The Slow Force Response to Stretch: Controversy and Contradictions

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Abstract

When exposed to an abrupt stretch, cardiac muscle exhibits biphasic active force enhancement. The initial, instantaneous, force enhancement is well explained by the Frank-Starling mechanism. However, the cellular mechanisms associated with the second, slower, phase remains contentious. This review explores hypotheses regarding this 'slow force response' with the intention of clarifying some apparent contradictions in the literature. This review is partitioned into three sections. The first section considers pathways that modify the intracellular calcium handling to address the role of the sarcoplasmic reticulum in the mechanism underlying the slow force response. The second section focuses on extracellular calcium flux and explores the identity and contribution of the stretch-activated, non-specific, cation channel as well as signalling cascades associated with G-protein coupled receptors. The final section briefly introduces promising candidates for the mechanosensor responsible for detecting the stretch perturbation.

Glossary

ADAM12	A disintegrin and metalloprotease 12
Ang II	Angiotensin II
ATR	Angiotensin receptor
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
EGFR	Epidermal growth factor receptor
$ERK_{1/2}$	Extracellular signal-regulated kinase
ET	Endothelin
ETR	Endothelin receptor
FAK	Focal adhesion kinase
GPCR	G-protein coupled receptor
HB-EGF	Heparin-binding-EGF
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MEK	MAPK kinase
mK_{ATP}	Mitochondrial potassium ATP channel
MLCK	Myosin light chain kinase
MP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NHE_1	Sodium-hydrogen exchanger
NO	Nitric oxide
NOS	Nitric oxide synthase
PI3K	Phosphatidylinositol-3-OH kinase
PKA	Protein kinase A
PKG	Protein kinase G
PLC	Phospholipase C
PLN	Phospholamban
RIRR	ROS-induced ROS release
NCX	Sodium calcium exchanger
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SAC_{NSC}	Stretch-activated, non-specific, cation channel
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SFR	Slow force response
SNAP	S-Nitroso-N-Acetyl-D,L-Penicillamine
SR	Sarcoplasmic reticulum
TRPC	Transient receptor potential canonical
TRPV	Transient receptor potential vanilloid

Introduction

Cardiac muscle is mechanosensitive. When exposed to an abrupt stretch, it immediately exhibits active force enhancement. This rapid response is mediated by the well-described Frank-Starling Mechanism (33): greater muscle length increases calcium sensitivity and myofilament overlap. Should this stretch be maintained, there is a slower secondary force increase referred to as the 'slow force response' (SFR). This secondary response was first observed in papillary muscle (112), and has subsequently been observed in atrial trabecula (75), single myocyte (20), and whole-heart (92) preparations. Such observations have been made in the absence of humoral and neural control (92) suggesting that the response is an intrinsic regulatory mechanism of cardiac myocytes. It is known that this secondary behaviour is independent of the Frank-Starling mechanism (133); rather, it is thought to be the adaptive phase of the Anrep Effect (164). The SFR occurs in response to a gradual augmentation of calcium transient magnitude (3, 58) during which there is no apparent increase in diastolic calcium (5, 58, 85), even when the calcium handling of the sarcoplasmic reticulum (SR) is compromised (69).

Calcium transients are the basis of force generation of cardiac muscle. Every heartbeat occurs as a consequence of a synchronised increase of cytosolic calcium via the process of excitation-contraction coupling. Depolarisation of cardiomyocyte sarcolemma during an action potential causes an extracellular calcium influx through the L-type calcium channel (referred to as the 'calcium current') (13). This process induces further calcium release from the SR through ryanodine receptors (RyR). Calcium-myofilament interaction enables the contractile elements of cardiac muscle to produce force. Unlike skeletal muscle, where a greater number of motor units are recruited when contracting against a greater load (102), every cardiomyocyte is activated every beat and so cardiac muscle must rely on alternative endogenous mechanisms for modulating contractile force. Modification of calcium handling, whether it be myofilament calcium sensitivity or calcium transient magnitude, is critical to the mechanisms underlying the transient force-changes associated with phenomena such as the SFR.

Due to its link with cardiac hypertrophy (38, 41), the SFR is clinically relevant. Yet despite several decades of investigation the mechanism(s) underlying the SFR remains contentious. This review aims to clarify apparent contradictions while exploring evidence presented for each hypothesised cellular pathway that drives the SFR. The final section of this review briefly introduces possible mechanosensors responsible for detecting the stretch perturbation – a particularly understudied area in the SFR literature.

Stretch-Activated Signalling Pathways

Since the calcium transient magnitude increases throughout the SFR (69), most studies have focussed on components of signalling pathways known to modulate calcium flux from two distinct sources: the extracellular space and the SR.

Extracellular Calcium Influx

The L-type calcium channel, being the main channel responsible for transporting extracellular calcium into the cytosol, was an initial candidate for mechanosensitive augmentation of the calcium transient. While L-type calcium may have some role in mechanosensitivity in the case of osmotic-swelling (101), its activity is neither sensitive to axial stretch (11, 58, 134), nor required for eliciting a SFR (153, 166). Instead, two alternative membrane bound transport proteins involved with extracellular calcium flux have been linked with the SFR: the stretch-activated, non-specific, cation channel and the reverse-mode sodium calcium exchanger (NCX).

Stretch-Activated Channels

Stretch-activated, non-specific, cation channels (SAC_{NSC}) would appear to be a logical constituent of the SFR mechanism as these sarcolemmal channels open in response to mechanical stretch (for reviews see: Bustamante *et al.* (19), Peyronnet *et al.* (118), and Sachs *et al.* (129)). Their involvement within the SFR appears likely in guinea pig (11) and mouse (30, 171) tissue. However, they almost certainly do not contribute to the SFR in human (74, 75, 166) or rabbit (167) tissues and in rat tissues, SAC_{NSC} contribution is controversial.

Mechanistic Studies

The currents associated with SAC_{NSC} are blocked by the pharmacological agents gadolinium (Gd^{3+}) (184) and streptomycin (11, 108). Use of these agents can either prevent (20, 171) or have no effect on (74, 84, 117, 153) the SFR. These directly conflicting findings are due to limitations with a number of these experiments. For example, one of the papers that claimed Gd^{3+} has no effect on the SFR quantified the magnitude of the response using the calcium spark rate (117). Such a metric is inappropriate for quantifying the SFR given the focus on SAC_{NSC}, as the channels would affect transarcolemmal calcium entry, not SR sensitivity. For a case where SAC_{NSC} blockade abrogates the SFR (171), the concentration of streptomycin used would have wider, non-specific, inhibitory action (9).

A further complication arises when the blockade inflicted by these pharmacological agents is shown to be heavily dose-dependent (193), thus making comparison between investigations difficult. In fact, both Gd^{3+} and streptomycin themselves have significant limitations. Gd^{3+} was initially thought to specifically block SAC_{NSC} (184) but it has since been found to be inhibitory to both the L-type calcium channel (82) and the NCX (191) at dosages typically used for inhibiting SAC_{NSC}. Similarly, streptomycin interacts antagonistically with the L-type calcium channel and so, at higher doses, hinders muscle shortening and reduces the calcium transient (9, 10). The non-specificity and dose-dependent action of these two agents on SAC_{NSC} have been overcome with the use of a much more specific peptide isolated from tarantula venom, GsMTx-4 (148). Its use in murine (30, 171), but not in human (74, 75), tissues abrogates the SFR.

While the experimental investigations regarding SAC_{NSC} involvement are burdened with controversy, the results from mathematical modelling investigations appear to be in better agreement. Models consistently predict that SAC_{NSC} are an integral component of the SFR (106, 154, 187), where their sodium permeability, and not calcium permeability, drives the increase of calcium transient amplitude via enhancement of the calcium flux through the reverse-mode activity of NCX (155, 187).

SAC_{NSC} Identification

There are a number of candidates that have been considered as the channel referred to the SAC_{NSC}, but the true molecular identity remains elusive. Many of them have come from the transient receptor potential canonical (TRPC) family of channels (for reviews see: Fliniaux *et al.* (42) and Inoue *et al.* (60)). TRPC are non-selective cation channels that can be activated by mechanical strain and phospholipase C (PLC) activity. In support of their candidacy, these channels are also specifically inhibited by GsMTx-4 (145).

Confocal imaging has indicated that TRPC1, TRPC3, and TRPC6 channels are abundant within ventricular tissue (48, 171). TRPC3 is colocalised with NCX and Na^+/K^+-ATPase but not with the SR (48) – a finding that was clarified by a computational model of the SFR by Yamaguchi *et al.* (181). Labelling of TRPC1 and TRPC6 revealed that both channels were distributed in a striated pattern within the myocytes rather than on the surface sarcolemma, strengthening the hypothesis that they are located in the t-tubules (75, 171). As a result, confirmation of the involvement of these channels via patch clamping would be difficult. Given that no members of the TRPC family are located on the surface sarcolemma, it seems unlikely that they would be activated by longitudinal stretch, as is the case in the SFR.

Regardless of whether or not a TRPC channel is the SAC_{NSC}, inhibiting TRPC channels directly with BTP-2 (181) or indirectly with PKG activators (136) (since cGMP inhibits TRPC (72, 76)) prevents the SFR. On that note, although the TRPC channels are activated by stretch, it may not be their main physiological means of activation given that TRPC3 and TRPC6 are also activated by G-protein/PLC activity (57, 181) and inhibition of PLC blocks the SFR (181). Animal models lacking TRPC6 express a substantially blunted SFR but those lacking TRPC 3 present conflicting results (136, 181).

As for how the pathway in which TRPC channels could contribute to the SFR, Rosker *et al.* (125) found that NCX interacts with the cytosolic C terminus of TRPC3. The resultant Na^+ influx through the channel drives reverse-mode activity of NCX – augmenting cytosolic calcium (125), in keeping with modelling predictions (106, 154, 187).

In addition to the TRPC family of mechanically sensitive channels, a member of the transient receptor potential vanilloid (TRPV) family is another possible candidate. TRPV4 is a channel that has been linked with enhanced calcium transients in response to hypo-osmotic stress (65). Calcium influx associated with TRPV4 channel activation is thought to activate both PI3K and integrins (185). It is unlikely that the stretch perturbation associated with a SFR would trigger such a flux so it is therefore improbable that it is the SAC_{NSC} but its connection to integrin signalling (see 'Mechanosensor' sections) warrants its consideration.

More work is required to elucidate the extent of SAC_{NSC} involvement within the SFR mechanism. While all mouse studies have demonstrated SAC_{NSC} involvement, evidence for other species is less convincing. There is also a need to identify the channel referred to as SAC_{NSC}, since consensus is lacking in the literature.

G-Protein Coupled Receptor Pathway

In addition to the SAC_{NSC} pathway, it has been proposed that mechanical stretch activates G-coupled protein-coupled angiotensin II receptors (ATR), and the resultant signalling cascade enhances the sodium-hydrogen exchanger (NHE_1) activity (Figure 1). As above, the resultant increase of intracellular sodium drives reverse-mode NCX activity and thus amplifies the calcium transient. The activity of NHE_1 also decreases cytosolic H^+ concentration in exchange for increased intracellular $[Na^+]$ (see 'Sodium Hydrogen Exchanger' section). However, pH is maintained when bicarbonate-containing bath solutions are used due to the activity of the Na^+-independent Cl^-/HCO_3^- exchanger (115).

Angiotensin II and Endothelin

Blockade of the G-protein coupled receptors (GPCR) ATR and endothelin receptor (ETR) has been shown to blunt or eliminate the SFR (5, 24, 27, 181). While ETR blockade prevents an inotropic response to exogenous angiotensin II (Ang II) application, ATR blockade does not do the same in the case of exogenous endothelin (ET) application, indicating a directionality of interaction (27).

151 There are two classes of ATR: ATR_1 and ATR_2 (31). Caldiz *et al.* (24) found that specific blockade of
152 ATR_1, but not ATR_2, abrogated the SFR. Even in the absence of Ang II, ATR_1 is activated when a muscle
153 sample is exposed to a length change (198). Additionally, ATR_1 expression is increased in response to
154 sustained stretch (91). Mechanical activation of ATR_1 triggers ET release (6) without the additional intra-
155 cellular effects associated with Ang II. However, preformed Ang II has been found to release in both
156 neonatal (131) and adult (91) cell-culture media in response to sustained stretch. This preformed Ang II is
157 released from ventricular granules rather than fibroblasts (131).

158 As for the specifics of the ETR, blockade of ETR_A but not ETR_B blunts the SFR (6, 182). Investigations
159 have also focused on elucidating the specific ET isoform that activates ETR in the SFR. Ennis *et al.* (39)
160 measured the level of mRNA expression in response to stretch and found that, while ET_1 and ET_2 mRNA
161 levels were unaffected, the mRNA expression of ET_3 was significantly upregulated. Others have
162 corroborated the lack of stretch-induced ET_1 release (131, 172). However, exogenous Ang II application
163 (163) and stretch (62, 182) have also been found to upregulate ET_1, the corresponding mRNA expression,
164 and a number of ET_1 precursors (29). Hence, assuming that ET is involved, further studies are required to
165 elucidate the specific isoform that is integral to the SFR signalling pathway.

166 ETR and ATR activation are not without controversy or apparent species differences. ATR and ETR
167 blockade has no effect on the SFR in rabbit (167), human ventricular tissue (75, 166), nor rat tissue (140).
168 The SFR in ferret tissue was also found to be immune to AT blockade yet significant SFR blunting
169 occurred in the presence of an ET blocker (22). Though the ventricular tissue of human myocardium is
170 independent of Ang II/ET, the opposite is true within the atrial tissue, thereby demonstrating not only
171 species difference but also an intra-species difference between chambers (75).

172 **Prostaglandins**

173 It seems that the release of myocardial autocrine/paracrine factors is a necessary component of the stretch
174 response. Tucci *et al.* (160) found that the SFR was attenuated if blood pumped out of a whole-heart was

filtered using a haemodialyser and recirculated. They suggested that filtering removed secreted ET or Ang II (160). Yet, using liquid chromatography-mass spectrometry, Ward *et al.* (172) measured no change in Ang II or ET concentration in the coronary effluent collected for the first minute of the stretch. This could be a consequence of the time scales used, as other studies (91, 131) have shown that Ang II concentration increases only after ten minutes of sustained stretch. From observation, the force enhancement associated with the SFR begins within a few twitches post-stretch suggesting that if Ang II is involved, it is not the sole agonist. Despite the time difference, Ward *et al.* (172) observed a 'slow force enhancement' in a second, unstretched, trabecula superfused with the collected coronary effluent. The contributing agents, as determined by liquid chromatography-mass spectrometry, were prostaglandins (PGF2α and PGE2), a finding that has since been affirmed, as blockade of NHE_1 prevents prostaglandin-induced inotropy (141). Similarly, indomethacin, a preventer of prostaglandin synthesis, reduces the magnitude of stretch-induced SFR (141).

Mineralocorticoid Receptor

ATR_1 activation is known to stimulate aldosterone production (142). Although contentious (50), stretch-induced endogenous aldosterone production has been observed in cardiac tissue (152). Since aldosterone is a mineralocorticoid, it activates the mineralocorticoid receptor, which is linked with mitogen activated protein kinases – signalling proteins also linked with the SFR (54, 90) (see the Mitogen Activated Protein Kinases section). Work in this area is limited, but a study by Caldiz *et al.* (23) found that blockade of the mineralocorticoid receptor prevented the SFR, suggesting an involvement of mineralocorticoids in its genesis.

Epidermal Growth Factor Receptor

A downstream effect of ATR_1 and ETR_A activation is the activation of epidermal growth factor receptor (EGFR) (6, 7) following interactions with tyrosine kinases and matrix metalloproteinase (MP) (174). Thus, investigations have examined the role that EGFR transactivation plays in the SFR. EGFR

phosphorylation measurably increases in response to stretch (6), and in those cases where EGFR or the signalling pathway components immediately upstream or downstream are inhibited (17, 23, 163), the SFR is blunted. The SFR (163) and increased EGFR phosphorylation (150) are prevented when the Src tyrosine kinase is blocked. This enzyme, activated by GPCR, is thought to activate MP and results in the extracellular proteolytic cleavage of pro-heparin-binding EGF to form heparin-binding EGF (HB-EGF), an activator of EGFR. EGFR activation in response to GPCR is prevented by compromising the MP, a disintegrin and metalloprotease 12 (ADAM12), but not in response to HB-EGF stimulation (7). This finding indicates that HB-EGF release is responsible for EGFR transactivation and that ADAM12 is the specific MP involved in this pathway. In support of this finding, MP inhibition can prevent HB-EGF secretion in response to strain (6). Nevertheless, most of these studies induce a 'pseudo'-SFR using pharmacological interventions and, as such, further investigations in this area should focus on strain-induced responses.

NADPH Oxidase-Derived Reactive Oxygen Species

EGFR (79, 163) and GPCR (29, 107, 132, 137) agonists increase nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) production (23, 32, 189, 190, 194). Compromising EGFR or its mediators suppresses Ang II/ET-1 induced ROS formation (17, 163) and, more relevantly, ROS production increases in response to stretch (61, 121, 123). Prosser *et al.* (123) demonstrated that this stretch-induced upregulation of NADPH oxidase activity was likely due, at least in part, to mechanical transduction of stretch via microtubules as well as the aforementioned GPCR pathway. Such a finding is in keeping with data from previous work (6) where ROS production was also observed upstream of ET agonism.

Agonism of EGFR via the GPCR pathway results in increased activity of the GTPase, Rac (2), leading to NADPH oxidase activation (137, 149, 174). Each of the constituent sub-units of NADPH oxidase has been observed within ventricular myocytes (177). When activated, NADPH oxidase produces ROS. Since ROS is an unstable free radical (8), a high concentration of ROS has deleterious effects on cell viability

and is linked to apoptosis (195), but at low concentrations it is thought to act as a signalling molecule (123). ROS is linked to modification of RyR sensitivity (123, 195), augmenting mitogen activated protein kinase activity (132, 173), as well as EGFR and ETR activation (6, 47, 49). Downstream effectors of ROS appear to underpin the SFR as the use of ROS scavengers blocks the SFR (23, 24, 29, 32, 132). Even so, the inotropic effects of ROS are controversial (132, 173).

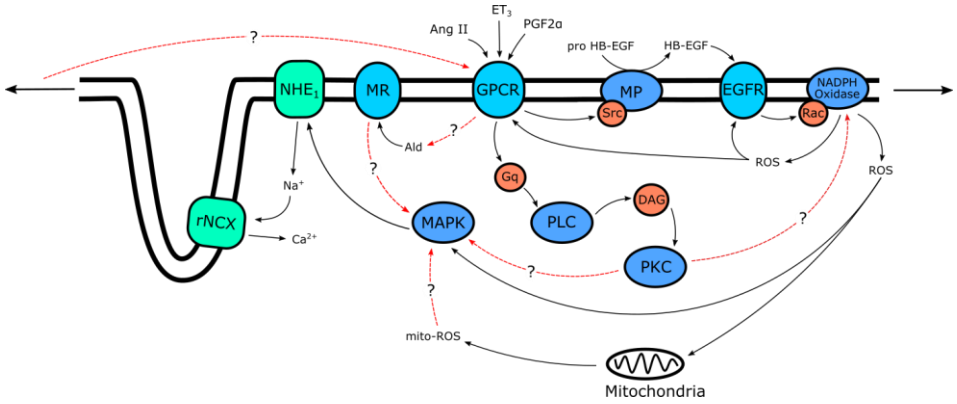

Figure 1: Putative intracellular SFR signalling pathway. Mechanical stretch (as indicated by the peripheral arrows) directly activates GPCR as well as causing the release of the GPCR agonists: Ang II, ET_3, and PGF2α. Activation of this receptor triggers multiple signalling pathways that culminate in the activation of MAPK. The downstream result of this cascade is the augmentation of NHE_1 activity and the resultant increased $[Na^+]_i$ causes NCX to operate in reverse-mode (indicated as rNCX), increasing the calcium transient. Red-dashed arrows with a question mark indicate pathway uncertainty.

Mitochondria-Derived ROS

ROS is also produced as a consequence of inefficiencies in the electron transport chain within mitochondria, with complexes I and III being the largest generators (146). Inhibition of respiratory complex I or mitochondrial potassium ATP channels (mK_{ATP}) prevents GPCR-dependent ROS production, suggesting mitochondrial dependency (23, 32, 163). MK_{ATP} are channels located on the mitochondrial membrane that, when open, enable movement of potassium into the mitochondria (99). The act of opening these channels is known to increase ROS production (43, 71, 99, 109, 110), although the precise mechanism remains unclear. Krenz *et al.* (78) linked it specifically to the movement of potassium

into the mitochondria but the connection between this and increased ROS production remains enigmatic. Such a flux of potassium would depolarise the mitochondrial membrane, which should decrease ROS production due to the well-established positive correlation between membrane potential magnitude and ROS production (52, 119). However, it appears that the extent of depolarisation is negligible and that the accompanying fluid movement into the mitochondria matrix may slow respiration, as a consequence of inorganic phosphate depletion, leading to increased ROS production from complex III (77, 99, 109). ROS can open mK_{ATP} channels via modification of sulfhydryl groups on the channel protein (188). Caldiz *et al.* (24) hypothesised that ROS induces opening of mK_{ATP} channels and the resultant augmentation of ROS production is a necessary step in the SFR, given that a blockade of mK_{ATP} abrogates the SFR (32). Caldiz *et al.* (24) referred to this as 'ROS-induced ROS release' (RIRR) which may represent a mechanism that contributes to the SFR (Figure 2). During RIRR, ROS build-up within the mitochondria increases the probability of mitochondrial permeability transition pore (mPTP) expression (195). The sudden depolarisation associated with mPTP formation is accompanied by a burst of ROS production (for reviews, see: Zorov *et al.* (196, 197)). When mPTP formation is prevented, intra-mitochondrial ROS accumulates and the SFR is blunted (163). In contrast to this finding, others have reported that mitochondrial ROS accumulation following stretch is negligible (61, 123, 172) and that it does not interact with MAPK (70, 177). This conundrum could be resolved by determining whether mitochondrial ROS acts permissively, (i.e. a small amount is required for a step in the SFR mechanism to occur) or if there is a feedback mechanism between cytosolic and mitochondrial ROS to ascertain why mK_{ATP} or mPTP blockade effects the SFR.

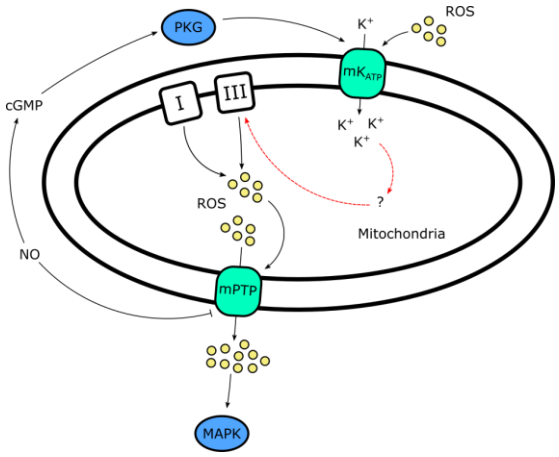

Figure 2: Mitochondrial ROS-induced ROS release. ROS, produced as a consequence of GPCR activation (described in Figure 1), and PKG activity increase mK_{ATP} channel activity. The intramitochondrial movement of potassium ions through this channel causes increased ROS production by respiratory complexes I and III. Accumulation of ROS within the mitochondria induces mPTP are induced allowing the ROS to be released. NO prevents the formation of mPTP by reacting with the thiols. The released ROS is thought to trigger MAPK.

Nitric oxide (NO) can undergo S-nitrosylation with mPTP thiols, preventing their formation and thus slowing RIRR (195). Perhaps NO acts as a negative control to regulate amplification of the ROS signal, as RIRR has been linked with deleterious levels of ROS production and apoptosis (196). Nevertheless, NO-dependent stimulation of cGMP, and therefore PKG, results in an mK_{ATP}-dependent production of ROS (109, 180).

As well as mK_{ATP} activation and RIRR, stretch can directly modify the mitochondrial membrane potential and thus, ROS production. Iribe *et al.* (61) found that mitochondrial membrane potential hyperpolarises in response to muscle stretch independently of NADPH oxidase activity. Hyperpolarisation promotes electron leakage from respiratory complexes I and III, resulting in greater ROS production (109, 169). In direct contrast, Liao *et al.* (95) observed membrane depolarisation when applying a length-change of greater magnitude. Similarly, exogenous application of Ang II and ROS also depolarise the mitochondrial

membrane (70). Such contradictions highlight the need for measuring mitochondrial membrane potential throughout a SFR.

Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPK) belong to the large family of serine/threonine kinases that participate in phosphorylation cascades typically involved in the promotion of various aspects of cell development (e.g. division, differentiation, and apoptosis) (35). The extracellular signal-regulated kinases ($ERK_{1/2}$), p38, and c-Jun N-terminal kinases (JNK) are involved in the MAPK cascades (24, 28, 116, 127, 161), but only $ERK_{1/2}$ has been linked specifically with the SFR (24).

Ras(86), a GTPase that activates the $ERK_{1/2}$ pathway, can be activated by strain-induced-ROS-dependent S-glutathionylation, where a glutathione group is added to its cysteine residue, at cys_{118} (100, 120, 161, 177). However, when the expression of the dominant negative form of Ras is increased, the activation of $ERK_{1/2}$ is prevented (120). It is also observed that when MAPK kinase (MEK), one of the downstream targets of Ras activity, is blocked, the effect of exogenous ROS on $ERK_{1/2}$ phosphorylation (81, 126, 128, 132, 173) is prevented. Similarly, MEK blockade prevents the positive inotropic effect of GPCR agonism (132, 150). In the stretch-case though, activation of $ERK_{1/2}$ occurs via both Ras-dependent (MEK) and Ras-independent (PI3K-Akt) mechanisms following EGFR transactivation (36) (Figure 3).

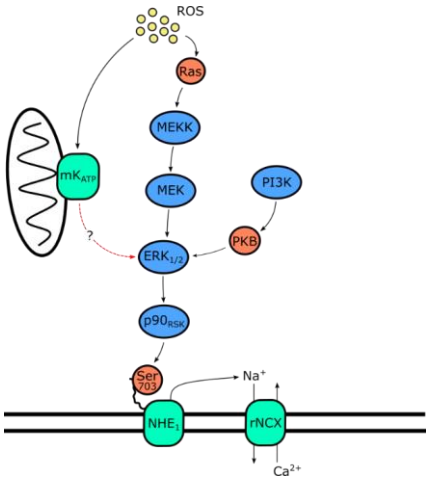

Figure 3: ROS-induced activation of sarcolemmal sodium transporters. The MAPK phosphorylation cascade culminates in the activation of $p90_{RSK}$ which phosphorylates NHE_1 at Ser_{703}. Phosphorylation at this site enables greater Na^+ transport by increasing the proton binding affinity of NHE_1.

Given the apparent importance of EGFR transactivation in the stretch-activation of $ERK_{1/2}$, it follows that inhibition of EGFR or its upstream mediator, Src kinase, significantly blunts stretch-induced $ERK_{1/2}$ phosphorylation (137, 163). Further, exogenous application of GPCR agonists is known to stimulate the action of MAPKs (16, 91, 150) and stretch-activation of $ERK_{1/2}$ is weakened when ATR_1 receptors are compromised (24, 198). Hence, it is likely that the stretch-activation of MAPKs operates, in part, via the GPCR-EGFR pathway outlined in earlier sections.

It was mentioned in the previous section that there is some controversy surrounding mitochondrial ROS-MAPK communication. While blockade of mK_{ATP} channels has been shown to reduce (163, 180) $ERK_{1/2}$ phosphorylation, it has also been reported to have no effect (71). However, given that these investigations studied the effect of mK_{ATP} blockade on $ERK_{1/2}$ phosphorylation during a pharmacological rather than a stretch intervention, little can be inferred regarding mitochondrial ROS-$ERK_{1/2}$ interaction during an actual stretch-induced SFR.

312 $ERK_{1/2}$ phosphorylation also increases cardiac muscle contractility (66) by phosphorylating the myosin
313 light chain kinase (MLCK) (73). As expected, MLCK phosphorylation increases in response to stretch
314 (74); preventing this phosphorylation by inhibiting MLCK blocks the SFR (74). Repeat studies in which
315 calcium transients are measured should be undertaken to affirm this observation.

316 **Protein Kinase C**

317 Activation of ATR_1 is also linked to protein kinase C (PKC) activation via the G-protein-PLC-DAG-PKC
318 pathway (34). As expected, PKC is activated in response to stretch (183). MAPK (16, 111) and NADPH
319 oxidase activation (137) are two downstream targets of PKC activity.

320 While inhibition of PKC prevents alkalinisation (27) and $ERK_{1/2}$ phosphorylation (150) associated with
321 the SFR, PKC inhibition does not appear to prevent the actual force augmentation in response to stretch
322 (75, 192). On the other hand, it does prevent the inotropic effect of aforementioned prostaglandin F2α
323 (141). However, this could be interpreted as the application of prostaglandin F2α activating a different
324 force-enhancing pathway to that activated within the SFR. Neves *et al.* (104) found that while the SFR
325 was still present during PKC inhibition, force-augmentation was not maintained for the same duration.
326 This contradicts previous findings regarding the time-course of PKC activity (137) where it had the
327 greatest effect within the first minute of Ang II induced inotropy.

328 Given the mechanism of PKC activation and its downstream effects, it seems unlikely that it is has no
329 contribution to the SFR. It could be, instead, that its contribution to indirectly phosphorylating NHE_1 is
330 insignificant in comparison to the ROS and EGFR pathways.

331 **Sodium Hydrogen Exchanger**

332 NHE_1 is an active transporter that is heavily involved in pH regulation due to the proton-extruding
333 component of its action (56, 114, 162). Some SFR experiments using bicarbonate-free solutions observed
334 alkalisation in response to stretch, indicating an enhanced NHE_1 activity (5, 27, 97). Alternatively, Shen
335 *et al.* (140) recorded cytosolic acidification, proposing that increased force production is mirrored by an

increased production of protons, and that the upregulation of NHE_1 activity occurs in order to maintain intracellular pH. These discrepant findings regarding pH regulation also conflict temporally as observations of alkalinisation occurred once the maximal SFR force was reached (5, 27, 97), whereas acidification commenced almost immediately post-stretch (140). However, there is general agreement between these studies that the Na^+ influx associated with NHE_1 activity plays a role in the SFR and that $[Na^+]_i$ increases during sustained stretch-interventions (20, 24). In modelling studies, the influx of sodium ions is predicted to occur through SAC_{NSC} rather than via NHE_1 (106) as concluded experimentally.

Blockade of NHE_1 with pharmacological agents (24, 75, 140, 141, 165, 186) or silencing NHE_1 gene expression (116) blunts the SFR substantially in a number of species. However, NHE_1 involvement is not ubiquitous, as its blockade was inconsequential in studies using both human atria (74, 75) and murine tissue (171). A modelling study (106) also predicted that NHE_1 activation is not required for the SFR to occur. It is likely that discrepancies regarding the contribution of this channel can be attributed to species difference given that the lack of a SFR in rainbow trout myocardium is attributed to a less robust NHE_1 than are found in its mammalian equivalents (113).

Takahashi *et al.* (151) first demonstrated that, in the absence of the amino acid Ser703 (144) on its regulatory carboxyl tail, NHE_1 no longer gains enhanced binding affinity for H^+ in response to serum application. The same group subsequently found that the phosphorylation of Ser703 results in the formation of a binding site for 14-3-3 proteins (88). The binding of these proteins increases the binding affinity of NHE_1 for protons and thus increases its activity (88). Vargas *et al.* (161) took advantage of 14-3-3 protein-NHE_1 interactions to estimate the extent of Ser703 phosphorylation with a phosphor-Ser-14-3-3 binding antibody and found that phosphorylated Ser703 increased substantially after a sustained period of stretch (23, 161, 163).

One of the kinases that phosphorylates Ser703, p90 ribosomal s6 kinase, is a downstream target of the $ERK_{1/2}$ pathway (126, 130, 132, 150, 151). As expected, blockade of upstream mediators of $ERK_{1/2}$

activation prevents NHE_1 phosphorylation (126, 132, 163, 173). However, blockade of an alternate MAPK, p38, has no effect (126, 132, 173).

Reverse-mode NCX

Normally, NCX operates in its forward mode to extrude calcium during twitch relaxation by exchanging three extracellular sodium ions for one intracellular calcium ion, thereby aiding relaxation (14). An increase of intracellular sodium, as would be the case with a greater NHE_1 activity, is thought to reverse the activity of the NCX (14). When operating in its reverse-mode, NCX contributes to the influx of calcium – augmenting the calcium transient. Modelling studies have consistently predicted that the sodium-driven reverse mode operation is the mechanism to explain augmentation of the calcium transient (154, 187). Empirically, the increased action potential duration associated with the SFR is thought to favour rNCX activity (11, 194).

Replacement of sodium ions with lithium ions in the bathing solution has enabled investigators to isolate NCX within sodium-coupled contractions, since other sodium transporters remain unaffected (44). Under these conditions, the SFR is completely abolished in cat papillary muscles (115). However, sodium-lithium replacement is limited in that it disables the NCX in both forward and reverse-modes. Thus, there are two possibilities for its involvement: either the forward mode is slowed, enabling greater loading of the SR, thereby resulting in increased magnitude of calcium-transients, or reverse-mode augments calcium influx. SFR magnitude reduction in response to selective inhibition of the reverse-mode configuration suggests the latter (115).

The use of pharmacological agents enables specific blockade of the reverse-mode. Blocking reverse-mode NCX activity almost completely silences the SFR in cat papillary muscles (115), but only blunts the response in rabbit (97, 167) and human ventricle (75, 166). To add to the confusion, reverse-mode NCX blockade in rabbits has also been found to silence the response (104). In the extreme case, inhibition of reverse-mode NCX has no effect whatsoever on the SFR in human atrial tissue (75). As the concentration

and the agent used are consistent between these studies, discrepancies cannot be attributed to dose-dependency of the pharmacological agent. The studies in which reverse-mode NCX blockade prevented the SFR used a lower [Ca^{2+}] in their superfusing solution compared to those where channel blockade had only blunting effects. It should be noted that intracellular calcium also increases if NCX is slowed rather than reversed. Therefore, blockers of the reverse mode configuration may not eliminate the SFR.

Intracellular Calcium Flux

As well as extracellular calcium influx, calcium transient magnitude can be augmented as a consequence of increased SR calcium release. The calcium content of the SR is dependent on SERCA calcium uptake and RyR channel calcium release. Many signalling pathways interact with SERCA and RyR but SFR literature has identified cyclic adenosine monophosphate (cAMP) and NO as the most likely to modulate the activity of these channels within the response.

Cyclic Adenosine Monophosphate

Muscle stretch increases cAMP levels in frog ventricle (143) and ferret papillary muscles (21), but not in rat atrial tissues (156). Given that both the ferret and rat preparations exhibited SFRs, it is possible that this was due to a combined effect of a difference between both species and heart chambers.

In the transient sense, cAMP concentration increases during the SFR, mirroring the active force augmentation (158). Use of a cAMP-antagonist (Rp-8-Br-cAMPS) also reduces the SFR magnitude (21), though the precise mechanism remains enigmatic. A hypothesised pathway is the cAMP-dependent modulation of SERCA activity; under basal conditions, phospholamban (PLN) inhibits SERCA activity (46) and the cAMP-dependent protein kinase A (PKA) (170) phosphorylates the Ser_{16} and Thr_{17} residues of PLN, reducing this inhibition (46), thereby enhancing the calcium loading of the SR. However, PLN is not phosphorylated during the SFR indicating that, if cAMP concentration increases, it does so in a compartment inaccessible to the SR (21). Mice with a transgenic human $NOX2^{i}$ sub-unit did present partial PLN phosphorylation (189), though, excluding Ser_{16}, no other known targets of PKA were

phosphorylated. PLN phosphorylation is observed only in studies utilising inotropic agents, not when stretch is the inotropic trigger. Therefore, if cAMP is involved in the SFR, it is unlikely that the mechanism of action of cAMP within the response involves modification of the SR calcium content.

In fact, cAMP may have an inhibitory effect on the SFR. Caffeine and theophylline (26), both phosphodiesterase inhibitors (157), reverse the SFR, when they would be expected to prevent the breakdown of cAMP. Chuck and Parmley (26) expected greater reversal when using theophylline, as it is a more potent phosphodiesterase inhibitor than caffeine, but the same reversal magnitude was observed. This led them to suggest cAMP independence, as the secondary effects of caffeine and theophylline are equivalent. However, samples treated with isoprenaline, a beta-adrenergic agonist, which also increases intracellular cAMP, exhibit reversed SFRs (68). Isoprenaline has also been shown to block the SFR while beta-blockers have no effect (92, 158). As such, it could be that a threshold cAMP level is required for this reversal to be expressed rather than the dose-dependent reversal originally expected(26). Hence, it appears that the mechanism, while not cAMP-dependent, involves cAMP in an inhibitory fashion.

Nitric Oxide

In addition to cAMP, NO also interacts with the SR. NO increases the fractional calcium release of the SR (64) through the process of S-nitrosylation (147, 179) where a NO molecule attaches to a RyR cysteine residue, which undergoes a conformational change. NO is generated by the enzyme nitric oxide synthase (NOS) which has three isoforms: endothelial NOS, inducible NOS, and neuronal NOS (178). Should RyR S-nitrosylation be a step within the SFR, neuronal NOS would be the prime candidate for NO generation given its proximity to the SR (178). However, myocytes isolated from mice lacking this isoform still exhibit a SFR (190). Furthermore, neuronal NOS-derived-NO is associated with attenuating inotropy by inhibiting L-type calcium channels (135) and augmenting lusitropy by promoting SERCA activity (18) – neither of which supports enhanced calcium transients. While NO produced by neuronal NOS has been linked to increased calcium spark rate (117), this is more likely to promote arrhythmia than a SFR (64).

Given the dissociation of neuronal NOS from the SFR, the endothelial NOS isoform was the next target to be investigated as it has been localised to small invaginations in the cardiomyocyte plasma membrane (caveolae) that are involved in signal transduction (40). Indeed, phosphorylation of endothelial NOS has been observed following a stretch (117). In support of the involvement of endothelial NOS in SFR, mice lacking the isoform no longer exhibit an augmented calcium transient after a sustained stretch (117).

A pharmacological NOS inhibitor, L-NAME, has been used in the investigation of the SFR (20, 75, 117), but the findings are inconsistent, perhaps as a consequence of various experimental conditions coupled with different L-NAME concentrations and the variety of species used. A 1 mM L-NAME solution abrogates the SFR in both mouse myocytes and rat trabeculae at 30 ^{o}C (117), but has no effect on the SFR at 22 ^{o}C to 25 ^{o}C (20). A study (75) using half the concentration of L-NAME at body temperature also failed to prevent the SFR in human samples. Such discrepancies mean that no firm conclusions regarding NO involvement can be drawn from these studies; it remains unclear as to why the conflicting results occur.

Although NO concentration is enhanced in response to stretch (117, 122), its relation to the SFR is unknown. Since NO is a free radical, it is unstable. This means that it has a very limited range of effect and is unlikely to interact with RyR directly. Hence, any mechanistic effect is more likely to be the result of a NO-initiated signalling pathway (64). NO production within the SFR was initially ascribed to the following pathway: stretch activates phosphatidylinositol-3-OH kinase (PI3K) which phosphorylates protein kinase B, resulting in activation of the endothelial nitric oxide synthase (30, 117). However, subsequent studies of rat and human tissues revealed that PI3K blockade has no effect on the magnitude of the SFR (20, 74, 75). These findings collectively suggest that the PI3K-protein kinase B interaction, although an essential step of the hypertrophy signalling pathway (124), is not involved in the SFR.

An alternate NO pathway begins with the binding of NO to guanylyl cyclase, which increases cyclic guanosine monophosphate (cGMP) and activates protein kinase G (PKG), resulting in RyR phosphorylation (51). This pathway is not involved in the stretch-induced augmentation of RyR

sensitivity, as calcium spark rate is unaffected by the blockade of both PKG and guanylyl cyclase (117). NO-dependent PKG activation initiates a signalling cascade that results in the opening of mK_{ATP} channels, elevating ROS generation (180). The stretch-dependence of this pathway requires further investigation as these data were generated in response to exogenous application of S-Nitroso-N-Acetyl-D, L-Penicillamine (SNAP). SNAP is an NO-donor and therefore causes a global increase of NO concentration. Given the spatial specificity of NO, using SNAP to approximate stretch-induced NO release is counterproductive, as it risks activation of other, irrelevant, pathways.

Given the evidence, it seems unlikely that NO is centrally involved in the SFR. To the authors' knowledge, only one study (117) has presented data where NOS blockade influences the stretch response. Yet, there does seem to be sufficient evidence to warrant further investigation into the endothelial NOS isoform given its link with calcium transient magnitude (64).

Sarcoplasmic Reticulum

In addition to investigations of the signalling pathways that target the calcium handling of the SR, a number of studies have examined whether a functional SR is actually necessary for the SFR (15, 20, 69, 166, 167). Such studies typically reason that if the SR is involved, then the SR load and/or its fractional release of calcium should increase. However, results regarding SR loading during the SFR are controversial. While SR loading has been observed throughout the SFR in rabbit (15, 167) and failing human myocardium (166), it has not been observed in wild-type rat (117) or mouse (64, 189) myocardia. Transgenic mice with human NOX2 do exhibit SR loading during Ang II induced inotropy (189), highlighting the possibility that species may be differentially ROS-dependent.

The SFR magnitude is consistently dependent on the steady-state load of the SR (64, 159, 168, 189). The greatest relative magnitude of SFR^{ii} occurs when the SR is least loaded (140). This is due either to a greater potential for increasing SR calcium content or the enhanced extracellular calcium availability for calcium transient augmentation. Such an observation corroborates the finding that cAMP inhibits the

SFR, as greater cytosolic cAMP concentrations would increase SR calcium content and therefore reduce SFR magnitude.

Of course, an increase of the SR load is not the sole prerequisite for enhanced SR calcium release. This is evident from the amplified fractional calcium release observed during Ang II induced inotropy (64). Yet enhanced SR calcium release would eventually deplete the SR store unless compensated by an increase of calcium loading (37). SERCA phosphorylation does increase during exogenous Ang II application but, this may be a consequence of non-specific pharmacological activation, and may not be exhibited during a stretch-induced response. Another theory for maintaining SR calcium content is that the activity of reverse-mode NCX replenishes the SR (64). This is relevant only if a functional SR is required to elicit an SFR.

One can test the necessity of a functional SR by inhibiting its calcium handling. There are two main elements to the calcium handling of the SR: calcium uptake (through SERCA) and calcium release (through RyR). Inhibition of calcium release alone has no effect on the relative magnitude of the SFR (15, 59, 153). A more complete inhibition of the SR, by blocking both uptake and release, also has no effect of the relative magnitude of the SFR (15, 20, 69). In a failing human tissue study, complete SR blockade abolished the SFR (166) but, as this was a diseased case, discrepancies may have arisen as a consequence of pathology. In most cases, then, it would seem that a functional SR is not required for an SFR to occur.

Mechanosensors

Despite the advances made in elucidating the signalling pathways that augment the calcium transient during the SFR, there is little consensus on the cellular mechanosensor that ‘senses’ or transduces the stretch into an increased calcium transient. Some hypotheses posit an intrinsic mechanosensor (e.g. stretch-activated channels) but most contain no explicit explanatory mechanism describing how the SFR is initiated. This section briefly introduces mechanosensors that have been investigated and/or show promise. A few factors have been considered in compiling this section: samples still demonstrate a SFR

505 when they are transiently stretched only during diastole (4, 105) and, due to the connection between the
506 SFR and the Anrep effect, the mechanosensor is activated by a stretch within the 'physiological' range.
507 That aside, Ward *et al.* (171) found that the SFR magnitude correlates positively with the extent of
508 stretch, and though not empirically defined, a stretch-perturbation of 10% L_{max} is typically used as the
509 minimum length change required.

510 ### Stretch-Sensitive Receptor

511 Zou *et al.* (198) proposed that ATR_1 undergoes a conformational change upon stretch of the membrane
512 thereby enabling activation even in the absence of its agonist Ang II. Certainly, many studies link ATR_1
513 activation with the SFR but little more has been done to corroborate this finding of direct mechanical
514 activation. Hence, we consider that elucidation of how this proposed conformational change occurs would
515 be necessary for its acceptance.

516 ### Microtubules

517 Microtubules are structural proteins that are also linked with G-protein activity (175). Further, and more
518 relevantly, they have been linked with mechanotransduction of stretch signals. Microtubular integrity is
519 required for stretch-induced mitochondrial hyperpolarisation (61, 103) and enhanced NOX2 activity
520 (123). Given the evidence regarding ROS as a signalling molecule in the SFR, the microtubular system,
521 which has recently been shown to be involved in mitochondrial motility and function (139, 176), may be
522 important.

523 ### Caveolae

524 Caveolae are small invaginations in the sarcolemma that have stretch-sensitive properties. Disruption of
525 caveolae prevents stretch-induced dissociation and activation of RhoA and Rac1 G-proteins (67). Despite
526 the fact that these G-proteins are known to be upstream effectors of $ERK_{1/2}$ activity (87), $ERK_{1/2}$
527 phosphorylation is not affected by caveolae disruption (67). Instead, G-protein activation has been linked
528 to stretch-induced alignment of actin fibres. Though $ERK_{1/2}$ phosphorylation is not affected by caveolae

disruption, stretch-induced $ERK_{1/2}$ translocation from the cytoplasm to nucleus does not occur and is deemed to be actin-alignment-dependent (67). The reason why this would elicit translocation is wanting. Such translocation is likely to be more relevant to hypertrophy rather than the SFR.

Thrombospondin-4

Thrombospondin-4 has been linked to regulating collagen mRNA and its absence results in enhanced deposition of extracellular matrix as well as fibrosis (45). Mice lacking this protein lack a SFR (30), have compromised stretch-induced $ERK_{1/2}$ phosphorylation, as well as reduced ability to deal with sudden pressure loads (45). However, a mechano-sensing role for collagen within the SFR has not since been considered.

Titin

The titin molecule is the major contributor to the passive properties of striated muscle (55). Recent evidence has suggested that titin may also operate as a stretch-sensitive modifier of active force generation (93). This suggestion is supported by studies showing a direct correlation between the active magnitude of the SFR and titin strain (1). As an aside, the observation that passive force decreases over the time course of the SFR has recently been attributed to increased titin phosphorylation via the cGMP-PKG activity in response to NO production (89).

Titin kinase (A-band) (53) and N2B/N2A (I-band) (96, 138) are examples of mechano-sensitive regions of titin. Of particular interest, and most likely to be involved in detecting stretch within the SFR, is the N2B element that arises from the interaction between the extensible N2B unique sequence and upstream regulators of $ERK_{1/2}$ activity (138). The particulars of the mechano-sensor remain enigmatic; however, it is likely that mechano-sensitivity is correlated with titin phosphorylation and, hence, titin compliance (80).

Integrin-Signalling

Integrins are transmembrane receptors predominantly involved in membrane adhesion (25). The cytosolic β1 integrin subunit is known to activate in response to stretch and its expression is enhanced by the activity of Ang II (63). Focal adhesion kinase (FAK), a downstream effector of β1, phosphorylates $ERK_{1/2}$ via G-protein dependent and independent pathways (94). However, β1 can also activate MAPK via FAK-independent mechanisms (83). There is cross-talk between AT and FAK, where FAK activation, depending on whether ATR is compromised or not, either augments or diminishes $ERK_{1/2}$ phosphorylation, respectively (83).

β1 integrin also interacts with integrin-linked kinase, a serine/threonine protein kinase localised at costameres and sarcomeric z-disks (12). Stretch is detected by a network consisting of integrin-integrin linked kinase-β-parvin increasing the expression of EGF and, thus, augmenting the calcium transient (12). There are a number of candidates for the molecular mechanosensor (for a comprehensive review see: Lyon *et al.* (98)) but no consensus has been reached yet.

Conclusions

From the work presented in this review there appears to be a general agreement that stretch activates an autocrine/paracrine response that triggers a signalling pathway that includes GPCR. There is also agreement that intracellular $[Na^+]_i$ is increased, and that the final step in the SFR involves reversal of NCX activity leading to the augmentation of intracellular $[Ca^{2+}]$ levels. However, we propose that the intricate details of the signalling pathway remain obfuscated because of the quest for a singular pathway.

The majority of SFR work to date seeks to find a ‘simple’ mechanistic signalling pathway i.e. one of Ang II, SAC_{NSC}, NO, etc. However, we propose that an explanation for the extent of contention within the SFR literature is the existence of a more complex ‘hybrid’ mechanism – which is to say, multiple mechanisms synergistically contributing to the augmentation of calcium during the SFR. It is possible that blockade of one signalling pathway is compensated by others as a form of natural ‘redundancy’, thereby leading to

conflicting data. Of course, such a complex mechanism would be difficult to test but it is necessary if this field of physiological interest is to progress.

Studies that induce positive inotropy via pharmacological agents, in the absence of stretch, are common within the SFR literature. Similarly, there is a heavy reliance on pharmacological agents and blockers in determining the mechanisms associated with the SFR. Very few are specific, and so, inadvertently, they may interrupt pathways other than the one under investigation, increasing the risk of arriving at invalid conclusions. Hence, conclusions from studies that use 'fail-safe' techniques are of more importance. In the same vein, due to the mechano-sensitivity of cardiac tissue, application of external force to change the length may trigger alternative endogenous inotropic mechanisms. To increase confidence that the triggered behaviour is the SFR, calcium should be measured in addition to muscle force production.

While there is still work to be done to determine the underlying mechanism(s) of the SFR, even more questions surround the mechanosensor(s) that detects the stretch-perturbation. This review presents only a small selection of promising mechanosensors in the hopes of guiding future research. Little can be concluded regarding the nature of the SFR mechanosensor, as literature in this area is lacking, but it is one burgeoning with potential.

Commented [AT1]: Wow. It's a hell of a review! Very thorough, and very well written. Dense in parts, mostly due to my lack of familiarity with the terminology.

However, I was hoping that stronger conclusions/recommendations might have been arrived at by the time we got to the final paragraph. Is there a more definite series of statements that can direct the reader as to the best way forward?

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Figure Captions

Figure 4: Putative intracellular SFR signalling pathway. Mechanical stretch (as indicated by the peripheral arrows) directly activates GPCR as well as causing the release of the GPCR agonists: Ang II, ET_3, and PGF2α. Activation of this receptor triggers multiple signalling pathways that culminate in the activation of MAPK. The downstream result of this cascade is the augmentation of NHE_1 activity and the resultant increased $[Na^+]_i$ causes NCX to operate in reverse-mode (indicated as rNCX), increasing the calcium transient. Red-dashed arrows with a question mark indicate pathway uncertainty.

Figure 5: Mitochondrial ROS-induced ROS release. ROS, produced as a consequence of GPCR activation (described in Figure 1), and PKG activity increase mK_{ATP} channel activity. The intramitochondrial movement of potassium ions through this channel causes increased ROS production by respiratory complexes I and III. Accumulation of ROS within the mitochondria induces mPTP are induced allowing the ROS to be released. NO prevents the formation of mPTP by reacting with the thiols. The released ROS is thought to trigger MAPK.

Figure 6: ROS-induced activation of sarcolemmal sodium transporters. The MAPK phosphorylation cascade culminates in the activation of $p90_{RSK}$ which phosphorylates NHE_1 at Ser_{703}. Phosphorylation at this site enables greater Na^+ transport by increasing the proton binding affinity of NHE_1.

Footnotes

[i] NOX2 is a sub-unit of NADPH oxidase

[ii] For a stretch perturbation between ~90 % L_{max} and ~L_{max}.