The Journal of Experimental Biology 200, 2377–2386 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB1031

REVIEW

THE FIRST PEPTIDE-GATED ION CHANNEL

GLEN A. COTTRELL*

School of Biomedical Sciences, University of St Andrews, Fife, KY16 9TS, UK and *Whitney Laboratory, University of Florida, St Augustine, FL 32086, USA*

Accepted 10 July 1997

Summary

Patch-clamp experiments on the C2 neurone of *Helix aspersa* have shown that the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) directly gates a Na^+ channel. The channel is amiloride-sensitive. Activation of this channel is responsible for the fast excitatory action of the peptide. Using primers based on amiloride-sensitive epithelial Na^+ channels, a complete cDNA sequence (*FaNaCh*) was cloned and sequenced from a *Helix* library. The sequence is predicted to have just two membrane-spanning regions and a large extracellular loop. When expressed in *Xenopus laevis* oocytes, the channel responded to FMRFamide. Taken together, these data provide the first evidence for a peptide-gated ion channel. Comparison of the properties of the expressed *FaNaCh* with the native neuronal channel show small differences in the sensitivities to some drugs and in channel conductance. It is not yet clear whether the native channel is a homo-oligomer or comprises other subunits. The peptide FKRFamide is an effective antagonist of FMRFamide on the expressed and neuronal channels. Nucleotide sequences encoding similar channel proteins occur in neurones of species as dissimilar as man and *Caenorhabditis elegans*. Some channels are thought to be associated with mechano-sensation, at least one is a proton-gated channel and others may also be ligand-gated channels.

Key words: peptide-gated, ion channel, amiloride-sensitive, Na^+ channel, FMRFamide, *Helix aspersa*, ligand-gated, neuropeptide.

Introduction

Neuropeptide intercellular messengers occur widely in invertebrate and vertebrate species. They exert many different effects, generally slow or modulatory and frequently dependent on GTP-binding proteins (G-proteins). In some identified neurones of the the snail *Helix aspersa*, however, one endogenous neuropeptide, phenylalanyl-methionyl-arginyl-phenylalanyl-NH_2 (FMRFamide), exerts a fast excitatory response accompanied by an increase in membrane conductance (Cottrell *et al.* 1984). This fast response is readily distinguished from other, slower neuronal responses in *Helix aspersa*, such as those resulting from an increase in K^+ conductance and the suppression of Ca^{2+} conductance (Cottrell *et al.* 1984; Colombaioni *et al.* 1985).

The peptide FMRFamide was discovered by Price and Greenberg (1977) in the clam *Macrocallista nimbosa*. The snail *Helix aspersa* also contains FMRFamide and in addition more than 10 related peptides, all of which are formed from two precursor proteins: one precursor incorporates FMRFamide, FLRFamide (phenylalanyl-leucyl-arginyl-phenylalanyl-NH_2) and pQFYRFamide (*p*-glutamyl-phenylalanyl-tyrosyl-arginyl-phenylalanyl-NH_2), the other many related peptides with N-terminal extensions (Lutz *et al.* 1992; Price *et al.* 1996*b*; Cottrell *et al.* 1994).

Discovery of a peptide-gated channel

Electrophysiological experiments on Helix aspersa *neurones*

The fast FMRFamide response desensitizes rapidly and shows a marked preference for the tetrapeptide amides (FMRFamide and FLRFamide) rather than the N-terminally extended forms (Cottrell and Davies, 1987). Unlike similar depolarizing responses evoked by acetylcholine, 5-hydroxytryptamine (5-HT) and dopamine, it is not blocked by tubocurarine. The first evidence that the peptide directly gates a channel was obtained using outside-out membrane patches from the *Helix* C2 neurone (Cottrell *et al.* 1990). Inward unitary currents were observed when FMRFamide was applied to the external membrane surface of isolated outside-out patches, even when the recording pipette contained guanosine 5′-*O*-(2-thiodiphosphate), which blocks G-protein-coupled responses (Green *et al.* 1994). These results suggested that there is a tight receptor/channel coupling with no involvement of a G-protein. The channels are much more permeable to Na^+ than to K^+ or Ca^{2+} and have a relatively low conductance of approximately 4 pS. It was also observed that the response is blocked with amiloride. Often, in addition to the major fast component, a slower component of the total response is seen. This slower component is less susceptible to amiloride and is

*e-mail: gac@st-and.ac.uk and cottrell@aug.com

potentiated by extracellular CPT-c-AMP, but not extracellular cyclic AMP (Falconer *et al.* 1993; K. A. Green and G. A. Cottrell, unpublished data). The slower component is variable, but also probably results from the direct gating of an ion channel (Green *et al.* 1994).

Cloning, sequencing and expression of the FaNaCh

The cDNA of an FMRFamide-gated receptor/channel protein (*FaNaCh*) was recently cloned and sequenced from a *Helix aspersa* cDNA library (Lingueglia *et al.* 1995). The observation that the neuronal peptide response is blocked by amiloride facilitated cloning, because cDNAs for the amiloride-sensitive epithelial Na^+ channel proteins, *ENaCh*s, had already been cloned from rat colon (Canessa *et al.* 1993, 1994; Lingueglia *et al.* 1993; and see Canessa *et al.* 1995). Sequence data from the *ENaCh*s provided clues for designing degenerate polymerase chain reaction (PCR) primers to search for, and finally to sequence, the cDNA for the channel, *FaNaCh*. The predicted structure of the protein encoded by *FaNaCh* has a long extracellular loop (including a cysteine-rich region) and two membrane-spanning domains with both termini located intracellularly (Lingueglia *et al.* 1995). The predicted structure is different from the well-characterized class of ligand-gated channels typified by the nicotinic acetylcholine (ACh) receptors (each subunit of which possesses several transmembrane domains). A diagrammatic representation of the peptide channel (*FaNaCh*) is shown in Fig. 1.

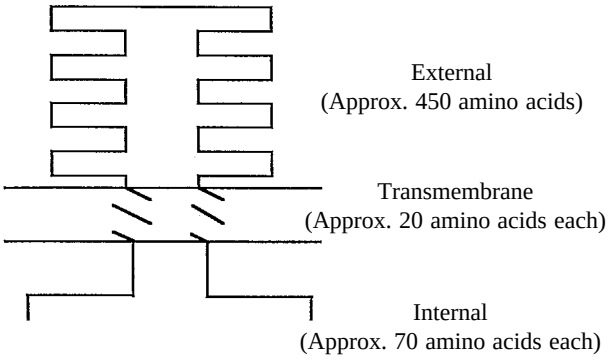

Fig. 1. Diagram of the predicted structure of the peptide-gated ion channel *FaNaCh*. There is a cysteine-rich domain in the large extracellular loop and just two predicted membrane-spanning regions. The style of representation is similar to that used in a recent review by North (1996), in which other channels with two hydrophobic segments are described.

cDNAs with sequences or features similar to the *FaNaCh*

Epithelial Na^+ channels

Sections of the *FaNaCh*, notably the predicted membrane-spanning regions and part of the extracellular cysteine-rich domains, show similarity to the cloned epithelial Na^+ channel subunits (*ENaCh*s) (Canessa *et al.* 1993, 1994; Lingueglia *et al.* 1993). The naturally occurring channels, which occur on the apical surface of epithelial cells in the kidney, distal colon and lung of mammals, are thought to comprise three different subunits deriving from different messages: α-, β- and γ-*ENaCh*s (Canessa *et al.* 1995; Lingueglia *et al.* 1994). Epithelial Na^+ channels are highly selective for Na^+ and are rate-limiting for Na^+ reabsorption. Under normal conditions they are open, but they can be blocked by submicromolar concentrations of the diuretic amiloride. Biochemical analysis has provided evidence that the channel proteins comprise two transmembrane α-helices and a large extracellular domain of approximately 500 amino acid residues, and that both the amino and carboxy termini are located within the cytoplasm (Renard *et al.* 1994).

Mammalian cDNA sequences expressed in neurones

cDNA for an amiloride-sensitive channel has been cloned from human and rat brain neurones. This cDNA was designated *BNC1* by Price *et al.* (1966*c*) and *MDeg* by Waldmann *et al.* (1996). When expressed, the channel was closed, but it was activated by a point mutation in an equivalent location that results in neuronal death with the degenerins of *Caenorhabditis elegans* (i.e. glycine-430 is replaced with a valine or phenylalanine) (Waldmann *et al.* 1996). This mammalian neuronal sequence (*BNC1* i.e. *MDeg*) shows the greatest sequence similarity to *FaNaCh*. Waldmann *et al.* (1996) suggest that it too may be a ligand-gated channel, but the natural ligand is not known. García-Añoveros *et al.* (1997) have also cloned a related mammalian sequence (*BNaC2*) as well as *BNaC1* (i.e. *BNC1* or *MDeg*). Both sequences appear to be widely expressed in neurones of the central nervous system, although their patterns of expression vary slightly. The most recent development is the discovery of a related rat sequence, *ASIC*, which has the properties of a proton-gated channel when expressed in *Xenopus laevis* oocytes (Waldmann *et al.* 1997). *ASIC* is expressed in neurones of the dorsal root ganglia and different regions of the brain. It appears to be important in sensing extracellular pH variations and translating them into depolarizations.

Predicted amiloride-sensitive Na^+ channels from Caenorhabditis elegans

Genetic analysis has identified a group of related genes, e.g. *Mec-4* and *Mec-10* (presumed mechano-sensory) and *Deg-1* (all of which, when mutated, result in neuronal degeneration – hence 'degenerins') in *C. elegans* (see García-Añoveros *et al.* 1995). On the basis of genetic data from mutations affecting degeneration of specified mechano-sensory neurones in *C. elegans*, and some analogy with the epithelial Na^+ channel, Huang and Chalfie (1994) have proposed that *Mec-4*, *Mec-10* and another sequence, *Mec-6*, form a complex in the membrane of the mechano-sensitive neurones needed for mechano-sensation. Several other related sequences occur, some of which may also play a role in mechano-sensation (see Tavernarakis *et al.* 1997). So far, none of the *C. elegans* sequences has been expressed to test directly the view that they encode Na^+ channels. However,

Waldman *et al.* (1995) have expressed chimeric proteins from synthetic RNAs that incorporate the MI and/or the MII (presumed transmembrane domains) from *Mec-4* within synthetic RNAs of the α-*ENaCh* (from which the corresponding regions had been deleted) and shown that they conducted Na^+ and Li^+.

ATP-P2X receptors

Hydropathicity plots of predicted proteins of the recently cloned cDNAs of ATP-P2X receptor subunits (Brake *et al.* 1994; Valera *et al.* 1994; Chen *et al.* 1995; Lewis *et al.* 1995) are similar to those of the *FaNaCh* protein, suggesting that they all have two membrane-spanning domains. Further, the putative extracellular loop of P2X receptor proteins is rich in cysteine residues, as is the *FaNaCh* protein. However, there is little or no similarity in the P2X sequences with *FaNaCh*. Unlike *FaNaCh*, P2X receptors are not selective for Na^+ or blocked by amiloride. Thus, the ATP-P2X receptors do not fall into the same group as those described above, although they too are predicted to have two membrane-spanning domains (see North, 1996).

What relationship does the cloned sequence *FaNaCh* have to the neuronal peptide-gated Na^+ channel?

It is likely that the neuronal peptide-gated channel incorporates the protein derived from the nucleotide sequence *FaNaCh*. If this assumption is correct (it can be verified by determining whether the message for *FaNaCh* occurs in responsive neurones), does the natural receptor comprise just one type of subunit (i.e. *FaNaCh*)? Evidence for the existence of three different subunits in epithelial Na^+ channels was obtained by observing increased Na^+ conductance by co-expression of the three different subunits (Canessa *et al.* 1994). It is well known that receptors for ACh and γ-aminobutyric acid (GABA) comprise many different subunits (see Barnard, 1996). However, only one subunit has been found for the 5-HT_3 receptor (Maricq *et al.* 1991).

One way to address the question of subunit composition is to compare the properties of expressed *FaNaCh* with those of the neuronal peptide-gated channel. Marked differences would suggest that the native receptor consists of more than one type of subunit (compared with the native epithelial Na^+ channel; see Canessa *et al.* 1994). Pharmacological experiments on the neuronal peptide-gated channel are technically more difficult than studies on the expressed *FaNaCh* in *Xenopus laevis* oocytes. Thus, pharmacological studies have proceeded on the oocyte-expressed receptor, and selected comparisons have been made with responses in the C2 neurone.

Studies on the heterologously expressed FaNaCh

When the cDNA sequence *FaNaCh* is transcribed and the message injected into *Xenopus laevis* oocytes, applied FMRFamide evokes an inward Na^+ current. The properties of this current (Lingueglia *et al.* 1995) resemble in many respects those of the native channel in *Helix* neurones (Green *et al.* 1994). However, the expressed *FaNaCh* does not show the same degree of desensitization as the faster (i.e. major and amiloride-sensitive) component of the FMRFamide response of the C2 neurone. Activation of the expressed channel by FMRFamide (and also by FLRFamide) appears to be cooperative, with an apparent Hill coefficient of 1.6 (Lingueglia *et al.* 1995), indicating that at least two molecules interact with the receptor complex (presumably a homo-oligomer in the oocyte membrane). Lingueglia *et al.* (1995) also showed that some other molluscan peptides (SDPFLRFamide, GDPFLRFamide) and the mammalian peptide FLFQPQRFamide did not activate the channel.

The effects of several other peptides have recently been tested on *FaNaCh* expressed in *Xenopus* oocytes [see Table 2; G. A. Cottrell, M. C. Jeziorski, P. A. V. Anderson, D. A. Price, K. E. Doble, M. J. Greenberg, R. M. Greenberg and A. Edison, unpublished data; further data from the *Helix* C2 neurone (K. A. Green and G. A. Cottrell, unpublished data) are included in Table 2 and also shown in Fig. 5].

Differing effects of FMRFamide, FLRFamide and pQFYRFamide (peptides formed from the precursor protein that incorporates FMRFamide)

cDNA for the FMRFamide precursor protein also encodes the peptides FLRFamide and pQFYRFamide (Lutz *et al.* 1992; Price *et al.* 1996*b*). Extensive comparison of the potencies of FLRFamide and FMRFamide on oocyte-expressed *FaNaCh* confirmed that FLRFamide has a lower apparent affinity than FMRFamide, as described by Lingueglia *et al.* (1995) and, in addition, when both were tested on the same oocytes, showed that the maximum effect of FLRFamide is always smaller (40–60 %) than that of FMRFamide (Fig. 2A; Table 1). These data strongly suggest that FLRFamide is a partial agonist on expressed *FaNaCh* (Cottrell, 1997). Hill plots constructed for FMRFamide, FLRFamide and also for FnorLRFamide (see below) gave values ranging from 1.4 to 1.7, providing additional evidence that at least two molecules of each peptide bind to the oocyte-expressed channel.

Unexpectedly, pQFYRFamide (the other neuropeptide encoded on the precursor protein) did not evoke the Na^+ current, but antagonized both FMRFamide and FLRFamide (Cottrell, 1997). An example of this antagonistic effect is shown on the FLRFamide response (Fig. 2C). Such receptor antagonism by an endogenous ligand has not been described before; its possible significance is considered in the Discussion.

Relationship between the peptide sequence and biological activity

Table 1 shows the relative potencies of several peptides on *FaNaCh* expressed in *Xenopus* oocytes. Some tentative conclusions can be drawn from the data.

(i) There is a requirement for a non-polar aromatic amino acid (phenylalanine or tryptophan) at amino acid positions 1 and 4. Biological activity is markedly reduced with peptides containing non-aromatic amino acids or a more polar aromatic amino acid, such as tyrosine, in either of these positions. The reduced activity of D-FMRFamide also suggests that the aromatic ring needs to be correctly orientated. Non-amidated analogues are much less

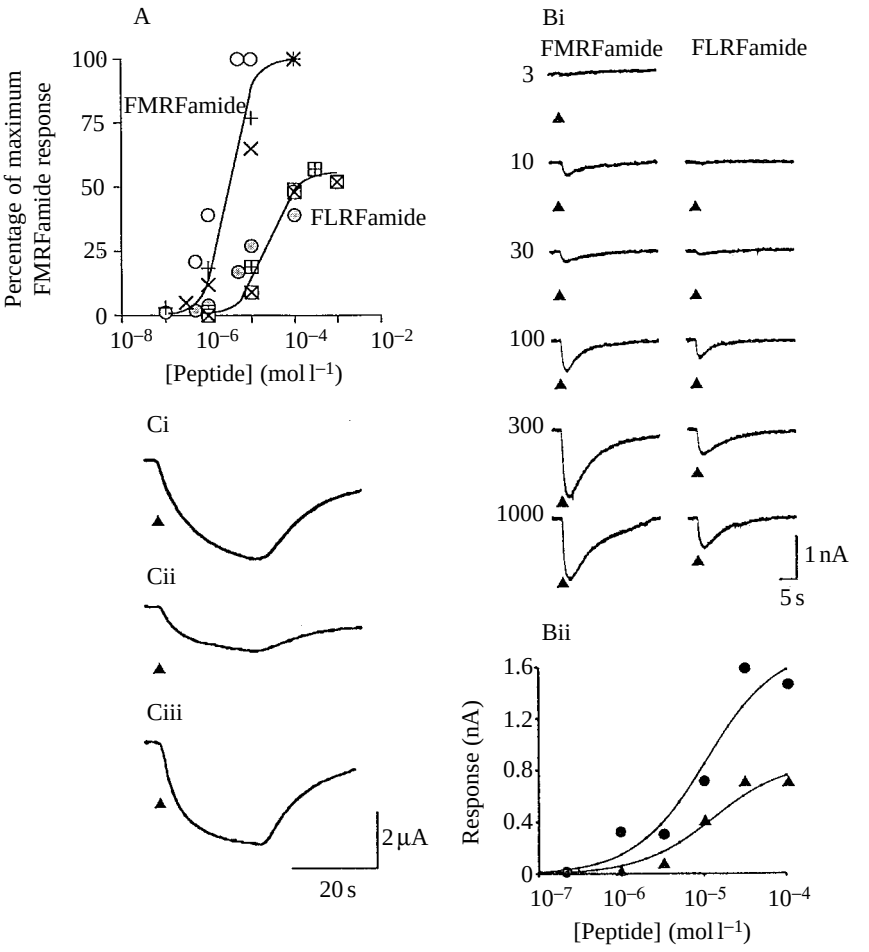

Fig. 2. (A) Comparison of dose–response relationships of FMRFamide and FLRFamide on oocyte-expressed *FaNaCh*. The effects of both peptides were tested on three oocytes, and the data are expressed as a percentage of the maximum response observed with each oocyte. The maximum response to 1000 µmol l^{-1} FLRFamide was only approximately half that to FMRFamide at 100 µmol l^{-1}. (Bi,ii) Responses to FMRFamide and FLRFamide recorded from the same *Helix* C2 neurone. As with the heterologously expressed receptor, FMRFamide evokes a much larger maximum response than does FLRFamide. Concentrations are given in µmol l^{-1}. (C) The endogenous peptide pQFYRFamide (100 µmol l^{-1}, Cii), encoded on the same precursor as FMRFamide, antagonises the response to FLRFamide (50 µmoll l^{-1}, Ci–iii) on the oocyte-expressed channel. The ratio of [pQFYRFamide] to [FLRFamide] showing this degree of antagonism is the same as that predicted for the N-terminal domain of the precursor (i.e. 2:1; see text).

active (compare FMRF with FMRFamide), as first shown on muscle responses by Price and Greenberg (1977).

(ii) Replacing methionine by leucine (FLRFamide) results in considerable loss of activity. There is reduction in the maximum response as well as a reduction in apparent affinity. FnorLRFamide, in contrast, is as potent as FMRFamide, and the dose–response relationship is very similar (see also WnorLRFamide). Thus, the chain of four carbons (with norleucine) appears to be as effective as the similar chain incorporating one atom of sulphur (methionine) (see Fig. 3).

(iii) Other amino acid substitutions in position 2 cause pronounced changes in biological activity. Some substitutions (e.g. with aspartic acid, arginine or lysine) result in antagonist activity. The most potent competitive antagonist detected so far is FKRFamide (phenylalanyl-lysyl-arginyl-phenylalanyl-NH_2) with an ID_{50} of approximately 7 µmol l^{-1}. FKRFamide also blocks the neuronal response (see below). A stick-figure representation of FKRFamide is compared with FMRFamide in Fig. 3.

(iv) Substituting the positively charged arginine in amino acid position 3 with isoleucine, D-arginine or even lysine also results in considerable loss of activity, suggesting that a positively charged residue is required in precisely the correct location and orientation. Some ionic interaction may occur between the amino acid in this position and the ligand-recognition site of the channel.

(v) As observed with the neuronal response (Cottrell and Davies, 1987), none of the peptides with an N-terminal extension is as active as FMRFamide. The synthetic peptide tyrosyl-glycyl-glycyl-phenylalanyl-methionyl-arginyl-phenylalanyl-NH_2 (YGGFMRFamide, related to met-enkephalin) was approximately 300 times less active than FMRFamide, and no effect could be detected with another endogenous peptide, asparagyl-glycyl-histidyl-tyrosyl-methionyl-arginyl-phenylalanyl-NH_2 (NGHYMRFamide), which is encoded by the cDNA sequence for the 'heptapeptide' FMRFamide-like peptides (Cottrell *et al.* 1994). Several other related peptides did not activate the current (see Lingueglia *et al.* 1995).

Unitary current recordings

The conductance of *FaNaCh* expressed in *Xenopus* oocytes was 13 pS in cell-attached patches (with 140 mmol l^{-1} Na^+ in the recording pipette); the channel showed a slight increase in open probability as the potential on the inner surface of the membrane was increase from −60 to −100 mV. With a concentration of FMRFamide of 30 µmol l^{-1}, the frequency distribution of the open state was fitted with a time constant of 4 ms, and that of

Table 1. *Approximate potencies of several peptides relative to the potency of FMRFamide.*

Peptide (*denotes endogenous)	Effect/No effect	Potency cf. FMRFamide = 1 (FMRFamide* $EC_{50} \approx 3\,\mu mol\,l^{-1}$)	Partial agonist or antagonist
Position 1			
WMRFamide	Effect	1	
Desamino-FMRFamide	Effect	0.5–1	
YMRFamide	Effect	0.005	
D-FMRFamide	Effect	0.005	
IMRFamide	Effect	0.003	
GMRFamide	No effect	<0.003	
Position 2			
Fn**L**RFamide	Effect	1	
Wn**L**RFamide	Effect	1	
F**L**RFamide*	Effect	≈0.1	Partial agonist
F**W**RFamide	Effect	0.01	
F**R**RFamide	Effect	0.01	Antagonist; $ID_{50} \approx 10\,\mu mol\,l^{-1}$
F**C**RFamide	Effect	0.002	
F**G**RFamide	Effect	0.003	
F**D**RFamide	No effect	<0.005	Antagonist; $ID_{50} > 10\,\mu mol\,l^{-1}$
F**K**RFamide	No effect	<0.005	Antagonist; $ID_{50} \approx 7\,\mu mol\,l^{-1}$
F**Q**RFamide	No effect	<0.005	
F**T**RFamide	No effect	<0.005	
F**D**-**M**RFamide	No effect	0.001	
F**S**RFamide	No effect	<0.001	
F**Y**RFamide	(No effect on neurone)		Antagonist; $ID_{50} \approx 30\,\mu mol\,l^{-1}$
Position 3			
FM**K**Famide	Effect	0.05	
FM**I**Famide	Effect	0.05	
FM**D**-**R**Famide	Effect	0.01	
Position 4			
FMR**W**amide	Effect	1–2	
FMR**Y**amide	Effect	0.1	
FMR**L**amide	Effect	0.07	
FMR**M**amide	Effect	0.03	
FMR**F**	Effect	0.01	
FMR**I**amide	No effect	<0.01	
FLR**F**	Effect	≈0.003	
FMR**G**amide	No effect	<0.001	
Other related peptides			
WLR**W**amide	Effect	0.01	
YGGFMRFamide	Effect	0.003	
pQF**Y**RFamide*	No effect		Antagonist; $ID_{50} \approx 65\,\mu mol\,l^{-1}$
GSLLRFamide	No effect	<0.0005	
AcFn**L**RFamide	(No effect on neurone)		
pQDPF**L**RFamide*	(No effect on neurone)		
PQFYRFamide	No effect	<0.005	
FLFQPQRFamide	No effect	<0.01	
ALAGDHFFRFamide	No effect	<0.005	
GSLFRFamide	No effect	<0.005	
NGH**Y**MRFamide*	No effect	<0.0001	
MRFamide	No effect	<0.001	

Many of the peptides were too inactive to obtain complete dose–response curves. In these cases, effects were bracketed with known concentrations of FMRFamide on at least two preparations.

It is possible that some of these peptide, such as FLRFamide, are also partial agonist. Comparisons of dose–response curves, however, suggest that the following are all full agonists: WMRFamide, FnLRFamide, FMRWamide and FMRYamide.

nL, norleucyl; pQ, pyroglutamyl; P, prolyl; all of the amino acids are the L-form, except where specified (**D**).

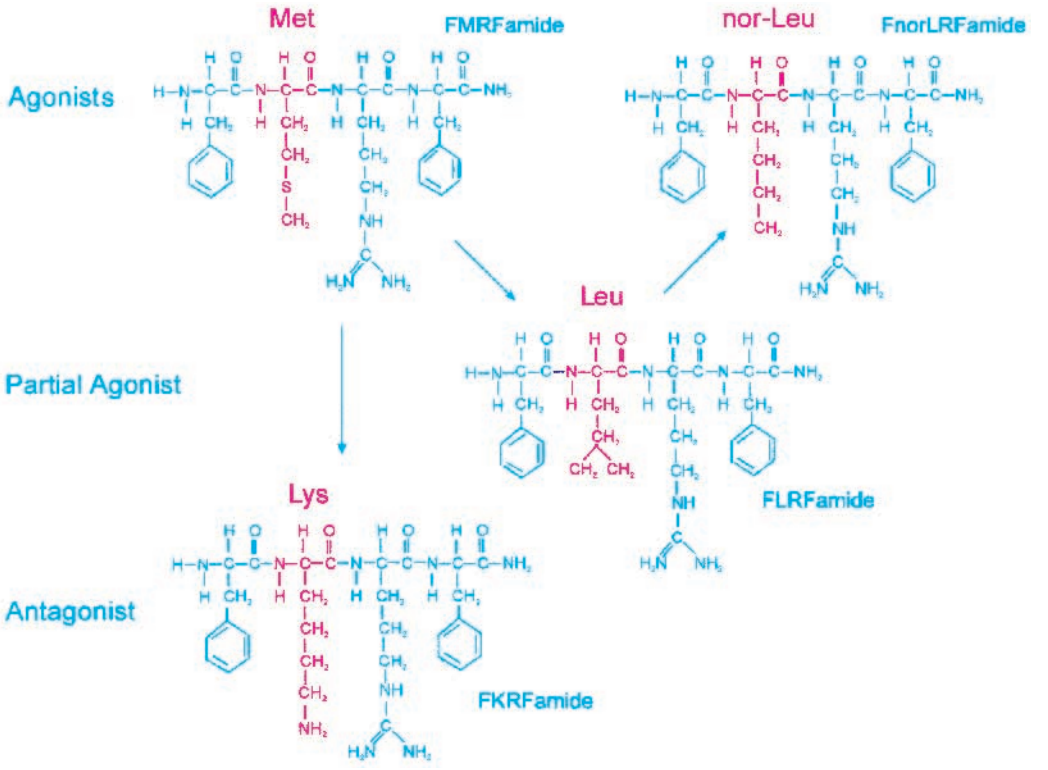

Fig. 3. Stick figure representations of the peptides FMRFamide, FLRFamide, FnorLRFamide and FKRFamide. The natural peptide FLRFamide is much less potent than FMRFamide on the oocyte-expressed *FaNaCh* and also on the neuronal channel response (see Fig. 2B). Replacement of leucine with nor-leucine increases activity so that the peptide is indistinguishable from FMRFamide on expressed-channel and neuronal responses. In contrast, substitution with lysine (FKRFamide) results in antagonist activity on the expressed channel (ID_{50} approximately 7 μmol l^{-1}) and also on the neuronal response (see Fig. 5).

the closed state with two time constants of 8 ms and 30 ms. It was noted that open states lasting several seconds occurred rarely. 100 μmol l^{-1} amiloride effectively blocked the unitary currents in outside-out patches (Lingueglia *et al.* 1995).

The single-channel conductance was similar with FLRFamide on oocyte-expressed *FaNaCh*, but at all concentrations tested the open-state time constant was shorter; 1.9 ms for FLRFamide compared with 3.9 ms for FMRFamide with the concentrations used in Fig. 4 (A. B. Zhainazarov and G. A. Cottrell, unpublished data), which at least partly accounts for its smaller maximum effect on the intact oocyte. With both peptides, open states lasting for several seconds were occasionally observed, as noted by Lingueglia *et al.* (1995) for FMRFamide.

Comparison of the properties of the heterologously expressed nucleotide sequence, FaNaCh, *with the neuronal peptide-gated ion channel*

Some properties of the neuronal- and oocyte-expressed channel are compared in Table 2. The most notable difference is the rate of desensitization in response to FMRFamide; the C2 neurone response rapidly desensitized with repeated FMRFamide applications, but desensitization of the oocyte-expressed *FaNaCh* is very slow. There is also a difference in potency of approximately threefold. This concentration difference may, however, be exaggerated because the neuronal response could decay in accessible regions of the large neurone (as a result of the marked desensitization), while access is being gained to more remote regions. This could influence the apparent affinity of the peptide. Peptide solutions are usually applied locally from a fine-tipped pipette onto the neurone, to reduce desensitization, whereas bath application is used with the oocytes. At present, the basis of neuronal desensitization is not known. Desensitization is far less pronounced, and sometimes not seen, in isolated outside-out neuronal patches. Thus, the observed differences in potency and also desensitization do not necessarily represent major differences between the neuronal channel and oocyte-expressed *FaNaCh*.

The single-channel conductance of the neuronal channel is 4 pS, and it is 11–13 pS for the expressed *FaNaCh*. Can this difference be accounted for in terms of a difference in environment within the lipid membrane of the *Helix* C2 neurone compared with the oocyte? We have recently determined that the conductance of *FaNaCh* is 12 pS in a cell line permanently expressing *FaNaCh*, kindly supplied by Drs M. Lazdunski and E. Lingueglia. Thus, in the cell membrane of two different cell types, the heterologously expressed *FaNaCh* protein has a similar conductance, highlighting the difference from the native

Table 2. *Comparison of the properties of the heterologously expressed* FaNaCh *with the neuronal peptide-gated channel*

	Oocyte	Neurone
FMRFamide ED_{50} (μmol l^{-1})	2	≈10
Amiloride ID_{50} (μmol l^{-1})	1	1–10
Single-channel conductance (ps)	11–13	4
Rate of desensitization	Very slow	Fast
Blockade by FKRFamide	Yes	Yes
FLRFamide-partial agonist	Yes	Yes

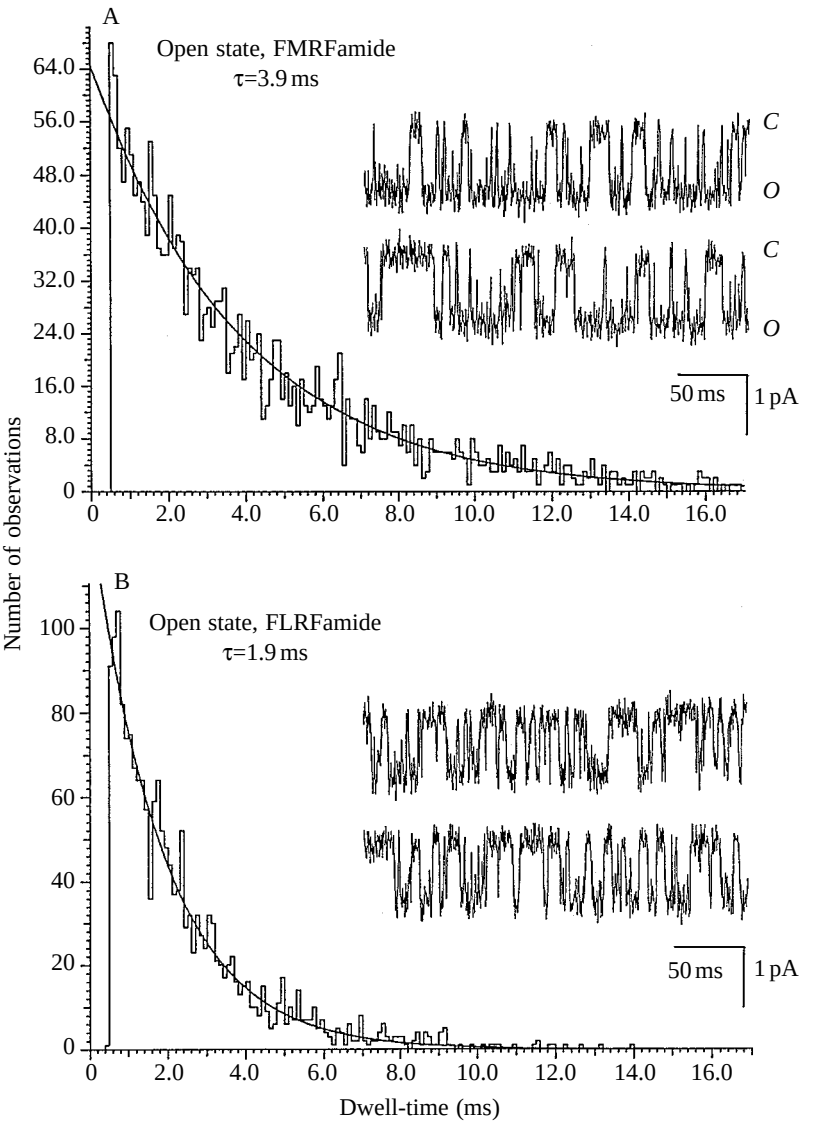

Fig. 4. FMRFamide and FLRFamide evoked a different pattern of single-current activity with *FaNaCh* in outside-out membrane patches of *Xenopus laevis* oocytes. (A) At 30 μmol l^{-1} FMRFamide (a concentration giving a maximum response), relatively long openings (*O*) with fast closings (*C*) were observed. The open-time histogram shown was fitted with a single exponential with a time constant τ of 3.9 ms. (B) Openings in the presence of 180 μmol l^{-1} FLRFamide (a concentration giving a maximum response with this peptide) were shorter; the FLRFamide open-time histogram was fitted with a time constant of 1.9 ms. Similar differences in open times were observed with lower concentrations of the peptides. Thus, the greater potency of FMRFamide may result from its ability to evoke longer channel open times (see text). The patch electrodes contained KF in place of KCl (Methfessel *et al.* 1986).

channel. Perhaps the difference is due to a different subunit composition. Alternatively, there may be a factor within the *Helix* neurones that influences the channel's conductance.

With both the expressed *FaNaCh* and the neuronal channel, maximal responses to FLRFamide were always smaller than to FMRFamide, suggesting that FLRFamide is a partial agonist. The time constant of the open state of the oocyte-expressed channel with FLRFamide was approximately half that of FMRFamide. Some data recently obtained from the neuronal channel also indicate that the open-state time constant for FLRFamide is much less than for FMRFamide (Fig. 5).

Accurate determination of the amiloride ID_{50} is more difficult on the neurone than the oocyte because of the technical problem of maintaining long-term recordings from the C2 neurone and also because of the presence of the second slower component of the neuronal response. Earlier experiments suggested an ID_{50} of approximately 10 μmol l^{-1}; more recently, data have yielded a value of 1 μmol l^{-1}. The latter is the same as that for amiloride on the heterologously expressed *FaNaCh*.

FKRFamide (phenylalanyl-lysyl-arginyl-phenylalanyl-NH_2) antagonised the action of FMRFamide on the *Helix* C2, as observed on the expressed *FaNaCh*. A blocking effect has been observed both on the whole neurone response and on activated unitary currents (see Fig. 5C). FKRFamide is the first effective competitive antagonist found for an FMRFamide receptor. Further 'molecular roulette' is likely to yield even more effective antagonists now that more is known about the structural requirements for activation.

Discussion

The FMRFamide-gated ion channel of *Helix aspersa* neurones is the first peptide-gated ion channel to be discovered. For a ligand-gated channel, it is unique in terms of its Na^+-selectivity. Previous work on transmitter-gated channels shows that they are generally selective for small cations or Cl^-. The significance of the Na^+-selectivity is not known.

It is not yet clear whether the natural channel is a homo-

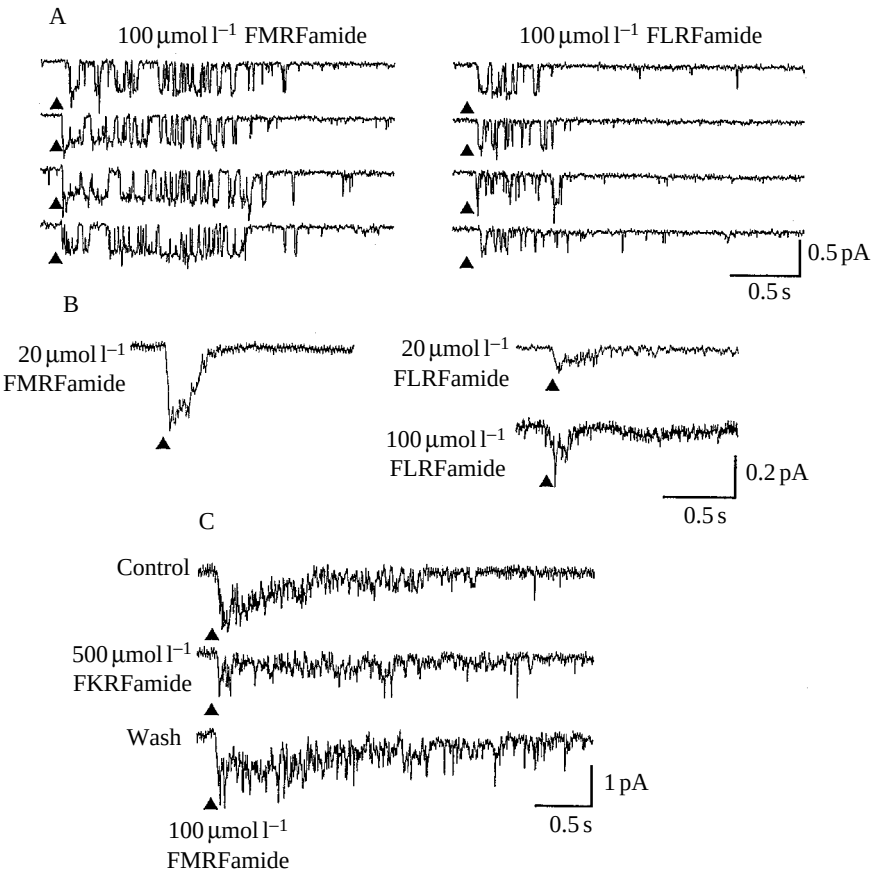

Fig. 5. Single-channel recordings from the *Helix aspersa* C2 neurone with a patch electrode containing KF in place of KCl. (A) Sample responses to applied FMRFamide on the left and FLRFamide on the right. It was noted that the pattern of openings evoked by the two peptides differed; opening durations were longer in the presence of FMRFamide, as with the expressed receptor, and they frequently persisted longer. (B) Ensemble single-channel responses, summed from several peptide applications, also show a marked difference in effects of the two peptides. The summed ensemble response to 20 µmol l^{-1} FMRFamide was much greater than that to 20 µmol l^{-1} FLRFamide and even greater than that to 100 µmol l^{-1} FLRFamide, in agreement with the view that longer openings are evoked by FMRFamide. (C) The response of an outside-out patch of neuronal membrane to FMRFamide was antagonised with a high dose of FKRFamide, which itself did not activate the channel.

oligomer of expressed *FaNaCh* or if it comprises other subunits, as with the epithelial Na^+ channels. The comparisons described above, of the expressed *FaNaCh* and the neuronal responses, show that they are not identical. However, the differences are not great and some can be explained in terms of differences in desensitization (notably not seen in some isolated neuronal patches). The differences are very much less than those observed with the *ENaCh* subunits, where current was increased by a factor of 100 when three subunits (α, β and γ) were co-expressed rather than one alone. Considered in terms of such marked differences, there is little compelling evidence at present for more than one subunit of the peptide-gated Na^+ channel. However, detailed studies on the response of the *Helix* C2 neurone have provided evidence for a second, slower component of the FMRFamide response and suggest that this component also results from the gating of a channel (Green *et al.* 1994). This channel may be related to *FaNaCh*.

Is the peptide-activated channel involved in fast synaptic transmission? Evidence at present is indirect: (a) FMRFamide exerts a fast response; (b) the cerebral ganglia, in which the C2 neurones occur, are rich in FMRFamide; (c) the peptide is detected in neuronal somata and axon processes (Cottrell *et al.* 1983); (d) neuronal release of FMRFamide has been detected in the periphery (see Bewick *et al.* 1990). Nevertheless, blockade of the synaptically evoked fast FMRFamide reponse remains to be demonstrated. This may now be possible using FKRFamide or better antagonists, if they can be found.

Unlike the other proposed receptors for FMRFamide, the peptide-gated Na^+ channel shows a high selectivity for FMRFamide. Such selectivity may partly explain the marked preponderance of FMRFamide (compared with the less readily oxidised FLRFamide) within precursor proteins from the molluscs so far studied (Greenberg and Price, 1992); the large number of FMRFamide copies could relate to the higher concentration of peptide required for activation of the ligand-gated channel compared with G-protein-coupled responses.

The physiological importance, if any, of the unique receptor-antagonism by the endogenous peptide pQFYRFamide is not known. The antagonism is weak and appears to be insignificant considered in terms of the many copies of FMRFamide encoded and processed from the precursor protein. However, some natural antagonistic role for pQFYRFamide can be envisaged if the precursor domain that encodes all the FMRFamide molecules (plus one of FLRFamide) is processed and targetted separately from the domain encoding the two pQFYRFamide molecules and the other molecule of FLRFamide. The two domains are separated by a furin-recognition sequence (see Hosaka *et al.* 1991), likely to be the first cleavage site, so such a scheme, although speculative, is feasible. Another identified *Helix* neurone, the C3 neurone, contains FMRFamide (Cottrell *et al.* 1983). It is interesting to

note that all three peptides can be detected in the C3 neurone in approximately the ratio predicted from the cDNA for the precursor protein i.e. 10 FMRFamide: 2 FLRFamide: 2 pQFYRFamide (Price *et al.* 1996*a*). It may also be relevant that pQFYRFamide is more potent than FMRFamide in evoking the slow increase in K^+ conductance.

Finally, the discovery of the *Helix* peptide-gated channel raises the question of whether there are similar ligand-gated channels in other groups of animals (others almost certainly occur in other molluscan species). At present, *FaNaCh* is the only example of a peptide-gated, amiloride-sensitive channel of the group typified by epithelial Na^+ channels (see North, 1996; García-Añoveros *et al.* 1997). *MNCh* (i.e. *MDeg* or *MNaC1*) and *MNaC2* are expressed in mammalian neurones, but their roles are not known. So far, the only related mammalian neuronal sequence (*ASIC*) whose role appears to be established is a proton-gated channel (Waldmann *et al.* 1997). The genetic studies on *C. elegans* predict many different sequences related to the amiloride-sensitive mammalian epithelial channels that are expressed in neurones and muscle cells, possibly at different stages of development. Other animals may similarly express many related sequences in neurones. It is, however, already clear that channels of this group serve diverse roles: in transporting epithelia, they regulate Na^+ transport; in neurones, at least one (the *Helix FaNaCh*) is peptide-gated, another (*ASIC*) is a proton-gated channel and yet others are probably involved in mechano-sensation.

I thank Drs Michel Lazdunski and Eric Lingueglia for supplying clones of *FaNaCh*, the Wellcome Trust for financial support and Dr M. Lazdunski for helpful discussions.

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