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Studies on the distribution of calcitonin gene-related peptide-like and substance P-like immunoreactivities in rat hind limb muscles

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Summary

The tibialis anterior, extensor digitorum longus and soleus muscles in the rat were examined with respect to the presence of calcitonin gene-related peptide-like as well as substance P-like immunoreactivity. In some of the motor endplates in these muscles, identified by staining for acetylcholinesterase activity, calcitonin gene-related peptide-like immunoreactivity was detected, but in others it was not. Calcitonin gene-related peptide-like immunoreactivity was found to coexist with substance-P-like immunoreactivity in nerve fibres located outside and inside the capsule of the muscle spindles, as well as in nerve fibres located in nerve fascicles. These fibres presumably represent sensory nerve fibres. Calcitonin gene-related peptide-like immunoreactivity, but not substance P-like immunoreactivity, was also detected in cap-like structures located on the surface of the intrafusal muscle fibres in the polar regions of the spindles, structures which are likely to correspond to motor plate endings. The observations suggest that calcitonin gene-related peptide is heterogeneously present in the endplates of rat hind limb muscles, and gives for the first time immunohistochemical evidence for the presence of calcitonin gene-related peptide and substance P in the innervation of muscle spindles.

Introduction

Calcitonin gene-related peptide has been generated via alternative processing of the mRNA transcribed from the calcitonin gene (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Calcitonin gene-related peptide frequently coexists with substance P in sensory nerve fibres (e.g. Wiesenfeld-Hallin *et al.*, 1984; Gibbins *et al.*, 1985). By use of immunohistochemical methods, calcitonin gene-related peptide has also been observed in the perikarya of motoneurons (e.g. Gibson *et al.*, 1984; Fontaine *et al.*, 1986; New & Mudge, 1986; Marti *et al.*, 1987; Gibson *et al.*, 1988a, b; Villar *et al.*, 1988, 1989; Rethelyi *et al.*, 1989; Hietanen *et al.*, 1990; Merighi *et al.*, 1990) and in motor endplates (e.g. Takami *et al.*, 1985a, b; Cadieux *et al.*, 1986). However, calcitonin gene-related peptide immunoreactivity is not always detected in motoneuron perikarya (e.g. Gibson *et al.*, 1988b; Villar *et al.*, 1989; Hietanen *et al.*, 1990; Newton *et al.*, 1990). Calcitonin gene-related peptide immunoreactivity is also heterogeneously present in the endplates of the rat tongue (Takami *et al.*, 1985a) and bulbocavernosus muscle (Popper & Micevych, 1989) and has been found in only a small portion of the endplates of human intercostal muscles (Mora *et al.*, 1989). Furthermore, calcitonin gene-related peptide immunoreactivity has never been observed in the motor endplates of the rat gastrocnemic and diaphragm muscles (Bööj *et al.*, 1989). To our knowledge, it is not known whether calcitonin gene-related peptide is also absent in the motor endplates of hind limb muscles other than the gastrocnemic. Moreover, nothing is known of the presence of calcitonin gene-related peptide in the innervation of muscle spindles, the specialized sensory organs which are associated with the coordination of muscle activity and the maintenance of muscle tone.

In the present study we have studied three hind limb muscles of the rat – the tibialis anterior, the extensor digitorum longus and the soleus – for the presence of calcitonin gene-related peptide by the use of immunohistochemical methods, with particular emphasis on the motor endplates and the muscle spindles. The presence of calcitonin gene-related peptide was compared with that of substance P.

Materials and methods

Tissue preparation

Six male Sprague–Dawley rats (300–350 g) were anaesthetized with sodium pentobarbital (40 mg kg^{-1} i.p.) and perfused transcardially with an ice-cold solution of 4% formaldehyde in

0.1 M phosphate buffer, pH 7.0, for 5 min. Following perfusion, the extensor digitorum longus, the tibialis anterior and the soleus muscles were dissected out and further fixed by immersion in the same fixative for 24 h at 4°C. The specimens were then thoroughly washed in Tyrode's solution, containing 10% sucrose, at 4°C overnight. Slices of the muscles were thereafter cut perpendicularly to the longitudinal axis of the muscles and mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL) and frozen in propane chilled with liquid nitrogen.

Sectioning and histochemical procedures

Series of 8–10 µm-thick sections were cut using a cryostat. The sections were mounted on slides pre-coated with chrome-alum gelatine, dried and processed for immunofluorescence, strained for the demonstration of acetylcholinesterase (AChE) activity or stained for Gomori Trichrome. The sections processed for AChE were postfixed in buffered formol–sucrose fixative (Karnovsky, 1964) for 1 h at 4°C, and treated with tetraisopropyl-pyrophosphoramide (iso-OMPA) (10^{-4} M) as an inhibitor of non-specific cholinesterase in a 30 min pre-incubation. Gomori's medium (Gomori, 1952), pH 6.0, was used for the incubation with acetylthiocholine iodide (20 mg in 10 ml of Gomori's medium) as substrate. After incubation, which proceeded for 60–120 min, the sections were washed in distilled water, developed in fresh 1% ammonium sulphide, counterstained for 30 s in Methyl Green, washed in distilled water, and mounted in glycerol jelly.

AChE was used as a marker for the endplates and the muscle spindles. The character of the AChE reaction was influenced by the length of incubation. The muscle spindles were also easily identified in sections stained for Gomori Trichrome.

Immunohistochemical procedures

Sections were processed for indirect immunofluorescence (for details of the procedures used, see Forsgren & Söderberg, 1987). In brief, the sections were incubated with a 1% solution of Triton X-100 (Kebo Lab, Stockholm) in 0.01 M phosphate-buffered saline (PBS), pH 7.2, rinsed in PBS and incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were thereafter incubated with antiserum against calcitonin gene-related peptide or substance P, diluted in PBS with BSA, in a humid atmosphere. Incubation was performed for 1 h at 37°C. After incubation with the primary antiserum, the sections were washed in PBS and incubated in swine anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Dakopatts, Copenhagen), diluted 1:40, for 30 min at 37°C, in a moist chamber. The sections were again washed in PBS and then coverslipped in a 1:1 mixture of glycerol and PBS. The sections were examined with a Leitz Orthoplane Photomicroscope equipped with epi-illumination.

Double labelling

Some sections were subjected to double labelling. These sections were first stained for substance P or calcitonin gene-related peptide, examined and photographed, and were then treated with acid potassium permanganate for 2 min for the removal of the antibodies (cf. Tramu *et al.*, 1978). The sections were then incubated with FITC-conjugated swine anti-rabbit IgG as described above and examined under the microscope, verifying the disappearance of specific immunoreactivity. The sections that had been initially incubated with substance P antiserum were then stained with calcitonin gene-related peptide antiserum, and vice versa. The areas of the sections previously photographed were identified and re-photographed. Some sections were stained twice with the calcitonin gene-related peptide antiserum. Examination of these sections revealed that the intensity of calcitonin gene-related peptide immunoreactivity seen after sequential staining was similar to that seen after the initial staining. Still other sections were processed for calcitonin gene-related peptide, washed with PBS and stained for demonstration of AChE activity.

Antisera

The calcitonin gene-related peptide antiserum was purchased from Amersham International (Buckinghamshire, UK). It is a rabbit polyclonal antiserum raised against synthetic α-calcitonin gene-related peptide conjugated to bovine serum albumin (code: RPN 1842). Usual tests for dilution were made, and an appropriate working dilution was found to be 1:100. The substance P antiserum is a rabbit polyclonal antiserum raised against synthetic substance P conjugated to bovine thyroglobulin (code: 675/002); its source was UCB (Brussels). The working dilution of the substance P antiserum was 1:500. The suppliers report that neither antiserum cross-reacts with other known neuropeptides. Specific immunoreaction was not obtained in sections incubated with antisera pre-absorbed overnight with the respective peptide (calcitonin gene-related peptide or substance P) (1.5×10^{-5} M; Sigma). Calcitonin gene-related peptide-like immunoreactivity was unaffected by the addition of substance P (1.5×10^{-5} M) to calcitonin gene-related peptide antiserum and substance P-like immunoreactivity was unaffected after pre-absorption of substance P antiserum with calcitonin gene-related peptide (1.5×10^{-5} M).

Results

Motor endplates

The motor endplates of the extrafusal muscle fibres were easily identified by their marked AChE activity (Figs 1b and 2b). They were then carefully analysed immunohistochemically in adjacent serial sections (cf. Figs 1 and 2). Calcitonin gene-related peptide-like immunoreactivity was detected in some of the endplates (Fig. 1a, c), whilst this immunoreactivity was not observed in others (Fig. 2a, c). This was the case for all three muscles examined. On no occasion was substance P-like immunoreactivity observed in the endplates (Fig. 3a). The endplates displayed an autofluorescence reaction, and such a reaction was also observed at and beneath the muscle fibre membranes (cf. Figs 2a, c and 3a). By the use of elution–restaining, after which procedure the autofluorescence reactions were greatly diminished, it was verified that calcitonin gene-related peptide-like immunoreactivity was present in some of the endplates (Figs 3 and 4).

Muscle spindles

The muscles spindles appeared as bundles of muscle fibres enclosed in a capsule in sections stained for

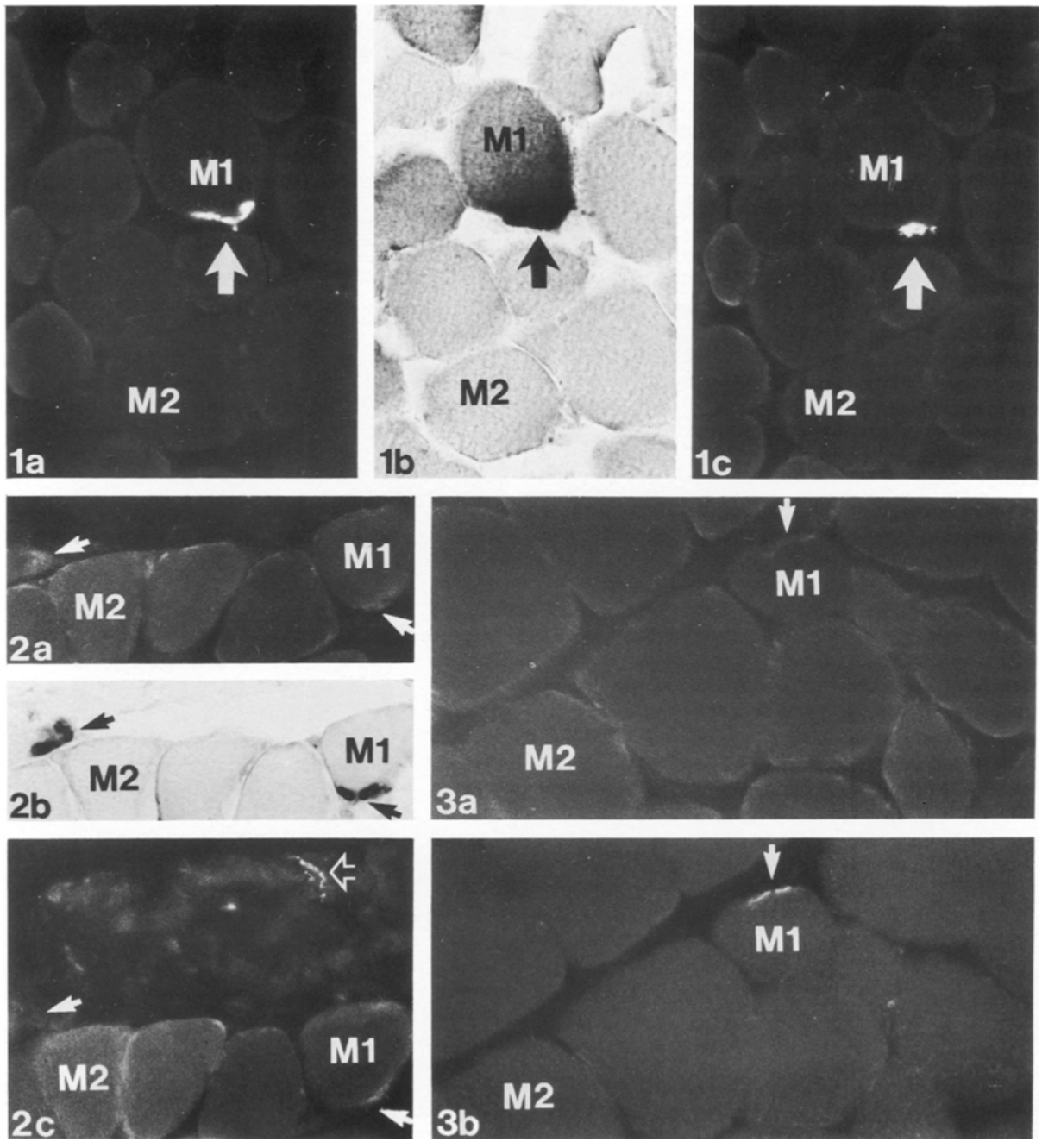
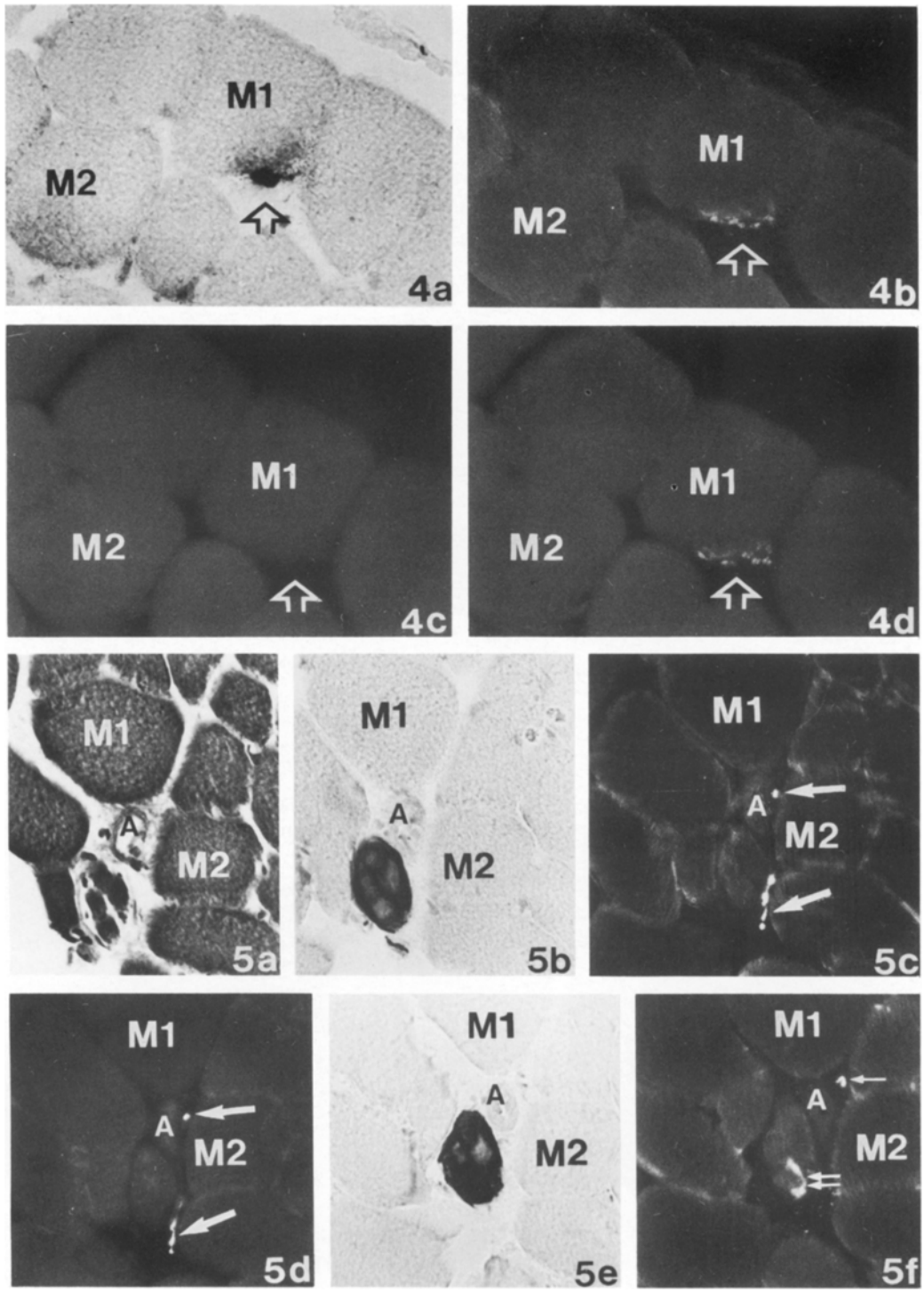


Fig. 1. Three consecutive serial sections of an extensor digitorum longus muscle, the sections being stained for calcitonin gene-related peptide (a, c) and AChE (b). One motor endplate is observed (arrows). Calcitonin gene-related peptide-like immunoreactivity is observed in the endplate. Corresponding myofibres: M1 and M2. × 310.

Fig. 2. Three consecutive serial sections of a soleus muscle. The sections had been processed for calcitonin gene-related peptide (a, c) and AChE (b). Two motor endplates are observed (solid arrows). No specific immunoreactivity was detected in these endplates. Autofluorescence is emanating from mitochondria located beneath the sarcolemma in the muscle fibres and in the endplates. A nerve fibre exhibiting calcitonin gene-related peptide-like immunoreactivity is observed in the connective tissue in (c) (open arrow). M1 and M2 are corresponding myofibres. × 310.

Fig. 3. A section of a tibialis anterior muscle subjected to elution restaining, the section first being processed for substance P (a) and then for calcitonin gene-related peptide (b). A motor endplate identified by showing AChE activity (not shown) does not exhibit substance P-like immunoreactivity but shows calcitonin gene-related peptide-like immunoreactivity (arrows, a and b). M1 and M2 are corresponding myofibres. Autofluorescence reactions in peripheral parts of myofibres are clearly more marked in (a) than in (b). × 310.



Gomori Trichrome (Fig. 5a) and showed a strong AChE activity (Fig. 5b, e). Calcitonin gene-related peptide-like immunoreactivity was detected in nerve fibres located just outside the spindle (Fig. 5d) and at the surface of intrafusal muscle fibres in the polar regions of the spindles, forming cap-like structures (Fig. 5f). Calcitonin gene-related peptide-like immunoreactivity was also detected in nerve fibres located at the capsule of the spindles (Fig. 7) as well as inside the capsule in the polar (Figs 6 and 8c) and equatorial and juxtaequatorial regions of the spindles. Substance P-like immunoreactivity was present in nerve fibres located outside and inside the capsule but was not detected in cap-like structures on the surface of the muscle fibres. Double-labelling studies showed that there was a high degree of coexistence of substance P-like immunoreactivity and calcitonin gene-related peptide-like reactivity in the nerve fibres associated with the spindles (Figs 5c, d and 8).

Nerve fascicles, blood vessels, free nerve endings

Nerve fascicles were located in the connective tissue both around and within the muscles. Nerve fibres exhibiting calcitonin gene-related peptide-like immunoreactivity as well as substance P-like immunoreactivity were observed in these fascicles. Sequential double staining revealed that all nerve fibres in the fascicles that showed substance P-like immunoreactivity exhibited calcitonin gene-related peptide-like immunoreactivity as well (Fig. 9). However, a few fibres in the fascicles exhibited calcitonin gene-related peptide-like immunoreactivity but not substance P-like immunoreactivity (Fig. 9).

Nerve fibres showing a positive AChE reaction occurred in the fascicles. The fibres exhibiting calcitonin gene-related peptide-like immunoreactivity showed a distribution in the fascicles different from those displaying a strong AChE activity. Sequential double staining showed that the latter fibres did not exhibit calcitonin gene-related peptide-like immunoreactivity (Fig. 10).

Nerve fibres showing substance P-like immunoreactivity and calcitonin gene-related peptide-like immunoreactivity occurred in the walls of blood vessels as well as in the connective tissue adjacent to these vessels (Fig. 11). This included the blood vessels that were located in the vicinity of the muscle spindles (Fig. 5c, d, f). Some finely varicose nerve fibres exhibiting calcitonin gene-related peptide-like immunoreactivity, as well as such fibres exhibiting substance P-like immunoreactivity, coursed in between myofibres (Fig. 12).

Discussion

In contrast to the situation in the rat gastrocnemic (Bööj *et al.*, 1989), the present study shows that calcitonin gene-related peptide-like immunoreactivity is present in motor endplates of the extrafusal muscle fibres in rat tibialis anterior, extensor digitorum longus and soleus muscles. One explanation for this discrepancy may be the method of sampling as calcitonin gene-related peptide-like immunoreactivity was detectable in only some of the endplates in the muscles studied in the present study. Other explanations may be differences in fixation and the sensitivity of the calcitonin gene-related peptide antisera.

The observations of a heterogeneity in the presence of calcitonin gene-related peptide-like immunoreactivity in motor endplates made in the present study conform to similar observations made in other skeletal muscles (Takami *et al.*, 1985a; Mora *et al.*, 1989; Popper & Micevych, 1989). Furthermore, there is a heterogeneity in the presence of calcitonin gene-related peptide-like immunoreactivity in motoneuron perikarya (Gibson *et al.*, 1988b; Villar *et al.*, 1989; Hietanen *et al.*, 1990; Newton *et al.*, 1990). All these observations suggest that calcitonin gene-related peptide may have varying importance for different muscle fibres. The exact functional role of calcitonin gene-related peptide in motor neurons is not clearly established, but it has been suggested that calcitonin gene-related peptide enhances the contraction of muscle fibres elicited by acetylcholine at the neuromuscular junction (Takami *et al.*, 1985b). It has also been shown that the peptide increases the synthesis of acetylcholine receptors in cultured chick muscle cells (Fontaine *et al.*, 1986; New & Mudge, 1986), which may be at least partly related to an increase in intracellular cAMP (Laufer & Changeux, 1987). On the whole, calcitonin gene-related peptide is suggested to act as a 'trophic' factor to control processes of differentiation of skeletal muscle fibres (see Villar *et al.*, 1989).

Calcitonin gene-related peptide-like immunoreactivity was observed in nerve fibres associated with blood vessel walls, as has been previously shown for rabbit skeletal muscle (Öhlén *et al.*, 1987). Calcitonin gene-related peptide-like immunoreactivity was also present in nerve fibres located in nerve fascicles. Calcitonin gene-related peptide-like immunoreactivity never coexisted with a marked AChE reaction in these fascicles. It is likely that the AChE-positive fibres observed in these structures represent the preterminal parts of the motoneurons. As calcitonin gene-related peptide is transported with anterograde axonal transport in the motoneurons (Bööj

Fig. 4. (a, b) Two consecutive sections of a tibialis anterior muscle, processed for AChE (a) and calcitonin gene-related peptide (b). A motor endplate exhibiting calcitonin gene-related peptide-like immunoreactivity is observed (open arrows). Corresponding myofibres: M1 and M2. (c, d) After elution of calcitonin gene-related peptide-antiserum, and incubation with swine anti-rabbit IgG, the section is shown in (b); the same section after re-staining for calcitonin gene-related peptide (d). No specific immunoreactivity is observed in the endplate in (c) (open arrow), but immunoreactivity is again visible (open arrow, d) after re-staining for calcitonin gene-related peptide. Autofluorescence reactions seen in peripheral parts of myofibres in (b) have, in principle, vanished in (c) and (d). × 310.

Fig. 5. Different levels in the series of sections of a muscle spindle and adjacent myofibres, the sections being stained for Gomorii Trichrome (a), AChE (b, e), substance P (c) and calcitonin gene-related peptide (d, f). A muscle spindle is clearly observed in all sections. A small arteriole (A) is located close to the spindle. M1 and M2 are corresponding myofibres. The same section subjected to double labelling is shown in (c) and (d). Substance P-like immunoreactivity and calcitonin gene-related peptide-like immunoreactivity are observed in nerve profiles located outside the spindle (arrows, below c, d) and in the wall of the arteriole (arrows, above d, e, f), substance-P-like immunoreactivity and calcitonin gene-related peptide-like immunoreactivity partly coexisting in these profiles (c, d). In (f), calcitonin gene-related peptide-like immunoreactivity is also present on the surface of one of the intrafusal muscle fibres (double arrow). × 310.

et al., 1989; Kashihara *et al.*, 1989), at least some of the motor axons in the fascicles were expected to show calcitonin gene-related peptide-like immunoreactivity. One explanation for the apparent non-existence of calcitonin gene-related peptide-like immunoreactivity in the preterminal parts of the motor axons may be the very rapid transport rate of the peptide (Bööj *et al.*, 1989), rendering the amount of calcitonin gene-related peptide too small to become detectable with the methodology used here. In accordance with such a suggestion, very little or no calcitonin gene-related peptide-like immunoreactivity is detectable distally to a crushed ventral root, as well as in uncrushed roots, whereas granular calcitonin gene-related peptide-like immunoreactivity accumulates in axons showing a positive AChE reaction – i.e., the presumable motor axons – proximal to the crush (Bööj *et al.*, 1989).

The calcitonin gene-related peptide-like immunoreactivity detected in nerve fascicles frequently coexisted with substance P-like immunoreactivity. A coexistence of calcitonin gene-related peptide-like immunoreactivity and substance P-like immunoreactivity has previously been shown for cell bodies in sensory ganglia and nerve fibres in various parts of the body in several mammalian species (e.g. Wiesenfeld-Hallin *et al.*, 1984; Gibbins *et al.*, 1985; Lundberg *et al.*, 1985; Skofitsch & Jacobowitz, 1985; Morishima *et al.*, 1986), including nerve fibres located in the limb muscles of the guinea pig (Gibbins *et al.*, 1987) and rabbit (Öhlén *et al.*, 1987). These calcitonin gene-related peptide- and substance P-immunoreactive nerve fibres are likely to represent sensory nerve fibres, as such fibres in various parts of the body are sensitive to treatment with the neurotoxin capsaicin (e.g. Lundberg *et al.*, 1983; Martling *et al.*, 1988; Domeij *et al.*, 1991). The observations in the present study of the existence of a few fibres showing calcitonin gene-related peptide-like immunoreactivity, but not substance P-like immunoreactivity, in the nerve fascicles, may be related to the fact that the number of calcitonin gene-related peptide-immunoreactive cells in the dorsal root ganglia is somewhat higher than that of the substance P-immunoreactive ones (e.g. Gibbins *et al.*, 1985; Lee *et al.*, 1985).

The muscle spindles are mechanoreceptors that receive both motor and sensory innervation (for review see Boyd, 1980; Barker & Banks, 1986; Hunt, 1990). In the present study it has been shown for the first time that nerve structures exhibiting calcitonin gene-related peptide-like immunoreactivity and substance P-like immunoreactivity are present in the spindles. Calcitonin gene-related peptide-like immunoreactivity was found to coexist with substance P-like immunoreactivity in nerve fibres located outside as well as inside their capsule. These fibres are likely to represent sensory nerve fibres since, as discussed above, a coexistence of calcitonin gene-related peptide-like immunoreactivity and substance P-like immunoreactivity is typical for sensory nerves. By the use of silver impregnation techniques, it has previously been shown that afferent nerve fibres course along the spindle (Boyd & Smith, 1984). Generally one single primary afferent (group I) axon innervates all intrafusal fibres in a spindle in the equatorial region. In addition, one or more secondary (group II) axons make contacts in the juxtaequatorial region, predominantly innervating nuclear chain fibres (Barker & Banks, 1986). Further studies are needed to clarify

Figs 6 and 7. Muscle spindles and adjacent tissue in sections processed for calcitonin gene-related peptide. The spindles are cut at the polar (Fig. 6) and juxtaequatorial (Fig. 7) levels. The capsule is marked with asterisks. Varicose nerve fibres are present at the capsule (small arrow, Fig. 7), just inside the capsule (arrow, Fig. 6) and in the surrounding connective tissue (large arrow, Fig. 7). × 400.

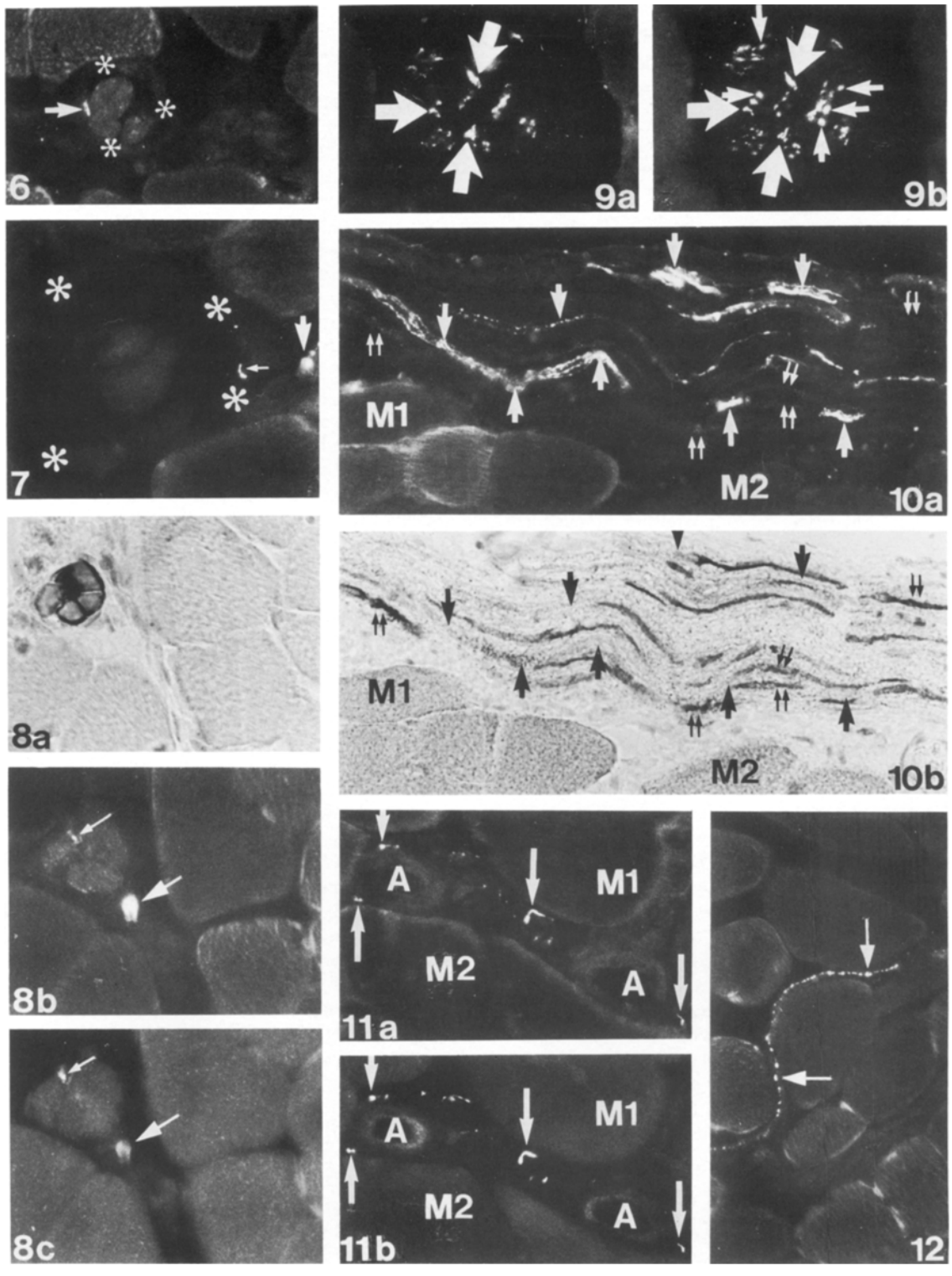
Fig. 8. A muscle spindle and adjacent tissue in a section stained for AChE (a) and a consecutive section in the series first processed for substance P (b) and then for calcitonin gene-related peptide (c). Spindle at the top left. Substance P-like immunoreactivity and calcitonin gene-related peptide-like immunoreactivity are present at similar locations in association with the spindle (small and large arrows). × 400.

Fig. 9. A section of a small nerve fascicle located within an extensor digitorum longus muscle. The section had been initially stained for substance P (a) and was then processed for calcitonin gene-related peptide (b). Most of the nerve profiles showing calcitonin gene-related peptide-like immunoreactivity also show substance P-like immunoreactivity (large arrows). Some profiles are immunolabelled only in (b) (small arrows). × 400.

Fig. 10. A section of a nerve fascicle located in the epimysium of an extensor digitorum longus muscle. The section was first processed for calcitonin gene-related peptide (a) and then for AChE (b). The fascicle contains fibres that show an intense calcitonin gene-related peptide-like immunoreactivity and a weak or negligible AChE reaction (single arrows) and fibres that exhibit a strong AChE reaction and no calcitonin gene-related peptide-like immunoreactivity (double arrows). M1 and M2 are corresponding myofibres. × 310.

Fig. 11. Two arterioles A and adjacent tissue in an extensor digitorum longus muscle in a section processed first for substance P (a) and then for calcitonin gene-related peptide (b). Fine nerve fibres showing both substance P-like immunoreactivity and calcitonin gene-related peptide-like immunoreactivity are observed (arrows). M1 and M2 are corresponding myofibres. × 310.

Fig. 12. A section of a tibialis anterior muscle incubated with calcitonin gene-related peptide antiserum. A varicose nerve fibre courses in between myofibres (arrows). × 310.



whether both group I and group II axons show calcitonin gene-related peptide-like immunoreactivity and substance-P-like immunoreactivity. The present study also shows that calcitonin gene-related peptide-like immunoreactivity but not substance P-like immunoreactivity was detectable in cap-like structures located on the surface of the intrafusal muscle fibres. These structures were located in the polar regions of the spindles. As the motor endings of the gamma motoneurons are distributed on both bag and chain fibres in this area (Kucera *et al.*, 1991), it is tentatively suggested that the cap-like structures showing calcitonin gene-related peptide-like immunoreactivity may represent motor plate endings of gamma motor neurons, or alternatively endings of β motor neurons.

In conclusion, the present study shows that calcitonin gene-related peptide-like immunoreactivity can be detected in a proportion of the motor endplates of the extrafusal fibres in rat tibialis anterior, extensor digitorum longus and soleus muscles and gives new information on the presence of a calcitonin gene-related peptide/substance P innervation in muscle spindles.

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