When activated skeletal muscles are stretched, the force increases significantly. After the stretch, the force decreases and reaches a new steady state, which is higher than the force produced at the corresponding length during purely isometric contractions. This phenomenon, referred to as residual force enhancement, has been observed for more than 50 years, but the mechanism remains elusive, generating considerable debate in the literature. This paper reviews studies performed with single muscle fibres, myofibrils and sarcomeres to investigate the mechanisms of the stretch-induced force enhancement. First, the paper summarizes the characteristics of force enhancement and early hypotheses associated with non-uniformity of sarcomere length. Then, it reviews new evidence suggesting that force enhancement can also be associated with sarcomeric structures. Finally, this paper proposes that force enhancement is caused by: (i) half-sarcomere non-uniformities that will affect the levels of passive forces and overlap between myosin and actin filaments, and (ii) a Ca$^{2+}$-induced stiffness of titin molecules. These mechanisms are compatible with most observations in the literature, and can be tested directly with emerging technologies in the near future.

Keywords: force enhancement; cross-bridge kinetics; titin; sarcomere

1. INTRODUCTION
For many years, it has been known that skeletal muscles which are stretched during activation present a significant increase in force [1–4], while the energy consumption decreases [5,6]. After the stretch, the force decays and reaches a new steady state, which is higher than the force produced at the corresponding length during purely isometric contractions [1,2]. This increase in force has been referred to as ‘residual force enhancement’, and has been the focus of much research after the original studies were published [7–12]. Incredibly enough, the cellular mechanism behind the residual force enhancement is still elusive, and has been the topic of intense debate. This form of force regulation is particularly challenging as it cannot be readily explained by changes in overlap between myosin and actin filaments and the classical force–length relationship [13], and thus must be associated with fundamental properties of muscle contraction.

In recent years, the emergence of new techniques has allowed investigators to evaluate the force enhancement with a high time and spatial resolution in isolated fibres, myofibrils and single sarcomeres, which has brought new insights into the phenomenon, ranging from the basic characteristics to the proposed mechanisms. While some of these studies focused on the residual force enhancement [10,11,14], others aimed to evaluate the molecular aspects of muscle contraction [15–18]. The latter studies still added important data for a better understanding of the mechanism of the force enhancement. This paper reviews these new results in light of earlier studies and proposed hypotheses, and suggests a testable mechanism for the residual force enhancement, which is consistent with most evidence from the literature.

2. CHARACTERISTICS AND NEW STUDIES OF THE STRETCH-INDUCED FORCE ENHANCEMENT
There are many studies on the residual force enhancement, with varying results and interpretations, at times contradictory. Since this review focuses on the mechanisms of force enhancement, it will concentrate only on studies developed with isolated muscle fibres, myofibrils or sarcomeres, in which the average or individual sarcomere length was measured or controlled during the experiments. Studies with whole muscles and human subjects may offer insights into potential physiological roles, but add little information on the mechanisms of force enhancement.

A typical experiment showing residual force enhancement is shown in figure 1a. This experiment was performed with an intact single fibre from the frog in the laboratory of Dr Paul Edman—who has extensively investigated this phenomenon—and summarizes most of the characteristics of the force enhancement. During the stretch of an activated fibre, the force increases significantly. When the stretch is performed at slow velocities ($<2L_0$, s$^{-1}$), the increase in force has two components. There is a sharp increase in force that happens over approximately 9 nm
In past years, myofibrils, which are preparations that allow measurements of individual sarcomeres [28], have been used to investigate the effects of stretch on force production [10,11,14,18]. These studies brought much information to the field of muscle mechanics, but are inconclusive and their interpretations vary largely. The reason for such variability may be the inconsistency of the experimental approaches used in different laboratories, including a lack of repeatable control and isometric contractions for comparisons with stretch contractions (e.g. [14,29]), probably owing to the inherent difficulty of performing mechanical measurements in myofibrils.

There is only one study, as far as this author knows, that has investigated the residual force enhancement in myofibrils in a situation similar to what has been done in single fibres [11]. The authors investigated small segments of myofibrils, and observed force enhancement levels between approximately 10 and 50 per cent, consistent with most studies in the field (force enhancement = steady-state force after stretch/steady-state force during an isometric contraction at the corresponding length). The same study showed that force enhancement is also present in mechanically isolated sarcomeres at levels of approximately 10 per cent [11]—a particularly important finding as it shows that force enhancement cannot be entirely associated with structural arrangements between sarcomeres in series. The traces recorded during the experiments with single sarcomeres are similar to those obtained with intact fibre preparations (figures 1b and 2a). Interestingly and new to the literature, the study also showed a population of sarcomeres in which force enhancement was not present after stretch (figure 2b), an observation with implications for the mechanism of force enhancement, as will be discussed later in this review.

3. MECHANISMS OF FORCE ENHANCEMENT

The proposed mechanisms of force enhancement can be classified into those involving: (i) sarcomere length non-uniformities, or (ii) sarcomeric structures, including (a) myosin cross-bridges and (b) passive elements, most recently associated with titin molecules. These mechanisms are not necessarily mutually exclusive, as length non-uniformities would affect contractile and passive properties of sarcomeres. It will be suggested later in this review that force enhancement results from these mechanisms working in parallel—the reason why several attempts to isolate one mechanism have not been successful.

(a) Sarcomere length non-uniformity

It is well accepted that muscle fibres develop significant sarcomere length non-uniformities upon activation [9–11,28,30–34]. Non-uniformities are associated with several experimental observations: the force creep in contractions produced at long sarcomere lengths [31,35], differences in force obtained when fibres are activated with sarcomere length control or fibre length control [13,31,36], and the kinetics of force development and relaxation [34,37,38]. Historically, sarcomere length non-uniformity has been incorporated into a hypothesis to explain the residual force enhancement [39,40]. This hypothesis has been a topic of much debate in the muscle field, with heated discussions that extended from scholarly
publications to letters/responses to editors of scientific journals [41–43].

According to this hypothesis, when muscle fibres are stretched along the descending limb of the force-length relationship, mechanical instability will lead to differences in the yield tensions of sarcomeres, initiating a process of length non-uniformities. As a result, sarcomeres will stretch with varying lengthening velocities—the weakest sarcomeres will lengthen very rapidly at the expense of sarcomeres that will lengthen slowly. At one point, the weak sarcomeres that are elongating rapidly will become unable to hold the tension; they will lose all filament overlap and will ‘pop’, to be supported by passive forces. The process will be repeated with all weakest sarcomeres, which would also stretch beyond filament overlap to stabilize higher force by means of passive forces.

Although first attempts to characterize the relation between force enhancement and large non-uniformities were not successful [7,12], the most important evidence supporting this hypothesis was obtained in studies in which muscles were frozen after stretch and analysed with electron microscopy [44,45]. These studies showed sarcomere length non-uniformities in several sections of the fibres, and striation patterns that suggest displacements of the thick filaments relative to the centre of the sarcomeres, between successive Z-lines [44,45]. Some sarcomeres and half-sarcomeres were extended to long lengths, which would support the notion of popping sarcomeres. However, close analysis of the pictures in these papers demonstrate that non-uniformities are scattered along small sections of the frozen samples, and most importantly sarcomeres elongated to a point in which they would lose all filament overlap and pop are not evident—the reader is encouraged to look at the pictures and judge.

In recent years, studies with isolated myofibrils and single sarcomeres have cast more doubt into the popping sarcomere hypothesis [10,11,28,34]. These experiments confirmed that stretch in fact leads to the development of sarcomere length non-uniformity, but without sarcomere popping—sarcomeres never stretched to the point where they would lose all filament overlap [18,28]. Furthermore, force enhancement was observed on the ascending limb and above the plateau of the force-length relationship [10], which is inconsistent with the idea of instabilities leading to popping sarcomeres.

In summary, activated muscle fibres and myofibrils develop significant sarcomere-length non-uniformity upon activation, which may increase significantly with an imposed

Figure 2. Experiments performed with one sarcomere isolated from the rabbit psoas muscle. (a,b) Force produced during an isometric contraction (red) and a contraction in which the sarcomere was stretched (black). In the experiment shown in (a) force enhancement was observed and in (b) force enhancement was not observed. (c,d) Sarcomere length changes during the stretches (black) and the isometric (red) phases of the contractions. Note that upon relaxation the sarcomere stretches slightly, returning to the pre-activation length. (e,f) Half-sarcomere length changes during the activation and stretch (black) or during isometric contraction (red). In both cases, the half-sarcomeres change length during the isometric period of contraction.
stretch, but popping sarcomeres have never been observed. Recently, such features and findings were extended to suggest that force enhancement might be explained by half-sarcomere length non-uniformities [11,18].

(i) Half-sarcomere length non-uniformities
Conceptually, non-uniformity in half-sarcomere lengths induced at the beginning of activation would increase throughout contractions, as the thick filaments would be pulled towards the ends of the sarcomeres. Half-sarcomere non-uniformities during isometric contractions have been observed in myofibrils [18,34] and isolated sarcomeres [46] (figure 2). In the case of single sarcomeres, A-band displacements follow a characteristic pattern that resembles the force–length relationship [46].

Can half-sarcomere length non-uniformities lead to a residual force enhancement after stretch? Recently, a study showed little correlation between force enhancement and A-band displacements in single sarcomeres, but a very strong relationship when experiments were conducted with at least three sarcomeres in series [11] (figure 2). Again, ‘popping’ was not observed in half-sarcomeres, as they never moved all the way towards the end of the sarcomeres, staggering adjacent to the Z-lines, a finding in line with previous myofibrils studies that investigated this phenomenon in a shorter time scale [18]. The maximal movement of A-band displacements observed was approximately 50 nm per half-sarcomere, suggesting that some structure is preventing the A-bands from moving towards the ends of sarcomeres. Based on these findings, it seems likely that force enhancement has two components: (i) non-uniformity among half-sarcomeres, and (ii) a sarcomeric component.

Half-sarcomere non-uniformity may lead to a complex behaviour, as displacements of A-bands would result in variable amounts of filament overlap. There would be more cross-bridges interacting with actin and thus more active force production in strong half-sarcomeres. Titin filaments would be overstretched and become stiffer in weaker half-sarcomeres, increasing the sarcomere strain and balancing opposing forces in the strong halves. Such a mechanism has been suggested in a different context by Edman and Tsuchiya [8], but it could explain the length dependence of the force enhancement. Force enhancement increases when measurements are carried out at sarcomere lengths up to approximately 20–30% longer than the plateau of the force–length relation [7,8], a region where A-band displacement is significant [11] and passive forces start playing a role in most skeletal muscles. Simultaneous with the increase in passive strain and increase in filament overlap, A-band displacements would cause cross-bridges to constantly stretch while the half-sarcomeres are not stabilized [11,34,46], which could add to the force enhancement by imposing resistance to the stretch—a phenomenon similar to what happens during muscle fibre stretch.

A recently published computation model strengthens the relation between force enhancement and half-sarcomere length non-uniformity [47]. In the model, the authors show that the development of a small degree of half-sarcomere heterogeneity causes force enhancement at levels close to those observed in most published experiments (approx. 5–13%). The model also predicts that force enhancement is dependent on the stretch magnitude but independent of the stretch velocity, consistent with findings in the literature.

(b) Sarcomeric structures
Since: (a) force enhancement is observed in sarcomeres that do not show A-band displacements after stretch and in situations where variability of sarcomere lengths is small [10,11], and (b) the development of half-sarcomere non-uniformities (when present) induces changes in the properties of sarcomeric structures, there must be a mechanism of force enhancement that is contained within the half-sarcomere. The mechanism must be associated with: (a) cross-bridge kinetics, and/or (b) passive elements, more specifically titin molecules.

(i) Myosin cross-bridges
During the stretch of muscle fibres, there is an increase in fibre stiffness, a putative measurement of the number of cross-bridges attached to actin. The levels of increase in stiffness vary between 10 and 60 per cent [19,20,48,49], raising controversy over the actual involvement of cross-bridges. Some authors suggest that the increase in force is caused by an increased mean force produced by the cross-bridges [50,51] and a redistribution of cross-bridges between pre-powerstroke and post-powerstroke states [19,22,23,26,52]. Others suggest the increase in force is caused by an increase in the number of cross-bridges attached to actin [49,53], which may include the involvement of a second, adjacent cross-bridge that shares the same myosin neck fragment. Regardless of the actual mechanism, the increase in stiffness vanishes quickly after stretch, to levels between 0 and 7 per cent (between 10 and 300 ms after the stretch) [19,49], which weakens the possibility that cross-bridges contribute to the residual force enhancement.

Furthermore, studies that measured the stiffness during the steady-state force enhancement period observed that it decreased after stretch, reaching levels similar to those observed during isometric contractions [9,12]. There is one study that showed an increase in stiffness after stretch when compared to isometric contractions produced at corresponding lengths [16]. However, the increase in stiffness was attributed to non-cross-bridges structures (see §3a(ii)).

Finally, a study showed that when myofibrils are activated with different levels of MgADP, which causes an increase in the proportion of cross-bridges strongly bound to actin, the levels of force enhancement were comparable to myofibrils activated with Ca2+ [10], strengthening the notion that force enhancement is not associated with cross-bridge kinetics.

(ii) Passive elements and titin
In recent years, it has been suggested that force enhancement may be caused by non-cross-bridge, visco-elastic structures and most specifically titin molecules [11,16,23,26,54,55]. Titin spans the half-sarcomeres, attaching to the Z-lines, thick and thin filaments, and acts as a molecular spring providing most of the passive force in muscle fibres in elongated sarcomeres. In skeletal muscles, titin is present in one major N2A isoform, which can however be expressed in different lengths; in rabbit soleus the isoform is 3.7 MDa, and in rabbit psoas it has 3.7 and 3.4 MDa forms [56]. According to this hypothesis, when muscle fibres are activated, an increase in the intracellular Ca2+ concentration (either
as a result of electrically induced action potentials in intact fibres or changes in external Ca\(^{2+}\) concentrations in permeabilized fibres) would trigger not only myosin—actin interactions, but also a stiffening of titin molecules. The increase in titin stiffness could increase force considerably when the sarcomeres are stretched.

Although direct evidence that Ca\(^{2+}\)-induced ‘activation’ of titin is responsible for force enhancement is still lacking, studies conducted independently in different laboratories provide data supporting this hypothesis. The most significant results include the presence of stiffness in the sarcomere that is not associated with cross-bridges—the static stiffness—and changes in the passive force–sarcomere length relation with Ca\(^{2+}\).

Studies have been reporting for a few years the presence of a ‘static’ stiffness and tension in skeletal muscle fibres, which is activated by stretch [57–60] (figure 3a). When intact muscle fibres are activated in the presence of different myosin inhibitors and stretched, the stiffness increases sharply, independently of cross-bridge attachment to actin. This static stiffness remains elevated for as long as activation persists, i.e. a static tension is present after stretch. The static tension has characteristics that are similar to the residual force enhancement: it increases with the amplitude of stretch and initial sarcomere length, but is independent of the velocity of stretch [57–59]. A recent study conducted with intact fibres isolated from the mouse showed that the static stiffness is greater in digitorun longus (fast) muscle than in soleus (slow) muscle [61]. The muscle type dependence strengthens the possibility that static stiffness is caused by titin, as the two muscles have different titin. Finally, a study performed with permeabilized fibres from mammalian muscles, in which extracellular structures and events associated with Ca\(^{2+}\) release are not involved in stiffness or force measurements, confirmed the presence of the static tension, which was directly associated with the residual force enhancement [16]. In a few studies, the static tension is observed even a few seconds after activation stops [55,62,63], although the nature of this persistent passive force enhancement, is unknown, and it is not always observed.

Studies with single fibres in which myosin–actin interaction is inhibited with chemical interventions or with depletion of troponin and thin filaments show that increasing Ca\(^{2+}\) concentrations cause an upward shift in the sarcomere length–passive force relationship [16,17,63] (figure 3b). The levels of increase in passive forces range between 5 and 10 per cent, which is in line with the levels of force enhancement observed in isolated sarcomeres that do not show half-sarcomere length non-uniformities.

(iii) Mechanisms of titin regulation of force enhancement

The increase in passive forces with Ca\(^{2+}\) can hardly be ignored when one evokes a mechanism of force enhancement after stretch. Obviously, it is important to understand how titin regulates the increases in passive forces in the presence of Ca\(^{2+}\) after stretch. In this regard, there are two proposed mechanisms: a direct effect of Ca\(^{2+}\) on titin stiffness, or an effect of Ca\(^{2+}\) on the titin–actin interactions.

During activation, an elevation in intracellular Ca\(^{2+}\) concentration can increase the stiffness of the Pro-Glu-Val-Lys (PEVK) element of titin—the region believed to

Figure 3. (a) Force response to a stretch (amplitude 29.7 nm per half-sarcomere, duration 1.2 ms) applied during the tetanus in a single fibre isolated from the frog anterior tibialis muscle, treated with 2,3-butanedione monoxime solution (6 mM). The picture shows: a, average sarcomere length; b, tetanus with stretch; c, isometric tetanus; and d, the force trace obtained by subtracting the passive force response (not shown) and trace c from trace b. The ‘static tension’ was measured after the end of the fast transient, as indicated by the arrow. Adapted from Bagni et al. [57] with permission. (b) Consecutive stretches performed with a permeabilized fibre from the rabbit psoas muscle in pCa\(^{2+}\) 9.0 and 4.5. An increase in Ca\(^{2+}\) concentration caused an increase in forces produced by the fibre during and after stretch. The passive force–sarcomere length relation in a series of experiments (n = 7) performed before and after treatment of fibres with rigor-EDTA, gelsolin and blebbistatin, in pCa\(^{2+}\) 9.0 (open symbols) and pCa\(^{2+}\) 4.5 (closed symbols). The passive forces after the stretches increase at pCa\(^{2+}\) 4.5.

be Ca\(^{2+}\) sensitive. In a comprehensive study using different fragments of titin, Labeit et al. [17] observed that Ca\(^{2+}\) binding to the PEVK region of the molecules caused a decrease in its persistence length. A decrease in the persistence length is associated with an increase in stiffness, and consequently passive force production. The authors also showed that the minimal titin fragment that responded to Ca\(^{2+}\) contained a central E-rich domain with glutamates flanked by PEVK repeats. Since skeletal muscle titin isoforms contain a variable number of PEVK repeats and E-rich motifs [64], their result is consistent with the idea that Ca\(^{2+}\) affects the conformation of the PEVK motifs. As a result, when muscles are activated with Ca\(^{2+}\) and
then stretched, the response of titin would be enhanced when compared to stretch without the presence of Ca\(^{2+}\).

Another mechanism by which Ca\(^{2+}\) could regulate the influence of titin is by increasing its binding to actin, which would increase the overall sarcomere stiffness. This hypothesis is tempting, given the proximity between titin and actin filaments in the I-band of the sarcomeres [65–68] and the malleability of the PEVK domain of titin, which may transit among different conformational states [69] and bind F-actin [70–73]. It has been shown that the binding of the PEVK domain of titin to actin can be modulated by S100A1, a member of the S100 family of EF-hand Ca\(^{2+}\)-binding proteins [73] which is present at high concentrations in striated muscles [74].

While one study showed that titin inhibited significantly the sliding of the actin filaments on \textit{in vitro} motility assays in the presence of Ca\(^{2+}\) [65], subsequent studies using recombinant titin fragments failed to detect binding between the tandem immunoglobulin segments of titin and actin [70,73]. In fact, in one of these studies it was suggested that S100A1–PEVK binding alleviates the PEVK–actin interaction and providing the sarcomere with a mechanism to free the thin filament from titin before contraction, thereby reducing the titin-based force. This latest finding confirmed speculations of Stuyvers \textit{et al.} [75], who demonstrated that the titin–actin interactions-based stiffness of rat cardiac trabeculae increases when Ca\(^{2+}\) levels decay during relaxation, opposite to what would have been expected if Ca\(^{2+}\) were to increase the titin–actin stiffness. At the cellular level, a recent study has compared muscle fibres treated for
removal of tropinin C with fibres treated for the removal of actin [16]. The two treatments produced similar results: an increase in static stiffness and tension that was directly associated with low levels of force enhancement, suggesting that the increase in force is associated with changes in titin, and not titin–actin interactions.

In conclusion, force enhancement is caused partially by an increase in titin stiffness, a process that is regulated by binding of Ca\(^{2+}\) to the PEVK domain of the molecule, independently of titin–actin interactions.

(iv) Other mechanisms

Other structural proteins may influence the levels of force enhancement after stretch. Nebulin (approx. 700–800 kDa) is a protein that spans the entire thin filament and attaches to the Z-line. Nebulin acts as a ruler to maintain the length of the thin filament, and new evidence shows that it also regulates myosin–actin interactions during contractions [76]. If stretch induces conformational changes in nebulin, it could lead to an increased number of myosin–actin interactions. Since an increase in stiffness after stretch is not commonly observed (as explained earlier in this review), the role of nebulin in force enhancement is not likely, although it deserves investigation. Desmin (approx. 52 kDa) is a protein responsible for keeping adjacent myofibrils in register across the muscle fibre, through lateral connections at the Z-discs, linking the contractile apparatus to the sarcolemma. If desmin filaments were strained during and after stretch, they could increase the overall stiffness of the sarcomeres, contributing to force enhancement in fibre preparations. Such a possibility also deserves investigation, but since force enhancement is present in isolated sarcomeres and myofibrils, it could not be explained entirely by a desmin-related mechanism.

4. SUMMARY OF THE PROPOSED MECHANISM FOR FORCE ENHANCEMENT

Based on the evidence presented in the literature, this review proposes that the mechanisms for the residual force enhancement are associated with: (i) non-uniformity among half-sarcomeres, which will influence both the stiffness of titin and the overlap between myosin and actin in half-sarcomeres, and (ii) a Ca\(^{2+}\)-regulated increase in stiffness during activation and stretch (figure 4). These mechanisms represent a variation of the original non-uniformity hypothesis, but without popping sarcomeres. It also includes the role of Ca\(^{2+}\)-activation of titin, a recently exploited phenomenon that may explain force enhancement in conditions in which sarcomeres are maintained mostly uniform through activation and stretch.

These mechanisms explain the following experimental features observed in studies using single fibres, isolated myofibrils and sarcomeres that used force and sarcomere length measurements simultaneously and that controlled the experiments for potential artefacts and intra-sample variability:

— force enhancement is present in single fibres, myofibrils and sarcomeres if titin isoforms are responsive to Ca\(^{2+}\). Since there are different lengths of the skeletal muscle titin isoforms, there may be cases where force enhancement is absent in isolated sarcomeres;

— the level of force enhancement in single sarcomeres that do not present half-sarcomere length non-uniformities and are dependent on a Ca\(^{2+}\)-induced increase in titin stiffness is smaller than preparations presenting large levels of half-sarcomere length non-uniformities;

— the level of force enhancement is dependent on the magnitude of stretch and initial sarcomere length, but not on the velocity of stretch;

— force enhancement can be observed along the ascending limb and plateau of the force–length relationship if some half-sarcomeres increase filament overlap after stretch and a Ca\(^{2+}\)-induced stiffer titin supports the passive forces at the other halves (which may be at the descending limb of the force–length relation); and

— force enhancement is present and enhanced by sarcomere length non-uniformities without the occurrence of popping sarcomeres. Sarcomeres may present considerably different lengths after stretch without showing large mechanical instabilities [28,33].

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