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# **A calmodulin antagonist reveals a calmodulin-independent inter-domain interaction essential for activation of inositol 1,4,5-trisphosphate receptors**

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Running title: Inhibition of IP<sub>3</sub> receptors by a CaM antagonist

Abbreviations used: CaM, calmodulin; CaMKII, Ca<sup>2+</sup>-CaM-dependent protein kinase II; CIRB3, Ca<sup>2+</sup>-CaM-IP<sub>3</sub>R-binding domain of TRPC3; CLM, cytosol-like medium; DT40-IP<sub>3</sub>R1 (2-3), DT40 cells expressing only recombinant IP<sub>3</sub>R1 (2-3); EC<sub>50</sub>, half-maximally effective concentration; IBC, IP<sub>3</sub>-binding core; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IC<sub>50</sub>, half-maximally inhibitory concentration; IP<sub>3</sub>R, IP<sub>3</sub> receptor; MLCK, myosin light chain kinase; NT, N-terminal (residues 1-604 of IP<sub>3</sub>R1); RyR, ryanodine receptor; SD, suppressor domain (residues 1-224 of IP<sub>3</sub>R1).

Calmodulin (CaM) has been implicated in the regulation of inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) and a recent report suggested that CaM tightly tethered to IP<sub>3</sub>R was essential for IP<sub>3</sub>R activation (Nadif Kasri, N. *et al.*, (2006) *J. Biol. Chem.* 281, 8332-8338). We confirm that a CaM-binding peptide derived from myosin light chain kinase (MLCK) inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release via all three IP<sub>3</sub>R subtypes. However, inhibition by MLCK peptide is not mimicked by other CaM antagonists that effectively block regulation of IP<sub>3</sub>R by CaM. Inhibition by MLCK peptide is rapid, fully reversible, and occurs under conditions where there is no CaM associated with IP<sub>3</sub>R. MLCK peptide stimulates IP<sub>3</sub> binding to IP<sub>3</sub>R1 and to its bacterially expressed N-terminal, but not after removal of the suppressor domain (residues 1-224). We suggest that MLCK peptide mimics a sequence within the suppressor domain that is similar to a 1-8-14 CaM-binding motif. The peptide may thereby unzip an interdomain interaction that is essential for IP<sub>3</sub>R activation. We conclude that CaM is not essential for IP<sub>3</sub>R activation, and that MLCK peptide is a selective antagonist of IP<sub>3</sub>R that binds directly to the N-terminal to uncouple IP<sub>3</sub> binding from channel gating. Our results highlight the importance of the suppressor domain in IP<sub>3</sub>R activation and suggest that MLCK peptide may provide a route to novel non-competitive antagonists of IP<sub>3</sub>R.

Key words: myosin light chain kinase, calmodulin, IP<sub>3</sub> receptor, Ca<sup>2+</sup> signalling, Ca<sup>2+</sup> channel.

## INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) are ubiquitously expressed intracellular Ca<sup>2+</sup> channels [1-3]. They mediate both the initial release of Ca<sup>2+</sup> from intracellular stores evoked by receptors that stimulate IP<sub>3</sub> formation and the regenerative propagation of intracellular Ca<sup>2+</sup> signals [4]. The latter depends upon Ca<sup>2+</sup> regulating the gating of IP<sub>3</sub>R. Indeed, even IP<sub>3</sub> works by regulating the Ca<sup>2+</sup> sensitivity of IP<sub>3</sub>R [2, 5]. The ability of Ca<sup>2+</sup> to both stimulate and inhibit IP<sub>3</sub>R is an important feature of all three vertebrate IP<sub>3</sub>R subtypes, but the structural basis of the Ca<sup>2+</sup> regulation is unresolved. There are several cytosolic Ca<sup>2+</sup>-binding sites within the IP<sub>3</sub>R itself [6], but their physiological roles are undefined. A likely endogenous Ca<sup>2+</sup>-sensor lies within the central regulatory domain of the IP<sub>3</sub>R and includes a highly conserved Glu residue (Glu<sup>2100</sup> in IP<sub>3</sub>R1), mutation of which affects the sensitivity to Ca<sup>2+</sup> [7-9]. Ca<sup>2+</sup>-binding sites within the N-terminal have also been implicated in Ca<sup>2+</sup> regulation of IP<sub>3</sub>R [6, 10].

The effects of Ca<sup>2+</sup> on IP<sub>3</sub>R might also be mediated by accessory proteins [11]. Calmodulin (CaM), a ubiquitous intracellular Ca<sup>2+</sup>-sensor, was proposed to mediate Ca<sup>2+</sup> inhibition of IP<sub>3</sub>R [12], but the evidence is conflicting [2, 11, 13]. In the presence of Ca<sup>2+</sup>, CaM inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release via each of the three IP<sub>3</sub>R subtypes [14], but the inhibition does not require binding of Ca<sup>2+</sup> to CaM [15]. CaM is unlikely therefore to provide a Ca<sup>2+</sup>-sensor for the IP<sub>3</sub>R, but Ca<sup>2+</sup>-evoked changes in the IP<sub>3</sub>R may facilitate its interaction with CaM and thereby lead to inhibition.

Ca<sup>2+</sup>-CaM binds to a central site in IP<sub>3</sub>R1 and IP<sub>3</sub>R2 [16-18] (Figure 1A), but the consequences are unclear because a mutation (Trp<sup>1577</sup>Ala) that eliminates Ca<sup>2+</sup>-CaM binding affects inhibition of IP<sub>3</sub>R1 function by neither Ca<sup>2+</sup> nor CaM [19, 20]. An additional Ca<sup>2+</sup>-CaM binding site is unmasked after removal of the S2 splice site (Figure 1A) [17], but it is unlikely to underlie the effects of CaM after mutation of Trp<sup>1577</sup>Ala because mutant IP<sub>3</sub>R with the S2 site respond normally [19, 20]. CaM also binds, in a Ca<sup>2+</sup>-independent manner to IP<sub>3</sub>R1 to cause inhibition of IP<sub>3</sub> binding [18, 21]. CaM or CaM<sub>1234</sub> likewise cause Ca<sup>2+</sup>-independent inhibition of IP<sub>3</sub> binding to an N-terminal fragment of IP<sub>3</sub>R1 (residues 1-582) [22, 23]. Within this N-terminal sequence, CaM binding straddles two regions (residues 50-82 and 107-129 of rat IP<sub>3</sub>R1) [10] (Figure 1A). The first of these sites also binds CaBP1 [24, 25]. Although the C-terminal region (residues 1565-1586) of the central CaM-binding site binds only Ca<sup>2+</sup>-CaM, additional N-terminal residues (1555-1586) allow it also to bind apo-CaM [14, 15]. The roles of these CaM-binding sites in mediating the Ca<sup>2+</sup>-dependent [14] and Ca<sup>2+</sup>-independent [18, 21] effects of CaM on IP<sub>3</sub>R behaviour have not been resolved [15, 26] and nor has the relationship between CaM and Ca<sup>2+</sup>-regulation of IP<sub>3</sub>R.

A similar situation prevails for ryanodine receptors (RyR), where neither the sites through which Ca<sup>2+</sup> biphasically regulates RyR gating nor the role of CaM are entirely resolved [27]. Ca<sup>2+</sup>-CaM and apo-CaM bind to overlapping sites on RyR1 (residues 3614-3643), probably via the C-lobe of CaM, while its N-lobe binds to a sequence (residues 1975-1999) on an adjacent RyR subunit [28]. RyR1 also has its own intrinsic CaM-like structure (residues 4064-4210), and this can bind to the C-lobe CaM-binding site [29]. Peptides derived from either region can interfere with activation of RyR suggesting that interactions between the CaM-like and CaM-binding domains might normally contribute to activation of RyR [29-31]. CaM itself might compete with these intra-receptor interactions.

These observations from RyR, which are structurally and functionally related to IP<sub>3</sub>R, suggest that the perplexing effects of CaM on IP<sub>3</sub>R might in part reflect disruption of a similar interaction between an intrinsic CaM-like domain or tethered CaM, with CaM-binding sites on the IP<sub>3</sub>R. A recent report lent support to this suggestion by demonstrating that a CaM-binding peptide derived from myosin light-chain kinase (MLCK) massively inhibited IP<sub>3</sub>-

evoked  $\text{Ca}^{2+}$  release, leading the authors to conclude that endogenously bound CaM was required for activation of  $\text{IP}_3\text{R}$  [32]. Here, we re-assess that interpretation and provide evidence that rather than causing dissociation of CaM from  $\text{IP}_3\text{R}$ , MLCK peptide specifically disrupts the coupling of  $\text{IP}_3$  binding to channel gating by binding directly to the N-terminal of the  $\text{IP}_3\text{R}$ . MLCK peptide is a non-competitive antagonist of the  $\text{IP}_3\text{R}$  that acts independently of CaM. We speculate that it disrupts an interdomain interaction between the suppressor domain and  $\text{IP}_3$ -binding core of the  $\text{IP}_3\text{R}$  that is essential for receptor activation.

## EXPERIMENTAL

### Materials

Cell culture materials were from Gibco, except for foetal bovine serum (Sigma). CaM purified from bovine brain was from Calbiochem. All other reagents were from Sigma unless otherwise stated. The sequences of the peptides, their codes and suppliers are listed in Supplementary Table S1.

### Culture and transfection of DT40 cells

DT40 cells in which the genes for all three  $\text{IP}_3\text{R}$  subtypes have been disrupted (DT40-KO) [33] and DT40 cells stably expressing only rat  $\text{IP}_3\text{R1}$  (Accession number: NP\_001007236), mouse  $\text{IP}_3\text{R2}$  (NP\_112308) or rat  $\text{IP}_3\text{R3}$  (NP\_037270) (DT40- $\text{IP}_3\text{R1-3}$ , respectively) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% heat-inactivated chicken serum, 2mM L-glutamine and 50 $\mu\text{M}$  2-mercaptoethanol. Cells were grown in suspension in 175 $\text{cm}^2$  flasks at 37°C in an atmosphere of 95% air and 5%  $\text{CO}_2$ . They were used or passaged when they reached a density of  $\sim 2 \times 10^6$  cells/ml.

### $\text{Ca}^{2+}$ release from the intracellular stores of permeabilized cells

The  $\text{Ca}^{2+}$  content of the intracellular stores of permeabilized cells was measured using a luminal low-affinity  $\text{Ca}^{2+}$  indicator as previously reported [34]. Briefly, DT40 cells ( $4 \times 10^7$  cells/ml) were suspended in hepes-buffered-saline (HBS: 135mM NaCl, 5.9mM KCl, 11.6mM hepes, 1.5mM  $\text{CaCl}_2$ , 11.5mM glucose, 1.2mM  $\text{MgCl}_2$ , pH 7.3) containing 1mg/ml bovine serum albumin, 0.4mg/ml Pluronic F127 and 20 $\mu\text{M}$  Mag-fluo-4-AM (Invitrogen). After incubation for 1h at 20°C in the dark with gentle shaking, cells were centrifuged (650 $\times g$ , 2min) and resuspended ( $10^7$  cells/ml) in  $\text{Ca}^{2+}$ -free cytosol-like medium (CLM: 20mM NaCl, 140mM KCl, 1mM EGTA, 20mM pipes, 2mM  $\text{MgCl}_2$ , pH 7.0) containing 20 $\mu\text{g}/\text{ml}$  saponin. After incubation at 37°C with gentle shaking for 4min, the permeabilized cells were centrifuged (650 $\times g$ , 2min) and resuspended in  $\text{Mg}^{2+}$ -free CLM, supplemented with  $\text{CaCl}_2$  buffered with 1mM EGTA and/or 1mM HEDTA to give final free  $[\text{Ca}^{2+}]$  of between 220nM and 4.2 $\mu\text{M}$  after addition of 1.5mM MgATP. The free  $[\text{Ca}^{2+}]$  of CLM were initially calculated using the MaxChelator program (<http://www.stanford.edu/~cpatton/>) and then measured using either fluo-3 ( $K_D^{\text{Ca}} = 325\text{nM}$ ) or Mag-fluo-4 ( $K_D^{\text{Ca}} = 22\mu\text{M}$ ). The cells were then washed, resuspended in  $\text{Mg}^{2+}$ -free CLM containing 10 $\mu\text{M}$  FCCP and distributed into a 96-well plate ( $10^6$  cells in 50 $\mu\text{l}$  CLM/well). After centrifugation of the plate, fluorescence from the luminal indicator was recorded using a FlexStation (Molecular Devices) equipped to allow automated additions [34]. In all experiments, the intracellular stores were allowed to load to steady-state with  $\text{Ca}^{2+}$  after addition of MgATP, and  $\text{IP}_3$  was then added with thapsigargin (1 $\mu\text{M}$ , to inhibit  $\text{Ca}^{2+}$  reuptake).

### Expression of N-terminal fragments of $\text{IP}_3\text{R}$

The N-terminal (NT, residues 1-604) and  $\text{IP}_3$ -binding core (IBC, residues 224-604) of  $\text{IP}_3\text{R1}$  were amplified by PCR from the full-length receptor clone lacking the S1 splice region. The

S1 region was re-introduced using a QuikChange mutagenesis kit (Stratagene) [35]. The fragments were ligated into pTrcHis A (Invitrogen) to allow expression of N-terminally His<sub>6</sub>-tagged proteins in *E. coli* strain BL21(DE3). The sequences of all constructs were confirmed by DNA sequencing. Before use for [<sup>3</sup>H]IP<sub>3</sub> binding, proteins were cleaved from the His<sub>6</sub>-tags using biotinylated thrombin (Novagen) at the engineered thrombin-cleavage site [35]. Complete cleavage was verified by western blotting using an anti-His antibody. The proteins were used for [<sup>3</sup>H]IP<sub>3</sub> binding without further purification.

### [<sup>3</sup>H]IP<sub>3</sub> binding

Equilibrium-competition binding assays were performed at 4°C for 5 min in CLM (500 µl) containing [<sup>3</sup>H]IP<sub>3</sub> (18 Ci/mmol, 0.75–1.5 nM) (PerkinElmer Life Sciences), bacterial lysate (10 µg protein for IBC, 100 µg for NT) or cerebellar membranes (50 µg protein), and competing ligands [36]. Non-specific binding was defined by addition of 10 µM IP<sub>3</sub>. Bound and free [<sup>3</sup>H]IP<sub>3</sub> were separated by centrifugation (20,000 × g, 5 min), after addition of polyethylene glycol (15% final concentration) for soluble proteins [36]. Results were analysed by fitting to a 4-parameter logistic equation (GraphPad Prism) from which the IC<sub>50</sub> and thereby the K<sub>D</sub> was calculated [18].

### Western blotting

Intact or permeabilized DT40 cells were prepared as for the Ca<sup>2+</sup> release assays and resuspended (8 × 10<sup>7</sup> cells/ml) in Ca<sup>2+</sup>-free CLM supplemented with protease inhibitors (Roche, 1 complete protease inhibitor mini-tablet/10 ml). The cells were lysed with 1% Triton-X-100, three freeze-thaw cycles, and vigorous vortexing. Proteins were separated using SDS-PAGE pre-cast mini-gels (Invitrogen) and transferred to a PVDF membrane using an Iblot dry-transfer apparatus (Invitrogen). The primary antibodies used were to CaM (monoclonal, 1:5000, Upstate) and to a peptide unique to the C-terminal of IP<sub>3</sub>R1 (rabbit, 1:1000) [37]. HRP-conjugated secondary antibodies (anti-rabbit 1:5000, AbCam; anti-mouse 1:5000, Promega) and the Super Signal West Pico chemiluminescence reagent (Pierce) were used to detect immunoreactivity. Bands were quantified using GeneTools software (Syngene).

### Immunoprecipitation

Permeabilized DT40-IP<sub>3</sub>R1 cells (10<sup>8</sup> cells) prepared as above were solubilized in lysis medium (1 ml: 140 mM NaCl, 10 mM Tris, 1 mini-protease inhibitor tablet/10 ml, pH 7.4) containing 1% Triton-X-100 at 4°C for 1 h. After centrifugation (21,000 × g, 4°C, 15 min), the supernatant was incubated with the IP<sub>3</sub>R1 antiserum (1:1000) for 1 h at 4°C on a rotating platform. Protein A/G agarose plus beads (50 µl, Santa Cruz Biotechnology) were then added. After 12 h at 4°C with constant rotation, the sample was centrifuged (650 × g, 1 min) and washed five times in lysis medium containing 0.1% Triton-X-100. The pellet and supernatant fractions were characterized by western blotting.

## RESULTS AND DISCUSSION

### Inhibition of IP<sub>3</sub>R1 by Ca<sup>2+</sup>-CaM

Ca<sup>2+</sup>-CaM inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release *via* all three IP<sub>3</sub>R subtypes, although the evidence presently derives from studies of native cells expressing predominantly a single IP<sub>3</sub>R subtype [14, 38, 39]. In each case, exogenous CaM in the presence of a free [Ca<sup>2+</sup>] (200 nM) similar to that of an unstimulated cell caused a modest (typically ~2-fold) reduction in IP<sub>3</sub> sensitivity without affecting the maximal response [14, 32, 39]. In DT40 cells expressing only IP<sub>3</sub>R1 (DT40-IP<sub>3</sub>R1), IP<sub>3</sub> caused a concentration-dependent release of the intracellular Ca<sup>2+</sup> stores.



CaM (10 $\mu$ M) inhibited IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by increasing the half-maximally effective concentration (EC<sub>50</sub>) for IP<sub>3</sub> from 41  $\pm$  6nM to 84  $\pm$  15nM without affecting the maximal Ca<sup>2+</sup> release (Figure 1B). It has been suggested that endogenous CaM may be pre-associated with IP<sub>3</sub>R [32] and perhaps thereby reduce the effects of added CaM. Permeabilized cells were pre-treated (100 $\mu$ M, 10-20min) with a Ca<sup>2+</sup>-CaM-binding peptide derived from MLCK (Supplementary Table S1) and then washed. Under these conditions, the inhibition by CaM of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release was indistinguishable from that observed in naive cells: the EC<sub>50</sub> for IP<sub>3</sub> increased from 46  $\pm$  8nM to 97  $\pm$  18nM in the presence of CaM (10 $\mu$ M, not shown). This inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by CaM was blocked by a variety of CaM antagonists, namely W-7 (20 $\mu$ M) and the peptides, camstatin (100 $\mu$ M) and CIRB3 (100 $\mu$ M) (Figure 1C and Supplementary Table S1).

These and previous results [14, 32, 39] establish that Ca<sup>2+</sup>-CaM inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by reducing the sensitivity of the intracellular stores to IP<sub>3</sub>, inhibition by added CaM appears not to be diminished by endogenously bound CaM, and the inhibition is reversed by a variety of CaM antagonists (Figure 1C).

### **Inhibition of all IP<sub>3</sub>R subtypes by MLCK peptide**

In DT40-IP<sub>3</sub>R1 cells, MLCK peptide ( $\leq$  100 $\mu$ M) affected neither the Ca<sup>2+</sup> content of the intracellular stores nor their sensitivity (EC<sub>50</sub>) to IP<sub>3</sub>, but the maximal response to IP<sub>3</sub> was significantly reduced (Figures 2A and B). The inhibitory effect of a submaximal concentration of MLCK peptide (10 $\mu$ M) was similar whether cells were pre-incubated with it for 2.5min (40.6  $\pm$  2.2% inhibition, Supplementary Table S2) or 10-20min (36.1  $\pm$  1.2%, Figure 2C). A maximal concentration of IP<sub>3</sub> and the longer pre-incubation period were used to assess the concentration-dependence of the inhibition by MLCK peptide. The results show that the half-maximal inhibitory concentration (IC<sub>50</sub>) of MLCK peptide was 19.8  $\pm$  4.5 $\mu$ M. A mutant peptide (MLCK<sup>C</sup>, 100 $\mu$ M), which differs from MLCK peptide by only two residues (Supplementary Table S1) and which binds to Ca<sup>2+</sup>-CaM with  $>10^6$ -fold lower affinity [40], was ineffective (Figure 2C). A scrambled MLCK peptide (MLCK<sup>S</sup>, 100 $\mu$ M, Supplementary Table S1) was also ineffective (Figure 2C).

For most analyses of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release, thapsigargin was added with IP<sub>3</sub> to allow the effects of IP<sub>3</sub> on unidirectional Ca<sup>2+</sup> release to be determined (see Experimental). To examine the kinetics of the effects of MLCK peptide, the experiments shown in Figure 2D were performed without thapsigargin. The results demonstrate that addition of MLCK peptide (100 $\mu$ M) after the stores have been emptied by IP<sub>3</sub> caused them rapidly to refill. The half-time (t<sub>1/2</sub>) for this refilling (7.4  $\pm$  0.3s, n = 3) was indistinguishable from that of empty stores loading with Ca<sup>2+</sup> after addition of ATP (7.6  $\pm$  0.1s, n = 3). These results establish that the time course of inhibition of IP<sub>3</sub>R by MLCK peptide is faster than the rate of Ca<sup>2+</sup> pumping by the SERCA: MLCK peptide maximally inhibits IP<sub>3</sub>R activity within 8s.

MLCK peptide is very basic (isoelectric point, pI = 14; Supplementary Table S1) and so might have inhibited IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by binding to IP<sub>3</sub>, but inhibition by a submaximal concentration of MLCK peptide (30 $\mu$ M) was unaffected by increasing the IP<sub>3</sub> concentration to 100 $\mu$ M (not shown). Furthermore, neither MLCK<sup>S</sup> peptide (pI = 14) nor MLCK<sup>C</sup> peptide (pI = 12.4) was effective (Figures 2C and D). These results with rat IP<sub>3</sub>R1 expressed in DT40 cells are similar to those obtained with rat IP<sub>3</sub>R1 over-expressed in fibroblasts, where the effects of MLCK peptide were shown not to result from disruption of CaM interactions with either calcineurin or CaMKII [32]. They differ, however, in that the inhibition was earlier reported to develop slowly ( $\sim$ 6min), whereas in our experiments inhibition was very rapid ( $<$ 8s, Figure 2D). It is, however, noteworthy that although Nadif Kasri *et al.* [32] observed a modest increase in the sensitivity to MLCK peptide when the

incubation was extended from 2 to 6min, they did not examine the effects of briefer incubations.

IP<sub>3</sub> caused a concentration-dependent release of a similar fraction (~75%) of the intracellular Ca<sup>2+</sup> stores from DT40 cells expressing only IP<sub>3</sub>R2 (EC<sub>50</sub> = 20 ± 5nM) or IP<sub>3</sub>R3 (EC<sub>50</sub> = 392 ± 70nM) (Figure 3A). As with IP<sub>3</sub>R1, MLCK peptide significantly inhibited IP<sub>3</sub>-evoked Ca<sup>2+</sup> release via IP<sub>3</sub>R2 and IP<sub>3</sub>R3 (Figure 3B) by reducing the amount of Ca<sup>2+</sup> released. The latter was shown earlier [32], as was inhibition of sea urchin IP<sub>3</sub>R by MLCK peptide. But the MLCK peptide has not hitherto been shown to inhibit homomeric IP<sub>3</sub>R2. It seems likely from these results that MLCK peptide inhibits all IP<sub>3</sub>R.

We conclude that both exogenous Ca<sup>2+</sup>-CaM [14, 32, 39] and MLCK peptide inhibit IP<sub>3</sub>-evoked Ca<sup>2+</sup> release *via* each of the three mammalian IP<sub>3</sub>R subtypes, but by different mechanisms. Ca<sup>2+</sup>-CaM reduces IP<sub>3</sub> sensitivity, while MLCK peptide reduces only the magnitude of the Ca<sup>2+</sup> release. The results are surprising because MLCK peptide is an antagonist of CaM and might therefore have been expected simply to reverse the effects of CaM. In subsequent experiments, we address the mechanisms of inhibition by MLCK peptide by focussing on its interactions with IP<sub>3</sub>R1.

### Ca<sup>2+</sup>-dependent inhibition of IP<sub>3</sub>R1 by MLCK peptide

In DT40-IP<sub>3</sub>R1 cells, increasing the cytosolic [Ca<sup>2+</sup>] inhibited IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by causing a concentration-dependent decrease in both the fraction of the stores released by IP<sub>3</sub> and their sensitivity (EC<sub>50</sub>) to IP<sub>3</sub> (Figures 4A and B). An increase in cytosolic [Ca<sup>2+</sup>] also increased the sensitivity of IP<sub>3</sub>R1 to inhibition by MLCK peptide (Figure 4C). The IC<sub>50</sub> for MLCK peptide decreased significantly as the cytosolic [Ca<sup>2+</sup>] was increased without affecting the maximal inhibition (~90%). The Ca<sup>2+</sup>-sensitivity of the inhibition by MLCK peptide is consistent with its ability to bind selectively to Ca<sup>2+</sup>-CaM [41], and consistent with the observation that MLCK peptide failed to inhibit IP<sub>3</sub>-evoked Ca<sup>2+</sup> release in Ca<sup>2+</sup>-free medium [32].

Camstatin (100μM), a peptide that binds with high affinity to Ca<sup>2+</sup>-CaM and apo-CaM [42], had no effect on IP<sub>3</sub>-evoked Ca<sup>2+</sup> release. The latter despite Camstatin reversing CaM-inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release (Figure 1C) and IP<sub>3</sub> binding [18]. These results contrast with the suggestion that peptides derived from CaM-binding sites of RyR1 and IP<sub>3</sub>R1 inhibit IP<sub>3</sub>-evoked Ca<sup>2+</sup> release, although the inhibition by these peptides was far less than with MLCK peptide [32]. W-7 (20μM), a non-peptide antagonist that binds only to Ca<sup>2+</sup>-CaM [43], abolished the inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by CaM (Figure 1C), but alone it had no effect on IP<sub>3</sub>-evoked Ca<sup>2+</sup> release (Figure 4D). These results establish that not all effective inhibitors of CaM inhibit IP<sub>3</sub>-evoked Ca<sup>2+</sup> release.

We conclude that although some features of the inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by MLCK peptide are consistent with it binding to Ca<sup>2+</sup>-CaM, there are many inconsistencies. MLCK peptide and Ca<sup>2+</sup>-CaM are both inhibitory, but with very different characteristics. Furthermore, inhibition by MLCK peptide is not reproduced by all antagonists that effectively block the inhibitory effect of CaM.

### Reversible inhibition of IP<sub>3</sub>R1 by MLCK peptide

Permeabilized DT40-IP<sub>3</sub>R1 cells were incubated with MLCK peptide (100μM) for 10-20min under conditions identical to those used to demonstrate its inhibitory effect on IP<sub>3</sub>-evoked Ca<sup>2+</sup> release (Figure 2C). The cells were then washed by centrifugation (650xg, 5min) and resuspended in CLM with or without MLCK peptide, before addition of MgATP and then IP<sub>3</sub>. The results demonstrate that the inhibition by MLCK peptide reverses completely when it is removed. Furthermore, a maximal or submaximal concentration of MLCK peptide evokes exactly the same inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release whether added to naïve IP<sub>3</sub>R1 or those

that have previously been fully inhibited by MLCK peptide (Figure 5A). These results contradict an earlier report [32], in which inhibition by MLCK peptide did not reverse after its washout. It may be significant that our analysis involved more thorough washing steps. Furthermore, our results are incompatible with the suggestion that MLCK peptide causes inhibition of IP<sub>3</sub>R1 by promoting dissociation of bound CaM.

In both our experiments and those reported earlier [32], permeabilized cells were incubated in the absence of CaM for prolonged periods (~20min for us, and ~60min for [32]) with intervening washes before addition of IP<sub>3</sub>. Any CaM that remains associated with the IP<sub>3</sub>R must therefore dissociate extremely slowly. If MLCK peptide works by antagonizing the effect of CaM bound tightly to IP<sub>3</sub>R [32], it is difficult to understand how CaM that remains tethered to IP<sub>3</sub>R for >20min after cell permeabilization can be released within 8s of addition of MLCK peptide (Figure 2D). We know of no precedent for Ca<sup>2+</sup>-CaM antagonists promoting CaM dissociation from its targets. The antagonists can only sequester Ca<sup>2+</sup>-CaM after it has dissociated and thereby prevent its reassociation [40]. Under these circumstances, it seems improbable that residual CaM tightly associated with most IP<sub>3</sub>R could dissociate within 8s (Figure 2D; or 2min [40]) of addition of MLCK peptide.

The results so far are not consistent with MLCK peptide displacing CaM from a simple CaM-binding site on the IP<sub>3</sub>R. But they leave open the possibility that a more complex tethering of CaM, such as occurs with RyR1 [28, 44] or voltage-gated Ca<sup>2+</sup> channels [45], where each lobe of CaM is separately anchored to its target, might be disrupted by MLCK peptide. Subsequent experiments eliminate this possibility.

### **CaM is not tethered to IP<sub>3</sub>R1**

Most endogenous CaM ( $98.5 \pm 0.8\%$ , Figure 5B) or heterologously expressed green fluorescent protein (GFP, 30kDa, not shown) was released from DT40 cells when they were permeabilized. Furthermore, heparin (13.5-15kDa), a competitive antagonist of IP<sub>3</sub> [36], caused the expected concentration-dependent ( $K_D = 15\mu\text{g/ml}$ , from Schild analysis) inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release from permeabilized DT40 cells (not shown). These results establish that free CaM is not retained by permeabilized cells and that exogenous CaM and other molecules of similar (heparin) or larger size (GFP) can reach the intracellular stores (Figures 1, B and C). However, these results do not exclude the possibility that some CaM remains tethered within cells.

After permeabilization of DT40-IP<sub>3</sub>R1 cells under conditions identical to those used for Ca<sup>2+</sup>-release assays, we detected  $4000 \pm 320$  tetrameric IP<sub>3</sub>R/cell and  $4500 \pm 700$  molecules of CaM/cell (Figures 5B and C). Similar amounts of CaM ( $4990 \pm 1100$  molecules/cell) were associated with permeabilized DT40-KO cells that lack IP<sub>3</sub>R. The density of IP<sub>3</sub>R was measured by quantitative comparison of scanned western blots of DT40-IP<sub>3</sub>R1 cells with cerebellar membranes in which the density of IP<sub>3</sub>R was determined by [<sup>3</sup>H]IP<sub>3</sub> binding [37] (not shown), and CaM expression was determined by comparison of scanned western blots loaded with DT40 cells or purified bovine brain calmodulin. We conclude that even if all CaM remaining within permeabilized cells were associated with IP<sub>3</sub>R, there could be only 1 CaM/IP<sub>3</sub>R, but that is inconsistent with there being similar amounts of CaM in permeabilized cells with and without IP<sub>3</sub>R. Furthermore, we failed to detect any association of CaM with IP<sub>3</sub>R1 after immunoprecipitation of IP<sub>3</sub>R1 (Figure 5D). The supernatant contained  $113 \pm 10\%$  of the CaM detected in the cell lysate, and the immunoprecipitate contained  $29 \pm 1.5\text{fmol}$  of IP<sub>3</sub>R1 (Figure 5D) and  $<5.9\text{fmol}$  of CaM (the sensitivity threshold of our western blot for CaM). These results establish that CaM is not stoichiometrically tethered to IP<sub>3</sub>R. We conclude that inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by MLCK peptide results from neither complete dissociation of CaM from IP<sub>3</sub>R nor reversible displacement of tethered CaM from an essential binding site.



### **An endogenous CaM-like region of IP<sub>3</sub>R1, F2v, does not mediate the effects of MLCK peptide**

Another possibility is that MLCK peptide might bind to an endogenous CaM-like structure within the IP<sub>3</sub>R, analogous to the CaM-like structure within RyR [29], although even this interaction would not alone provide an obvious explanation for the inability of other CaM antagonists to reproduce the effects of MLCK peptide. A protein-fold prediction program (3D-PSSM, <http://www.sbg.bio.ic.ac.uk/3dpssm>) that identified the CaM-like domain of RyR1 [29] failed to identify any EF-hand-like motifs in any of the rat IP<sub>3</sub>R subtypes. Nor was there any significant sequence similarity between the CaM-like domains of RyR and the equivalent region immediately before the transmembrane domains of IP<sub>3</sub>R.

A C-terminal region (CIRB domain) of the TrpC3 protein (Ser<sup>764</sup>-Asp<sup>793</sup>) and of other TrpC proteins binds both Ca<sup>2+</sup>-CaM and an N-terminal region of all three IP<sub>3</sub>R subtypes (Figure 6A), such that Ca<sup>2+</sup>-CaM and IP<sub>3</sub>R compete for binding to the CIRB domain [46]. Ca<sup>2+</sup>-CaM and the isolated peptides from IP<sub>3</sub>R3 (F2v: Glu<sup>681</sup>-Asp<sup>698</sup>) [47] and TrpC3 (CIRB3: Ser<sup>764</sup>-Asp<sup>793</sup>) interact directly [46]. These observations suggest that residues 684-702 of IP<sub>3</sub>R1 may, at least insofar as they are recognized by TrpC proteins, mimic CaM (Figure 6A), although the sequence itself has no obvious resemblance to CaM. We therefore considered the possibility that this region (684-702) of IP<sub>3</sub>R1 might be the site through which MLCK peptide causes inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release. However, CIRB3 (≤30μM) had no effect on the fraction of the Ca<sup>2+</sup> stores released by a maximal concentration of IP<sub>3</sub> (Figure 6B), although it did reduce the steady-state loading of the Ca<sup>2+</sup> stores. We have not explored the latter further, but it is noteworthy that F2v peptide (100μM), the fragment of IP<sub>3</sub>R3 that competes with CaM for binding to CIRB3, fully reversed the inhibition of Ca<sup>2+</sup> uptake by CIRB3 (30μM) (Figure 6B). This suggests that the peptides interact appropriately with each other [46]. Nevertheless, F2v has no effect on the ability of MLCK peptide to inhibit IP<sub>3</sub>-evoked Ca<sup>2+</sup> release (Figure 6B). We conclude that it is unlikely that MLCK peptide inhibits IP<sub>3</sub>R by interacting with the site through which IP<sub>3</sub>R competes with CaM for binding to the CIRB domain of TrpC proteins.

### **MLCK peptide directly stimulates IP<sub>3</sub> binding to IP<sub>3</sub>R1**

MLCK peptide (30μM) increased specific binding of [<sup>3</sup>H]IP<sub>3</sub> to cerebellar IP<sub>3</sub>R1 (Figure 7A). This is consistent with previous analyses of cerebellar microsomes, although the same study reported that MLCK peptide did not affect [<sup>3</sup>H]IP<sub>3</sub> binding to recombinant IP<sub>3</sub>R1 [32]. However, the single high concentration of [<sup>3</sup>H]IP<sub>3</sub> used (10nM) for the latter experiments may have obscured any modest increase in the affinity of IP<sub>3</sub>R1 (K<sub>D</sub> for IP<sub>3</sub> = 9.6 ± 1.9nM under the conditions used [37]).

MLCK peptide also stimulated IP<sub>3</sub> binding to the N-terminal fragment (NT, residues 1-604) of IP<sub>3</sub>R1 (Figures 7A-D), but not to the IP<sub>3</sub>-binding core alone (IBC, residues 224-604) (Figure 7A). The MLCK<sup>C</sup> and MLCK<sup>S</sup> peptides were ineffective (Figure 7C). The effect of MLCK peptide was due entirely to an increase in the affinity of the NT for IP<sub>3</sub>: the K<sub>D</sub> for IP<sub>3</sub> was 29 ± 6nM in the presence of 30μM MLCK peptide, and 53 ± 10nM in its absence (Figure 7B). CaM (10μM) modestly inhibited specific binding of [<sup>3</sup>H]IP<sub>3</sub> to the NT (Figure 7C), consistent with a previous report [10]. In keeping with results from the functional assays (Figures 1C and 2), other CaM antagonists (CIRB3, camstatin and W-7) had no effect on specific [<sup>3</sup>H]IP<sub>3</sub> binding to the NT. Furthermore, the sensitivity to MLCK peptide of [<sup>3</sup>H]IP<sub>3</sub> binding to the NT (EC<sub>50</sub> = 21 ± 9μM, Figure 7D) and of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release (IC<sub>50</sub> = 20 ± 4μM, Figure 2C) were indistinguishable. These results with the bacterially expressed NT of IP<sub>3</sub>R1 confirm that MLCK peptide interacts directly and specifically with IP<sub>3</sub>R1 in the complete absence of CaM.

### MLCK peptide selectively uncouples IP<sub>3</sub> binding from channel gating

We have confirmed that MLCK peptide massively inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release via each of the three IP<sub>3</sub>R subtypes [32] (Figures 2 and 3). Other antagonists that effectively reverse the effects of exogenous CaM do not mimic MLCK peptide (Figures 1C and 4D), the inhibition by MLCK peptide is very rapid (Figure 2D) and it is entirely independent of CaM (Figures 4D, 5 and 7). The inhibition is specific in that it is not mimicked by other CaM antagonists, by a scrambled MLCK peptide (MLCK<sup>S</sup>) or by a peptide (MLCK<sup>C</sup> peptide) that differs by only two residues from MLCK peptide (Figures 2, C and D, 7C and Supplementary Table S1). We conclude that endogenously bound CaM is not essential for activation of IP<sub>3</sub>R [32], but that MLCK peptide directly interacts with the N-terminal of the IP<sub>3</sub>R to uncouple IP<sub>3</sub> binding from channel gating.

MLCK peptide stimulates IP<sub>3</sub> binding only when the suppressor domain (SD) is present (Figure 7A). This is significant because the SD is required for activation of IP<sub>3</sub>R [48], possibly by providing the link between IP<sub>3</sub> binding to the IP<sub>3</sub>-binding core (IBC) and the cytosolic loop linking transmembrane domains 4 and 5 (TMD4-5) that may directly gate the channel [49, 50]. Because some of the free energy provided by binding of IP<sub>3</sub> to the IBC is used to evoke the conformational changes that lead to channel gating, disrupting the conformational changes can lead to an increase in IP<sub>3</sub> affinity (Rossi, A. M. *et al.*, unpublished observations). One such example, is the increase in IP<sub>3</sub> affinity associated with removal of the SD [51, 52] and another is provided by the demonstration that disruption of the TMD4-5 loop increases IP<sub>3</sub> affinity [50]. We speculate that MLCK peptide disrupts effective communication between the IBC and SD and thereby prevents IP<sub>3</sub> from diverting its binding energy into the conformational changes that lead to channel opening, causing both an increase in IP<sub>3</sub> affinity and a loss of channel gating by IP<sub>3</sub> (Figure 8A). But why should MLCK peptide so selectively disrupt IP<sub>3</sub>R activation?

Binding of MLCK peptide to CaM [41] occurs via a well characterized positively charged  $\alpha$ -helical “1-8-14” CaM-binding motif [53], where the numbers denote the positions of conserved hydrophobic/aromatic residues (Figures 8B and C). By sequence alignment and manual inspection, we identified a similar sequence within the first apo-CaM-binding site of rat IP<sub>3</sub>R1 (residues 50-82) [10] (Figure 8B). The sequence (residues 51-66), which is conserved in all IP<sub>3</sub>R (Figure 8B), retains the critical arrangement of hydrophobic residues, it has the same propensity as MLCK peptide to form an  $\alpha$ -helix (<http://calcium.uhnres.utoronto.ca>) and the predicted orientations of the key hydrophobic and basic residues are similar for both peptides (Figures 8C and D). None of the inactive peptides retains the 1-8-14 motif. MLCK<sup>C</sup> peptide has two of the key hydrophobic residues replaced by glutamate (Supplementary Table S1) and Camstatin and CIRB bind to CaM via an “IQ motif” and “1-5-10 motif, respectively [53]. We note also that the CaM-binding peptide derived from RyR1, which rather ineffectively inhibited IP<sub>3</sub>R [15], has appropriate hydrophobic residues at positions 1 and 14, but position 8 is occupied by a basic residue (Lys). In isolation, the putative 1-8-14 peptide from IP<sub>3</sub>R1 is predicted to be  $\alpha$ -helical (Figure 8C), but within the SD it is not  $\alpha$ -helical. It is, however, striking that within this native setting the positions of the key hydrophobic residues replicate those within MLCK peptide (Figure 8D).

We speculate that the intrinsic pseudo 1-8-14 motif within the SD of all IP<sub>3</sub>R plays an essential role in facilitating communication between the IBC and pore. One possibility is that CaM may bind directly to this motif [10], while MLCK peptide may bind to an endogenous site (as yet unidentified) that shares some critical features with CaM and which normally interacts with the 1-8-14 motif. By analogy with similar inter-domain interactions within RyR [31], we suggest that both CaM and MLCK peptide may “unzip” an essential interaction

between two domains that are required for IP<sub>3</sub>R activation (Figure 8F). We recognise that our scheme is presently too simple because MLCK peptide and CaM do not have identical inhibitory effects, but the differences may arise from additional sites of interaction between CaM and the IP<sub>3</sub>R [10]. Our interpretation is, however, consistent with the effects of mutations within the SD (highlighted in Figure 8E) that interfere with communication between the IBC and SD [51]. These residues form a continuous surface with the putative 1-8-14 motif.

We conclude that MLCK peptide is a non-competitive antagonist of all IP<sub>3</sub>R that acts entirely independently of CaM to uncouple IP<sub>3</sub> binding from channel gating. We speculate that MLCK peptide mimics a motif within the SD that resembles a 1-8-14 CaM-binding motif and thereby ‘unzips’ an interdomain interaction (possibly between the SD and IBC) that is essential for IP<sub>3</sub>R activation. Our results emphasise the importance of the SD in IP<sub>3</sub>R activation and suggest that MLCK peptide may provide a route to novel, non-competitive antagonists of IP<sub>3</sub>R.

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### Figure 1 CaM inhibits $\text{Ca}^{2+}$ release via all $\text{IP}_3\text{R1}$

(A) Key features of  $\text{IP}_3\text{R1}$ , including the sites (open bars) to which CaM binds, a residue ( $\text{E}^{2100}$ ) suggested to form part of an essential  $\text{Ca}^{2+}$ -regulatory site, and the site from which the peptide F2v derives. (B)  $\text{Ca}^{2+}$  release from DT40- $\text{IP}_3\text{R1}$  cells evoked by  $\text{IP}_3$  alone or after pre-incubation (10min) with  $10\mu\text{M}$  CaM in CLM (free  $[\text{Ca}^{2+}]$  of  $200\text{nM}$ ). (C)  $\text{Ca}^{2+}$  release from DT40- $\text{IP}_3\text{R1}$  cells evoked by maximal ( $10\mu\text{M}$ , open bars) or submaximal ( $40\text{nM}$ , solid bars) concentrations of  $\text{IP}_3$  alone or after pre-incubation (10min) with the indicated combinations of CaM ( $10\mu\text{M}$ ), W-7 ( $20\mu\text{M}$ ), camstatin ( $100\mu\text{M}$ ) or CIRB3 ( $30\mu\text{M}$ ). Results are means  $\pm$  SEM,  $n = 3$ ; \*  $p < 0.05$ .

### Figure 2 Inhibition of $\text{IP}_3\text{R1}$ by MLCK peptide

(A) Typical recording of the  $\text{Ca}^{2+}$  content of the intracellular stores of permeabilized DT40- $\text{IP}_3\text{R1}$  cells incubated with MgATP ( $1.5\text{mM}$ ),  $\text{IP}_3$  ( $10\mu\text{M}$ , added with  $1\mu\text{M}$  thapsigargin to block  $\text{Ca}^{2+}$  re-uptake) and MLCK peptide ( $10\mu\text{M}$ ) as shown. Results are means  $\pm$  SEM of 3 replicates from a single experiment. In all subsequent experiments,  $n$  refers to the number of such independent experiments. (B) Concentration-dependent release of the intracellular  $\text{Ca}^{2+}$  stores by  $\text{IP}_3$  alone or after pre-incubation (2.5min) with MLCK peptide. Results are means  $\pm$  SEM,  $n = 3-7$ . (C) Permeabilized cells pre-incubated (10-20min) with the indicated concentrations of MLCK, MLCK<sup>S</sup> or MLCK<sup>C</sup> peptide were stimulated with  $\text{IP}_3$  ( $10\mu\text{M}$ , in the continued presence of the peptide). Results (means  $\pm$  SEM,  $n = 3-5$ ) show the  $\text{Ca}^{2+}$  content of the stores before addition of  $\text{IP}_3$ , and the  $\text{Ca}^{2+}$  release evoked by  $\text{IP}_3$ . (D) Typical recording showing that addition of MLCK peptide ( $100\mu\text{M}$ ), but not MLCK<sup>C</sup> peptide ( $100\mu\text{M}$ ), causes rapid refilling of stores that were emptied by  $\text{IP}_3$  ( $10\mu\text{M}$ , added without thapsigargin). Results are means  $\pm$  SEM of 3 replicates from a single experiment, itself typical of 3 experiments (RFU, relative fluorescence units).

### Figure 3 Inhibition of $\text{IP}_3\text{R2}$ and $\text{IP}_3\text{R3}$ by MLCK peptide

(A) Concentration-dependent release of  $\text{Ca}^{2+}$  by  $\text{IP}_3$  from the intracellular stores of DT40 cells expressing  $\text{IP}_3\text{R2}$  or  $\text{IP}_3\text{R3}$ . (B) Permeabilized cells were stimulated with  $\text{IP}_3$  alone ( $10\mu\text{M}$ , solid bars) or after pre-incubation (10-20min) with MLCK peptide ( $30\mu\text{M}$ , open bars). Results (means  $\pm$  SEM,  $n = 3$ ) show the  $\text{Ca}^{2+}$  content of the stores before addition of  $\text{IP}_3$  (circles) and the  $\text{Ca}^{2+}$  release evoked by  $\text{IP}_3$  for DT40 cells expressing  $\text{IP}_3\text{R1-3}$  (bars).

### Figure 4 $\text{Ca}^{2+}$ -dependent inhibition of $\text{IP}_3\text{R1}$ by MLCK peptide

(A) Concentration-dependent effect of  $\text{IP}_3$  on  $\text{Ca}^{2+}$  release in the presence of the indicated free  $[\text{Ca}^{2+}]$ . (B) Results from similar experiments summarize the effects of  $\text{Ca}^{2+}$  on the maximal  $\text{Ca}^{2+}$  release and the  $\text{EC}_{50}$  for  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release. (C) Concentration-dependent inhibition of  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release by MLCK peptide in the presence of the indicated free  $[\text{Ca}^{2+}]$ . (D)  $\text{Ca}^{2+}$  release by the indicated concentrations of  $\text{IP}_3$  in CLM containing  $0.2$  or  $2.5\mu\text{M}$  free  $[\text{Ca}^{2+}]$  alone or with  $20\mu\text{M}$  W-7. Results are means  $\pm$  SEM,  $n \geq 3$ .

### Figure 5 Reversible inhibition of IP<sub>3</sub>R1 by MLCK peptide in the absence of CaM

(A) Permeabilized DT40-IP<sub>3</sub>R1 cells were incubated alone or with MLCK peptide (100 μM) for 10-20 min under conditions identical to those used to demonstrate inhibition of IP<sub>3</sub>R function (Figure 2). The cells were then washed (650xg, 5 min) before re-suspending them in CLM and assessing the response to IP<sub>3</sub> (10 μM) after a further incubation (10-20 min) with the indicated concentrations of MLCK peptide. Results (means ± SEM, n = 3) show the % of the intracellular stores released by IP<sub>3</sub>. (B) Western blot (with CaM antibody) loaded with the indicated amounts of bovine CaM (ng), intact or permeabilized DT40-IP<sub>3</sub>R1 cells (numbers of cells × 10<sup>3</sup> are shown). The lower panel shows the same blot overexposed to highlight the faint bands detected in permeabilized cells. Blots were quantified using GeneTools software. Results typical of >3 experiments show that >98% of CaM is released when cells are permeabilized. (C) Typical blot comparing amounts of CaM in intact or permeabilized DT40-IP<sub>3</sub>R1 and DT40-KO cells (9 × 10<sup>5</sup> cells/lane). The lower blot is an over-exposed version of the upper one to reveal the faint CaM bands in permeabilized cells. (D) Permeabilized DT40-IP<sub>3</sub>R1 cells were solubilized and immunoprecipitated with an antiserum to IP<sub>3</sub>R1. Lanes were loaded with material from equivalent numbers of cells from the total lysate (T), pellet (P) or supernatant (S) and western blotted (WB) with antisera to IP<sub>3</sub>R1 or CaM (the band with slightly higher M<sub>r</sub> than CaM in the P fraction is the light-chain of IgG). Results are typical of 3 similar experiments.

### Figure 6 A CaM-like domain, F2v, within IP<sub>3</sub>R1 does not mediate the effects of MLCK peptide

(A) Interaction between CaM and the CIRB domain of TrpC with F2v regions of IP<sub>3</sub>R. (B) Permeabilized cells were pre-incubated (10-20 min) with MLCK peptide (30 μM), CIRB3 (30 μM) or F2v (100 μM) peptides as indicated, before addition of IP<sub>3</sub> (10 μM). Results (means ± SEM, n ≥ 3) show the Ca<sup>2+</sup> content of the stores before addition of IP<sub>3</sub> (circles) and the Ca<sup>2+</sup> release evoked by IP<sub>3</sub> (bars).

### Figure 7 MLCK peptide directly interacts with IP<sub>3</sub>R1

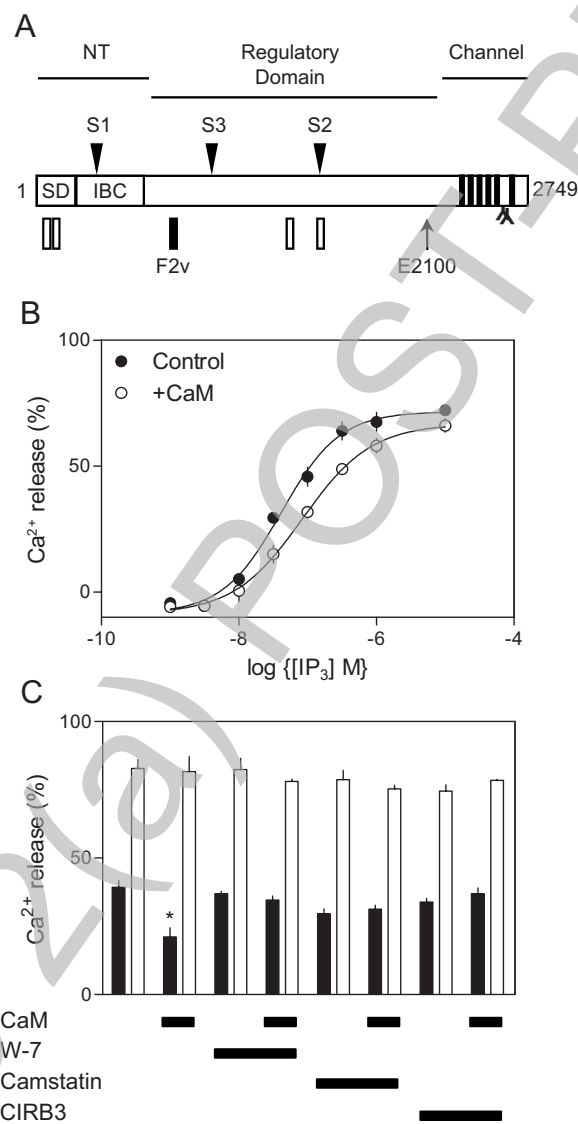
(A) Specific binding of [<sup>3</sup>H]IP<sub>3</sub> (1.5 nM) to full-length cerebellar IP<sub>3</sub>R and bacterially expressed NT and IBC from IP<sub>3</sub>R1 is shown alone (solid bars) and with 30 μM MLCK peptide (open bars); \*p < 0.05, \*\*p < 0.01. (B) Concentration-dependent effect of IP<sub>3</sub> on specific [<sup>3</sup>H]IP<sub>3</sub> binding to the NT alone, and in the presence of 30 μM MLCK peptide. Specific binding is shown as % of maximal specific [<sup>3</sup>H]IP<sub>3</sub> binding in the absence of peptide. (C) Specific binding of [<sup>3</sup>H]IP<sub>3</sub> (1.5 nM) to NT from IP<sub>3</sub>R1 is shown alone, and with CaM (10 μM), MLCK, MLCK<sup>C</sup>, MLCK<sup>S</sup>, CIRB3 or camstatin peptides (100 μM or each) or W-7 (20 μM) as indicated. (D) Concentration-dependent effect of MLCK peptide on specific [<sup>3</sup>H]IP<sub>3</sub> (1.5 nM) binding to NT. Results (A-D) are means ± SEM, n > 3).

### Figure 8 A conserved 1-8-14 CaM-binding motif within the suppressor domain may allow MLCK peptide to inhibit IP<sub>3</sub>R by unzipping a critical inter-domain interaction

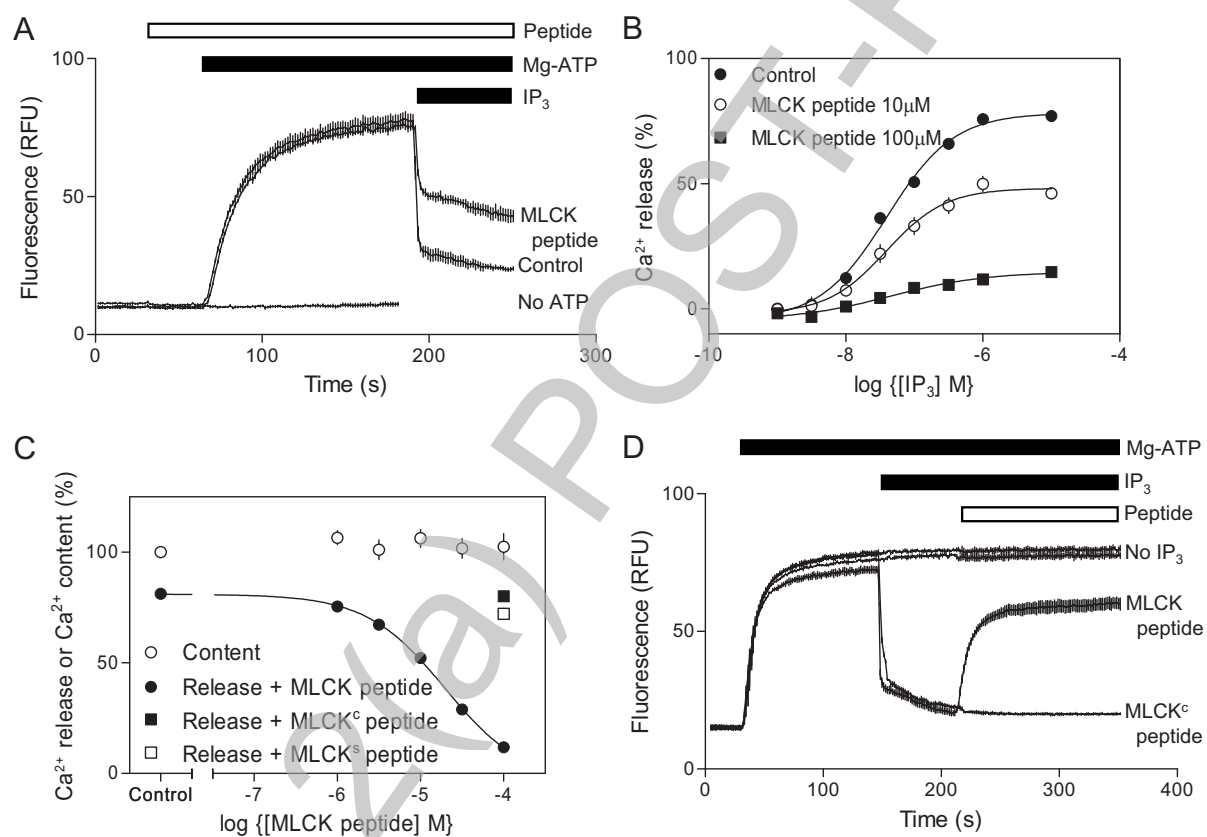
(A) Others have suggested that the SD may interact directly with the TMD4-5 loop to allow gating of the IP<sub>3</sub>R pore [50]. We suggest that MLCK peptide binds directly to the SD, uncoupling its interaction with the IBC, and so causing both an increase in IP<sub>3</sub> affinity and a loss of IP<sub>3</sub>-gated channel activity. (B) Within the SD of all IP<sub>3</sub>R, there is a sequence in which



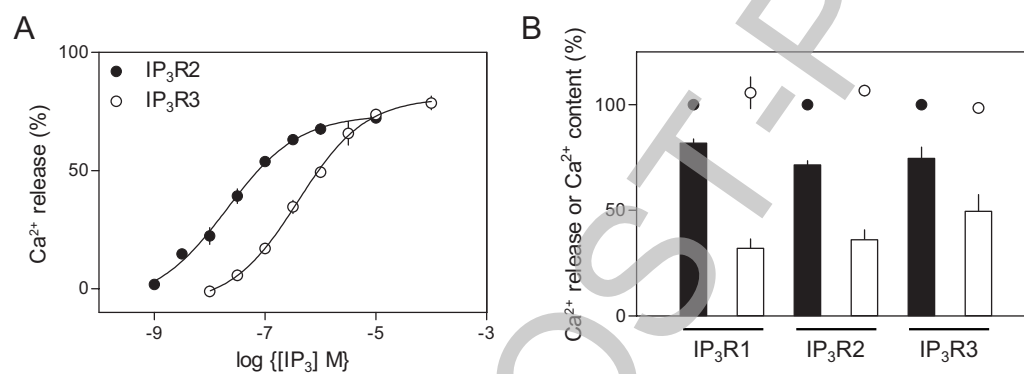
the essential features of a 1-8-14 CaM-binding motif [53] are conserved. The essential and appropriately spaced (1-8-14) hydrophobic/aromatic residues are highlighted in blue. A net positive charge (+3 to +6) provided by basic residues (red) is another key feature of the motif. **(C)** Helical wheel representations of MLCK peptide and the putative 1-8-14 motif from IP<sub>3</sub>R1 (residues 51-66). **(D)** Structure of MLCK peptide in complex with Ca<sup>2+</sup>-CaM (but with CaM not shown; PDB: 1QTX) is shown at the top with the key hydrophobic residues highlighted. The lower structure shows the SD of IP<sub>3</sub>R1 (PDB: 1XZZ) with the putative 1-8-14 motif shown in red and the key hydrophobic/aromatic residues in blue. **(E)** Structures of the SD with the putative 1-8-14 motif in red. Residues (Leu<sup>30</sup>, Leu<sup>32</sup>, Val<sup>33</sup>, Asp<sup>34</sup>, Arg<sup>36</sup>, Arg<sup>54</sup> circled, Lys<sup>127</sup>) shown to disrupt the interaction between the IBC and SD [51] (highlighted in orange) form a continuous surface with the putative 1-8-14 motif. **(F)** Speculative scheme in which an endogenous interaction between a pseudo 1-8-14 motif (residues 51-66) and an endogenous CaM-like motif is required for activation of the IP<sub>3</sub>R. MLCK peptide and CaM are proposed to “unzip” this essential interaction, although CaM also makes additional contacts with the IP<sub>3</sub>R.



Sun & Taylor Figure 1

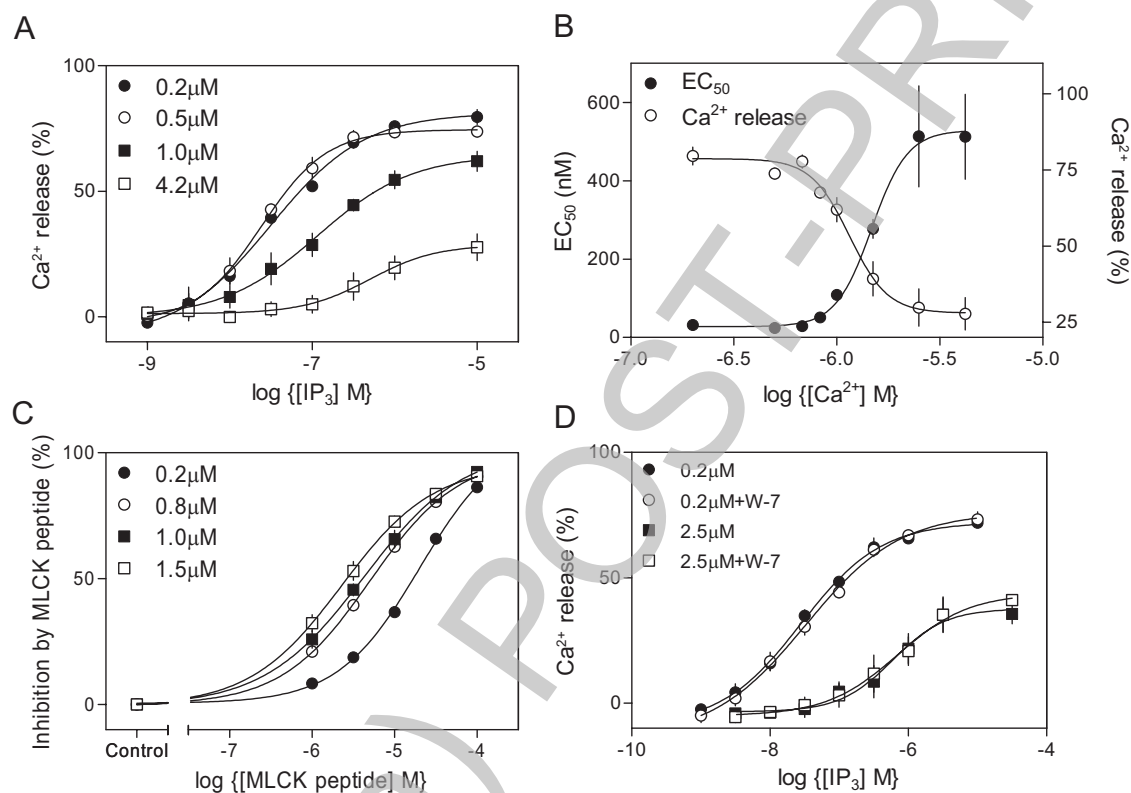


Sun & Taylor Figure 2

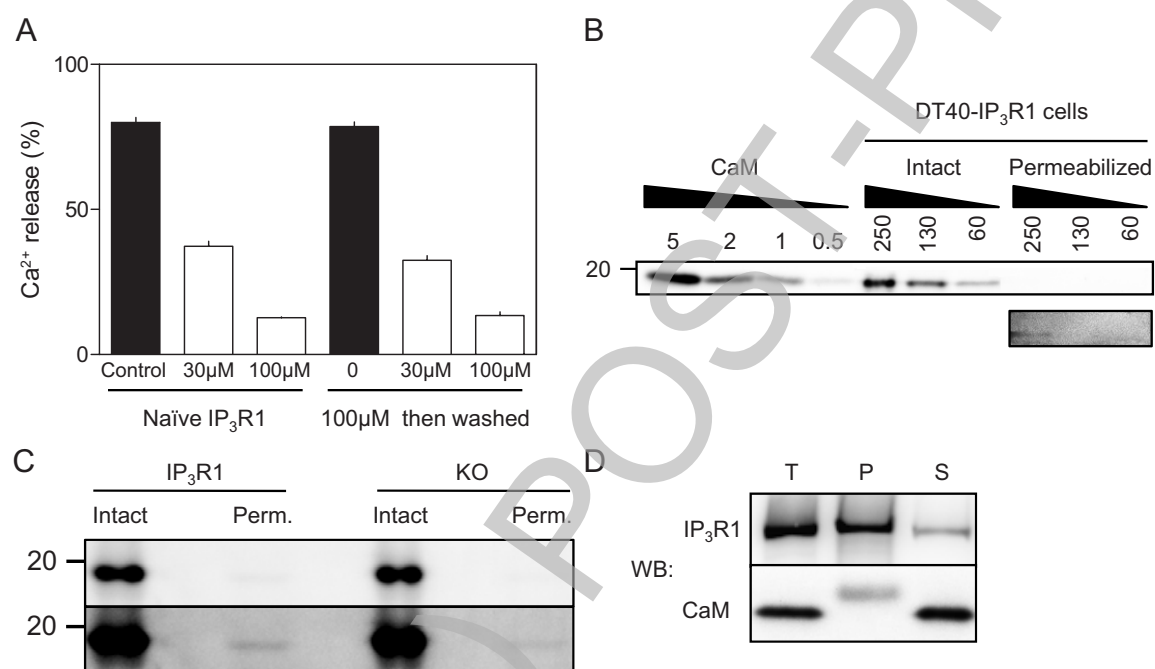


Sun & Taylor Figure 3

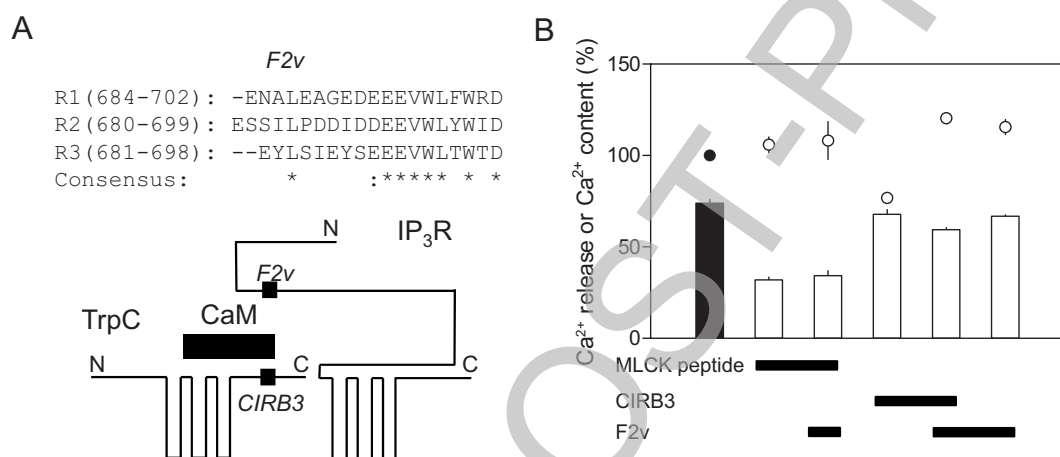




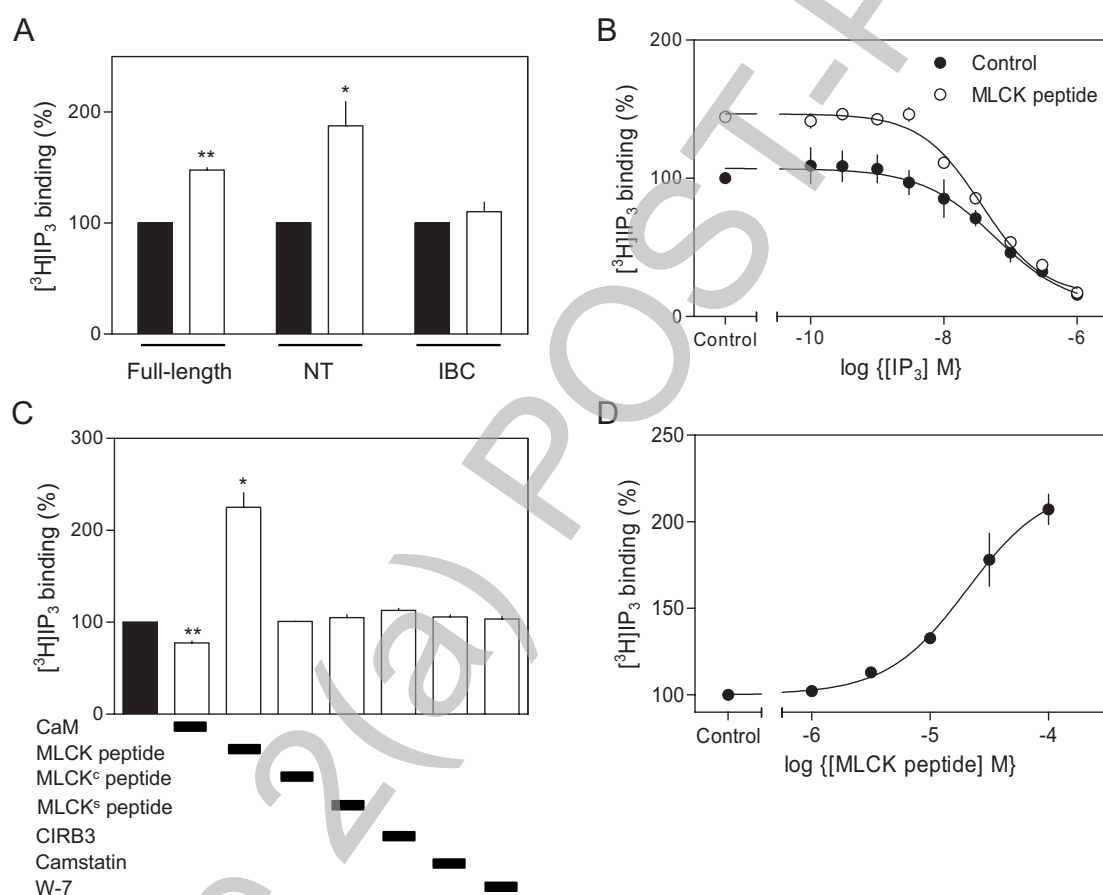
Sun & Taylor Figure 4



Sun & Taylor Figure 5

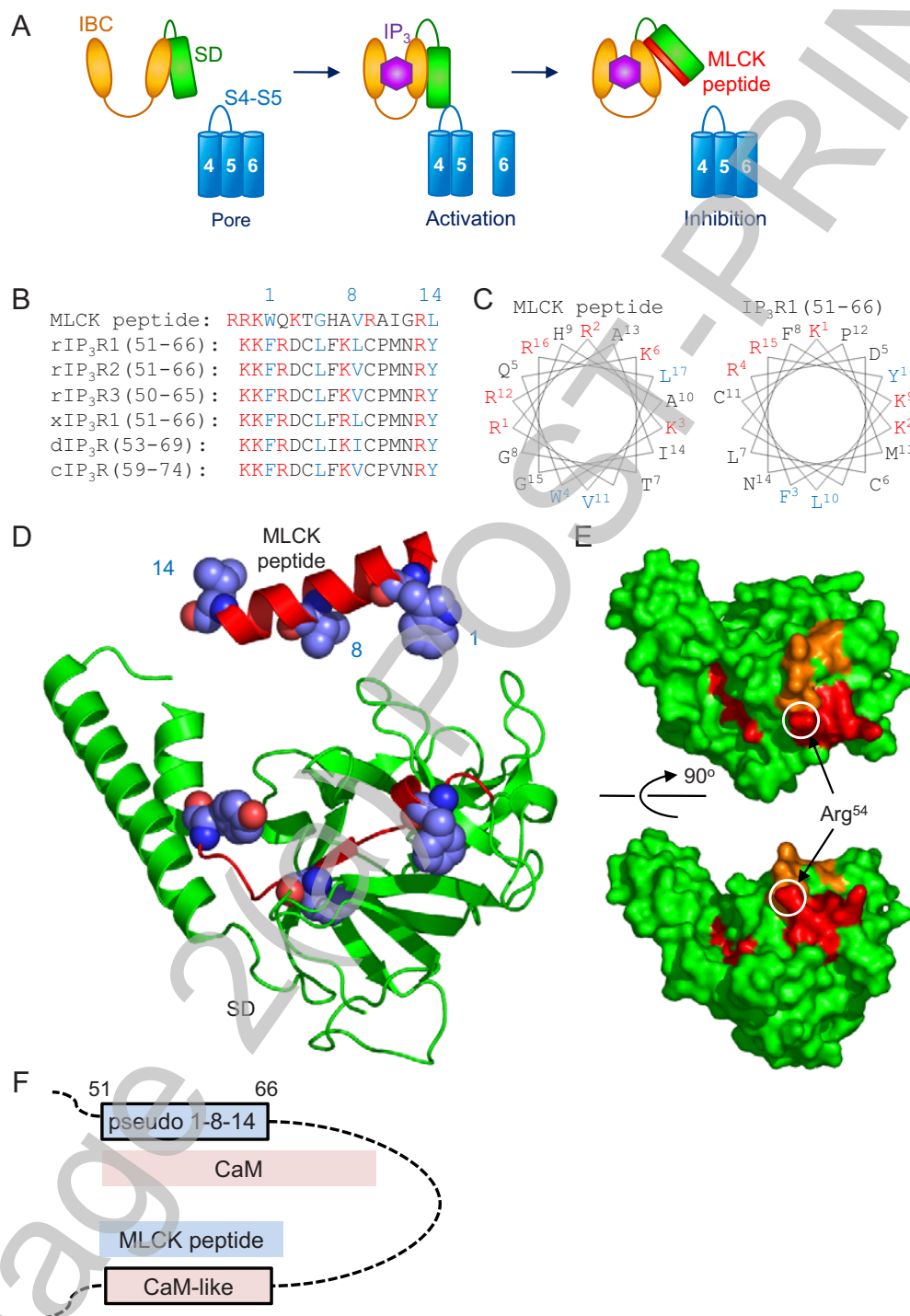


Sun & Taylor Figure 6



Sun & Taylor Figure 7





Sun & Taylor Figure 8