

THE FAST RESPONSE MEDIATED BY THE C3 MOTONEURONE OF *HELIX* IS NOT ATTRIBUTABLE TO THE CONTAINED FMRFamide

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Summary

The C3 neurone of *Helix aspersa* is known to induce contraction of the ipsilateral tentacle retractor muscle. It is immunoreactive for peptides of the FMRFamide series and immunoreactive FMRFamide nerve fibres occur in the muscle.

Here we found that FMRFamide-like immunoreactivity was released from the isolated muscle following depolarization with high-potassium saline and that such release was calcium-dependent. HPLC characterization of the immunoreactive material in the muscle showed that FMRFamide itself, rather than the other FMRFamide-related peptides known from *Helix*, accounted for most of the immunoreactivity. Parallel radioimmunoassay (RIA) also showed that FMRFamide predominates in the C3 neurone.

The nicotinic acetylcholine (ACh) receptor antagonists benzoquinonium (10^{-5} mol l⁻¹) and trimetaphan (5×10^{-5} mol l⁻¹) reversibly reduced tension evoked in the muscle by C3 stimulation. These drugs also blocked the muscle response to bath-applied ACh. Physostigmine (5×10^{-8} mol l⁻¹), an acetylcholinesterase antagonist, potentiated the C3-evoked muscle response. Depolarizing excitatory junction potentials recorded in the muscle cells in response to C3 neurone stimulation corresponded 1:1 with action potentials in the C3 neurone. Whereas locally applied ACh evoked a depolarizing response in the muscle fibres, similar to that evoked by C3 neurone activation, locally applied FMRFamide, even at very high doses, did not affect the membrane potential of the muscle cells. Also, exposure of the cerebral ganglia to high-Mg²⁺/low-Ca²⁺ saline did not block the C3 neurone-evoked muscle tension. Taken together, these findings indicate that the C3 neurone makes monosynaptic connections with the tentacle retractor muscle cells and that the contraction of the muscle it induces is due, at least in part, to the release of ACh at the neuromuscular junction. The role of the FMRFamide contained in the neurone is not yet clear.

Introduction

The C3 neurone, located in each cerebral ganglion of *Helix aspersa*, evokes

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contraction of the ipsilateral tentacle retractor muscle (TRM), and is known to react with antisera prepared against FMRFamide (Cottrell *et al.* 1983*b*). Of the five FMRFamide-related peptides which occur in *Helix*, the two tetrapeptides, FMRFamide and FLRFamide, usually cause contraction of the TRM, but the three heptapeptides of the form XDPFLRFamide, where X can be pyroglutamate (pQ), serine (S) or asparagine (N), primarily cause relaxation (Lehman and Greenberg, 1987; Cottrell *et al.* 1988). Some recent bioassay data suggest that the C3 neurone also contains and can synthesize ACh, another compound known to activate the muscle (G. P. Xu, G. S. Bewick and G. A. Cottrell, in press).

We have undertaken experiments designed to establish which of the FMRFamide peptides known to occur in *Helix* is found in the C3 neurone and whether it is released. We have also tested the hypothesis that the C3 neurone is cholinergic. The results provide clear evidence that this neurone contains FMRFamide itself and that FMRFamide is released within the muscle. However, as at least part of the response of the muscle to C3 neurone activation is due to the release of ACh, the precise role of the contained peptide is not yet clear.

Materials and methods

Determination of FMRFamide-related peptides

Radioimmunoassay (RIA)

Two different antisera for RIA were raised in rabbits to carbodiimide-mediated thyroglobulin-peptide conjugates. Antiserum Q2 was raised to a conjugate of pQDPFLRFamide, but boosted with a conjugate of DDPFLRFamide. This antiserum reacts better with those FMRFamide-related peptides containing leucine than with those containing methionine. Antiserum S253 was raised to YGGFMRFamide, and has been extensively characterized (Price, 1983; Lehman and Price, 1987). One trace, pQYPFLRFamide iodinated by the chloramine T method (see Price, 1982, for iodination method), was used for both RIAs. Use of this trace results in a somewhat more sensitive assay with S253 than that previously described using iodinated YGGFMRFamide. Otherwise the RIA protocols used were as previously described (Price, 1982; Lehman and Price, 1987).

Individual C3 neurones or tissue fragments (1–3 mg) were put into 100 μ l of acetone and stored at -20°C until assayed. Samples were transferred to RIA tubes and dried with a stream of air.

HPLC fractionations

Tissue fragments were extracted in acetone at -20°C overnight. The extract was decanted, filtered through a 0.45 μ m pore size Nylon syringe filter, and dried. The aqueous solution remaining was again filtered, and placed onto a NovaPak C18 column (Waters) and eluted with a gradient of acetonitrile from 16 to 32% in

water containing 0.1% trifluoroacetic acid (TFA) over 20 min. 0.5 min fractions (equivalent to 1 ml) were collected and samples taken for RIA.

FMRFamide release

A method similar to that devised for detecting FMRFamide release from *Helix* heart (W. Lesser, personal communication) was used. Three or four tentacle retractor muscles were tied to the end of a thin glass cannula through which *Helix* saline flowed at about $100 \mu\text{l min}^{-1}$, superfusing the muscles. Each drop of saline (about $40 \mu\text{l}$) was collected in a separate test tube which was used directly in the S253 RIA. Salines containing elevated levels of potassium (replacing some sodium), reduced levels of calcium (nominally zero calcium with equimolar substitution of magnesium for calcium), or both, were used to test for calcium-dependent, depolarization-induced release of FMRFamide-like immunoreactivity. The use of *Helix* saline instead of RIA buffer as the sample diluent reduced the sensitivity of the assay somewhat, but compensation for this was made by running the standard curve in *Helix* saline. Since the RIA data showed some variation, a sliding average was used to smooth the data points shown in Fig. 3. The first level shown is an average of the first three data points, and succeeding levels are produced by averaging the set of three data points produced by adding one new data point, and dropping the oldest.

Physiological experiments

The cerebral ganglia, tentacles and nerve connectives were isolated and pinned out in a three-chambered organ bath (Fig. 1) with a silastic rubber (Sylgard, Dow Corning) base.

The cerebral ganglia were placed in the central chamber and the tentacles placed one in each of the two lateral chambers. The nerve connectives passed through slots connecting the chambers and the slots were then sealed with petroleum jelly. A physiological solution was then placed in each chamber. The solution had the following composition (mmol l^{-1}): NaCl, 80; KCl, 5; CaCl_2 , 7; MgCl_2 , 5; HEPES, 20; pH 7.5. The C3 neurones were exposed using sharpened watch-makers' forceps and impaled with glass microelectrodes of 4–10 M Ω impedance, filled with 150 mmol l^{-1} KCl. Electrical activity in the C3 neurones was simultaneously displayed on an oscilloscope (Tektronix 5113) and one channel of a pen recorder (Gould, Brush 220). Tension evoked in the tentacle retractor muscle by C3 neurone stimulation was recorded by hooking a bent insect pin through the small portion of columellar muscle left attached to the distal insertion of the tentacle retractor muscle. The pin was connected to a tension transducer (Grass, FT03C) via a weak spring (3 g cm^{-1}), allowing contractions of the muscle to be recorded. Drugs were made up to the required concentration in 5 ml of physiological solution and the contents (2 ml) of the appropriate tentacle bath were completely exchanged with the test solution. All experiments were performed at room temperature. The following drugs were used: an anticholinesterase, physostigmine sulphate (BDH) and the acetylcholine receptor blockers

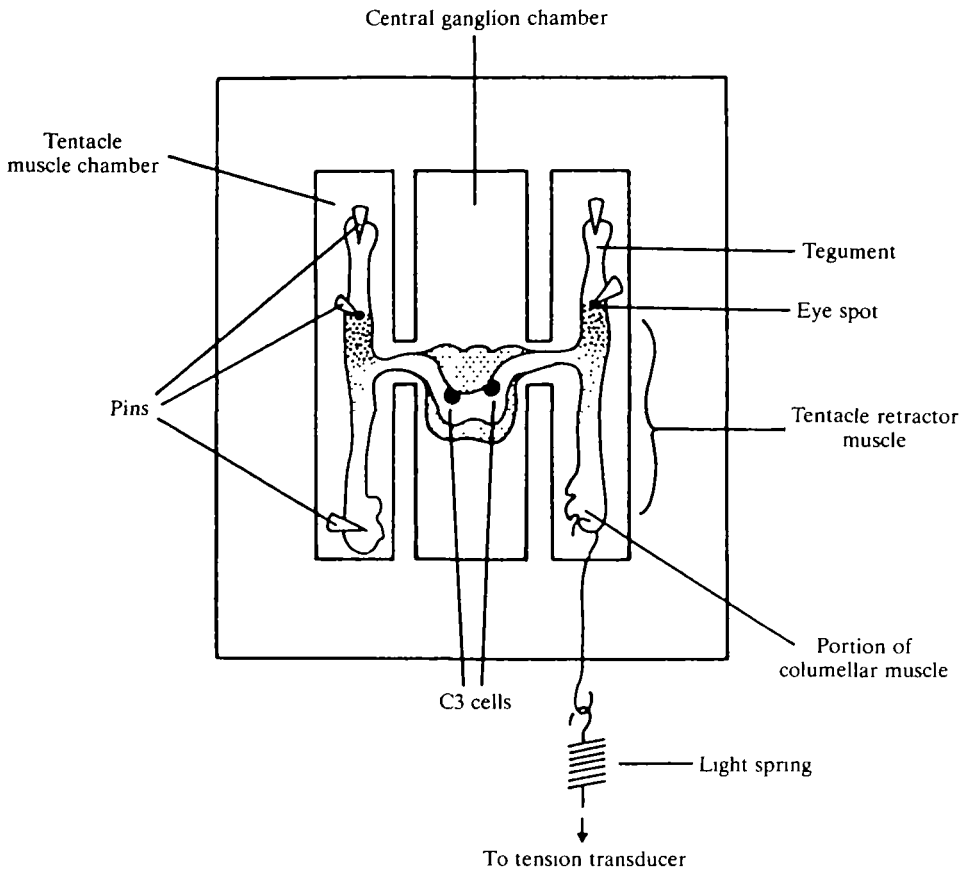


Fig. 1. The recording arrangement for measuring tension evoked in the tentacle retractor muscle. The ipsilateral C3 neurone was impaled with a microelectrode to record and elicit electrical activity. Petroleum jelly was injected into the slots between the chambers, sealing the central well from the two lateral wells and allowing application of different solutions to the separate chambers. Tension generated by the muscle was transmitted to the tension transducer *via* a fine insect pin formed into a hook and connected to a light spring. The black part of the muscle, indicated by the stipples, abutted the tegument. The hook was fastened to the white portion.

benzoquinonium (Stirling Winthrop), trimetaphan camsylate (Roche), α -bungarotoxin (Sigma) and Erabutoxin A (kindly supplied by Dr N. Tamiya). Intracellular recordings from tentacle retractor muscle cells were obtained using glass microelectrodes of 40–100 M Ω impedance filled with 3 mol l⁻¹ KCl. The output from the tension transducer (or muscle intracellular microelectrode) was displayed on the second channel of the pen recorder. The effects of locally applied ACh and FMRFamide were tested on the membrane potential of muscle fibres by iontophoresis from electrodes containing 10⁻³ mol l⁻¹ ACh or 10⁻² mol l⁻¹ FMRFamide. A separate micro-iontophoresis programmer (WPI, model 160) was used for each drug.

Results

Analysis of FMRFamide peptides

The potencies of several synthetic peptides in the Q2 and S253 assays are presented in Table 1. As can be seen, the S253 assay reacted almost equally well with most of the FMRFamide-related peptides known from *Helix*, the two exceptions being FLRFamide and oxidized FMRFamide, which were underestimated by four- to fivefold. Thus, the level of FMRFamide equivalents found by this assay approximates to the unweighted sum of all the FMRFamide-related peptides if oxidation is not extensive and if FLRFamide is, as usual in molluscs (Price *et al.* 1987), a minor component. In contrast, the Q2 assay detects primarily those peptides containing FLRFamide. Oxidized FMRFamide is for all practical purposes unreactive and even unoxidized FMRFamide is about 20-fold less reactive than the FLRFamide-containing analogues.

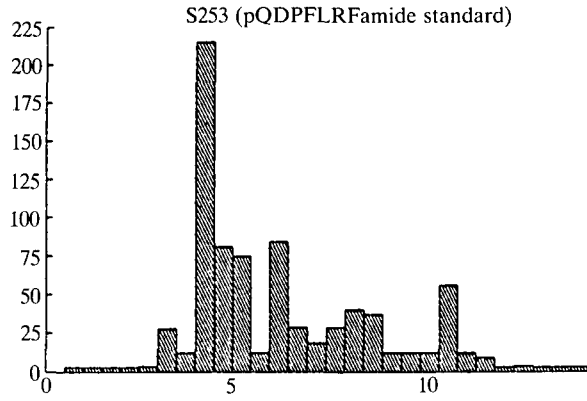
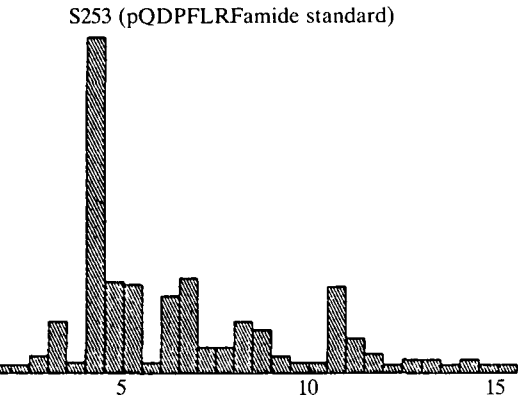
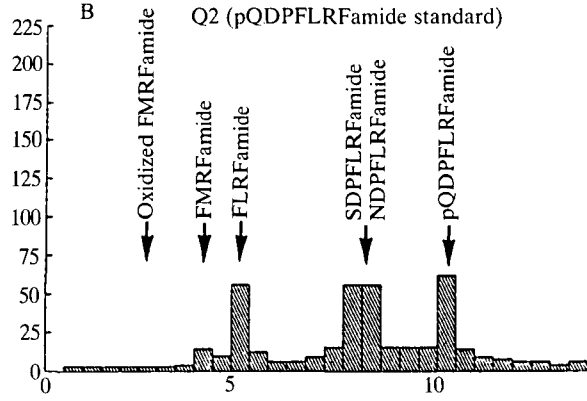
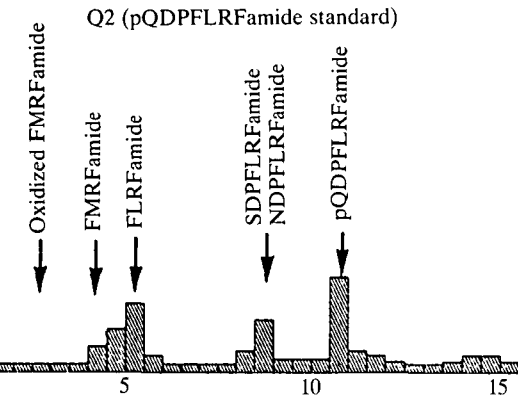
When the two RIAs are to be used in parallel, they must be standardized with the same peptide, and we have used both FMRFamide and pQDPFLRFamide as standards at different times. The values from the S253 assay are fairly independent of which standard is used, but the Q2 assay gives about 20-fold different values depending on the standard. When standardized with FMRFamide, the Q2 values will be too high unless only FMRFamide is present. When standardized with pQDPFLRFamide, the values will be too low unless no FMRFamide is present.

The FMRFamide-like peptides of the tentacle muscle and tegument were quantified with both RIA methods after HPLC separation. In our first experiment, the pigmented (black) and non-pigmented (white) regions of the tentacle muscle were run separately through the HPLC. The black and white portions of the tentacle muscle showed very similar HPLC patterns of immunoreactivity (Fig. 2A, B). FMRFamide itself was the predominant peptide of its family detected in both regions. However, some immunoreactivity was present at the expected elution positions of all the FMRFamide-related peptides known from *Helix*. Some of the FMRFamide was oxidized, as shown by the peak in the S253 assay of both muscle regions (Fig. 2A,B). Since the S253 antiserum only reacts about one-quarter as well with the oxidation product as with native FMRFamide, these peaks

Table 1. *Potencies of synthetic peptides in the two radioimmunoassays*

Peptide	IC ₅₀ (pmol tube ⁻¹)	
	S253	Q2
FMRFamide	0.05	1.5
Oxidised FMRFamide	0.25	15.0
FLRFamide	0.20	0.15
pQDPFLRFamide	0.04	0.05
NDPFLRFamide	0.06	0.10
SDPFLRFamide	0.08	0.12

IC₅₀, concentration required to produce 50% inhibition.



Time (min)

HPLC fractionation of tentacle retractor muscle extracts on a NovaPak C18 column (3.9 mm×150 mm). Samples were used to determine the immunoreactive FMRFamide content by RIA with two different antisera. Both RIAs were standardized with pQDPFLRFamide, and the immunoreactivity in each fraction is plotted. (A) Unpigmented (white) part of the muscle; (B) pigmented part of the muscle.

actually contain significant amounts of FMRFamide. Taking the oxidized peptide into account, we estimate that FMRFamide makes up about 90% of the total FMRFamide-related peptides in the tentacle retractor muscle.

Since the two portions of the tentacle retractor muscle did not differ significantly, they were pooled in a second experiment and compared with the tegument. Again FMRFamide accounted for most of the immunoreactivity in the tissues. The heptapeptides accounted for only about one-tenth of the total immunoreactivity in the muscles. However, in the tegument as much as one-third of the total immunoreactivity was due to heptapeptides.

Extracts of each of the tentacle regions were also tested directly in both RIAs. In one series of experiments, samples were taken for both assays from extracts of individual tissue pieces. Both assays were standardized with FMRFamide, and the levels of immunoreactivity given by the two assays were compared using a paired *t*-test (Table 2). (Since the measurements are paired values – one measurement with each assay – the paired *t*-test is a more sensitive test of interassay difference than a comparison of the means.) This test showed that the two RIAs gave significantly different values (at the 0.1% level) for all three of the tissues tested. The difference was minimal for the white part of the muscle; the means were not significantly different. However, the differences for the tegument and black part of the muscle were sufficiently great that even their means were significantly different. This finding indicates that FMRFamide itself must account for the vast majority of the immunoreactivity present in the muscle, since FLRFamide-containing analogues will contribute 20 times their true level to the immunoreactivity detected using the Q2 antiserum. The tegument, however, must contain significant amounts of FLRFamide-containing analogue in addition to FMRFamide.

Table 2. *Parallel RIA of FMRFamide-like immunoreactivity*

	Muscle extracts						Neuronal extracts	
	White part (pmol mg ⁻¹)		Black part (pmol mg ⁻¹)		Tegument (pmol mg ⁻¹)		C3 neurone (fmol cell ⁻¹)	
	S253	Q2	S253	Q2	S253	Q2	S253	Q2
	13.31	23.63	8.92	22.20	19.93	56.33	189.00	304.00
	24.25	26.90	11.68	22.30	7.55	45.02	145.00	199.00
	11.47	4.92	14.14	15.54	21.75	56.75	185.00	244.00
	11.25	8.38	11.94	20.51	10.29	35.92	165.00	119.00
	7.68	21.15	7.64	11.89	12.12	43.28	186.00	164.00
	14.63	17.27	25.79	36.47	22.00	66.86	151.00	202.00
	26.15	21.82	23.31	39.11	24.30	90.28		
Mean	15.53	17.72	14.77	24.00	16.85	56.35	170.17	205.33
S.E.	2.63	3.08	2.66	3.84	2.52	6.86	7.86	26.10
<i>t</i> -value	-1.90		-18.07		-11.32		-0.36	

The different parts of the tentacle used are as indicated in Fig. 1.

Thus, the results of measurement of unfractionated extracts confirmed the results of HPLC separation. This gave us confidence that the parallel RIA method was basically sound. Experiments had previously been made using the S253 antiserum in parallel with the W2 antiserum of Weber *et al.* (1981) as a parallel RIA. Though these two antisera differ somewhat in their reactivity with the FMRFamide-related peptides of *Helix*, they did not give reliable results with tissue extracts because the extent of oxidation was variable (D. A. Price, unpublished observations).

For six C3 neurones, we determined the level of immunoreactive FMRFamide in both RIAs using FMRFamide as the standard (Table 2). There was no significant (at the 0.1% level) interassay difference. This is the expected result if FMRFamide was the only immunoreactive peptide in the neuronal extracts.

FMRFamide-like immunoreactivity was found in the superfusate of the tentacle retractor muscles (Fig. 3). The rate of release was greatly increased by a 10-fold elevation of the potassium concentration. However, when calcium was omitted from the saline, a 10-fold increase in potassium concentration only increased release slightly. These results indicate that the release is depolarization-sensitive and calcium-dependent.

Response of the muscle to activation of the C3 neurone

Stimulation of the C3 neurone causes the tentacle retractor muscle to contract to

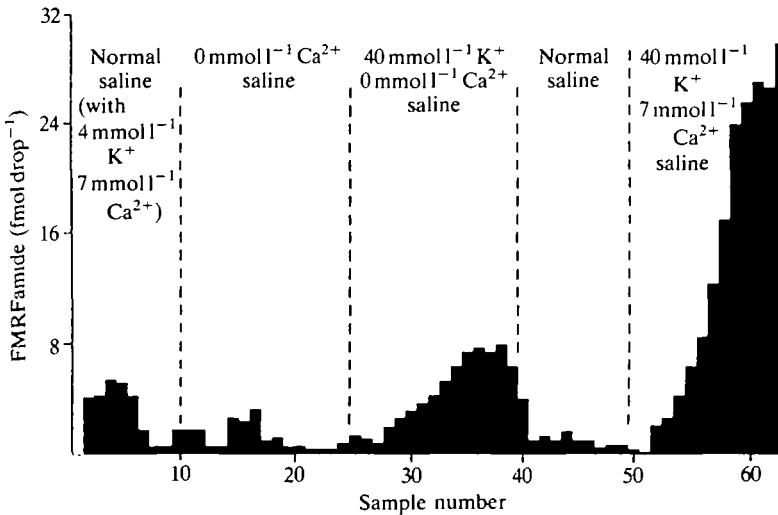


Fig. 3. Release of immunoreactive FMRFamide from the tentacle retractor muscle (TRM). Four TRMs were superfused with saline at a rate of about 4 drops min^{-1} . After washing for 2 min, each drop was collected in an RIA tube. The saline was switched to one with nominally zero calcium (calcium replaced with magnesium, no chelators) and then to one with no calcium, but with elevated potassium. It was then switched back to normal saline before the flow was switched to a saline with 10 times the normal potassium level. A sliding average of the immunoreactivity (fmol drop^{-1}) of three drops is plotted against drop number.

an extent that is dependent upon both the frequency of stimulation and the number of stimuli applied (Cottrell *et al.* 1983*b*). The relationship between stimulation frequency and the maximum tension produced at that frequency was investigated more fully in the present experiments. Impulse activity in the C3 neurone at frequencies above 2 Hz resulted in a smooth contraction of the muscle. The tentacle retractor muscle neither twitched in response to single impulses nor produced any tension in response to repetitive stimulation at frequencies less than 1 Hz.

The relationship between stimulation frequency and tension production between 2 and 10 Hz was steep, with a greater contraction being elicited at higher stimulation frequencies. The maximum muscle tension was usually produced between 7.5 and 15 Hz. At higher stimulation frequencies, the tension evoked often decreased slightly, but between 20 and 50 Hz this slightly sub-maximal tension was well maintained, suggesting the decrease in tension was not due to an inability of the neurone to follow the stimulation at high frequencies.

Effect of ACh receptor antagonists on tentacle muscle tensions

Benzoquinonium

The response of the muscle to ACh appeared to be mediated by a 'nicotinic-type' receptor, but the receptor properties did not parallel those of the vertebrate neuromuscular junction (see Colquhoun *et al.* 1987). Of the antagonists effective at the vertebrate neuromuscular junction, benzoquinonium, a synthetic nicotinic antagonist (Hoppe, 1951), was the most effective in antagonizing the response of the muscle to C3 neurone activation. The threshold for antagonism was in the low micromolar range; a 10^{-5} mol l⁻¹ solution markedly suppressed the response in six of seven preparations. The results of one such experiment are shown in Fig. 5.

The response of the muscle to 50 stimuli at 3 Hz was totally abolished in the presence of 10^{-5} mol l⁻¹ benzoquinonium in the muscle chamber. This blockade was partially overcome at higher frequencies of stimulation, as expected for a competitive antagonist. The preparation recovered on washing. Unexpectedly, higher concentrations of the drug in the tentacle muscle chamber markedly increased the excitability of the C3 neurone, making control of the electrical activity of the cell very difficult. Consequently, it was not possible to obtain experimental results for concentrations of benzoquinonium higher than 10^{-5} mol l⁻¹.

Fig. 5B summarizes the results of application of 10^{-5} mol l⁻¹ benzoquinonium to the muscle on C3-cell-evoked tension at a range of stimulation frequencies. In this preparation, suppression of tension production at C3 stimulation frequencies of 2 and 3 Hz was complete. Higher frequencies of stimulation always partially reversed the blockade, suggesting some competitive interaction of the released transmitter with benzoquinonium at the synaptic junctions. Benzoquinonium at

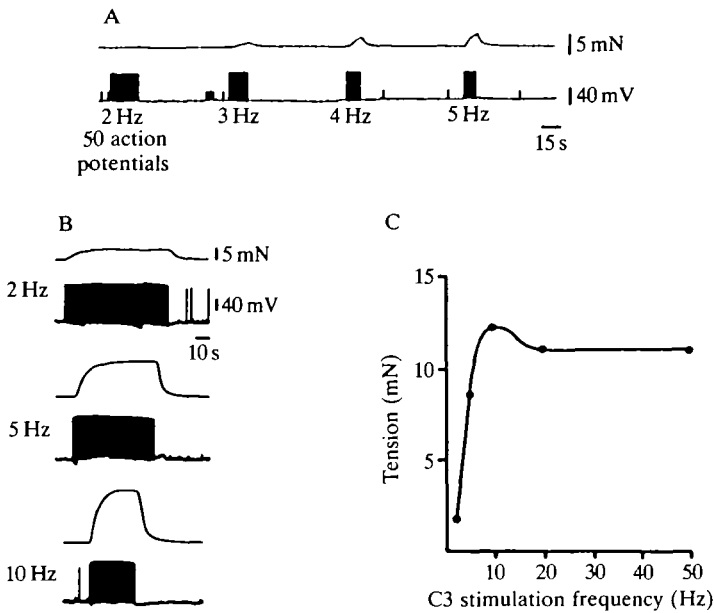


Fig. 4. Tension–frequency relationship for C3-stimulation-evoked responses of the tentacle retractor muscle (TRM). (A) A fixed number of stimuli in the C3 neurone (lower trace) elicits progressively greater rates of tension production in the tentacle muscle (upper trace) with increasing impulse frequency. The peak tension also increased with higher impulse frequencies. (B) When the C3 neurone was stimulated at a constant frequency, the TRM contracted until it reached a maximum tension, which was maintained until stimulation ceased. The maximum tension achieved increased with increasing impulse frequency, up to approximately 10 Hz. At higher stimulation frequencies, the plateau tension produced was either equal to or slightly less than that at 10 Hz. (C) The relationship between plateau tension and C3 impulse frequency in a representative preparation.

$10^{-5} \text{ mol l}^{-1}$ also inhibited the tension elicited by the direct addition of ACh to the tentacle muscle, as shown in Fig. 5C. This effect was also reversed with washing.

Trimetaphan

Trimetaphan, an antagonist of nicotinic receptors in vertebrate ganglia, was also effective in suppressing tension production in the tentacle retractor muscle. Concentrations of trimetaphan as low as $10^{-7} \text{ mol l}^{-1}$ reduced tension production considerably in response to low-frequency stimulation of C3 in some preparations; more usually, however, a concentration of 10^{-5} – $10^{-4} \text{ mol l}^{-1}$ was required (Fig. 6A). Trimetaphan also inhibited tension production in response to direct ACh application, as shown in Fig. 6B.

α -Bungarotoxin and Erabutoxin A

Neither α -bungarotoxin ($10^{-6} \text{ mol l}^{-1}$) nor Erabutoxin A ($3 \times 10^{-5} \text{ mol l}^{-1}$)

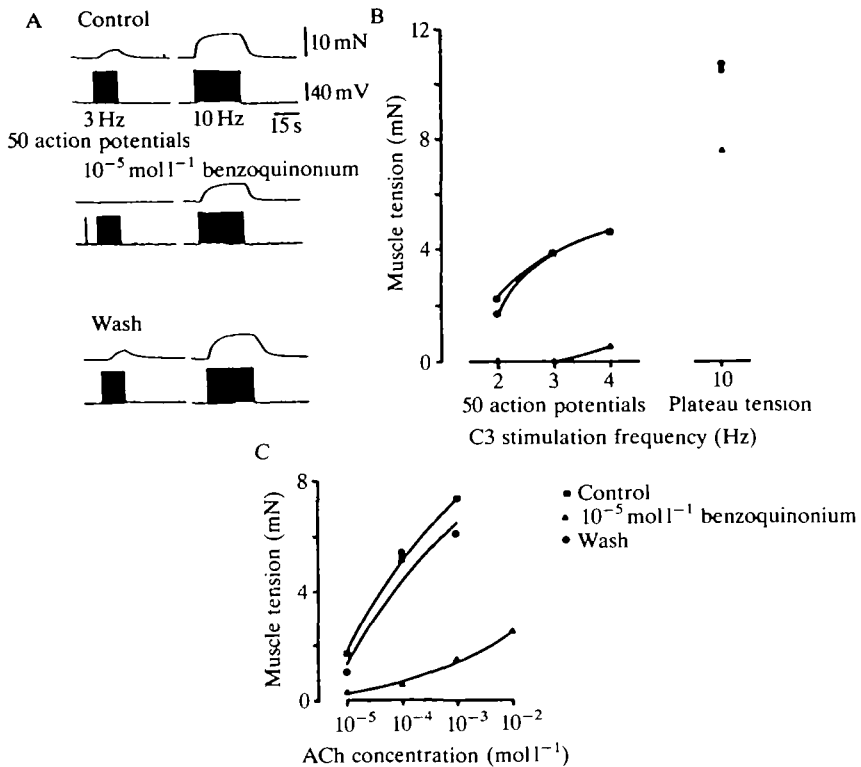


Fig. 5. The effect of application of $10^{-5} \text{ mol l}^{-1}$ benzoquinonium to the tentacle retractor muscle on the tension evoked by C3 neurone stimulation. (A) At low impulse frequencies (3 Hz) the contractile response was totally abolished in the presence of the drug. At 10 Hz, however, the preparation produced 70% of its control tension, partially overcoming the inhibition. This effect was reversed with washing. (B) The effect of benzoquinonium on the tension responses of the tentacle muscle to stimulation of the C3 neurone at different frequencies. In this preparation, tension evoked in response to 50 stimuli at 2 and 3 Hz was completely suppressed in the presence of $10^{-5} \text{ mol l}^{-1}$ benzoquinonium. The tension generated at 4 Hz was reduced to only 14% of the control tension. For the maximum attainable tension, at higher frequencies, the inhibition of tension was less. In this case, at a frequency of 10 Hz the response in the presence of the drug was only reduced to 74% of the control value. (C) The response of the tentacle retractor muscle to bath application of acetylcholine in the presence and absence of benzoquinonium. There was a very marked reduction in the tension generated by the muscle in the presence of $10^{-5} \text{ mol l}^{-1}$ benzoquinonium. Symbols in part B are the same as in C.

inhibited the response to C3 activation or the response to ACh application, even after prolonged exposure (40–120 min). These results, together with those for trimetaphan, suggest that the ACh receptors in the muscle are more similar to the nicotinic receptors of vertebrate autonomic ganglia than to those of the vertebrate neuromuscular junction (Colquhoun *et al.* 1987).

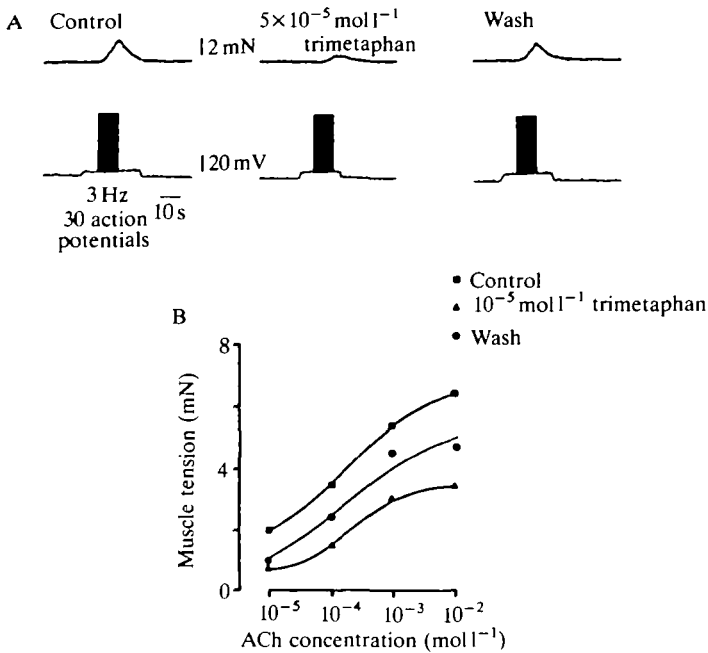


Fig. 6. (A) The addition of $5 \times 10^{-5} \text{ mol l}^{-1}$ trimetaphan camsylate to the muscle chamber resulted in a reduction of the tension produced in response to C3 stimulation. This C3 neurone tended to fire action potentials spontaneously and was therefore kept hyperpolarized until just prior to stimulation. The suppression of tension produced by the drug was reversed upon washing with fresh physiological saline. (B) The effect of $10^{-5} \text{ mol l}^{-1}$ trimetaphan on the dose-response curve of the tentacle retractor muscle after ACh application of acetylcholine. Trimetaphan reversibly reduced the contractile responses of the tentacle muscle at all the acetylcholine concentrations tested.

Effect of an acetylcholinesterase antagonist on tentacle muscle tension

Physostigmine (eserine), an acetylcholinesterase inhibitor, produced an enhancement of the tension evoked by C3 neurone stimulation. In four of the five preparations tested, a reversible increase in tentacle muscle tension was produced (Fig. 7). The threshold dose for such a response was approximately $10^{-8} \text{ mol l}^{-1}$. Such effective potentiation of the response to C3 activation with low concentrations of an anticholinesterase is strong evidence for the involvement of ACh in transmission.

The results of the pharmacological experiments are summarized in Table 3.

Reduction of synaptic transmission in the cerebral ganglia does not block the muscle response

Axons from the C3 neurone pass along the ipsilateral tentacle nerve and FMRFamide-immunoreactive processes are present among the tentacle muscle cells (Cottrell *et al.* 1983b). However, it has not been determined whether the C3 neurone makes direct synaptic connections with tentacle muscle cells.

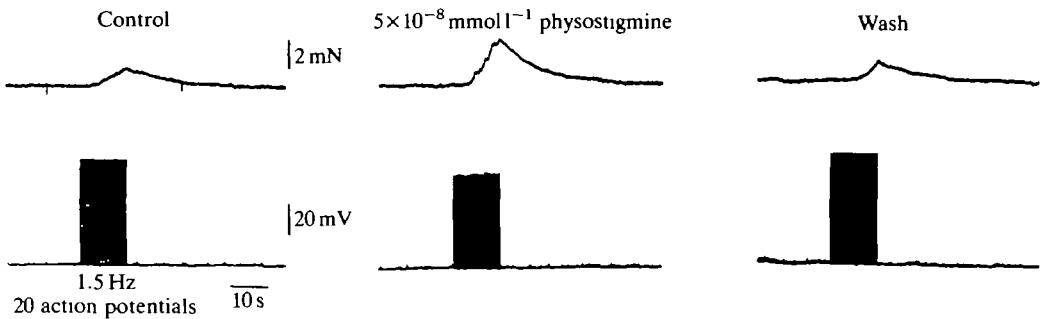


Fig. 7. The acetylcholinesterase inhibitor physostigmine potentiated C3-evoked tension in the tentacle retractor muscle. Physostigmine at $5 \times 10^{-8} \text{ mol l}^{-1}$ doubled the tension evoked by low-frequency stimulation.

To test this possibility, the cerebral ganglia were exposed to a physiological solution containing $15 \text{ mmol l}^{-1} \text{ Mg}^{2+}$ and $2 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ in the central ganglion chamber. This solution blocked synaptic activity in the ganglia. The outer tentacle chambers with the tentacle muscles contained normal physiological solution. When C3 neurones were impaled under these conditions, the synaptic activity recorded in the cell body was at a very low level compared to normal. However, the tension evoked in the tentacle muscle by stimulation of the C3 neurone was of normal amplitude (Fig. 8A). On changing the ganglion bath solution to normal *Helix* saline, synaptic activity in the cell body increased markedly, indicating that previously blocked synapses were now functional, but tension production was not increased (Fig. 8B). The C3 neurone, therefore, does not need to synapse with other neurones in the cerebral ganglion in order to elicit tension production in the muscle.

Intracellular recording from the tentacle retractor muscle cells during C3 stimulation

Intracellular recordings were made from the tentacle retractor muscle fibres while stimulating the C3 neurone. Excitatory junction potentials (EJPs) were found to occur, with a latency of about 80 ms (Fig. 9). Although the latency for the EJPs appeared to be constant at any one recording site, the EJPs were not of a

Table 3. Results of pharmacological experiments

Drug	Effect			Number of preparations	Doses tested (mol l^{-1})
	Increase	Decrease	None		
Benzoquinonium	1	6	0	7	10^{-8} – 10^{-4}
Trimetaphan	0	4	0	4	10^{-7} – 10^{-4}
Erabutoxin A	0	0	2	2	10^{-9} – 3.5×10^{-5}
α -Bungarotoxin	0	0	1	1	10^{-6}
Physostigmine	4	1	0	5	10^{-8} – 10^{-6}

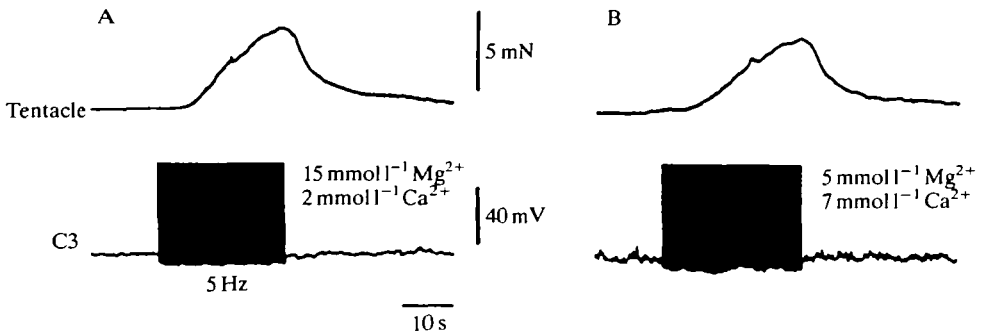


Fig. 8. The presence of high- Mg^{2+} /low- Ca^{2+} saline in the cerebral ganglia chamber was without effect on the tension produced by the tentacle retractor muscle in response to C3 neurone stimulation. (A) The lower trace shows a very low level of synaptic activity recorded in the C3 cell bathed in $15 \text{ mmol l}^{-1} Mg^{2+}$ / $2 \text{ mmol l}^{-1} Ca^{2+}$. The solid rectangular area indicates when the cell was stimulated. As can be seen in the upper trace, the tentacle muscle still contracted under these conditions. (B) On replacing the high- Mg^{2+} /low- Ca^{2+} saline with normal *Helix* saline, there was a marked increase in synaptic activity (increase in baseline noise) but the tentacle muscle contraction in response to C3 neurone stimulation was not increased.

consistent size or shape when recordings were made from different muscle fibres. Some (Fig. 9A) had very sharp rising phases and were of large amplitude, suggesting that the recording electrode was very close to synaptic junctions. Others, however, were of a much smaller amplitude and slower rise time, indicating that in these cases the junction was at some distance from the recording electrode, perhaps even in another, electrically coupled, fibre. It is also possible, however, that the slower events represent a second class of synaptic response. Mixtures of the responses were also observed in some instances; see, for example, Fig. 9B, where a slow EJP was triggered by C3 neurone activation and a 'spontaneous' fast EJP occurred, possibly due to the firing of another (unidentified) motoneurone during the second C3-neurone-evoked EJP. Repetitive stimulation of the C3 neurone produced EJPs in the tentacle muscle cells on a 1:1 basis (Fig. 9C).

Facilitation occurred at the neuromuscular junction. This was investigated further (Fig. 9D). A fixed number of stimuli was applied to the C3 neurone and the response to increasing frequencies was recorded. At a stimulation frequency of 1 Hz, 20 action potentials in the C3 neurone induced a depolarization of only 0.5 mV in the muscle cells whereas, at 5 Hz, the same number of stimuli resulted in a depolarization of 3.0–3.5 mV. The amplitude of the EJPs was also increased with increasing frequency, ultimately resulting in muscle cell action potentials at stimulation frequencies above 3 Hz. Thus, facilitation and potentiation of the synaptic transmission occurred in response to repetitive stimulation.

Ionophoresis of acetylcholine and FMRFamide

Representative responses of the muscle cells to ionophoresed ACh and to

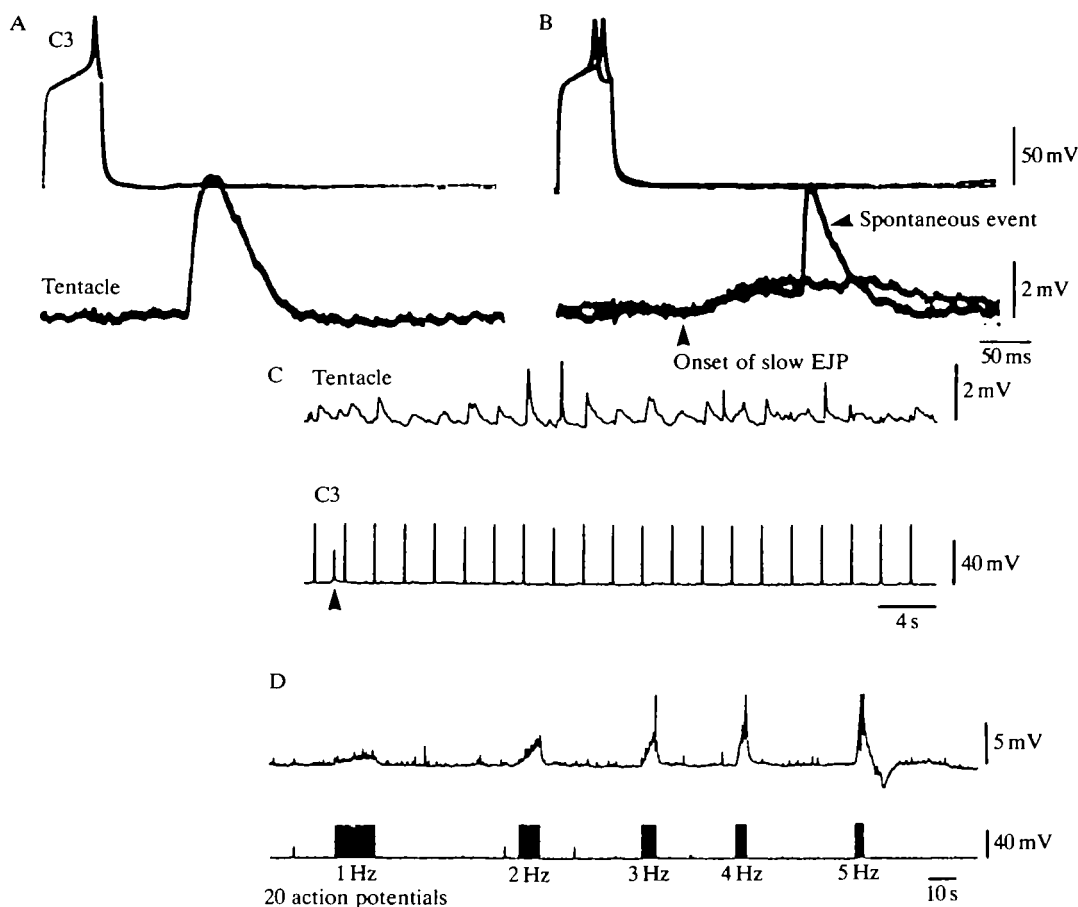


Fig. 9. Intracellular recording of excitatory junction potentials (EJPs) in the tentacle retractor muscle during C3 neurone stimulation. (A) The upper trace shows the C3 neurone being depolarized by a square pulse of depolarizing current, which results in a single action potential. In the lower trace, after a latency of some 80 ms, a single fast rise-time EJP was recorded. (B) Example of other responses. Two sweeps are shown superimposed. An impulse in the C3 neurone resulted in a slow EJP, possibly because the junction was at some distance from the recording electrode. During the second evoked EJP a faster EJP was observed, possibly originating from 'spontaneous' activity of another unidentified neurone. The latencies of the slow EJPs were both around 80 ms. (C) A 1:1 relationship was observed between C3 neurone action potentials and the EJPs recorded in the tentacle muscle. The C3 neurone was stimulated at 1.5 Hz (lower trace). The resulting EJPs are seen in the upper trace. C3 neurone impulses always resulted in an EJP, even when an impulse arose without stimulation (filled arrowhead). (D) Facilitation and potentiation of the electrical response to C3 stimulation. In the lower record, bursts of 20 impulses were evoked in the C3 neurone at progressively higher frequencies. Both the EJP amplitude and the underlying depolarization increased with increasing frequency, resulting in muscle cell action potentials at higher frequencies of stimulation. The downward deflection of the muscle trace following stimulation at 5 Hz was probably an electrical artefact due to contraction of the muscle.

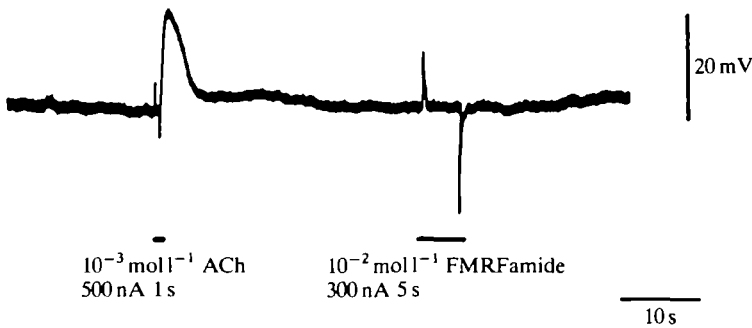


Fig. 10. A 1 s ionophoretic pulse of acetylcholine applied to an impaled tentacle muscle cell caused a large rapid depolarization. A 5 s pulse of $10^{-2} \text{ mol l}^{-1}$ FMRFamide to the same area of membrane, however, had no effect on the resting membrane potential. A similar effect of acetylcholine and lack of effect of FMRFamide were recorded from several muscle cells.

FMRFamide are shown in Fig. 10. A brief pulse of ACh caused a large and rapid depolarization. However, despite the higher mechanical sensitivity of the entire muscle to FMRFamide (Cottrell *et al.* 1983*b*), no effect was observed on the resting membrane potential even at a pipette concentration of $10^{-2} \text{ mol l}^{-1}$ FMRFamide. Thus, it seems likely that the evoked EJPs recorded in tentacle muscle cells following C3 neurone activation are primarily the result of the release of ACh, and not FMRFamide, from the nerve terminals.

Discussion

Earlier work has suggested that the C3 neurone is a motoneurone and has established that it contains one or more of the FMRFamide series of peptides (Cottrell *et al.* 1983*b*). Though FMRFamide has a contractile effect on the tentacle retractor muscle, some other FMRFamide-like peptides cause relaxation. Of the five FMRFamide-like peptides known from *Helix*, the three heptapeptides pQDPFLRFamide, NDPFLRFamide and SDPFLRFamide are the most potent relaxing agents (Lehman and Greenberg, 1987; Cottrell *et al.* 1988). FLRFamide, like FMRFamide, has little relaxing ability, but causes contractions (Cottrell *et al.* 1988). Therefore, we wished to determine which FMRFamide-related peptides were present in the C3 neurone and the tentacle retractor muscle.

Analysis of fractions of tentacle muscle using HPLC showed that FMRFamide itself is the major FMRFamide-related peptide. Simple parallel RIA led to the same conclusion. The usefulness of this simple parallel RIA method depends on both the selectivity of the antisera and the relative tissue levels of the various peptides.

Functionally, the pulmonate FMRFamide-like peptides seem to fall into two classes: the tetrapeptides and the heptapeptides. Price *et al.* (1987) have argued

that the tetrapeptides and heptapeptides also have distinct precursors. If this is the case, individual neurones would probably contain either tetrapeptides or heptapeptides, but not both. Therefore, we would like our parallel RIA to distinguish between the class of heptapeptides and the class of tetrapeptides. Based on its reactivity with synthetic peptides, our parallel RIA distinguishes mainly between the class with leucine between the phenylalanine and arginine residues and the class with methionine in this position. Yet since FLRFamide always seems to be a relatively minor component of the FMRFamide immunoreactivity (see Price *et al.* 1987), our parallel RIA, in practice, does make the desired distinction, and we can use it to determine which class of peptides is contained in the C3 neurone.

Parallel RIA analysis of extracts of the C3 neurone indicates that primarily FMRFamide is present. The Q2 antiserum value was slightly, though not significantly, higher than the S253 antiserum value, suggesting that the cell could have a very small amount of an FLRFamide-containing analogue. The ratios between the Q2 and S253 values were very similar for the neurone and the white part of the TRM, indicating similar peptide compositions. The same assays as those run on the C3 neurones, when run on identified groups of neurones in the visceral and right parietal ganglia (Cottrell *et al.* 1988), gave much higher values with the Q2 than the S253 antiserum (D. A. Price and G. A. Cottrell, unpublished observations), indicating high levels of the heptapeptides relative to FMRFamide.

The release of FMRFamide-like immunoreactivity from tissues of *Helix* can be measured fairly easily. Since FMRFamide is rapidly destroyed by blood (Lehman and Price, 1987) and tissue (Payza, 1987), we have tried to minimize its contact with them. Blood can be eliminated from the tissue by washing with saline before starting to collect samples, and tissue contact can be minimized by perfusing or superfusing the tissue. If these precautions are taken, then no protease inhibitors are needed. The release of immunoreactivity is dramatically increased by high-potassium depolarization (Fig. 3) and this increase is calcium-dependent. Thus, FMRFamide appears to meet the presynaptic requirements for an extracellular messenger.

The results of the physiological experiments support the view that the C3 neurone is a motoneurone. It innervates the tentacle retractor muscle directly, eliciting EJPs with a constant latency on a 1:1 basis with the action potentials generated in the perikaryon. Contraction of the tentacle muscle was not blocked by bathing the cerebral ganglia in high-Mg²⁺/low-Ca²⁺ saline, which suggests that central connections of the C3 neurone with other neurones are not necessary for the muscle response. Observations of dye-filled preparations (Cottrell *et al.* 1983b) have allowed processes of the neurone to be traced along the tentacle nerve and into the muscle mass. The C3 motoneurone makes direct synaptic contact with the tentacle retractor muscle cells.

The results from these experiments also suggest that the ACh is released as a neurotransmitter at the nerve-muscle junction. The tentacle muscle contracted in response to directly applied ACh and the responses were blocked by benzoquinonium, a nicotinic antagonist, and trimetaphan, a ganglionic nicotinic antagonist.

These same antagonists also blocked the C3 stimulation-evoked muscle contraction. In addition, the C3-evoked contractions were enhanced by physostigmine, an acetylcholinesterase antagonist. Ionophoretic application of ACh and FMRFamide showed that only ACh evoked a depolarization in the muscle cells, as seen with C3 neurone activation. These results, together with the experimental results which indicate that the C3 motoneurone also contains ACh and the enzyme for its synthesis, choline acetyltransferase (see Xu *et al.* 1989), strongly suggest that ACh is the neurotransmitter responsible for eliciting the EJPs and also at least part of the smooth contraction in the tentacle retractor muscle when the C3 motoneurone is stimulated.

The effect of C3 neurone activation could not be completely blocked with either trimetaphan or benzoquinonium. This could be taken to imply that some other factor, such as FMRFamide, also plays a part in the total mechanical response, particularly perhaps with high-frequency stimulation. It is also possible, however, that the peptide plays some other, as yet undefined, role. In this regard, it should be noted that neither trimetaphan nor benzoquinonium completely blocked the effect of applied ACh on the muscle. Blockade with trimetaphan and the lack of effect of α -bungarotoxin (and also Erabutoxin A) suggest that the ACh receptor involved is more like that of vertebrate autonomic ganglia than that of the vertebrate neuromuscular junction.

What, then, is the role of the FMRFamide? FMRFamide-immunoreactive neuronal processes are observed in the TRM (Cottrell *et al.* 1983*b*). It is not clear what proportion of these fibres originates from the C3 neurones, but it has been established that the varicose endings of fine terminal branches of C3 neurones maintained in culture do possess FMRFamide immunoreactivity (B. Powell and G. A. Cottrell, unpublished observations). FMRFamide is released within the muscle in a calcium-dependent way in response to depolarizing concentrations of potassium. Further, FMRFamide at concentrations of 10^{-9} mol l⁻¹ and above stimulates the TRM but, unlike ACh, it evokes rhythmic contractions and there is a marked delay in the onset of the response after adding the peptide (Cottrell *et al.* 1983*a*). All these observations suggest an important role for FMRFamide, in addition to ACh, at the neuromuscular junction, but this role is at present unclear.

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