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NEW FRONTIERS IN MUSCLE RESEARCH

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'CURRENT' ADVANCES IN MECHANICALLY-SKINNED SKELETAL MUSCLE FIBRES

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Summary

In skeletal muscle, excitation-contraction (E-C) coupling describes a cascade of cellular events initiated by an action potential (AP) at the surface membrane which ultimately results in muscle contraction. Being able to specifically manipulate the many processes that constitute E-C coupling as well as the many factors that modulate these processes has proved challenging. One of the simplest methods of gaining access to the intracellular environment of the muscle fibre is to physically remove (mechanically skin) the surface membrane. In doing so the myoplasmic environment is opened to external manipulation. Surprisingly, even though the surface membrane is absent, it is still possible to activate both twitch and tetanic force responses in a mechanically-skinned muscle fibre by generating an AP in the transverse tubular system. This proves that all the key steps in E-C coupling are retained in this preparation. By using this technique, it is now possible to easily manipulate the myoplasmic environment and observe how altering individual factors affects the normal E-C coupling sequence. The effect of important factors, such as the redox state of the cell, parvalbumin, and the sarcoplasmic reticulum Ca^{2+}-ATPase, on twitch and tetanic force can now be specifically investigated independent of other factors.

1. Overview of excitation-contraction coupling in skeletal muscle

Given the extensive literature on E-C coupling in skeletal muscle, a detailed examination of each step in the process is beyond the scope of this article. Instead, recent reviews are cited where appropriate and only a brief account of E-C coupling is given here to help the reader appreciate certain points raised later in this review. In this article, I will give examples of the recent use and possible future contributions of mechanically-skinned fibre technique towards the understanding certain parts of the E-C coupling cascade, namely: a) the spread of excitation within the transverse tubular (t-) system; b) the mechanisms of communication between the voltage-sensors in the t-system and the Ca^{2+} release channels of the terminal cisternae of the sarcoplasmic reticulum (SR); and c) Ca^{2+} handling by the SR.

In skeletal muscle, the AP at the surface membrane rapidly spreads down into the t-system of the muscle fibre where the associated depolarization is sensed by the voltage-sensors (dihydropyridine receptors - DHPRs) (Schneider, 1994; Melzer *et al.*, 1995). The DHPRs of skeletal muscle are modified L-type Ca^{2+} channels in which the Ca^{2+} channel function is virtually redundant because entry of Ca^{2+} into the cell is not necessary to initiate contraction (Rios & Pizzaro, 1991; Dulhunty, 1992; Melzer *et al.*, 1995). The DHPR consists of five subunits, with the α_1 subunit playing the primary role in E-C coupling. The α_1 of the DHPR is composed of four repeats (I-IV), each with six hydrophobic intramembranous segments (s1-s6). The fourth segment (s4) of each repeat contains a series of positive charges which are thought to be the voltage-sensitive elements that underlie the voltage-dependent asymmetric charge movement observed originally by Schneider & Chandler (1973). Connecting each repeat are hydrophilic peptide loops, with the myoplasmic loop joining repeats II and III being essential for signal transmission to the SR in vertebrate skeletal muscle (Tanabe *et al.*, 1990).

The DHPRs co-localize in arrangements of four, termed tetrads (Block *et al.*, 1988), and are located immediately adjacent to alternate Ca^{2+}-release channels (ryanodine receptors - RYRs) in the adjacent SR. Activation of the DHPRs subsequently leads to the activation of the RYRs by a mechanism that is not fully understood. The RYRs are large homotetrameric Ca^{2+} channels which tightly bind the plant alkaloid ryanodine. The RYRs specific to skeletal muscle are termed RYR1 and are arranged in closely packed arrays *in vivo* (Block *et al.*, 1988). In mammalian muscle, every RYR1 appears to be functionally identical at the biochemical level (Ogawa, 1994), although the properties of DHPR coupled versus DHPR uncoupled RYR1s *in vivo* may differ. In amphibian muscle, the RYR1s are composed of two isoforms, α and β, of which the properties of the β-isoform may or may not be differ from amphibian α and mammalian RYR1 (Ogawa, 1994; Franzini-Armstrong & Protasi, 1997; Ogawa *et al.*, 1999). A distinct feature of the various RYR1s of both amphibian and mammalian skeletal muscle is the strong inhibition of channel activity by physiological levels of Mg^{2+} (~1 mM), millimolar concentrations of Ca^{2+}, and the ability of ATP to stimulate channel activity even in the absence of Ca^{2+} (Lamb, 2000). These features are essential for the type of E-C coupling observed in skeletal muscle as opposed to cardiac and smooth muscle cells.

Precisely how the DHPR and the RYR1 interact has not been established, although a direct interaction between these two channels is thought to occur (Melzer *et al.*, 1995; Meissner & Lu, 1995; Franzini-Armstrong & Protasi, 1997). Activation of the RYR allows Ca^{2+} stored in the SR to enter the myoplasm where it binds to the contractile apparatus to initiate force production (Melzer *et al.*, 1995). The release of Ca^{2+} is tightly controlled by the DHPRs (Rios & Pizzaro, 1991; Melzer *et al.*, 1995). The cessation of Ca^{2+} release upon deactivation of the DHPRs leads to relaxation of force as the Ca^{2+} initially released is resequestered back into the SR through the activity of the SR Ca^{2+}-ATPases and in fast-twitch fibres, relaxation may be aided by the binding of Ca^{2+} to parvalbumin (Rall, 1996).

2. Techniques for investigating E-C coupling in skeletal muscle

Many techniques have been used in the study of E-C coupling and all have both advantages and disadvantages. Whole intact cell preparations have the advantage that they retain normal physiological function. However, the usefulness of these preparations is to some extent limited by difficulties in controlling and measuring intracellular processes. One way around this problem is to use molecular biology techniques, such as the knockout of a specific gene. In this way the influence of a specific protein can be removed and the effect of this studied in the intact system. However, given the complex interaction between many cellular constituents, the removal of a specific component may cause some unintentional change in the function of other components. In contrast, the biochemical approach involves the study of key components in well controlled artificial environments, such as the isolation of a single channel in an artificial lipid bilayer. In this way the basic function of a particular component can be determined in isolation, although the effect of complex interactions with other cellular components that may normally occur *in vivo* is lost. Bridging the gap between intact fibre and biochemical techniques are the skinned muscle fibre preparations. The key advantage of these preparations is that the myoplasmic environment can be easily manipulated whilst in certain skinned fibre preparations (see below), all the essential elements in the E-C coupling cascade also remains intact.

3. Types of skinned fibre preparations

There are a two main ways to permeablise a skeletal muscle fibre – chemically and mechanically. However, these differ in the consequences they have on the various structures of the muscle fibre.

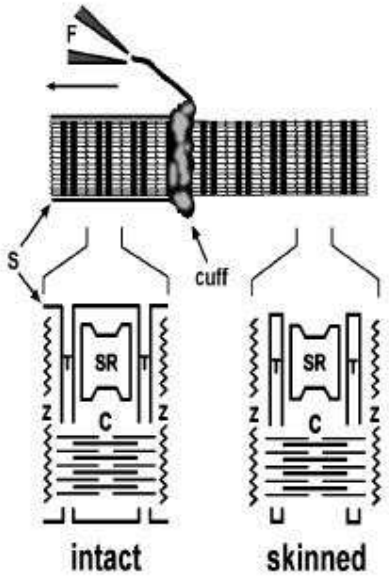

Figure 1. Intact and mechanically-skinned portion of a skeletal muscle fibre. *Schematic diagram showing the skinning of a single skeletal muscle fibre by rolling back the surface membrane (sarcolemma - S) with a pair of forceps (F), forming a 'cuff'. The transverse tubular system (T) seals off to form a closed compartment after skinning. SR, sarcoplasmic reticulum; Z, Z-line; C, contractile apparatus. (Modified from Posterino et al., 2000).*

a) Chemical skinning

This involves the use of a number of chemical reagents that permeablise the various membranes of the fibre – some are more selective than others. Commonly used reagents are saponin, β-escin, glycerol and triton-X 100. The more selective permeablising agents (e.g. saponin and β-escin) are thought to act primarily on the surface membranes (sarcolemma) and t-tubules by binding cholesterol which is largely absent from the SR. However, this selectivity is not as precise as first thought and significant effects on the SR have been observed (Launikonis & Stephenson, 1997, 1999). Other non-specific reagents (such as triton-X 100 and glycerol) destroy all the membrane structures and leave only the contractile machinery intact. The type of permeablising reagent that is most appropriate depends on the particular cellular process the investigator wishes to examine. For examination of the properties of the contractile apparatus, it is often best to remove all membrane structures to ensure that they do not interfere with the measurements. For example, the presence of a functional SR can greatly affect the Ca^{2+} gradients within the fibre and this may significantly affect the force-[Ca^{2+}] relationship unless the [Ca^{2+}] was buffered very strongly. If on the other hand the purpose is to investigate the Ca^{2+}-handling properties of the SR, one needs to use the more selective reagents that leave the SR intact and functional - and this may not be possible with even the more selective reagents used (cf. Launikonis & Stephenson, 1997, 1999). Thus, to avoid any undesired effects of permeablising reagents it is perhaps best to use mechanically-skinned fibres.

b) Mechanical-skinning

The mechanically-skinned fibre technique (originally termed Natori-type fibres) was first developed by Natori in 1954. He showed that it was possible to gain access to intracellular environment by physically rolling back the surface membrane of a single muscle fibre with a pair of fine needles under paraffin oil (see Fig. 1.). Unlike chemically-skinned fibres, in which the surface

membrane, t-system and SR are perforated by various chemical agents, in mechanically-skinned fibres the t-system seals off to form an intact, fully functional compartment (see later). The SR also remains intact and fully functional. There are other variations of this technique, such as splitting the fibre, however, the t-system in this preparation does not seal off which limits its experimental application. The functional integrity of mechanically-skinned fibres is one of the key advantages of this preparation and this can be seen by its use in the examination of many aspects of E-C coupling.

Examination of the contractile apparatus: One of the earliest uses of mechanically-skinned fibres was in the study of the various properties of the contractile apparatus in which a number of models derived from earlier biochemical studies could be tested in a more physiological preparation (Gordon *et al.*, 2000). These fibres were often also treated with membrane permeablising reagents, such as triton X-100, and are thus better termed chemically-skinned fibres. Nevertheless, the specific role of Ca^{2+}, Mg^{2+}, ATP and other compounds in the regulating contraction has been studied extensively by exposing the myofilaments of both mechanically-skinned and chemically-skinned fibres to various buffered solutions (Stephenson, 1981; Gordon *et al.*, 2000). Mechanically-skinned fibres are useful in understanding the properties of the contractile apparatus as it has been shown that the intrinsic contractile properties of intact fibres can be observed in mechanically-skinned fibres provided that the buffering of various factors in solution is firmly controlled (Moisescu, 1976). More recently, a study comparing the Ca^{2+}-activation properties (i.e. the Ca^{2+}-sensitivity and Hill coefficient) of both intact and mechanically-skinned fibres further demonstrate that contractile function remains unchanged following skinning (Konishi & Watanabe, 1998).

Examination of SR properties: Biochemical assays have provided enormous insight into the function of the SR and its key molecules. However, during preparation of these assays, the structure of the SR and associated proteins may be compromised and no longer representative of their state *in vivo*. Furthermore, the normal constraints and relationships between RYR1s and other key molecules present *in vivo* are typically lost (Favero, 1999). The importance of such constraints and relationships between molecules for their normal function is becoming clearer. As mentioned earlier, RYR1s are arranged in a closely packed array *in vivo* and it has been shown recently that RYRs will spontaneously form these arrangements in solution (Yin & Lai, 2000). Furthermore, RYRs in bilayers have been observed to open and close in synchrony (termed coupled gating, Marx *et al.*, 1998). This phenomena suggested that there is physical cooperativity between such channels and this could well be important for normal Ca^{2+} release. There is also a close association between RYR1 and DHP receptors of the t-system (Block *et al.*, 1988) and recent studies have suggested that RYR1 can bind DHP receptors *in vitro* (Murray & Ohlendiek, 1997). The close proximity of the DHP receptors and RYR1 appears to directly influence their individual functions (Nakai *et al.*, 1996). Associated proteins of the RYR1, such as FKBP-12, also help to link and control neighbouring channels (Marx *et al.*, 1998) whilst other associated molecules, such as calmodulin and calsequestrin, appear to regulate channel activity at an individual level (Franzini-Armstrong & Protasi, 1997). Mechanically-skinned fibres retain these relationships and structural constraints and are thus ideal for the examination of the activity of the RYR1 and the Ca^{2+}-ATPase in their native state. The endogenous Ca^{2+} content of the SR, which is reported to regulate the activity of the RYR1 (Sitsapesan & Williams, 1997), can also be assayed and controlled in this preparation. By using a number of RYR agonists and antagonists, such as caffeine and ryanodine, as well as antagonists of the Ca^{2+}-ATPase, such as 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (TBQ), the properties of the SR and related molecules can be examined (see later).

Examination of voltage-dependent Ca^{2+} release: Considering the apparent importance of maintaining normal structural integrity, how do we really know that mechanically-skinned fibres accurately describe events that occur *in vivo* given that the surface membrane is removed in this preparation?

One of the most important features of mechanically-skinned fibres is that activation of Ca^{2+} release from the SR can be elicited by activation of the voltage-sensors (DHP receptors) present in the t-system just as it is in an intact fibre. That is, the normal E-C coupling mechanism is retained in this preparation. What is the evidence for this? The first indication came from experiments conducted by Natori in the fifties. He showed that small contractions could be elicited in mechanically-skinned fibres when large electrical stimuli (>110 V cm^{-1}) were applied via electrodes to fibres under oil (Natori, 1954) (see later section). Later, Costantin & Podolsky (1967) showed that both electrical stimulation and raising the $[Cl^-]$ in the myoplasm of mechanically-skinned fibres produced contraction. At the time, not much was known about the mechanism of E-C coupling and these authors could only conclude that such contractions arose from depolarisation of some internal membrane compartment in the skinned fibre and favoured the idea that it involved both the t-system and SR. However, from this point in time electrical stimulation of skeletal muscle was not pursued further. Instead, the focus was directed towards the mechanism of Cl^--induced activation of mechanically-skinned fibres. This led to the discovery that the t-system sealed off after skinning to form a separate compartment (see Fig 1). It was subsequently shown that by forming a separate compartment that is isolated from the myoplasmic environment of the fibre (as it is normally in an intact fibre), the t-system of a mechanically-skinned fibre could be polarized if the fibre was bathed in a solution that mimics the normal myoplasm (e.g. high $[K^+]$, some Na^+, 8 mmol l^{-1} ATP, 10 mmol l^{-1} creatine phosphate, 1 mmol l^{-1} free Mg^{2+}, 0.1 umol l^{-1} Ca^{2+}, pH 7.1) (Donaldson, 1985; Stephenson, 1985; Fill & Best, 1988; Lamb & Stephenson, 1990). Repolarization of the sealed t-system was possible due to the presence of functional Na^+-K^+ pumps that reestablish the normal Na^+-K^+ gradient (Donaldson, 1985; Stephenson, 1985; Fill & Best, 1988; Lamb & Stephenson, 1990). Some control of the t-system potential was then possible by simply changing the $[K^+]$ bathing the fibre (Fill & Best, 1988; Lamb & Stephenson, 1990; Posterino & Lamb, 1998a). If all the K^+ in the bathing solution was rapidly removed, it was possible to depolarize the t-system. The K^+ ion was often replaced with Na^+, although in some instances, the K^+ was replaced with choline chloride, in which the simultaneous increase in the $[Cl^-]$ helped to further depolarize the t-system (Lamb & Stephenson, 1990). Such depolarization led to the activation of force in mechanically-skinned fibres which was subsequently shown to involve the activation of both the DHPRs in the t-system and the RYR1s of the SR because such responses were: a) inhibited by antagonists of DHP receptors, such as nifedipine and verapamil (Posterino & Lamb, 1998a; Lamb & Stephenson, 1990); and b) completely blocked by ryanodine and ruthenium red, specific antagonists of RYRs (Lamb & Stephenson, 1990). The transient force responses observed following depolarization of the t-system in this manner last a few seconds and are graded by the myoplasmic $[K^+]$. These results confirm that mechanically-skinned fibres retain functional E-C coupling and it is clear that such force responses are analogous to K^+ contractures generated in intact fibres (i.e. when extracellular $[K^+]$ is increased). These results also showed that the essential elements involved in E-C coupling must be very robust as they are retained following mechanical-skinning and after the normal myoplasmic constituents are replaced with a minimal physiological solution (Lamb, 2000).

Nevertheless, despite many useful properties mentioned above, the mechanically-skinned fibre technique does have several limitations. One is that the t-system membrane potential can not be directly measured and importantly, can not be accurately controlled. A second, and perhaps the most important, is the slow depolarization of the t-system associated with diffusion of the bathing solution into the fibre. This prevents the study of rapid voltage-dependent Ca^{2+} release from the SR. Depolarization-induced force responses elicited by solution substitution occur with a rise time of some 500 ms, with the whole response lasting some 2-3 s. Consequently, force responses elicited in this manner may not be as sensitive to changes in Ca^{2+} release as a more rapid physiological response seen during a single twitch or a tetanus.

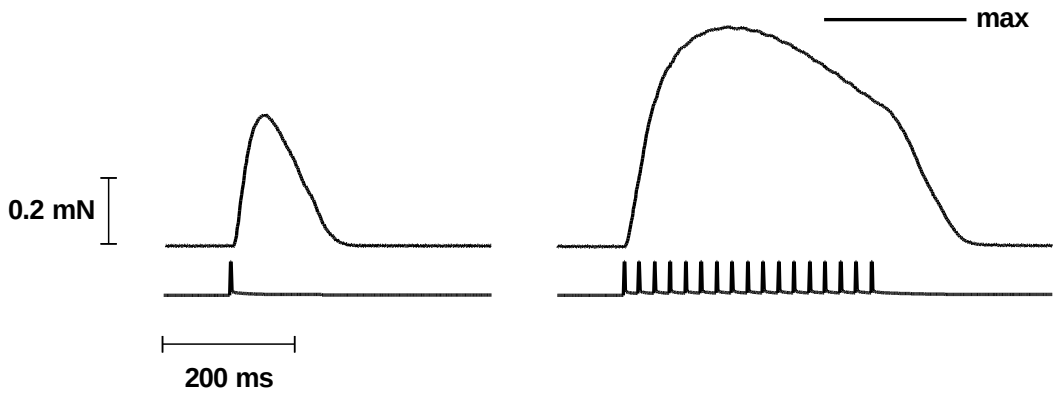

Figure 2. *Twitch and tetanic (50 Hz) force responses elicited by applying 50 V cm^{-1} field stimulation (2 ms duration) to a segment of a mechanically-skinned EDL muscle fibre of the rat bathed in a solution mimicking the normal myoplasmic environment (ie. high [K^+]; see Section 3). The applied stimulus is simultaneous recorded below each force response. Maximum Ca^{2+}-activated force (max) was elicited in the same fibre using a heavily buffered Ca-EGTA solution and is indicated by the solid bar above the tetanic force response.*

4. Electrical stimulation of mechanically-skinned fibres

As mentioned earlier, electrical stimulation of mechanically-skinned fibres has not been re-examined for some 25 years since the last experiments performed by Costantin. At the time large electrical stimuli were required to elicit relatively weak force responses. The reason for this may be due to the fact that mechanically-skinned fibres were stimulated under oil which left the t-tubules in a poorly polarised state. Furthermore, fibres were typically stimulated with a longitudinal electric field and with very large voltages that may have damaged the fibre. Recently, Posterino et al. (2000) revisited the idea of electrically-stimulating mechanically-skinned fibres, modifying both the solutions used to bath mechanically-skinned fibres and the orientation of the electric field. Thin platinum wire electrodes were positioned parallel with the long axis of the muscle fibre at a distance of 4 mm apart and along the whole length such that a uniform stimulus was applied. Fibres were bathed in a physiological solution that mimics the normal myoplasmic environment ensuring that the t-tubules were well polarized (Posterino & Lamb, 1998a; Lamb, 2000). A brief 2 ms, 20-25 V stimulus was applied giving a field strength of 50-60 V cm^{-1}. In this manner, Posterino et al. (2000) were able to elicit reproducible twitch and tetanic force responses in mechanically-skinned fibres (see Fig 2). Precise positioning of the fibre between the electrodes was not necessary and twitch and tetanic force could be elicited in both mammalian fast-twitch fibres (Posterino *et al.*, 2000) or amphibian twitch fibres (unpublished data).

Twitch or tetanic force responses in mechanically-skinned fibres are initiated by the generation of APs in the sealed t-tubules and by the activation of voltage-dependent processes that underlie normal E-C coupling (Posterino *et al.*, 2000). Thus, even the first step in E-C coupling is retained in mechanically-skinned fibres – the ability to generate an AP. The evidence for this is threefold. Firstly, it was noted that the twitch response in fibres was steeply dependent on the applied voltage and exhibited a sharp threshold in which the transition between zero force and 70% of maximum twitch size required only a 10% increase in the applied electric field. Secondly, chronic depolarisation of the t-system prevented any twitch or tetanic response from being elicited by field stimulation. This is consistent with the inactivation of voltage-dependent processes that underlie both the AP and activation of the voltage-sensors of the t-system. And thirdly, the presence of 10 μM TTX in the

sealed t-tubules strongly inhibited such responses proving that activation of Na^+ channels (and the generation of an AP) in the t-system is essential in triggering further steps in the cascade.

The characteristics of both twitch and tetanic force responses elicited in mechanically-skinned fibres closely resemble the responses observed in intact fibres (Fryer & Neering, 1988; Schwaller *et al.,* 1999). The peak amplitude of the twitch response in fast-twitch mammalian and twitch amphibian muscle is between 30% and 60% of maximum Ca^{2+}-activated force. Tetani (50 Hz) elicits force responses between 80-100% of maximum Ca^{2+}-activated force. The twitch-tetanus ratio in mammalian fast-twitch skinned fibres ranges between 0.40 and 0.60 which is larger than that observed in intact fibres (~0.30) (Schwaller *et al.,* 1999). The larger twitch-tetanus ratio and the ability to achieve near maximal force during a tetanus, is possibly due at least in part to the loss of parvalbumin in mechanically-skinned fibre preparations (Stephenson *et al.,* 1999).

The clear functional similarities between intact and mechanically-skinned fibres highlight the potential of the mechanically-skinned fibre technique in the study of skeletal muscle physiology. Nevertheless, there are a few differences in the responses observed between mechanically-skinned fibres and intact fibres. One such difference is that tetanic force in fast-twitch mechanically-skinned fibres declines much more rapidly during high frequency stimuli than in intact fibres, with force fading after ~200 ms of stimulation (termed fade; see Fig. 2). The cause of this phenomenon is not certain. Posterino et al. (2000) attributed this to a gradual build up of K^+ in the sealed t-system with repeated APs leading to depolarization; this would not normally occur in an intact fibre where the t-system is open to the extracellular environment. However, we have recently observed that this phenomena appears to be related to the fibre length, as fade was largely eliminated in fibres that were stretched from between 120% to 140% of their resting length (unpublished results). This is currently being further examined. Another difference between mechanically-skinned fibres and intact fibres is that the absence of a surface membrane means the intracellular Ca^{2+} content of the fibre can vary from the endogenous level. The comparatively large volume of solution bathing the mechanically-skinned fibre means that a substantial amount of Ca^{2+} can be gained from (or lost to) the bulk solution. In order to maintain the normal endogenous level of SR Ca^{2+}, both over time and with repeated responses, it is necessary ensure that the free [Ca^{2+}] of the bathing solution is buffered to the normal resting myoplasmic concentration (pCa 7.0). This limits the amount of Ca^{2+} loading. It is noteworthy that little variability is observed between successive twitch responses in mechanically-skinned fibres which suggests that the SR Ca^{2+} content remains relatively stable under the conditions used. However, a more precise determination of changes in the SR Ca^{2+} content during repeated stimulation is still needed as well as a better way of clamping the SR Ca^{2+} content.

5. Recent contributions and future directions

The ability to electrically stimulate mechanically-skinned fibres helps bridge the gap between biochemical and whole cell studies. Some recent findings illustrate the current and future potential of this technique towards the understanding of E-C coupling in skeletal muscle.

a) Mechanisms and pathways involved in the initial spread of excitation

The first step in E-C coupling involves the initiation and spread of the AP throughout the muscle fibre. It is generally accepted that the spread of excitation into the t-system in amphibian skeletal muscle involves an AP (Costantin, 1970; Bezanilla *et al.,* 1972; Nakajima & Gilal, 1980). In mammalian skeletal muscle, it was not known if the spread was either passive or active, although it was assumed to involve an AP. As indicated earlier, the ability to generate an AP in mechanically-skinned fibres by electrical stimulation now provides direct evidence that excitation also spreads down the t-tubules via an active process in mammalian muscle (Posterino *et al.,* 2000). This observation is unambiguous as there is no surface membrane present. Furthermore, it is apparent that other

characteristics of the AP in the t-system can also be determined using this preparation. By using the twitch response as an indirect measure of the AP, it is possible to estimate the relative refractory period of the AP. If two single stimuli are elicited in close succession (less than 6 ms) no summation of the twitch force response was observed. However, if the second stimulus is applied 6 ms (or later) after the first, the twitch response is potentiated. This indicates that the refractory period of the AP is at most 6 ms long (unpublished data). Refinement of this measure could be achieved by examining the Ca^{2+} transient rather than force.

Experiments with electrically-stimulated mechanically-skinned fibres have also revealed the role of a previously identified structure in skeletal muscle. The observation that contractions (either spontaneous or elicited electrically) have the ability to propagate over hundreds of sarcomeres in mechanically-skinned fibres revealed a mechanism that allows excitation to spread throughout a skeletal muscle fibre independent of the surface membrane (Natori, 1954; Costantin & Podolsky, 1967; Posterino *et al.,* 2000). It was found that an AP(s) could propagate along the entire length of skinned fibre segment (without the presence of a surface membrane) and could cause relatively synchronous activation of a large proportion of the fibre (>70%) travelling with an estimated velocity of some 13 mm s^{-1} (Posterino *et al.,* 2000). It was suggested that the structure involved in the spread of the AP must be the longitudinal tubular system (LTS) which has been observed with electron microscopy (EM) (Franzini-Armstrong & Jorgenson, 1988; Stephenson & Lamb, 1992) and by confocal imaging of mechanically-skinned fibres in which a fluophore was trapped in the t-system (Peachey, L.D., 1965). These findings in mechanically-skinned fibres revealed a fundamental property that is likely to be important in the spread of the AP throughout a fibre, in fatigue and during myogenesis.

b) Internal transmission of excitation and control of Ca^{2+} release

Data obtained from mechanically-skinned fibres in which functional E-C coupling is retained have also provided strong evidence for and against a number of ideas regarding the mechanism by which the DHPRs and the RYR1s communicate. It is clear from some experiments in mechanically-skinned fibres that the link between these two channels does not involve a diffusible second messenger such as inositol 1,4,5-trisphosphate (Walker *et al.,* 1987; Posterino *et al.,* 1998b) or Ca^{2+} (Endo, 1985; Meissner *et al.,* 1986; Lamb & Stephenson, 1991; Owen *et al.,* 1997; Lamb & Laver, 1998). The link was also previously thought to involve the transient formation of disulphide bonds between the DHPRs and the RYRs (Salama *et al.,* 1992). However, recent experiments in mechanically-skinned fibres have showed that strong sulphydryl reducing reagents do not interfere with normal E-C coupling(Posterino & Lamb, 1996). Nevertheless, various sulphydryl oxidants have been shown to modulate the RYR1 function (Dulhunty *et al.,* 1996) indicating that the cellular redox state may effect E-C coupling in a more complex manner that is dependent on the precise ratio of endogenous redox reagents (ie. ratio of glutathione to reduced glutathione) both within the myoplasm and the lumen of the SR Feng *et al.,* 2000). Here again we can see the potential advantage of mechanically-skinned fibres in addressing this question which can not readily be observed in an intact fibre preparation.

c) Ca^{2+} handling

As mentioned earlier, mechanically-skinned fibres rapidly loose their parvalbumin after skinning and this may account for some of the dynamic differences between twitch responses observed between mechanically-skinned fibres and intact fibres. The absence of parvalbumin in mechanically-skinned fibres allows the examination of the functional role of this protein in SR Ca^{2+} handling. Most previous studies examining the role of parvalbumin have been limited by the inability to remove the effects of parvalbumin completely. Only one recent study using mice in which the parvalbumin gene has been knocked out has been able to examine the precise contribution of parvalbumin on twitch and tetanic

characteristics (Fryer & Neering, 1988). This study showed that twitch-tetanus ratio was greater in fibres from parvalbumin knockout mice (PKM) than from wildtype mice. Interestingly, the characteristics of the twitch response from PKM greatly resemble those from mechanically-skinned fibres. Nevertheless, it is possible that the absence of parvalbumin in PKM may have effected the other constituents important in E-C coupling and Ca^{2+} handling. The unique properties of mechanically-skinned fibres allows the examination of parvalbumin more precisely without the problem of non-specific effects that may arise from gene knockout. Parvalbumin can be simply added to and removed from the bathing solution of mechanically-skinned fibres and the effects on the twitch and tetanus observed in the same fibre. Apart from parvalbumin, mechanically-skinned fibres can allow the precise examination of the role of the SR Ca^{2+} ATPase in contraction and relaxation. In intact fibre studies, the role of the SR Ca^{2+} ATPase is often examined by using specific inhibitors such as TBQ and thapsigargin (Westerblad & Allen, 1994; Caputo *et al.*, 1999). However, it is difficult to be sure that complete block of the pump has taken place and that there are no complicating effects of increased resting Ca^{2+}. Similarly, the intact fibre studies must also take into account any effects of parvalbumin. An advantage of mechanically-skinned fibres is that distinct qualities of the SR Ca^{2+} ATPase can be examined in isolation of parvalbumin and without changes to resting myoplasmic $[Ca^{2+}]$.

Conclusion

To date, mechanically-skinned fibres have been a useful tool in the study of many aspects of E-C coupling in skeletal muscle. The controlled nature of the myoplasmic environment of skinned fibres, the presence of functional E-C coupling that can be now be activated in the same manner and with a similar time course as an intact fibre, and the fact that the key structures involved in E-C coupling obviously remain as they were *in vivo*, demonstrate the potential of this technique in further aiding our understanding of E-C coupling in skeletal muscle.

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References

Bezanilla, F., Caputo, C., Gonzalez-Serratos, H. & Venosa, R.A. (1972) Sodium dependence of the inward spread of activation in isolated twitch muscle fibres of the frog. *Journal of Physiology*, 223, 507-523.

Block, B.A., Imagawa, T., Campbell, K.P. & Franzini-Armstrong, C. (1988) Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *Journal of Cell Biology*, 107(6/2), 2587-2600.

Caputo, C., Bolanos, P. & Escobar, A.L. (1999) Fast calcium removal during single twitches in amphibian skeletal muscle fibres. *Journal of Muscle Research & Cell Motility*, 20, 555-567.

Costantin, L.L. & Podolsky, R.J. (1967) Depolarization of the internal membrane system in the activation of frog skeletal muscle. *Journal of General Physiology*, 50, 1101-1124.

Costantin, L.L. (1970) The role of sodium current in the radial spread of contraction in frog muscle fibres. *Journal of General Physiology*, 55, 703-715.

Donaldson, S.K.B. (1985) Peeled mammalian skeletal muscle fibres. Possible stimulation of Ca^{2+} release via a transverse tubule-sarcoplasmic reticulum mechanism. *Journal of General Physiology*, 86, 501-525.

Dulhunty, A.F. (1992) The voltage-activation of contraction in skeletal muscle. *Progress in Biophysics and Physiology*, 53, 1-16.

Dulhunty, A.F., Junankar, P.R., Eager, K.R., Ahern, G.P. & Laver, D.R. (1996) Ion channels in the sarcoplasmic reticulum of striated muscle. *Acta Physiologica Scandanavica*, 156, 375-385.

Endo, M. (1985) Calcium release from the sarcoplasmic reticulum. *Current Topics in Membranes and Transport*, 25, 181-230.

Favero, T.G. (1999) Sarcoplasmic reticulum Ca^{2+} release and muscle fatigue. *Journal of Applied Physiology*, 87(2), 471-483.

Feng, W., Guohua, L., Allen, P.D. & Pessah, I.N. (2000) Transmembrane redox sensor of ryanodine receptor complex. *Journal of Biological Chemistry*, 275, 35902-35907.

Fill, M.D. & Best, P.M. (1988) Contractile activation and recovery in skinned frog muscle stimulated by ionic substitution. *American Journal of Physiology*, 254, C107-114.

Franzini-Armstrong, C. & Jorgenson, A.O. (1988) Discrimination between fast and slow twitch fibres of guinea pig skeletal muscle using the relative surface density of junctional transverse tubule membrane. *Journal of Muscle Research and Cellular Communication*, 9, 403-413.

Franzini-Armstrong, C. & Protasi, F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiological Reviews*, 77, 699-729.

Fryer, M.F. & Neering, I.R. (1988) Actions of caffeine on fast- and slow-twitch muscles of the rat. *Journal of Physiology*, 416, 435-454.

Gordon, A.M., Homsher, E. & Regnier, M. (2000) Regulation of contraction in striated muscle. *Physiological Reviews*, 80(2), 853-924.

Konishi, M. & Watanabe, M. (1998) Steady state relation between cytoplasmic free Ca^{2+} concentration and force in intact frog skeletal muscle fibres. *Journal of General Physiology*, 111, 505-519.

Lamb, G.D. & Laver, D.R. (1998) Adaption, inactivation and inhibition in ryanodine receptors. In: *The Structure and Function of Ryanodine Receptors.* Ed. Sitsapesan, R. & Williams, A.J. Ch. 14. London: Imperial College Press.

Lamb, G.D. & Stephenson, D.G. (1990) Calcium release in skinned muscle fibres of the toad by transverse tubule depolarization or by direct stimulation. *Journal of Physiology*, 423, 495-517.

Lamb, G.D. & Stephenson, D.G. (1991) Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *Journal of Physiology*, 434, 507-528.

Lamb, G.D. (2000) Excitation-contraction coupling in skeletal muscle: comparisons with cardiac muscle. *Clinical and Experimental Physiology and Pharmacology*, 27, 216-224.

Launikonis, B.S. & Stephenson, D.G. (1997) Effect of saponin treatment on the sarcoplasmic reticulum of rat, cane toad and crustacean (yabby) skeletal muscle. *Journal of Physiology*, 504.2, 425-437.

Launikonis, B.S. & Stephenson, D.G. (1999) Effects of β-escin and saponin on the transverse-tubular system and the sarcoplasmic reticulum membranes of rat and toad skeletal muscle. *Pflügers Archiv*, 437, 955-965.

Marx, S.O., Ondrias, K. & Marks, A.R. (1998) Coupled gating between individual skeletal muscle Ca^{2+} release channels (ryanodine receptors). *Science*, 281, 818-821.

Meissner, G. & Lu, X. (1995) Dihyropyridine receptor-ryanodine receptor interactions in skeletal muscle excitation-contraction coupling. *Bioscience Reports*, 15(5), 399-408.

Meissner, G., Darling, E. & Eveleth, J. (1986) Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+} and adenine nucleotides. *Biochemistry*, 25, 236-244.

Melzer, W., Herrmann-Frank, A. & Lüttgau, H.Ch. (1995) The role of Ca^{2+} ions in excitation-contraction coupling in skeletal muscle fibres. *Biochimica et Biophysica Acta*, 1241, 59-116.

Moisescu, D.G. (1976) Kinetics of reaction of calcium-activated skinned frog muscle fibres. *Nature*, 262, 610-613.

Murray, B.E. & Ohlendiek, K. (1997) Cross-linking analysis of the ryanodine receptor and α_1-dihydropyridine receptor in rabbit skeletal muscle triads. *Biochemical Journal*, 324, 689-696.

Nakai, J., Dirksen, R.T., Nguyen, H.T., Pessah, I.N., Beam, K.G. & Allen, P.D. (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature*, 380, 72-75.

Nakajima, S. & Gilal, A. (1980) Radial propagation of muscle action potentials along the tubular system examined by potential-sensitive dyes. *Journal of General Physiology*, 76, 751-762.

Natori, R. (1954) The property and contraction process of isolated myofibrils. *Jikei-Kai Medical Journal*, 1, 119-126.

Ogawa, Y. (1994) Role of ryanodine receptors. *Critical Reviews in Biochemistry and Molecular Biology*, 29(4), 229-274.

Ogawa, Y., Murayama, T. & Kurebayashi, N. (1999) Comparison of properties of Ca^{2+} release channels between rabbit and frog skeletal muscles. *Molecular and Cellular Biochemistry*, 190, 191-201.

Owen, V.J., Lamb, G.D., Stephenson, D.G. & Fryer, M.F. (1997) Relationship between depolarization-induced force responsese and Ca^{2+} content in skeletal muscle fibres of rat and toad. *Journal of Physiology*, 498, 571-586.

Peachey, L.D. (1965) The sarcoplasmic reticulum and the transverse tubules of the frog's sartorius. *Journal of Cell Biology*, 25, 209-231.

Posterino, G.S. & Lamb, G.D. (1996) Effects of reducing agents and oxidants on excitation-contraction coupling in skeletal muscle fibres of rat and toad. *Journal of Physiology*, 496.3, 809-825.

Posterino, G.S. & Lamb, G.D. (1998a) Effect of nifedipine on depolarization-induced force responses in skinned skeletal muscle fibres of rat and toad. *Journal of Muscle Research & Cell Motility*, 19, 53-65.

Posterino, G.S. & Lamb, G.D. (1998b) Investigation of the effect of inositol trisphosphate in skinned skeletal muscle fibres with functional excitation-contraction coupling. *Journal of Muscle Research & Cell Motility*, 19, 67-74.

Posterino, G.S., Lamb, G.D. & Stephenson, D.G. (2000) Twitch and tetanic force responses and longitudinal propagation of action potentials in skinned skeletal muscle. *Journal of Physiology*, 527.1, 131-137.

Rall, J. (1996) The role of parvalbumin in skeletal muscle relaxtion. *News in Physiological Sciences*, 11, 249-255.

Rios, E. & Pizzaro, G. (1991) Voltage-sensor of excitation-contraction coupling in skeletal muscle. *Physiological Reviews*, 71, 849-908.

Salama, G., Abramson, J.J. & Pike, G.K. (1992) Sulphydryl reagents triggers Ca^{2+} release from the sarcoplasmic reticulum of skinned rabbit psoas fibres. *Journal of Physiology*, 454, 389-420.

Schneider, M.F. & Chandler, W.K. (1973) Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature*, 242, 244-246.

Schneider, M.F. (1994) Control of caclium release in functioning muscle fibres. *Annual Review of Physiology, 56, 463-484.*

Schwaller, B., Dick, J., Dhoot, G., Carroll, S., Vrbova, G., Nicotera, P., Pette, D., Wyss, A., Bluethmann, H., Hunziker, W. & Celio, M.R. (1999) Prolonged contraction relaxation cycle of fast-twitch muscles in parvalbumin knockout mice. *American Journal of Physiology*, 276, C395-403.

Sitsapesan, R. & Williams, A.J. (1997) Regulation of current flow through ryanodine receptors by luminal Ca^{2+}. *Journal of Membrane Biology*, 159, 179-185.

Stephenson, D.G. & Lamb, G.D. (1992) Confocal imaging of key intracellular compartments in muscle fibres. *Proceedings of the Australian Physiological and Pharmacological Society*, 23, 244P.

Stephenson, D.G., Nguyen, L.T. & Stephenson, G.M.M. (1999) Glycogen content and excitation-contraction coupling in mechanically skinned muscle fibres of the cane toad. *Journal of Physiology*, 519(1),177-187.

Stephenson, E.W. (1981) Activation of fast skeletal muscle: contributions of studies on skinned fibres. *American Journal of Physiology*, 240, C1-C19.

Stephenson, E.W. (1985) Excitation of skinned muscle fibres by imposed ion gradients. I. Stimulation of ^{45}Ca efflux at constant [K][Cl] product. *Journal of General Physiology*, 86, 813-832.

Tanabe, T., Beam, K.G., Adams, B.A., Nicodome, T. & Numa, S. (1990) Regions of skeletal muscle dihydropyridine receptor critical fro excitation-contraction coupling. *Nature*, 346, 567-569.

Walker, J.W., Somlyo, A.V., Goldman, Y.E., Somlyo, A.P. & Trentham, D.R. (1987) Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5-trisphosphate. *Nature*, 327, 249-252.

Westerblad, H. & Allen, D.G. (1994) The role of sarcoplasmic reticulum in relaxation of mouse muscle; effects of 2,5-di(tert-butyl)-1,4-benzohydroquinone. *Journal of Physiology*, 474.2, 201-301.

Yin, C.C. & Lai, F.A. (2000) Intrinsic lattice formation by the ryanodine receptor calcium-release channel. *Nature Cell Biology*, 2, 669-671.

THE POWER OF SINGLE CHANNEL RECORDING AND ANALYSIS:
Its application to ryanodine receptors in lipid bilayers

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Summary

Since the inception of the patch-clamp technique, single channel recording has made an enormous impact on our understanding of ion channel function and its role in membrane transport and cell physiology. However, the impact of single channel recording methods on our understanding of intracellular Ca^{2+} regulation by internal stores is not as broadly recognized. There are several possible reasons for this. First, ion channels in the membranes of intracellular organelles are not directly accessible to patch pipettes, requiring other methods, which are not as widely known as the patch-clamp techniques. Secondly, bulk assays for channel activity have proved very successful in advancing our knowledge of Ca^{2+} handling by intracellular stores. These assays include Ca^{2+} imaging, ryanodine binding assays and measurements of muscle tension and Ca^{2+} release and uptake by vesicles that have been isolated from internal stores. This review describes methods used for single channel recording and analysis, as applied to the calcium release channels in striated muscle, and details some of the unique contributions that single channel recording and analysis have made to our current understanding of the release of Ca^{2+} from the internal stores of muscle. With this in mind, it focuses on three aspects of channel function and shows how single channel investigations have led to an improved understanding of physiological processes in muscle. Finally, it describes some of the latest improvements in membrane technology that will underpin future advances in single channel recording.

Regulation of intracellular [Ca^{2+}] by internal stores in striated muscle

In many cell types the intracellular free calcium ion concentration is altered by the uptake and release of calcium from internal stores such as the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). In striated muscle, intracellular calcium concentration, and hence muscle force and cardiac output, is regulated by release of calcium from the SR via ryanodine receptor calcium channels (RyRs) and uptake via the Ca-ATPase. Ca^{2+} fluxes across the SR often take place in the presence of a changing cytoplasmic milieu during episodes of metabolic challenge such as that seen during hypoxia, ischaemia and fatigue. For example, the large changes in the concentration of cytoplasmic anions such as inorganic phosphate, phosphocreatine, and ATP seen during muscle fatigue have profound effects on SR Ca^{2+} handling. In striated muscle the depolarization of the surface membrane and transverse-tubular (T) system by an action potential (the T-system is an invagination of the surface membrane) triggers calcium release from the SR by a process known as excitation-contraction coupling (EC coupling). Dihydropyridine receptors (DHPRs, L-type calcium channels in the T-system) act as voltage-sensors that detect depolarization due to an action potential. Depolarisation induced activation of DHPRs somehow activates RyRs in the apposing SR membrane. In cardiac muscle the influx of Ca^{2+} through DHPRs is believed to activate RyRs (Nabauer *et al.*, 1989) whereas in skeletal muscle DHPRs are mechanically coupled to RyRs (Tanabe *et al.*, 1990) so that Ca^{2+} influx through the DHPRs receptors is not a prerequisite for muscle contraction (Ashley *et al.*, 1991). However, the specific details of EC coupling are not understood, specifically how it is modulated or limited by various cytoplasmic and luminal factors.

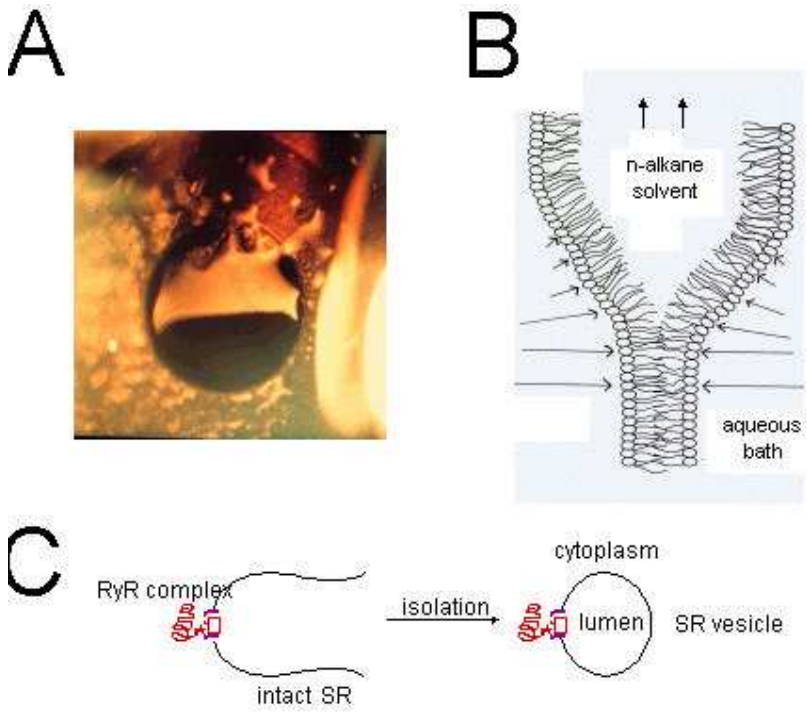

Figure 1. *Formation of lipid bilayers and incorporation of RyRs. (A) A photograph of a lipid bilayer (bottom half of aperture) during its formation from a thick lipid film (top half). The lipid film was spread across an aperture (~100mm diameter) in a delrin septum. The thick lipid film strongly reflects the incident light whereas the bilayer, which shows the black background, is totally transparent. The bilayer portion of the film spreads across the entire aperture in a few seconds leaving a region of thick film at the periphery. (B) A schematic diagram of the process of lipid bilayer formation showing the lipid monolayers that the two oil-water interfaces. The lengths of the arrows, which are shown in each aqueous phase, indicate the relative strengths of the Van der Waals compressive forces between the adjacent water phases. This compressive force squeezes the oil (n-alkane in this case) out from between the monolayers. (C) The procedure for incorporating ion channels form the SR (RyRs in this case) into lipid bilayers. Vesicles of SR membrane (~0.1 mm diameter) are isolated from muscle tissues using differential centrifugation methods. SR vesicle containing ion channels are added to the bath near the lipid bilayer. Fusion of the vesicles with the bilayer carries ion channels into the bilayer membrane.*

The RyR is a homotetramer of ~560 kDa subunits containing ~5035 amino acids. Electron microscope image reconstruction shows RyRs to have four-fold symmetry with a large cytoplasmic domain (the foot region) and a relatively small transmembrane region that forms the Ca^{2+} pore (Orlova *et al.*, 1996). The trans membrane pore is comprised of the ~1000 C-terminal amino acids (aa 4000-5000) and the remaining amino acids form the foot region. In mammals, three isoforms of RyRs have been cloned and sequenced: namely, ryr-1 found in skeletal muscle and brain, ryr-2 most abundantly found in brain and cardiac muscle and ryr-3, though originally found in the brain, is the major isoform in smooth muscle (Ogawa, 1994).

The Bilayer method

At the same time that Neher and Sakmann were developing the patch-clamp technique (Neher and Sakmann, 1976), Miller and Racker (1976) discovered that SR vesicles isolated from muscle could be fused with artificial lipid bilayers and so incorporate ion channels from muscle membranes into artificial membranes. Artificially produced, planar, bimolecular lipid membranes (bilayers) were originally used as model systems for studying cell membrane structure. Their large area (they can be produced with diameters ranging from 1 μm to 1 cm) and planar geometry made them particularly convenient membrane models for electrical and mechanical measurements. For the purposes of studying ion channels, bilayers are usually formed using a modification of the film drainage method developed by (Mueller *et al.*, 1962). A solution of lipids in a hydrophobic solvent (usually n-decane) is smeared across a hole in a plastic septum (eg. Delrin, polycarbonate or Teflon) to produce a thick lipid film separating two baths (Fig. 1A). The bilayer forms spontaneously from this thick lipid film. During bilayer formation the surface-active lipids aggregate into monolayers at the oil-water interfaces on each side of the thick film. The solvent drains away from between the two monolayers thus allowing their apposition and formation of the bilayer structure (Fig. 1B).

Incorporation of ion channels into bilayers is usually simply done by adding ion channel protein to one of the baths and stirring. Ion channel incorporations occur spontaneously and can be detected by conductance changes in the bilayer membrane. For studying the SR ion channels, the bilayers are produced with a diameter of ~100 μm. SR vesicles are added to a final concentration of 1-10 μg/ml and the bath is stirred until channel activity indicates vesicle fusion with the bilayer. The side of the bilayer to which the vesicles are added is usually defined as the *cis* side (Fig. 1C). Conditions that promote vesicle fusion are: 1) a gradient in osmotic potential across the membrane (*cis* high), 2) *cis* $[Ca^{2+}]$ at mM concentrations and 3) vigorous stirring of the *cis* bath. The cytoplasmic side of the SR membrane, when fused with the bilayer, faces the *cis* bath and the luminal side faces the *trans* bath. Stirring of the *cis* and *trans (*cytoplasmic and luminal*)* chambers is usually done using magnetic stirrers.

Cesium methanesulfonate (CsMS) is commonly used as the principal salt in the bathing solutions. This is to prevent current signals from other ion channels from interfering with RyR recordings: the RyR is quite permeable to Cs^+ whereas other ion channels in the SR do not conduct MS^- and Cs^+. Experiments also use a $[Cs^+]$ gradient across the bilayer (250 mM *cis* and 50 mM *trans*) to promote vesicle fusion (see above).

When a vesicle has fused with a bilayer the ion channels embedded in the vesicle membrane become incorporated into the bilayer. Once this happens it is possible to determine the ionic conductance of a single channel and to monitor it's opening and closing (gating) by measuring the current through the membrane in response to an applied electrochemical gradient. The bilayer technique allows considerable flexibility in manipulating the experimental conditions. One can examine the response of channels to a variety of substances in the cytosolic and luminal baths as well as changes in the composition of the bilayer itself. One can also measure channel function under steady-state conditions or when solutions are rapidly and transiently altered. With this experimental technique, like with the patch-clamp technique, it is possible to obtained very detailed information about mechanisms determining channel conductance and gating.

Single channel analysis.

This section is devoted to examining the most commonly used methods for describing the properties of ion channel signals and inferring their mechanisms of function. Single channel signals generally appear as a series of stochastic current jumps between stationary current levels (e.g. see Fig. 2A). For most channel types the jumps are mainly between the closed state and a maximum level (the

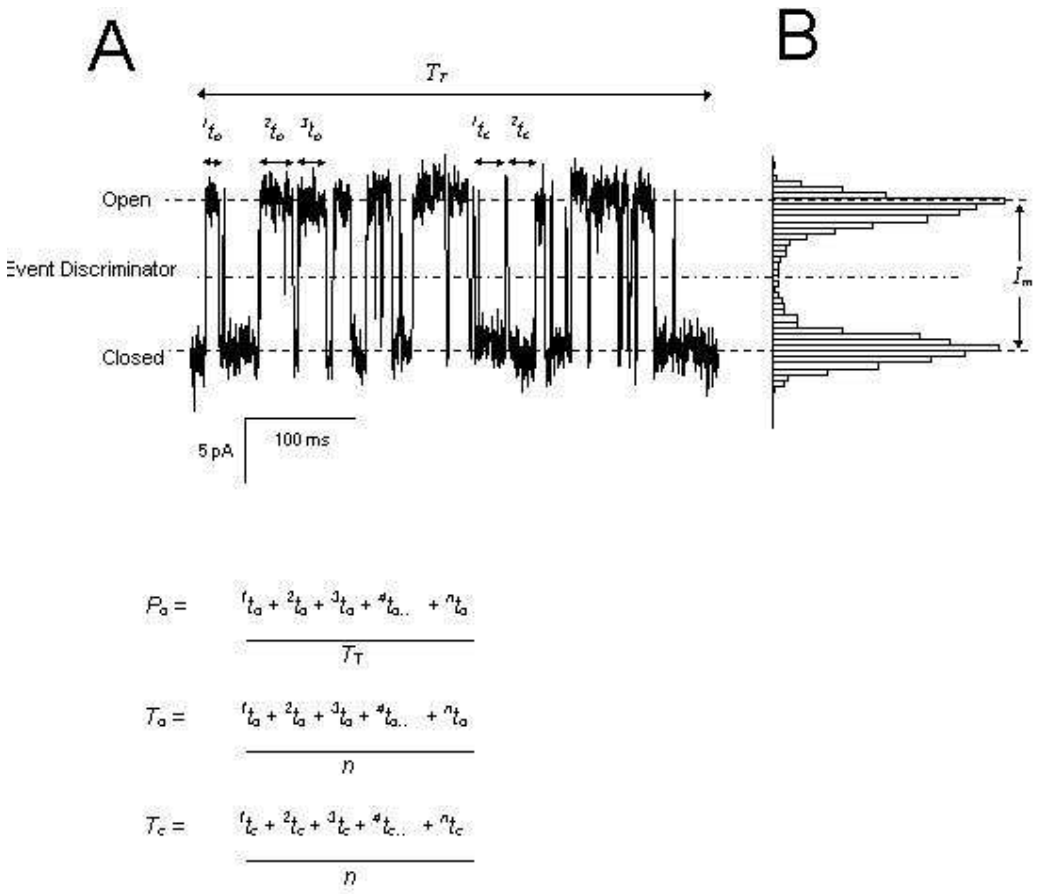

Figure 2. *Analysis of single channel parameters. (A) A typical example of a current signal from a single RyR (left) where channel openings are marked by upward transitions in the current. The dotted lines indicate the current levels corresponding to the open and closed channel. The dashed line shows the current threshold, which defines open and closed events in the analysis. (B) An amplitude histogram of the data in part A which shows a bimodal distribution with peaks corresponding to the stationary current levels (i.e. Open and Closed). The maximum unitary current is I_m. The open and closed dwell times are given by the parameters, ${}^nt_o$ and ${}^nt_c$ respectively. The equations show how open probability, P_o, mean open dwell-time, T_o and mean open dwell-time, T_c, are calculated from the dwell-times.*

unitary current, I_m). Intermediate current levels correspond to subconductance (substates) of the channel.

Channel function is broadly characterised by the amplitudes and durations of stationary current levels. The most popular method for visualizing the different current levels from a channel signal is the all-points amplitude histogram. This is a histogram of all data points grouped according to their amplitude (Fig. 2B). Peaks in the histogram correspond to sustained current levels in the record. The width of the peaks is proportional to the size of the background noise and the area under each peak is proportional to the total time spent at that level. An overall measure of the channel activity can be obtained from the open probability, P_o and the fractional, mean current, *I*. P_o is the fraction of time the channel is in a conducting state and is calculated from the ratio of the number of data points in conducting levels and the total number of points in the record (assuming equally spaced data samples). The fractional, mean current is equal to the time-average of the current amplitude divided by I_m. A value of one indicates that the channel is never closed and a value of zero indicates that the channel is never open. For ion channels with only one open conductance level P_o and *I* give the same value.

Although these parameters provide an overall picture of channel activity, they do not provide much more information than what can be obtained from bulk assays of channel activity.

A more detailed and rewarding analysis of channel activity can be derived from the statistics of amplitudes and durations (i.e. dwell-times: the times spent at each current level before it jumps to a new value). An overall picture of channel gating rates is encapsulated in the mean open and closed dwell-times (T_o and T_c, see Fig. 2). Frequency histograms of open and closed dwell-times graphically show the kinetic signature of the gating mechanism and provide clues to the underlying gating mechanisms (see below). The frequency distributions of dwell-times can be well described by the sum of decaying exponential functions. There are several graphical methods for displaying these histograms, two of which are shown in Figure 3A. The most useful types of plot is that developed by Sigworth and Sine (1987) which is shown in Figure 3B. The data are grouped into bins that are equally spaced on a log scale. In this log-binned scale the broadening of the bins at longer times increases the number of counts in each bin which tends to counter the exponential decline seen in uniformly binned distributions. Distributions that are exponentially distributed on the linear scale form peaked distributions in log-binned histograms where each peak corresponds to an exponential component of the distribution. Probability distributions of dwell-times can be obtained from frequency distributions by dividing the data by the total number of events in the distribution (i.e. the area under the curve becomes one). In addition when the square root of the probability is plotted, the statistical scatter on the data becomes uniform across the entire distribution.

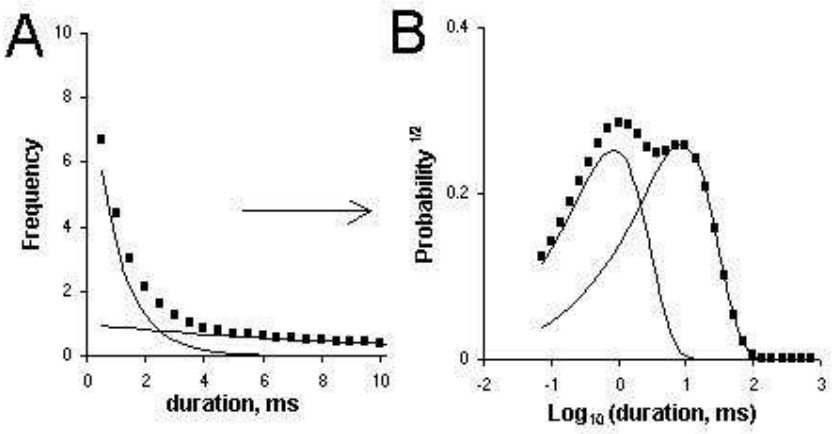

Figure 3. *Frequency histograms of open and closed dwell-times. (A) A fictitious distribution of dwell times, typical of that obtained from single channel recordings, which is comprised of two exponential components with distinctly different decay constants (time constants). (B) The probability distribution from the same dwell-time data is plotted using log-spaced bins (i.e. they appear equally spaced in the log-time scale). The wider bins (in absolute terms) at the right end of the scale tend to collect more data counts than the narrower bins at the left. The probability distribution is calculated by normalizing the frequency distribution according to the area under the curve (see text). Hence the two exponential decays in part A assume a double peaked distribution in part B. The locations of the peaks on the time-scale approximately correspond to the time constants of each exponential.*

From the kinetic signature it is possible to make inferences about the mechanisms underlying the gating processes of the channels. This is illustrated here with a simulated single channel recording based on a six-state gating scheme with three open and three closed states (Fig. 4A). The timing of transitions between states is calculated from the reaction rates using a stochastic algorithm. Figure 4B (circles) shows probability distributions (log-binned) of open and closed dwell-times obtained from the simulated recording. These open and closed probability distributions show three exponential time constants, which are manifest as three peaks in the distributions. Each peak corresponds to a different open or closed state of the reaction scheme. In this simple case, each exponential time constant in Figure

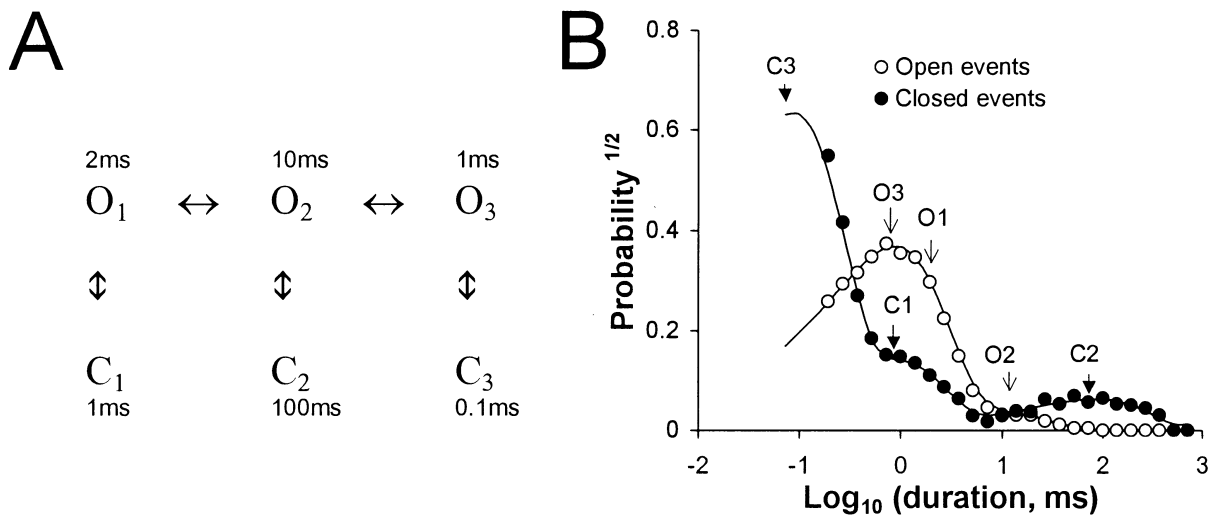

Figure 4. (A) *A reaction scheme describing some of the aspects of RyR gating in which the channel can adopt three different open and closed states. Rather than showing all the reaction rates just the mean lifetimes of each state is shown here. (B) A probability (the square root) distribution of open (○) and closed (●) dwell-times obtained from a simulated, single channel recording which was generated from a gating mechanism given by the Scheme shown in part A (see text). The arrows indicate the peaks in the distributions that correspond to the various states in the gating scheme. The solid lines are theoretical probability functions derived from the gating scheme in part A, using the method of Colquhoun and Hawkes (1981).*

4B (arrows) corresponds approximately in value to the average time spent in each state in Figure 4A. Generally, the number of exponential components observed in the open and closed dwell time distributions gives a lower estimate of the number of different states (i.e. protein conformations) associated with channel open and closed events respectively. It is also possible to predict theoretical probability distributions from the reaction rates using methods detailed by Colquhoun and Hawkes (1981). The theoretical predictions from the gating scheme are shown as solid curves in Figure 4B. Thus by fitting the data with these predictions it is possible to model the data in terms of rate constants between different conformational states of the channel.

A Study of Ca^{2+} and Mg^{2+} regulation of RyRs: An example of single channel analysis

The gating of RyRs depends on cytoplasmic Ca^{2+} and Mg^{2+} concentrations. RyRs from skeletal and cardiac muscle (ryr-1 and ryr-2 respectively) are activated by μM Ca^{2+} and inhibited by mM Ca^{2+} and Mg^{2+}. Several studies show that Mg^{2+} is a strong inhibitor of Ca^{2+} release in skeletal muscle (Owen *et al.*, 1997) and plays an important role in EC coupling (Lamb & Stephenson, 1991, Lamb & Stephenson, 1992). $^{45}Ca^{2+}$ release from skeletal SR vesicles suggests that regulation of RyRs by Mg^{2+} and Ca^{2+} is tied to two common mechanisms (Meissner *et al.*, 1986) but it was recognized that confirmation of that hypothesis awaited detailed single channel experiments. This section will show how single channel measurements of the Mg^{2+}- and Ca^{2+}-dependent gating kinetics in skeletal and cardiac RyRs identified two mechanisms for Mg^{2+}-inhibition. Details of this work can be found in (Laver *et al.*, 1997a, Laver *et al.*, 1997b, Laver *et al.*, 1995).

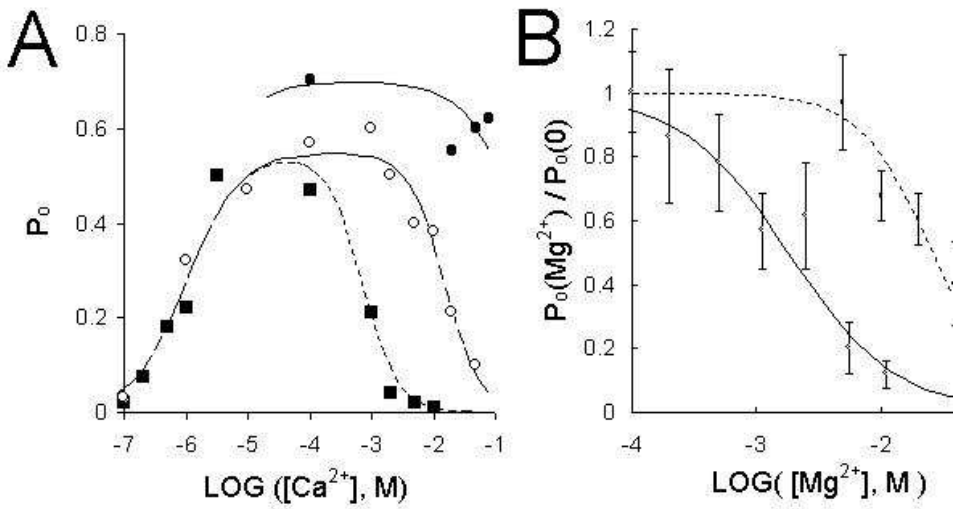

Figure 5. (*A) The dependence of the P_o on the cytoplsmic [Ca^{2+}] from three groups of RyR. (●)-sheep cardiac RyRs treated with CHAPS or high [CsCl] that were insensitive to inhibition by mM cis Ca^{2+}, (○)- native cardiac RyRs which could be inhibited by mM cis Ca^{2+}, (■)- rabbit skeletal RyRs. (B) The [Mg^{2+}]-dependence of the P_o of cardiac RyR in the presence of 1 mM Ca^{2+} (○) or 1 mM Ca^{2+} (●). The lines show Hill fits to the data (see below) using the following parameters: (solid line)- H = 1, K_m = 1.8 mM. (dashed line)- H = 1.5, K_m = 26 mM. The Hill equation used here relates the degree of channel inhibition by Mg^{2+} (P_o/P_ocontrol) to its binding affinity (K_m), Hill coefficient (H) and concentration:*

Analysis of the effects of non-physiological [Ca^{2+}] and [Mg^{2+}] can identify multiple mechanisms that are difficult to distinguish under physiological conditions.

It has long been recognized that there are two distinct Ca^{2+} regulation mechanisms in RyRs: one that activates them at μM cytoplasmic [Ca^{2+}] and another that inhibits them at mM [Ca^{2+}] (Meissner, 1994). This case study focuses on three groups of RyRs: Rabbit skeletal RyRs (■), sheep cardiac RyRs(○) and modified cardiac RyRs(●) which have been exposed to 500 mM CsCl or CHAPS detergent and so have lost their sensitivity to Ca^{2+} inhibition. An overall picture of the regulation of the three types of RyR by cytoplasmic Ca^{2+} is obtained from measurements of channel open probability (P_o) in Figure 5A. The three RyR types were similarly activated by cytoplasmic [Ca^{2+}] of ~ 1μM but they were differently inhibited by Ca^{2+}. Cardiac RyRs are, on average, 10 fold less sensitive to Ca^{2+} inhibition than skeletal RyRs, and cardiac RyR treated with CHAPS were not significantly inhibited by [Ca^{2+}], even at 100 mM. Mg^{2+} inhibited the three RyR groups. Once again, measurements of P_o give the overall picture of this inhibition. Increasing concentrations of Mg^{2+} progressively reduce the activity of RyRs (Figs. 5B and 6). Mg^{2+} differently inhibited the three RyR groups. Figure 7A shows the concentration of Mg^{2+} needed to reduce the P_o of RyRs by 50%, plotted against the Ca^{2+} concentration. The data in Figure 7 shows two broad features in the [Ca^{2+}] dependence of Mg^{2+} inhibition. One of these is an ascending limb where increasing [Ca^{2+}] causes the channel to become less sensitive to inhibition by Mg^{2+}. At low [Ca^{2+}] the Ca^{2+}-dependence of Mg^{2+} inhibition of the cardiac RyRs (both native and treated with CHAPS or CsCl) clearly show this ascending limb. There is also some indication of this with the skeletal RyRs at very low [Ca^{2+}]. The other feature is the plateau region, which occurs at higher [Ca^{2+}] where Mg^{2+} inhibition is insensitive to [Ca^{2+}]. This is clearly seen in the data from skeletal and cardiac RyRs in Figure 7A, although this was not apparent in the data obtained from RyRs treated with CHAPS or CsCl. A model for explaining the P_o data is shown schematically in Figure 8 in which there are two Ca^{2+} and Mg^{2+} dependent gates acting in series. One gate opens (activation gate) when the [Ca^{2+}] increases above 1 μM giving rise to Ca^{2+}-

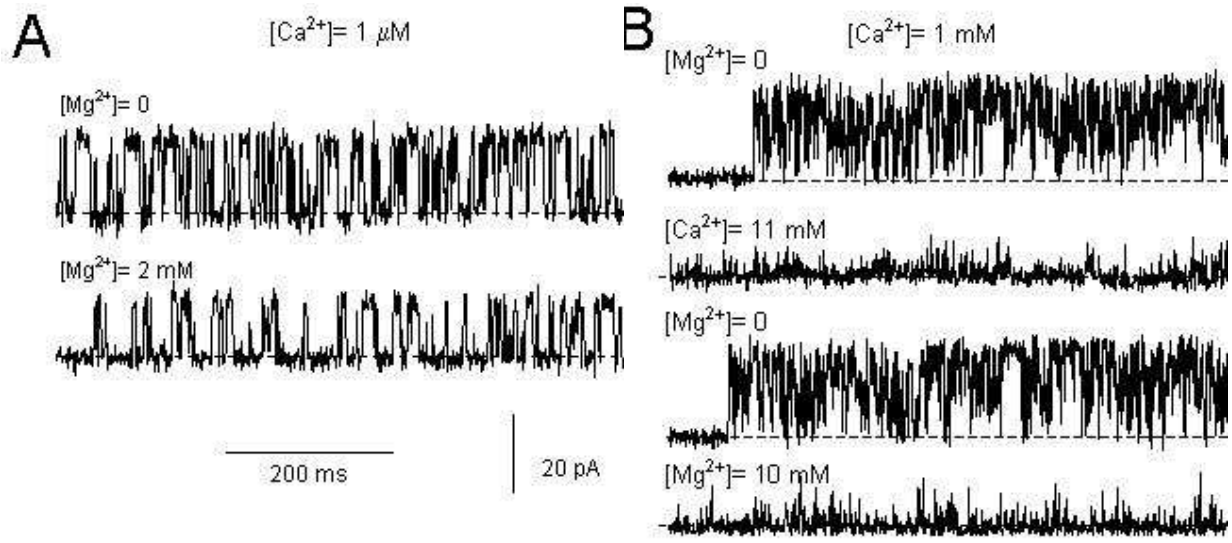

Figure 6. *Single channel recordings of sheep cardiac RyRs in lipid bilayers showing the effects of Mg^{2+}-inhibition on the pattern of channel gating. The cis bath contained 250 mM CsCl and the trans bath contained 50 mM CsCl. The potential difference across the bilayer is 40 mV (cis-trans) and the current baseline is at the bottom of each trace (dashed lines). (A) Mg^{2+}-inhibition in 1 mM Ca^{2+} appears to increase the duration of channel closures. (B) 10 mM Ca^{2+} was added to a RyR initially in 1 mM Ca^{2+} and this inhibits the channel. The cis bath was flushed with solutions containing 1mM Ca^{2+} and maximal channel activity was restored. Then 10 mM Mg^{2+} was added to the cis bath. Gating pattern of the RyR inhibited by 10 mM Mg^{2+} + 1 mM Ca^{2+} appeared to be the same as that inhibited by 11 mM Ca^{2+} alone. Mg^{2+}-inhibition in 1 mM Ca^{2+} also appeared to induce a more flickery gating pattern than in 1 mM Ca^{2+}.*

activation of the RyR. Another gate closes (inhibition gate) when [Ca^{2+}] rises to mM levels. Mg^{2+} at the activation gate reduces P_o by competing with Ca^{2+} for activation sites. However, Mg^{2+} is unable to open the channel. At the inhibition gate, Mg^{2+} and Ca^{2+} can each bind at the inhibition site and cause channel closure by a common mode of action. In this model the two gating mechanisms are assumed to operate concurrently and independently. The combined effect of both gates is such that the open probability of the channel is equal to the product of the open probabilities of each gate. It follows that the gating of the channel will tend to be dominated by the gate that is least open (see Fig. 7B) which explains why different inhibition mechanisms become apparent at low and high [Ca^{2+}].

Analysis of single channel gating kinetics can provide a stringent test for gating models.

If Ca^{2+} and Mg^{2+} do inhibit via a common mode of action then the kinetic signatures of Ca^{2+} and Mg^{2+} inhibition should be identical in the plateau region of the data (see Fig. 7). Moreover, the kinetic signature of Mg^{2+} inhibition in the plateau region should be different to that seen in the ascending limb since it is assumed that these two features arise from different mechanisms. Figure 9 shows that this is the case. Single channel recordings of native cardiac RyR inhibited by Mg^{2+} in the presence of low (1 μM) and high (1 mM) [Ca^{2+}] are shown in Figure 6 (different inhibition mechanism should be apparent at low and high [Ca^{2+}] see above). Inhibition by 2 mM Mg^{2+} in the presence of 1 μM Ca^{2+} reduced P_o to 50% of the control value and inhibition by10 mM Mg^{2+} in the presence of 1 mM Ca^{2+} also reduced P_o by 50%. Even though Mg^{2+} produced the same degree of inhibition in both cases, inspection of the pattern of gating in these records shows that Ca^{2+} and Mg^{2+} have very different effects on channel gating. Moreover, at high [Ca^{2+}], addition of either Ca^{2+} or Mg^{2+} produced

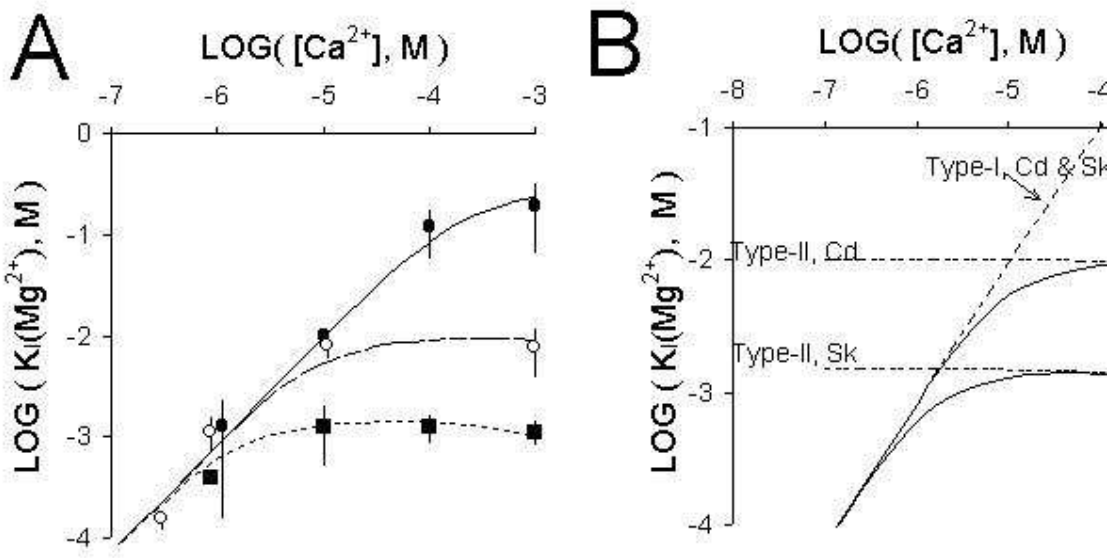

Figure 7 *(A) The mean (± sem) cis [Mg^{2+}] causing 50% inhibition of RyRs, $K_I(Mg^{2+})$, plotted against cis [Ca^{2+}] for three groups of RyR, namely, (●)-sheep cardiac RyRs treated with CHAPS or 500 mM [CsCl] that were insensitive to inhibition by mM cis Ca^{2+}, (○)- native cardiac RyRs which could be inhibited by mM cis Ca^{2+}. (■)- Rabbit skeletal RyRs. Three model predictions for the Ca^{2+}-dependence of $K_I(Mg^{2+})$, shown in part B, are compared with the data. (B) Model predictions for $K_I(Mg^{2+})$ showing the relative contributions of Mg^{2+}-inhibition at the activation and inhibition gates of cardiac (Cd) and skeletal (Sk) RyRs. The solid lines are two of the model fits to the data in Part A. The only difference between the two fits are due to differences in the affinity of Ca^{2+} and Mg^{2+} at the inhibition gate. The thick dashed lines show the Ca^{2+}-dependence of the [Mg^{2+}] required to halve the open probability of the activation and inhibition gates separately. It can be seen that for the cardiac RyRs (upper solid line) $K_I(Mg^{2+})$ at high and low [Ca^{2+}] extremes are similar to that expected solely from each inhibition mechanism separately.*

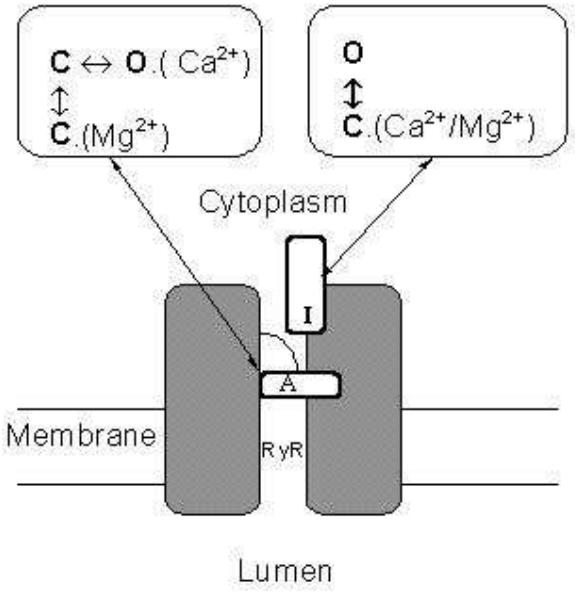

Figure 8. *A schematic diagram of a RyR which illustrates the main aspects of the model for Mg^{2+}-inhibition of RyRs. Gating mechanisms for Ca^{2+}-activation and Ca^{2+}-inhibition of the RyR are labelled (A) and (I) respectively. These gates are assumed to operate independently and such that both gates must be open for the channel to conduct. At the activation gate inhibition occurs when Mg^{2+} binds and prevents opening of the activation gate by competing with Ca^{2+} for the activation site. However, unlike Ca^{2+} binding (~1 mM affinity) the binding of Mg^{2+} at this site (~1 mM affinity) does not open the channel. At the inhibition gate, inhibition occurs with the binding of Mg^{2+} or Ca^{2+} (~ mM affinity) at a common set of sites.*

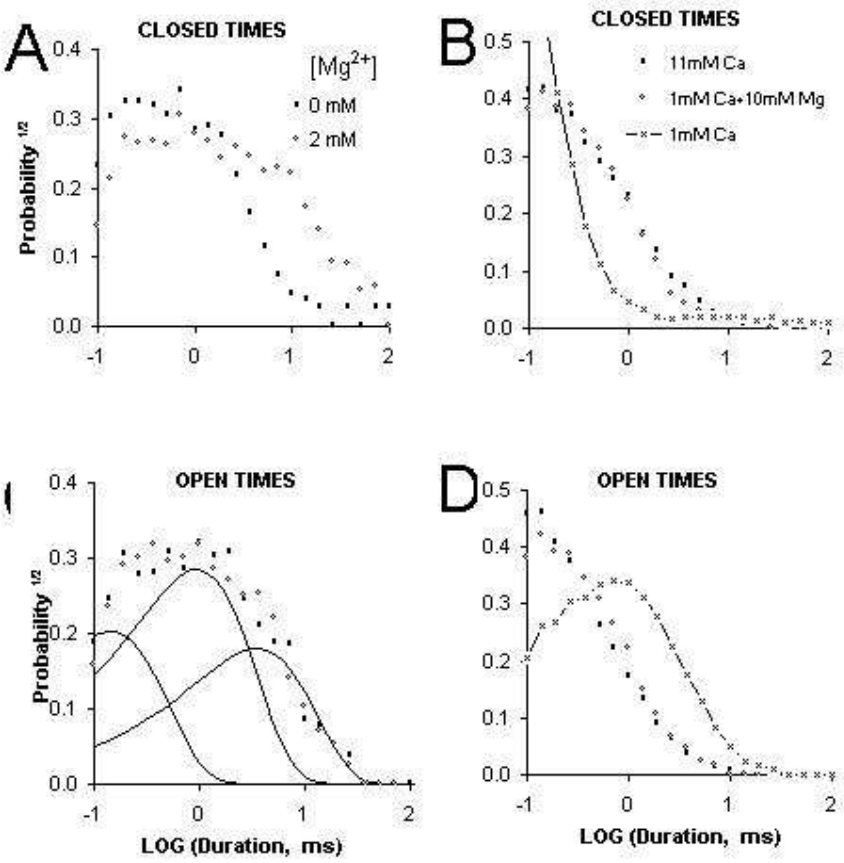

Figure 9. *Probability distributions of open and closed dwell times obtained from the same records as shown in Figure 6. (A&C) The effect of Mg^{2+} on the probability distributions closed (A) and open (C) dwell-times of cardiac RyRs in the presence of 1 mM cis [Ca^{2+}]. The probabilities were calculated from number of events/ bin divided by the total number of events (~1500 events in each record). The histograms were extracted from single channel recordings of ~15 second duration. The dwell-times are "log binned" and displayed using the approach of Sigworth and Sine (1987). The distributions of open and closed dwell-times could be fit by the sum of three exponentials shown separately by the three curves in part C. (B&D) The effect of Mg^{2+} on the probability distributions of channel closed (B) and open (D) dwell-times of cardiac RyRs in the presence of 1 mM [Ca^{2+}]. The gating of a single cardiac RyR was initially measured when the cis bath contained 1 mM $CaCl_2$ (æ xæ). The measurement was repeated after 10 mM $CaCl_2$ was added to the bath (open circles). The cis bath was then flushed with solution containing 1 mM $CaCl_2$. The cis [Mg^{2+}] was increased to 10 mM before the final measurement was made (closed circles). In both experiments a 50% inhibition of the RyR, by the addition of either 10mM Ca^{2+} or Mg^{2+}, had identical effects on channel gating.*

inhibition with a similar gating pattern. Probability histograms of open and closed dwell-times quantify these Mg^{2+}-inhibition effects on the channel gating. Inhibition by Mg^{2+} at low [Ca^{2+}] significantly increased the probability of long closed dwell-times (Fig. 9A) but produced no significant change in the open dwell-time distribution (Fig. 9C). In contrast, Mg^{2+} inhibition a high [Ca^{2+}] both increased the probability of long closed dwell-times (Fig. 9B) and increased the probability of short open dwell-times (Fig. 9D). Thus Mg^{2+} inhibition clearly has a different kinetic signature at high [Ca^{2+}] than at

low [Ca^{2+}]. However, the kinetic signatures of Ca^{2+} and Mg^{2+} inhibition at high [Ca^{2+}] are identical indicating that Ca^{2+} and
Mg^{2+} inhibit RyRs by modulating the same gating mechanism. It is highly unlikely that different mechanisms as complex as these (complex meaning that they are described by 6 exponentials and11 independent parameters) could, by coincidence, produce the same gating pattern.

Biophysical characterization of RyR regulation mechanisms has physiological relevance.

The fact that Ca^{2+} and Mg^{2+} share a common inhibitory mechanism provides an answer to the question of why RyRs are inhibited by mM cytoplasmic Ca^{2+} when [Ca^{2+}] never reach this level in muscle. Once it is realized that Mg^{2+} inhibition shares a common mechanism with Ca^{2+} and that Mg^{2+} is present at mM concentrations the answer becomes apparent. Thus it is likely that inhibition by Mg^{2+} is the physiologically relevant mechanism (as suggested by Lamb, 1993) and by focusing on Ca^{2+}-inhibition of RyRs one misses the physiologically important process. This interpretation sheds a new light on the molecular basis of Malignant Hyperthermia. Malignant hyperthermia (MH) is an inherited skeletal muscle disorder of humans and pigs that can be triggered in susceptible individuals by anesthetics, such as halothane, and by certain other agents and even by stress (Mickelson & Louis, 1996). The disorder is due to abnormal regulation of intracellular [Ca^{2+}] in the muscle cells due to over active RyRs. If an MH episode is initiated, it results in muscle rigidity, severe metabolic changes and excessive heat production, often leading to death if untreated. Porcine Malignant Hyperthermia is associated with the Arg615Cys mutation in the RyR, which alleviates RyR inhibition at mM [Ca^{2+}] (Mickelson & Louis, 1996). Because cytoplasmic [Ca^{2+}] never reaches mM levels it was not clear how the RyR mutation caused Malignant Hyperthermia. However, once it is realized that Ca^{2+} and Mg^{2+} share the same inhibitory mechanism then alleviation of Mg^{2+} inhibition becomes a plausible mechanism for altered Ca^{2+} release by RyRs. At physiological [Mg^{2+}] (~1 mM) RyRs from Malignant Hyperthermia susceptible muscle are less depressed by Mg^{2+} than normal RyRs. Therefore MH RyRs are more readily activated by any stimulus because cytoplasmic Mg^{2+} does not hold them as tightly shut as normal RyRs and this is a likely cause of the abnormally high Ca^{2+} release associated with this myopathy (Laver *et al.,* 1997b).

Variations between individual RyRs and different RyR types provide additional information about regulation mechanisms.

It is commonly found in single channel studies that the gating properties of ion channels differ to some extent from one channel to the next and RyRs are no exception to this. Several studies have focused on the heterogeneity of RyRs in bilayers (eg. Laver *et al.,* 1995, Copello *et al.,* 1997). While RyRs of one type all share common regulation mechanisms, there are substantial variations in RyR sensitivity to regulatory ligands such as ATP (Laver *et al.,* 2000b), Ca^{2+} (Laver *et al.,* 1995), Mg^{2+} (Laver *et al.,* 1997b, Laver *et al.,* 1997a) and pH (Laver *et al.,* 2000a). This variability provides an opportunity to examine correlations between different properties of the RyR that would suggest common underlying mechanisms. For example, if Ca^{2+} and Mg^{2+} do inhibit RyRs by a common mechanism then their sensitivity to Ca^{2+} should be correlated with their sensitivity to Mg^{2+}. The Mg^{2+} sensitivity of cardiac RyRs varied by an order of magnitude between individual channels. Half inhibition by Mg^{2+} in the presence of high [Ca^{2+}] (in the plateau region, see Fig. 7) occurred at [Mg^{2+}] ranging from 2 to 10 mM. The sensitivity to Ca^{2+} and Mg^{2+} inhibition in the variable RyR population were compared in the same RyR as shown in Figure 10, which shows a good correlation and equality between half inhibiting concentrations of Ca^{2+} and Mg^{2+}. In addition to variations between individual RyRs of one group there are systematic differences in the mean sensitivity of different groups of RyRs to Ca^{2+} and Mg^{2+} inhibition. In this regard, cardiac RyRs are less sensitive than skeletal RyR and skeletal RyRs with the MH mutation are less sensitive than normal skeletal RyRs. When the sensitivity

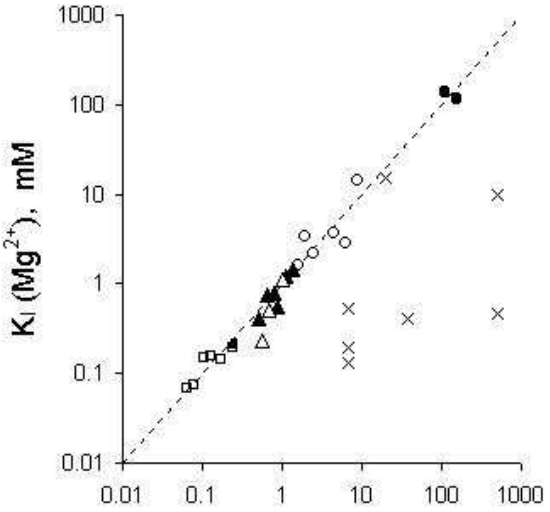

Figure 10. *Correlation between the sensitivity to inhibition by Ca^{2+} and Mg^{2+} in individual RyRs. Half inhibition by Mg^{2+}, $K_I(Mg^{2+})$, and by Ca^{2+}, $K_I(Ca^{2+})$, was determined on the same RyRs. The correlation between $K_I(Mg^{2+})$ and $K_I(Ca^{2+})$ is also shown for different groups of RyR which have, on average, different sensitivities to Ca^{2+} and Mg^{2+} inhibition. Within each group there is significant variation in these properties. Unless otherwise stated cis $[Cs^+]$=250 mM; (○)- cardiac RyRs in the presence of 1mM Ca^{2+} where they showed normal Ca^{2+}-inhibition, (●)-cardiac RyRs in the presence of 1mM Ca^{2+} where they showed reduced Ca^{2+}-inhibition because they had been exposed to cis 500 mM CsCl or CHAPS for one minute prior to measurements of divalent ion inhibition, (□)- normal pig skeletal RyRs in the presence of 50 mM Ca^{2+} and 100 mM CsCl (■)-MHS RyRs in the presence of 50 mM Ca^{2+} and 100 mM CsCl. (△)-normal pig skeletal RyRs in the presence of 50 mM Ca^{2+}, (▲)-MHS pig skeletal RyRs in the presence of 50 mM Ca^{2+}. (X)- cardiac RyR in the presence of less than 100 mM Ca^{2+}.*

of individual RyRs to Ca^{2+} and Mg^{2+} inhibition are compared across a range of RyR types there is a tight correlation and equality between the half inhibiting concentrations of Ca^{2+} and Mg^{2+} over three orders of magnitude (correlation coefficient, r = 0.96 or coefficient of determination, r^2=0.9). In contrast to this was the lack of any correlation (r = 0.17) between Ca^{2+} and Mg^{2+} inhibition when Mg^{2+} inhibition was measured in the presence of low $[Ca^{2+}]$. This clearly shows that the Mg^{2+}/Ca^{2+} equivalence does not apply under low $[Ca^{2+}]$ conditions suggesting that Mg^{2+} inhibition at low $[Ca^{2+}]$ are due to a different mechanism to Ca^{2+} inhibition.

Functional interactions between RyRs and other endogenous proteins.

Several proteins are now known to have effects on the activity of the RyR in muscle. The most notable of these is the DHPR (see above). In addition there is also calmodulin, calsequestrin, triadin, junctin and the FK506 binding protein (FKBP). These proteins are believed to form part of a large complex that is the machinery for EC coupling in muscle. With bilayer methods it has been possible to dismantle the EC-coupling machine to gain clues as to how the individual components contribute to its overall function. With the likely exception of the DHPR, native RyRs in lipid bilayers appear to remain associated with the above-mentioned co-proteins. The effects of these co-proteins on RyR activity have been studied in lipid bilayers by dissociating these proteins while simultaneously recording channel activity. Biochemical methods such as SDS-PAGE, Western blots and radio active labeling have been used to confirm that the treatments used to dissociate RyR co-proteins in lipid bilayer experiments are specific to the protein of interest. This approach has been used to study interactions between RyRs and calmodulin (Tripathy *et al.*, 1995), calsequestrin (Beard *et al.*, 2000, Beard *et al.*, 1999) and FKBP (Ahern *et al.*, 1997). So far this approach has not proved successful for studying the effect of DHPRs on RyRs in lipid bilayers. However, inroads into this area have been made using less direct means. The DHPR and RyR in skeletal muscle are believed to interact via the cytoplasmic loop region between transmembrane repeats II and III of the DHPR α_1 subunit (aa 666-791, Tanabe *et al.*, 1990). The isolated skeletal II-III loop has been added to single RyRs in bilayers and was found to activate them (Lu *et al.*, 1994). Also, synthetic peptides, encompassing regions of the skeletal II-III loop have been applied to RyRs and were found to regulate RyR activity (Dulhunty *et al.*, 1999).

An example of how single channel experiments have been used to study the functional interactions between RyRs and other proteins is the investigation of calsequestrin. Calsequestrin is a protein found in the lumen of the SR, which acts as a moderate affinity (K_D=1 x 10^{-5} M) Ca^{2+} binding protein that buffers the luminal free [Ca^{2+}] (MacLennan & Wong, 1971). Two other proteins, triadin and junctin, span the SR membrane and these proteins bind to both calsequestrin and the RyR (Knudson *et al.,* 1993, Kanno & Takishima, 1990, Zhang *et al.,* 1997, Jones *et al.,* 1995, Guo *et al.,* 1996). SDS-PAGE and Western Blotting techniques have shown that calsequestrin can be dissociated from the RyR complex by exposing SR membranes to raised ionic strength (ie 500 mM as apposed to 250 mM), or by exposing them to higher than usual [Ca^{2+}] (13 mM as apposed to 1 mM). By applying similar solutions changes to RyRs in bilayers it was possible to see the effects of a calsequestrin dissociation event during recordings of RyR activity. To do this the ionic strength or [Ca^{2+}] of the luminal bath in bilayer experiments was increased like that in the SDS-PAGE experiments. This caused an increase in RyR activity that could only be reversed by addition of purified calsequestrin to the luminal bath. The presence or absence of calsquestrin on the RyR in bilayers was confirmed using an anti-calsequestrin antibody that inhibits channel activity when it binds to the calsequestrin-RyR complex. The data were consistent with an overall picture in which calsequestrin dissociation enhanced native RyR activity and calsequestrin binding suppressed channel opening. The next step would be to assess the combined effects of triadin, junctin and calsequestrin on RyR activity. Junctin and triadin can be dissociated from the RyR by solublising the SR membranes with CHAPS detergent and purifying the RyR. Thus by applying triadin, junctin and calsequestrin, in various combinations, to purified RyRs in bilayer experiments it will be possible to systematically piece together these components of the EC coupling machinery and measure their effects on RyR activity.

RyR function and its relationship to its tetrameric structure

Electron microscope image reconstruction shows RyRs to have four-fold symmetry (Orlova *et al.,* 1996). This four-fold symmetry means that each of the four subunits must somehow contribute equally to ligand binding and channel gating. Therefore the mechanisms regulating channel gating are likely to be complex. Single channel studies are now starting to give an insight into how homotetrameric structures like the RyR control channel activity.

Four-fold structure inferred from channel conductance.

Telltale signs of the contribution of four subunits to channel conductance appeared not long after the discovery of the RyR (Ma *et al.,* 1988, Smith *et al.,* 1988). RyRs displayed multiple conductance levels with subconductances near 25%, 50% and 75% of the maximum (ie equally spaced levels) conductance level. These were interpreted a current flow through pores formed by the activation of different numbers of subunits; 25% corresponding to one, 50% to two subunits etc. Very clear and sustained substate activity at approximately equally spaced levels has been observed in RyRs that were modified either by the binding of ryanodine (Ma & Zhao, 1994) or the stripping of the FKBP, a co protein to the RyR (Ahern *et al.,* 1997). Amplitude histograms of channel activity revealed that the spacing of substates was not exactly equal. Nonetheless, these studies interpreted the substates as the opening of four separate conducting pathways in the channel. These must gate cooperatively and simultaneously in order to produce the observed gating of the RyR complex between closed and maximum conductance levels in a single transition. More recently mutations in the RyR have been discovered that alter channel conductance and experiments with hybrid RyRs containing these mutations have shed more light on subunit contributions to channel conductance. A highly conserved 10 amino acid segment within the pore-forming region about aa4824 was found to be the determinant of channel conductance (Zhao *et al.,* 1999). Single channel studies showed that the glycine to alanine substitution, G4824A, reduced channel conductance by 97%. Co-expression of mutant and wild-type

subunits produced a range of hybrid channel types with six different maximal conductance levels that lie between those of the homozygous mutant and wild-type RyRs. The number of different conductance levels corresponds to the number of subunit combinations that are possible with two types of subunits in a tetramer. The fact that six conductance levels could be observed suggest that the four subunits somehow contribute to a single conduction pathway through the RyR as apposed to the idea that each subunit possesses a separate pathway.

Four-fold structure inferred from steady state channel gating.

The gating of RyRs has exhibited phenomena that suggest mechanisms stemming from a tetrameric structure. The Arg615Cys mutation (MH mutation in pigs, see above) in the RyR alleviates RyR inhibition at mM [Ca^{2+}] and [Mg^{2+}]. Shomer *et al.* (1995) found that RyRs expressed in heterozygous pigs gave rise to hybrid RyRs with gating properties intermediate to those of the wild type and mutant. This indicated that each subunit in the RyR tetramer has an influence over the Ca^{2+} sensitivity of the channel. More recently, Chen *et al.* (1998) discovered an alanine to glutamate substitution, E3885A, which decreased the sensitivity of RyR to Ca^{2+} activation by four orders of magnitude such that mM levels of cytoplasmic Ca^{2+} were required to activate the channel. Co-expression of subunits containing altered sites for Ca^{2+}-activation with wild-type subunits produced five or six types of RyRs with sensitivities to Ca^{2+} activation ranging between those seen for the mutant and wild type homotetramers. Chen *et al.* (1998) suggested that all four subunits contribute to each Ca^{2+} activation site. A more detailed interpretation of these experiments awaits the development of methods for linking the stoichiometry of the hybrid channels with the observed channel function.

Four-fold structure inferred from non-steady state channel gating.

Aspects of the tetrametric structure of RyRs may also show up in the response of RyRs to rapid changes in the concentration of regulatory ligands. It is recognized that during muscle contraction the RyRs respond to rapid (~ms) step increases in [Ca^{2+}] resulting from Ca^{2+} flow through nearby dihydropyridine and ryanodine receptors. Consequently there has been considerable interest in the effects of rapid [Ca^{2+}] transients on the activity of RyRs in bilayers (see perspectives by Sitsapesan & Williams, 2000, Lamb *et al.*, 2000, Fill *et al.*, 2000). Now there is also a growing interest in the effects of rapid application of other regulatory ligands such a protons (Laver *et al.*, 2000a). The effect of steady cytoplasmic pH on RyRs has been addressed in several studies (Rousseau & Pinkos, 1990, Shomer *et al.*, 1994, Ma *et al.*, 1988), which show acid pH inhibits RyRs; with half inhibition at pH6.5 and with near total inhibition below pH6. However, rapid changes in pH reveal new characteristics of RyR gating not apparent in steady state recordings. The pH was increased from ~5.5 (inhibiting pH) to above 7 (activating pH) over a 500 ms period by squirting solutions from a perfusion tube placed in the vicinity of the bilayer. Rather than activating in a graded manner reflecting the continuous change of pH over this period, the RyRs activate in a stepwise manner. As the RyRs activate their open probability RyRs increases in what appears to be different gating modes with ascending P_o (see Fig. 11A). Amplitude histograms of RyR P_o (Fig. 11B) show four of these gating modes as four distinct peaks. The four gating modes correspond in number to what would be expected if RyR subunits activated separately in response to proton dissociation. Thus the first, lowest P_o mode would be when one subunit is active and higher P_o modes would occur as more of the four subunits activated. If this hypothesis were correct then the stability of ligand mediated channel openings would depend on the number of subunits that have bound ligand molecules. This phenomenon has been seen in the cyclic nucleotide gated channel (Ruiz & Karpen, 1997).

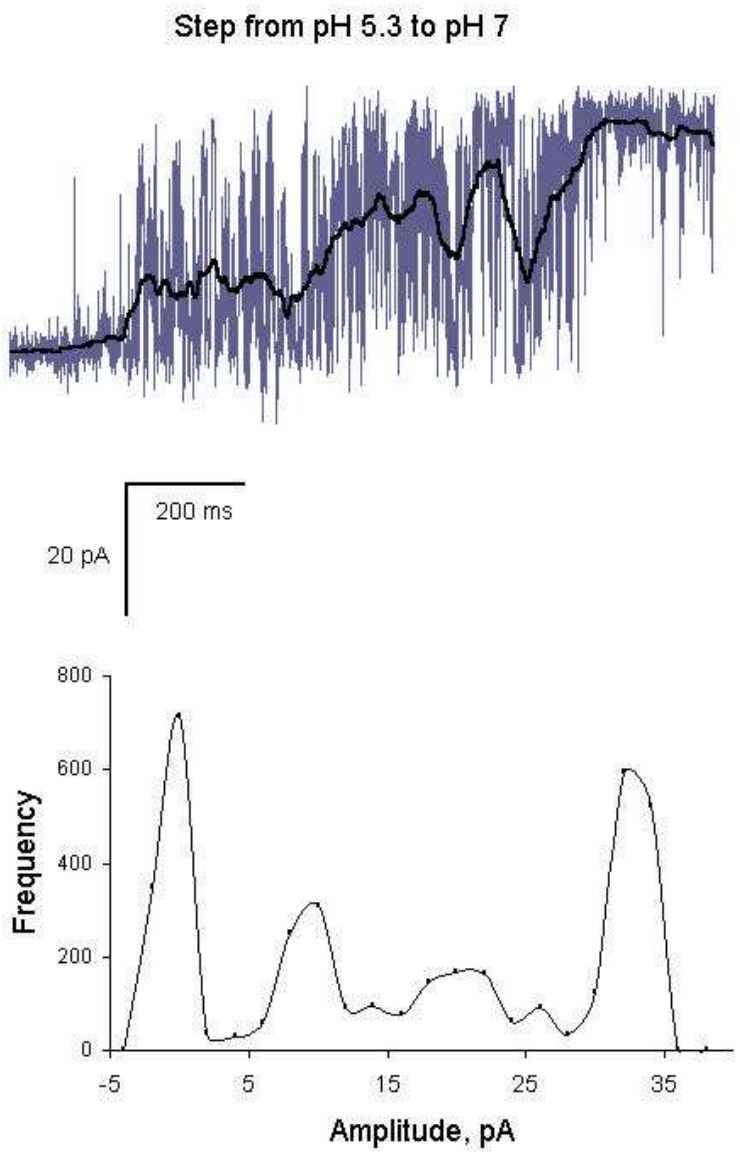

Figure 11. (*A) Recording of a single RyR showing recovery from inhibition at low pH. The cytoplasmic pH was rapidly raised (~1s) from 5.3 to 7 by moving a solution stream from a tube onto and away from the bilayer. The onset of solution change occurred at the beginning of the trace. In the first 200 ms the channel showed slight activation (P_o~0.01) that jumped to a P_o of 0.3 at 200ms, 0.6 at 400 ms and 1.0 at 800 ms into the record. The heavy line shows a running average of the current record, which rises in a stepwise manner as the channel activates. (B) The amplitude histogram of the running average showing peaks, which correspond to relatively stationary segments of activity in part A. The weakest activation seen in the first 200 ms produced the peak at zero current and the other three modes of activity give rise to peaks at 6pA, 20pA and 34 pA.*

Future directions in single channel recording

The rate at which single channel recording techniques have advanced our knowledge is generally limited by the rate at which good recordings can be obtained from either membrane patches or artificial bilayers. Single channel recording is a slow and tedious process where progress is constrained by a range of technical limitations. First, the techniques are notoriously hit and miss in that they do not provide control over the number and types of channels observed. It is common for

experiments to be confounded by the appearance of more than a single channel, the appearance of the wrong channel type or the non-appearance of any channel. Secondly, these membrane systems are extremely fragile and rupture of membrane patches and bilayers terminate experiments within minutes. This severely restricts the range of experimental protocols that can be used for single channel recordings. Any way of overcoming these problems would provide a major advance in single channel recording techniques. This section describes a number of new methods for producing robust, long-lived membrane systems that could be applied to studying individual ion channels.

As described above, the most commonly used method of making planar bilayers is the film drainage method, which was developed by (Mueller *et al.,* 1962). Tien, one of the authors on the original bilayer paper, has published several new methods for forming robust lipid bilayers using solid supports (Ottova & Tien, 1997). As before, bilayers are formed from solutions of lipids in hydrophobic solvents such as n-decane. However, instead of producing lipid films that are suspended across an aperture, bilayer membranes are formed from lipid films spread across solid or gel surfaces. The viscous support offered by a nearby solid surface boosts enormously the stability of the bilayer. Doping these stable membranes with various substances has been shown to endow these membranes with a range of useful properties such as ion selectivity (by incorporation of ionophors such as gramicidin), immunologic reactivity (with antibodies), electron transporters (with C_{60} fullerenes) and photosensitivity (with Zn-phthalocyanine) (Ottova & Tien, 1997, Tien *et al.*, 1991). However, the advantages of supported bilayer systems have not yet been realised in single channel studies. If one could incorporate RyRs, for example, into bilayers on agar supports then conventional electrophysiology experiments could be carried out on a single channel over extended periods of time (hours). Moreover, because the supported bilayers are robust and usually made on the end of electrodes, they are remarkably manoeuvrable. Hence, these bilayers could be aligned within complex measuring apparatus such as those using confocal microscopy or rapid perfusion. The rate and quality of data acquired this way would be far superior to that obtained using conventional bilayer methods.

More recently solid substrate bilayer methods were further improved by developing bilayer structures that could be chemically anchored to a gold surface (Cornell *et al.,* 1997). These were developed for the purpose of making a new generation of biosensors. These systems are remarkably robust: being able to be stored dehydrated for periods of many months. In principle, ion channels could be embedded in these membranes though no successful attempts to do this have been published. Future developments of this system could revolutionise bilayer-based methods of single channel recording. In the future one might purchase bilayers off the shelf and perform weeks of experiments on a single ion channel. Moreover, the hit and miss aspect of single channel methods (see above) may soon be a thing of the past. It is recognised in the biosensor industry that biosensors based on arrays of detectors can offer vastly superior combination of detection sensitivity and speed. Once these bilayer array platforms are produced then the scope for single channel recording is truly enormous. Incorporation of ion channels onto arrays of 10,000 or more electrically isolated bilayers will allow an experimenter to select bilayers in the array that contain the desired number and type of ion channels. One could easily work with mixtures of ion channels as found, for example, in SR membranes because one could choose particular bilayers that contained only the ion channels of interest. It will also be possible to get information from individual ion channels at the same time as obtaining measurements of the average response of many channels by averaging signals from many bilayers. In fact, a highly parallel solid-state electrode array has been developed for patch-clamping that makes possible multiple, simultaneous, single-cell electrical recordings (Axon instruments press release). Thus by layering cells onto these arrays one can simultaneously obtain single-channel and whole-cell currents. So it appears that even now the former limits to data acquisition are giving way to new frontiers in which the rate-limiting factor will be the rate at which data can be acquired with massively parallel detection systems.

Concluding remarks

The regulation of RyR channels by intracellular metabolites such as ATP, Ca^{2+}, Mg^{2+} and pH is a complex interplay of several regulation processes. Overlayed on this is the fact that these mechanisms are modulated by oxidation and phosphorylation mediated modifications of the RyR and by RyR interactions with a variety of co-proteins. Consequently, in order to understand how RyRs are regulated by normal metabolism or during muscle fatigue and myocardial ischemia it is necessary to deal with the very complex problem of understanding RyR function. Single channel recording and analysis methods are powerful enough to examine RyRs in sufficient detail to dissect the complex regulation mechanisms operating in RyRs. The enormous scope with the bilayer method for manipulating the quaternary structure of the EC-coupling machinery have allowed us to probe the effects of DHPRs, calmodulin, calsequestrin, triadin and the FKBP on RyR function. Three examples of forays into these areas by single channel studies have been described here. First, the analysis of [Ca^{2+}] and [Mg^{2+}] regulation of RyRs showed how it is possible to identify and characterise multiple mechanisms by which a single ligand can regulate RyRs and how this leads to an improved understudying of physiological processes in muscle. Secondly, experiments with RyRs in which co-proteins such as calsequestrin and triadin were systematically removed and replaced show how it is possible to understand the functional importance interactions between RyRs and co-proteins. Finally, investigations of the relationship between structure and function of native and mutated RyRs showed several aspects of RyR that reflects the four-fold symmetry of the RyRs structure. However, this area is still at an early stage and it is not yet clear how the four identical subunits of the RyR cooperate in regulating RyR gating.

In spite of the power of single channel recording the bilayer method has an Achilles heel. The fact that RyRs are studied in isolation means that it is not possible to examine their function in the physiological context. Hence one cannot directly apply RyR phenomena in bilayers to the physiological situation. However, the underlying mechanisms for RyR regulation identified from bilayer measurements are likely to apply to the *in vivo* situation and so contribute to our understanding of EC-coupling. On the other hand, studies of more intact muscle preparations such as suspensions of SR vesicles and mechanically skinned fibres are well suited for addressing the physiological situation. However, these systems are complex and it is difficult to identifying underlying mechanisms from the experimental data. Parallel experiments on lipid bilayers and muscle preparations in which the machinery for EC coupling is still intact have proved to be a powerful tool for elucidating mechanisms of Ca^{2+} regulation in muscle. In this collaboration of techniques the bilayer studies identify the basic mechanisms of channel function and the experiments on intact systems show the outworking of these mechanisms in the physiological situation.

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References

Ahern, G.P., Junankar, P.R. & Dulhunty, A.F. (1997) Subconductance states in single channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. *Biophysical Journal,* 72, 146-162.

Ashley, C.C., Mulligan, I.P. & Lea, T.J. (1991) Ca^{2+} and activation mechanisms in skeletal muscle *Quarterly Reviews in Biophysics,* 24, 1-73.

Beard, N.A., Dulhunty, A.F. & Laver, D.R. (2000) The effect of increasing luminal calcium on skeletal muscle *Proceedings of the Australian Physiological and Pharmacological Society,* 31, 22P.

Beard, N.A., Laver, D.R. & Dulhunty, A.F. (1999) Regulation of skeletal muscle ryanodine receptors by calsequestrin *Proceedings of the Australian Physiological and Pharmacological Society*, 30, 43P.

Chen, S.R., Ebisawa, K., Li, X. & Zhang, L. (1998) Molecular identification of the ryanodine receptor Ca^{2+} sensor. *Journal of Biological Chemistry*, 273, 14675-14678.

Colquhoun, D. & Hawkes, A.G. (1981) On the stochastic properties of single ion channels *Proceedings Royal Society London Biol*, 211, 205-235.

Copello, J.A., Barg, S., Onoue, H. & Fleischer, S. (1997) . Heterogeneity of Ca^{2+} gating of skeletal muscle and cardiac ryanodine receptors. *Biophysical Journal*, 73, 141-156.

Cornell, B.A., Braach-Maksvytis, V.L., King, L.G., Osman, P.D., Raguse, B., Wieczorek, L. & Pace, R.J. (1997) A biosensor that uses ion-channel switches. *Nature*, 387, 580-583.

Dulhunty, A.F., Laver, D.R., Gallant, E.M., Casarotto, M.G., Pace, S.M. & Curtis, S. (1999) Activation and inhibition of skeletal RyR channels by a part of the skeletal DHPR II-III loop: effects of DHPR Ser687 and FKBP12 *Biophysical Journal*, 77, 189-203.

Fill, M., Zahradnikova, A., Villalba-Galea, C.A., Zahradnik, I., Escobar, A.L. & Gyorke, S. (2000) . Ryanodine receptor adaptation. *Journal of General Physiology*, 116, 873-82.

Guo, W., Jorgensen, A.O. & Campbell, K.P. (1996) Triadin, a linker for calsequestrin and the ryanodine receptor. *Society of General Physiologists Series*, 51, 19-28.

Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A.O. & Kelley, J. (1995) Purification, primary structure, and immunological characterization of the 26-kDa calsequestrin binding protein (junctin) from cardiac junctional sarcoplasmic reticulum. *Journal of Biological Chemistry*, 270, 30787-96.

Kanno, T. & Takishima, T. (1990) Chloride and potassium channels in U937 human monocytes. *Journal of Membrane Biology*, 116, 149-161.

Knudson, C. M., Stang, K. K., Jorgensen, A. O. & Campbell, K. P. Biochemical characterization and ultrastructural localization of a major junctional sarcoplasmic reticulum glycoprotein (Triadin). *Journal of Biological Chemistry*, (1993) 268, 12637-12645.

Lamb, G.D. (1993) Ca^{2+} inactivation, Mg^{2+} inhibition and malignant hyperthermia [news]. *Journal of Muscle Research and Cell Motility*, 14, 554-556.

Lamb, G.D., Laver, D. R. & Stephenson, D.G. (2000) Questions about Adaptation in Ryanodine Receptors. *Journal of General Physiology*, 116, 883-890.

Lamb, G.D. & Stephenson, D.G. (1991) Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *Journal of Physiology*, 434, 507-528.

Lamb, G.D. & Stephenson, D.G. (1992) Importance of Mg^{2+} in excitation-contraction coupling in skeletal muscle. *News in Physiological Sciences*, 7, 270-274.

Laver, D.R., Baynes, T.M. & Dulhunty, A.F. (1997a) *Journal of Membrane Biology*, 156, 213-229.

Laver, D.R., Eager, K.R., Taoube, L. & Lamb, G.D. (2000a) *Biophysical Journal*, 78, 1835-1851.

Laver, D.R., Lenz, G.K.E. & Lamb, G.D. (2000b) *Proceedings of the Australian Physiological and Pharmacological Society*, 31, 7P.

Laver, D.R., Owen, V.J., Junankar, P.R., Taske, N.L., Dulhunty, A.F. & Lamb, G.D. (1997b) Reduced inhibitory effect of Mg^{2+} on ryanodine receptor-Ca^{2+} release channels in malignant hyperthermia. *Biophysical Journal*, 73, 1913-1924.

Laver, D.R., Roden, L.D., Ahern, G.P., Eager, K.R., Junankar, P.R. & Dulhunty, A.F. (1995) Cytoplasmic Ca^{2+} inhibits the ryanodine receptor from cardiac muscle. *Journal of Membrane Biology,* 147, 7-22.

Lu, X., Xu, L. & Meissner, G. (1994) Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. *Journal of Biological Chemistry,* 269, 6511-6.

Ma, J., Fill, M., Knudson, C. M., Campbell, K. P. & Coronado, R. (1988) Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science,* 242, 99-102.

Ma, J. & Zhao, J. (1994) . Highly cooperative and hysteretic response of the skeletal muscle ryanodine receptor to changes in proton concentrations. *Biophysical Journal,* 67, 626-633.

MacLennan, D. H. & Wong, P. T. (1971) Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proceedings of the National Academy of Sciences USA,* 68, 1231-1235.

Meissner, G. (1994) Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annual Review of Physiology,* 56, 485-508.

Meissner, G., Darling, E. & Eveleth, J. (1986) Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry,* 25, 236-244.

Mickelson, J. R. & Louis, C. F. (1996) Malignant hyperthermia: Excitation-contraction coupling, Ca^{2+} release channel, and cell Ca^{2+} regulation defects. *Physiological Reviews,* 76, 537-592.

Miller, C. & Racker, E. (1976) Ca^{++}-induced fusion of fragmented sarcoplasmic reticulum with artificial planar bilayers. *Cell,* 9, 283-300.

Mueller, P., Rudin, D. O., Tien, H. T. & Westcott, W. C. (1962) . Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature,* 194, 979-981.

Nabauer, M., Callewaert, G., Cleemann, L. & Morad, M. (1989) Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science,* 244, 800-803.

Neher, E. & Sakmann, B. (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature,* 260, 799-802.

Ogawa, Y. (1994) Role of ryanodine receptors. *Critical Reviews of Biochemistry and Molecular Biology,* 29, 229-74.

Orlova, E. V., Serysheva, I. I., Van Heel, M., Hamilton, S. L. & Chiu, W. (1996) Two structural configurations of the skeletal muscle calcium release channel. *Nature Structural Biology,* 3, 547-552.

Ottova, A. L. & Tien, H. T. (1997) Self-assembled bilayer lipid membranes: from mimicking biomembranes to practical applications. *Bioelectrochemistry and Bioenergetics,* 42, 141-152.

Owen, V. J., Taske, N. L. & Lamb, G. D. (1997) Reduced inhibitory effect of Mg^{2+} on Ca^{2+} release in porcine muscle fibers with ryanopdine receptor mutation for malignant hyperthermia *American Journal of Physiology,* 272, C203-211.

Rousseau, E. & Pinkos, J. (1990) pH modulates conducting and gating behaviour of single calcium release channels. *Pflugers Archiv,* 415, 645-647.

Ruiz, M. L. & Karpen, J. W. (1997) Single cyclic nucleotide-gated channels locked in different ligand- bound states. *Nature,* 389, 389-392.

Shomer, N. H., Mickelson, J. R. & Louis, C. F. (1994) Caffeine stimulation of malignant hyperthermia-susceptible sarcoplasmic reticulum Ca^{2+} release channel. *American Journal of Physiology,* 267, C1253-C1261.

Shomer, N. H., Mickelson, J. R. & Louis, C. F. (1995) Ca^{2+} release channels of pigs heterozygous for malignant hyperthermia. *Muscle Nerve,* 18, 1167-1176.

Sigworth, F. J. & Sine, S. M. (1987) Data transformations for improved display and fitting of single- channel dwell time histograms. *Biophysical Journal,* 52, 1047-1054.

Sitsapesan, R. & Williams, A. J. (2000) Do inactivation mechanisms rather than adaptation hold the key to understanding ryanodine receptor channel gating?*Journal of General Physiology*, 116, 867-72.

Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P. & Coronado, R. (1988) Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *Journal of General Physiology*, 92, 1-26.

Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T. & Numa, S. (1990) . Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature*, 346, 567-569.

Tien, H. T., Salamon, Z. & Ottova, A. (1991) . Lipid bilayer-based sensors and biomolecular electronics. *CriticalReviews in Biomedical Engineering*, 18, 323-340.

Tripathy, A., Xu, L., Mann, G. & Meissner, G. (1995) Calmodulin activation and inhibition of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *Biophysical Journal*, 69, 106-119.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y. M. & Jones, L. R. (1997) . Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. *Journal of Biological Chemistry*, 272, 23389-23397.

Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R. J. & Chen, S. R. (1999) . Molecular identification of the ryanodine receptor pore-forming segment. *Journal of Biological Chemistry*, 274, 25971-25974.

GENE TRANSFER: MANIPULATING AND MONITORING FUNCTION IN CELLS AND TISSUES

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Summary

1. The ectopic expression of genes has proved to be an extremely valuable tool for biologists. The most widely used systems involve electrically or chemically mediated transfer of genes to immortalized cell lines and, at the other end of the spectrum, transgenic animal models. As would be expected, there are compromises to be made when employing either of these broad approaches. Immortalised cell lines have limited "physiological relevance" and transgenic approaches are costly and out of the reach of many laboratories. There is also significant time required for the *de novo* generation of a transgenic animal.
2. As a viable alternative to these approaches we describe the use of recombinant adenovirus and Sindbis virus to deliver genes to cells and tissues.
3. We exemplify this approach with studies from our laboratories. i) An investigation of Ca^{2+} handling deficits in cardiac myocytes of hypertrophied hearts using infection with recombinant adenovirus encoding either green fluorescent protein (GFP) or the sarcoplasmic/endoplasmic reticulum calcium-ATPase (Serca2a). ii) A study of the mechanism of macrophage/microglial migration by infection of embryonic phagocytes with a GFP encoding virus and co-culture with brain slices to then track the movement of labelled cells. ii) We are also exploiting the natural tropism of the Sindbis virus to label neurones in hippocampal brain slices in culture to resolve high-resolution structure and to map neuronal connectivity.
4. Further development of these approaches should open new avenues of investigation for the study of physiology in a range of cells and tissues.

Introduction

Gene transfer, a simple and attractive concept, involves the transfer of DNA to cells of interest. Progress in gene transfer technology has made it a potentially powerful tool for the treatment of a wide variety of diseases (Romano *et al.*, 2000). Early gene transfer techniques using chemical methods such as calcium phosphate or liposomes and physical methods such as electroporation were successfully exploited for basic research but had limited use for gene therapy. Gene transfer technology for gene therapy approaches is based on the ability to efficiently deliver the therapeutic gene to relevant target cells. Efficient delivery in turn is dependent upon type of gene delivery vehicles. Recent advances in gene therapy research and vector technology have led to the development of variety of viral and non-viral vector systems to efficiently deliver genes to cells, tissues and organs by *ex vivo* and *in vivo* strategies (Mountain, 2000). Efficient gene transfer systems represent useful tools for basic research and provide new opportunities to study gene function at the cellular and molecular level in a wide variety of cells, tissues, organotypic cultures and whole animals. At this stage integration of gene transfer technology with physiological genomics to study the function of gene products in context of the whole organism and its environment or in a particular cell type at a specific stage of development will play a major role in physiology and medicine.

Gene Transfer Systems

An important challenge to gene transfer technology is the development of a single gene delivery system that can adequately satisfy all of the following criteria: 1) Efficient and targeted cell-specific delivery 2) High levels and long term expression of the transgene 3) Low toxicity for *in vivo* delivery with minimal side effects 4) Non-immunogenicity. Although existing viral and non-viral vector systems can fulfil some of these criteria, none can single-handedly provide all of the necessary functions. Control of gene expression and targeting to specific cells or tissues is currently an intensive area of gene transfer research. Some of the genetic elements that are being incorporated into the design of new and improved vectors include promoters that are cell-type specific, cell-cycle regulated or tumor selective promoters as well as promoters that respond to radiation, chemotherapy or are heat induced (Nettelbeck *et al.*, 2000).

Non Viral Delivery Systems

Non-viral vectors using mechanical or chemical approaches can efficiently transfect cells *in vitro*. Mechanical methods involve direct injection or the use of "gene gun technology" to introduce the plasmid DNA (Yang *et al.*, 1996). Low levels of gene expression and inability to use these methods for systemic administration due to the presence of serum nucleases has limited their applications to tissues that are easily accessible such as skin and muscle cells. Electroporation using electrical mediated disruption of cell membranes to effect transfection is used mainly for *in vitro* applications. Chemical methods are divided into two classes: different formulations of cationic liposomes and cationic polymers such as polylysine, protamine, DEAE dextran or polyethyleneimine (PEI). Classical liposomes (positively charged) have been used to deliver encapsulated drugs and transfer genes into cells in culture (Gao & Huang, 1995). Problems with encapsulation of DNA have led to the development of different formulations of cationic liposomes that are able to interact spontaneously with negatively charged DNA. The transfection efficiency of the liposome/DNA complexes *in vivo* is very low and can sometimes be cytotoxic *in vitro*. New and improved formulations of cationic lipids that have enhanced the cellular internalization and transfection efficiency are being used for human gene therapy. The success of non-viral delivery will be greatly dependent on the ability to design systems that can transfect cells with high efficiency, increased stability in presence of serum proteins and reduced toxicity to cells both *in vitro* and *in vivo*. One advantage of this system is they have no constraints on size of the gene that can be delivered.

Viral Delivery Systems

Viruses are naturally evolved vehicles that efficiently transfer their genes into host cells. This ability has made them attractive as tools for gene delivery purposes. Viral vectors that have been extensively studied and genetically manipulated for safety concerns in laboratory research and for *in vivo* gene transfer protocols include retroviruses, adenoviruses, herpes simplex viruses, lentiviruses, adeno associated viruses and Sindbis viruses. Each of the viral vectors has their own individual advantages, problems, and specific applications. Choice of viral vectors is dependent on gene transfer efficiency, capacity to carry foreign genes, tropism, toxicity, stability, immune responses towards viral antigens and potential viral recombination. For functional studies of different transgenes our laboratory is using recombinant adeno and Sindbis viruses for gene transfer purposes and this paper will discuss and review literature related to only these viral gene delivery systems.

Adenoviral Mediated Gene Transfer

Adenoviral genome consists of double stranded linear DNA of ~36 kb in length (Graham & Prevec, 1995). Adenoviral vectors can infect a wide variety of cells and tissues. They can transfer genes to both proliferating and quiescent cells and express the transgene at very high levels. Transgene expression is transient, as these viruses do not integrate into the host genome. To generate replication defective viruses early genes such as E1A, E1B, E2, E3 and E4 involved in adenoviral gene transcription, DNA replication and host cell immune suppression can be deleted. The first-generation recombinant adenoviruses were constructed using a two-plasmid system. The "shuttle" plasmid contains part of the viral genome where the E1A and E1B genes are replaced by a transgene driven by its own regulatory system. The "helper" plasmid contains a complete but unpackageable viral genome. On cotransfection of the two plasmids, homologous recombination takes place to generate a recombinant adenovirus encoding the transgene. Infectious viral particles can be generated in permissive host cells such as HEK293 in which E1A proteins are provided *in trans*.

First generation recombinant adenoviral vectors have several drawbacks. Transgenes of only 4-5 kb can be packaged. They cause significant cytotoxicity in infected cells and immunological responses in the infected animal. Low efficiency of homologous recombination in HEK293 cells made the process of generating recombinant adenovirus very tedious. To increase the packaging capacity of the foreign gene (~ 7 kb), viral vectors in which E1A and E3 or E4 are deleted were developed (Bett *et al.,* 1995; Gao *et al.,* 1996). To reduce the cytotoxicity *in vivo* and prolong the transgene expression, a second generation of adenoviral vector with E1 and E2 deletions/mutations was developed (Engelhardt *et al.,* 1994a,b). To simplify the process of generating recombinant viruses, Vogelstein and group (He *et al.,* 1998) developed the AdEasy method that has the significant advantage in that homologous recombination is carried out in *E. coli* bacterial cells. This made the process of screening for recombinants less cumbersome. Successful recombinants are isolated and then transfected into HEK293 cells to generate replication deficient infectious viral particles. These recent advances in adenoviral technology have made them attractive tools for physiological studies, functional genomics and gene therapy purposes (Wang & Huang, 2000).

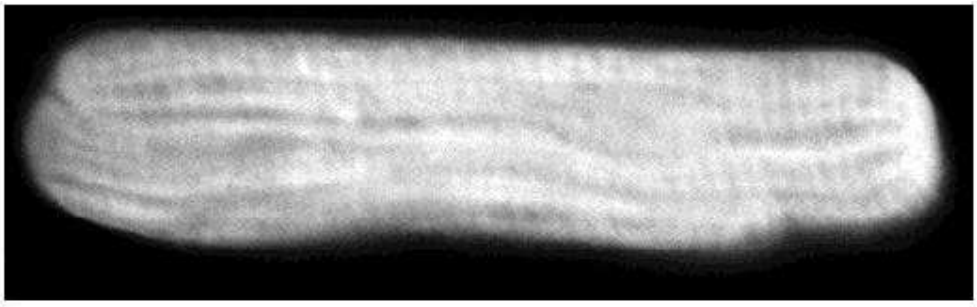

Figure 1. *Laser scanning confocal microscopy image of AdSERCA2a-EGFP infected myocyte from rat right ventricle. Expression of EGFP is apparent throughout the cytosol of the cell which exhibits a longitudinal banding pattern reflecting exclusion of expressed protein from the mitochondria, sarcoplasmic reticulum and T-tubules (cell length 120 mm).*

Ca^{2+} Handling In Cardiac Hypertrophy

In our laboratory recombinant adenoviruses were generated using the AdEasy system. Within 24 hrs of infection with a recombinant adenovirus carrying GFP, high levels of GFP expression (as evidenced by specific fluorescence emission), was observed in majority (95%) of adult rat cardiac myocytes possessing the rod-shaped morphology characteristic of healthy Ca^{2+}-tolerant cardiac myocytes (Fig. 1). This gene transfer method was then used to manipulate the Ca^{2+} handling system

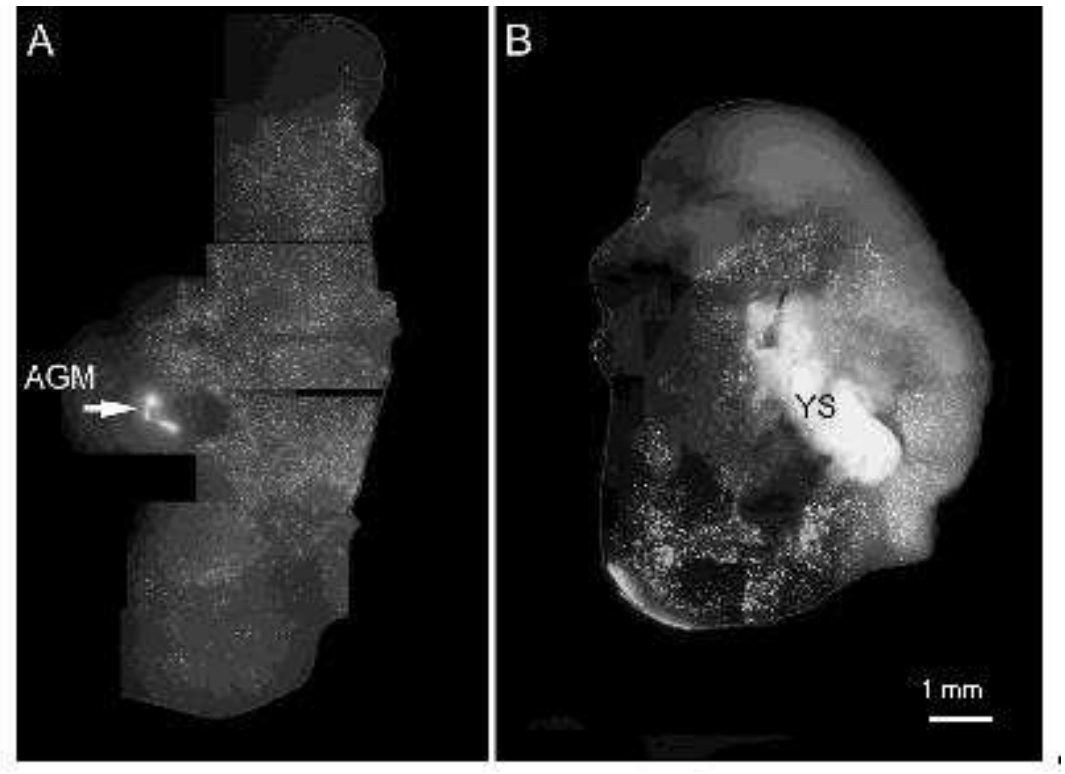

Figure 2. *Brain slices from a post-natal day 4 rat pup cultured for 6 days with GFP-labelled embryonic haemopoietic tissues. A) Aorta-gonad-mesonephros, AGM. B) The yolk sac, YS. These tissues where placed in the ventricles of the brain slice. In both cases cells from the haemopoietic tissues have migrated into the brain slice forming a dense distribution.*

of isolated cardiac myocytes from rats suffering cardiac hypertrophy and heart failure (Reilly *et al.*, 2001). The rate of Ca^{2+} sequestration of the sarcoplasmic reticulum (SR) ATP dependent Ca^{2+} pump is a major determinant of cardiac relaxation and it is clear that reductions in the expression of pumps underlie the prolonged calcium (Ca^{2+}) transients and consequent reduced contractile performance seen in human cardiac hypertrophy and heart failure. As such, modulation of intracellular Ca^{2+} levels, Ca^{2+} kinetics or Ca^{2+} sensitivity is the focus of many current therapeutic approaches to improve contractile performance in the hypertrophic or failing heart. While there are three highly homologous genes encoding SR Ca^{2+} pumps, SERCA2a is the specific isoform found in cardiac (and slow-twitch skeletal) muscle, and is abundantly expressed in both atrial and ventricular compartments of mammalian myocardium (Kiriazis & Kranias, 2000).

In freshly isolated cardiac myocytes from rats with monocrotaline-induced right ventricular hypertrophy, Serca2a gene transfer resulted in a marked restoration of Serca2a protein expression levels and completely normalised the timecourse of the stimulated Ca^{2+} responses in these cells without altering diastolic Ca^{2+} values or Ca^{2+} transient amplitudes. These results highlight the importance of Serca2a deficiencies in the hypertrophic phenotype of cardiac muscle and outline a simple, effective viral approach for manipulation and improvement of complex cardiac functions.

Origins Of Microglia

We have used adenovirus modified to express GFP to label cells in organotypic co-culture experiments which were designed to establish the developmental potential of embryonic haemopoietic cells in rats. Before the bone marrow develops to become the source of all blood cells a number of

transient haemopoietic sites exist at different times in the developing embryo. To determine whether haemopoietic cells of the yolk sac and the aorta-gonad-mesonephros region had the potential to develop into the ramified phagocytic cells in the brain (the microglia) these embryonic tissues were isolated and co-cultured with organotypic brain slices taken from neonatal rats.

Microglia are considered to be the macrophages of the central nervous system and are seen in the developing brain before bone marrow haemopoiesis. Because the neonatal brain slice is known to provide an environment that supports the differentiation of microglia from macrophage-like precursors it was anticipated that the co-culture conditions would allow the potential of the transient embryonic sites to generate microglia cells to be examined. To identify any cells in the brain slice that are derived from the co-cultured yolk sac and AGM, these haemopoietic tissues were infected with GFP-expressing adenovirus after their removal from embryos (but prior to co-culturing with brain slices).

We examined brain slices after 1 week of co-culture and typically found vast numbers of cells derived from the haemopoietic tissues had invaded the brain slice (Fig. 2). Depending on the age of the embryos from which the tissues were taken, both of the embryonic haemopoietic tissues examined had the capacity to populate brain slices with numerous cells of microglial morphology (numerous ramified processes).

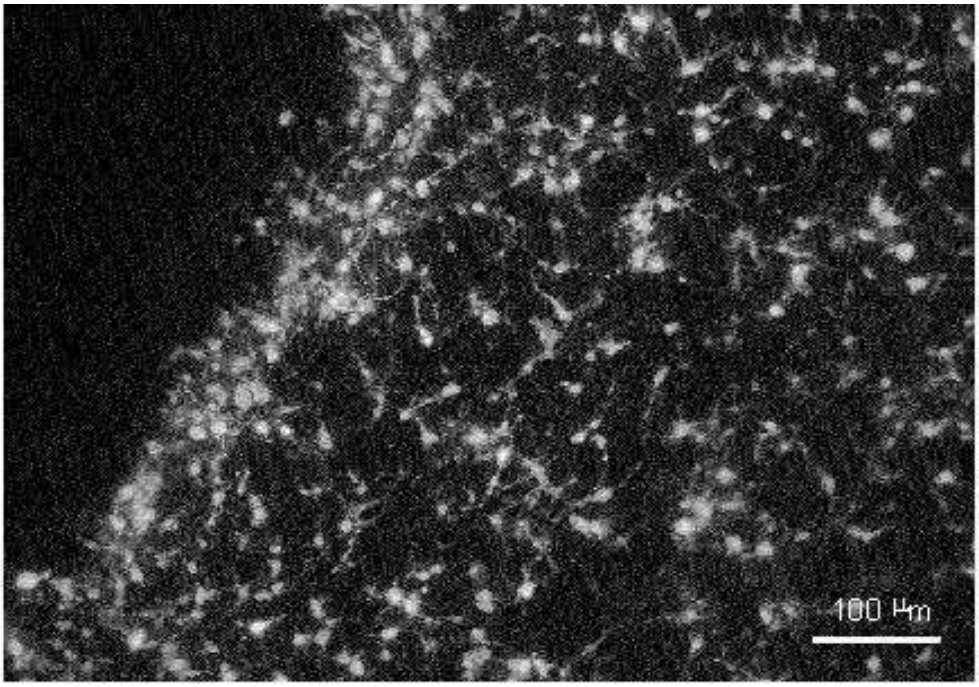

Figure 3. *Detail of a region of slice invasion by the GFP labelled cells from the embryonic haemopoietic tissues showing a ramified phenotype typical of microglia.*

The adenovirus-mediated GFP labelling allowed detailed examination of the morphology of these cells, enabling us to confirm their ramified state indicative of microglia (Fig. 3). Although we have not confirmed by experiment, our finding of very large numbers of donor tissue derived cells in the slice suggests that considerable proliferation of the GFP labelled population had occurred. If this was the case it would appear that the GFP fluorescence was retained in daughter cells after division.

Adeno-GFP infection of heamopoietic tissues provided intense and apparently robust GFP fluorescence in cells derived from these tissues, allowing the details of the cell morphology to be established. The ease of use and efficacy of this cell labelling method makes it an attractive approach to use in other cell tracking experiments in organ culture or *in vivo*.

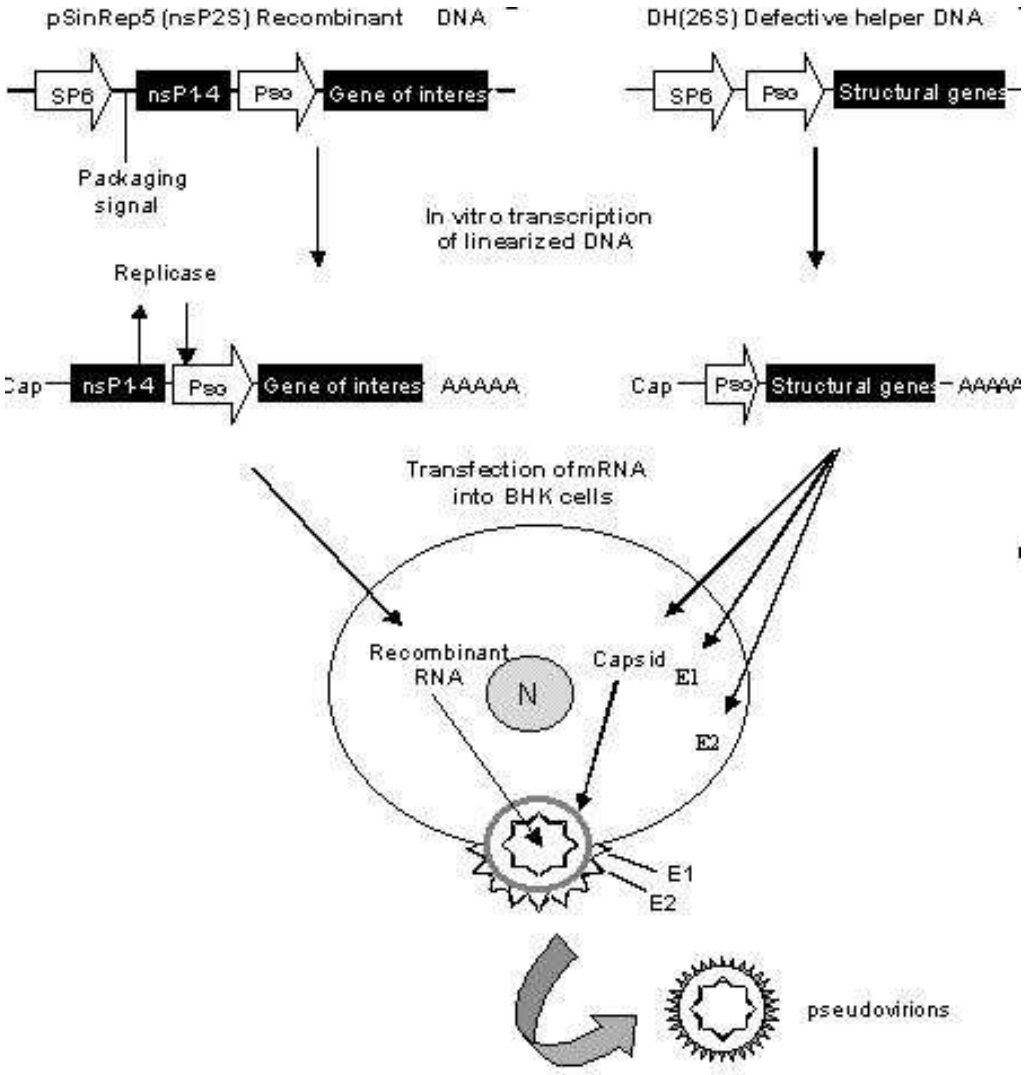

Figure 4. *Generation of recombinant Sindbis virus. pSinRep5 (nsps2S) is the recombinant plasmid containing the viral nonstructural genes (nsP1-4) and the subgenomic promoter (P_{SG}) that controls the transcription of the gene of interest. The defective helper plasmid (DH26S) contains the viral structural genes under the control of the P_{SG} promoter. In vitro transcription of linearized pSinRep5 and DH26S plasmids results in RNA that has a Cap at the 5' end and poly A tail at the 3' end. The transcripts are cotransfected into BHK cells. Translation of the nonstructural genes produces replication enzymes that replicate the recombinant RNA. Capsid protein and E1 and E2 glycoproteins translated from the structural genes of the helper viral RNA, package the recombinant RNA and cause release of the pseudovirions into the medium.*

Sindbis Virus mediated Gene Transfer

Sindbis virus is an alphavirus containing a 12-kb single-stranded, positive sense, capped and polyadenylated RNA genome. Introduction of the RNA genome into cells produces infectious virus that is cytopathic. This virus causes encephalitis in mice and rash and arthritis in humans. In mice its primary target is the neurons of the central nervous system (Lustig *et al.*, 1988) and causes neuronal death by inducing apoptosis (Griffin & Hardwick, 1997). Neural infection is dependent on the age of mice and the strain of the virus. The ability to infect a broad range of host cells, small genome size

and to amplify and transcribe their genome exclusively in the cytoplasm without affecting host chromosomal machinery are some of the properties that have made Sindbis viruses attractive tools for reengineering purposes and gene transfer. Genetic manipulation of the viral genome, use of two vector system and development of packaging cell lines stably transformed with inducible structural protein expression cassette (Polo *et al.*, 1999) has resulted in new Sindbis vectors that are noncytopathic in mammalian cells, express the transgene at very high levels and reduced the generation of contaminating replication competent virus.

We generated recombinant Sindbis virus using the two-vector system (Fig 4). Briefly, the plasmid pSinRep5 (nsP2S) is used to generate recombinant RNA molecules for transfection and infection. This plasmid contains the viral nonstructural protein genes (nsP1-4) required for replicating RNA transcripts *in vivo* (in the cell), the SP6 promoter for in vitro transcription, packaging signal and subgenomic promoter (P_{SG}) for transcription of the subgenomic RNA containing the gene of interest. To reduce the cytopathic effects of the Sindbis virus a single mutation (P726S) has been introduced in the nsP2 gene (Dryga *et al.*, 1997). The defective helper plasmid DH(26S) that provides the structural proteins in trans contains the SP6 promoter for in vitro transcription and the P_{SG} promoter for transcription of the viral capsid and E1 and E2 genes. Both the pSinRep5 (nsp2S) and DH(26S) plasmids are linearized and then *in vitro* transcribed to generate corresponding mRNA that has a Cap at the 5'end and a poly A tail at the 3'end. The transcripts are cotransfected into BHK cells. In the cell cytoplasm, the recombinant RNA is translated to produce the replication enzymes that synthesize the recombinant RNA. Translation of the structural genes from the defective helper RNA produce the capsid protein and E1 and E2 glycoproteins that package the recombinant RNA and subsequently cause release of the viral particles into the medium.

Neuronal structure revealed by sindbis-GFP.

Recombinant Sindbis virus carrying the GFP gene efficiently infects neuronal cells from hippocampus of neonatal rat brain (Fig. 5). Brain slices were grown on cell culture inserts (Millipore) for 1-2 days and then microinjected with the Sindbis virus. About 14-18 hours following infection GFP fluorescence was visualised in the cell body and the dendritic processes of the neurons. In many cases nerve processes could be traced for several millimetres suggesting that this technique can be used for tracing long-range nerve projections in whole brain. Further support for this approach comes from a recent report (Chen *et al.*, 2000), that employed *in vivo* injection of Sindbis-EGFP to obtain high-resolution images of neurones both in slices and *in vivo*. These studies thus suggest the potential use of this virus for studying dynamic changes and neural connectivity during development.

Viral Transfer For Physiological Studies - The Future

A number of other exciting possibilities are currently under exploration in our own laboratories including the use of *in vivo* viral infection to deliver genes to organs and tissues in whole animals. Using this approach our goal is to create genetically modified animals to study the physiological consequences of gene expression. The use of antisense genes may delete endogenous gene expression, akin to a "knockout" animal. Dominant mutant genes that cause disease in humans can be expressed in animals and the phenotype assessed, similar to the approach employed by transgenic and knockin models. Such studies are not only less expensive and more rapid to develop but may also be used as a screening method for the creation of *useful* genetically modified animal models. Furthermore the infection with multiple dominant mutant genes may be used to examine the more difficult polygenic disorders such as hypertension and mental disease.

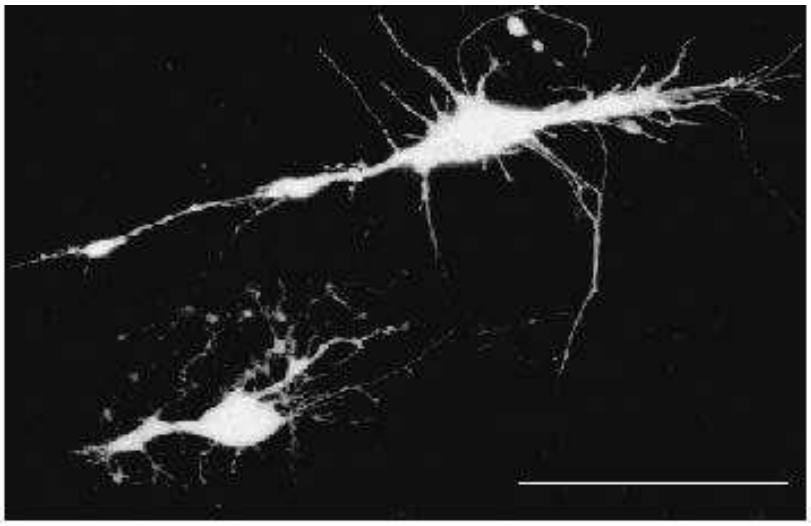

Figure 5. *Hippocampal neurones infected with Sindbis encoding GFP. Tissue slices were made from a post natal day 7 rat brain and cultured overnight. Recombinant Sindbis (50 nl of pseudovirions) was injected into the slice using a Drummond nanoject II and the slices examined on a Zeiss confocal LSM510 after 24 hrs in culture. For this image 20 confocal planes were projected onto the Z-axis. Scale bar is 50 mM.*

A final application under study in our laboratories is the use of viral vectors for the *in vivo* delivery of genetically encoded reporter genes. We have employed two such sensors in our own studies, cameleons (Miyawaki *et al.*, 1997, 1999) and camgaroos (Baird *et al.*, 1999). Both these are GFP-based sensors that convert Ca^{2+} levels into readily measurable fluorescence light signals. We have modified them for targeting to organelles or cellular compartments of interest (Petrou *et al.*, 2000) that obviates the need for high spatial resolution and results in a commensurate gain in temporal resolution.

The completion of the first draft of the human genome sequence heralded a new era in genomics and for physiology, opened new vistas. While the sequence and basic function of many genes will soon be resolved, elucidation of the physiological role of these genes is a major new challenge facing modern physiologists. The science of Physiological Genomics is ultimately concerned with solving this challenge and we envisage that viral mediated gene transfer provides physiologist with an important tool for this emerging discipline.

References

Baird, G.S., Zacharias, D.A. & Tsien, R.Y. (1999) Circular permutation and receptor insertion within green fluorescent proteins. *Proceedings of the National Academy of Sciences USA*, 96, 11241-11246.

Bett, A.J., Krougliak, V. & Graham, F.L. (1995) DNA sequence of the deletion/insertion in early region 3 of Ad5 dl309. *Virus Research,* 39, 75-82.

Chen, B.E., Lendvai, B., Nimchinsky, E.A., Burbach, B., Fox, K. & Svoboda, K. (2000) Imaging high-resolution structure of GFP-expressing neurons in neocortex In vivo. *Learning and Memory*, 7, 433-441.

Dryga, S.A., Dryga, O.A. & Schlesinger, S. (1997) Identification of mutations in a Sindbis virus variant able to establish persistent infection in BHK cells: the importance of a mutation in the nsP2 gene. *Virology*, 228, 74-83.

Engelhardt, J.F., Litzky, L. & Wilson, J.M. (1994a) Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a. *Human Gene Therapy*, 5, 1217-1229.

Engelhardt, J.F., Ye, X., Doranz, B. & Wilson, J.M. (1994b) Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proceedings of the National Academy of Sciences USA*, 91, 6196-6200.

Gao, G.P., Yang, Y. & Wilson, J.M. (1996) Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *Journal of Virology*, 70, 8934-8943.

Gao, X. & Huang, L. (1995) Cationic liposome-mediated gene transfer. *Gene Therapy*, 2, 710-722.

Graham, F.L. & Prevec, L. (1995) Methods for construction of adenovirus vectors. *Molecular Biotechnology*, 3, 207-220.

Griffin, D.E. & Hardwick, J.M. (1997) Regulators of apoptosis on the road to persistent alphavirus infection. *Annual Review of Microbiology*, 51, 565-592.

He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W. & Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. *Proceedings of the National Academy of Sciences USA*, 95, 2509-2514.

Kiriazis, H. & Kranias, E.G. (2000) Genetically engineered models with alterations in cardiac membrane calcium-handling proteins. *Annual. Review of Physiology*, 62, 321-351.

Lustig, S., Jackson, A.C., Hahn, C.S., Griffin, D.E., Strauss, E.G. & Strauss, J.H. (1988) Molecular basis of Sindbis virus neurovirulence in mice. *Journal of Virology*, 62, 2329-2336.

Miyawaki, A., Griesbeck, O., Heim, R. & Tsien, R.Y. (1999) Dynamic and quantitative Ca2+ measurements using improved cameleons. *Proceedings of the National Academy of Sciences USA*, 96, 2135-2140.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. & Tsien, R.Y. (1997) Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature*, 388, 882-887.

Mountain, A. (2000) Gene therapy: the first decade. *Trends in Biotechnology*, 18, 119-128.

Nettelbeck, D.M., Jerome, V. & Muller, R. (2000) Gene therapy: designer promoters for tumour targeting. *Trends in Genetics*, 16, 174-181.

Petrou, S., Bowser, D.N., Nicholls, R.A., Panchal, R.G., Smart, M.L., Reilly, A.M. & Williams, D.A. (2000) Genetically targeted calcium sensors enhance the study of organelle function in living cells. *Clinical and Experimental Pharmacology and Physiology*, 27, 738-744.

Polo, J.M., Belli, B.A., Driver, D.A., Frolov, I., Sherrill, S., Hariharan, M.J., Townsend, K., Perri, S., Mento, S.J., Jolly, D.J. et al. (1999) Stable alphavirus packaging cell lines for Sindbis virus and Semliki Forest virus-derived vectors. *Proceedings of the National Academy of Sciences USA*, 96, 4598-4603.

Reilly, A.M., Petrou, S., Panchal, R.G. & Williams, D.A. (2001) Restoration of calcium handling properties of adult cardiac myocytes from hypertrophied hearts (submitted).

Romano, G., Michell, P., Pacilio, C. & Giordano, A. (2000) Latest developments in gene transfer technology: achievements, perspectives, and controversies over therapeutic applications. *Stem Cells*, 18, 19-39.

Wang, I. & Huang, I. (2000) Adenovirus technology for gene manipulation and functional studies. *Drug Discovery Today*, 5, 10-16.

Yang, N.S., Sun, W.H. & McCabe, D. (1996) Developing particle-mediated gene-transfer technology for research into gene therapy of cancer. *Molecular Medicine Today*, 2, 476-481.

HYBRID SKELETAL MUSCLE FIBRES: A RARE OR COMMON PHENOMENON?

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Summary

1. The main aim of this review is to raise awareness of the molecular complexity of single skeletal muscle fibres from 'normal' and 'transforming' muscles, in recognition of the many types of hybrids that have been observed in vertebrate skeletal muscle. The data used to illustrate various points made in the review were taken from studies on mammalian (mostly rat) and amphibian muscles.
2. The review provides a brief overview of the pattern and extent of molecular heterogeneity in hybrid muscle fibres and of the methodological problems encountered when attempting to identify and characterise such fibres. Particular attention is given to four types of skeletal muscle hybrids: myosin heavy chain (MHC) hybrids, mismatched MHC-myosin light chains (MLC) hybrids, mismatched MHC-regulatory protein hybrids and hybrids containing mismatched MHC-sarcoplasmic reticulum protein isoforms.
3. Some of the current ideas regarding the functional significance, origin and cognitive value of hybrid fibres are critically examined.

Introduction

Skeletal muscle contraction is the net result of a series of cellular events known collectively as the excitation-contraction-relaxation (E-C-R) cycle. The major events in the E-C-R cycle were summarised by Stephenson *et al* (1998).

Major events in the E-C-R cycle in the vertebrate skeletal muscle include: (i) initiation and propagation of an action potential along the sarcolemma and transverse (T)-tubular system, (ii) transmission of the T-system depolarisation signal from the T-tubule to the sarcoplasmic reticulum (SR) membrane, (iii) release of calcium ions (Ca^{2+}) from the SR, (iv) transient rise of myoplasmic [Ca^{2+}], (v) binding of Ca^{2+} to the regulatory protein troponin C (Tn C), (vi) transient activation of the regulatory system and contractile apparatus, (vi) dissociation of Ca^{2+} from Tn C and (vii) Ca^{2+} reuptake by SR mediated by SERCA.

The key roles in these events are played by Ca^{2+} (the activation ion) and by a large number of proteins/protein complexes located in several subcellular compartments. It is now widely accepted that many of the proteins involved in events of the E-C-R cycle exist as multiple forms (isoforms), which can be distinguished and identified by biochemical methods such as gel electrophoresis and immunochemistry (Moss *et al.*, 1995). Polymorphous skeletal muscle proteins of the E-C-R cycle include: the α-subunit of the dyhydropyridine receptor (DHPR), ryanodine receptor/SR calcium release channel (RyR), sarco (endoplasmic) reticulum Ca^{2+}-ATPase (SERCA), calsequestrin, myosin heavy chain (MHC), myosin light chain (MLC) and the regulatory proteins troponin C (TnC), troponin I (TnI), troponin T (TnT) and tropomyosin (Tm) (Pette & Staron, 2000, 1997, 1990). It is important to point out that the list of isoforms of skeletal muscle proteins (particularly myofibrillar proteins) has been increasing in parallel with the development/refinement of protein separation/identification techniques and with the application of these techniques to a wider range of muscles and animal species. Updated versions of this list can be found in reviews produced regularly by major contributors

to the field (Pette & Staron, 2000; Schiaffino & Salviati, 1998; Pette & Staron, 1997; Schiaffino & Reggiani, 1996; Pette & Staron, 1990; Swynghedauw, 1986).

It is noteworthy that the number of isoforms varies markedly between various skeletal muscle proteins. For example, according to a recent count, mammalian skeletal muscle expresses as many as ten MHC isoforms, but only two TnC isoforms [one typical of fast-twitch muscle (TnC-f), the other of slow-twitch muscle (TnC-s) (Pette & Staron, 2000; 1997). The ten MHC isoforms* include four isoforms present in adult mammalian muscle (slow-twitch isoform MHCI or MHCβ/slow and fast-twitch isoforms MHCIIa, MHCIId/x and MHCIIb), two isoforms present in developing and regenerating muscles (MHC-emb, MHC-neo), and four isoforms present in some highly specialized muscles [extraocular and jaw closing muscles (MHC-exoc). Currently it is not known whether there is any relationship between the number of isoforms of a muscle protein, its cellular function and/or the molecular mechanisms responsible for its molecular diversity.

So far, the terms 'hybrid muscle fibres†', 'polymorphic fibres', or 'MHC hybrids' have been used interchangeably to define fibres that co-express more than one MHC isoform. However, there is now compelling evidence to suggest that this meaning of the term 'hybrid fibres' is highly inadequate because it does not apply to fibres displaying patterns of molecular heterogeneity with respect to other proteins. A population of such fibres, expressing only one MHC isoform (MHCIIa) and both fast- and slow-twitch isoforms of the MLC subunits has been detected in rat soleus (SOL) in an early study by Mizusawa *et al.* (1982) and in a very recent study by Bortolotto *et al.* (2000a). Based only on MHC composition, these fibres would be classified as 'pure', but such classification would be incorrect because it would not provide information on the molecular heterogeneity of the fibres with respect to MLC composition. Two other major groups of hybrids not covered by the traditional meaning of the term 'hybrid fibres' include fibres in which two or several proteins are expressed as isoforms (e.g. fibres containing several isoforms of MLC and several isoforms of tropomyosin) and fibres in which one isoform is expressed as a protein and another as a mRNA species. It is important to note that the sets of isoforms detected so far in fibres co-expressing isoforms of two or more muscle proteins have been found to be either of the same type (matched) or of different types (mismatched). Matched sets of MHC and MLC isoforms would comprise, for example, fast-twitch MHC isoforms MHCIIa and MLCIIb and fast-twitch MLC isoforms $MLC1_f$, $MLC2_f$ and MLC3, while mismatched sets would comprise fast-twitch MHC isoforms MHCIIa and MHCIIb, slow-twitch MLC isoform $MLC1_s$ and fast-twitch MLC isoforms $MLC1_f$, $MLC2_f$ and MLC3. In recognition of the many kinds of hybrids that have been observed so far in vertebrate skeletal muscles, the hybrid fibres discussed in this review will be described by terms that indicate both the muscle protein(s) whose isoforms are being considered (e.g. MHC-MLC) and the relationship (matched or mismatched) between the sets of isoforms co-expressed in the fibre.

It is worth pointing out that hybrid fibres were once regarded as a rare phenomenon and often discarded from studies concerned with the functional characteristics of single fibre preparations (Danieli-Betto *et al.*, 1990). More recently, however, hybrid muscle fibres have started to attract considerable interest from a broad range of cell biologists. This can be explained, in part, by the finding that MHC hybrids represent the dominant biochemical phenotype even in skeletal muscles that were previously thought to be 'pure' in terms of fibre type composition (Bortolotto *et al.*, 2000a).

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*In this review a **fibre type** is identified by a roman numeral and a capital letter (e.g. IIA), while the **MHC isoform** expressed in the fibre is identified by a roman numeral and the corresponding lower case letter (i.e. MHCIIa). This nomenclature, which has been introduced by Pette's laboratory (e.g. Hämäläinen & Pette, 1995), has not been adopted consistently by other laboratories working in the field, causing a certain degree of confusion among readers of articles and reviews on MHC isoforms and MHC-based fibre types.

†Term coined by Pette's laboratory about a decade ago to describe fibres expressing more than one MHC isoform and to distinguish them from fibres expressing only one MHC isoform ('pure fibres').

Moreover, hybrid muscle fibres are now seen as valuable experimental tools for gaining further insights into two major areas of research: (i) the physiological role of muscle protein isoforms and (ii) the regulation of gene expression in multinucleated cells (see last section).

This is the first review to focus on skeletal muscle hybrid fibres. Its main aim is to raise awareness of the molecular complexity of single muscle fibres, particularly among physiologists concerned with basic and applied aspects of skeletal muscle function. The data used to illustrate various points made in the review were taken from studies of mammalian (mostly rat) and amphibian muscle. The general background sections provide a brief overview of the pattern and extent of molecular heterogeneity in hybrid muscle fibres from 'normal' and 'transforming' muscles and of the methodological problems encountered when attempting to identify and characterise such fibres. Particular attention is given to four types of skeletal muscle hybrids: MHC hybrids, mismatched MHC-MLC hybrids, mismatched MHC-regulatory protein hybrids and mismatched MHC-SR protein hybrids. The last section of the review comprises a critical examination of some of the current ideas regarding the functional significance, origin and cognitive value of hybrid fibres.

MHC Hybrids

Methods used for the detection and characterisation of MHC hybrids.

It is now quite clear that neither of the three methods traditionally employed to distinguish fibre types in skeletal muscles [light microscopy, myosin/myofibrillar ATPase (mATPase)-based or metabolic enzyme-based histochemistry] can be used effectively for the identification and characterisation of MHC hybrids (Hämäläinen & Pette, 1995; Schiaffino & Reggiani, 1996). The most suitable methods for detecting MHC polymorphism in individual muscle fibres, at protein or mRNA level, include MHC-based immunohistochemistry (MHC- IHChem), single fibre polyacrylamide gel microelectrophoresis under denaturing conditions (SDS-PAGE $_{sf}$), pyrophosphate gel electrophoresis of myosin isoenzymes in single fibre segments, reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridisation (for review see Pette & Staron, 2000; Pette *et al*., 1999; Schiaffino & Salviati, 1998; Hämäläinen & Pette, 1995). This methodological point is well illustrated by the bar graph shown in Figure 1, which allows a quick comparison of the fibre type composition of rat extensor digitorum longus (EDL) (A,B) and SOL (C,D) muscles reported by Armstrong and Phelps (1984), on the basis of mATPase-based histochemistry (A, C) and that found in more recent studies using SDS-PAGE$_{sf}$ only (EDL; Bortolotto *et al.*, 2000a) (B) or a combination of SDS-PAGE$_{sf}$ and MHC-IHChem (SOL, Bottinelli *et al.*, 1994a) (D). A significant conclusion emerging from these data is that the largest proportion (~70%) of fibres in rat EDL are hybrid IIB+IID fibres, rather than IIA or IIB fibres, as previously thought. The newly discovered heterogeneity of rat EDL muscle and muscle fibres has important theoretical and methodological implications because rat EDL has been for many years the preferred experimental model in physiological investigations of mammalian fast-twitch muscle contractility.

It is important to note that even methods such as MHC-based IHChem and SDS-PAGE$_{sf}$ have intrinsic limitations when used for the detection and characterisation of MHC hybrids. Some of these limitations have been pointed out by Pette *et al*.(1999) and Schiaffino & Salviati (1998). For example, MHC-based IHChem is limited by the availability and specificity of anti-MHC isoform antibodies and it does not always detect fibres containing both MHCIId and MHCIIb isoforms (IID + IIB hybrids; Rivero *et al.*, 1998). Furthermore, while MHC-based IHChem can provide considerable information about the proportion and intramuscular distribution of hybrid fibres and about the intracellular distribution of the co-expressed MHC isoforms (Dix & Eisenberg, 1988), it does not allow for the quantification of MHC isoforms co-expressed in individual hybrid fibres. By comparison, SDS-PAGE$_{sf}$ combined with scanning densitometry, enables the researcher to separate and quantify the relative proportion of MHC isoforms co-expressed in a single fibre, and to relate these results to other

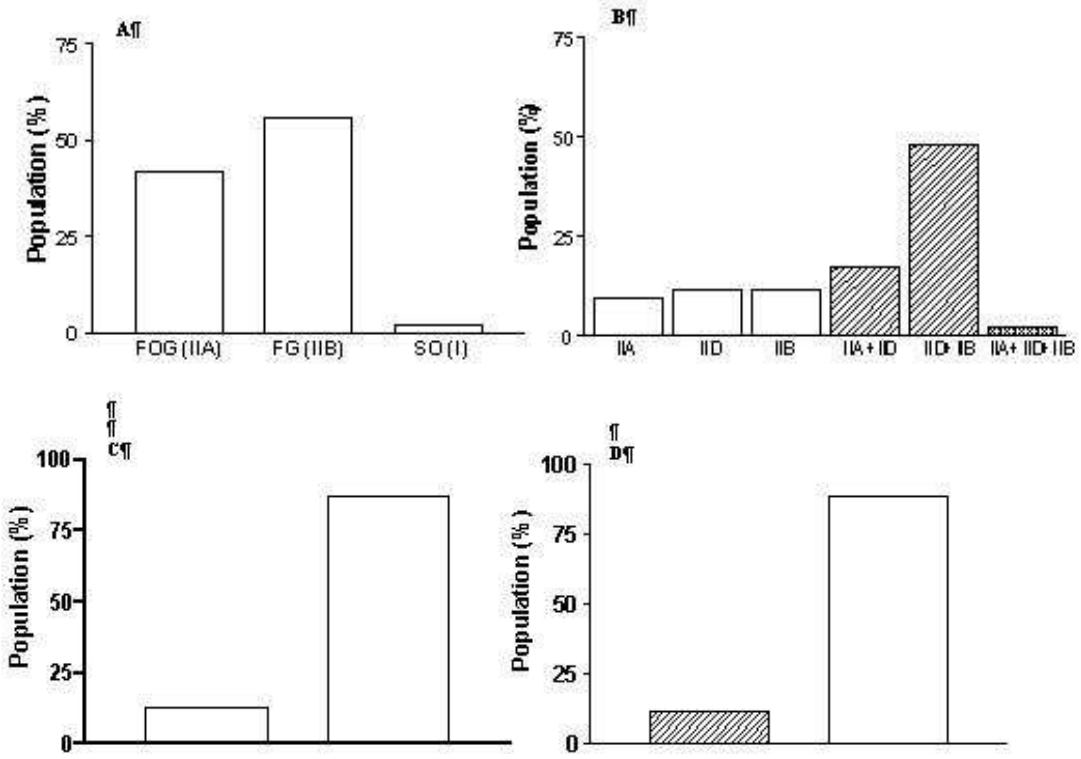

Figure 1. *The fibre type composition of rat EDL (A,B) and SOL (C,D) muscles, as determined by mATPase histochemistry (A, C) and single fibre SDS-PAGE (B, D). The graphs were plotted using the data reported by Armstrong & Phelps (1984) (A, C), Bortolotto et al. (2000a) (B) and Botinelli et al. (1994) (D). FOG, fast oxidative glycolytic; FG, fast oxidative; SO, slow oxidative.*

physiological or biochemical parameters determined in the fibre prior to incubation in the sample solubilising buffer. However, a notable limitation of SDS-PAGE$_{sf}$ is that it does not provide information on the intramuscular distribution of hybrid fibres. Also, it has to be stressed that when applied to MHC isoform analyses, SDS-PAGE$_{sf}$ displays a high degree of variability with respect to the effectiveness of separation of MHC isoform bands, a problem which has not been eliminated despite intense efforts made in several laboratories. In practice, this means that that each fibre sample has to be electrophoresed, at the same time with a MHC isoform marker (a reference sample containing all MHC isoforms), on several gels, until the separation of MHC isoform bands allows meaningful densitometric analyses to be carried out. Moreover, a SDS-PAGE$_{sf}$ protocol that separates well MHC isoforms in one species may not be so effective when applied to another species (Nguyen & Stephenson, 1999). The low specificity of anti-MHC isoform antibodies and the difficulties related to the electrophoretic resolution of MHC isoform bands are probably due to the high degree of molecular homology (78-98% aminoacid identity in striated muscles; Weiss & Leinwand, 1996) displayed by MHC isoforms and therefore cannot be eliminated in a simple manner.

Since many functional parameters of a hybrid fibre are not tightly correlated to its MHC isoform expression, neither MHC-based IHChem nor SDS-PAGE$_{sf}$ analyses of MHC isoform composition can accurately predict/characterise the functional phenotype of hybrid fibres (e.g. Bortolotto *et al.*, 2000a). Thus, in order to gain further insights into the structural and functional complexity of MHC hybrids, one needs to combine creatively existing microanalytical, microphysiological and microhistochemical methods and/or develop new methods for single fibre analysis (Pette *et al.*, 1999). For example, by

TABLE I. EVIDENCE THAT COEXISTENCE OF MULTIPLE MHC ISOFORMS IN A SINGLE FIBRE IS A COMMON MOTIF ACROSS A BROAD SPECTRUM OF NORMAL VERTEBRATE SKELETAL MUSCLES AND THAT THE PATTERN OF MHC POLYMORPHISM IS MUSCLE AND ANIMAL SPECIES SPECIFIC.

Muscle[†]	**Species**	**Proportion of hybrids**	**Number of MHC isoforms detected**	**Pattern of MHC isoform co-expression in hybrid fibres**	**Reference [method used**[‡]**]**
EDL DPH	rat	67% 30%	2 or 3 2 or 3	IIa+IId; IId+IIb (majority); IIa+IId+IIb I+IIa; ***I+IId***; IIa+IId; IIa+IId+IIb; I+IIa+IId	Bortolotto et al., (2000) **[a]**
Plantaris TA-superficial SOL	rat	~50% ~30% 11%	2 or 3 2 2	IIa+IId; IIb+IId (majority); IIa+IIb+IId IId+IIb I+IIa	Bottinelli et al., (1994a) **[a,b]**
MG	rat	12%	2	I+IIa; IIa+IId (majority); IIb+IId	Rivero et al., (1998) **[b]**
PCA*	rat	~ 35%	2, 3, or 4	IId+IIb (majority); IIIb+exoc; I+IIa+IId; IIa+IId+IIb; IIa+IIb+exoc; IId+IIb+exoc; I+IIa+IId+IIb; IIa+IId+IIb+exoc	Wu et al., (2000a) **[a]**
ThA*	rat	~90%	2 or 3	IId+IIb; IIb+exoc; IId+IIb+exoc	
AM	rabbit	not available	2	IIb+IId; IId+IIa; IIa+I	Aigner et al., **1993** (mATPase & SDS-PAGE$_{sf}$)
LC*	dog	41%	2 or 3	IIa+IId; I+IIa; I+IIa+IId	Wu et al., **1998** (SDS-PAGE$_{sf}$)
PCA*	dog	~20%	2, 3 or 4	IIa+IId (majority); IIa+IIb; IId+IIb;I+IIa+IId; IIa+IId+IIb; I+IIa+IId+IIb	Wu et al., **2000b** (SDS-PAGE$_{sf}$)
CT*		<10%	2, 3 or 4	I+IIa; IIa+IId; IId+IIb;I+IIa+IId+IIb	
ThA*		30-40%	2, 3 or 4	IIa+IId and IId+IIb (majority); I+IIa+IId;I+IId+IIb; IIa+IId+IIb; I+IIa+IId+IIb	
Rectus abdominis	cane toad	~65%	2 or 3	HCT+HC1 ; HCT+HC3 ; HC1+HC2 ; HC2+HC3 ; HCT+HC1+HC3 ; HCT+HC2+HC3 ; HC1+HC2+HC3	Nguyen & Stephenson, (in preparation) **[a]**

[†]Muscles: abbreviations as in text except *laryngeal muscles: PCA, posterior cricoarytenoid; LC, lateral cricoarytenoid; CT, cricothyroid; ThA, thyroarytenoid

[‡]Methods: [a], single fibre SDS-PAGE: [b], Immunohistochemistry: [c], mATPase histochemistry.

I+IId, atypical combination of MHC isoforms; HCT, HC1, HC2 and HC3, MHC isoforms associated with tonic, type 1, type 2 and type 3 fibres in cane toad skeletal muscles (see Nguyen & Stephenson, 1999).

applying three different biochemical methods (*in situ* hybridization, MHC-based IHChem and mATPase histochemistry) to serial cryosections from human vastus lateralis muscles (prior and post-37 day period of bed rest), Andersen *et al.* (1999) discovered a novel population of MHC hybrids, in which the protein of one MHC isoform (MHCI) coexisted with the mRNA of another (MHCIId). The discovery of MHC hybrids with mismatched protein-mRNA species adds a new meaning to the concept of skeletal muscle cell heterogeneity and prompts the obvious question whether vertebrate skeletal muscles contain any fibres that are genuinely 'pure'.

MHC hybrids detected in 'normal' muscles*

Fibres co-expressing the slow-twitch and fast-twitch MHC isoforms MHCI and MHCIIa (I+IIA or I/IIA fibres) are the earliest examples of MHC hybrids reported to occur in normal mammalian skeletal muscle. These fibres, previously referred to as IC or IIC fibres on the basis of the most abundant MHC isoform expressed (MHCI or MHCIIa, respectively) (Pierobon-Bormioli *et al.*, 1981), are still the easiest to identify and characterise both by MHC-based IHChem and SDS-PAGE$_{sf}$. This is because the currently available monoclonal antibodies (Mabs) against MHCI and MHCIIa have a high degree of specificity and the electrophoretic bands corresponding to the MHCI and MHCIIa proteins are separated quite effectively and reproducibly by all SDS-PAGE$_{sf}$ protocols developed and/or used in different laboratories. As described in detail in a recent study by Bortolotto *et al.* (2000a), type I+IIA hybrid fibres can also be identified by the physiological fibre typing method of Fink *et al.* (1986), because they produce characteristic staircase-like force-pSr curves ('composite' curves).

A large number of data generated over the last two decades by MHC-based IHChem and/or SDS-PAGE$_{sf}$, strongly suggest that normal muscles from mammals and amphibians contain a sizeable proportion of MHC hybrids, which co-express, at the protein level, two, three and even four MHC isoforms. A small sample of these data (Table I) shows that the pattern of MHC polymorphism (as indicated by the proportion of hybrids, the number of isoforms co-expressed and the combination of MHC isoforms detected in individual fibres) is muscle and animal species specific. For example in adult rat, soleus muscle (SOL) was found to contain about 11% MHC hybrids, all of which co-expressed two MHC isoforms (I and IIa), while laryngeal thyroarytenoid (ThA) muscle was found to contain about 90% MHC hybrids, some of which co-expressed combinations of two or even three MHC isoforms. In the dog, however, ThA muscle was found to contain a smaller proportion (30-40%) of MHC hybrids and many of these hybrids co-expressed <u>all</u> 4 major MHC isoforms commonly found in mammalian muscle (I, IIa, IId and IIb). MHCexoc, a tissue specific isoform co-expressed with MHCIId and MHCIIb in rat ThA muscle was not detected in dog ThA muscle.

MHC hybrids detected in muscles in transformation

It is now widely accepted that the proportion of MHC hybrids and their molecular complexity (as judged by the number of MHC isoforms co-expressed and the pattern of co-expression) is higher in muscles undergoing molecular and functional transformation than in normal muscles (see review by Pette *et al.*, 1999). As seen in Table II, soleus muscles of rats subjected to four week unloading by hindlimb suspension (a strategy inducing a slow to fast transition in muscle phenotype) contained 4 times more hybrid fibres than the controls (Oishi *et al.*, 1998). Moreover, most hybrid fibres in the transforming soleus co-expressed three MHC isoforms (I, IIa and IId) , while all hybrids in the control soleus co-expressed two MHC isoforms only (I and IIa). Some MHC isoform combinations, such as I+ IId, which are seen only rarely in fibres from normal muscles, have been found to occur fairly

* The term 'normal' muscles is used to indicate muscles from animals free of disease, muscles from adult animals or muscles that had not been subjected to 'transforming conditions' (e.g. changes in the neural impulse pattern or hormone level).

TABLE II. EXAMPLES OF MHC POLYMORPHISM ASSOCIATED WITH EXPERIMENTALLY INDUCED MUSCLE TRANSFORMATION

Muscle (method used to induce muscle transition)	**Species**	**Proportion of hybrids-experimental (proportion of hybrids-control)**	**Number of MHC isoforms detected in hybrids (vs control)**	**Pattern of MHC isoform co-expression in hybrid fibres (vs control)**	**Reference (method† used)**
<u>slow→fast</u>					
SOL (4 wk-unloading by HS)	rat	32% (7%)	2 or 3 (2)	I+IIa; IIa+IId; **I+IIa+IId***; IIa+IId+IIb (I+IIa)	Oishi et al., (1998) **[a]**
SOL (60 days post SCT)	rat	~88% (~4%)	2 or 3 (2)	I+IIa; **I+IId**; IIa+IId; I+IIa+IId (I+IIa)	Grossman et al., (1998) **[b]**
SOL (4 wk-thyroid hormone treatment)	rat (male) rat (female)	99% (ni) 63% (ni)	2 & 3 2 & 3	**I+IIa**; I+IIa+IId (ni) **I+IIa**; IIa+IId; αcl+IIa; I+IIa+IId (ni)	Yu et al., (1998) **[b]**
<u>fast →faster</u>					
MG-deep region (60 days post SCT)	rat	~55% (~15%)	2,3 or 4 (2)	I+IIa; I+IId; IIa+IId; **IId+IIb**; IIa+IId+IIb; I+IIa+IId; I+IIa+IId+IIb (I+IIa; **IIa+IId**; IId+IIb)	Roy et al., (2000) **[b]**
MG-superficial region (60 days post SCT)	rat	~7% (~22%)	2 or 3 (2 or 3)	**IIa+IId**; IId+IIb; IIa+IIb+IId (**IIa+IId**; IIa+IId+IIb)	ditto
<u>fast→slow</u>					
EDL (28 d low frequency stimulation)	rat	?	2 and 4 (2)	I+IIa; IIa+IId; **I+IIa+IId+IIb** (**IIb+IId**)	Termin et al., (1989) **[a,c]**
TA (30 d low frequency stimulation)	rabbit	70% (ni)	2 or 3 (ni)	IIa+Iα; IIa+Iα+dev; **IIa+I**a **+I**; IIa+I; Iα+I; Iα+I+dev;I+dev (ni)	Peuker et al., (1999) **[b]**

HS, hindlimb suspesion ; SCT, spinal cord transection ;

†Methods: [a], single fibre SDS-PAGE; [b], immunohistochemistry; [c], mATPase histochemistry

*bolding indicates that the respective pattern of MHC isoform expression was detected in the most abundant type of hybrid fibres; ni, not clearly indicated.

αcl (Iα), α-cardiac like MHCisoform ; dev, MHC_{dev} ; Note: Peuker et al.(1999) give no information on the relationship between MHC_{dev} and the two MHC isoforms found in developing and regenerating muscles known as MHC_{neo} and MHC_{emb}

frequently in fibres from transforming muscles (Pette & Staron, 2000, 1997; Talmadge, 2000). For example, Bortolotto *et al.* (2000a) detected only two I + IID hybrids in a population of 43 fibres dissected from 8 normal rat diaphragms, but I + IID hybrids were reported to make up the most abundant fibre-type population in SOL muscles of adult rat, 60 days post spinal cord transection (see Table II; Grossman *et al.*, 1998).

The transition of a muscle from one 'steady-state' to another, through a process which may involve the replacement or addition of muscle protein isoforms in one or several intracellular compartments, has been found to accompany several physiopathological conditions/factors (Table III). These include growth and development of an organism from embryonic to adult stage, ageing, muscle degeneration/regeneration and changes in the hormonal level (thyroid hormone being the classical example) (Pette & Staron, 2000, 1997). For example, La Framboise *et al.* (1991) reported that before reaching the adult state, the rat diaphragm muscle contained some fibres that co-expressed as many as four MHC isoforms.

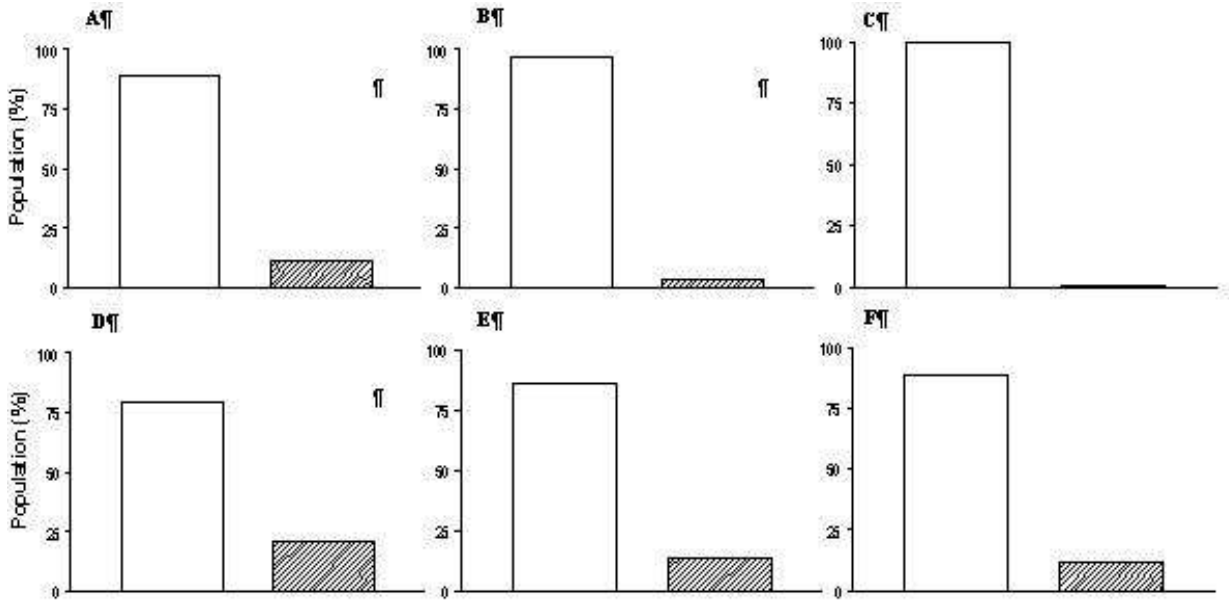

Figure 2. *The proportion of pure and hybrid fibres in soleus muscles from age-matched normotensive WKY rats (A,B,C) and spontaneously hypertensive rats (D,E,F) at three stages of development of hypertension: 4 weeks (A,D), 16 weeks (B,E) and 24 weeks (C,F). , pure fibres (I, IIA) , hybrid fibres: I+IIA (in WKY rats) I+IIA; IIA+IID (in SHRs). The raw data used to plot the bar graphs can be found in Table 3 in Bortolotto et al. (1999). The number of animals used and the number of fibres analysed for each group were : 7; 73 (A); 8; 85 (B); 7; 79 (C); 7; 75 (D); 6; 73 (E); 7; 87 (F).*

Recently, Bortolotto *et al.* (1999) observed, using SDS-PAGE$_{sf}$, that the populations of fibres dissected from SOL muscles of spontaneously hypertensive rats (SHR) at three different stages in the development of hypertension (4, 16-18 and 24 weeks) contained a higher proportion of hybrid fibres than the homologous muscles from age matched normotensive (WKY) controls (Fig.2). Does this mean that hypertension, a pathological condition not generally associated with skeletal muscle pathology, causes transformation in rat soleus muscles? Not necessarily, because the higher proportion of MHC hybrids found in SHR soleus may be due to strain-related differences in the fibre type composition of individual muscles. To address this possibility we are now examining the MHC isoform expression in soleus muscles from several normotensive and hypertensive rat strains (J. Kemp, S. Bortolotto & G. M.M. Stephenson, unpublished data).

TABLE III. FACTORS/CONDITIONS THAT HAVE BEEN REPORTED TO TRIGGER SKELETAL MUSCLE TRANSITION IN NON-HUMAN MAMMALS.

Factor/ condition	Direction of phenotype shift
• muscle growth and development to neonatal stage	embryonic → neonatal
• maturation and ageing	fast → slow
• spontaneous hypertension (model for hypertension)	slow → fast
• thyroid hormone administration (hyperthyroidism)	slow → fast
• muscle degeneration	
• muscle regeneration	
- post myotoxic treatment	
- post denervation-devascularization	
• lab induced-decrease in neuromuscular activity	
- spinal cord transection (model for : spinal cord injury, orthopedic injury)	slow → fast
- limb immobilization in a shortened position	slow → fast
- hindlimb suspension (model for weightlessness during spaceflight)	slow → fast
- blockage of motoneuron action potential conduction by tetrodotoxin	fast → slow
• lab-induced increase in neuromuscular activity	
- chronic electrical stimulation	fast → slow
- functional overload induced by synergist ablation	
- endurance training	
- strength (resistance) training	fast → slow
• lab induced depletion in energy rich phosphates	
• induction of null mutations in muscle protein isoform genes	

The main aim of the study by Bortolotto et al. (1999) was to compare the MHC isoform and fibre type composition of soleus muscles from SHR and WKY rats; however, since the study involved the use of animals at three different developmental stages, the data generated offer also an insight into the effect of rat maturation on the proportion of hybrid fibres present in this muscle. As seen in Figure 2, the proportion of hybrids detected in the fibre populations dissected from SOL muscles of WKY rats aged 4 weeks, 16 weeks and 24 weeks decreased from 11% (4 weeks) to 3.5% (16 weeks) and 0% (24 weeks). A decrease in the proportion of hybrid fibres with an increase in animal age was also noted in the soleus muscles from SHRs. In contrast, the rectus abdominis muscles of 'adult'cane toads (bw ~250 g) produced a larger proportion of MHC hybrids than the homologous muscles from 'juvenile' (bw ~15g) toads (L. Nguyen & G.M.M. Stephenson, unpublished data). This difference in the effect of animal maturation on the proportion of hybrid fibres present in rat soleus and toad rectus abdominis muscle suggests that muscle transformation associated with development may be animal species- and/or muscle-specific.

A limited survey of the literature on vertebrate skeletal muscle reveals that, over the last two decades, there has been a flurry of activity in the field of muscle transformation. This, together with the breadth of journals that published this information (for recent reviews see Pette & Staron, 2000;

Talmadge, 2000; Pette & Staron, 1997), indicate that, muscle plasticity, like muscle heterogeneity, is currently viewed as a 'hot' research topic by biomedical researchers from a broad range of disciplines (molecular biology, biochemistry, cell physiology, medical practice and exercise physiology).

Other Types of Hybrid Fibres Detected in Vertebrate Skeletal Muscles

Methodological issues

It is important to point out that highly complex hybrid fibres, such as those containing matched or mismatched sets of isoforms for two or more proteins, are more difficult to detect than MHC hybrids, which are heterogeneous with respect to MHC isoform composition only. This is because many muscle proteins, such as the proteins of the sarcotubular system, are present in the fibre only in very small amounts and therefore cannot be easily visualised on SDS-polyacrylamide gels using current staining protocols. Even when the concentration of a given protein in a single fibre segment is large enough to allow for easy visualisation, as is the case for most myofibrillar proteins, there may be problems related to the electrophoretic separation of the protein bands of interest (see comments made earlier regarding the separation of MHC isoforms). A classic example is that of the fast-twitch and slow twitch isoforms of MLCs, troponin subunits and tropomyosin, which either co-migrate or migrate very closely on SDS-polyacrylamide gels prepared according to common protocols. In such cases, the accurate identification of hybrid fibres requires further refinement of SDS-PAGE$_{sf}$ protocols, the combined use of SDS-PAGE$_{sf}$ and IHChem or the combined use of several biochemical and physiological methods of single fibre analysis. The following sub-sections focus on three groups of hybrid fibres co-expressing mismatched sets of protein isoforms and highlight, when appropriate, the methodological approaches that led to their discovery.

Mismatched MHC-MLC hybrids

Single muscle fibres that express only one MHC isoform, but are heterogeneous with respect to their myosin light chain complement represent a relatively common example of MHC-MLC polymorphism (for review see Pette & Staron, 1997). Thus, a small number of mismatched MHC-MLC hybrids have been detected among fibres dissected from diaphragm muscles of adult normotensive (WKY) rats (Bortolotto *et al*., 2000a), indicating that mixed expression of MHC and MLC isoforms occurs in normal muscle fibres. In Figure 3 are shown the electrophoretograms of two of the fibres analysed by Bortolotto *et al.* (2000a) for MHC and MLC composition: one displaying full correlation (MHCI + MLC1$_s$ + MLC2$_s$; left lane) and the other no correlation between the myosin subunit isoforms present in the fibre (MHCIIa + MHCIId + MLC1$_s$ + MLC1$_f$ + MLC2$_f$; right lane). Another interesting observation made in the study of Bortolotto *et al.* (2000a) is that, in the rat soleus muscle, fibres expressing only fast MHCIIa isoforms contained the slow isoform MLC1$_s$ in addition to the fast isoforms MLC1$_f$ and MLC2$_f$. This result is in agreement with earlier data by Mizusawa *et al.* (1982) and Salviati *et al.* (1982) who showed also that type IIA fibres isolated from soleus muscles of the rat (Mizuzawa) and rabbit (Salviati) co-expressed various combinations of fast and slow MLC isoforms.

Mismatched MHC-MLC hybrids have been detected not only in normal, but also in transforming muscles. For instance, 'pure' slow-twitch (type I) soleus fibres from female rats, treated for 4 weeks with the thyroid hormone (T_3), were found by Yu *et al*. (1998) to contain both slow and fast MLC isoforms, in different combinations and in varying proportions.

Notwithstanding their presence in many of the commonly studied muscles and the relative ease with which they are detected by SDS-PAGE$_{sf}$, the physiological significance of MHC-MLC hybrids remains largely unknown.

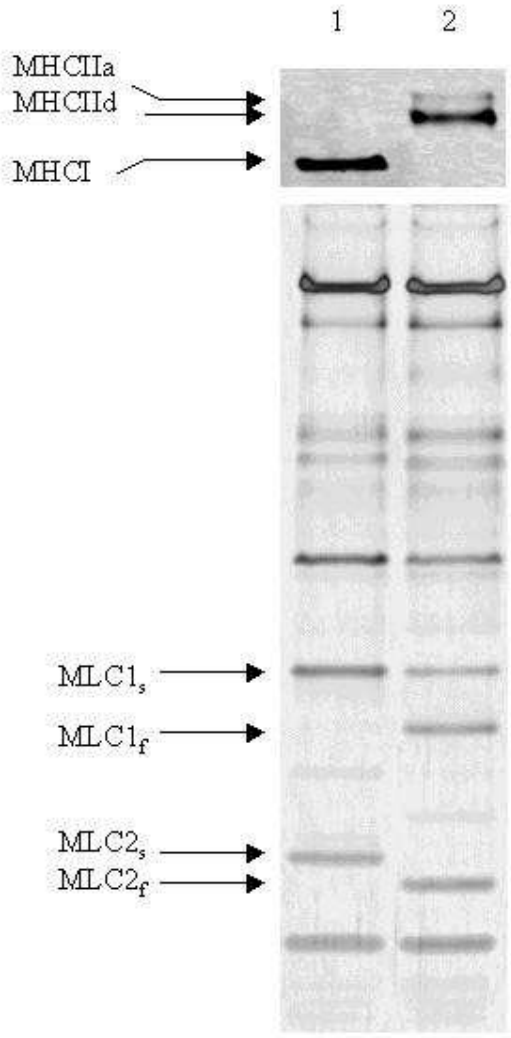

Figure 3. *Representative electrophoretograms of myofibrillar proteins from single rat skeletal muscle showing matched (lane 1) and mismatched (lane 2) MHC-MLC isoform composition. Upper panel: MHC isoforms; lower panel: MLC isoforms. Lane 1, fibre type I; Lane 2, fibre type IIA + IID.*

Mismatched MHC-regulatory protein hybrids

To date, reports of mixed MHC-regulatory protein hybrids are few and far between. For example two decades ago, Salviati et al. (1982) described a small population of rabbit masseter muscle fibres, which contained fast-twitch and slow-twitch isoforms of MHCs, MLCs, TnT and Tn I, but only the fast-twitch isoform of TnC. Mismatched MHC-TnC hybrids co-expressing either MHCI (slow), TnC-s (slow) and TnC-f (fast) or MHCI and TnC-f were also detected in rat diaphragm muscle by Danieli-Betto *et al.* (1990) and Geiger *et al.* (1999), respectively. Since adult normal animals were used in all three studies, these data suggest that single fibres from normal muscles co-express mismatched sets of MHC and TnC isoforms.

At present it is not clear whether mismatched MHC-regulatory protein hybrids occur also in transforming skeletal muscles. Kischel *et al.* (2001) did not detect any fibres containing mismatched MHC and TnC isoforms in rat soleus muscles undergoing a shift from slow- to fast-twitch phenotype, but in this study the MHC isoform composition was 'deduced' from MLC isoform composition rather than determined directly. Given that in rat skeletal muscle fibres there is no tight correlation between MLC and MHC isoform expression (see previous section), it is possible that the MHC isoform composition in some single fibres was not correctly assessed and therefore mismatched MHC-TnC hybrids were overlooked.

As mentioned previously, the visualisation and identification of Tn subunit isoforms on SDS polyacrylamide gels are fraught with problems, because the electrophoretic bands are not well separated from each other and from MLC2 isoform bands. This point is well illustrated in the study of Kischel *et al.* (2001), because the authors combined three methods in order to positively identify the TnC isoforms expressed in their fibre preparations: SDS-PAGE$_{sf}$, Western Blotting and measurements of the sensitivity of chemically skinned fibre segments to activation by Sr^{2} and Ca^{2+} (in the presence/absence of the fibre-type dependent Ca^{2+} sensitiser molecule bepridil).

Mismatched MHC - SR protein hybrids

According to the current dogma, SR protein complexes such as the Ca^{2+} release channel/ryanodine receptor (RyR) and SERCA play key roles in skeletal muscle contraction and relaxation by regulating the concentration of activating ions ($[Ca^{2+}]$) in the myoplasm (see review by Stephenson et al., 1998). Mammalian skeletal muscles have been shown to express two different RyR isoforms (predominantly RyR 1 and some RyR 3; Csernoch, 1999) and three different SERCA isoforms (SERCA 1a, SERCA 1b and SERCA 2a; Loukianov et al., 1998). SERCA 1a is present in typical fast-twitch fibres and SERCA 2a is present in typical slow-twitch fibres.

There are very few reports of mixed MHC - SR protein hybrids in the literature concerned with skeletal muscle heterogeneity. For example, in a paper on the fibre-specific regulation of Ca^{2+}-ATPase isoform expression by thyroid hormone in rat skeletal muscle, the authors (Van der Linden *et al.*, 1996) describe a small population of fast-twitch fibres, dissected from soleus muscles of euthyroid ('normal' muscles) and hypothyroid rats (muscles 'in transition'), which expressed the fast-twitch isoform MHC IIa and both fast-twitch SERCA1a and slow-twitch SERCA2a isoforms. Other examples of mixed MHC-SR protein hybrids can be found in a recent study by Bortolotto et al. (2001), who detected in soleus muscles of spontaneously hypertensive rats (SHR) a population of type I (slow-twitch) fibres displaying fast-type SR characteristics, and another population of type II (fast-twitch) fibres displaying slow-type SR characteristics (see Fig. 4).

Once again it is interesting to note the methodological approaches that allowed the identification of the mismatch between the protein isoform composition of the myofibrillar compartment and that of the SR. The MHC-SERCA hybrids described in the study of Van der Linden *et al.* (1996) were identified by IHChem with a protocol using fluorescence labelled antibodies against MHCI, MHCII, SERCA1a and SERCA2a, while the MHC-SR protein hybrids described in the study of Bortolotto et al. (2001), were identified by a combination of biochemical (SDS-PAGE$_{sf}$) and physiological methods (measurements of caffeine thresholds for contraction in mechanically skinned single fibre preparations).

Functional Significance, Origin and Experimental Value of Hybrid Skeletal Muscle Fibres

Functional significance of hybrid fibres

As it has been already discussed, hybrid fibres exist in both transforming and normal skeletal muscles, and in some muscles they represent the predominant phenotype. What is unclear, however, is whether hybrid fibres play a major role in the mechanical performance of a muscle or whether they are incompletely differentiated muscle cells, and as such, functionally redundant entities. The issue of the functional significance of hybrid fibres is further complicated by compelling evidence that some hybrid fibres are persistent rather than transitory cellular species (Lutz & Lieber, 2000; Bortolotto et al., 2000a; Talmadge, 2000; Talmadge et al., 1999)

At present, the prevailing view is that hybrid fibres enable a muscle to fine tune its efficiency for the wide range of forces, velocities, levels of endurance and levels of resistance to fatigue it is required to generate (Pette & Staron, 2000; Pette et al., 1999; Galler *et al.*, 1994; Botinelli *et al.*, 1994a,b; Danieli-Betto *et al.*, 1986). This view, which does not distinguish between hybrid fibres from normal

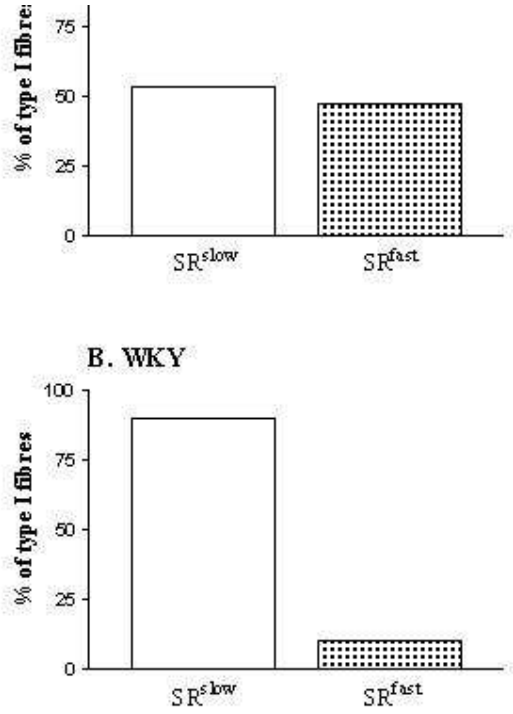

Figure 4. *MHC-SR protein hybrids* (**.....**) *detected in soleus muscles from adult SHR (panel A) and WKY (panel B) male rats, based on caffeine thresholds for contraction. Methodological details and the data which were used to plot these graphs can be found in Bortolotto et al. (2000b).*

and transforming muscles, is based largely on data showing that some contractile characteristics of MHC hybrids lie between the contractile characteristics of the corresponding pure fibres. For example, in adult rat skeletal muscle, hybrid and pure fibre types have been found to display a continuum of values with respect to stretch-activation kinetics (Galler *et al.,* 1994), maximum/unloaded shortening velocity (Botinelli *et al.,* 1994a), myofibrillar ATPase and tension cost (the ratio between ATPase activity and isometric tension; Botinelli *et al.,* 1994b).

Support for the idea that hybrid fibres enable a muscle to respond more effectively to functional demands comes not only from studies of contractile properties in fibres from normal and transforming muscles, but also from data on the fibre type composition of very specialised normal muscles. For example, Dammeijer *et al.* (2000) found that all fibres in rat stapedius, a small muscle believed to prevent the auditory receptors in the inner ear from injury by intense noise, co-expressed more than one MHC isoform. According to Dammeijer *et al.* (2000), this unusual fibre type composition enables the stapedius muscle to contract fast and fatigue slowly (thereby stiffening the middle ear bone chain) at acoustic stimulation stronger that 80 dB.

There are reasons to believe that 'the contractile properties continuum' paradigm tells only part of the story of the functional significance of hybrid fibres. Since it has been developed mainly on the basis of results obtained with MHC hybrids co-expressing two MHC isoforms only, the paradigm does not explain the role of hybrid fibres co-expressing three or four MHC isoforms (Talmadge, 2000) or displaying other types of polymorphism.

There is no doubt that far more work will have to be carried out in order to understand the functional significance of hybrid fibres in skeletal muscle. From a methodological point of view, this will mean in first instance applying the aforementioned strategies to more functional parameters, more muscles and more species. One can envisage, however, that this approach will not be sufficient and that other methods will have to be developed/refined later on. In this context, a study by Acakpo et al. (1997) in which mice carrying null mutations in members of the MHC gene family were used to examine the functional role of MHCIIb and MHC IId in mouse EDL and diaphragm may be regarded as a trendsetter.

Molecular mechanisms underlying the hybrid fibre phenomenon

Related to the functional significance of hybrid fibres is the issue of their origin. Some obvious questions that can be asked in connection with this issue are: (i) what are the initiating signal(s) and cellular pathway(s) involved in the appearance of hybrid fibres in transforming muscles? (ii) are the cellular events associated with muscle transformation muscle-specific or transforming factor/condition-specific? (iii) when and how do hybrids appear in normal muscles? (iv) is muscle protein polymorphism related to the presence of multiple nuclei in the muscle cell? (v) why do hybrids

from transforming muscles have a more complex pattern of MHC isoform co-expression than hybrids from normal muscles? (vi) is the muscle protein complement expressed in a hybrid fibre predetermined or does it result from various inductive influences exerted on a naive cell? Once again, it is important to stress that our current understanding of the origin of hybrid fibres (particularly of those present in normal muscles) is very limited.

According to currently available data, most proteins involved in the E-C-R cycle are encoded by unique genes and their isoforms are generated by alternative splicing of the primary RNA transcript through a process mediated by muscle-specific factors. The most frequently cited examples of muscle protein isoforms produced by alternative splicing are the two fast-twitch isoforms of myosin light chains, $MLC1_f$ and MLC3 (Wade & Kedes, 1989).

Some muscle proteins (such as MHC_s) are encoded, however, by multiple genes (referred to as a 'multi-gene family'or 'isogenes') and their isoforms results from the differential expression of the gene-family members. It is interesting to note that isogenes can be located on the same chromosome (in tandem or as a cluster), but also on different chromosomes, and that their expression is regulated by muscle-specific *cis*-acting sequences (such as the E-box elements) and *trans*-acting factors (Talmadge 2000; Weiss & Leinwand, 1996).

Based on this insight into the molecular mechanisms underlying muscle protein polymorphism, it is reasonable to suggest that, in transforming muscles, the hybrid fibre phenotype is the product of a series of coordinated or independent transcriptional events initiated by transforming factors/conditions. In a recent review, Talmadge (2000) raised also the possibility that MHC hybrid fibres observed in skeletal muscle after alterations in electrical activity result from the differential responsiveness of individual myonuclei in a muscle fibre to regulators of MHC isoform gene expression. The idea of nuclei of transforming fibres not working in synchrony, which has been canvassed earlier by Staron & Pette (1987), provides an interesting perspective when inquiring into the origin of hybrid fibres in muscles in transition.

It has been suggested that the molecular heterogeneity of hybrid fibres can derive not only from pre-translational , but also from translational and even post-translational events. In the former case, the resulting hybrid fibres may contain one isoform expressed as a protein, and another expressed as a matched or mismatched mRNA species (Andersen *et al.*, 1999; Barton & Buckingham, 1985). In the latter case, the presence of certain isoforms in a fibre may be the result of relatively slow rates of degradation. Thus, Staron & Pette (1993) argued that the MHCIIb isoform detected in hybrid fibres from rat fast-twitch muscles undergoing fast-to-slow transition is not a newly expressed protein, but a transient species with a half life of about 14.7 days. If the rates of degradation of muscle proteins are slow and if they are isoform-specific it is quite clear that after a sudden change in certain conditions there would be an isoform-specific lag between the time when the synthesis of a protein isoform stops and the time when the protein disappears completely from the cell. In this case, however, one would have to wonder about the functional status of the isoform that is in the process of being replaced by a newly synthesized one.

Experimental value of hybrid fibres.

In this review it has been argued that identifying and characterising hybrid fibres is not an easy task. Indeed, each of the methods used so far for this purpose has been found to be fraught with technical difficulties and, under certain conditions, to be of limited effectiveness. So, why study hybrid fibres?

The reasons for studying hybrid fibres become clearer if one considers a small sample of research questions that have already benefited or are likely to benefit in the future from studies of hybrid skeletal muscle fibres (Table IV). These questions, whose scope may at times overlap, belong loosely to two major fields of inquiry: one concerned with the relationship between the structure of

TABLE IV. EXAMPLES OF RESEARCH QUESTIONS THAT HAVE BENEFITED OR ARE LIKELY TO BENEFIT FROM STUDIES OF HYBRID FIBRES

Research question
Questions related to the relationship between the structure and function of proteins involved in the E-C-R cycle.
1a. What is the distribution of hybrid fibres in a given muscle and how does it relate to the overall muscle function?
2a. Do motor units contain hybrid fibres?
3a. When several MHC isoforms are present in the same muscle fibre, are they expressed simultaneously or is there a programmed gene switching process
4a. Do MLCs play a role in mATPase activity?
5a. Is the pattern of muscle protein co-expression in a hybrid fibre constant along the length of the fibre?
6a. What are the relative rates of synthesis/degradation of various muscle protein isoforms in skeletal muscle?
7a. Are MHC and MLC isoforms independently regulated?
8a. What is the origin of hybrid fibres in normal muscles?
9a. Are there any molecular differences between hybrid fibres from transforming muscles and normal muscles?
10a. Are interactions between mismatched isoforms of myosin subunits (eg. MHCI and $MLC1_f$) different from those between matched isoforms (eg. MHCI and $MLC1_s$)?
11a Can the proportion or type of hybrid fibres act as an indicator of skeletal muscle pathology?
Questions related to the mechanisms of regulation of gene expression in complex eukaryotic cells
1b. What is the subcellular distribution of isoform-specific mRNA species?
2b. Are events involved in the synthesis of various muscle protein coordinated?
3b. Are events involved in the degradation of various muscle protein coordinated?
4b. What are the superior elements controlling the coordinated expression of genes regulated by gene switching and by alternative splicing
5b. How do different nuclei work in a multinucleated cell?

muscle proteins and their specific roles in events of the E-C-R cycle (1a-11a), the other, of more general interest, concerned with mechanisms of regulation of gene expression in mammalian cells (1b –5b). Let us consider, for example, the paradigm of MHC gene-switching pathway in mammalian skeletal muscle. This is a paradigm that has emerged as a result of the discovery of hybrid fibres and is continuously modified to reflect the data generated by ongoing research on the diversity and plasticity of skeletal muscle fibres and on muscle protein polymorphism. Thus, shortly after the first reports of MHC isoform co-expression in single fibres, Danieli-Betto et al. (1986) hypothesised that during transition from slow to fast phenotype, MHC genes in adult rat skeletal muscle are activated in the sequence I $\rightarrow$ IIa $\rightarrow$ IIb. As a result of 14 years of intense investigations on muscles in transition, the paradigm has been modified to include the newly discovered fast-twitch MHCIId/x isoform (I$\rightarrow$ IIa $\rightarrow$ IId $\rightarrow$ IIb; Talmadge, 2000) and to reflect the reversible nature of fibre transition (MHCI $\leftrightarrow$ MHC IIa $\leftrightarrow$ MHC IId $\leftrightarrow$ MHC IIb; Pette & Staron, 2000). As emphasized by Oishi *et al.* (1998), learning from

hybrid fibres the MHC composition in a fibre, at the start of the transforming process, is essential when defining the specific steps in the MHC activation sequence.

There is little doubt that, in terms of attention received from muscle researchers, the 'hybrid' fibre (regardless of its type) has reached the big time. This is not surprising, for as Pette *et al.* (1999) stated recently, these fibres 'offer unique opportunities for relating molecular patterns of protein expression to functional properties and for elucidating mechanisms controlling gene expression in muscle'.

Conclusions

The major points made in this review can be summarized as follows:

- Hybrid fibres are present in both normal and transforming skeletal muscle, but their proportion and molecular complexity is higher in the latter. The frequency of their occurrence in various muscles and species suggests that hybrid fibres are not a rare phenomenon.
- To date, the phrase 'hybrid fibres' has been used to describe only fibres co-expressing several MHC isoforms. MHC hybrids represent, however, only one of the many types of hybrid fibres that exist in vertebrate skeletal muscles. Therefore, in order to facilitate progress in the area concerned with muscle fibre diversity and plasticity, a new set of terms is required. This new terminology should be able to include all the hybrid fibre types discovered so far, as well as those that are likely to be discovered in the future.
- To detect and characterize hybrid muscle fibres one needs to combine creatively existing microanalytical, microphysiological and microhistochemical methods and to develop new methods for single fibre analysis.
- Regardless of their type, hybrid fibres have the potential to become valuable tools for the pursuit of knowledge related to events of the E-C-R cycle and to the regulation of gene expression in multinucleated cells.

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References

Acakpo-Satchivi, L.J.R., Edelmann, W., Sartorius, C., Lu, B.D., Wahr, P.A., Watkins, S.C., Metzger, J., Leinwand, L & Kucherlapati, R. (1997) Growth and muscle defects in mice lacking adult myosin heavy chain genes. *Journal of Cell Biology*, 139, 1219-1229.

Aigner, S., Golsch, B., Hämäläinen, N., Staron, R.S., Uber, A., Wehrle, U. & Pette, D. (1993) Fast myosin heavy chain diversity in skeletal muscles of the rabit: heavy chain IId, not IIb predominates. *European Journal of Biochemistry*, 211, 367-372.

Andersen, J.L., Gruschi-Knudsen, T., Sandri, C., Larsson, L. & Schiaffino, S. (1999) Bed rest increases the amount of mismatched fibres in human skeletal muscles. *Journal of Applied Physiology*, 86, 455-460.

Armstrong, R.B. & Phelps, R.O. (1984) Muscle fibre type composition of the rat hindlimb. *American Journal of Anatomy*, 171, 259-272.

Barton, P.J. & Buckingham, M.E. (1985) The myosin light chain proteins and their genes. *Biochemical Journal*, 231, 249-261.

Bortolotto, S.K., Cellini, M., Stephenson, D.G.& Stephenson, G.M.M. (2000a) MHC isoform composition and Ca^{2+}- or Sr^{2+}-activation properties of rat skeletal muscle fibres. *American Journal of Physiology*, 279, 1564-1577

Bortolotto, S.K., Stephenson, D.G.& Stephenson, G.M.M. (2000b) Caffeine thresholds for contraction in electrophoretically typed, mechanically skinned muscle fibres from SHR and WKY rat. *Pflügers Archiv* (in print).

Bortolotto, S.K., Stephenson, D.G.& Stephenson, G.M.M. (1999) Fibre type populations and Ca^{2+}- activation properties of single fibres in soleus muscles from SHR and WKY rats. *American Journal of Physiology*, 276, C628-C637.

Bottinelli, R., Betto, R., Schiaffino, S & Reggiani, C. (1994a) Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle. *Journal of Muscle Research and Cell Motility*, 15, 413-419.

Bottinelli, R., Canepari, M., Reggiani, C. & Stienen, G.J.M. (1994b) Myofibrillar ATP ase activity during isometric contraction and isomyosin composition in rat single skinned muscle fibres. *Journal of Physiology*, 481.3, 663-675.

Csernoch, L. (1999) Regulation of the ryanodine receptor calcium release channel of the sarcoplasmic reticulum in skeletal muscle. *Acta Physiologica Hungarica*, 86, 77-97.

Dammeijer, P.F.M., Van Mameren, H., Van Dijk, P., Moorman, A.F.M., Habets, P., Manni, J.J. & Drukker, J. (2000) Stapedius muscle fibre composition in the rat. *Hearing Research*, 141, 169-179.

Danieli-Betto, D., Betto, R. & Midrio, M. (1990) Calcium sensitivity and myofibrillar protein isoforms of rat skinned skeletal muscle fibres. *Pflügers Archiv*, 417, 303-308.

Danieli-Betto, D., Zerbato, E. & Betto, R. (1986) Type I, 2A, and 2B myosin heavy chain electrophoretic analysis of rat muscle fibres. *Biochemical and Biophysical Research Communications*, 138, 981-987.

Dix, D.J. & Eisenberg, B.R. (1988) In situ hybridization and immunohistochemistry in serial sections of rabbit skeletal muscle to detect myosin expression. *Journal of Histochemistry and Cytochemistry*, 36, 1519-1526.

Fink, R.H., Stephenson, D.G. and Williams, D.A. (1986) Calcium and strontium activation of single skinned muscle fibres of normal and dysstrophic mice. *Journal of Physiology*, 373, 513-525.

Galler, S., Schmitt, T.L. & Pette, D. (1994) Stretch activation, unloaded shortening velocity, and myosin heavy chain isoforms of rat skeletal muscle fibres. *Journal of Physiology*, 478, 513-521.

Geiger, C.G., Cody, M.J. & Sieck, G.C. (1999) Force-calcium relationship depends on myosin and troponin isoforms in rat diaphragm muscle fibres. *Journal of Applied Physiology*, 87, 1894-1900.

Grossman, E.J., Roy, R.R., Talmadge, R.J., Zhong, Hui. & Edgerton, V.R. (1998) Effects of inactivity on myosin heavy chain composition and size of rat soleus fibres. *Muscle & Nerve*, 21, 375-389.

Hämäläinen, N. & Pette, D. (1995) Patterns of myosin isoforms in mammalian skeletal muscle fibres. *Microscopy Research and Technique*, 30, 381-389.

Kischel, P., Bastide, B., Stevens, L. & Mounier, Y. (2001) Expression and functional behaviour of troponin C in soleus muscle fibres of rat after hindlimb unloading. *American Journal of Applied Physiology*, (In press).

La Framboise, *W.A.*, Daood, M.J., Guthrie, R.D., Schiaffino, S., Moretti, P., Brozanski, B., Ontell, M.P., Butler-Browne, G.S., Whalen, R.G & Ontell, M. (1991) Emergence of the mature myosin phenotype in the rat diaphragm muscle. *Developmental Biology*, 144, 1-15.

Loukianov, E., Ji, Y., Baker, D.L., Reed, T., Babu, J., Loukianova, T., Greene, A., Shull, G & Periasamy, M. (1998) Sarco(endo)plasmic reticulum Ca2+ ATPase isoforms and their role in muscle physiology and pathology. *Annals of the New York Academy of Sciences*, 853, 251-259.

Lutz, G.J. & Lieber, R.L. (2000) Myosin isoforms in anuran skeletal muscle: their influence on contractile properties and in vivo muscle function. *Microscopy Research and Technique*, 50, 443-457.

Mizusawa, H., Takagi, A., Sugita, H. & Toyokura, Y. (1982) Coexistence of fast and slow types of myosin light chains in a single fiber of rat soleus muscle. *Journal of Biochemistry (Tokyo)*, 91, 423-425.

Moss, R.L., Diffee, G.M. & Greaser, M.L. (1995) Contractile properties of skeletal muscle fibres in relation to myofibrillar protein isoforms. *Reviews in Physiology, Biochemistry and Pharmacology*, 126, 2-63.

Nguyen, L.T. & Stephenson, G.M.M. (1999) An electrophoretic study of myosin heavy chain expression in skeletal muscles of the toad *Bufo marinus. Journal of Muscle Research and Cell Motility*, 20, 687-95.

Oishi, Y., Ishihara, A., Yamamoto, H. & Miyamoto, E. (1998) Hindlimb suspension induces the expression of multiple myosin heavy chain isoforms in single fibres of the rat soleus muscle. *Acta Physiologica Scandinavica*, 162, 127-134.

Pette, D., Peuker, H. & Staron, R.S. (1999) The impact of biochemical methods for single muscle fibre analysis. *Acta Physiologica Scandinavica*, 166, 261-277.

Pette, D. & Staron, R.S. (2000) Myosin isoforms, muscle fibre types and transitions. *Microscopy Research and Technique*, 50, 500-509.

Pette, D. & Staron, R.S. (1997) Mammalian skeletal muscle fibre type transitions. *International Review of Cytology*, 170, 143-223.

Pette, D. & Staron, R.S. (1990) Cellular and molecular diversities of mammalian skeletal muscle fibres. *Reviews of Physiology, Biochemistry and Pharmacology*, 116, 2-76.

Peuker, H., Conjard, A., Putman, C.T. & Pette, D. (1999) *Journal of Muscle Research and Cell Motility*, 20, 147-154.

Pierobon-Bormioli, S., Sartore, S., Libera, L.D., Vitadello, M. & Schiaffino, S. (1981)"Fast" isomyosins and fiber types in mammalian skeletal muscle. *Journal of Histochemistry and Cytochemistry*, 29, 1179-1188.

Rivero, J.L., Talmadge, R.J. & Edgerton, V.R. (1998) Fibre size and metabolic properties of myosin heavy chain-based fibre types in rat skeletal muscle. *Journal of Muscle Research and Cell Motility*,19, 733-742.

Roy, R.R., Kim, J.A., Grossman, E.J., Bekmezian, A., Talmadge, R.J., Zhong, H. & Edgerton, V.R. (2000) Persistence of myosin heavy chain-based fiber types in innervated but silenced rat fast muscle. *Muscle & Nerve*, 23, 735-747.

Salviati, G., Betto, R.& Danieli-Betto, D. (1982) Polymorphism of myofibrillar proteins of rabbit skeletal muscle fibres. *Biochemical Journal*, 207, 261-272.

Schiaffino, S. & Salviatti, G. (1998) Molecular diversity of myofibrillar proteins: isoforms analysis at the protein and mRNA level. *Methods in Cell Biology*, 52, 349-369

Schiaffino, S. & Reggiani, C. (1996) Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiological Reviews*, 76, 371-423.

Staron, R.C. & Pette, D. (1987) Nonuniform myosin expression along single fibres of chronically stimulated and contralateral rabbit tibialis anterior muscles. *Pflügers Archiv*, 409, 67-73.

Staron, R.C. & Pette, D. (1993) The continuum of pure and hybrid myosin heavy chain based fibre types in rat skeletal muscles. *Histochemistry*, 100, 149-153.

Stephenson, D.G., Lamb, G.D. & Stephenson, G.M.M. (1998) Events of the excitation-contraction-relaxation cycle in fast-and slow-twitch mammalian muscle fibres relevant to muscle fatigue. *Acta Physiologica Scandinavica*, 162, 229-245.

Swynghedauw, B. (1986) Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiological Reviews*, 66, 710-771.

Talmadge, R.J. (2000) Myosin heavy chain expression following reduced neuromuscular activity: potential regulatory mechanisms. *Muscle & Nerve*, 23, 661-679.

Talmadge, R.J., Roy R.R. & Edgerton, V.R. (1999) Persistence of hybrid fibres in rat soleus after spinal cord transection. *Anatomical Records*, 255, 188-201.

Termin, A., Staron, R.S. & Pette, D. (1989) Changes in myosin heavy chain isoforms during chronic low-frequency stimulation of rat fast hindlimb muscles. *European Journal of Biochemistry*, 186, 749-754.

Van der Linden, G.C., Simonides, W.S., Muller, A., Van der Laarse, W.J., Vermeulen, j.L., Zuidvijk, M.J., moorman, A.F. & van Hardeveld, C. (1996) Fibre-specific regulation of Ca^{2+} - ATPase isoform expression by thyroid hormone in rat skeletal muscle. *American Journal of Physiology*, 271, C1908-C1919.

Wade, R. & Kedes, L. (1989) Developmental regulation of contractile protein genes. *Annual Review of Physiology*, 51, 179-188.

Weiss, A. & Leinwand, L.A. (1996) The mammalian myosin heavy chain gene family. *Annual. Review of Cellular and Developmental Biology*, 12, 417-39.

Wu, Y.Z., Baker, M.J., Crumley, R.L., Blanks, R.H. & Caiozzo, V.J. (1998) A new concept in laryngeal muscle: multiple myosin isoform typesin single muscle fibers of the lateral cricoarytenoid. *Otolaryngology of the Head Neck Surgery*, 118, 86-94.

Wu, Z.Y., Baker, M.J., Crumley, R.L. & Caiozzo, V.J. (2000a) Single-fiber myosin heavy chain isoform composition of rodent laryngeal muscle. Modulation by thyroid hormone. *Archives of Otolaryngological Head and neck Surgery*, 126, 874-880.

Wu, Y.Z., Crumley, R.L. & Caiozzo, V.J. (2000b) Are hybrid fibers a common motif of canine laryngeal muscles? *Archives of Otolaryngological Head and Neck Surgery*, 126, 865-873.

Yu, F., Degens, H., Li, X & Larsson, L. (1998) Gender- and age-related differences in the regulatory influence of thyroid hormone on the contractility and myosin composition of single rat soleus muscle fibres. *Pflügers Archiv*, 437, 21-30.

DOES Ca^{2+} RELEASE FROM THE SARCOPLASMIC RETICULUM INFLUENCE THE HEART RATE?

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Summary

The review summarises the evidence that Ca^{2+} release from sarcoplasmic reticulum (SR) is an important contributor to the systolic rise in $[Ca^{2+}]_i$ (the Ca^{2+} transient) and influences the pacemaker firing rate. We believe that mechanism whereby $[Ca^{2+}]_i$ influences firing rate is through the dependence of the Na^+-Ca^{2+} exchanger on $[Ca^{2+}]_i$. Ca^{2+} extrusion by the electrogenic Na^+-Ca^{2+} exchanger produces an inward current which contributes to the pacemaker currents. Confocal images of Ca^{2+} indicate the distribution of $[Ca^{2+}]_i$ and Ca^{2+} sparks add to the evidence that the Ca^{2+} release from SR is involved in pacemaker activity. The normal pathway for increased heart rate is sympathetic activation; we discuss the evidence that part of the chronotropic effect of β -adrenergic stimulation is through the modulation of SR Ca^{2+} release. These studies show that Ca^{2+} handling by the pacemaker cells makes an important contribution to the regulation of pacemaker activity.

Introduction

The heart rate is determined by the firing rate of a small group of specialised pacemaker cells, which are located in the sinoatrial node in mammals and sinus venosus in amphibians. Early electrophysiological studies established that the spontaneous firing of pacemaker cells was due to a period of spontaneous diastolic depolarisation, known as pacemaker potential, which preceded the action potential. The pacemaker action potential has a relatively slow upstroke and it has long been recognised that traditional I_{Na} makes relatively little contribution (Yamagishi & Sano, 1966). Instead the L-type Ca^{2+} current provides the positive feedback for the rise of the action potential and the delayed rectifier potassium current is mainly responsible for repolarization. The inward currents which contribute to the slow diastolic depolarization are the key to understanding the pacemaker activity and the currents involved are still the subject of debate (Campbell *et al.*, 1992). The hyperpolarization-activated cation current (I_f) has been proposed as the most important pacemaker current (DiFrancesco, 1993). However, pacemaker cells are still able to firing after blockage of I_f (Zhou & Lipsius, 1992) indicating that other mechanisms are involved. Several other inward currents with proposed or established roles in pacemaking include the T-type Ca^{2+} current (Hagiwara *et al.*, 1988); the Na^+-Ca^{2+} exchange current (Brown *et al.*, 1984), background Na^+ current (Hagiwara *et al.*, 1992); persistent Na^+ current (Ju *et al.*, 1995) and the sustained inward current (Guo *et al.*, 1995). At present there is no consensus on which of these currents makes the major contribution to pacemaking activity (compare DiFrancesco, 1993; Irisawa et al, 1993).

Given the uncertainty about which membrane current is the true pacemaker current, there is growing interest in the influence of intracellular Ca^{2+} on the pacemaker activity. One important issue is the possible role of Ca^{2+} release from the sarcoplasmic reticulum (SR) in pacemaker function. In this short review we first provide the evidence that cane toad pacemaker cells contain SR which is capable of Ca^{2+} release and contributes to the Ca^{2+} transient in pacemaker cells. We then try to establish answers to the following questions. Can spontaneous action potentials be generated in the absence of SR Ca^{2+} release? What is the membrane current that underlies the Ca^{2+}-dependence of

pacemaker firing rate? Is the increase in firing rate caused by β -adrenergic stimulation also mediated by the increase in Ca^{2+} transients that they cause?

Evidence that intracellular Ca^{2+} influences the firing rate of pacemaker cells.

It has long been recognised that changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) affect some of the pacemaker currents and may therefore potentially affect the firing rate (DiFrancesco & Noble, 1985; Campbell *et al.*, 1992). For instance the following potential pacemaker currents are affected by $[Ca^{2+}]_i$; L-type Ca current (Irisawa *et al.*, 1993); I_f (Hagiwara & Irisawa, 1989); delayed rectifier potassium current (Nitta *et al.*, 1994); sustained inward current (Guo *et al.*, 1995). However the discovery that ryanodine, which interferes with Ca^{2+} release from the SR, slows the firing rate of pacemaker cells has been a major factor in the increased interest in Ca^{2+}-dependent mechanisms (Rubenstein & Lipsius, 1989; Rigg & Terrar, 1996; Hata *et al.*, 1996; Satoh, 1997).

The realisation that $[Ca^{2+}]_i$ may affect firing rate of pacemaker cells has led to new interest in measuring $[Ca^{2+}]_i$ in pacemaker cells (Hancox *et al.*, 1994; Li *et al.*, 1997; Huser *et al.*, 2000). We began to study intracellular Ca^{2+} in spontaneously firing toad sinus venosus (SV) pacemaker cells in 1996. There are several reasons for using toad pacemaker cells. Firstly, the sinus venosus is easy to identify in amphibian heart and provides a relatively large number of homogeneous pacemaker cells. Secondly, amphibian pacemaker cells from the toad *Bufo marinus* like those from the bullfrog lack I_f (Shibata & Giles, 1985; Ju *et al.*, 1995) demonstrating that I_f is not the sole pacemaker current and providing an impetus to identify the role of other pacemaker mechanisms. Thirdly, there are quantitative amphibian models of pacemaker activity which offer the possibility of determining the relative contribution of various pacemaker currents (Rasmusson *et al.*, 1990).

Single cells were isolated and loaded with the acetoxy-methyl ester form of indo-1. Pacemaker action potential and $[Ca^{2+}]_i$ signal were simultaneously recorded by using nystatin perforated-patch technique as shown in Figure 1. Note the rapid transient rise of $[Ca^{2+}]_i$ (the Ca^{2+} transient) following the spontaneous action potential. The minimum $[Ca^{2+}]_i$ during diastole was around 200 nM while the peak of the Ca^{2+} transient was around 600 nM (Ju & Allen, 1998). Although the $[Ca^{2+}]_i$ rise was associated with action potential, the source of Ca^{2+} was uncertain. $[Ca^{2+}]_i$ rise could entirely due to the influx of Ca^{2+} from extracellular space though voltage-sensitive Ca^{2+} channels in amphibian preparations (as discussed below). Therefore, it is important to demonstrate whether there are contributions from SR Ca^{2+} release or other possible sources, such as the reverse mode of the Na^+/Ca^{2+} exchanger (Na^+ extrusion, Ca^{2+} entry).

Is SR in the amphibian pacemaker cell capable of releasing Ca^{2+}?

In pacemaker cells, morphological studies show the SR is relatively sparse (Duvert & Barets, 1979) and there is debate in the literature as to whether Ca^{2+}-induced Ca^{2+} release exists in amphibian heart. Fabiato demonstrated Ca^{2+}-induced Ca^{2+} release using skinned cardiac cells from a variety of species but it was notably absent from frog ventricular myocytes (Fabiato, 1982). Consistent with this finding, voltage clamp studies of frog ventricle showed that the Ca^{2+} involved in the activation of tension arose primarily from the extracellular space (Morad & Cleemann, 1987). Subsequently studies in frog atrial cells using ryanodine and caffeine suggested that some Ca^{2+} was stored and capable of release from SR (Tunstall & Chapman, 1994). Nevertheless the prevalent view remains that in amphibian heart tissue the SR is not a major source of Ca^{2+} during the normal contraction (Rasmusson *et al.*, 1990).

In order to identify whether SR is capable of storing Ca^{2+} in cane toad pacemaker cells, we used rapid application of caffeine. Caffeine increases the frequency and duration of SR Ca^{2+} release channel opening (Rousseau & Meissner, 1989) and therefore rapidly depletes the SR of Ca^{2+}

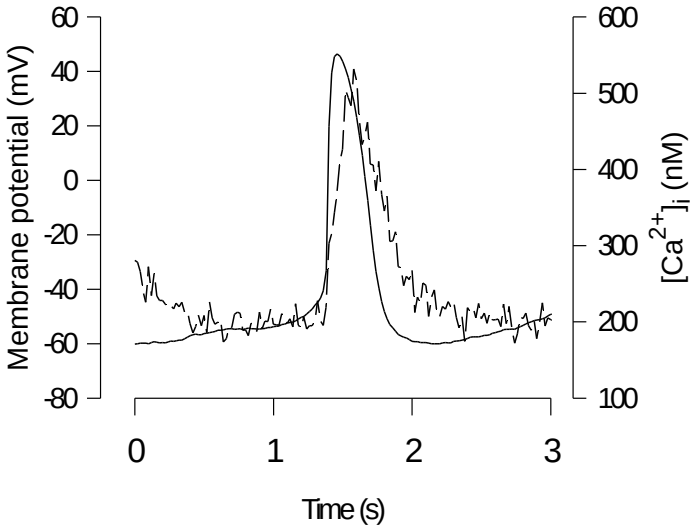

Figure 1. *Simultaneously recorded action potential (solid line) and $[Ca^{2+}]_i$ signals (dashed line) from single spontaneously firing toad pacemaker cell. Action potential was recorded using the nystatin perforated-patch technique. The cell was loaded with Ca^{2+} indicator, indo-1 AM (from Ju & Allen, 1998).*

(Callewaert *et al.*, 1989). These properties have made caffeine a popular tool to measure SR Ca^{2+} content in mammalian cardiac tissues (Diaz *et al.*, 1997). In toad pacemaker cells caffeine caused a larger and rapid rise in $[Ca^{2+}]_i$ which then fell spontaneously in the continuing presence of caffeine (Fig. 2). The peak of caffeine-induced $[Ca^{2+}]_i$ signal was about 5 times the spontaneous $[Ca^{2+}]_i$ transient induced by the action potential (Ju & Allen, 1999a). It is interesting that after application of caffeine, spontaneous firing stopped. The time for recovery of firing was about 20s. This time might reflect the duration of SR refilling with Ca^{2+} (Hussain & Orchard, 1997) and suggested that spontaneous firing was at least partly dependent on SR Ca^{2+} content.

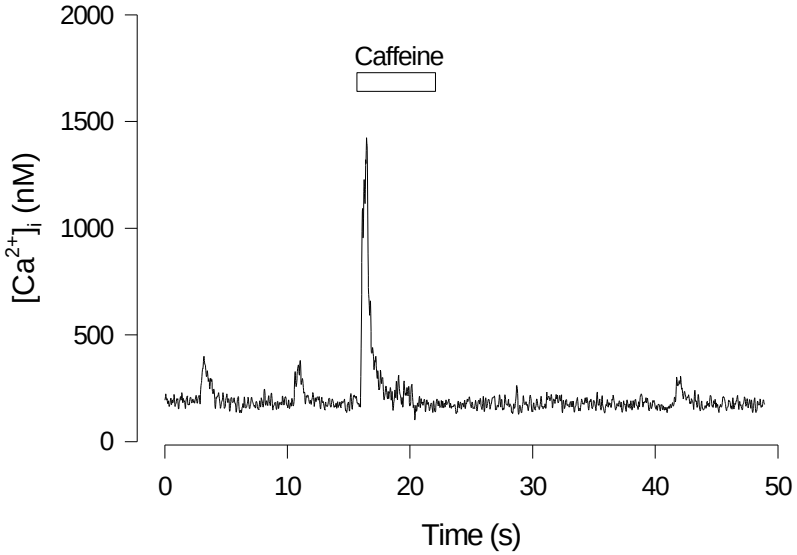

Figure 2. *Effect of rapid application of caffeine on $[Ca^{2+}]_i$ and firing rate in an isolated pacemaker cell. Caffeine caused a large increase in $[Ca^{2+}]_i$ which spontaneously declined in the continuing presence of caffeine. After caffeine was washed off the cell did not fire spontaneously for about 20 s. The firing rate is indicated by the frequency of Ca^{2+} transients (From Ju & Allen, 1999).*

Is SR Ca^{2+} release involved in pacemaker activity?

Although the experiments with caffeine above demonstrate that SR of toad pacemaker cells are capable of releasing Ca^{2+}, they do not identify whether release of Ca^{2+} from the SR occurs during the normal action potential. To test this possibility, we used ryanodine which is an SR Ca^{2+} release channel blocker (Fleischer & Inui, 1989). We found that 5 min after application of 10 μM ryanodine, the peak of the Ca^{2+} transient decreased to 50% of control level (Fig. 3). Cells were still able to firing at this stage though at a reduced frequency. After 30 min exposure to ryanodine, spontaneous firing ceased. This effect of ryanodine on pacemaker activity is consistent with the idea that the Ca^{2+} transients consist of a component of Ca^{2+} release from SR. Decreasing SR Ca^{2+} release slows the heart rate. The caffeine experiments show that when the SR is emptied of Ca^{2+} firing temporarily ceases while the ryanodine experiments show that preventing SR Ca^{2+} release also slows pacemaker firing. Thus normal SR Ca^{2+} release seems to be needed for regular firing of the pacemaker cells. Furthermore, the argument for involvement of the SR is strengthened by recent observations of single Ca^{2+} release events (Ca^{2+} sparks) during pacemaker action potential (Huser *et al.*, 2000; Ju & Allen, 2000a) (as described below) .

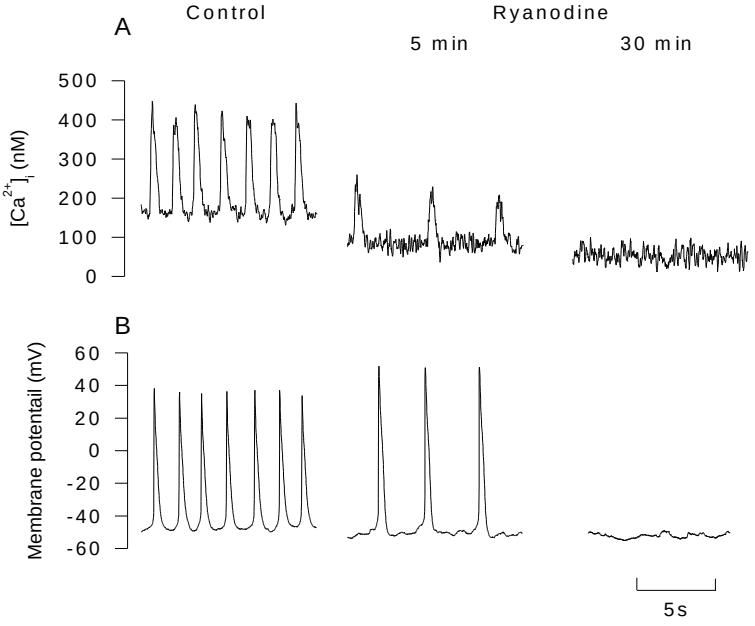

Figure 3. *The effects of 10 mM ryanodine on $[Ca^{2+}]_i$ and the spontaneous action potential. A, $[Ca^{2+}]_i$ transient recorded under control and after 5, and 30 min exposure to ryanodine (10 mM). B, the effects of ryanodine on spontaneous action potential were recorded from a non-indo loaded cell to avoid the possible effect of indo-1 AM loading on pacemaker activity (from Ju & Allen, 1998).*

The role of Na^{+}-Ca^{2+} exchanger in pacemaker activity

We have established that SR Ca^{2+} release occurs in pacemaker cells and that when it is prevented firing rate slows. However, the nature of the link between Ca^{2+} release from SR and diastolic depolarisation needs to be established. How does Ca^{2+} release from SR generate an inward current during the pacemaker potential? It is known that Na^{+}-Ca^{2+} exchanger exist in most cardiac cells. It is also known that the Na^{+}-Ca^{2+} exchanger generates a electrogenic current, since the coupling

ratio for Na^+-Ca^{2+} is 3 Na^+/Ca^{2+} (Reeves & Hale, 1984). The amplitude and the direction of exchanger current depend most directly on the membrane potential and on $[Ca^{2+}]_i$. Under most normal condition the exchanger extrudes Ca^{2+} from the cell and therefore generates an inward current (Brown *et al.*, 1984; Zhou & Lipsius, 1993). Although the possibility for I_{NaCa} to have a role in pacemaker activity is clear the actual importance remains controversial (Janvier & Boyett, 1996).

To establish the role of Na^+-Ca^{2+} exchanger toad pacemaker cells we first demonstrated that there is a very active Na^+-Ca^{2+} exchanger by monitoring $[Ca^{2+}]_i$ in response to Na^+ free extracellular solution (Ju & Allen, 1998). To quantify the amplitude of exchanger current that is generated by Ca^{2+} release from SR we simultaneously recorded $[Ca^{2+}]_i$ and the inward current induced by a rapid application of caffeine (Fig. 4). The application of caffeine produced an increase in $[Ca^{2+}]_i$ and an inward current. The shape and time course of the two are similar. In the presence of the Na^+-Ca^{2+} exchanger blocker Ni^{2+}, the caffeine-induced inward current was largely suppressed and the time course of decay of $[Ca^{2+}]_i$ became much slower. These results are consistent with the current and the decline of $[Ca^{2+}]_i$ both being caused by I_{NaCa}. By plotting the caffeine-induced inward current *versus* $[Ca^{2+}]_i$, we estimated that exchanger would produce about 20-27 pA inward I_{NaCa}, at the early diastolic $[Ca^{2+}]_i$ level (250-300 nM), 12 pA at the late diastolic $[Ca^{2+}]_i$ level (200 nM) (Ju & Allen, 1998). Since pacemaker cells have very high input resistance, this amount of inward current would make a substantial contribution to diastolic depolarisation (DiFrancesco, 1993).

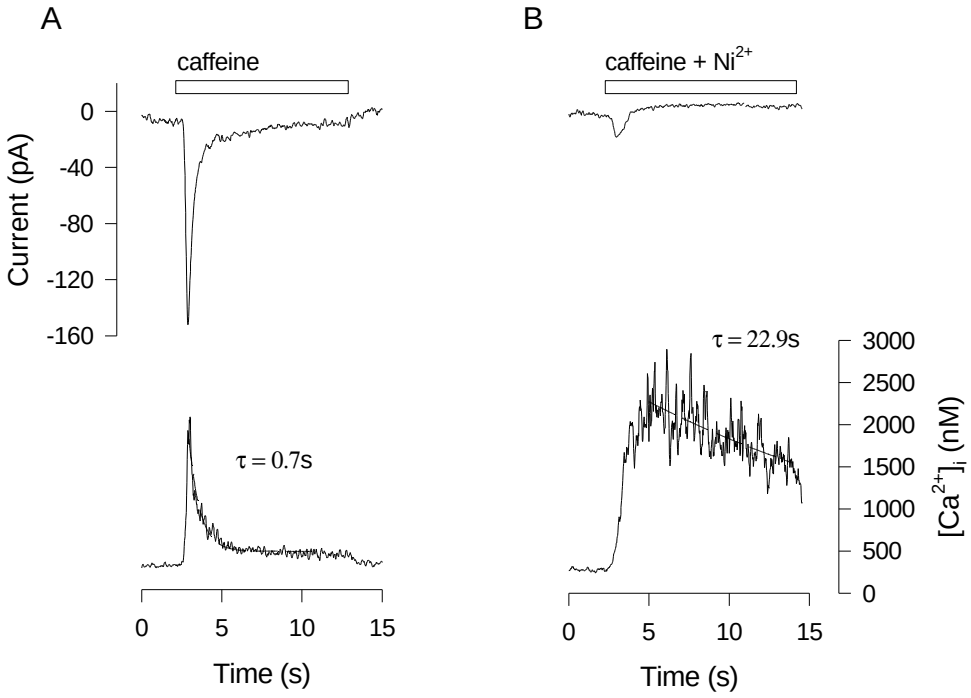

Figure 4. *The inward Na^+-Ca^{2+} exchanger current induced by Ca^{2+} release from SR. A, An indo-1 loaded cells was voltage- clamped at -60 mV. Rapid application of 10 mM caffeine induced an inward current (upper panel) with the time course similar to that of $[Ca^{2+}]_i$ (lower panel). Line drawn through declining phase of $[Ca^{2+}]_i$ is an exponential fit whose time constant (t) is shown. B, Caffeine and 5 mM Ni^{2+} applied simultaneously. The inward current was largely blocked while the $[Ca^{2+}]_i$ increase was larger but declined more slowly. Exponential fit to early $[Ca^{2+}]_i$ decline is shown by line and time constant (t) (from Ju & Allen, 1998).*

Distribution of $[Ca^{2+}]_i$ during pacemaking

Given that SR Ca^{2+} release contributes pacemaker function at least in part through stimulating the Na^+-Ca^{2+} exchanger, it becomes of interest to know the distribution of $[Ca^{2+}]_i$ during the action potential. This is because the Na^+-Ca^{2+} exchanger is situated in the surface membrane and is sensitive only to the near membrane $[Ca^{2+}]_i$. This issue was examined using confocal microscopy during spontaneous firing of isolated pacemaker cells. For these studies, pacemaker cells were loaded with fluo-3. Surprisingly, given that the pacemaker cells have no T-tubules we found that the distribution of Ca^{2+} release during an action potential was uniform (Ju & Allen, 2000a). This is surprising because one would expect the Ca^{2+} distribution resulting from L-type Ca channels to be localised around the edges of the cell. In fact Ca^{2+} reached a similar peak in the centre of the cell as at the edge and there was no detectable delay in the rise of Ca in the middle of the cell compared to the edge. One explanation for these findings is that SR is uniformly distributed across the cell and the triggering mechanism is so fast that no detectable decay occurs between edge and centre of these small 4 μm diameter cells.

Confocal studies of $[Ca^{2+}]_i$ are also capable of localised, spontaneous Ca^{2+} release from SR release channels (Ca^{2+} sparks) which provide further information about Ca^{2+} release from the SR. Ca^{2+} sparks were detected in cane toad cells and become smaller in magnitude and longer in duration in the presence of 250 nM of ryanodine (Ju & Allen, 2000a). This finding is consistent with the ability of low concentration of ryanodine to cause the SR Ca^{2+} channels to enter an intermediate conductance state with long openings (Rousseau *et al.*, 1987). A novel finding was that the frequency of Ca^{2+} sparks increased just before an action potential. A recent study in mammalian pacemaker cells has confirmed this finding and suggested that the mechanism involved is that T-type Ca^{2+} current triggers Ca^{2+} sparks from SR close to the membrane (Huser *et al.*, 2000). We do not believe this is the only mechanism involved because in our experiments the increased frequency of sparks was also observed in the middle of the cell.

What is a trigger for SR Ca^{2+} release in pacemaker cells?

In order to study the mechanism underlying SR Ca^{2+} release in pacemaker cells, we simultaneously voltage-clamped the cells and measured $[Ca^{2+}]_i$. In the present of SR Ca^{2+} pump inhibitor 2,5-di(tert-butyl)-1,4-hydroquinone (TBQ), which would be expected to deplete the SR of Ca^{2+}, Ca^{2+} transients were reduced to 34% while there was no significant effect on the peak inward current. This result suggests that about 66% of Ca^{2+} contributing to the Ca^{2+} transient is released from SR, which is consistent with previous observation in spontaneous firing cells with ryanodine. In response to a series of membrane depolarisations we found that the amplitude of the Ca^{2+} transient is not simply related to the size of inward current (Ju & Allen, 2000b). Ca^{2+} transients increased continuously as membrane potential was increased whereas the current-voltage relationship of the inward current was bell-shaped. By using various channel blockers we found that not only L-type Ca^{2+} current but also reversal mode Na^+-Ca^{2+} exchanger current could trigger Ca^{2+} SR release in pacemaker cells (Ju & Allen, 2000b). The results pose the question whether reversal model Na^+-Ca^{2+} exchanger induces Ca^{2+} induced Ca^{2+} release during the spontaneous pacemaker action potentials. However, lack a specific Na^+-Ca^{2+} exchanger blocker prevents us addressing this issue directly at precent.

Is the increase heart rate by adrenaline related to the change of SR Ca^{2+} release?

It is generally thought that the increase in the heart rate after *b*-adrenergic stimulation is caused by modulation of ionic current, such as L-type Ca^{2+} current (Noma *et al.*, 1980) and I_f (DiFrancesco, 1981). It is also known that *b*-adrenergic stimulation increase the amplitude of Ca^{2+} transients in cardiac myocytes (Allen & Blinks, 1978; Hussain & Orchard, 1997; Hancox *et al.*, 1994). We have

found that in toad pacemaker cells various aspects of Ca^{2+} handling were modified by *b*-adrenergic stimulation, including increases in the L-type Ca^{2+} current, the SR Ca^{2+} content, and the magnitude of Na^{+}-Ca^{2+} exchanger current (Ju & Allen, 1999a). We also found that increased Na^{+}-Ca^{2+} exchange current could be explained by the increased $[Ca^{2+}]_i$ rather than changes in the intrinsic properties of exchanger (Ju & Allen, 1999b). Since adrenaline changed several potential pacemaker currents in addition to having multiple effects on the $[Ca^{2+}]_i$ handling, it is difficult to identify the exact basis of the chronotropic effect. However, one intriguing observation suggests that SR Ca^{2+} release has a critical role in *b*-adrenergic stimulation. We found that isoprenaline was able to restore spontaneous firing in the cells treated with a high concentration of ryanodine but not in the cells treated with a low concentration of ryanodine (Ju & Allen, 1999a). It is known that different concentrations of ryanodine have different effect on the SR Ca^{2+} release channel (Fleischer & Inui, 1989). Low concentration of ryanodine lead to channels open in the subconductance state whereas high concentration of ryanodine close the channels. Thus, we expect the SR to be empty of Ca^{2+} at low ryanodine concentrations but loaded with Ca^{2+} at high ryanodine concentration and this prediction was confirmed by caffeine exposures. It appears that isoprenaline was able to overcome the inhibition of Ca^{2+} release caused by high ryanodine concentration and that spontaneous firing could resume provide SR Ca^{2+} release could occur. In contrast, when intracellular Ca^{2+} store were emptied by low concentration of ryanodine, spontaneous firing was unable to occur.

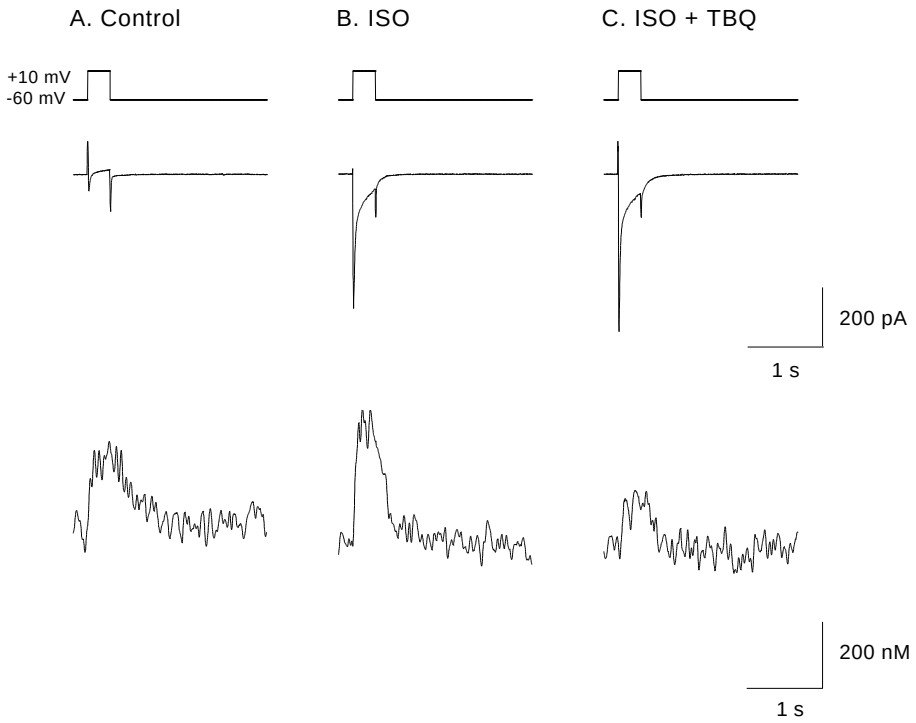

Figure 5. *Membrane current and $[Ca^{2+}]_i$ in a voltage-clamped pacemaker cells showing effects of isoprenaline and TBQ. Cell was loaded with indo-1 AM. Perforated patch technique was used to voltage clamp cells. Depolarisation to 0 mV from holding potential -60 mV evoked an inward current associated with an $[Ca^{2+}]_i$ transient in the control condition (A). B, 2mM isoprenaline caused a lager increase of inward current and $[Ca^{2+}]_i$ transient. C, after 5min application of 10 mM TBQ in the continuing presence of isoprenaline. The amplitude of $[Ca^{2+}]_i$ transient was greatly decreased while the amplitude of inward current remained the same (from Ju & Allen, 1999).*

The above experiments suggest that SR Ca^{2+} release plays a specific role in response to β-stimulation. In order to separate the effects of β-stimulation on Ca^{2+} influx from that due to SR Ca^{2+} release we simultaneously recorded Ca^{2+} current and $[Ca^{2+}]_i$. We found that in the present of isoprenaline both Ca^{2+} current and $[Ca^{2+}]_i$ transients were increased (Fig. 5). TBQ was used to reveal the SR contribution. We found that application of TBQ had no significant effect on Ca^{2+} current enhanced by isoprenaline. However, $[Ca^{2+}]_i$ transient was greatly decreased. The similar result was obtained by using low concentrations of ryanodine. Such experiments suggest that SR Ca^{2+} release contributes about 50% of the Ca^{2+} transient both in the absence and presence of *b* - adrenergic stimulation (Ju & Allen, 1999a). Therefore the increase of $[Ca^{2+}]$ transient by β-stimulation is partly caused by increased SR Ca^{2+} release. In order to maintain the homeostasis of $[Ca^{2+}]_i$, the Na^{+}-Ca^{2+} exchanger would produce more inward current by extruding more Ca^{2+}. Thus increased inward current during the diastolic potential would accelerated the diastolic depolarisation, therefore increasing the heart rate.

Conclusion

The evidence is clear that $[Ca^{2+}]_i$ and SR Ca^{2+} release are in some way related to the firing rate of cane toad pacemaker cells. It is very likely that I_{NaCa} is at least part of the intermediary process which links the Ca^{2+} to the pacemaker current. However many other details are less clear; does $[Ca^{2+}]_i$ affect other pacemaker currents which have significant effects? Are Ca^{2+} sparks important in the pacemaker process and is the mechanism proposed by Huser *et al.* (2000) correct and applicable in other cell types? Does SR Ca^{2+} release have some special role over and above its contribution to the Ca^{2+} transients? The ryanodine experiments suggest that it may and studies by Cousins & Bramich (1998) also suggest there may be a class of Ca^{2+} store which is modulated only by neuronally-released adrenaline.

Cellular studies of pacemaker cells have been impeded by the small numbers of these cells and the difficulties in isolating them. There is increasing evidence that pacemaker function declines in the elderly and those with ischaemic heart disease (Benditt *et al.*, 1995) and understanding and treatment of these problems is dependent on increasing understanding of pacemaker function at a cellular and molecular level.

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References

Allen, D.G. & Blinks, J.R. (1978) Calcium transients in aequorin-injected frog cardiac muscle. *Nature*, 273, 509-513.

Benditt, D.G., Sakaguchi, S., Golstein, M.A., Lurie, K.G., Gornick, C.C. & Adler, S.W. (1995) Sinus Node Dysfunction:Pathophysiology, clinical feature, evaluation, and treatment. In: *Cardiac electrophysiology: from cell to bedside*.

Brown, H.F., Kimura, J., Noble, D., Noble, S.J. & Taupignon, A. (1984) The slow inward current, isi, in the rabbit sino-atrial node investigated by voltage clamp and computer simulation. *Proceedings of the Royal Society of London.Series B: Biological Sciences*, 222, 305-328.

Callewaert, G., Cleemann, L. & Morad, M. (1989) Caffeine-induced Ca^{2+} release activates Ca^{2+} extrusion via Na^{+}-Ca^{2+} exchanger in cardiac myocytes. *American Journal of Physiology*, 257, C147-52.

Campbell, D.L., Rasmusson, R.L. & Strauss, H.C. (1992) Ionic current mechanisms generating vertebrate primary cardiac pacemaker activity at the single cell level: an integrative view. *Annual Review of Physiology,* 54, 279-302.

Cousins, H.M. & Bramich, N.J. (1998) Effects of sympathetic nerve stimulation on membrane potential, $[Ca^{2+}]_i$ and force in the arrested sinus venosus of the toad, *Bufo marinus. Journal of Physiology,* 505, 513-527.

Diaz, M.E., Trafford, A.W., O'Neill, S.C. & Eisner, D.A. (1997) Measurement of sarcoplasmic reticulum Ca^{2+} content and sarcolemmal Ca2+ fluxes in isolated rat ventricular myocytes during spontaneous Ca^{2+} release. *Journal of Physiology,* 501 (Pt 1), 3-16.

DiFrancesco, D. (1981) A study of the ionic nature of the pace-maker current in calf Purkinje fibres. *Journal of Physiology,* 314, 377-393.

DiFrancesco, D. (1993) Pacemaker mechanisms in cardiac tissue. *Annual Review of Physiology,* 55, 455-472.

DiFrancesco, D. & Noble, D. (1985) A model of cardiac electrical activity incorporating ionic pumps and concentration changes. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences,* 307, 353-398.

Duvert, M. & Barets, A.L. (1979) Fine structure and organization of the sarcoplasmic reticulum in the sino-atrial fibres of the frog heart. *Zeitschrift fur Naturfurschung,* 34, 865-875.

Fabiato, A. (1982) Calcium release in skinned cardiac cells: variations with species, tissues, and development. *Federation Proceedings,* 41, 2238-2244.

Fleischer, S. & Inui, M. (1989) Biochemistry and biophysics of excitation-contraction coupling. *Annual Review of Biophysics & Biophysical Chemistry,* 18, 333-364.

Guo, J., Ono, K. & Noma, A. (1995) A sustained inward current activated at the diastolic potential range in rabbit sino-atrial node cells. *Journal of Physiology,* 483, 1-13.

Hagiwara, N. & Irisawa, H. (1989) Modulation by intracellular Ca2+ of the hyperpolarization- activated inward current in rabbit single sino-atrial node cells. *Journal of Physiology,* 409, 121-141.

Hagiwara, N., Irisawa, H. & Kameyama, M. (1988) Contribution of two type of calcium currents to the pacemaker potentials of rabbit sinoatrial node cells. *Journal of Physiology,* 395, 233-253.

Hagiwara, N., Irisawa, H., Kasanuki, H. & Hosoda, S. (1992) Background current in sino-atrial node cells of the rabbit heart. *Journal of Physiology,* 448, 53-72.

Hancox, J.C., Levi, A.J. & Brooksby, P. (1994) Intracellular calcium transients recorded with Fura-2 in spontaneously active myocytes isolated from the atrioventricular node of the rabbit heart. *Proceedings of the Royal Society of London - Series B: Biological Sciences,* 255, 99-105.

Hata, T., Noda, T., Nishimura, M. & Watanabe, Y. (1996) The role of Ca2+ release from sarcoplasmic reticulum in the regulation of sinoatrial node automaticity. *Heart and Vessels,* 11, 234-241.

Huser, J., Blatter, L. A. & Lipsius, S.L. (2000) Intracellular Ca2+ release contributes to automaticity in cat atrial pacemaker cells [see comments]. *Journal of Physiology,* 524 Pt 2, 415-422.

Hussain, M. & Orchard, C.H. (1997) Sarcoplasmic reticulum Ca^{2+} content, L-type Ca^{2+} current and the Ca^{2+} transient in rat myocytes during β-adrenergic stimulation. *Journal of Physiology,* 505, 385-402.

Irisawa, H., Brown, H.F. & Giles, W. (1993) Cardiac pacemaking in the sinoatrial node. *Physiological Reviews,* 73, 197-227.

Janvier, N.C. & Boyett, M.R. (1996) The role of Na-Ca exchange current in the cardiac action potential. *CardiovascularResearch,* 32, 69-84.

Ju, Y.K. & Allen, D.G. (1998) Intracellular calcium and Na^+-Ca^{2+} exchange current in isolated toad pacemaker cells. *Journal of Physiology*, 508, 153-166.

Ju, Y. K. & Allen, D.G. (1999b) Does adrenaline modulate the Na^+/Ca^{2+} exchanger in isolated toad pacemaker cells. *Pflügers Archiv European Journal of Physiology*, 438, 338-343.

Ju, Y.K. & Allen, D.G. (1999a) How does β-adrenergic stimulation increase heart rate? The role of intracellular Ca^{2+} release in amphibian pacemaker cells. *Journal of Physiology*, 516, 793-804.

Ju, Y.K. & Allen, D.G. (2000a) The distribution of calcium in toad cardiac pacemaker cells during spontaneous firing. *Pflügers Archiv European Journal of Physiology*, 441, 219-227.

Ju, Y.K. & Allen, D.G. (2000b) The mechanisms of sarcoplasmic reticulum Ca2+ release in toad pacemaker cells. *Journal of Physiology*, 525 Pt 3, 695-705.

Ju, Y.K., Saint, D.A., Hirst, G.D. & Gage, P.W. (1995) Sodium currents in toad cardiac pacemaker cells. *Journal of Membrane Biology*, 145, 119-128.

Li, J., Qu, J. & Nathan, R.D. (1997) Ionic basis of ryanodine's negative chronotropic effect on pacemaker cells isolated from the sinoatrial node. *American Journal of Physiology*, 273, H2481-H2489.

Morad, M. & Cleemann, L. (1987) Role of Ca2+ channel in development of tension in heart muscle. *Journal of Molecular and Cellular Cardiology*, 19, 527-553.

Nitta, J., Furukawa, T., Marumo, F., Sawanobori, T. & Hiraoka, M. (1994) Subcellular mechanism for Ca(2+)-dependent enhancement of delayed rectifier K+ current in isolated membrane patches of guinea pig ventricular myocytes. *Circulation Research*, 74, 96-104.

Noma, A., Kotake, H. & Irisawa, H. (1980) Slow inward current and its role mediating the chronotropic effect of epinephrine in the rabbit sinoatrial node. *Pflügers Archiv European Journal of Physiology*, 388, 1-9.

Rasmusson, R.L., Clark, J.W., Giles, W.R., Shibata, E.F. & Campbell, D.L. (1990) A mathematical model of a bullfrog cardiac pacemaker cell. *American Journal of Physiology*, 259, H352-H369.

Reeves, J.P. & Hale, C.C. (1984) The stoichiometry of the cardiac sodium-calcium exchange system. *Journal of Biological Chemistry*, 259, 7733-7739.

Rigg, L. & Terrar, D.A. (1996) Possible role of calcium release from the sarcoplasmic reticulum in pacemaking in guinea-pig sino-atrial node. *Experimental Physiology*, 81, 877-880.

Rousseau, E. & Meissner, G. (1989) Single cardiac sarcoplasmic reticulum Ca2+-release channel: activation by caffeine. *American Journal of Physiology*, 256, H328-H333.

Rousseau, E., Smith, J.S. & Meissner, G. (1987) Ryanodine modifies conductance and gating behavior of single Ca2+ release channel. *American Journal of Physiology*, 253, C364-C368.

Rubenstein, D.S. & Lipsius, S.L. (1989) Mechanisms of automaticity in subsidiary pacemakers from cat right atrium. *Circulation Research*, 64, 648-657.

Satoh, H. (1997) Electrophysiological actions of ryanodine on single rabbit sinoatrial nodal cells. *General Pharmacology*, 28, 31-38.

Shibata, E.F. & Giles, W.R. (1985) Ionic currents that generate the spontaneous diastolic depolarization in individual cardiac pacemaker cells. *Proceedings of the National Academy of Sciences USA*, 82, 7796-7800.

Tunstall, J. & Chapman, R.A. (1994) The effect of ryanodine on the contraction of isolated frog atrial trabeculae is triggered by caffeine. *ExperimentalPhysiology*, 79, 435-444.

Yamagishi, S. & Sano, T. (1966) Effect of tetrodotoxin on the pacemaker action potential of sinus node. *Proceedings of the Japan Academy,* 42, 1194-1196.

Zhou, Z. & Lipsius, S.L. (1992) Properties of the pacemaker current (If) in latent pacemaker cells isolated from cat right atrium. *Journal of Physiology,* 453, 503-523.

Zhou, Z. & Lipsius, S.L. (1993) Na^{+}-Ca^{2+} exchange current in latent pacemaker cells isolated from cat right atrium. *Journal of Physiology,* 466, 263-285.