J. Mar. Biol. Ass. U.K. (1999), **79**, 1111–1116
Printed in the United Kingdom

Calycophoran siphonophore muscle fibres without any sarcoplasmic reticulum but with tubular invaginations morphologically analogous to a T-system

Q. Bone*, C. Carré†, I. Tsutsui‡ and I. Inoue∫

*Marine Biological Association, The Laboratory, Citadel Hill, Plymouth, PL1 2PB. †Observatoire Océanologique, URA 231, 06230 Villefranche-sur-Mer, France. ‡National Institute of Physiological Sciences, Okazaki, 444-0867 Japan. ∫Institute for Enzyme Research, University of Tokushima, Tokushima, 770-0042 Japan.
*Corresponding author: q.bone@mba.ac.uk

Different marine invertebrates have different tubular or vesicular systems within their locomotor muscle fibres. The siphonophores *Chelophyes, Abylopsis* and *Muggeia* have invaginated tubules which are the morphological equivalent of the vertebrate invaginated tubular system, but lack a sarcoplasmic reticulum. In *Chelophyes* the previous suggestion that Ca^{2+} channels in the extensive invaginated tubule system allow ingress of Ca^{2+} is shown to be incorrect. Contraction of the swimming muscles in *Chelophyes* is not blocked by 20 μM ryanodine, nor is it induced by 10 mM caffeine, hence intracellular Ca^{2+} stores appear absent. Contraction is, however, maintained by replacement of the greater part of the usual external Na^+ by Li^+ or by or *N*-methyl-D-glucamine, although action potentials can still be evoked. Hence we conclude that following contraction, internal Ca^{2+} is reduced by a Na/Ca^{2+} exchange mechanism.

INTRODUCTION

The striated muscles of marine invertebrates offer interesting material to the comparative physiologist, for in different groups there are striking differences in fibre morphology, particularly in the internal vesicle and tubular Ca^{2+} systems linked to the contractile machinery. For example, the thin obliquely-striated locomotor muscle fibres of the small pelagic tunicate *Doliolum* lack any internal vesicle system equivalent to the sarcoplasmic reticulum (SR), and have no internal Ca^{2+} stores. The Ca^{2+} required to activate the contractile apparatus crosses the sarcolemma during the action potential: Ca^{2+} is subsequently removed by Na^+/Ca^{2+} exchange across the sarcolemma (Bone et al., 1997). In all invertebrate muscle fibres so far studied, external Ca^{2+} is required for contraction, hence contraction is abolished by Ca^{2+} channel blockers such as Co^{2+} (Inoue et al., 1994). Most invertebrate muscle fibres have a more or less extensive SR, but some, in addition to the SR, may have tubular invaginations of the sarcolemma, morphologically the equivalent of the vertebrate transverse tubular (T) system. Evidently, as in vertebrates, Ca^{2+} diffusion distances from the SR are required to be minimal for rapid simultaneous activation, hence larger-diameter invertebrate fibres require to have such T-system analogues. For example, in larger appendicularian tunicates, with thick muscle fibres, both SR and T systems are present, with the SR coupled to the T-tubules, whilst in small appendicularians with thinner fibres, only subsurface SR is found (Bone et al., 1979). Prosser (1982) gives an interesting discussion of the differences between large- and small-diameter muscle fibres, including the absence of T-tubule analogues in the small-diameter fibres. In chaetognaths, although the fibres are small diameter, both systems are present, and just as in vertebrate fibres, the SR is coupled to the T-system (Duvert & Salat, 1980), but chaetognath fibres are quite exceptional and will be considered elsewhere. Perhaps the most unexpected structural arrangement was found in the small rapidly-swimming siphonophore *Chelophyes* where the muscle fibres of the locomotor sheet lining the swimming bell possess an invaginated tubular system (the morphological equivalent of a T-system), yet lack an SR (Mackie & Carré, 1983). Not only does this pose the problem of the function of this T-system analogue but equally paradoxical was the observation (Bone, 1981) that uniquely amongst invertebrate muscle fibres so far studied, prolonged exposure to Co^{2+} in the external solution did not block contraction. We have therefore re-examined the situation in the *Chelophyes* muscle sheet.

MATERIALS AND METHODS

Most of our physiological observations were made on the diphyid calycophoran siphonophore *Chelophyes appendiculata* (Eschscholtz), a few were made on the larger *Abylopsis tetragona* (Otto). They were collected in plankton tows made in the Bay of Villefranche near Nice. *Chelophyes* could be kept in good condition for some days in a cold room, but most animals were used on the day they were collected. In addition, some histological observations were made on *Muggeia atlantica* (Eschscholtz) collected from the Plymouth plankton.

For the morphology of the myoepithelial cells, cut open nectophores were fixed in 5% glutaraldehyde in seawater buffered to pH 7.0 with sodium cacodylate, post-fixed in

2% osmium tetroxide and dehydrated and embedded in Spurr resin. Sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300.

Two kinds of experiments were made. Either the entire animal, or the anterior nectophore alone were placed in various solutions in small dishes, and tested at intervals for locomotor activity, or anterior nectophores alone were pinned to the Sylgard base of a small dish in such a way that their movements could be recorded by a fine probe attached to a strain gauge (N801, SensoNor, Norway) when various solutions were added. For some of these experiments, the anterior nectophores were partially cut open to permit access of microelectrodes to the locomotor muscle fibres so that simultaneous records of muscle electrical activity and tension could be obtained. Conventional KCl-filled microelectrodes (15–30 MΩ) with long flexible shanks were made with a Livingstone-type puller and led via a laboratory-made follower amplifier to a PCM recorder (PC204, Sony, Japan) for analysis with a pCLAMP system (Axon Instruments, USA). The strain gauge output was led to a second channel on the recorder. The preparations were stimulated at the basal nerve rings using fine polyethylene suction electrodes fed by a Grass S48 stimulator via an isolating unit.

For some experiments, and for recovery after different solutions, either seawater (SW) or artificial seawater (ASW) were used. The ASW had the following composition (in mM): NaCl: 500, $CaCl_2$: 10, KCl: 10, $MgCl_2$: 30 and was buffered to pH 7.8 with Tris-HEPES: 10. For experiments in Na^+-free ASW, Na^+ was replaced with Li^+ or *N*-methyl-D-glucamine (NMG). Caffeine solutions were made up containing 10% glycerol for isosmolarity with seawater. The experiments were carried out at the room temperature of 23°C, and although this was somewhat higher than the sea temperature, the preparations lasted in good condition for many hours.

OBSERVATIONS

Calycophoran siphonophores are jet-propelled animals, swimming by contraction of a sheet of myoepithelial cells lining the jet chamber of the nectophore. Different species vary in size and activity, for example the diphyids *Muggeia* and *Chelophyes* are much more active than the larger abylid *Abylopsis* (Figure 1). Most histological and physiological studies have been made upon *Chelophyes* which is a fast-swimming animal, reaching instantaneous velocities up to 30 cms^{-1} (Bone & Trueman, 1982). Here the much larger anterior nectophore (Figure 1A) is up to 1.2 cm long, shaped like a missile, with external flanges. The elongate internal cavity is lined with a sheet of thin closely-coupled myoepithelial cells which are shaped like elongate parallelopipeds. As Mackie & Carré (1983) showed, these have an external mitochondrial layer, and an inner myofilament layer, some 0.75–1.0 μm thick. On their internal face, apposed to an underlying thin mesogloeal layer, the myoepithelial cells have invaginations that branch to form a very regular single or double array of longitudinal tubules within the myofilament zone, spaced more or less regularly at intervals of 0.5–1.0 μm (Figure 2A). In *Muggeia* these myoepithelial cells are closely similar both in dimensions and structure (Figures 1B & 2B). *Abylopsis* (Figure 1C) is a larger and much slower-swimming animal (Bone & Trueman (1982), the muscle sheet is considerably thicker (around 6 mm, Figure 1C), and instead of a single row of tubules, there are a large number (Figure 2C,D) which are spaced regularly within the myofibrillar layer. These invaginated tubules, running parallel to the myofibrils, are therefore morphologically equivalent to the transverse tubular (T) system of vertebrate skeletal muscles, although they are longitudinal rather than transverse. Their lumina seem to be more or less filled with material similar to that of the mesogloea, for in electron micrographs, the material has the same electron density as the mesogloea, and the tubules open to the basal mesogloeal layer, as Mackie & Carré (1983) have previously demonstrated. In our preparations as seen in Figure 2D, the tubules often appear somewhat shrunken within the spaces of the myofibrillar array within which they lie, and the presence of occasional dual profiles suggests that they branch as they run along parallel to the myofibrils.

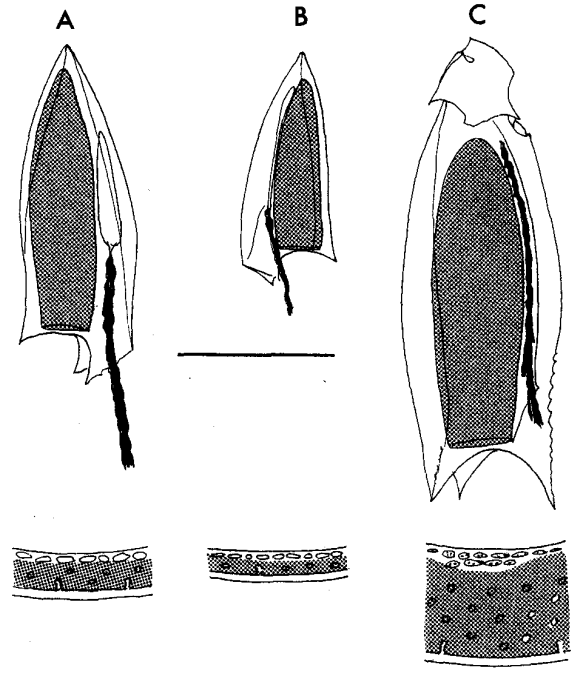

Figure 1. The three siphonophores examined (shown to same scale). The jet chamber lined by the myoepithelial sheet is shown stippled. (A) *Chelophyes;* (B) *Muggeia;* (C) *Abylopsis.* Below each is shown the relative size and structure of their myoepithelial sheets, the myofilament zone stippled. In *Abylopsis* the myoepithelium thickness is 6 μm. Scale bar: 0.5 cm.

The two faces of each muscle cell are thus different, one being closely linked to the underlying mesogloea, the other directly exposed to external solutions. A cathamnal endodermal layer lies under the electron dense mesogloeal layer, and then there is a wide less electron-dense mesogloeal zone produced into longitudinal flanges and finally covered with an epidermal epithelial layer. This wide mesogloeal zone is apparently different in composition from that adjacent to the muscle sheet, staining differently, and containing fibrils perpendicular to the outer surface of the animal. Two nerve rings at the base of the nectophore (which is rimmed by a small sphincter

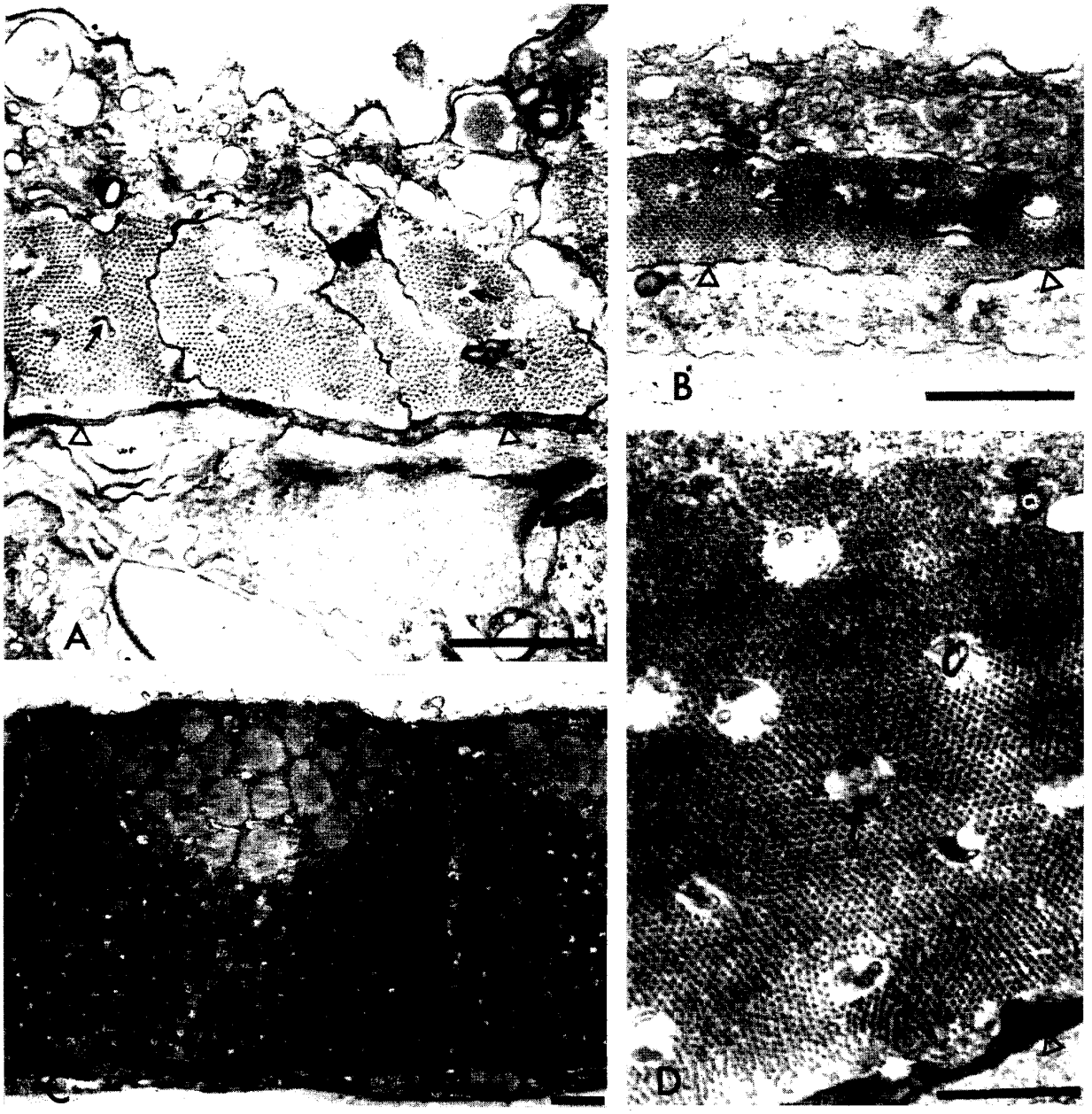

Figure 2. Transverse sections of myoepithelial cells. At top, the surface exposed to seawater (SW) inside the nectophore; at bottom cathamnal endoderm cell layer. Arrows, 'T'-system invaginations of basal sarcolemma. Triangles, electron-dense mesogloea separating base of myoepithelial cells from cathamnal layer below. (A) *Chelophyes*. The 'T'-tubules are shrunken in this preparation. (B) *Muggeia*. Similar section to (a), showing thinner myoepithelial and mesogloeal layer than in *Chelophyes*, and below cathamnal layer, less dense mesogloea composing the nectophore. (C) *Abylopsis*. Low power micrograph showing thicker myofibrillar zone array of 'T'-tubules above black mesogloeal layer separating myoepithelial cells from underlying thin cathamnal layer. (D) *Abylopsis*. Myofibrillar zone showing spaces occupied by 'T'-tubules, somewhat shrunken. The electron-dense mesogloeal layer separates the cathamnal layer at bottom right from the myoepithelial cells. Scale bars: A & B, 1.0 μm; C, 2.0 μm; D, 0.5 μm.

muscle) innervate the muscle sheet which is not innervated elsewhere (Mackie & Carré, 1983). Small suction electrodes attached to the sphincter muscle can therefore conveniently be used to evoke muscle action potentials across the sheet.

We have found it almost impossible to obtain intracellular records from the thicker myoepithelial cells of *Abylopsis*, despite numerous trials. The few that we have observed show similar features to those of *Chelophyes*. We do not know why this should be, for although the cells of the myoepithelial sheet in *Chelophyes* are only around 1.5–2.0 μm thick, stable penetrations are possible for long periods, even during bursts of contractions (Chain et al., 1981; Bone, 1981). It is therefore possible to follow the effects of external ionic changes upon the relations between action potentials and contractions. The action

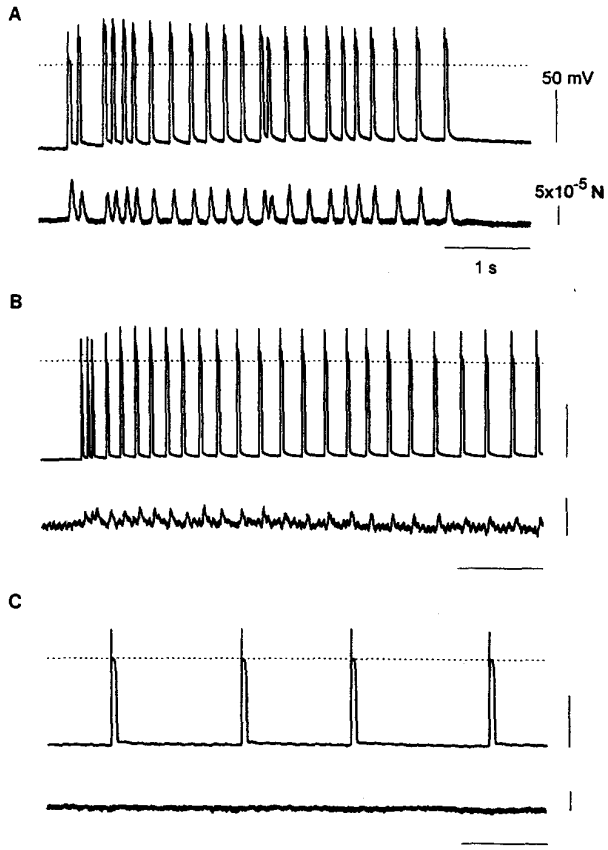

Figure 3. *Chelophyes.* Effects of Co^{2+} on spontaneous action potentials and twitches. (A) injected into the mesogloea; (B) only in the external solution; and (C) in both the mesogloea and external solution, demonstrating that twitch was blocked only when Co^{2+} was applied from both (basal and external) sides of the muscle fibres. Records (A) and (C) were obtained from the same animal (9830402), and (B) from animal (9831001).

potentials that pass across the muscle fibre sheet are complex events, as Chain et al. (1981) noted, but for the present, it suffices to note that they are evidently mainly carried by Na^+ since they can be evoked in solutions where Li^+ replaces Na^+, in OCaSW, and are abolished in low Na^+ solutions.

1. The effects of Co^{2+}

As previously observed (Bone, 1981) addition of Co^{2+} to ASW or SW solutions to make the external concentration up to 15 mM has little or no effect on the contractions of the muscle cells, Figure 3A. Note that although the contractions recorded in Figure 3B are apparently less powerful than those in Figure 3A, the output of the strain guage differed from preparation to preparation, depending upon the extent to which the animal was dissected, its size, and the placement of the tip of the guage probe. Only for the same preparation where the probe tip remained in the same place do the outputs indicate the relative force of contraction.

The reverse condition, i.e. when the inner or basal faces of the myoepithelial cells are exposed to Co^{2+} by injecting small volumes of Co^{2+} (50 mM) solutions into the mesogloea (via a micro-electrode with a broken tip)

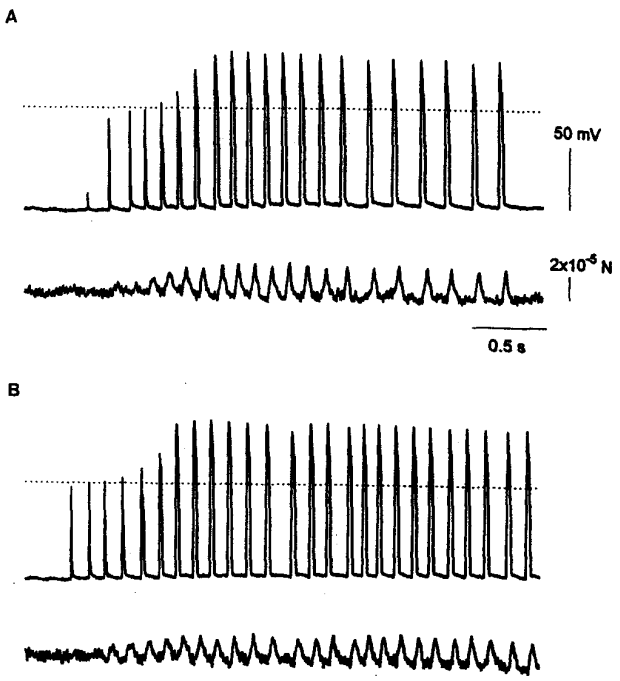

Figure 4. *Chelophyes.* Spontaneous action potentials and twitches recorded before (A) and 5 min after (B) application of 10 mM caffeine to the bath. Animal no. 9830902.

to permit Co^{2+} to gain access to the inner face of the muscle cells (Figure 3B), also evokes no block of stimulated or spontaneous contractions when the outer face of the sheet is exposed to SW or ASW in the bath.

However, addition of Co^{2+} to the external solution after Co^{2+} had previously been injected into the mesogloea caused complete block of contractions (Figure 3C). These effects are reversible, that is, replacement of the external solution containing Co^{2+} in the case of the animal shown in Figure 3A & C by SW produced recovery of normal contractions. It is also possible to dissect much of the mesogloea away from underneath the muscle sheet, leaving a flap with little underlying mesogloea. In this kind of preparation, 15 mM Co^{2+} added to the external solution causes reversible block of contractions.

These observations indicate that Ca^{2+} channels are present in the membranes of both the inner and outer face of the muscle cells, and explain the earlier anomalous results (Bone, 1981), where Co^{2+} at 15–20 mM in the external solution did not block contractions of the muscle sheet in intact animals.

2. Caffeine

Caffeine at 10 mM in the external solution had no effect upon the muscle sheet of *Chelophyes*. In particular, it did not evoke any contracture. Figure 4A shows a control burst of spontaneous contractions, and Figure 4B a similar burst 5 min after the addition of caffeine. If there are internal Ca^{2+} stores, they are not accessible to caffeine.

3. Replacement of Na^+ by Li^+

Replacement of external SW or ASW containing Na^+, by Li^+ ASW to give a solution containing around 90% Li^+ causes contractions to summate until the muscle

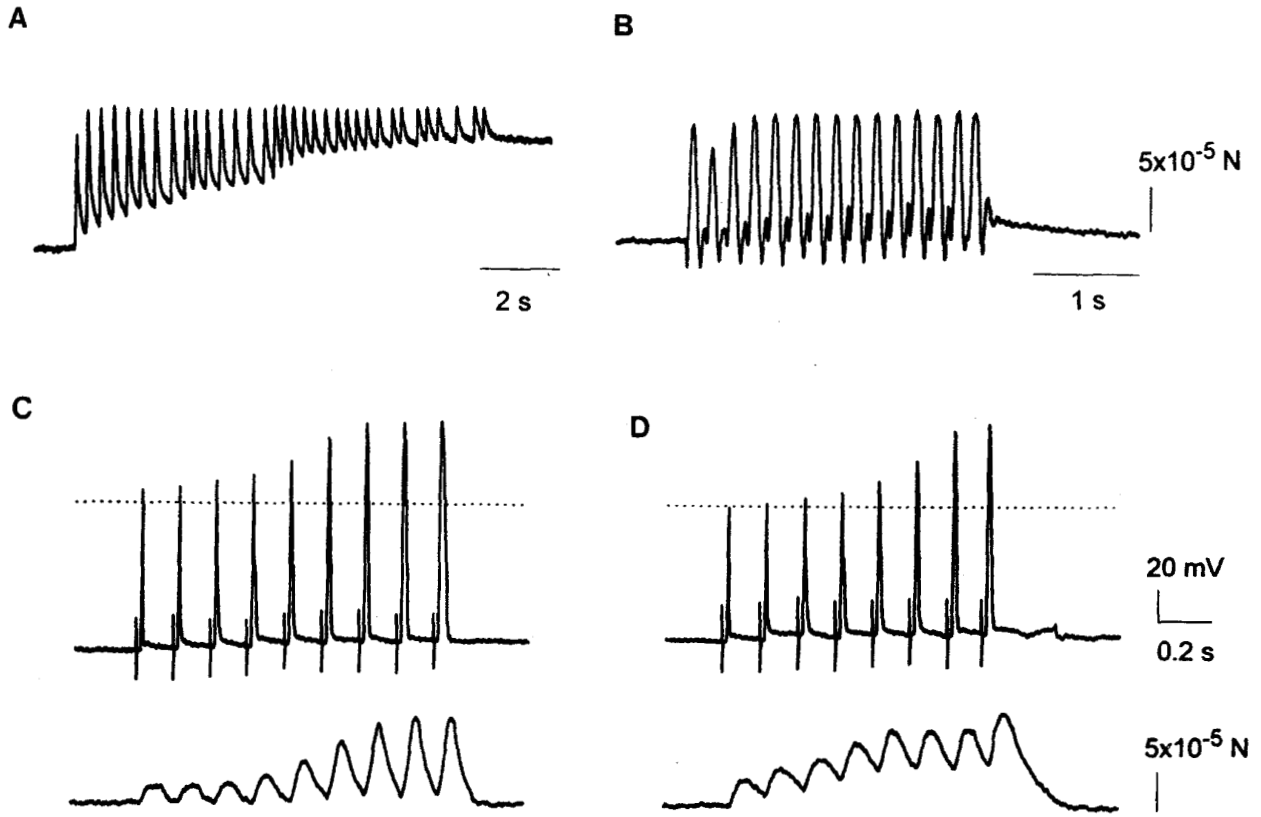

Figure 5. *Chelophyes*. (A) Muscle contracture (upward deflection) and decline of spontaneous muscle twitches after replacement of 90% of Na^+ in the external solution with Li^+ in LiASW. (B) Spontaneous muscle twitches after recovery from the contracture by changing the 90% Li^+ ASW back to the original ASW. (C) Action potentials and twitches evoked by a train of electrical stimuli recorded in ASW and (D) after replacing 50% Na^+ with Li^+ showing that the relaxation phase after the twitches slowed in the 50% Li^+ ASW. The time constant of the relaxation phase of the last twitch in (C) obtained by fitting with a single exponential function is 51 ms, and that of the last twitch in (D) 110 ms. Animal no. 9830701.

sheet is completely contracted and shrunken (Figure 5A). Washing in SW or ASW leads to complete recovery, and shrinkage disappears, each contraction returning to the same baseline (Figure 5B). After recovery in SW, stimulation evoked a series of action potentials and contractions (Figure 5C). The contractions returned to the baseline in each case, the final contraction returning with a time constant of 51 ms.

When Li^+ was now added to the external solution to make 50% Li^+, successive contractions summated and the final contraction returned to baseline with a time constant of 110 ms, indicating slowing down of the rate of Na^+/Ca^{2+} exchange.

4. Replacement of Na^+ by N-methyl-D-glucamine (NMG)

Figure 6 shows the results of replacing 50% of the Na^+ in the external solution by NMG. After an initial burst of 12 spontaneous twitches 50 s before the record shown in Figure 6, which served to exchange the dead space of fluid within the jet chamber lined by the muscle sheet, a burst of 27 twitches led to maintained increase of tension, augmented at each single contraction which followed until the muscle was much contracted and shrunken. Return to SW produced complete recovery and return to the normal unshrunken state.

Figure 6. *Chelophyes*. Spontaneous twitches and contractures in 50% NMG ASW. Animal no. 9831102. Note increase in resting tension following each contraction (see text).

5. The effects of ryanodine at 20M

There were no effects observable when an anterior nectophore of *Chelophyes* was placed in for 30 min ryanodine solution in SW. The animal occasionally swam spontaneously, and could be induced to do so by gentle mechanical stimulation.

DISCUSSION

It seems clear from our own electron micrographs, and from those of Mackie & Carré (1983), that there are no vesicles within the myoepithelial cells that could represent an SR. The lack of effect of caffeine and

ryanodine on *Chelophyes* support this, and we conclude that the myoepithelial cells lack intracellular Ca^{2+} stores and ryanodine receptor end feet coupling such stores to the sarcolemma. The paradox of an invaginated tubule system in cells without an SR is now resolved, because the invaginated tubules seemingly containing mesogloea, presumably act simply to anchor the myoepithelial cells to the underlying mesogloea, and play no part in regulating internal Ca^{2+}.

In all other invertebrates so far examined, (Inoue et al., 1996) Ca^{2+} influx across the sarcolemma is required for contraction, which is therefore blocked by external Co^{2+}. The previous anomaly that external Co^{2+} did not block contractions in *Chelophyes* is explained by Co^{2+} only gaining access to the external face of the myoepithelial sheet, whilst Ca^{2+} can continue to enter from the unexposed internal face. Once access is provided to the inner face of the sheet by dissection of the underlying mesogloea, Co^{2+} causes complete contraction block (as does nominally Ca^{2+} free ASW). *Chelophyes* is like the small tunicate *Doliolum* in lacking an SR involved in Ca^{2+} regulation, and the results obtained by replacement of Na^+ with NMG or Li^+ are most simply explained by assuming the existence of a Na^+/Ca^{2+} exchange mechanism across the sarcolemma, as in *Doliolum* (Bone et al., 1997).

It seems therefore that both show the simplest type of Ca^{2+} regulation within muscle fibres where internal Ca^{2+} stores are absent: external Ca^{2+} enters across the sarcolemma, and is removed across it solely by a Na^+/Ca^{2+} exchange mechanism. It seems likely that in fibres where an SR is present, there will be a spectrum of stages in the relative importance of the Na^+/Ca^{2+} exchange mechanism and the internal stores, at one extreme vertebrate skeletal muscle, where Ca^{2+} influx across the sarcolemma is no longer required for contraction and Ca^{2+} movements linked to contraction are predominately internal. Although a Na^+/Ca^{2+} exchange mechanism in vertebrate skeletal muscle for rapidly decreasing internal Ca^{2+} following contraction is unimportant under normal conditions (Caputo & Balanõs, 1995); Gonzales-Serratos et al. (1996) have shown that a Na^+/Ca^{2+} exchange mechanism operates to extrude the constant leakage of Ca^{2+} into the fibres, as it presumably does in many cell types. It is probable that a range of muscle fibres will be found in different animals, where a Na^+/Ca^{2+} exchange mechanism is of greater or lesser importance in the rapid reduction of internal Ca^{2+} following contraction.

Whilst the different mechanisms for reducing free Ca^{2+} may be imagined in a general way as steps in a gradual process leading from small fibres dependent on external Ca^{2+} to the organization of internal Ca^{2+} stores, and their coupling to T-system invaginations with increase in muscle fibre diameter, it is important to recognize that these steps are not necessarily related to taxonomic status. For instance the cnidarian *Chelophyes* has the same simple arrangement as the tunicate *Doliolum*, and in the mollusc *Pecten* Ca^{2+} uptake to intracellular stores plays a much more significant role than does a Na^+/Ca^{2+} exchange mechanism (Inoue et al., 1994).

I.I. and I.T. were supported by Japanese Ministry of Culture and Education grants 09044229 (I.I.) and 09044243 (I.T.). C.C. and Q.B. were supported by NATO grant no. CRG 970127. The generous hospitality of the Director and staff of the Station Zoologique was much appreciated by I.I, I.T. and Q.B. Figure 1 was based in part on data kindly supplied by Dr D.Carré.

REFERENCES

Bone, Q., 1981. The relation between the form of the action potential and contractions in the subumbrellar myoepithelium of *Chelophyes* (Coelenterata: Siphonophora). *Journal of Comparative Physiology*, **144**, 555–558.

Bone, Q., Flood, P.R., Mackie, G.O. & Singla, C.L., 1977. On the organisation of the sarcotubular systems in the caudal muscle cells of larvaceans (Tunicata). *Acta Zoologica*, **58**, 187–196.

Bone, Q., Inoue, I. & Tsutsui, I., 1997. Contraction and relaxation in the absence of a sarcoplasmic reticulum: muscle fibres in the small pelagic tunicate *Doliolum*. *Journal of Muscle Research and Cell Motility*, **18**, 375–380.

Bone, Q. & Trueman, E.R., 1982. Jet propulsion of the calycophoran siphonophores *Chelophyes* and *Abylopsis*. *Journal of the Marine Biological Association of the United Kingdom*, **62**, 263–276.

Caputo, C. & Bolanõs, P., 1995. Na^+/Ca^{2+} exchange in amphibian skeletal muscle fibre. *Journal of Physiology*, **487P**, 160P.

Chain, B.M., Bone, Q. & Anderson, P.A.V., 1981. Electrophysiology of a myoid epithelium in *Chelophyes* (Coelenterata: Siphonophora). *Journal of Comparative Physiology* **143**, 329–338.

Gonzalez-Serrato, H., Hilgemann, D.W., Rozycka, M., Gauthier, A. & Rasgado Flores, H., 1996. Na/Ca exchange studies in sarcolemmal skeletal muscle. *Annals of the New York Academy of Science*, **779**, 556–560.

Inoue, I., Tsutsui, I., Bone, Q. & Brown, E.R., 1994. Evolution of skeletal muscle excitation-contraction coupling and the appearance of di-hydropyridine-sensitive intramembrane charge movement. *Proceedings of the Royal Society* B, **255**, 181–187.

Inoue, I., Tsutsui, I., Brown, E.R. & Bone, Q., 1996. A phylogenetic approach to understanding the molecular mechanism of excitation contraction coupling in striated muscle. In *Basic neuroscience in invertebrates* (ed. H. Koike et al.), pp. 45–57. Tokyo: Japan Scientific Societies Press.

Mackie, G.O. & Carré, D., 1983 Coordination in a diphyid siphonophore. *Marine Behaviour and Physiology*, **9**, 139–170.

Prosser, D.L., 1982. Diversity of narrow-fibered and wide-fibered muscles. In *Basic biology of muscles: a comparative approach* (ed. B.M. Twarog et al.), pp. 381–397. New York: Raven Press.

Submitted 11 June 1999. Accepted 15 September 1999.