IP$_3$R Ca$^{2+}$ release shapes the cytosolic Ca$^{2+}$ transient for hypertrophic signalling in cardiomyocytes

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Abstract

Calcium plays a central role in mediating both contractile function and hypertrophic signalling in cardiomyocytes. While L-type Ca$^{2+}$ channels (LCCs) trigger beat-to-beat release of calcium through ryanodine receptors (RyRs) for cellular contraction, inositol 1,4,5-triphosphate receptors (IP$_3$Rs) have been implicated in releasing Ca$^{2+}$ to modify beat-to-beat calcium to concurrently signal translocation of transcription factor NFAT into the nucleus to initiate hypertrophy. Details of both the generation and delivery of this translocation signal are unclear however. A recent study by Hannanta-anan and Chow has indicated that NFAT integrates the calcium signal over time. Their experiments revealed that an increase in the duration of elevated cytosolic calcium within each period of oscillation (increase in the duty cycle) increases NFAT-mediated gene transcription. However, it is not clear whether this mechanism exists in cardiac cells, or is even the mechanism underlying NFAT activation. Here we present a mathematical model of functional interactions between RyR and IP$_3$R channels which demonstrates that IP$_3$-mediated Ca$^{2+}$ release in the cytosol is indeed capable of increasing the duty cycle of the calcium signal in the cardiomyocyte. Through comparison with published experimental results we demonstrate the importance of appropriately representing IP$_3$R gating, verify that our model is capable of reproducing known disease phenotypes in the calcium transient through alteration of channel properties, and examine how sensitive the cardiac cell is to IP$_3$. A key result of our study is that increasing IP$_3$ concentration increases calcium transient duration but may not always lead to positive inotropy. This work, together with the recent study by Hannanta-anan and Chow, suggests a plausible mechanism for IP$_3$-dependent hypertrophic signalling in cardiomyocytes.

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Introduction

Calcium plays a central role as a second messenger in many intracellular signalling pathways, from development to division, contraction, growth, and death [8, 12]. How calcium fulfils each of these different roles, while also ensuring signal specificity, has been a key question for experimentalists and modellers alike for decades. This question is particularly pertinent in the heart because of the essential role calcium plays in excitation-contraction coupling (ECC) and signalling hypertrophic cell growth. A 10-fold change in cytosolic calcium precedes each cardiac contraction yet increases in cytosolic calcium have also been implicated in the signalling pathways that mediate hypertrophic growth [45, 14, 18]. These hypertrophic signals must be detected over the background noise in a way that doesn’t disrupt the normal processes of the heart, where even subtle changes to the cellular components involved in maintaining cytosolic calcium levels can lead to severe health outcomes including heart failure and arrhythmia [29, 44, 7].

The current hypothesis for the signalling cascade that results in hypertrophy follows several pathways, one of which is the CnA/NFAT signalling pathway [34, 13]. This pathway involves an extracellular agonist binding to a phospholipase C-coupled receptor on the cell membrane. Phospholipase C (PLC) is an enzyme that then cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce diacylglycerol and IP$_3$. This means that inositol 1,4,5-triphosphate (IP$_3$R) channels, IP$_3$ and Ca$^{2+}$ gated Ca$^{2+}$ channels, are active in the hypertrophic signalling pathway (Figure 1). It is believed that activating these channels modifies the ECC signal to initiate both contraction and hypertrophic cell growth. A downstream protein, NFAT (nuclear factor of activated T-cells), mediated by the calcium-activated proteins calmodulin (CaM) and calcineurin (CnA), detects this signal and triggers the expression of hypertrophic genes. Specifically, Ca$^{2+}$-dependent dephosphorylation of NFAT leads to nuclear translocation and subsequent gene expression. How this is achieved given the low mass of the Ca$^{2+}$ signal emanating from IP$_3$Rs relative to ECC is not clear.

The exact nature of the IP$_3$R-mediated Ca$^{2+}$ signal that causes hypertrophy in cardiomyocytes is unknown. We do know that some change in nuclear Ca$^{2+}$ and the presence of CnA is required for NFAT to enter the nucleus [42]. And changes to nuclear calcium must be sustained to maintain NFAT activity [30]. In other cell types, IP$_3$Rs and their role in mediating Ca$^{2+}$ signalling are well studied and modelled [16, 49, 47], and subcellular signal localisation and Ca$^{2+}$ oscillations have

Figure 1: Calcium signalling in the cardiac hypertrophic pathway. Blue arrows indicate the direction of calcium transport across membranes.
Experimental data on hypertrophy and IP$_3$

| Cell State    | No IP$_3$ | IP$_3$ | ET-1 |
|---------------|-----------|--------|------|
| Control       |           | ▲[50] ▲[46] | ▲[46] ▲[50] |
| Basal Ca$^{2+}$ | −         | −      | ▼[30] ▼[26] ▼[50] |
| SCTs:         | −         | ▲[38] ▲[25] | ▲[50] |
| Hypertrophic  | ▲[5] ▲[24] | ▲[25] | ▲[25] |
| Basal Ca$^{2+}$ | −         | ▲[25] | ▲[25] |
| SCTs:         | −         | ▲[25] | ▲[25] |
| Hypertrophic (HF) | ▲[9] ▲[29] | ▲[28] | − |
| Basal Ca$^{2+}$ | ▲[9] ▲[29] ▲[2] | − | − |
| SCTs:         | ▲[28] | − | − |

Table 1: Summary of experimentally verified changes to the calcium transient with addition of IP$_3$ and after hypertrophic remodelling. SCTs stands for spontaneous calcium transients. Dashes indicate no data found. Colours indicate species (e.g. rat, cat, human, ferret, and mouse). ▲ indicates an increase, ▼ a decrease, and ◆ no significant change detected. Faded symbols indicate that this wasn’t specifically mentioned in the paper but was gleaned from figures.

been described [5]. In cardiomyocytes, IP$_3$R-mediated Ca$^{2+}$ signalling is less extensively studied. Studies have reported the effects of stimulation of cardiomyocytes both directly with IP$_3$ and via an extracellular agonist known to activate the CnA/NFAT pathway and increase IP$_3$, such as endothelin-1 (ET-1) or angiotensin II (Ang II) [35, 46, 54, 56, 38, 25, 26, 50, 17]. IP$_3$Rs appear to be distributed across the whole cell [25] but more heavily distributed around the nucleus [3]. Additionally, the most obvious effects of these experiments was positive inotropy of the cardiac transient in control cardiomyocytes, even greater positive inotropy in hypertrophic cardiomyocytes, and greater frequency of spontaneous Ca$^{2+}$ release events (Table 1) leading to speculation that the hypertrophic signal could be conveyed in changes to the frequency or amplitude of Ca$^{2+}$ transients [6]. While there has been less of a focus on the effects of IP$_3$R activation on nuclear calcium, experimentalists have reported positive inotropy in the nuclear calcium transient of paced cells with stimulation by either IP$_3$ or ET-1 but little to no change between control and hypertrophic ventricular myocytes [26, 25]. In atrial myocytes, stimulation with either ET-1 or IP$_3$ have been shown to cause positive inotropy in electrically paced nuclear transients [57, 31].

Hannanta-anan and Chow [24] recently used optogenetic encoding of cytosolic calcium transients in HeLa cells to demonstrate that the transcriptional activity of NFAT is dependent on the ‘duty cycle’ - that is, the ‘cumulative load of calcium concentration elevated above basal levels with respect to time, normalized to a steady-state load of equal peak amplitude’ (Figure 2A, below). They showed that increasing the duty cycle had a proportionally greater effect on NFAT transcriptional activity than changing either the frequency or amplitude of cytosolic calcium oscillations while holding the duty cycle unchanged. This suggests increased Ca$^{2+}$ duty cycle as a possible mechanism by which NFAT is activated to induce hypertrophy during disease, and raises the question of whether IP$_3$R-dependent Ca$^{2+}$ flux can generate an increased duty cycle in the heart.

Here, we show through computational modelling that Ca$^{2+}$ flux through IP$_3$R channels is capable of increasing the duty cycle in cytosolic Ca$^{2+}$ transients in adult ventricular cardiomyocytes. Furthermore our model provides insights that have not yet been directly observed experimentally, and explains the variation in the changes effected by IP$_3$ stimulation on cardiac inotropy between species. To our knowledge, this is the first model based study of IP$_3$R-RyR calcium interaction in cardiomyocytes.
Methods

We developed a computational model of RyR and IP₃R mediated Ca²⁺ fluxes in the cardiomyocyte, described below. Model simulations were performed using the ode15s ODE solver from MATLAB 2017b (The MathWorks Inc., Natick, Massachusetts) with default relative and absolute tolerances (1e-3 and 1e-6 respectively). The model equations were simulated for 15 beats at 0.33 Hz, and the first 14 oscillations discarded to eliminate transient behaviours (Figure 2B). Initial conditions were set to the basal Ca²⁺ level of the model at dynamic equilibrium with inactive IP₃R channels, determined after running the model for 100 beats. All model equations and parameter values were taken from Hinch et al. [27], other than those described below relating to IP₃R channels and the fluorescent dye.

Model Equations

We constructed a compartmental model of the left ventricular myocyte based on the Hinch et al. [27] model of ECC. The calcium in the cytosol is governed by the following ODE:

\[
\frac{d[Ca^{2+}]_{cyt}}{dt} = \beta_{fluo} \cdot \beta_{CMDN} \cdot (I_{LCC} + I_{RyR} - I_{SERCA} + I_{IP₃R} + I_{other}) \tag{1}
\]

\[
I_{other} = I_{SR} + I_{NaCa} - I_{pCa} + I_{CaB} + I_{TRPN} \tag{2}
\]

A small calcium flux through the LCCs, \(I_{LCC}\), activates RyR channels to release calcium from the SR into the cytosol at a rate of \(I_{RyR}\). Calcium is resequestered into the SR by SERCA at a rate of \(I_{SERCA}\). \(\beta_{fluo}\) is the rapid buffer coefficient [55] for the fluorescent dye in the cytosol and \(\beta_{CMDN}\) is the rapid buffer coefficient for calmodulin in the cytosol. \(I_{other}\) includes other calcium fluxes such as exchange with the extracellular environment through NaCa exchangers, \(I_{NaCa}\); sarcolemmal Ca²⁺-ATPase, \(I_{pCa}\); and the background leak current, \(I_{CaB}\); as well as the SR leak current, \(I_{SR}\); and buffering from troponin, \(I_{TRPN}\) (Figure 1 for schematic).
When the simulation is run with IP$_3$ present, there is also a flux through the IP$_3$Rs.

$$I_{IP_3R} = k_f \cdot N_{IP_3R} \cdot P_{IP_3R} \cdot (Ca^{2+})_{cyt} \cdot ((Ca^{2+})_{SR} - (Ca^{2+})_{cyt}) / V_{myo}$$  \hspace{1cm} (3)$$

Here $k_f$ is the maximum total flux through each IP$_3$R channel, this was chosen to be 1.2 $\mu$m$^3$ms$^{-1}$ to maximise the increase in transient inotropy. $N_{IP_3R}$ is the number of IP$_3$R channels in the cell, this was set to 1/50th of the number of RyR channels [36]. $V_{myo}$ is the volume of the cell and $P_{IP_3R}$ is the $(Ca^{2+})$-dependent proportion of the IP$_3$R channels at saturating levels of IP$_3$. $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{SR}$ are the Ca$^{2+}$ concentrations in the cytosol and SR respectively.

$P_{IP_3R}$ is determined using the Siekmann-Cao-Sneyd model [48, 11, 52] which has an in-built delay in IP$_3$R response to changing Ca$^{2+}$ concentration along with several parameters governing channel activation and inactivation. This model describes the open probability of IP$_3$R channels, $P_{IP_3R}$, as

$$P_{IP_3R} = \beta / (\beta + k_\beta \cdot (\beta + \alpha))$$  \hspace{1cm} (4)$$

where parameter $k_\beta$ is a transition term derived from single-channel IP$_3$R type II data by Siekmann et al. [48], $\beta$ describes the rate of activation, and $\alpha$ the rate of inactivation:

$$\beta = B \cdot m \cdot h$$ \hspace{1cm} (5)$$
$$\alpha = (1 - B) \cdot (1 - m \cdot h_{\infty})$$ \hspace{1cm} (6)$$

where $h$ is time-dependent, and $B$, $m$, and $h/h_{\infty}$ describe the dependence on IP$_3$, the dependence on Ca$^{2+}$ and the Ca$^{2+}$-dependent delay in IP$_3$R gating, respectively. Mathematical expressions for these variables are as follows:

$$m = (Ca^{2+})^4 / (K_c^4 + (Ca^{2+})^4)$$ \hspace{1cm} (7)$$
$$\frac{dh}{dt} = ((h_{\infty} - h) \cdot (K_h^4 + (Ca^{2+})^4)) / (t_{max} \cdot K_t^4)$$ \hspace{1cm} (8)$$
$$h_{\infty} = K_h^4 / (K_h^4 + (Ca^{2+})^4)$$ \hspace{1cm} (9)$$

Here $K_c$ and $K_h$ determine the Ca$^{2+}$-dependence of IP$_3$R channel open probability, while $K_t$ and $t_{max}$ affect the IP$_3$R gating delay. $K_t$ determines the influence of $(Ca^{2+})$ on the delay, while $t_{max}$ is a temporal scaling factor. We illustrate the interaction between all four of these parameters on the IP$_3$R channel flux in Figure 3.

Several experimental studies have investigated IP$_3$R activity across a range of calcium concentrations with 1$\mu$M IP$_3$ [40, 19]. These studies suggest that IP$_3$R channels would be open, with an almost constant $P_0$, over the full range of cytosolic calcium concentrations experienced during ECC in the cardiomyocyte. If this were the case, IP$_3$ stimulation would result in a Ca$^{2+}$ flux equivalent to an SR leak. While an IP$_3$R-facilitated SR-Ca$^{2+}$ leak has been reported by Zima et al. [58] and Blanch i Salvador and Egger [10], our model indicates that the $P_1$ at base Ca$^{2+}$ concentrations must be much smaller than that at higher concentrations to simultaneously amplify ECC. Instead, as a commonly reported effect of IP$_3$ stimulation on the cardiac calcium transient is positive inotropy (Table 1), we tuned these parameters to achieve the same result in simulations with hypertrophic levels of IP$_3$R channels. In order for this to happen:

- IP$_3$R channels must only open when cytosolic calcium is elevated – otherwise they are merely a constant drain from the SR.
- IP$_3$R channels must be open at the peak of the calcium transient – to augment the release by RyRs

To achieve this, we used the values $K_c = 16\mu$M, $K_h = 2.2\mu$M, $K_t = 0.2\mu$M, and $t_{max} = 1s^{-1}$ in the subsequent simulations.
Figure 3: The effect of $[\text{Ca}^{2+}]$, $[\text{IP}_3]$, $K_c$, and $K_h$ on $P_{IP_3R}$ in the Siekmann-Cao-Sneyd IP$_3$R model [48, 11, 52]. The coloured bars on the side of each plot show the approximate proportion of IP$_3$R channels that will open for each set of parameters. This is an approximation based on the model equations that does not take into account the built in delay to changing calcium concentrations. For this model, we used the values $K_h = 2.2 \mu M$, $K_c = 16 \mu M$, and IP$_3 = 10 \mu M$.

Results

IP$_3$R activation can generate physiologically realistic Ca$^{2+}$ transients

Much of the literature on the effects of IP$_3$ on the cytosolic cardiac calcium transient indicate that it should increase peak amplitude [35, 38, 17, 50, 25] (see Table 1). There are a range of parameters within the Siekmann-Cao-Sneyd model [48, 11, 52] that result in an increased Ca$^{2+}$ peak after IP$_3$R activation. This increase in peak transient occurs when the model is tuned so that IP$_3$R channels only open at Ca$^{2+}$ concentrations greater than the baseline (approximately 0.1 $\mu M$) and are inhibited by Ca$^{2+}$ concentration greater than the usual peak (approximately > 1 $\mu M$). It is always accompanied by a small increase in transient duration but this change may be indiscernible at low sampling rates (Figure 4).

Interestingly, IP$_3$R activation not only increases the calcium peak, it also results in a pronounced delay in calcium decay back to resting levels (Figure 4). This delay appears due to the finite amount of Ca$^{2+}$ available in the SR. The initial rapid release into the cytosol is unsustainable and is followed by re-uptake as the SR refills via SERCA. Once SR stores have recovered enough, smaller, prolonged release (approx. 400ms) is observed from IP$_3$Rs which contributes to this delay. The slower release through IP$_3$R channels here is a result of lower $P_0$, that is, a smaller proportion of the channels are open at this stage.

Allowing IP$_3$R opening at base calcium levels and IP$_3$R calcium inhibition at calcium levels
below peak transient concentrations results in similar behaviour to that seen in cells with a greater SR leak.

![Graph showing Cytosol (Model Resolution), SR, and Flux (µM/ms) over time (ms)](image)

Figure 4: Simulated hypertrophic ECC transient and fluxes with IP$_3$ present. IP$_3$R gating is based on the Siekmann-Cao-Sneyd model [48, 11, 52] with parameters tuned for positive inotropy. IP$_3$R channels open only at Ca$^{2+}$ concentrations greater than 0.1µM. This results in increased peak in cytosolic Ca$^{2+}$ transients and depleted SR Ca$^{2+}$ stores.

**Altering the relative expression of RyRs and IP$_3$Rs generates realistic control and hypertrophic Ca$^{2+}$ transients**

Increasing the number of IP$_3$R channels in the cell increases the transient peak. This becomes more pronounced with higher levels of IP$_3$ in the cell as well as greater numbers of IP$_3$Rs (Figure 5). Hypertrophic cells have higher expression of IP$_3$Rs [43, 25]. To simulate the consequences of IP$_3$ signalling on the Ca$^{2+}$ transient in hypertrophic cells, we ran simulations with 3-fold more IP$_3$R channels [1, 25]. We compared these to our initial simulations and investigated the comparative effects in conditions where IP$_3$Rs are inhibited (Figure 5A), and IP$_3$ concentration is increased (Figure 5B) as done experimentally by Harzheim et al. [25] in cardiomyocytes in vitro. Additionally, we investigated the difference between our original simulations and those with both a 3-fold increase in IP$_3$Rs and a 30% decrease in RyRs, as might be the case in cardiomyocytes from heart failure subjects [21] (Figure 5C). In all cases, the model realistically simulated the expected transients from in vitro experiments [25, 9].
Figure 5: Comparison of Ca\(^{2+}\) transients in the model with varying expression of IP\(_3\)Rs and RyRs. The blue lines are representative of control cardiomyocytes while the orange lines in A and B represent hypertrophic cardiomyocytes. The hypertrophic cardiomyocytes have 3-fold more IP\(_3\)R channels than the control myocytes. A The difference between transients with and without the contribution of IP\(_3\)Rs at ‘low’ IP\(_3\) concentrations. B Effect of increasing the IP\(_3\) concentration on both transients. C In this plot, the orange transient instead represents a hypertrophic cardiomyocyte that is close to heart failure. The simulation was run with a 3-fold increase in IP\(_3\)Rs and a 30% reduction in RyRs.

**Increasing [IP\(_3\)] alters IP\(_3\)R gating and increases the effect of IP\(_3\)Rs**

In the model, the primary effect of increased IP\(_3\) concentration is an increase in the Ca\(^{2+}\) release by IP\(_3\)R channels. Increasing [IP\(_3\)] also increases the calcium concentration required to activate IP\(_3\)R channels resulting in less of an effect on the baseline calcium concentration at higher IP\(_3\) concentrations. Generally, greater IP\(_3\) concentrations will result in greater Ca\(^{2+}\) transient amplitude and duration. Above a certain concentration, IP\(_3\)Rs can activate at any point during the calcium transient. Sustained IP\(_3\)R Ca\(^{2+}\) release causes negative inotropy and increased transient duration (Figure 6). The perpetual IP\(_3\)R release counteracts the action of SERCA resulting in slower uptake dynamics and SR depletion. This, in turn, leads to reduced calcium release from RyR and IP\(_3\)R channels during ECC.
Figure 6: The effect of IP$_3$ concentration on the calcium transient with hypertrophic IP$_3$R expression levels. As IP$_3$ concentration increases, the effect of IP$_3$R activation on the calcium transient becomes more pronounced. Here the initial rise gets larger while the peak gets slimmer. This is because IP$_3$Rs are inhibited by the high Ca$^{2+}$ concentration and SERCA activity is increased at these concentrations. Under conditions of saturating IP$_3$, IP$_3$Rs are almost constantly open. As a consequence, SR is depleted leading to reduced Ca$^{2+}$ transient amplitude. Model parameters are the same as those used to simulate a hypertrophic cell in Figure 5, i.e. IP$_3$R gating parameters remain the same but there are 123% more IP$_3$R channels.

RyR and IP$_3$R interactions alter the ECC duty cycle

Hannanta-anan and Chow [24] found that, when comparing calcium oscillations of the same amplitude, oscillations with greater duty cycle had the greater effect on NFAT activation, and hence, NFAT-mediated gene expression, regardless of frequency or amplitude. In their study, duty cycle, $\gamma$, was calculated as the area under the curve, $U$, divided by the maximum area under the curve (for calcium oscillations of the same amplitude, $A$, and frequency, $T$), i.e. $\gamma = \frac{U}{AT}$.

In a parameter regime where the peak of the calcium transient is relatively unaffected by IP$_3$R activation, addition of IP$_3$ increases the duty cycle of the calcium transient as IP$_3$R activation increases the transient duration and/or baseline (Figure 7). Where IP$_3$R activation also increases the transient amplitude, the effect of adding IP$_3$ on the duty cycle becomes more variable. An increase in amplitude without a similar increase in transient duration like those commonly observed in experiments involving IP$_3$ (e.g. the hypertrophic transient in Figure 5B) has a much lower duty cycle than a shorter transient with a similar width.

Discussion

We have presented what is, to our knowledge, the first modelling study to investigate the effect of hypertrophy-initiated IP$_3$R channel activity on the cardiac ECC calcium transient. The model is able to replicate several experimentally observed effects of varying IP$_3$R activation, IP$_3$R expression and IP$_3$ on the ECC calcium transient. We find IP$_3$R activation increases the duration of the calcium transient but the effect on peak Ca$^{2+}$ is [IP$_3$]-dependent. The increase in transient duration means there is a corresponding increase in duty cycle. The implication of this observation from the model is that IP$_3$R activation is sufficient to provide a signal to drive NFAT translocation and hence hypertrophic gene expression in the manner described by Hannanta-anan and Chow [24].

With an increase in expression of IP$_3$Rs, key predictions from the model include: (i) an increase in peak height; (ii) a decrease in the time it takes to reach the peak (by 1-2 ms for every doubling of total active IP$_3$Rs ); (iii) a decrease in diastolic calcium concentration; (iv) an increase in the time it takes for the calcium transient to return to baseline (i.e. width at 10% of max; (v) and an increase in the transient duration but a decrease in full duration at half max (FDHM) predominantly because the peak rises further but is short-lived. This would change with higher IP$_3$R inhibition concentration ($K_h$). These changes increase almost exponentially up to approximately 10$\mu$M.
Figure 7: A Comparison of transients (blue lines), area under the curve, and duty cycle (γ) in a regime where IP₃Rs have no effect on transient amplitude. Both increased numbers of IP₃Rs and increased IP₃ concentration increase the duty cycle. B Duty cycle increases with both [IP₃] and IP₃R numbers.
Beyond this point, the effect of increased \([\text{IP}_3]\) changes. While the FDHM continues to increase as the release from the SR overwhelms the ability of SERCA to compensate, SR calcium stores deplete leading to negative inotropy. Interestingly, the same effect can be achieved at lower \([\text{IP}_3]\) concentrations when the number of \(\text{IP}_3\)Rs is increased above a certain point. Cardiac hypertrophy is associated with an increased transient FDHM. Prolonged duration of the calcium transient can lead to early after-depolarisations (EADs) [37].

This regime in which there is always some release through \(\text{IP}_3\)R channels is reminiscent of the \(\text{IP}_3\)R channels acting as an SR leak. This has been reported as a necessary adaptation to prevent SR \(\text{Ca}^{2+}\) overload [58, 10]. However, in hypertrophic cells, especially those close to heart failure, it has been observed that there is less SERCA function and correspondingly lower \(\text{Ca}^{2+}\) stores and release [2, 15]. This additional SR leak through \(\text{IP}_3\)R channels could potentially be exacerbating the problem. The importance of this leak pathway is likely to be species dependent. In species where a high proportion of ECC calcium originates in the SR, such as rats and mice, this leak should not be an issue. However, in species such as rabbits and humans where there is a much greater dependence on the sodium-calcium exchanger (NCX), \(\text{Ca}^{2+}\) lost from the SR would be extruded and a new steady state SR \(\text{Ca}^{2+}\) concentration would have to be reached. This would result in a reduced gradient between SR and cytosolic \(\text{Ca}^{2+}\).

**The choice of \(\text{IP}_3\)R model**

Parameters in the model were specifically chosen so that the calcium transients generated resemble \textit{in vitro} transients. However, as shown in Figure 6, stimulation with high \(\text{IP}_3\) can result in a completely different effect on the transient than stimulation at lower \(\text{IP}_3\) concentrations. Examination of the \(\text{IP}_3\)R flux at this concentration shows that \(\text{IP}_3\)R channels are constantly open, resulting in depletion of the SR, and thus the model behaves in much the same way as an SR leak.

Much of the \textit{in vitro} transient data used to fit the model was gathered from whole cell experiments in which cardiomyocytes were stimulated with an esterified, cell permeant form of \(\text{IP}_3\) (InsP3-BM) [25]. The exact \(\text{IP}_3\) concentration that \(\text{IP}_3\)R channels see in these experiments is not clear. It has been found that ET-1 stimulation of cardiomyocytes produces a similar positive inotropic effect on the calcium transient to that produces by \(\text{IP}_3\) stimulation, albeit more prominently in atrial myocytes than ventricular. Moravec et al. [35] found that the maximal positive inotropic effect produced by ET-1 on rat cardiomyocytes could be observed at concentrations of 50nM. A report by Remus et al. [43] found that free \(\text{IP}_3\) rises to only 30nM in rat ventricular myocytes after stimulation with 100nM ET-1. These global measurements of \(\text{IP}_3\) may not however reflect at the \(\text{IP}_3\)R. Indeed, ET receptors are located in the t-tubules which would result in higher \(\text{IP}_3\) at the \(\text{IP}_3\)R than that recorded.

Fitting the model to the same whole cell experiments but assuming a lower \(\text{IP}_3\) concentration may well result in a model in which excess \(\text{IP}_3\) leads similar transients to those generated by a SR leak. It is likely that the concentration of the 10µM \(\text{IP}_3\) ester that filters through the cell to the \(\text{IP}_3\)R channels in whole cell experiments is much less than the 1µM seen in single-channel experiments [41].

We have so far examined the implications of \(\text{IP}_3\)R activation leading to positive inotropy as is commonly reported, however a summary of the experimental literature is equivocal. While \(\text{IP}_3\) stimulation usually leads to positive inotropy, Smyrnias et al. [51] found that stimulation of adult rat ventricular myocytes with ET-1, which was thought to have a similar effect due to downstream \(\text{IP}_3\) production, can cause positive inotropy even while \(\text{IP}_3\)Rs are inhibited. Further, the same study found that activation of \(\text{IP}_3\)Rs via ET-1 appears to contribute to initial negative inotropy. How this new evidence can be reconciled with previous experiments involving only \(\text{IP}_3\) stimulation is unclear.

The changes to the calcium transient present in hypertrophic cells and the extent to which \(\text{IP}_3\) stimulation affects the calcium transient in healthy cells have also differed between studies. The lack of a consistent effect between studies may be due to differences in species studied and methods by which intracellular \(\text{IP}_3\) is elevated, and by limitations on calcium measurement. In particular small but significant variation in the duration of the \(\text{Ca}^{2+}\) transient may go undetected in
fluorescence imaging experiments using calcium-sensitive dyes [53]. Due to the different behaviour of Ca^{2+} dyes in the cytosol vs the nucleus, quantification of Ca^{2+} in the nucleus is also challenging.

We expect that a narrow, range of IP_3 concentrations exists that will result in peak positive inotropy within cardiomyocytes. In cells where IP_3 stimulation causes positive inotropy, increasing the amount of IP_3 used to stimulate the cardiomyocytes should eventually result in negative inotropy instead. Whereas in species where IP_3 stimulation causes negative inotropy, it is possible that stimulation with less IP_3 may cause positive inotropy instead. If not, this could be an indication that the sensitivity of IP_3R gating to IP_3 and Ca^{2+} concentrations is different in these species.

The biological significance of the duty cycle

It is unclear what duty cycle is in a biological sense. It has been shown to affect NFAT, which is a calcium integrator. Duty cycle increases the time NFAT spends in the dephosphorylated state, which is what it needs to both enter the nucleus and affect transcription. NFAT responds to changes in duty cycle while being insensitive to both amplitude and frequency changes. However, IP_3 stimulation, which has been implicated in the NFAT pathway, has been shown to lead to positive inotropy in cardiac cells but no significant change in duration. Based on the duty cycle equation, the overall effect on NFAT activation in this situation is negative. Hannanta-anan and Chow mention an alternative method of calculating the duty cycle involving spike width, \( \Delta \), here, \( \gamma = \frac{\Delta}{T} \). This definition makes more sense physically, and in the context of previous cardiac experiments. NFAT sensitivity to the duration of calcium over a certain threshold in each period of oscillation better explains both the biological mechanism and the presence of positive inotropy in the hypertrophic pathway. What effect any prolongation of the calcium transient would have on cell contraction and how this would contribute to EADs remains to be seen.

Experimental evidence of an increase in duty cycle?

An increase in duty cycle without an increase in frequency requires an increase in transient duration. While we see this increase in our simulations, it has not been reported in experiments involving IP_3 stimulation. The possible reasons for this are many and varied, however, as discussed earlier, small but significant variation in Ca^{2+} concentrations may be easily missed by many calcium sensitive dyes [53]. Hence it remains to be determined whether IP_3R-dependent Ca^{2+} flux leads to an increased Ca^{2+} duty cycle in cardiomyocytes.

Limitations of the model

In this work we have modelled only voltage driven calcium transients with deterministic gating models of each channel. As such there are no stochastic events such as sparks or puffs in this model. Instead of sparks, our deterministic model can only show a small Ca^{2+} leak. This means that we do not see any of the stochastic features of calcium transients. Regardless, the overall behaviour is representative of the average transient in a cardiomyocyte. While cell structure is known to play a role in cardiac calcium dynamics [20, 39], effects beyond the synchronising function of the dyad are beyond the scope of this model. As a compartmental model, we have ignored the spatial effects of IP_3R distribution in its formulation and assumed a uniform distribution over the SR. In the absence of conclusive data, we base this model primarily on data from rat ventricular myocytes and make no distinction between IP_3R channels located within or outside the dyad [32, 33]. These and/or other structural features of the cell would alter the [Ca^{2+}] visible to IP_3R channels and may be visible in the calcium transient.

Distinct effects of IP_3 signalling in the nucleus and cytosol are not considered in this model. Cytosolic Ca^{2+} is thought to drive NFAT into the nucleus while nuclear Ca^{2+} holds it there. Hence we have only investigated a small part of the calcium signalling within the CnA/NFAT pathway. Additionally, not all components of this pathway have been included in this study. Indeed Ca^{2+}/calmodulin-dependent kinases II and Class IIa histone deacetylases are both known
Ca\(^{2+}\)-mediated components of the hypertrophic pathway not included in this model. While we have entirely glossed over the role of calcineurin in NFAT activation. As calcium activates NFAT via calcineurin [4], we have treated them here as one combined calcium sensing entity. In this study we have investigated only the feasibility of IP\(_3\)R-mediated changes to the calcium signal increasing the transcriptional activity of NFAT.

**Conclusion**

The sensitivity of NFAT translocation to the calcium duty cycle demonstrated by Hamanta-anan and Chow [24] raises the question as to whether IP\(_3\)R flux can increase the calcium duty cycle in cardiomyocytes. Here we have shown using mathematical modelling that an increase in cytosolic calcium transient duration may occur following addition of IP\(_3\). Together, these results suggest a plausible mechanism for hypertrophic signalling via IP\(_3\)R activation in cardiomyocytes. While it cannot be ruled out that there are other components of this pathway that are not considered here, the computational evidence provided in this study along with the previous experimental findings suggests encoding of the hypertrophic signal through variation of the cytosolic duration of calcium oscillations to be a feasible mechanism for IP\(_3\) dependent hypertrophic signalling.

**Author Contributions**

EJC, VR, HLR and CS conceived of the study; EJC and VR supervised the project; HH, AT, VR and EJC developed the concepts and modelling approach. HH implemented the simulations. HLR and CS provided experimental data and critical feedback. All authors contributed to the writing the manuscript.

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