$ (MH^+) calculated 225.1603, found 225.1594.

(4b): δH (300 MHz, CD_3OD) 1.94 (4H, approx. quint., *J* = 6.5 Hz), 3.05 (4H, t, *J* = 7.0 Hz); 3.60 (4H, t, *J* = 6.0 Hz); 4.52 (4H, s), 7.27-7.36 (4H, m); m/z (ES^+) 275 MNa^+ (10%), 253 MH^+ (60%); HRMS $C_{14}H_{25}N_2O_2$ (MH^+) calculated 253.1916, found 253.1911.

(4c): δH (300 MHz, CD_3OD) 1.66-1.81 (8H, m), 2.94 (4H, t, *J* = 7.0 Hz); 3.53 (4H, t, *J* = 5.5 Hz), 4.50 (4H, s), 7.26-7.32 (4H, m); □C (75.5 MHz, CD_3OD) 139.9, 129.6, 128.3 (2 peaks), 73.9, 70.7, 40.7, 27.7, 25.8; m/z (ES^+) 303 MNa^+ (10%), 281 MH^+ (70%); HRMS $C_{16}H_{29}N_2O_2$ (MH^+) calculated 281.2229, found 281.2226.

(4d): δH (300 MHz, CD_3OD) 1.44-1.52 (4H, m), 1.61-1.72 (8H, m), 2.91 (4H, t, *J* = 7.5 Hz), 3.52 (4H, t, *J* = 6.5 Hz), 4.49 (4H, s), 7.25-7.32 (4H, m); δC (75.5 MHz, CD_3OD) 140.0, 129.5, 128.3, 128.2, 73.9, 71.1, 40.7, 30.2, 28.4, 24.3; 331 MNa^+ (10%), 309 MH^+ (90%); HRMS $C_{18}H_{33}N_2O_2$ (MH^+) calculated 309.2542, found 309.2544.

4.1.2 Scheme 2 (BIT 058, 061, 077, 090, 111, 131):

Compounds (2a), (2b): Compound (2a) was prepared as described in the literature [26]. This procedure was also used to prepare (2b). A sample of (2a) was further purified by treatment with Boc_2O (3.1 equiv.) and Et_3N (3.1 equiv.) in THF (overnight at room temperature), followed by flash chromatography on silica gel (7.5% MeOH in EtOAc). The derivatised material was treated with TFA in $CHCl_3$ to give the test sample.

(2a): δH (300 MHz, CD_3OD) 3.20 (6H, t, *J* = 6.0 Hz), 3.71 (6H, t, *J* = 6.0 Hz), 8.54 (3H, s); δC (75.5 MHz, CD_3OD) 169.4, 136.0, 130.6, 40.8, 38.9; m/z (ES^+) 337 MH^+ (40%); HRMS $C_{15}H_{25}N_6O_3$ (MH^+) calculated 337.1988, found 337.1975.

(2b): δH (300 MHz, CD_3OD) 1.78 (6H, quint., *J* = 7.0 Hz), 2.72 (6H, t, *J* = 7.0 Hz), 3.47 (6H, t, *J* = 7.0 Hz), 8.38 (3H, s); δC (75.5 MHz, CD_3OD) 168.7, 136.7, 129.8, 39.9, 38.5, 33.5; m/z (ES^+) 379 MH^+ (100%); HRMS $C_{18}H_{31}N_6O_3$ (MH^+) calculated 379.2458, found 379.2460.

Compound (4): *N*,*N*'-bis-t-Boc-2-Methyl-2-thiopseudourea (3 equiv.) and silver nitrate (3 equiv.) were added to a solution of 2a (1 equiv.) and Et_3N (6.1 equiv.) in dry DMF/DCM (2:1). The mixture was stirred for 4h at room temperature under an atmosphere of nitrogen, filtered through celite and the solvent removed. Derivative 3 was purified by flash chromatography on silica gel (2% MeOH in EtOAc) to give a white solid (49%),

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which was suspended in DCM. TFA (approx. 420 equiv.) was added and the mixture stirred overnight (17 h) at room temperature under nitrogen. The solvent was removed in vacuo and the residue washed with several portions of Et_2O followed by EtOAc to give 4 as a white solid (quant. yield if x = 3).

(4): δH (300 MHz, CD_3OD) 3.44 (6h, t, *J* = 6.0 Hz), 3.59 (6H, t, *J* = 6.0 Hz), 8.48 (3H, s); δC (75.5 MHz, CD_3OD) 169.2, 159.1, 136.3, 130.4, 41.9, 40.2; m/z (ES^+) 463 MH^+ (40%); HRMS $C_{18}H_{31}N_{12}O_3$ (MH^+) calculated 463.2642, found 463.2625.

Compounds 6a-c: Prepared by adapting the method of Wörl and Köster[26] to prepare 2a from trimethyl benzene-1,3,5-tricarboxylate (1). In these cases trimethyl cyclohexane-1,3,5-tricarboxylate (5) [24] and the diamine were heated at 30 °C for 4 days under nitrogen. After removal of the excess diamine the products were purified by precipitation from MeOH by addition of EtOAc or Et_2O. In the case of (6b) this material was treated with HCl/MeOH before purification.

(6a): δH (300 MHz, CD_3OD) 1.51-1.63 (3H, m), 1.86-1.93 (3H, m), 2.29-2.36 (3H, m), 2.68-2.72 (6H, m), 3.19-3.27 (6H, m); δC (75.5 MHz, CD_3OD) 177.8, 44.9, 42.5, 41.9, 32.9; m/z (ES^+) 343 MH^+ (100%), 365 MNa^+ (10%); HRMS $C_{15}H_{31}N_6O_3$ (MH^+) calculated 343.2458, found 343.2446.

(6b): δH (300 MHz, CD_3OD) 1.60 (3H, approx. q, *J* = 12.5 Hz), 1.83 (6H, approx. quint. *J* = 7.0 Hz), 1.92 (3H, d, *J* = 12.0 Hz), 2.41 (3H, t, *J* = 12.5 Hz), 2.92 (6H, t, *J* = 7.5 Hz), 3.27 (6H, t, *J* = 7.0 Hz); δC (75.5 MHz, CD_3OD) 178.2, 44.4, 38.3, 36.9, 32.9, 28.8; m/z (ES^+) 385 MH^+ (20%); HRMS $C_{18}H_{37}N_6O_3$ (MH^+) calculated 385.2927, found 385.2944.

(6c): δH (300 MHz, CD_3OD) 1.43-1.57 (12H, m), 1.64 (3H, approx. q, *J* = 13.0 Hz), 1.88 (3H, d, *J* = 13.0 Hz), 2.29 (3H, t, *J* = 12.5 Hz), 2.64 (6H, t, *J* = 7.0 Hz), 3.16 (6H, t, *J* = 6.5 Hz); δC (75.5 MHz, CD_3OD) 177.3, 45.1, 42.2, 40.1, 33.0, 30.9, 27.9; m/z (ES^+) 427 MH^+ (15%), 214 MH_2^{2+} (100%); HRMS $C_{21}H_{43}N_6O_3$ (MH^+) calculated 427.3397, found 427.3381.

4.1.3 Scheme 3 (BIT 092, 105, 112, 138, 144):

Compound (2). Prepared using the method of Wörl and Köster[26] with N,N-dimethylethylene diamine (Aldrich) in place of ethylene diamine. The product was purified by precipitation from MeOH / Et_2O.

(2): δH (300 MHz, CD_3OD) 2.31 (18H, s), 2.59 (6H, t, *J* = 7.0 Hz), 3.55 (6H, t, *J* = 7.0 Hz), 8.43 (3H, s); δC (75.5 MHz, CD_3OD) 168.7, 136.7, 130.0, 59.2, 45.6, 38.7; m/z (ES^+) 421 MH^+ (100%); HRMS $C_{21}H_{37}N_6O_3$ (MH^+) calculated 421.2927, found 421.2927.

Compound (4). Prepared as described in the literature [27].

Compound (6): Pyridine (2 μl, 0.025 mmol) was added to a suspension of benzene-1,3,5-tricarboxylic acid (60 mg, 0.286 mmol) in thionyl chloride (2 ml) and the mixture refluxed for 24 h under nitrogen. The solvent was removed *in vacuo*, the residue suspended in dry toluene (2 ml) and added dropwise to a cooled (ice bath) solution of N-Boc-piperazine[3] (373 mg, 2.01 mmol) in toluene (2 ml). The mixture was stirred for 1 h at room temperature and water (20 ml) added. The product was extracted into Et_2O (3 x 20 ml), washed with sat. NaCl (20 ml), dried (Na_2SO_4) and the solvent removed. The protected derivative 5 was purified by flash chromatography on silica gel (100% EtOAc) to give 104 mg, 51%. This derivative (55 mg) was suspended in DCM (5 ml) and TFA (0.5 ml) added. After stirring for 6 h at room temperature the solvent was removed and the residue washed with several portions of Et_2O to give 62 mg, (approx. quant. yield if x = 3). The test sample was further purified by recrystallization from MeOH / Et_2O.

(6): δH (300 MHz, CD_3OD) 3.20-3.40 (12H, br m), 3.60-4.10 (12H, br m), 7.74 (3H); δC (75.5 MHz, CD_3OD, amide rotamers) 170.3, 137.0, 128.8, 45.7 (br), 44.2, 40.2 (br); m/z (ES^+) 415 MH^+ (15%), 208 MH_2^{2+} (20%); HRMS $C_{21}H_{31}N_6O_3$ (MH^+) calculated 415.2458, found 415.2443.

Compound (8): Prepared using the method of Wörl and Köster[1] with dimethyl isophthalate used in place of trimethyl benzene-1,3,5- tricarboxylate. In this case the product was treated with HCl in MeOH and purified by recrystallization from MeOH.

(8): δH (300 MHz, CD_3OD) 3.19 (4H, t, *J* = 6.0 Hz), 3.69 (4H, t, *J* = 6.0 Hz), 7.61 (1H, t, *J* = 8.0 Hz), 8.06 (2H, dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz), 8.50 (1H, t, *J* = 1.5 Hz); δC (75.5 MHz, CD_3OD) 170.1, 135.3, 131.9, 130.0, 127.8, 41.0, 38.8; m/z (ES^+) 251 MH^+ (20%); HRMS $C_{12}H_{19}N_4O_2$ (MH^+) calculated 251.1508, found 251.1503.

Compound (11): This material was prepared from tribromomesitylene [28] by applying similar procedures to those described for related compounds in scheme 1.

(11): δH (300 MHz, CD_3OD) 3.14 (6H, t, *J* = 5.0 Hz), 3.69 (6H, t, *J* = 5.0 Hz), 4.61 (6H, s), 7.36 (3H, s); δC (75.5 MHz, CD_3OD) 139.6, 128.0, 74.0, 67.1, 40.7; m/z (ES^+) 298 MH^+ (100%); HRMS $C_{15}H_{28}N_3O_3$ (MH^+) calculated 298.2131, found 298.2127.

4.1.4 Scheme 4 (BIT 047, 070, 125, 145):

Compound (3): *N,N'*-bis-t-Boc-2-Methyl-2-thiopseudourea (100 mg, 0.345 mmol) and $AgNO_3$ (62 mg, 0.365 mmol) were added to a suspension of 3,6-diaminoacridine (36 mg, 0.172 mmol) in Et_3N (101 μl, 0.723 mmol) and DCM (10 ml). The mixture was stirred overnight at room temperature under nitrogen, filtered through celite and the solvent removed. The protected product (2) was purified by flash chromatography on silica gel (EtOAc/PE 1:4) to give 41 mg, 34%. This material (39 mg) was suspended in $CHCl_3$ (4 ml) and TFA (0.8 ml) added. The mixture was stirred overnight at room temperature under nitrogen. The solvent was removed and the residue washed with several portions of Et_2O to give (3) as a yellow solid (32 mg, approx. quant. yield if x = 2).

(3): δH (300 MHz, CD_3OD) 7.65 (2H, dd, *J* = 9.0 Hz, 2.0 Hz), 8.01 (2H, s), 8.35 (2H, d, *J* = 9.0 Hz), 9.37 (1H, s); δC (75.5 MHz, CD_3OD) 157.5, 146.9, 145.6, 144.4, 133.3, 125.0, 124.2, 111.5; m/z (ES^+) 294 MH^+ (15%), 147.5 MH_2^{2+} (100%); HRMS $C_{15}H_{16}N_7$ (MH^+) calculated 294.1467, found 294.1461.

Compounds (7a), (7b), (7c): Mono-protected diamines (5,a,b,c) were prepared as described by Krapcho and Kuell[29] and were condensed with cyanuric chloride using conditions described by Ebel *et al*[30]. for reaction of cyanuric chloride with ethyl 2-aminoethylcarbamate to prepare 5a. In this case protected derivatives (6a,b,c) were extracted into Et_2O and purified by flash chromatography on silica gel (MeOH/DCM 7:93) before deprotection by treatment with TFA in $CHCl_3$.

(7a): δH (300 MHz, CD_3OD) 3.21 (6H, br t, *J* = 5.5 Hz), 3.69-3.80 (6H, br m); m/z (ES^+) 256 MH^+ (100%); HRMS $C_9H_{22}N_9$ (MH^+) calculated 256.1998, found 256.1998.

(7b): δH (300 MHz, CD_3OD) 1.91-1.96 (6H, m), 2.96-3.04 (6H, m), 3.46-3.51 (6H, m); m/z (ES^+) 298 MH^+ (100%); HRMS $C_{12}H_{28}N_9$ (MH^+) calculated 298.2468, found 298.2465.

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(7c): δH **(**300 MHz, CD_3OD) 1.60-170 (12H, m), 2.93-2.96 (6H, m), 3.42-3.46 (6H, m); m/z (ES^+) 340 MH^+ (40%); HRMS $C_{15}H_{34}N_9$ (MH^+) calculated 340.2937, found 340.2943.

4.1.5 Scheme 5 (BIT 146, 151, 159, 182):

General : Biphenyl-3,3',5,5'-tetracarboxylic acid (2) was prepared from commercially available 3,3'5,5'-tetramethylbiphenyl (Lancaster) as described by Coles *et al*. [31] and converted to its methyl ester (3) by standard conditions[1]. 3,3'5,5'-Tetramethylbiphenyl (1) was converted to tetrabromide (5) by treatment with NBS in a procedure analogous to that used to produce dibromoxyxlene and tribromomesitylene in schemes 1 and 3. Potassium phthalimide was prepared as described previously and is also commercially available [32].

Compounds (4a), (4b), (4c): A suspension of tetra ester (3) (50 mg, 0.13 mmol), in ethylene diamine (4 ml, 59.8 mmol) was stirred at room temperature for 24 h under an atmosphere of N_2. The excess diamine was removed *in vacuo* (sample taken down several times from MeOH / toluene) and the residue dissolved in MeOH / HCl. The solvent was removed and the crude product purified by precipitation from MeOH / Et_2O to give 53 mg (63 % if x = 3) as a white solid. 4b and 4c were prepared in a similar manner except that 4c was purified by flash chromatography on silica gel (MeOH / DCM / Et_3N 20 : 75 : 5).

(4a): MP > 300°C; δH **(**300 MHz, CD_3OD) 3.25 (8H, t, *J* = 6.0 Hz), 3.76 (8H, t, *J* = 6.0 Hz), 8.56 (2H, s), 8.71 (4H, s); δC **(**75.5 MHz, DMSO-D_6) 165.9, 138.6, 134.9, 128.3, 127.1, 38.5, 37.2; m/z (ES^+) 499 MH^+ (80%); HRMS $C_{24}H_{35}N_8O_4$ (MH^+) calculated 499.2781, found 499.2774.

(4b): δH **(**300 MHz, D_2O) 1.99 (8H, approx. quint., *J* = 7.5 Hz), 3.06 (8H, approx. t, *J* = 7.5 Hz), 3.50 (8H, approx. t, *J* = 7.0 Hz), 8.10 (2H, s), 8.15 (4H, s); δC **(**75.5 MHz, CD_3OD) 169.1, 141.1, 136.4, 129.9, 127.9, 38.5, 37.6, 28.7; m/z (ES^+) 555 MH^+ (20%), 278 MH_2^{2+} (40%); HRMS $C_{28}H_{43}N_8O_4$ (MH^+) calculated 555.3407, found 555.3403.

(4c): δH **(**300 MHz, CD_3OD) 2.42 (24H, s), 2.73 (8H, t, *J* = 6.5 Hz), 3.62 (8H, t, *J* = 6.5 Hz), 8.37 (2H, s), 8.39 (4H, s); δC **(**75.5 MHz, CD_3OD) 169.1, 141.5, 136.9, 129.9, 127.1, 59.1, 45.3, 38.4; m/z (ES^+) 611 MH^+ (80%), 306 MH_2^{2+} (50%); HRMS $C_{32}H_{51}N_8O_4$ (MH^+) calculated 611.4033, found 611.4038.

Compound (7): Prepared from tetrabromide (5) by applying similar procedures to those described for related compounds in scheme 1.

(7): δH **(**300 MHz, CD_3OD) 3.17 (8H, t, *J* = 5.0 Hz), 3.73 (8H, t, *J* = 5.0 Hz), 4.68 (8H, s), 7.44 (2H, s), 7.60 (4H, s); δC **(**75.5 MHz, CD_3OD) 142.5, 140.0, 127.8, 127.3, 74.1, 67.1, 40.6; m/z (ES^+) 447 MH^+ (20%), 224 MH_2^{2+} (100%); HRMS $C_{24}H_{39}N_4O_4$ (MH^+) calculated 447.2971, found 447.2955.

Compound (9): A solution of tetrabromide (5) (110 mg, 0.209 mmol), potassium phthalimide (178 mg, 0.961 mmol) and 18-crown-6 (17 mg, 0.064 mmol) in dry toluene (17 ml) was stirred for 24 h at 100°C under N_2. The mixture was cooled to room temperature and water (30 ml) added. The product was extracted into DCM (4 x 20 ml), washed with sat. $NaHCO_3$ (20 ml), dried (Na_2SO_4) and the solvent removed. The product was purified by flash chromatography on silica gel (EtOAc / PE 8 : 2, then MeOH / DCM 20 : 80 to recover precipitated sample) to give 48 mg, 29% of protected derivative (8). This material (45 mg, 0.057 mmol) was suspended in EtOH / toluene (2:1, 4.5 ml). Hydrazine hydrate (15 mg, 0.25 mmol) was added and the mixture refluxed for 72 h under N_2. The solvent was removed and the product extracted into 1M HCl (aq) (4 x 1 ml). The combined extracts were filtered, freeze dried and the residue washed with Et_2O and cold MeOH to give 17 mg, 72% (if x = 4).

(9): δH **(**300 MHz, D_2O) 3.29 (8H, s), 7.51 (2H, s), 7.76 (4H, s); δC **(**75.5 MHz, D_2O) 141.0, 134.4, 128.7, 128.4, 42.8; m/z (ES^+) 271 MH^+ (10%), 254 MH^+-NH_3 (30%), MH_2^{2+}-$2NH_3$ (100%); HRMS $C_{16}H_{23}N_4$ (MH^+) calculated 271.1923, found 271.1911.

4.1.6 Scheme 6 (BIT 147, 148, 152, 153, 157, 227):

General: t-Butylphenylcarbonate was prepared using the procedure of Houlihan *et al*. [33] and is also commercially available (Aldrich). Di-protected triamines, such as (4), were prepared as described by Pittelkow *et al* [34].

Compound 6: Pyridine (2 μl, 0.025 mmol) was added to a suspension of benzene-1,3,5-tricarboxylic acid (30 mg, 0.286 mmol) in thionyl chloride (2 ml) and the mixture refluxed for 24 h under nitrogen. The solvent was removed *in vacuo*, the residue suspended in dry toluene (2 ml) and added dropwise to a cooled (ice bath) solution of di-protected triamine (4) (300 mg, 0.990 mmol) in toluene (2 ml). The mixture was stirred for 1 h at room temperature and water (20 ml) added. The product was extracted into Et_2O (3 x 20 ml), washed with sat. NaCl (20 ml), dried (Na_2SO_4) and the solvent removed. The protected derivative 5 was purified by flash chromatography on silica gel (5% MeOH / DCM) to give 86 mg, 57%. This derivative (73 mg) was suspended in DCM (6 ml) and TFA (1.8 ml) added. After stirring overnight at room temperature the solvent was removed and the residue washed with several portions of Et_2O to give 75 mg, (95% if x = 6).

(6): δH **(**300 MHz, D_2O) 3.17 (6H, approx. t, *J* = 7.5 Hz), 3.34 (6H, t, *J* = 6.5 Hz), 3.67 (6H, approx. t, *J* = 7.5 Hz), 3.87 (6H, t, *J* = 6.5 Hz), 7.79 (3H, s); δC **(**75.5 MHz, CD_3OD) 173.4, 137.8, 128.0, 44.9, 44.8, 39.4, 38.3; m/z (ES^+) 466 MH^+ (10%); HRMS $C_{21}H_{40}N_9O_3$ (MH^+) calculated 466.3254, found 466.3256.

Compounds (7a), (7b) and (8a), (8b), (8c): Prepared by applying similar methods to those described for (6) from commercially available (Aldrich) terephthalic and isophthalic acids.

(7a): MP 183-184°C; ν_{max} 3423, 2926, 1681, 1631, 1426, 1203, 1130, 834, 798, 723 cm^{-1}; δH **(**300 MHz, D_2O, amide rotamers[2]) 3.16 (4H, t, *J* = 7.0 Hz), 3.34 (4H, t, *J* = 6.0 Hz), 3.68 (4H, t, *J* = 7.0 Hz), 3.87 (4H, t, *J* = 6.0 Hz), 7.62 (4H, s); δC **(**75.5 MHz, D_2O, amide rotamers) 174.3, 136.1, 127.2, 46.7, 43.5, 37.7, 36.8; m/z (ES^+) 338 MH^+ (70%), 169 MH_2^{2+} (100%); HRMS $C_{16}H_{29}N_6O_2$ (MH^+) calculated 337.2352, found 337.2336; $C_{16}H_{28}N_6O_2.4CF_3CO_2H.H_2O$ calculated C 35.56, H 4.23, N 10.37, found C 35.51, H 4.09, N 10.28.

(7b) δH **(**300 MHz, D_2O, amide rotamers) 1.84-1.94 (4H, m), 2.05 (4H, quint. *J* = 7.0 Hz), 2.73-2.78 (4H, m), 3.06 (4H, t, *J* = 7.5 Hz), 3.37 (4H, t, *J* = 7.5 Hz), 3.61 (4H, t, *J* = 7.0 Hz), 7.52 (4H, s); δC **(**75.5 MHz, D_2O, amide rotamers) 173.6, 136.5,

[1] Thionyl chloride (7.2 equiv) was added dropwise to a suspension of (2) in dry MeOH and the mixture refluxed for 3 h and the solvent removed. The residue was suspended in DCM, washed with sat. $NaHCO_3$, dried (Na_2SO_4), the solvent removed and the product recrystallised from Et_2O.

[2] In the ^1H NMR spectrum the two multiplets at 3.16 and 3.34 and also the two at 3.68 and 3.87 coalesce at 85°C.

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126.8, 46.7, 42.1, 37.0, 36.7, 25.9, 24.9; m/z (ES^+) 393 MH^+ (30%); HRMS $C_{20}H_{37}N_6O_2$ (MH^+) calculated 393.2978, found 393.2976.

(8a): δH (300 MHz, D_2O, amide rotamers) 3.11 (4H, approx. t, *J* = 7.5 Hz), 3.31 (4H, t, *J* = 6.0 Hz), 3.63 (4H, approx. t, *J* = 7.5 Hz), 3.84 (4H, t, *J* = 6.0 Hz), 7.56 (1H, s), 7.61-7.65 (3H, m); δC (75.5 MHz, D_2O, amide rotamers) 174.1, 134.9, 130.2, 128.3, 124.2, 46.7, 43.5, 37.6, 36.7; m/z (ES^+) 337 MH^+ (10%); HRMS $C_{16}H_{29}N_6O_2$ (MH^+) calculated 337.2352, found 337.2351.

(8b): δH (300 MHz, D_2O, amide rotamers) 1.87 (4H, approx. quint., *J* = 8.0 Hz), 2.04 (4H, quint., *J* = 7.5 Hz), 2.75 (4H, approx. t, *J* = 8.0 Hz), 3.06 (4H, approx. t, *J* = 7.5 Hz), 3.34 (4H, approx. t, *J* = 7.5 Hz), 3.60 (4H, t, *J* = 7.0 Hz), 7.42 (1H, s), 7.51-7.64 (3H, m); δC (75.5 MHz, D_2O, amide rotamers) 173.4, 135.7, 129.9, 127.6, 123.7, 46.7, 42.3, 37.1, 36.7, 25.9, 25.0; m/z (ES^+) 393 MH^+ (20%), 197 MH_2^{2+} (70%); HRMS $C_{20}H_{37}N_6O_2$ (MH^+) calculated 393.2978, found 393.2968.

(8c): δH (300 MHz, D_2O, amide rotamers) 1.37-2.08 (12H, m), 2.72-2.82 (4H, m), 3.03-3.08 (4H, m), 3.25-3.36 (4H, m), 3.50-3.62 (4H, m), 7.39 (1H, s), 7.49-7.60 (3H, m); δC (75.5 MHz, D_2O, amide rotamers) 173.3, 172.9, 135.8, 129.8, 127.6, 127.5, 123.6, 49.0, 46.7, 44.8, 42.2, 39.2, 38.9, 37.0, 36.7, 25.9, 25.0, 24.3, 23.9; m/z (ES^+) 421 MH^+ (20%); HRMS $C_{22}H_{41}N_6O_2$ (MH^+) calculated 421.3291, found 421.3289.

4.1.7 Scheme 7 (BIT 180, 181, 241, 242, 228, 283):

General : *cis*-Stilbene-4,4'-dicarboxylic acid (1) and its *trans* isomer (2) are commercially available (Lancaster). Biphenyl-3,3'-dicarboxylic acid (6) was prepared by literature methods [35]. Di-protected triamines were prepared by known methods referenced in scheme 6.

Compound (3a): BOP (Benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate) (52 mg, 0.12 mmol) was added to a solution of *cis*-stilbene-4,4'-dicarboxylic acid (15 mg, 0.056 mmol) in dry DMF (1 ml) and the mixture stirred for 5 min at room temperature under N_2. [2-(2-*tert*-Butoxycarbonylaminoethylamino)ethyl]carbamic acid *tert*-butyl ester (36 mg, 0.12 mmol) and Et_3N (19 μl, 0.14 mmol) were added and stirring continued overnight. Water (30 ml) was added and the mixture extracted with EtOAc (3 x 20 ml). The organic extracts were washed with sat. NaCl (aq., 30 ml), dried (Na_2SO_4) and the solvent removed. The derivative (2a) was purified by flash chromatography on silica gel (2.5% MeOH / EtOAc) to give 31 mg, 66%. This material (28 mg) was suspended in DCM (8 ml) and TFA (1.2 ml) added dropwise. The mixture was stirred for 6 h at room temperature, the solvent was removed and the residue washed with several portions of Et_2O to give (3a) as a hygroscopic solid (26 mg, 87% if x = 4).

(3α): δH (300 MHz, D_2O, amide rotamers) 3.10 (4H, t, *J* = 7.0 Hz), 3.28 (4H, approx. t, *J* = 6.0 Hz), 3.66 (4H, t, *J* = 7.0 Hz), 3.80 (4H, approx. t, *J* = 6.0 Hz), 6.76 (2H, s), 7.30 (4H, d, *J* = 8.0 Hz), 7.36 (4H, d, *J* = 8.0 Hz); δC (75.5 MHz, D_2O, amide rotamers) 175.2, 139.4, 132.6, 130.7, 129.5, 126.5, 46.7, 43.4, 37.7, 36.8; m/z (ES^+) 439 MH^+ (20%); HRMS $C_{24}H_{35}N_6O_2$ (MH^+) calculated 439.2821, found 439.2823.

Compounds (3b), (3c), (5), (7a), (7b): Prepared by the applying the same procedure used to make (3a) to the appropriate diacid and triamine starting materials.

(3b): δH (300 MHz, D_2O, amide rotamers) 1.86 (4H, approx. quint., *J* = 7.5 Hz), 2.00 (4H, quint. *J* = 7.0 Hz), 2.72 (4H, approx. t, *J* = 7.5 Hz), 3.02 (4H, t, *J* = 7.5 Hz), 3.34 (4H, t, *J* = 7.5) Hz), 3.57 (4H, t, *J* = 7.0 Hz), 6.76 (2H, s), 7.24 (4H, d, *J* = 8.0 Hz), 7.34 (4H, d, *J* = 8.0 Hz); δC (75.5 MHz, D_2O, amide rotamers) 174.5, 139.9, 133.5, 130.5, 129.4, 126.2, 46.6, 42.0, 37.0, 36.7, 25.9, 24.9; m/z (ES^+) 495 MH^+ (20%); HRMS $C_{28}H_{43}N_6O_2$ (MH^+) calculated 495.3447, found 495.3445.

(3c): δH (300 MHz, D_2O, amide rotamers) 1.35-2.03 (12H, m), 2.69-2.80 (4H, m), 3.00-3.05 (4H, m), 3.26-3.36 (4H, m), 3.47-3.59 (4H, m), 6.76 (2H, s), 7.21-7.26 (4H, m), 7.33 (4H, d, *J* = 8.0 Hz); δC (75.5 MHz, D_2O, amide rotamers) 174.4, 174.0, 139.0, 133.9, 133.8, 130.7, 129.4, 129.3, 126.3, 126.2, 49.9, 48.9, 46.6, 44.6, 42.0, 39.2, 38.9, 37.0, 36.8, 25.9, 24.9, 24.3, 23.9; m/z (ES^+) 523 MH^+ (15%); HRMS $C_{30}H_{47}N_6O_2$ (MH^+) calculated 523.3760, found 523.3752.

(5): δH (300 MHz, D_2O, amide rotamers) 1.91 (4H, quint., *J* = 7.0 Hz), 2.05 (4H, quint., *J* = 7.0 Hz), 2.77 (4H, t, *J* = 7.5 Hz), 3.07 (4H, t, *J* = 7.0 Hz), 3.40 (4H, t, *J* = 7.5 Hz), 3.62 (4H, t, *J* = 7.0 Hz), 7.32 (2H, s), 7.42 (4H, d, *J* = 8.0 Hz), 7.70 (4H, d, *J* = 8.0 Hz); δC (75.5 MHz, D_2O, amide rotamers) 174.5, 138.9, 134.0, 129.2, 127.1, 126.8, 46.7, 42.1, 37.1, 36.7, 25.9, 24.0; m/z (ES^+) 495 MH^+ (5%); HRMS $C_{28}H_{43}N_6O_2$ (MH^+) calculated 495.3447, found 495.3443.

(7a): δH (300 MHz, D_2O, amide rotamers) 3.22 (4H, t, *J* = 7.0 Hz), 3.43 (4H, t, *J* = 6.0 Hz), 3.81 (4H, t, *J* = 7.0 Hz), 3.96 (4H, t, *J* = 6.0 Hz), 7.57 (2H, d, *J* = 7.5 Hz), 7.71 (2H, t, *J* = 8.0 Hz), 7.83 (2H, s), 7.91 (2H, d, *J* = 8.0 Hz); δC (75.5 MHz, D_2O, amide rotamers) 175.1, 140.5, 134.7, 129.9, 129.3, 125.6, 124.9, 46.8, 43.5, 37.8, 36.8; m/z (ES^+) 413 MH^+ (20%); HRMS $C_{22}H_{33}N_6O_2$ (MH^+) calculated 413.2665, found 413.2660.

(7b): δH (300 MHz, D_2O, amide rotamers) 1.92 (4H, approx. quint., *J* = 7.5 Hz), 2.08 (4H, approx. quint. *J* = 7.0 Hz), 2.77 (4H, t, *J* = 7.5 Hz), 3.10 (4H, approx. t, *J* = 7.5 Hz), 3.43 (4H, t, *J* = 7.5 Hz), 3.43 (4H, t, *J* = 7.0 Hz), 7.44 (2H, d, *J* = 7.5 Hz), 7.63 (2H, t, *J* = 7.5 Hz), 7.68 (2H, s), 7.82 (2H, d, *J* = 7.5 Hz); δC (75.5 MHz, D_2O, amide rotamers) 174.4, 140.5, 135.6, 129.8, 128.9, 125.5, 124.6, 46.7, 42.1, 37.1, 36.7, 25.9, 25.0; m/z (ES^+) 469 MH^+ (80%); HRMS $C_{26}H_{41}N_6O_2$ (MH^+) calculated 469.3291, found 469.3300.

4.1.8 Scheme 8 (BIT 160):

1,4,5,8-Tetramethylnaphthalene (3) was prepared by a known method [36] and isomerized to 1,3,5,7–tetramethylnapthalene (4) as described by Oku and Yuzen[37]. This material was brominated and converted to the final product (7) using similar procedures to those described in scheme 1 for related compounds prepared from *m*-xylene.

(7): δH (300 MHz, D_2O) 3.01-3.07 (8H, m), 3.62 (8H, t, *J* = 5.0 Hz), 4.65 (4H, s), 5.95 (4H, s), 7.54 (2H, s), 7.97 (2H, s); δC (75.5 MHz, CD_3OD) 136.5, 135.6, 132.8, 128.2, 124.6, 74.4, 72.6, 67.2, 67.0, 40.7; m/z (ES^+) 421 MH^+ (15%); HRMS $C_{22}H_{37}N_4O_4$ (MH^+) calculated 421.2815, found 421.2798.

***4.2 Isolation of SR vesicles*:** Cardiac SR was prepared from sheep heart [15]Vesicles were frozen and stored either in liquid N_2 or at -70°C. Skeletal SR was isolated from the back and leg muscles of New Zealand White rabbits, and heavy SR was collected from the 35-45% (wt/vol) interface of a discontinuous sucrose gradient, centrifuged and resuspended [38].

4.3 Ca^{2+} release. Extravesicular Ca^{2+} was monitored at 710 nm with the Ca^{2+} indicator, antipyrylazo III, using a Cary 3 Spectrophotometer[6]. Identical release experiments were performed at 790 nm, to detect Ca^{2+}-independent changes in optical density (OD) which would alter the Ca^{2+} release measurement. The cuvette solution was stirred continuously

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and temperature controlled at 25° C. Typical Ca^{2+} release experiments are shown in Figs. 2 and 6. Skeletal SR (100 μg of protein) was added to the cuvette solution (final volume of 2 ml), containing (mM): 100, KH_2PO_4 (pH = 7); 4, $MgCl_2$; 1, Na_2ATP; 0.5, antipyrylazo III). Ca^{2+},Mg^{2+}-ATPase activity was suppressed with thapsigargin (200 nM, [39]). The same solutions were used with cardiac SR except that an ATP regenerating system – phospho(enol)pyruvate (5 mM) and pyruvate kinase (25 μg /ml) – was added. OD calibration curves were obtained daily. Control experiments, performed either without SR vesicles or when the SR vesicles were blocked with thapsigargin, ruthenium red and ionophore, were performed to detect changes in OD that could be attributed to the compounds themselves. Such changes were detected with only one compound (BIT 262). This artefactual change in OD was taken into account in experimental design and data analysis.

4.4 Intact fibre techniques.

Entire soleus muscles were removed from adult female Wistar rats immediately after CO_2 euthanasia and the muscles placed in normal Kreb's Ringer solution [40]. For experimentation, bundles of intact muscle fibres were dissected to cross-sectional diameters of ≤1-2 mm and produced force of at least 20 N/cm^2. All experiments were conducted at ambient room temperature.

The fibre bundles were mounted in the experimental chamber between a stationary point and a force transducer (Transbridge 4M, WPI) by means of surgical suture. The length of bundles was adjusted to produce maximum twitch force. The fibres were stimulated (Grass S88 Stimulator) to produce twitches (5 ms pulses at 0.04Hz) and tetani (trains of 5 ms pulses at 50 Hz for 4 s, elicited every 10 min), and force was recorded (PowerLab System/Chart4, ADInstruments). The experimental BIT compound, or IPTX, was added 2.5 min after the control tetanus. Tetani were recorded at 5 and 15 min and then either at 25 min with short exposures to BIT 180, or at 75 and 85 min during longer exposures to IPTXA). The chamber was washed with several volumes of normal Kreb's ringer, and recovery monitored by recording tetani 10 and 20 minutes after the start of wash out.

4.5 Peptide Synthesis

Peptides A1 and A2(d-R18) were synthesized by the Biomolecular Resource Facility of the John Curtin School of Medical Research (Australian National University, Canberra, Australia) using an Applied Biosystems 430A peptide synthesizer and purified by reverse-phase HPLC on a Jupiter 300 C4 column. Peptides were eluted using a linear gradient from buffer A (deionized water and 0.1% TFA) and buffer B (acetonitrile and 0.1% TFA). Purified peptide fractions were identified by mass spectroscopy using an AB MDS Sciex 4800 MALDI-TOF-TOF mass analyzer. (IPTXA) was synthesized by Auspep Australia and folded using procedures outlined by Fajloun et al. [41].

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Key Words Ryanodine receptor, calcium release, peptidomimetics, scorpion toxin, muscle

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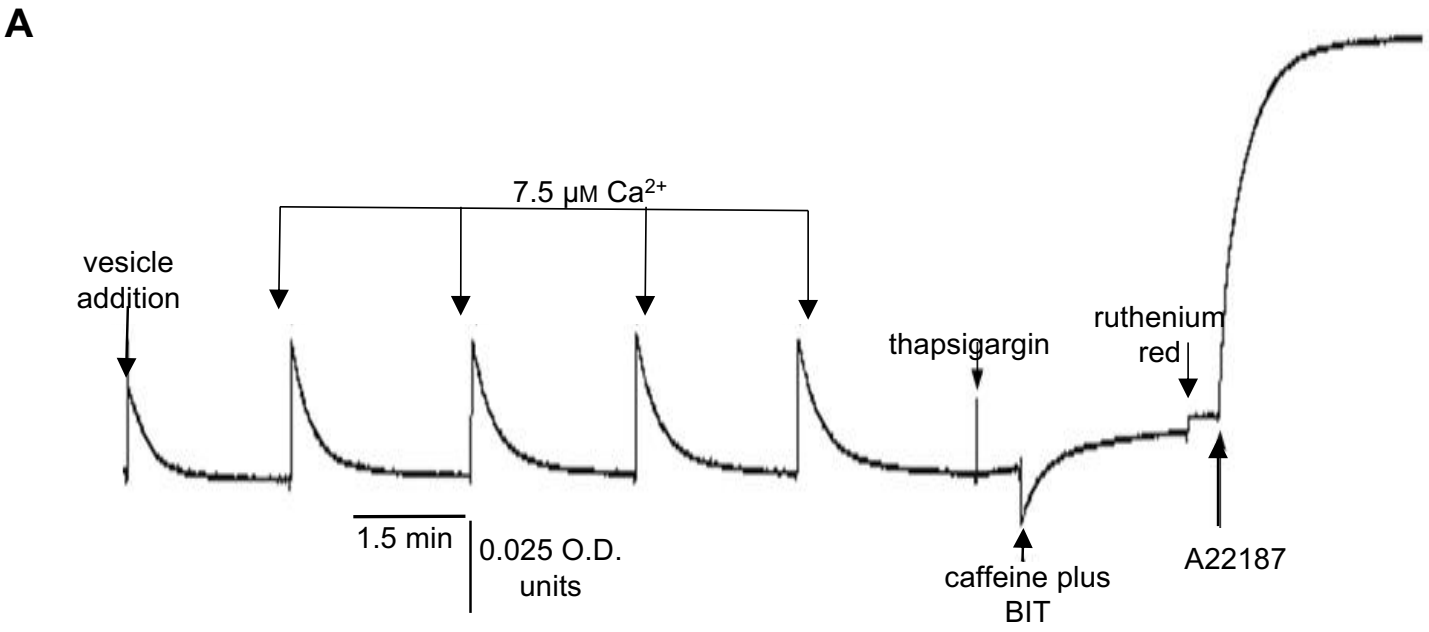

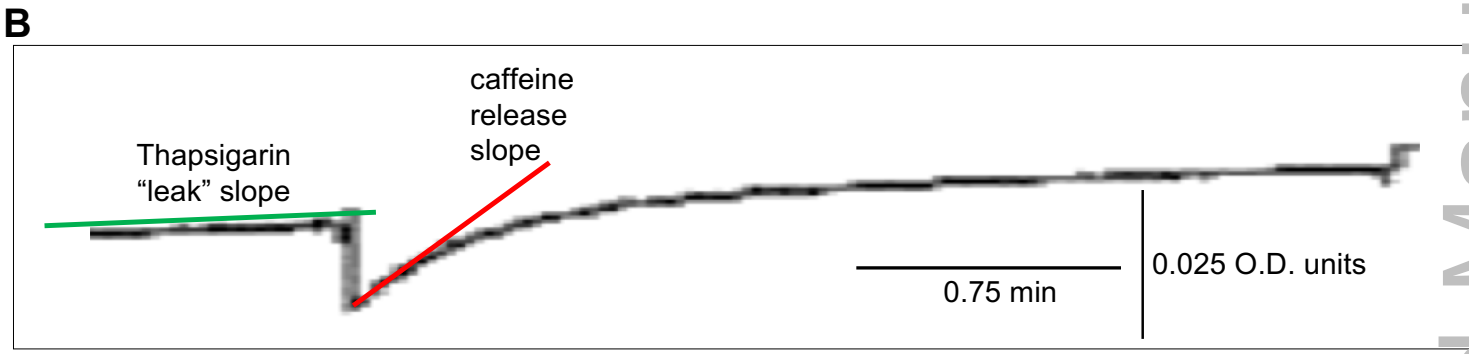

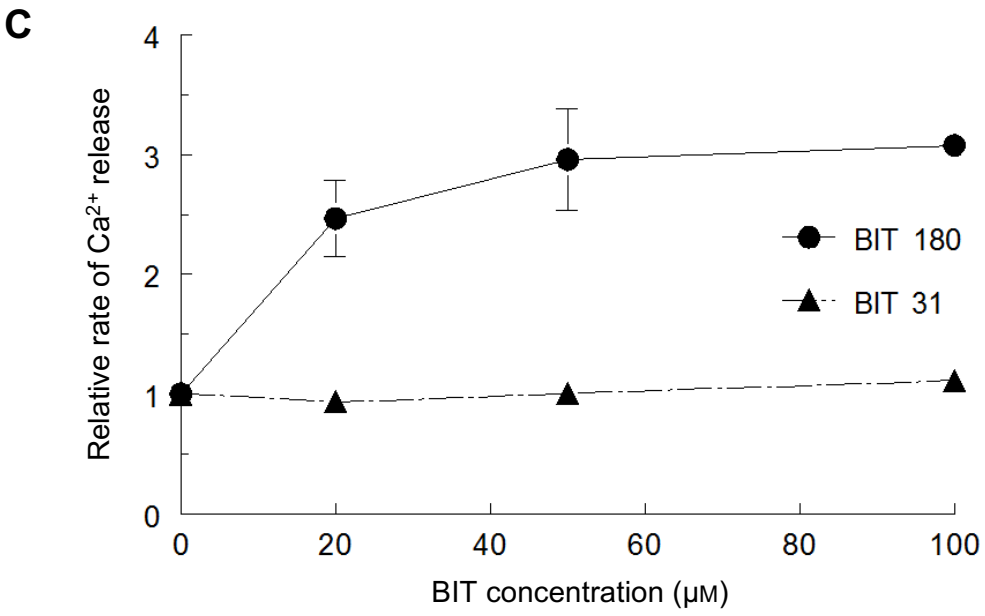

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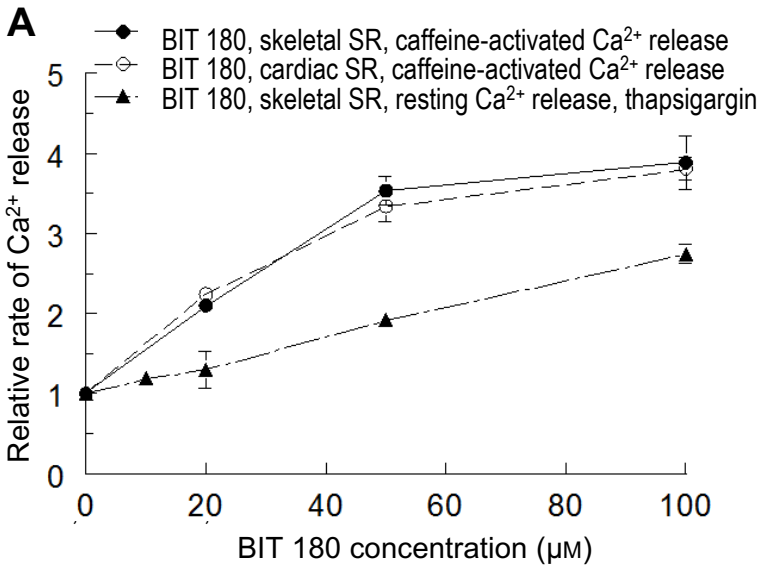

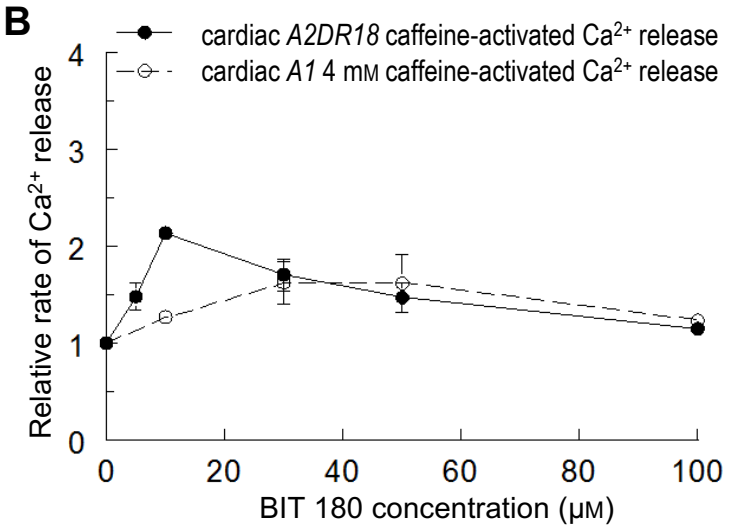

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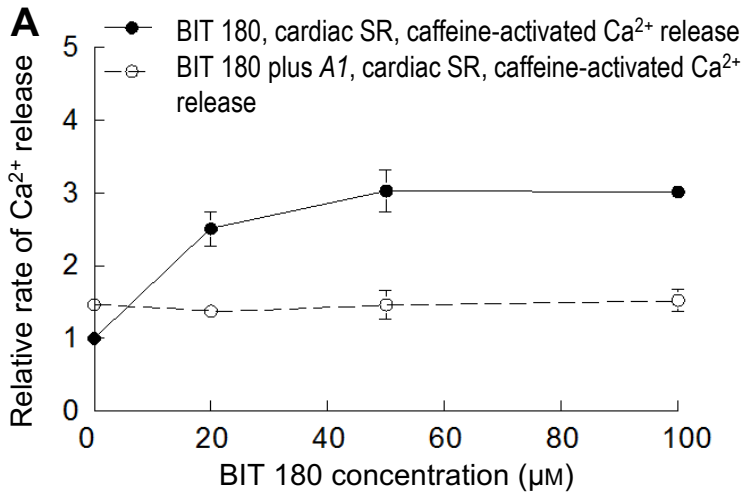

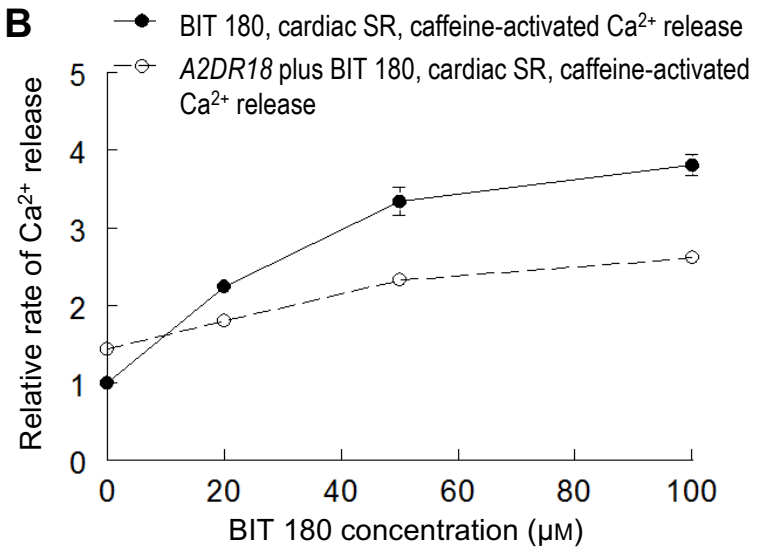

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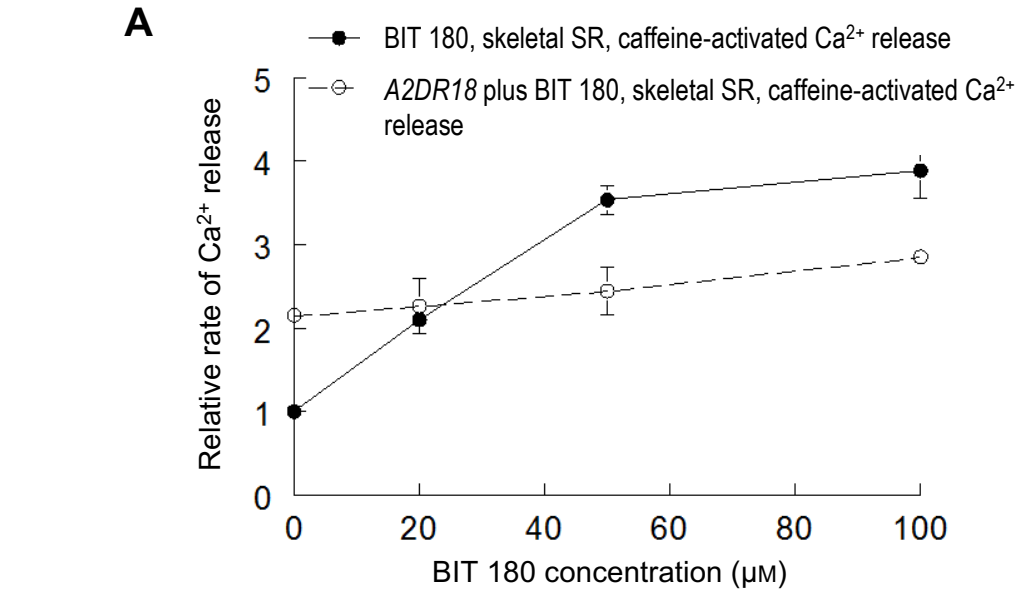

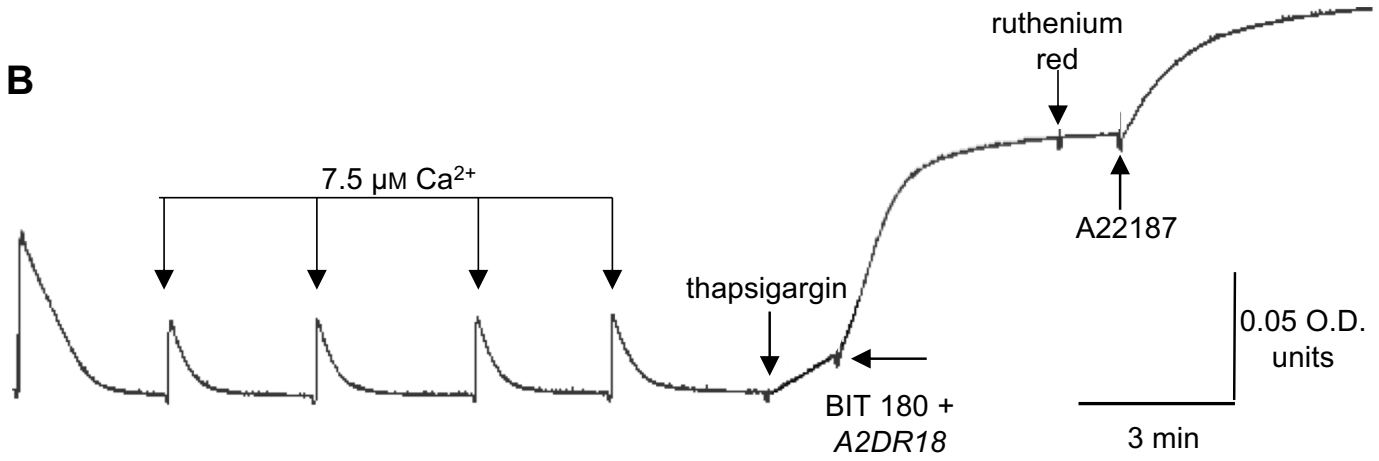

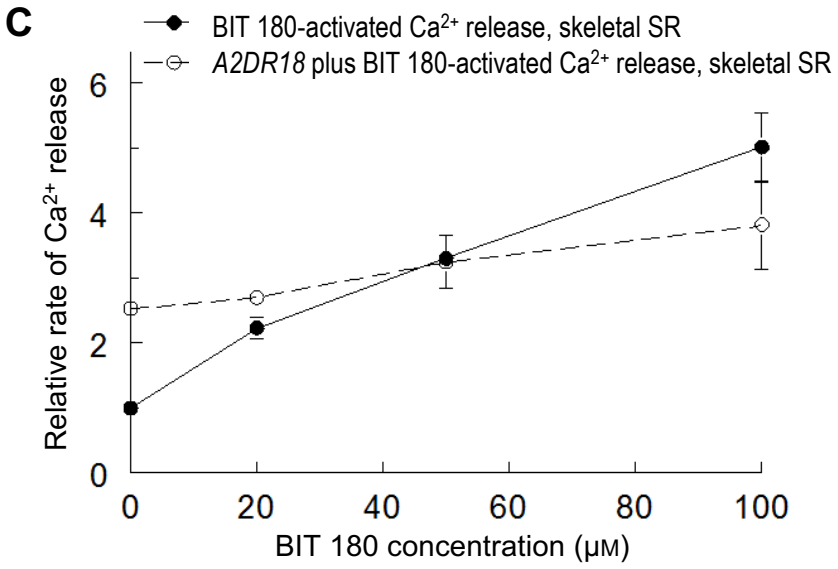

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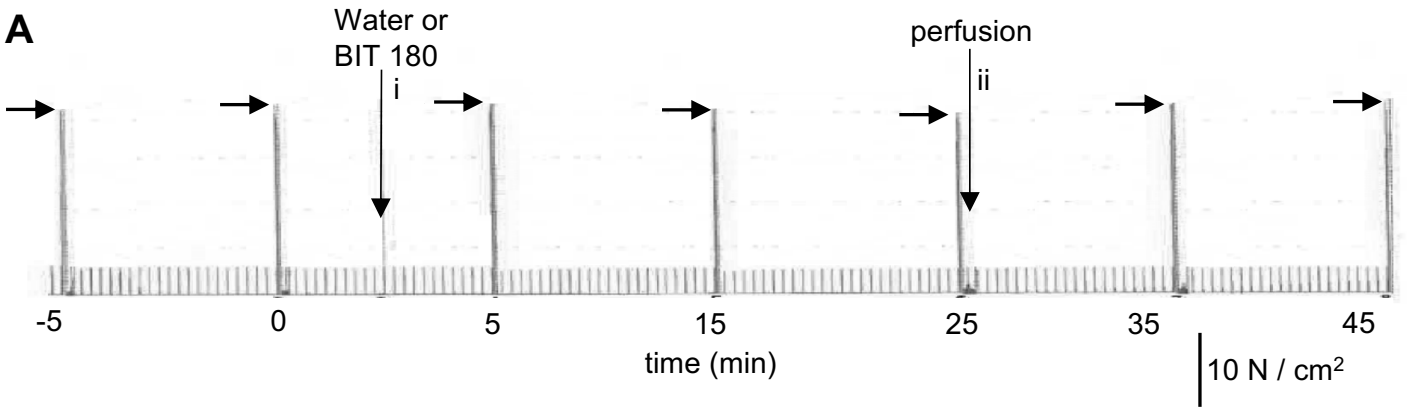

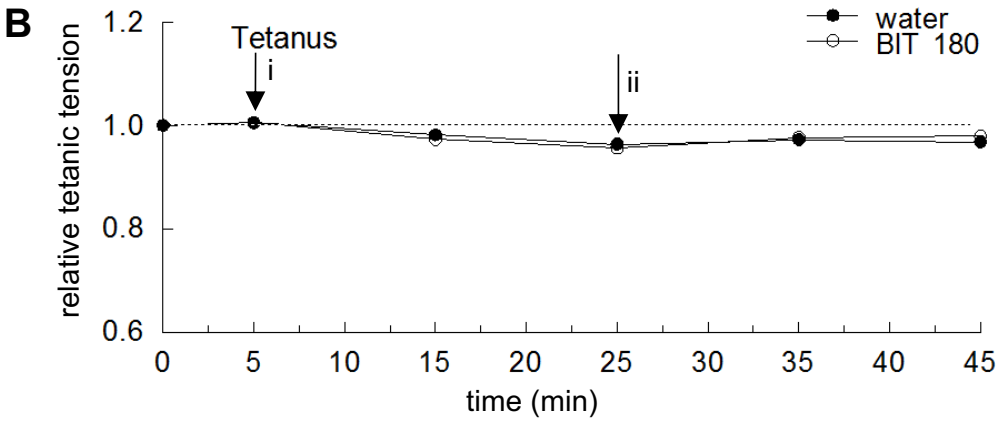

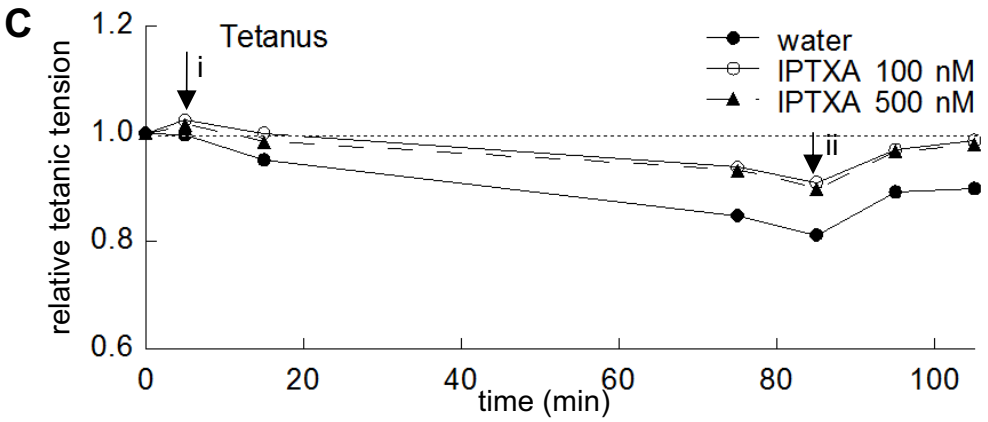

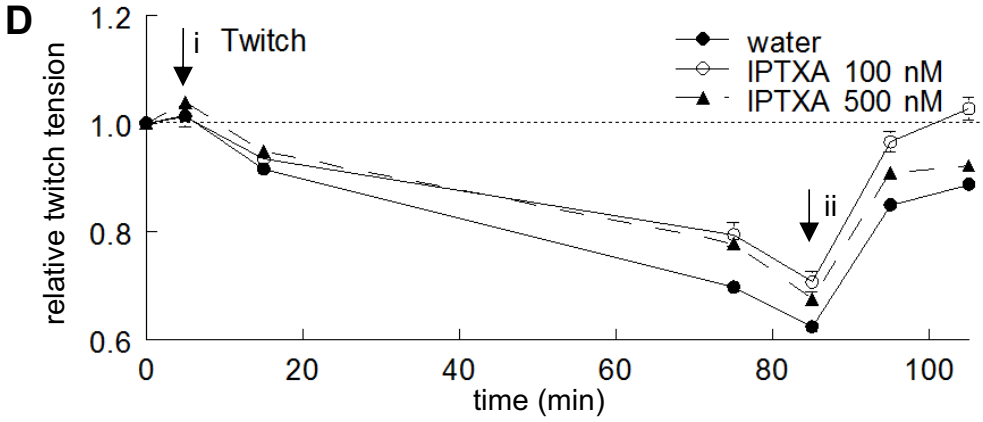

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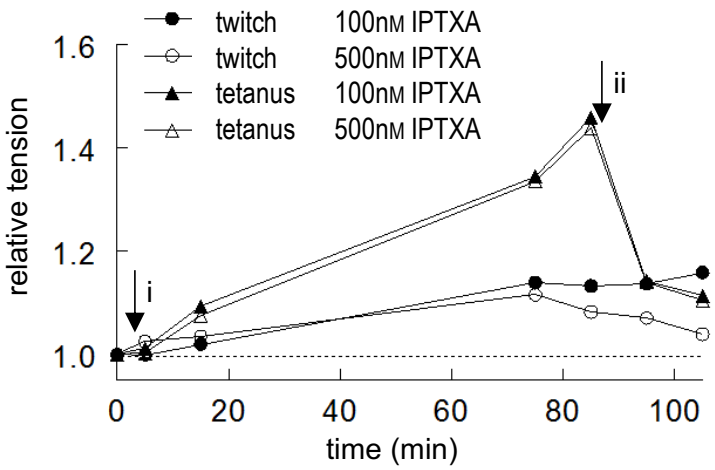

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