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# RELEASE OF CALCITONIN GENE-RELATED PEPTIDE FROM NERVE TERMINALS IN RAT SKELETAL MUSCLE

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SUMMARY

1. The amount of calcitonin gene-related peptide (CGRP) released from the isolated rat soleus muscle was measured by enzyme immunoassay.

2. When the soleus muscle was exposed to a solution containing high $K^+$ (20–100 mM) in the presence of tetrodotoxin, the amount of CGRP released into the bathing medium increased with an increase in the $K^+$ concentration.

3. The exposure to 100 mM-$K^+$ did not increase CGRP release from chronically denervated soleus muscles or from pieces of the soleus nerve separated from the muscle.

4. The amount of CGRP released from the isolated muscle by 100 mM-$K^+$ depended on the external $Ca^{2+}$ concentration. The slope of the relation between the amount of CGRP release and the $Ca^{2+}$ concentration was less than one on double logarithmic co-ordinates.

5. Following chronic section of the lumbar ventral roots, the mean amount of CGRP released from the soleus muscle by 100 mM-$K^+$ was reduced by 28%, compared with that observed in normal muscle.

6. Antidromic stimulation of the lumbar dorsal roots at an intensity three times the threshold for most excitable sensory fibres failed to induce CGRP release from the soleus muscle, whereas stimulation at intensities 50–100 times the threshold increased significantly the amount of CGRP release from the muscle.

7. Stimulation of the muscle nerve at an intensity sufficient to activate the $\alpha$-motor fibres did not release CGRP from the soleus muscle or from the diaphragm.

8. It is concluded that the major source of CGRP released from skeletal muscle is A$\delta$- and/or C sensory terminals and that the $Ca^{2+}$ dependence of CGRP release is less steep than that reported for acetylcholine release at neuromuscular junctions.

## INTRODUCTION

The presence of calcitonin gene-related peptide (CGRP) has been shown in a variety of central and peripheral neurones (Rosenfeld, Mermod, Amara, Swanson, Sawchenko, Rivier, Vale & Evans, 1983; Gibson, Polak, Bloom, Sabate, Mulderry, Ghatei, McGregor, Morrison, Kelly, Evans & Rosenfeld, 1984; Wiesenfield-Hallin, Hökfelt, Lundberg, Forssmann, Reinecke, Tschopp & Fischer, 1984). In the peripheral nerve, CGRP is transported in the somatofugal direction in both motor

and sensory fibres (Kashihara, Sakaguchi & Kuno, 1989). CGRP transported from the neurone cell bodies appears to accumulate at nerve terminals in muscle since the CGRP content in skeletal muscle is much greater than that expected from the amount of CGRP in the muscle nerve (Kashihara *et al.* 1989). In fact, intensive CGRP immunoreactivity has been found at motor nerve terminals (Takami, Kawai, Shiosaka, Lee, Girgis, Hillyard, MacIntyre, Emson & Tohyama, 1985*a*; Takami, Kawai, Uchida, Tohyama, Shiotani, Yoshida, Emson, Girgis, Hillyard & MacIntyre, 1985*b*; Matteoli, Haimann, Torri-Tarelli, Polak, Ceccarelli & De Camilli, 1988) as well as at sensory terminals in skeletal muscle (Takami *et al.* 1985*a*; Ohlen, Lindbom, Hökfelt, Cuello, Fischer & Hedqvist, 1987; Matteoli *et al.* 1988).

The release of CGRP from central terminals of primary sensory fibres has been demonstrated in the spinal cord slice preparation by the application of capsaicin or high-$K^+$ solutions (Saria, Gamse, Petermann, Fischer, Theodorsson-Norheim & Lundberg, 1986; Franco-Cereceda, Henke, Lundberg, Petermann, Hökfelt & Fischer, 1987). Stimulation of the peripheral nerve also causes to release CGRP in the dorsal horn of the spinal cord *in vivo* (Morton & Hutchison, 1989). Similarly, peripheral endings of sensory neurones may be capable of releasing CGRP. Thus, the application of capsaicin (Ohlen *et al.* 1987; Diez Guerra, Zaidi, Bevis, MacIntyre & Emson, 1988) or bradykinin (White, Leah & Zimmermann, 1989) has shown to induce CGRP release from peripheral sensory fibres. CGRP released from the peripheral sensory nerve is suggested to produce vasodilatation (Zaidi, Bevis, Girgis, Lynch, Stevenson & MacIntyre, 1985; Holzer, 1988; Louis, Jamieson, Russell & Dockray, 1989). However, it is not known whether CGRP can be released from peripheral sensory endings by terminal depolarization or by nerve stimulation.

CGRP released from motor nerve terminals has been suggested to display several trophic actions at neuromuscular junctions, including the regulation of acetylcholine (ACh) receptor synthesis (Fontaine, Klarsfeld, Hökfelt & Changeaux, 1986; New & Mudge, 1986) and of its desensitization rate (Mulle, Benoit, Pinset, Roa & Changeaux, 1988) as well as inhibition of sprouting of motor nerve terminals (Tsujimoto & Kuno, 1988). It has been reported that CGRP can be released at neuromuscular junctions of the diaphragm by nerve stimulation (Uchida, Yamamoto, Iio, Matsumoto, Wang, Yonehara, Imai, Inoki & Yoshida, 1990). However, because of the long pulse duration (10 ms) used for nerve stimulation in this study (also, see Results), identification of the site of CGRP release remains uncertain.

In the present study, CGRP release induced by high $K^+$ or nerve stimulation was examined in rat skeletal muscle. The principal question was whether CGRP can be released from motor and/or sensory nerve terminals in muscle, and if so, whether CGRP release is triggered by $Ca^{2+}$ in a manner similar to that reported for the release of classical transmitters. Our data suggest that CGRP is mainly released from the endings of high-threshold muscle sensory fibres.

## METHODS

### *Preparation*

Adult Wistar rats (about 6 weeks in age) were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). The soleus muscle and attached nerve were excised from the hindlimb. The muscle was slightly stretched and pinned at the fascia and tendon to the Sylgard-

lined bottom of a 60 mm plastic Petri dish. The preparation was bathed in 7 ml of standard Krebs solution of the following composition (in mM): NaCl, 130; KCl, 4·5; $CaCl_2$, 2·0; $NaHCO_3$, 10; glucose, 11·0. The bathing solution also contained 0·02% bovine serum albumin and was equilibrated with 95% $O_2$–5% $CO_2$.

In one series of experiments, the release of CGRP from the soleus muscle was induced by the application of a solution containing high $K^+$ concentrations (20–100 mM). In these cases, the soleus nerve was cut near its entry to the muscle, and the generation of action potentials was blocked by tetrodotoxin (TTX; 2 $\mu$M) added to the bathing solution. The NaCl concentration was fixed at 35 mM, and osmolarity was balanced by adding appropriate amounts of choline chloride according to the KCl concentration used for the experiment.

Attempts were also made to measure CGRP released from the soleus muscle in response to nerve stimulation. The soleus nerve was stimulated at 2 Hz with a suction electrode in the standard Krebs solution. Before the experiment, the threshold intensity for eliciting muscle contractions was determined. The nerve was then stimulated at an intensity 3 times or 50–100 times higher than the threshold in the presence of *d*-tubocurarine (dTC; 2 $\mu$M). In the case of stimulation at high intensities (50–100 times the threshold), the stimulus pulse duration was also increased from 0·1 to 0·5 ms in order to assure activation of A$\delta$- and unmyelinated C fibres (Harper & Lawson, 1985).

To examine the relative contributions of motor and sensory fibres to CGRP release, the measurements were made after degeneration of motor fibres in a few experiments. For this purpose, the ventral roots of the 4th and 5th lumbar segments were sectioned on one side. Four days after this operation, the amounts of CGRP released by high $K^+$ (100 mM) were compared between the soleus muscles excised from the experimental and contralateral, intact sides. Also, in another series of experiments, the soleus muscle was excised together with attached sciatic nerve and lumbar spinal roots, and CGRP released from the soleus muscle was measured in response to stimulation of the 4th and 5th lumbar ventral or dorsal roots. The threshold intensity for most excitable sensory fibres was determined by recording the compound action potential from the peripheral nerve branch evoked by antidromic stimulation of the dorsal roots. The amount of CGRP released from the soleus muscle was measured by stimulation of the dorsal roots again at an intensity 3 times the threshold and at an intensity 50–100 times the threshold.

In a separate series of experiments, the isolated diaphragm was also used for measurements of the content and release of CGRP. Technical details on this series of experiments are given in the appropriate section of Results.

*Recovery of the bathing solution for CGRP assay*

In the majority of experiments, two soleus muscles were placed in a bathing chamber in order to increase the amount of CGRP release, thereby facilitating the assay reliability. The isolated soleus muscles were first incubated in the standard solution for 30 min in the presence of dTC. This solution was discarded. The preparation was then briefly rinsed, and the chamber was filled with a fresh solution. The nerve was stimulated for 6 min, and the entire bathing solution was collected 1 min after nerve stimulation. The preparation was then rinsed briefly 3 times. This procedure was repeated several times with or without nerve stimulation. In the experiment with solutions containing different $K^+$ concentrations, the bathing solution was collected after exposure of the preparation to a given solution for 7 min. The bath temperature was maintained at 23–24 °C. CGRP release at other temperature ranges was not measured.

All the tubes containing the solutions collected after each trial were kept on ice till the end of the experiment. Each sample solution was lyophilized and redissolved in 0·3 ml of a buffer solution for assay. However, high salt contents in the sample were found to reduce the assay sensitivity. Therefore, before lyophilization, the sample solution was dialysed through molecular-porous membranes (SPECTRA/POR MWCD, 1000; Spectrum Inc., Los Angeles, CA, USA) against 1 M-acetic acid for 3 h at 4 °C.

*Enzyme immunoassay of CGRP*

The procedure of two-site enzyme immunoassay of CGRP has been described previously in detail (Kashihara *et al.* 1989). Briefly, the antiserum raised in rabbits by injecting synthetic rat $\alpha$-CGRP (Peptide Institute, Inc., Osaka, Japan) was purified by affinity chromatography with the rat CGRP linked to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Polystyrene beads (3·2 mm in diameter) were coated with the affinity-purified antibodies.

Individual antibody-coated beads were exposed to the test solution overnight at 4 °C. CGRP bound to the beads was then assayed by the anti-CGRP Fab' (Fragment with antigen binding) linked to horseradish peroxidase (HRP). The enzyme reaction to HRP initiated by the addition of $H_2O_2$ against 3-(*p*-hydroxyphenyl)propionic acid was quantified by the fluorescence intensity. The lower limit of this assay was between 1 and 2 pg/assay or 10–20 pg/ml. The CGRP immunoreactive material obtained from neural tissues coincided with synthetic rat CGRP in fractional distributions separated by gel filtration (Kashihara *et al.* 1989).

All the values presented in the text give the mean and its standard deviation. The statistical analysis was made by two-tailed *t* tests with the significance limit of $P < 0.05$.

## RESULTS

### *CGRP release induced by high-$K^+$ solutions*

Figure 1 shows the results obtained from two experiments (*A* and *B*) in which the soleus muscles were exposed to solutions containing different $K^+$ concentrations (4·5–100 mM) for 7 min in the presence of TTX and 2 mM-$Ca^{2+}$. In one experiment (*A*), the $K^+$ concentration of the bathing solution applied was progressively increased from 20 mM ($K_{20}$) to 100 mM ($K_{100}$), each test being alternated by the exposure to the normal $K^+$ level (4·5 mM indicated by R). The amount of CGRP measured in the solution (pg/muscle) increased with an increase in the $K^+$ concentration.

The same result was obtained regardless of the application order of different $K^+$ concentrations (Fig. 1*B*). However, it was not certain whether the amount of CGRP released by a given concentration of $K^+$ might be affected by previous exposure to high-$K^+$ solutions. Therefore, for quantitative measurements, only the CGRP assay made for the first application of high-$K^+$ solutions (20–100 mM) following the exposure to the initial standard $K^+$ fluid (4·5 mM-$K^+$) was adopted. Figure 2 summarizes the results in this series of experiments. The amount of CGRP release increased significantly at $K^+$ concentrations higher than 50 mM, whereas the amount of CGRP released by 20 mM-$K^+$ was not significantly different from that in the control solution.

When the soleus muscle had been denervated 4 days previously, the application of 100 mM-$K^+$ did not increase CGRP release from the muscle ($< 1.5$ pg/two muscles, $n = 3$). Also, following the exposure of two pieces of a 15 mm segment of the soleus nerve (without the muscle) to 100 mM-$K^+$, there was again no detectable CGRP in the bathing solution. Therefore, it seems reasonable to conclude that CGRP measured in the experiments shown in Fig. 2 originates from motor and/or sensory nerve terminals in the muscle.

### *Calcium dependence of CGRP release*

In order to test the effects of external $Ca^{2+}$ concentrations on CGRP release, two soleus muscles were again exposed to 100 mM-$K^+$ solutions which contained different concentrations of $Ca^{2+}$ in a range of 0·1–2 mM. As shown in Fig. 3*A*, a reduction of external $Ca^{2+}$ resulted in a decrease of the amount of CGRP released by high-$K^+$ solutions. The relation between CGRP release and the external $Ca^{2+}$ concentration appears to be almost linear. When the amount of CGRP release and the $Ca^{2+}$ concentration were plotted on double logarithmic co-ordinates, the slope of the regression line was 0·30 between 0·1 and 0·5 mM-$Ca^{2+}$ and 0·75 between 0·5 and 2 mM-$Ca^{2+}$ (Fig. 3*B*). It is not certain whether this difference in the slope at different $Ca^{2+}$

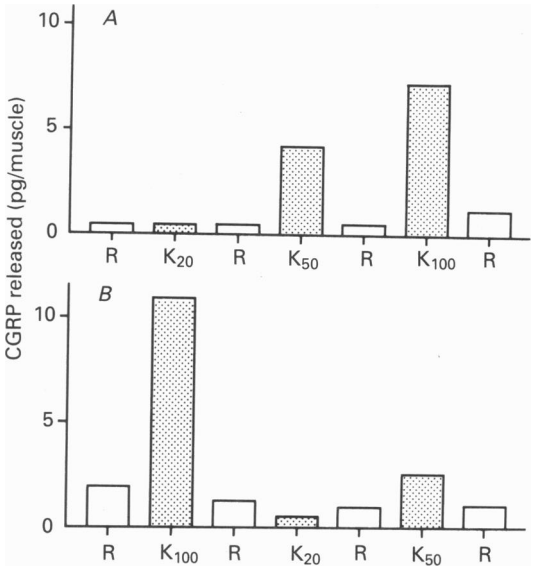


Fig. 1. The amounts of CGRP released from the soleus muscle in response to the exposure to solutions containing different concentrations of $K^+$ for 7 min. R, normal level of $K^+$ (4·5 mM). *A* and *B*, two different experiments. The test solutions were applied in sequence from the left to the right.

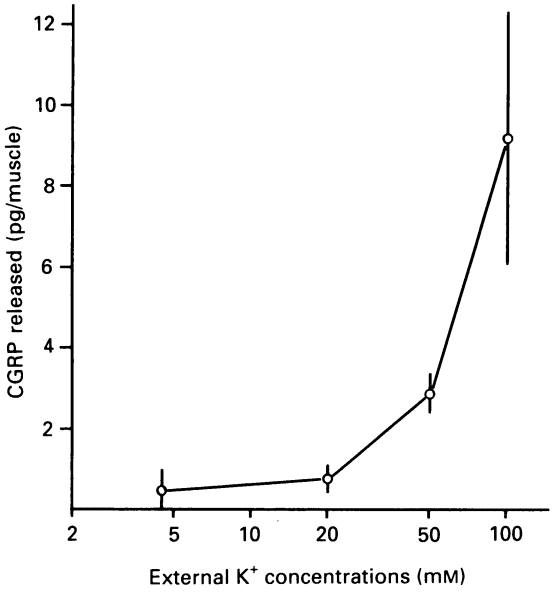


Fig. 2. The relation between the amount of CGRP released from the soleus muscle and the $K^+$ concentration in the solution to which the muscle was exposed for 7 min. In each experiment, the muscle was first exposed to a 4·5 mM-$K^+$ solution and then to one of the 20, 50 or 100 mM-$K^+$ solution. Each point is the mean of three experiments, except for the point at 4·5 mM which is the mean of nine observations. Vertical bars indicate $\pm$ S.D.

levels is genuine. However, the overall $Ca^{2+}$ dependence of CGRP release was considerably less steep than that reported for ACh release at neuromuscular junctions (see Discussion).

*CGRP release by stimulation of sensory fibres*

CGRP release from the soleus muscle by nerve impulses was first examined by stimulation of its muscle nerve. The soleus nerve was stimulated at 2 Hz for 6 min

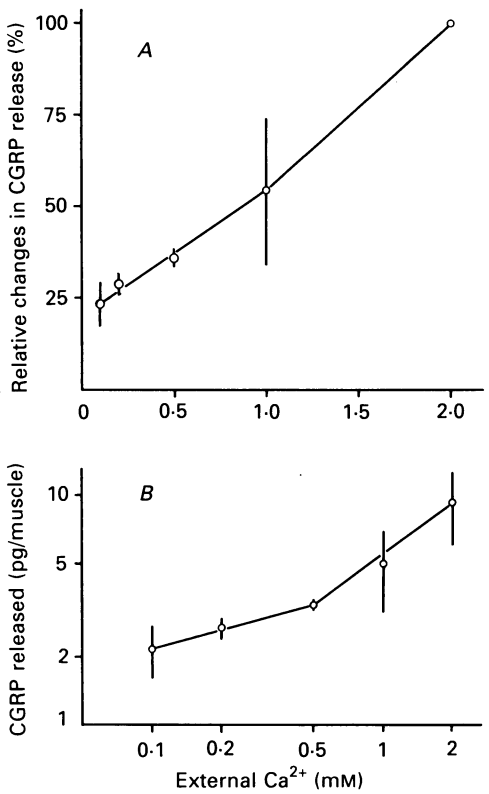


Fig. 3. Relations between the amount of CGRP released from the soleus muscle by 100 mM-$K^+$ and the external $Ca^{2+}$ concentration. *A*, changes in the amount of CGRP release relative to that observed at 2 mM-$Ca^{2+}$. *B*, same results plotted on double logarithmic co-ordinates. Each point is the mean of three experiments with S.D. (vertical bars).

in the standard Krebs solution. When the nerve was stimulated at an intensity three times the threshold for eliciting muscle contractions (the intensity sufficient to activate A$\alpha$-nerve fibres), there was no significant increase in the amount of CGRP collected from the bathing solution above the resting level. A significant increase in CGRP release was observed when the nerve was stimulated at an intensity 50–100 times the threshold for evoking muscle contractions (the intensity sufficient to activate A$\delta$- and C nerve fibres). This suggests that the major source of CGRP

released by stimulation of the peripheral nerve is high-threshold sensory fibres. In order to examine this possibility, CGRP release was measured in response to antidromic stimulation of the dorsal roots.

Figure 4 summarizes the results obtained by antidromic stimulation of the 4th and 5th lumbar dorsal roots. When the dorsal roots were stimulated at 2 Hz at an

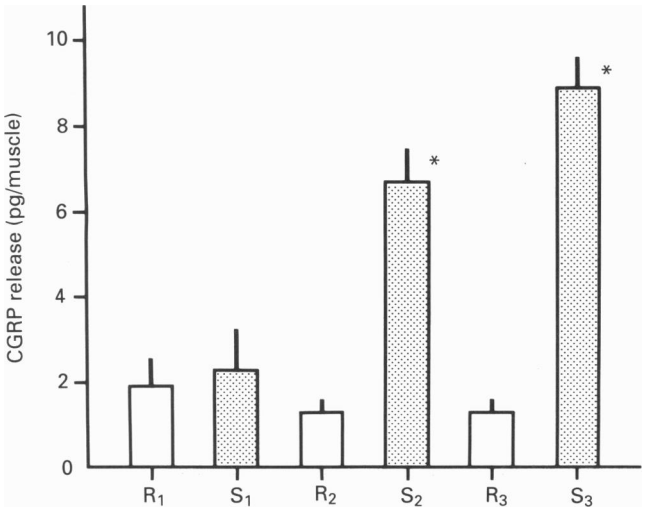


Fig. 4. The amounts of CGRP released from the soleus muscle in the standard Krebs solution with (stippled columns) or without (open columns) antidromic stimulation of the lumbar dorsal roots for 6 min. Each column is the mean of three observations with S.D. $S_1$, 2 Hz stimulation at an intensity three times the threshold for most excitable sensory fibres. $S_2$, 2 Hz stimulation at an intensity 50–100 times the threshold for most excitable sensory fibres. $S_3$, same as $S_2$, but at 10 Hz. In each experiment, the measurements were made in sequence from the left to the right. Asterisks indicate a significant difference from the amount of CGRP at rest, $R_1$.

intensity three times the threshold for most excitable sensory fibres (Fig. 4, $S_1$), the amount of CGRP collected from the bath ($2{\cdot}3 \pm 0{\cdot}93$ pg/muscle; $n = 3$) was not significantly different from those measured at rest before ($R_1$; $1{\cdot}9 \pm 0{\cdot}64$ pg/muscle) or after ($R_2$; $1{\cdot}3 \pm 0{\cdot}29$ pg/muscle) nerve stimulation. However, when the stimulus intensity was increased to 50–100 times the threshold for most excitable fibres, a significant increase in the amount of CGRP release ($S_2$; $6{\cdot}7 \pm 0{\cdot}75$ pg/muscle) was induced by stimulation of the dorsal roots at 2 Hz. The amount of CGRP release increased further when the dorsal roots were stimulated at 10 Hz with the same level of high intensity ($S_3$; $8{\cdot}9 \pm 0{\cdot}70$ pg/muscle). Thus, the sensory fibres responsible for CGRP release appear to be high-threshold muscle sensory fibres, probably A$\delta$- and/or C fibres.

*Lack of evidence for CGRP release by stimulation of motor fibres*

As described above, stimulation of the peripheral nerve at intensities sufficient to activate motor nerve (A$\alpha$-fibres) failed to increase CGRP release from the soleus muscle within the limit of detection by our assay. Stimulation of the 4th and 5th

lumbar ventral roots also did not increase the amount of CGRP released from the soleus muscle above the resting level. Thus, we could not obtain any evidence that CGRP can be released by stimulation of motor nerve fibres.

The contribution of motor fibres to CGRP release was also examined by comparing the amounts of CGRP released from the soleus muscle with and without degeneration

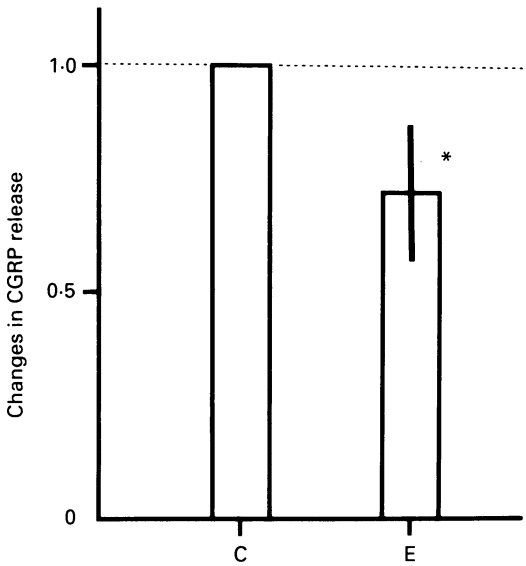


Fig. 5. Changes in the amount of CGRP released from the soleus muscle by 100 mM-$K^+$ following degeneration of motor nerve fibres. The 4th and 5th lumbar ventral roots had been sectioned, and the amounts of CGRP released from the soleus muscle were measured on the sectioned side (E) as well as on the contralateral, intact side (C) 4 days later. The amount of CGRP release on the sectioned side is expressed relative to that on the control side. The relative change on the experimental side is the mean of five experiments with S.D. Asterisk indicates a significant difference from the control value.

of the motor fibres. In order to minimize the variability in the amount of CGRP release from the soleus muscle in different animals, the 4th and 5th lumbar ventral roots were sectioned on one side, and 4 days later, CGRP release in the soleus muscle induced by 100 mM-$K^+$ was compared between the sectioned side and the contralateral, intact side. In five experiments, the mean amount of CGRP released from the intact soleus muscle ($10{\cdot}9 \pm 3{\cdot}2$ pg/muscle) was not significantly different ($0{\cdot}05 < P < 0{\cdot}10$) from that observed on the sectioned side ($7{\cdot}6 \pm 1{\cdot}9$ pg/muscle). However, the amount of CGRP measured was consistently lower on the sectioned side than on the paired, control side. As shown in Fig. 5, the mean ratio of the amounts of CGRP release between the two sides was $0{\cdot}72 \pm 0{\cdot}15$ (range, 0·59–0·90) which was significantly ($P < 0{\cdot}02$) different from unity. This 28% reduction in the amount of CGRP release induced by 100 mM-$K^+$ following chronic section of the ventral roots might correspond to the amount of CGRP released from motor terminals in the intact soleus muscle. Stimulation of A$\delta$- and C fibres at 2 Hz for 6 min increased CGRP release about 5·4 pg/muscle above the resting level, on

average (Fig. 4, $S_2$). Therefore, if 28% of the total CGRP released by nerve stimulation originated similarly from motor terminals, stimulation of the lumbar ventral roots would be expected to increase CGRP release by about 2·1 pg/muscle above the resting level, which should be detected by our assay. Thus, it appears that motor nerve terminals may be capable of releasing CGRP in response to prolonged depolarization induced with high $K^+$ but not to nerve stimulation (see Discussion). In the rat lumbar segments, the dorsal root ganglion and the ventral root are enclosed by a common connective sheath (Schmalbruch, 1986). Therefore, it is difficult to remove the dorsal root ganglia (deafferentation), leaving the ventral root fibres intact. Because of this difficulty, chronic deafferentation was not attempted.

*CGRP release from the diaphragm*

Recently, it has been reported that stimulation of the phrenic nerve induces the release of CGRP at neuromuscular junctions of the rat diaphragm (Uchida *et al.* 1990). Because of the uncertainty concerning CGRP release from motor nerve terminals in the soleus muscle, we reinvestigated the release of CGRP from the diaphragm. For this purpose, a 5 mm wide strip of the hemidiaphragm containing endplate regions was dissected along the nerve branch together with the phrenic nerve. The strip was placed in a bath filled with 1 ml of the standard Krebs solution. We confirmed that a significantly larger amount of CGRP ($6·4 \pm 1·2$ pg/strip; $n = 3$) than the resting level ($2·4 \pm 1·0$ pg/strip) is released by nerve stimulation (for 10 min at 10 Hz), *if the nerve is stimulated by electrical pulses with a duration of 10 ms* (5 V in intensity), as reported by Uchida *et al.* (1990). However, when the nerve was stimulated by pulses with a duration of 0·5 ms at intensities three to five times the threshold for evoking muscle contractions (for 10 min at 10 Hz), no significant amount of CGRP was released above the resting level ($n = 3$). Therefore, it seems clear that stimulation of the phrenic nerve at an intensity sufficient to activate the $\alpha$-motor fibres does not cause the release of CGRP from the muscle. It is then possible that stimulation of phrenic nerve with a long pulse duration (10 ms) might have produced current spread, thereby activating directly some neural tissues in the muscle strip.

In order to examine whether the site of CGRP release from the diaphragm is confined to the endplate region, a muscle strip isolated from the junctional region (endplate region) or from the non-junctional region was exposed to 100 mM-$K^+$ for 7 min. The mean amount of CGRP released in the non-junctional region ($0·11 \pm 0·018$ pg/mg tissue; $n = 3$) was found to be about 60% of that released in the junctional region ($0·18 \pm 0·028$ pg/mg tissue, $n = 3$). Similarly, the CGRP content in the non-junctional region was about 70% of that in the junctional region (Fig. 6*a*). These results indicate that the motor nerve terminals are not the only neural tissue which contains and releases CGRP in the diaphragm. Furthermore, 4 days after denervation of the diaphragm, 20–25% of the normal level of CGRP content was still found in the junctional and non-junctional regions (Fig. 6*b*, stippled columns). This is in contrast with the results from the soleus muscle in which 95% of CGRP in the muscle disappears after denervation (Kashihara *et al.* 1989). Since the diaphragm is lined with the parietal pleura on the thoracic surface and with the peritoneum on the abdominal surface, it is possible that these tissues may contain

nerve endings. To test this possibility, these membranes lining the diaphragm were carefully removed in the non-junctional region of the innervated diaphragm. This procedure reduced the CGRP content by more than 50% (Fig. 6*c*), compared with that of the sheathed diaphragm (Fig. 6*a*, non-junctional). It is likely that the

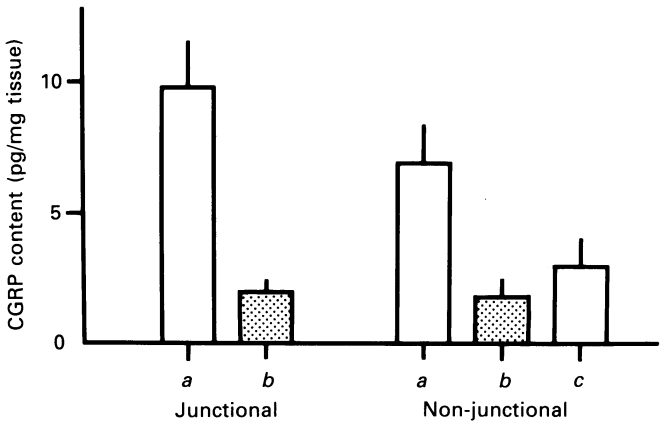


Fig. 6. CGRP contents in a strip of the diaphragm obtained from the junctional or non-junctional region: *a*, from the intact diaphragm; *b*, from the diaphragm which had been denervated 4 days previously (stippled columns); *c*, the same as in *a*, but from the normal diaphragm from which the pleura and peritonium were removed. Each columns represents the mean of three observations with S.D.

junctional region may also be lined with similar membranes which harbour CGRP-containing sensory nerve endings. The presence of a substantial amount of CGRP in the denervated diaphragm (Fig. 6*b*, stippled columns) suggests that sensory endings in the membranes lining the diaphragm may originate in part from non-phrenic nerves. The present results do not exclude the possibility of CGRP release from motor terminals in the diaphragm. However, at least, stimulation of the phrenic nerve at an intensity sufficient to produce vigorous contractions of the muscle did not cause the release of CGRP from the diaphragm. Stimulation of the phrenic nerve with a long pulse duration (and/or at extremely high intensities) might activate sensory nerve endings in the pleura or peritoneum covering the diaphragm. Therefore, CGRP release observed under these conditions may not necessarily be attributed to the release of CGRP from phrenic motor nerve terminals.

## DISCUSSION

The present study shows that CGRP can be released from skeletal muscle either by nerve stimulation or by membrane depolarization induced with high-$K^+$ solutions. The site responsible for CGRP release must be nerve terminals in muscle since the application of high-$K^+$ solutions does not increase CGRP release in chronically denervated muscles or in the muscle nerve alone (without the muscle).

The sensory neurones innervating skeletal muscle are known to show CGRP immunoreactivity (Molander, Ygge & Dalsgaard, 1987; Ohlen *et al.* 1987; O'Brien,

Woolf, Fitzgerald, Lindsay & Molander, 1989). In the present study, antidromic stimulation of the lumbar dorsal roots induced CGRP release from the soleus muscle. Judging from the intensity of effective nerve stimulation, the sensory fibres involved in CGRP release appear to be mainly A$\delta$- and/or C fibres. This is consistent with the observation that CGRP release in the dorsal horn of the cat spinal cord increases above the basal level in response to stimulation of high-threshold sensory fibres but not to stimulation of low-threshold sensory fibres (Morton & Hutchison, 1989). Also, CGRP immunoreactivity has been found to be associated largely with small myelinated and unmyelinated sensory fibres in the rat spinal cord (McNeill, Coggeshall & Carlton, 1988). However, McCarthy & Lawson (1990) have reported that 17% of the A$\alpha$-sensory neurones recorded (identified by their conduction velocities) show CGRP immunoreactivity. The relatively large standard deviation in the amount of CGRP released by antidromic stimulation of the dorsal roots at an intensity sufficient to activate only A$\alpha$-fibres (Fig. 4, $S_1$) might reflect the release of CGRP from a small population of A$\alpha$-sensory neurones.

CGRP is present within large dense-core vesicles in motor nerve terminals (Matteoli *et al.* 1988) as well as in synaptosomes isolated from the spinal cord (Fried, Franck, Brodin, Born, Fischer, Hiort & Hökfelt, 1989). Large dense-core vesicles have also been found in the peripheral ending of slowly conducting sensory fibres which respond to bradykinin (Kruger, Kumazawa, Mizumura, Sato & Yeh, 1988). When ACh and small clear vesicles had been depleted in frog nerve terminals by $\alpha$-latrotoxin isolated from the black widow spider venom, CGRP still remained in the nerve terminals (Matteoli *et al.* 1988). Therefore, the release of the content from small clear vesicles and the release from dense-core vesicles appear to depend on different mechanisms. It has also been shown that the stimulus frequency favourable for the release of neuropeptides differs from that optimum for the release of classical transmitters (Andersson, Bloom, Edwards & Järhult, 1982; Lundberg & Hökfelt, 1983; Agoston, Conlon & Whittaker, 1988). It is well known that the release of classical transmitters is triggered by $Ca^{2+}$ entering the nerve ending in association with terminal depolarization (Katz, 1969). In the dorsal horn of the spinal cord, the application of high $K^+$ fails to release CGRP in the absence of external $Ca^{2+}$ (Franco-Cereceda *et al.* 1987; also, see Mason, Peterfreund, Sawchenko, Corrigan, Rivier & Vale, 1984). However, from this observation alone, it is not certain whether the degree of $Ca^{2+}$ dependence of CGRP release is comparable to that of the classical transmitter. At neuromuscular junctions, evoked release of ACh occurs as a fourth-power functional of the external $Ca^{2+}$ concentration (Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968; Cull-Candy, Miledi, Trautmann & Uchitel, 1980). Transmitter release from Ia sensory neurones at central synapses on rat spinal motoneurones increases with a 1·6 power of the external $Ca^{2+}$ concentration (Kuno & Takahashi, 1986). The $Ca^{2+}$ dependence of CGRP release from nerve terminals in muscle (0·3–0·8 power) was substantially less steep than those reported for classical transmitters. Clear synaptic vesicles containing classical transmitters are accumulated in close proximity to active zones which harbour putative $Ca^{2+}$ channel particles (Pumplin, Reese & Llinás, 1981). In contrast, dense-core vesicles are dispersed widely within the terminal. Therefore, the concentration of $Ca^{2+}$ entering the terminal upon depolarization may considerably be diluted before reaching the

dense-core vesicles. This may account for the relatively low $Ca^{2+}$ dependence of CGRP release. The release of CGRP or other peptides containing in dense-core vesicles may be facilitated by accumulation of residual $Ca^{2+}$ in nerve terminals following repetitive nerve stimulation (see Lundberg & Hökfelt, 1983).

In spite of several trophic functions suggested for CGRP at neuromuscular junctions (see Introduction), the present study failed to show direct evidence for the release of CGRP from motor terminals by nerve impulses. Stimulation of a muscle nerve at an intensity sufficient to activate motor fibres did not increase the amount of CGRP release from the muscle above the resting level. Similarly, increased release of CGRP from the muscle was not detected following stimulation of the ventral roots. Also, the release of CGRP at neuromuscular junctions previously reported for the diaphragm (Uchida *et al.* 1990) could not be attributed to the release from motor nerve terminals. However, following degeneration of motor nerve fibres, the amount of CGRP release from the soleus muscle by 100 mM-$K^+$ was reduced by 28%, compared with that released from the normally innervated muscle. This suggests that motor nerve terminals may be capable of releasing CGRP upon prolonged depolarization. Two possible factors may be considered for uncertainty of CGRP release from motor nerve terminals. First, motor nerve terminals are considerably larger in size than varicosities formed by sensory endings. Thus, the intracellular concentration of $Ca^{2+}$ sufficient to induce CGRP release from motor nerve terminals may be achieved only by prolonged terminal depolarization (e.g. with high $K^+$) or by nerve stimulation at high frequencies. Second, motor fibres are considerably fewer in number than sensory fibres in a muscle nerve (Jenq, Hulsebosch, Coggeshall & Perez-Polo, 1984; Schmalbruch, 1986). Therefore, the amount of CGRP released from motor fibres might not significantly exceed the CGRP level at rest.

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