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AN INITIAL SCREEN OF A SERIES OF NEUROACTIVE PEPTIDES FOR ACTIVITY ON IDENTIFIED CENTRAL NEURONES OF *HELIX ASPERSA*

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Abstract—1. Intracellular recordings were made from identified neurones in the suboesophageal ganglia of *Helix aspersa*. Seven neuropeptides were tested for activity and their actions compared with acetylcholine and FMRFamide.

2. Three peptides isolated from nematodes, AF-1, AF-2 and PAN-1 had mainly inhibitory effects with thresholds of around 1 nM. This inhibition was due to an increase in potassium conductance.

3. The molluscan neuropeptides LSSFVRIamide, CARP and ACEP-1 were all active on certain neurones; the first two showed only inhibitory effects while ACEP-1 was mainly excitatory. The thresholds in each case were 0.1–10 μM. When norleucine replaced methionine in CARP, the potency was reduced by at least 100 times.

4. The echinoderm peptide, SALMF-1, only excited neurones but with a very low threshold, around 1.0 fM.

5. There was no obvious correlation between the action of these peptides and either acetylcholine or FMRFamide.

INTRODUCTION

There are an increasing number of peptides, identified in invertebrate tissue, which exhibit potent actions on peripheral tissues and central neurones of invertebrates (Kobayashi and Muneoka, 1990; Walker and Holden-Dye, 1991; Kits *et al.*, 1991). Among the first neuroactive peptides to be identified in invertebrates were FMRFamide and proctolin (Price and Greenberg, 1977; Brown and Starratt, 1975). Since the discovery of FMRFamide, a large family of related peptides has been identified in many phyla including chordates (Walker, 1992). For example, three RFamides have been identified in nematode, AF-1 (KNEFIRFamide), AF-2 (KHEYLRFamide), both from *Ascaris* and (SDPNFLRFamide), from *Panagrellus* (Cowden *et al.*, 1989; Cowden and Stretton, 1990; Bowman *et al.*, 1991). One of these, AF-1, has potent inhibitory actions on *Helix* central neurones (Walker *et al.*, 1991). Two Famides, S-1 (GFNSALMFamide) and S-2 (SGPYSFNSGLTFamide) have been identified in echinoderms (Elphick *et al.*, 1991a, b). Muneoka and his colleagues in Japan have isolated and tested a large number of neuroactive peptides including CARP (catch relaxing peptide of *Mytilus*, AMPMLRLamide) (Hirata *et al.*, 1987), ACEP-1 (*Achatina* cardioexcitatory peptide, SGQSWRPQGRFamide (Fujimoto *et al.*, 1990), Achatin-I (G^DFAD-OH) (Fujimoto *et al.*, 1991; Kim *et al.*, 1991) and the SSFVRIamide family (Ikeda *et al.*, 1991).

In the present study we have surveyed a wide range of neurones in the parietal and visceral ganglia of *Helix aspersa*, to determine the actions of AF-1, AF-2, PAN-1, LSSFVRIamide, ACEP-1, SALMF-1 and CARP. For comparison we have also tested acetylcholine and FRMFamide on the same neurones. The amino acid sequences of these peptides, together with their primary natural source, are shown in Table 1.

METHODS

All experiments were performed on the suboesophageal ganglia isolated from the garden snail, *Helix aspersa*. Animals were collected locally and kept in the laboratory until required for experimentation. The ganglia were removed from the animals, pinned in a Sylgard coated bath of volume 0.5 ml and perfused continuously with *Helix* saline (Walker, 1968) at about 4 ml per minute. The saline had the following composition: NaCl 100 mM; KCl 4 mM; $CaCl_2$ 7 mM; $MgCl_2$ 5 mM; Tris buffer 5 mM; final pH 7.5. Peptides were bath applied into the main perfusion system for one minute. For experiments involving ion substitution, chloride was replaced by acetate and sodium replaced by Tris. For low chloride experiments, all the NaCl was replaced by Na acetate. For high magnesium/low calcium experiments, 20 mM $MgCl_2$ and 0.5 mM $CaCl_2$ was used. For high potassium experiments the concentration of KCl was raised to 16 mM, and the NaCl reduced to 88 mM. All recordings were made from neurones in the right and left parietal ganglia and the visceral ganglion. The ganglia were lightly stained with Methylene Blue to aid identification which was based on the map of Kerkut *et al.* (1975). Intracellular recordings were made using glass microelectrodes filled with 2 molar potassium acetate, resistance 10–15 MΩ. Signals

Table 1. Table to show the amino acid sequences of the peptides used in this study together with the natural source of the peptide.

Name	Amino acid sequence		Source
AF-1	H-Lys-Asn-Glu-Phe-Ile-Arg-Phe-NH_2	KNEFIRFa	Nematode—*Ascaris*
AF-2	H-Lys-His-Glu-Tyr-Leu-Arg-Phe NH_2	KHEYLRFa	Nematode—*Ascaris*
PAN-1	H-Ser-Asp-Pro-Asn-Phe-Leu-Arg-Phe NH_2	SDPNFLRFa	Nematode—*Panagrellus*
LSSFVR1a	H-Leu-Ser-Ser-Phe-Val-Arg-Ile NH_2	LSSFVR1a	Prosobranch Mollusc—*Fusinus*
ACEP-1	H-Ser-Gly-Gln-Ser-Trp-Arg-Pro-Gln-Gly-Arg-Phe-NH_2	SGQSWRPQGRFa	Gastropod Mollusc—*Achatina*
SALMF-1	H-Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	GFNSALMFa	Echinoderm—*Asterias*
CARP	H-Ala-Met-Pro-Met-Leu-Arg-Leu-NH_2	AMPMLRa	Lamellibranch Mollusc—*Mytilus*
FMRFa	H-Phe-Met-Arg-Phe NH_2	FMRFa	Lamellibranch—*Macrocallister*

were recorded using a single electrode voltage clamp Dagan 8100–1 instrument and displayed on a Clevite Brush 220 pen recorder.

RESULTS

The results from this study represent an initial screen for activity and are summarised in Table 2 with examples of the types of responses shown in Figs 1–6. All the cells tested responded to acetylcholine, either hyperpolarization (H cells) or depolarization (D cells). Nearly all the cells also responded to FMRFamide and so these two compounds provide a useful comparison for the activity of the peptides. The actions of each peptide or group of peptides will be considered in turn.

AF-1, AF-2, PAN-1 peptides

As can be seen from Table 2, the overwhelming effect of the nematode peptides is one of inhibition on *Helix* central neurones. There is no obvious correlation between the actions of the nematode peptides and either acetylcholine or FMRFamide. Figure 1 shows an example from cell F-9 where all three nematode peptides, acetylcholine and FMRFamide are inhibitory and cause a hyperpolarization of the membrane potential. All three nematode peptides are over 100 times more potent than either FMRFamide or acetylcholine on this neurone. The thresholds for the three nematode peptides on this cell are around 0.1 nM. On cell F-2, where acetylcholine tends to be biphasic and FMRFamide, excitatory, AF-2 is inhibitory with a threshold of around 10 nM. Interestingly, AF-2 has no effect on this cell while PAN-1 is inhibitory but is slightly less potent than AF-1. The ionic mechanism associated with the inhibitory response of AF-1 has been investigated on cell F-2 and appears to be mainly associated with an increase in potassium permeability, Fig. 2. Reducing external chloride levels changes the firing pattern of the cell

Table 2. Table to summarise the effects of acetylcholine (ACh), FMRFamide, AF-1, AF-2, AF-3, LSSFVRIamide, ACEP-1 SALMFamide and CARP on different identified neurones. H indicates the cell was inhibited and hyperpolarized, D indicates the cell was excited and depolarized and O means the cell failed to respond to the peptide at the concentrations applied. A blank means the peptide was not tested. The numbers after H, D or O indicate the number of times that concentration was tested on the cell in different preparations.

Cell	ACh	FMRFa	AF1	AF2	PAN-1	LSS-FVRIa	ACEP-1	SALMF-1	CARP
Left parietal									
D1	H	D	H^{-5}(2)			D^{-7}(3)			
D4/5	H	H				H/H > D			
Visceral									
E2	D	H	H^{-6}(6)	H^{-5}(2)		H^{-7}(4)			
E4	H	D	O(3)				0(2)		
E8	D	0	0			0	D^{-7}		
E10	D	D/0				H^{-7}(3)			
E11	H	D	0(3)	0(3)		0(6)			
E12	H	H/0	H^{-5}(2)			H^{-7}(4)		D^{-6}	0
E13	D	H	H^{-7}(6)	H^{-5}(2)		H^{-7}(6)	$D > H^{-6}$	$?H^{-9}$	
E14	H	D	D^{-6}(4)	D^{-5}(3)	D^{-6}(5)	0(2)			
E16	D	H	0(3)	0(2)	0(3)	0(3)			
Right parietal									
F1	D	H	H^{-6}(9) /0(3)	H^{-5}(2) /0(4)	H^{-6}	0(2)	$D > H^{-6}$(8) /D^{-7}(3)	D^{-7}(4)	H^{-7}(3) /0(2)
F2	D	D	H^{-8}(7)	H^{-5}(3) /0(4)	H^{-6}(3)	0(3)		$D^{-15,-8}$	
F5/6	D	H	H^{-7}(2)	H^{-6}(3)		H^{-6}(4) /0(2)	D^{-7}		
F9	H	H	H^{-8}(4)	H^{-8}(4)	H^{-8}(2)	H^{-5}(2) /0(2)	H^{-6}(2)		H^{-8}(3)
F14	H	D	H^{-7}(2)		D^{-7}(2)				
F26	H	?	H^{-5}(3)	0(2)			0		0
F76	H	H	H^{-7}(2)						
F77	H	D	D^{-6}(4) /0(3)				0(2)		0(3)

The number of different cells tested with a peptide are indicated in brackets. In general a new cell came from a new preparation, different peptides were not applied on the same cell except for specific comparisons. ACh and FMRFamide were used at 10^{-6} or 10^{-5} Molar. The superscripts indicate the molarity of the peptide concerned.

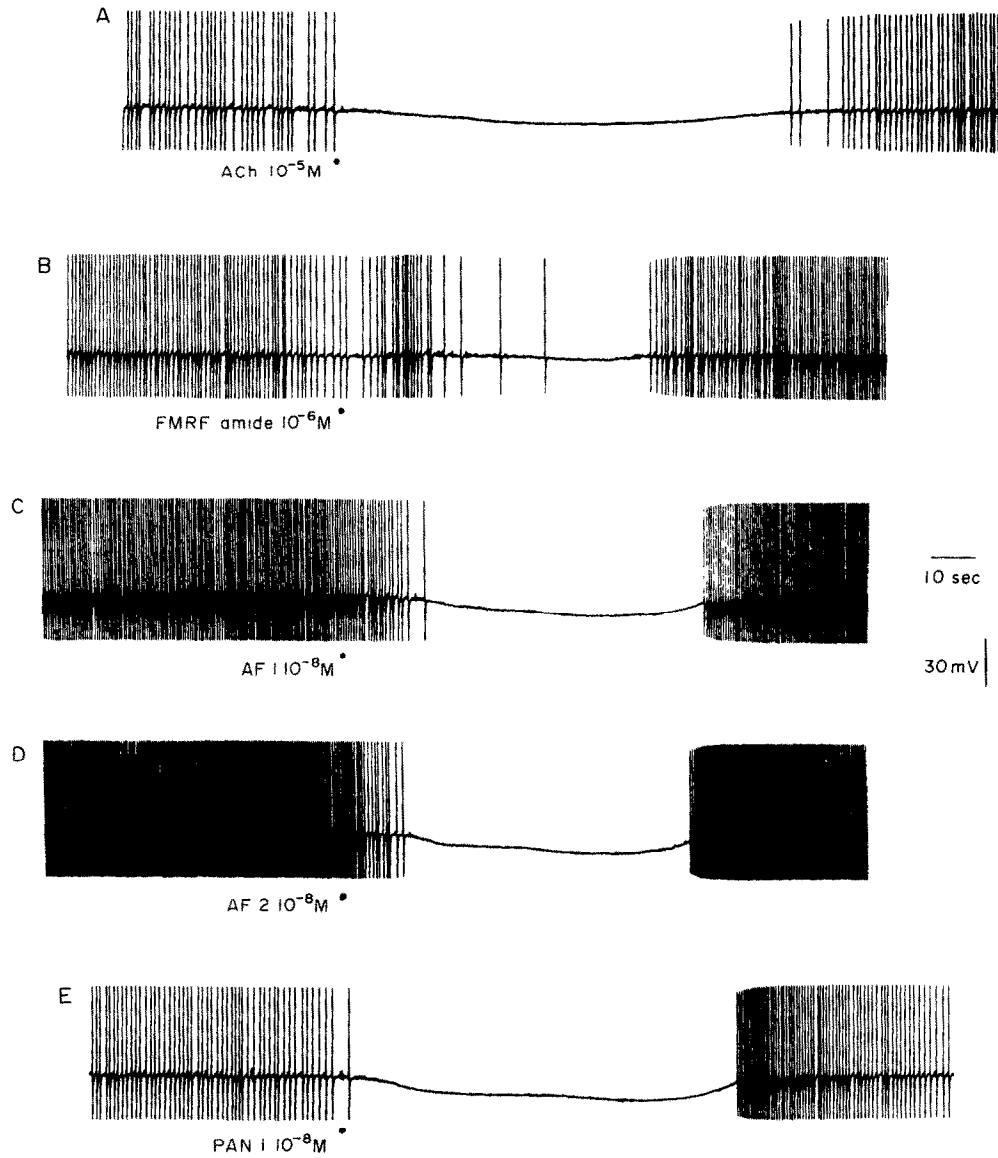

Fig. 1. Intracellular recordings from cell F-9 to compare the actions of the three nematode peptides. This cell is inhibited by both acetylcholine, 10 μM, and FMRFamide, 1 μM, traces A and B respectively. Traces C, D and E show the responses to the three peptides, all applied at 10 nM. The potencies of the three peptides on F-9 are similar.

and appears to reduce the hyperpolarization seen with AF-1, though both the hyperpolarizing and inhibitory phases of the AF-1 response remain. There is no sign of a reversal of the response to AF-1 in low external chloride. In this figure it can be seen also that in the presence of high magnesium/low calcium saline, the response to AF-1 still occurs, indicating it is a direct action on F-2. The action of AF-1 on cell F-2 is dose-dependent. Only a few cells, F-77 and in the E12/13 region, have been identified so far where AF-1 is excitatory, threshold 1.0 μM, considerably higher than the threshold for inhibition. AF-2 and PAN-1 have not been tested on cell F-77. Cell F-77 is inhibited by acetylcholine but excited by FMRF-amide. Cell F-14 (or 16) is inhibited by acetylcholine and excited by FMRFamide and while this cell is inhibited by AF-1, 0.1 μM, PAN-1 is excitatory at the same concentration. This excitation is dose-dependent. None of these peptides appear to alter acetylcholine responses.

LSSFVRIamide peptide

This peptide has only been found to have an effect on cells E-2, E-12, E-13, F-5 and F-9, where in all cases it is inhibitory, with a threshold of 1–100 nM. This inhibition is accompanied by hyperpolarization of the cell membrane potential and is dose-dependent, Fig. 3. There is no correlation between LSSFVRI-amide inhibition and the responses of cells to acetyl-choline or FMRFamide. The action of this peptide is

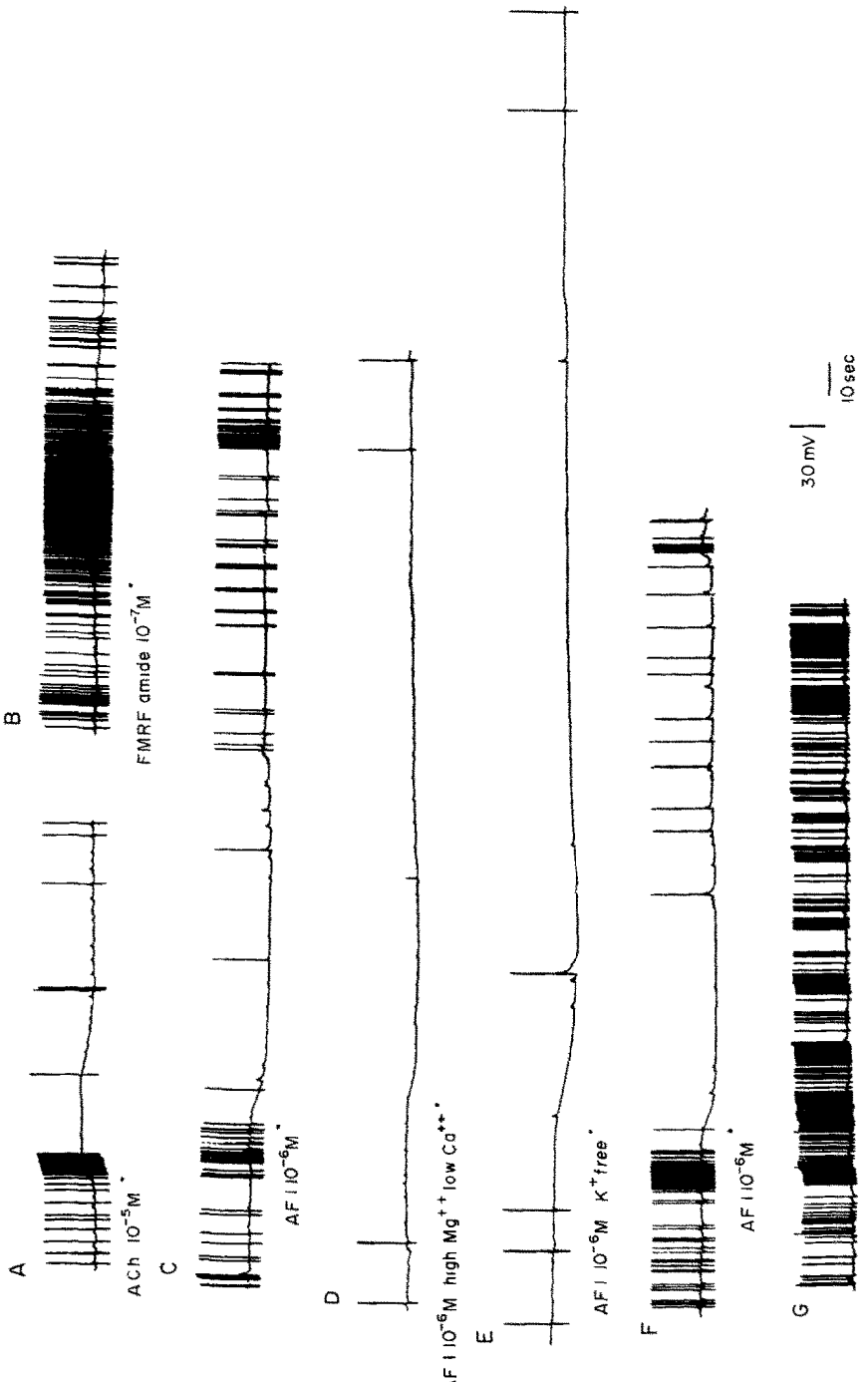

Fig. 2. Intracellular recordings from cell F-2 to show the effect of high magnesium/low calcium saline and changes external potassium on the response to AF-1. Traces A and B show the responses to acetylcholine, 10 μM, and to FMRFamide, 0.1 μM, respectively. Trace C shows that AF-1, 1.0 μM, hyperpolarizes the cell and this effect persists in 20 mM magnesium/0.5 mM calcium saline, trace D. In potassium-free saline, trace E, the response to AF-1 is enhanced while in high potassium, 16 mM, the response is virtually absent, trace F. On return to normal saline, the response is restored, trace G.

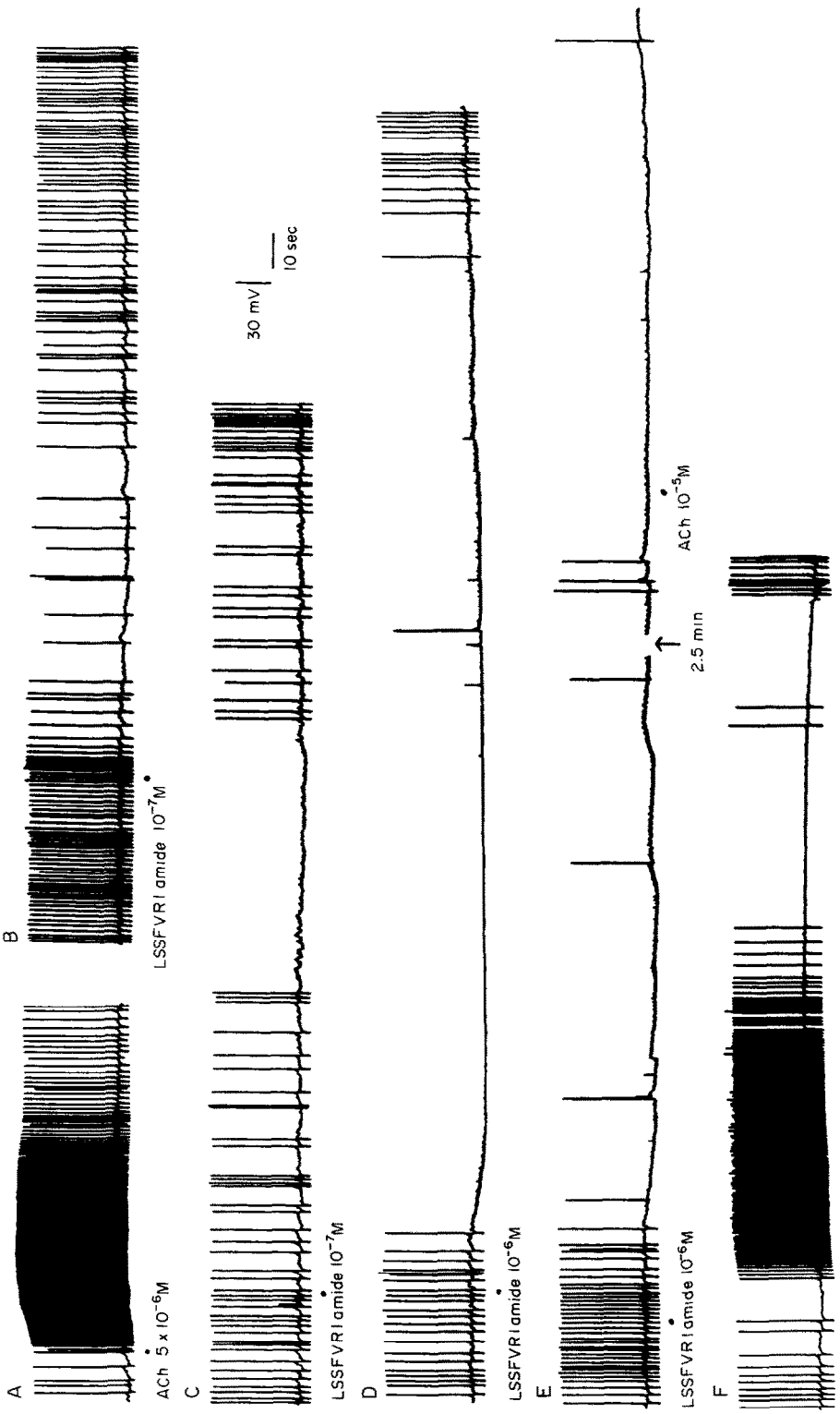

Fig. 3. Intracellular recordings from cell E-13 to show the effect of LSSFVRIamide on cell activity. Traces A and B show the actions of acetylcholine, 5.0 μM, and LSSFVRIamide, 0.1 μM, respectively. A higher application of peptide, 1.0 μM, trace D, shows a clear hyperpolarization. This concentration of peptide completely blocks the excitatory effect of 10 μM acetylcholine, trace E. Following prolonged washing, the acetylcholine excitation is partially restored, trace F.

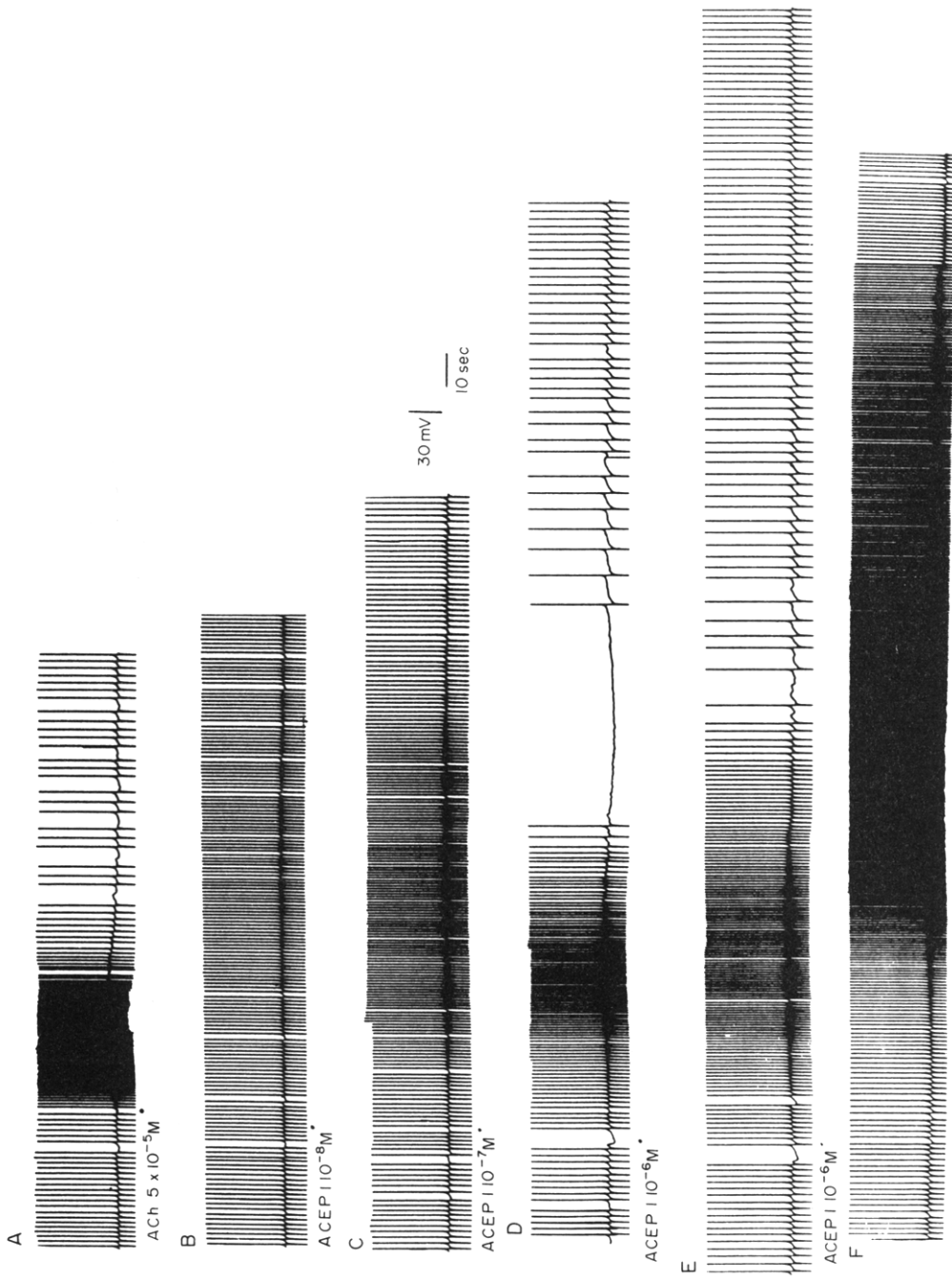

Fig. 4. Intracellular recordings from cell F-1 to show the effect of ACEP-1 on cell activity. Acetylcholine, 5.0 μM, trace A, and low concentrations of peptide, 10–100 μM, traces B and C, both excite the cell. A higher concentration of peptide, 1.0 μM, trace D, produces a biphasic effect, excitation followed by inhibition but the inhibitory phase tends to disappear with repeated applications of peptide, traces E and F.

direct on the cells studied since it is unaltered in the presence of high magnesium/low calcium saline. The size of the hyperpolarization to this peptide is enhanced in potassium-free saline and reduced in saline where external potassium has been raised above normal. Neither low sodium nor low chloride salines have any effect on the response. The responses to acetylcholine appear to be modified by LSSFVRI-amide in some cells since in the presence of the peptide, the acetylcholine excitatory response is

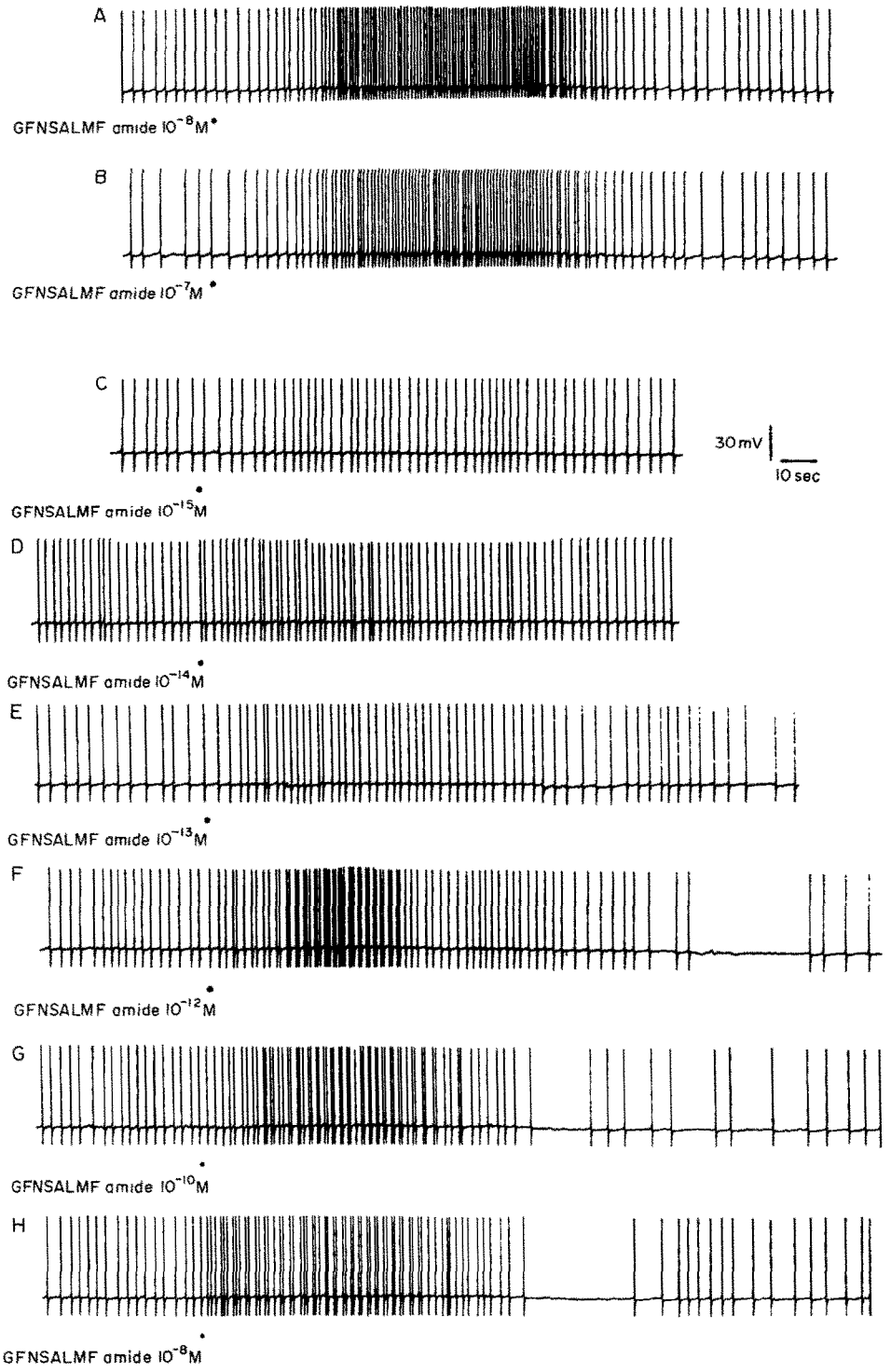

Fig. 5. Intracellular recordings from cell F-2 to show the effect of the echinoderm peptide GFNSALM-Famide on cell activity. This peptide only excited the cell with a threshold as low as 1–10 fM. The size of the excitation did increase with increasing concentration of peptide.

blocked, Fig. 3 trace E, and recovers only slowly following washing.

ACEP-1 peptide

The major effect of ACEP-1 is depolarization and excitation though in some cases the initial excitation is followed by inhibition, Fig. 4. The secondary inhibitory phase tends to fade on repeated application of the peptide; for example, compare traces D, E and G of Fig. 4. Threshold concentrations of peptide usually only produced excitation, traces B and C. The threshold for a response is around 10 nM. Low potassium saline has little or no effect on the initial excitatory

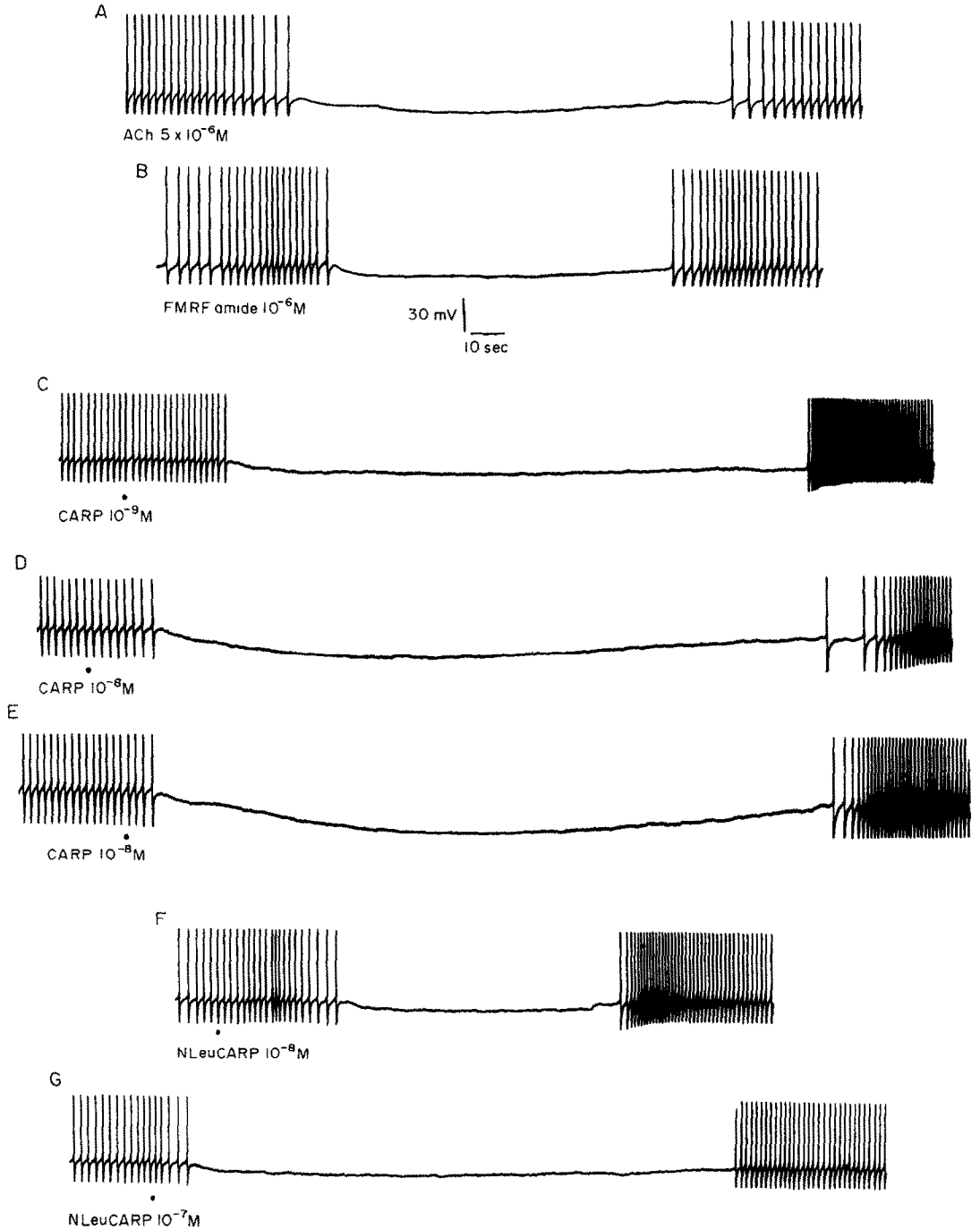

Fig. 6. Intracellular recording from cell F-9 to show the effect of CARP and a CARP analogue on cell activity. Traces A and B show the responses to acetylcholine, 5.0 μM, and FMRFamide, 1.0 μM, respectively. CARP, traces C, D and E show a clear dose-dependent hyperpolarization of cell activity. When methionine was replaced by norleucine, the response was considerably reduced, traces F and G.

phase but reduces or blocks the secondary inhibition. Low chloride saline appears to have little effect on the excitatory response.

SALMF-1 peptide

The echinoderm peptide, SALMF-1, is only excitatory on *Helix* neurones, Table 2 and Fig. 5. The threshold for this peptide is extremely low when compared with the other peptides tested in this study, threshold being around 1.0 fM, trace C. The response is dose-dependent though never excessively strong in terms of depolarization, trace B 100 nM. As with the other peptides, there is no apparent correlation between the response to SALMF-1 and either acetylcholine or FMRFamide.

CARP peptide

In this study CARP was tested on five different cell types and only exhibited inhibition accompanied by hyperpolarization, Fig. 6. This hyperpolarization could be large, for example, around 15 mV, trace E. On this cell, F-9, both acetylcholine and FMRFamide are inhibitory but CARP was far more potent than either with a potency ratio of at least 1000. An analogue of CARP, where methionine was replaced by norleucine, was also tested. It can be seen from traces F and G that this peptide is less potent than CARP by at least 100 times.

DISCUSSION

The present study extends the range of peptides which are active on *Helix* central neurones. The nematode peptide AF-1 has been tested on *Ascaris* neurones and found to inhibit slow oscillatory potentials (Cowden *et al.*, 1989), indicating an inhibitory role as also shown on *Helix* neurones. Both AF-1 and AF-2 blocked locomotory movements in *Ascaris* in the area of injection. The inhibitory action of AF-1 on *Helix* neurones appears to be through an increase in potassium conductance, which is the same mechanism of action as that shown by APGWamide, a peptide present in both *Fusinus* and *Lymnaea* (Kuroki *et al.*, 1990; Smit *et al.*, 1991), on both *Helix* and *Achatina* central neurones (Chen and Walker 1992; Liu *et al.*, 1991). Interestingly, cell F-2 in *Helix* is inhibited and hyperpolarized by both APGWamide and AF-1 and since they act through the same mechanism, evidence for any cross desensitization should be checked. Since the amino acid sequences of the two peptides are completely different, there is little likelihood they act via the same receptors. Neither peptide appear to have any significant effect on acetylcholine responses. The potent action of AF-1 on *Helix* neurones raises the question of the nature of the receptor it interacts with. For example, is it a receptor which only recognizes RFamide, the only feature common to all three nematode peptides? This could be so in the case of F-9 where ACEP-1 and FMRFamide are both inhibitory. However, on F-2, while AF-1 is inhibitory, FMRFamide is excitatory, making it unlikely they are acting on a common receptor. This raises the possibility the receptor which recognizes AF peptides recognizes a longer C terminal although AF-1 and AF-2 have only four amino acids in common and all three nematode peptides have only the first two C terminal amino acids in common. This would strongly suggest they act through separate receptors which appears to be likely in *Ascaris* where, eg, AF-1 and PAN-1 have different actions on muscle strips (Franks *et al.*, 1992). It would be of interest to see whether an AF peptide is present in *Helix*. Equally, it would be interesting to synthesize fragments of the peptides to determine the amino acid sequence necessary for the inhibitory response of *Helix* neurones.

LSSFVRIamide is a member of a large family of peptides recently identified in molluscs and echiuroids (Ikeda *et al.*, 1991). A total of seven peptides have been identified, three from *Urechis*, one each from *Fusinus* and *Helix* and two from *Achatina*. LSSFVRIamide can be either inhibitory or excitatory on various molluscan muscles but differs in its action from FMRFamide (Kuroki *et al.*, 1992). Since one of the family occurs in *Helix*, this group is likely to have a physiological role in this genus. The present study shows it can have a powerful blocking action against acetylcholine excitation and it will be interesting to pursue this in detail, both against acetylcholine and other putative transmitters. In earlier studies both CARP and argvasotocin have been shown to modify *Helix* central acetylcholine responses (Mat Jais *et al.*, 1990; Boyd *et al.*, 1987).

ACEP-1 peptide was isolated from the atria of *Achatina* (Fujimoto *et al.*, 1990) and found to have a potent excitatory effect on the heart ventricle but strangely relatively inactive against atrial contraction. Like FMRFamide, ACEP-1 potentiates tetanic contractions of the penis retractor muscle and buccal muscles of *Achatina*. However, on central neurones of *Achatina*, ACEP-1 excites neurones which are inhibited by FMRFamide (Kobayashi and Muneoka, 1990). Similarly on *Helix* neurones, ACEP-1 excites cells which are inhibited by FMRFamide, for example, E-13 and F-1. On the other hand, both peptides excite D-11, while on F-9, both peptides inhibit cell activity. Interestingly the C terminal portion of ACEP-1, that is, QGRFamide, is similar to a peptide isolated from coelenterates, anthoRFamide, pQGRFamide (Grimmelikhuijzen and Graff, 1986). This latter peptide should be tested on neurones which respond to ACEP-1. It is also possible that ACEP-1 and related peptides are present in other phyla.

SALMF-1, isolated from the echinoderm, *Asterias*, by Thorndyke's group (Elphick *et al.*, 1991a, b), has not previously been tested on central neurones. In the present study, it was consistently excitatory, with a very low threshold on, for example, cell F-2.

Although this cell is also excited by FMRFamide, another cell excited by SALMF-1, cell F-1, is inhibited by FMRFamide. This would suggest it is acting via a separate receptor and one which recognizes some portion of ALMFamide or even more of the peptide sequence. Again, this or related peptides may occur in molluscs.

The final peptide examined in this study was CARP, first identified in *Mytilus* (Hirata *et al.*, 1987). This peptide has potent relaxing actions on the ABRM (anterior byssus retractor muscle) of *Mytilus*. CARP has a similar amino acid sequence to the myomodulins, myomodulin modulating the action of the accessory radula closer (ARC) muscle of *Apylsia* (Cropper *et al.*, 1987). CARP has potent actions on various molluscan muscles and central neurones (Hirata *et al.*, 1989a, b; Kiss 1988; Mat Jais *et al.*, 1990). CARP immunoreactive neurones have been localized in the *Helix* central ganglia together with immunoreactivity in various peripheral organs, except for the heart (Hernadi *et al.*, 1992). These authors conclude CARP may have a role as an inhibitory or relaxing agent onto these organs. From a previous study it has been shown that CARP has powerful modulatory actions on the acetylcholine response of *Helix* neurones (Mat Jais *et al.*, 1990). Interestingly, myomodulin and acetylcholine are likely to be co-localized in cell B-16 of *Apylsia* where, at low concentrations, the peptide potentiates muscle contraction but at higher concentrations, it depresses contraction (Cropper *et al.*, 1987; Vilim *et al.*, 1989). Since CARP acts on both mammalian and echiuroid muscle, it is possible that either this peptide or a related one is present in non-molluscan phyla (Kobayashi and Muneoka, 1990).

Overall, the present study demonstrates that *Helix* central neurones provide an excellent model system to screen for neuropeptide activity in the central nervous system. It can also provide evidence concerning their mechanism of action and from structure-activity studies indicate the amino acid sequences essential for potent activity. It can also be used as a model in the development of possible antagonists to neuroactive peptides and to show possible modulatory roles against classical transmitters. This last point is particularly relevant since *Helix* neurones respond to a wide range of putative transmitters and possess specific receptors for them, although their pharmacological profiles may well be different from those found, for example, on vertebrate neurones.

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