The Journal of Experimental Biology 202, 2581–2593 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB2313

INOSITOL-1,4,5-TRISPHOSPHATE AND INOSITOL-1,3,4,5-TETRAKISPHOSPHATE ARE SECOND MESSENGER TARGETS FOR CARDIOACTIVE NEUROPEPTIDES ENCODED ON THE FMRFamide GENE

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Accepted 30 June 1999

Summary

This paper examines the importance of the calcium-mobilizing inositol phosphate pathway in mediating the effects of FMRFamide and its gene-related neuropeptides on the myogenic heart beat of the pond snail *Lymnaea stagnalis*. These peptides are encoded on a single exon of the FMRFamide gene and mediate diverse physiological effects in the isolated heart. The rate of production of inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] and inositol-1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], measured using an HPLC method, were both significantly elevated in a concentration-dependent manner by FMRFamide (and were also elevated by FLRFamide). The threshold for increasing inositol phosphate production was low (100 pmol l^{-1}) with a peak response occurring at 1 μmol l^{-1} FMRFamide. The shape of the dose–response curve for FMRFamide-induced elevation of heart-beat frequency, obtained in pharmacological experiments on the isolated whole heart, was similar to that for stimulation of inositol phosphate levels in homogenized heart tissue. FMRFamide and Ins(1,4,5)P_3 produced similar effects on the rate of heart beat in permeabilized whole hearts. In addition, the phospholipase C inhibitor, neomycin (2.5 mmol l^{-1}), blocked the stimulatory effects of FMRFamide on Ins(1,4,5)P_3 production in heart homogenate, and attenuated the excitatory effects of this neuropeptide in the isolated heart. The 'isoleucine' pentapeptides, EFLRIamide and pQFYRIamide, also encoded by the FMRFamide gene, produced no significant effects on inositol phosphate production when applied alone or in combination with FMRFamide. These results suggested that FMRFamide (and FLRFamide), but not EFLRIamide and pQFYRIamide, mediated their main effects on heart beat *via* the inositol phosphate pathway. The fifth peptide, SEQPDVDDYLRDVVLQSEEPLY ('SEEPLY') had no effect when applied alone but appeared to modulate the effects of FMRFamide by delaying the time-to-peak of the Ins(1,4,5)P_3 response from 5 s to 20 s by an unknown mechanism.

Key words: FMRFamide-related peptide, heart, inositol phosphate, multiple peptide signalling, *Lymnaea stagnalis*.

Introduction

Neuropeptides are now recognized as the largest and most diverse class of neurotransmitter molecules. Previous research has shown that these signalling molecules are commonly colocalized in neurones with classical neuroactive substances or other peptides (Cottrell, 1977; Lloyd, 1982; Osborne and Dockray, 1982). Neuropeptides are synthesized from genes, which often code for multiple structurally related or non-related peptides that may act together to mediate physiological effects directly following their release, or may modulate the effects of other coreleased messengers (Kupfermann, 1991; Weiss et al., 1993). At present the mechanisms by which coreleased neuropeptides interact at the cellular level is poorly documented. Most evidence suggests that neuropeptides act *via* G-proteins to up- or downregulate the production of intracellular second messengers. Identifying the second messenger targets for neuropeptides is therefore an important objective for studies aimed at understanding the mechanisms of action of coreleased peptides.

In this paper we identify a major second messenger pathway involved in mediating the physiological effects of the neuropeptide, FMRFamide (Phe-Met-Arg-Phe-NH_2), and some of its related peptides that are encoded on the same exon of a multi-exonic gene in the pond snail, *Lymnaea stagnalis*. Although FMRFamide was originally isolated from molluscs (Price and Greenberg, 1977), FMRFamide and related peptides have been found in species representing the whole spectrum of invertebrate and vertebrate phyla, including humans (Greenberg and Price, 1992; Yang et al., 1985; Perry et al., 1998). FMRFamide is a cardioexcitatory agent in many molluscan systems (Price et al., 1987) including *L. stagnalis*, in which it has previously been shown to enhance the beat frequency of the isolated auricle significantly. FMRFamide

produced less consistent stimulatory effects on the amplitude of individual heart beats and the underlying tonus of the heart (Buckett et al., 1990a). These studies concluded that FMRFamide was probably a primary peptide transmitter in the heart of this mollusc (Buckett et al., 1990b). However, it is now realized that a variety of other peptides are also likely to be involved in cardiac regulation in *L. stagnalis*. In addition to FMRFamide, four other peptides encoded on the FMRFamide gene of *L. stagnalis* are thought to be cotransmitters in a pair of cardioexcitatory motoneurons called the E_{he} (E heart excitor) neurons (Benjamin and Burke, 1994). Consequently, the heart of *L. stagnalis* has become a model system for investigating the mechanisms of action of multiple neuropeptides of common neuronal origin that act on a single target organ.

In *L. stagnalis*, FMRFamide and the four other peptides are encoded on exon II ('tetrapeptide' exon) of a complex multiexonic gene (Linacre et al., 1990; Kellett et al., 1994). The five peptides can be divided into three classes based on their amino acid composition and pharmacological effects: the tetrapeptides FMRFamide and FLRFamide, the pentapeptides EFLRIamide and pQFYRIamide (referred to as the 'isoleucine' peptides, due to the terminal isoleucine amino acid), and a 22-amino-acid peptide SEQPDVDDYLRDVVLQSEEPLY (abbreviated to 'SEEPLY'). The existence of the five peptides has been confirmed by sequencing and mass spectrometry (Ebberink et al., 1987; Santama et al., 1993, 1995b) and they have been localized to both the central nervous system (Santama et al., 1993, 1995a,b) and heart tissue (Buckett et al., 1990a; Santama et al., 1993, 1995b). Peptides in the heart are thought to be localized to the nerve terminals of the E_{he} motoneurons. These cells have been shown by *in situ* hybridization specifically to express exon II (Santama et al., 1995a) and FMRFamide/FLRFamide, SEEPLY and pQFYRIamide/EFLRIamide by immunocytochemistry (Buckett et al., 1990a; Santama et al., 1993, 1995b). The presence in the E_{he} motoneurons of all five exon II-specific neuropeptides has recently been confirmed by Maldi-ToF mass spectrometry (Worster et al., 1999). A third type of isoleucine peptide, pQFLRIamide, was discovered by Santama et al. (1995b), and although it is not encoded on the FMRFamide precursor (Linacre et al., 1990), it has been found in heart tissue. At present the origin of this peptide is unknown; however, its effects on the heart were tested in this study for comparison with the structurally related pentapeptides EFLRIamide and pQFYRIamide.

In contrast to the purely cardioexcitatory effects of FMRFamide, the 'isoleucines' are thought to produce more complex biphasic effects in the heart, consisting of both an inhibitory and an excitatory phase (Santama et al., 1995b). The third class of exon II-encoded peptide, SEEPLY, is believed to be purely modulatory. Recent experiments have shown that it can modulate the ability of FMRFamide to open Ca^{2+} channels in dissociated heart muscle cells of *L. stagnalis*, but has no effect when applied alone (Brezden et al., 1998).

At present the cellular and biochemical mechanism of action of multiple neuropeptides originating from a single gene (or exon) is poorly understood in any system, including the *L. stagnalis* cardioactive peptides. In the present study biochemical and pharmacological techniques were therefore combined to identify candidate second messenger targets for each of the three cardioactive peptide classes encoded on exon II of the FMRFamide gene. The functional roles of the intracellular signalling pathways in the heart of *L. stagnalis* were also examined by pharmacological methods. These results highlight the important role of the calcium-mobilizing inositol phosphate pathway in mediating the effects of FMRFamide and its exon II-related peptides.

Materials and methods

Experimental animals

Lymnaea stagnalis (25–40 mm shell length) were supplied by Blades biological (Kent, UK) or the Biology Department of the Vrije Universiteit (Amsterdam). All snails were maintained at 20 °C and subjected to a 12 h:12 h L:D cycle. Animals were fed *ad libitum* on lettuce.

Synthetic peptides and pharmacological agents

FMRFamide and FLRFamide were purchased from Sigma Chemicals Inc. (Dorset, UK). The peptides EFLRIamide, pQFYRIamide, pQFLRIamide, SEQPDVDDYLRDVVLQSE-EPLY ('SEEPLY') and EDRVYDSQPYLLDSEPDLEVQV ('RSEEPLY') were synthesized in our laboratory, and checked for purity by HPLC. All pharmacological agents were purchased from Sigma (Dorset, UK) unless stated otherwise. The radioisotope [^{3}H]*myo*-inositol was purchased from Amersham International (Bucks., UK). Liquid scintillant (Ecolite$^+$) was purchased from ICN Pharmaceuticals (Oxon., UK). ^{32}P-labelled inositol phosphate standards for Ins(1,3,4,5)P_4, Ins(1,4,5)P_3 and Ins(1,4)P_2 were kindly donated by Professor Robin Irvine and Dr Pete Cullen of the Babraham Institute (Cambridge, UK).

Extraction of inositol phosphates

Whole hearts from *L. stagnalis* were carefully dissected out, placed in Hepes-buffered saline (HBS) containing (in mmol l^{-1}): NaCl (24), KCl (2), $CaCl_2$ (4), $MgCl_2$ (2), NaH_2PO_4 (0.1), NaOH (35), glucose (1), Hepes (50) adjusted to pH 7.9 (Benjamin and Winlow, 1981). The hearts were chopped into 3 or 4 pieces (8–10 hearts were required to produce detectable levels of inositol phosphate production for each sample in an experiment). The chopped hearts were transferred to fresh saline and incubated with 200 µl of 14.7 µmol l^{-1} [^{3}H]*myo*-inositol plus 400 µl of HBS at 8 °C for 22 h and at 20 °C for an additional 2 h. This length of incubation was adequate for ^{3}H-inositol phosphate metabolites to reach an equilibrium with cytosolic [^{3}H]*myo*-inositol (Irvine et al., 1985). The tissue was washed 3 times with fresh HBS containing 10 mmol l^{-1} unlabelled inositol over a 30 min period. Incubation with unlabelled 10 mmol l^{-1} inositol was then continued for a further

60 min at room temperature. This was essential to chase labelled inositol from the tissue and prevent further inositol phospholipid labelling.

The chopped heart tissue was then homogenized and 200 µl samples placed into clean Eppendorf tubes. Samples were then centrifuged at 13 000 revs min^{-1} (approximately 8,000 ***g***) in a microcentrifuge for 5 min and the supernatant replaced with 100 µl of fresh HBS. Each resuspended sample of homogenate was then incubated with a further 100 µl of HBS containing the appropriate peptide or pharmacological agent plus 2 mmol l^{-1} ATP, for a set period of time (varying from 0 to 2 min). The incubation was terminated by the addition of 50 µl of 15 % w/v trichloracetic acid. The mixture was then washed with 2×50 µl of water-saturated diethyl ether and samples neutralized with 50 µl of 1 mol l^{-1} KOH (all samples were between pH 6.0 and 7.0). Each sample was evaporated to dryness using a Speed-Vac, and stored at −80 °C for up to 3 days until the time of HPLC separation.

HPLC separation of inositol phosphates

The column used for HPLC separation of the inositol phosphates was a Partisil Sax 10 anion exchange column (0.46 cm × 25 cm, packed by Technicol, Cheshire, UK). Buffer 'A' consisted of HPLC grade water. Buffer 'B' contained 1·5 mol l^{-1} ammonium formate buffered to pH 3.7 with orthophosphoric acid (i.e. the P_i content was approximately 0.5 mol l^{-1}). An elution programme was set up to control the flow of buffers 'A' and 'B' through the column during the 46 min that followed the injection of each sample. Details of this programme are as follows: 100 % buffer 'A', 0 % buffer 'B' was allowed to flow through the column for 4 min at 1.25 ml min^{-1}. This flow rate of 1.25 ml min^{-1} was maintained throughout the elution. A linear gradient was then passed through the column over a 20 min time period, rising from 100 % buffer 'A', 0 % buffer 'B', to 0 % buffer 'A', 100 % buffer 'B'. A flow of 100 % buffer 'B' through the column was maintained for a further 10 min. During the following 2 min the gradient was linearly returned to 100 % buffer 'A', 0 % buffer 'B'. A 10 min water elution (100 % buffer 'A') was then employed before the column was ready for the next sample injection.

Prior to HPLC analysis of the inositol phosphate extracts, preliminary trials were performed using adenine nucleotide markers AMP, ADP and ATP, which have previously been shown to co-elute with $InsP_1$, $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$, respectively. Their elution was followed by measuring their absorbance at 254 nm (Irvine et al., 1985). Each experimental sample was dissolved in 200 µl water and routinely spiked with a 50 µl mixture containing 3.3 nmoles of each nucleotide marker before loading on to the column. Routine spiking with nucleotides helped ensure that the elution of each sample from the column was normal by checking the absorbance of the nucleotide markers.

Fractions were collected at 0.5 min intervals throughout each sample run (finishing approximately 10 min after the ATP had eluted). Each fraction was diluted with 0.5 ml water and 0.5 ml methanol. Radioactivity within each fraction was counted for 1 min in a Beckman β-scintillation counter following the addition of 5 ml of liquid scintillant (Ecolite$^+$). The production of inositol-1,4,5-trisphosphate [$Ins(1,4,5)P_3$], inositol-1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$] or total inositol phosphate production [inositol monophosphates ($InsP_1$) + inositol bisphosphates ($InsP_2$) + $Ins(1,4,5)P_3$ + inositol-1,3,4-trisphosphate ($Ins(1,3,4)P_3$) + $Ins(1,3,4,5)P_4$] were expressed as a percentage change from controls.

Whole heart pharmacological experiments

The location of the heart was first determined following removal of the shell. The skin was cut to expose the heart, which was then isolated by cutting through the aorta at the base of the ventricle, and cutting as far above the auricle as possible. Once removed from the snail, the heart was maintained in a perspex organ bath and perfused by means of a cannula that passed into the auricle and was secured by a length of silk thread. The other end of the cannula was attached to a 2-way tap allowing rapid switching between drug solutions and HBS. The heart was constantly perfused with solution to maintain a steady heart beat. A small hook was passed through the ventricle of the heart and attached, *via* a length of silk thread, to an auxotonic force transducer.

The length of time over which peptide and drug solutions were perfused through the isolated heart varied between 30 s and 2 min depending on the type of experiment being performed. Normal HBS was perfused between each application of peptide or pharmacological agent for a minimum of 8 min to allow the heart beat to return to a steady rate. In addition the solution bathing the outside of the heart was constantly perfused using a peristaltic pump. Heart beat was permanently displayed on a chart recorder (Gould Instruments, Hainault, UK). A wide range of peptides and drugs were applied to the heart following their dilution in HBS. Non-membrane permeable drugs were diluted in HBS containing 0.1–1.0 % (v/v) dimethylsulphoxide (DMSO; a membrane permeabilizing agent) to facilitate their entry into the heart muscle cells.

Statistics

Data are reported as mean ± S.E.M. Means were compared using the Student's *t* test. $P<0.05$ was considered significant and *N* was the number of experiments carried out in the biochemical experiments, or the number of hearts used in the pharmacological experiments.

Results

Biochemical analysis of inositol phosphates

The inositol phosphate pathway present in heart tissue from *L. stagnalis* appeared to have similar components to that detailed in other systems (Berridge, 1993). Various metabolites of this pathway, including two identifiable isomers of $InsP_3$ and $Ins(1,3,4,5)P_4$, could be extracted from snail heart tissue and their levels detected using the sensitive HPLC separation

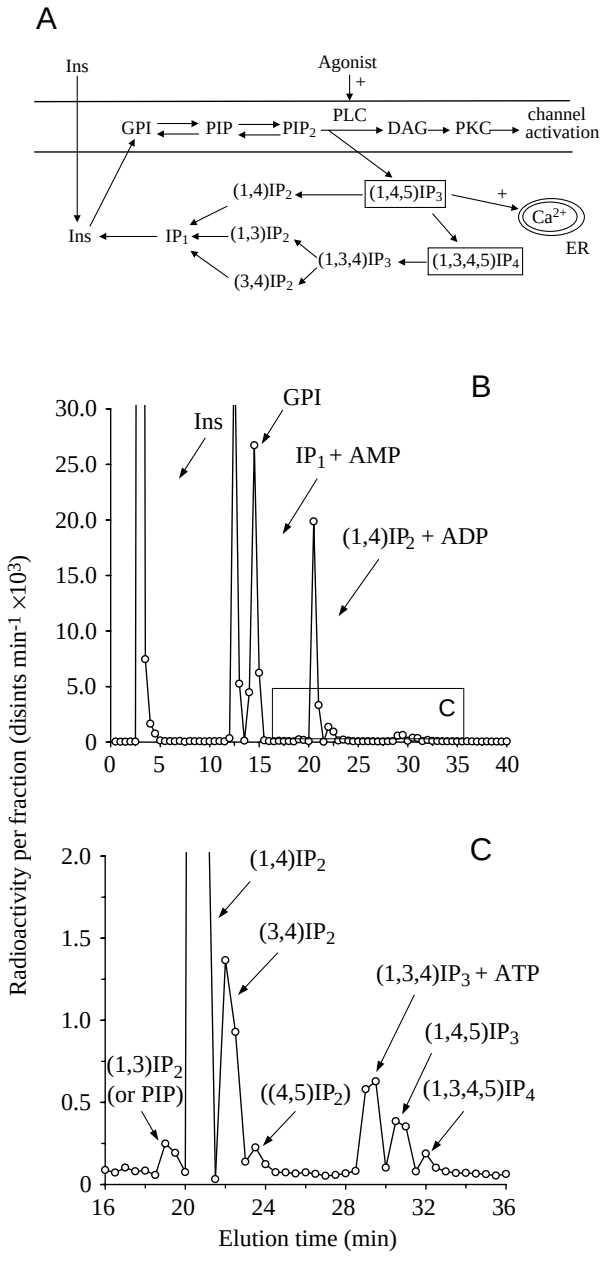

Fig. 1. HPLC separation of inositol phosphates from the heart tissue of *Lymnaea stagnalis*. (A) Details of the inositol phosphate cycle involved in the generation and degradation of inositol trisphosphate [Ins(1,4,5)P_3] and inositol tetrakisphosphate [Ins(1,3,4,5)P_4]. (B) A typical HPLC separation profile of inositol phosphates extracted from homogenized heart of *L. stagnalis*. The peaks were identified by comparing the times of elution with those of radioactive standards and adenine nucleotide markers. The boxed area in B is shown in greater detail in (C). All of the expected components of the inositol phosphate pathway can be identified. Ins, inositol; GPI, glycerol phosphoinositide; PIP, phosphatidyl inositol phosphate; PIP_2, phosphatidyl inositol bisphosphate; PLC, phospholipase C; DAG, diacyl glycerol; PKC, phosphokinase C; ER, endoplasmic reticulum; IP_1, inositol monophosphates; IP_2, inositol bisphosphates; IP_3, inositol trisphosphates, IP_4, inositol tetraphosphates.

method. Combined analysis of HPLC profiles using radioactive standards, nucleotide markers and time of elution enabled the identification of all of the major peaks expected from the inositol phosphate pathway (Fig. 1A). Peaks illustrated in Fig. 1B were identified as *myo*-inositol (Ins), the inositol monophosphates ($InsP_1$) and the inositol bisphosphates ($InsP_2$), which are all metabolites of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Glycerol phosphoinositide (GPI) is a phospholipid precursor for phosphatidyl inositol bisphosphate (PIP_2) (and consequently Ins(1,4,5)P_3 and diacyl glycerol, DAG), as indicated in Fig. 1A. Ins(1,3,4,5)P_4, Ins(1,4,5)P_3 and Ins(1,4)P_2 peaks were identified by their co-elution with ^{32}P-labelled standards. $InsP_1$ was identified by co-elution with AMP, Ins(1,4)P_2 co-eluted with ADP, and the Ins(1,3,4)P_3 peak was identified by its co-elution with ATP. Glycerol phosphoinositide and Ins(1,3)P_2, Ins(3,4)P_2 and Ins(4,5)P_2 peaks were identified by their position in the elution profile based on previous data (Irvine et al., 1985). A segment of the HPLC trace shown in Fig. 1B was enlarged in Fig. 1C and shows elution of the inositol phosphates of particular interest [i.e. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4], which are believed to play a secondary messenger role in the heart tissue. 2 mmol l^{-1} ATP was used in all experiments to enhance the levels of activity in the inositol phosphate pathway (Berridge, 1983).

Effects of the exon II-encoded peptides on inositol phosphate production

The effects of the exon II-encoded FMRFamide-related peptides on inositol phosphate production were analysed following a 5 s application of each of the peptide classes. 5 s was sufficient time for FMRFamide to mediate its maximum effects on the isolated heart preparation (Buckett et al., 1990a).

The tetrapeptides

HPLC analyses of heart extracts from *L. stagnalis* revealed that the rate of production of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in the tissue was elevated following applications of low concentrations of FMRFamide. Fig. 2A shows an example from one experiment where 1 μmol l^{-1} FMRFamide was applied to the heart homogenate. Rates of production of both isomers of $InsP_3$ (Ins(1,4,5)P_3, Ins(1,3,4)P_3) and Ins(1,3,4,5)P_4 were more than doubled compared to controls. FMRFamide-induced increases in both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were shown to be concentration-dependent (Fig. 2B, N=11). Statistically significant increases in FMRFamide-stimulated inositol phosphate production occurred above a threshold concentration of 100 pmol l^{-1} for Ins(1,4,5)P_3 and 10 nmol l^{-1} for Ins(1,3,4,5)P_4 (Fig. 2B). The peak response occurred following application of 1 μmol l^{-1} FMRFamide and declined at higher concentrations. Maximum increases at 1 μmol l^{-1} FMRFamide were greater for Ins(1,4,5)P_3 (250 % compared with controls; P<0.001, N=11) than Ins(1,3,4,5)P_4 (80 % compared with controls; P<0.001, N=11). If the increases in metabolite production due to FMRFamide stimulation were combined to give total inositol phosphate production ($InsP_1$+$InsP_2$+$InsP_3$+$InsP_4$), then a similar type of

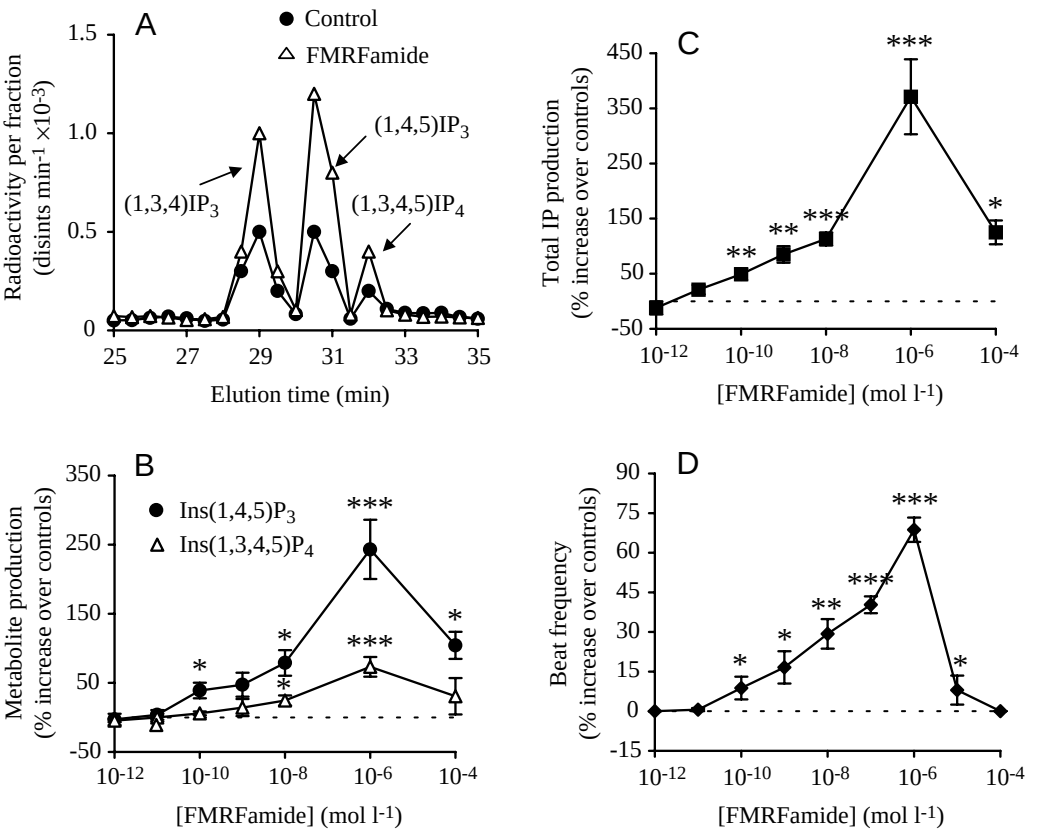

Fig. 2. FMRFamide increases $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ production in heart tissue homogenate. (A) A representative example of the HPLC separation of inositol phosphates extracted from homogenized heart tissue. The effects of a 5 s application of 1 μmol l^{-1} FMRFamide on production of both IP_3 isomers and also $Ins(1,3,4,5)P_4$ are compared to control levels. (B) Dose-dependent stimulation of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ production during FMRFamide application. Each point is plotted as mean percentage increase over control ± S.E.M. (N=11). (C) Dose–response curve for FMRFamide plotted against increase in total inositol phosphate production. (D) Dose–response curve for FMRFamide effects on beat rate plotted as percentage increase in maximum beat rate over controls. FMRFamide was applied for periods of 30 s in pharmacological experiments on the whole heart. Asterisks indicate values significantly greater than control; *P<0.05, **P<0.01 and ***P<0.001.

dose–response curve was obtained (Fig. 2C). The sensitivity was even greater, however, with a threshold concentration as low as 100 pmol l^{-1} (P<0.05) and a maximum response at 1 μmol l^{-1} FMRFamide of approximately 350 % compared with controls (P<0.001, N=11). This indicated that a proportion of $InsP_3$/$InsP_4$ had already been broken down to its various metabolites within the 5 s incubation period with FMRFamide. Analysis of the rate of production of $InsP_1$ and $InsP_2$ revealed an increase at 5 s of 1 μmol l^{-1} FMRFamide incubation. The rate of production of $InsP_1$ was increased by 8±3 %, and $InsP_2$ by 44±9 % (P<0.05, N=11) compared with controls.

If the $InsP_3$/$InsP_4$ pathway was mediating the pharmacological effects of FMRFamide on the snail heart then it might be expected that the dose–response curve for the inositol responses to FMRFamide would be similar to that resulting from FMRFamide application to the whole heart. The latter was plotted as the peak increase in beat frequency in response to a 30 s application of FMRFamide (N=22 hearts). Comparison of the pharmacological data (Fig. 2D) with that for total inositol phosphate production (Fig. 2C) revealed a striking similarity in the sensitivity of both parameters to FMRFamide application. Threshold and maximal doses of FMRFamide were the same in whole hearts as those values obtained by stimulating the inositol phosphate pathway in *L. stagnalis* heart homogenate (Fig. 2B,C). Other parameters of heart beat (i.e. force of contraction and tonus of the muscle) also had similar dose–response curves (data not shown).

The 'isoleucine' pentapeptides

Incubation of the heart tissue with either 1 μmol l^{-1} EFLRIamide, 1 μmol l^{-1} pQFYRIamide or 1 μmol l^{-1} pQFLRIamide did not significantly affect the rate of production of any of the inositol phosphate metabolites (N=6). These data were plotted in Fig. 3 as a percentage change in total inositol phosphate production (i.e. $InsP_1$+$InsP_2$+$InsP_3$+$InsP_4$). This result was independent of the period of incubation (5 s or 20 s). A 5 s incubation was chosen because it coincided with the point at which these peptides produced their maximum inhibitory effect in whole-heart physiological experiments. In contrast, a 20 s incubation period with the peptides corresponded to the delayed excitatory response of the heart to EFLRIamide, pQFYRIamide and pQFLRIamide (pharmacological data not shown). These results suggested that $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ were not involved in mediating the physiological effects of the 'isoleucine' peptides on the heart of *L. stagnalis*.

SEEPLY

The third class of exon II-encoded FMRFamide-related peptide, SEEPLY, was also found to have no effect on the inositol phosphate production when applied to heart

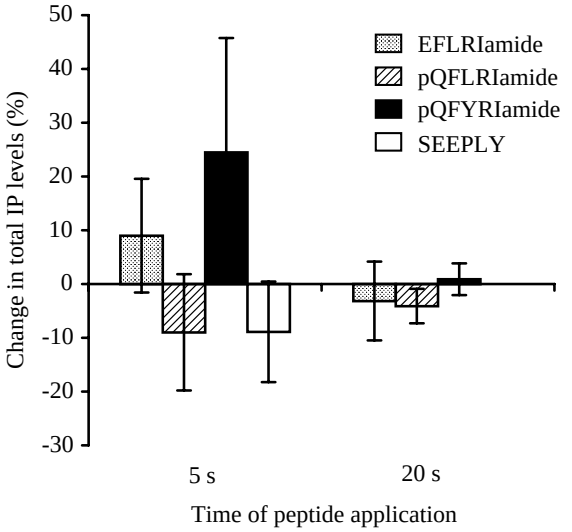

Fig. 3. The 'isoleucine' peptides and SEEPLY have no significant effects on inositol phosphate production. Data are presented as the percentage change in total inositol phosphate levels in experimental preparations compared with controls. Peptides were applied for 5 or 20 s. 1 μmol l^{-1} EFLRIamide, 1 μmol l^{-1} pQFLRIamide and 1 μmol l^{-1} pQFYRIamide did not significantly affect inositol phosphate production (N=6). Similarly a 5 s incubation of heart tissue with 10 μmol l^{-1} SEEPLY had no significant effects (N=4). All values are presented as mean ± S.E.M. (N=6).

homogenates. Fig. 3 shows that there was no significant difference in total inositol phosphate production following a 5 s incubation with 10 μmol l^{-1} SEEPLY compared with controls (N=4). Further experiments involving the application of different concentrations of SEEPLY varying between 100 nmol l^{-1} and 10 μmol l^{-1} for incubation times of 5, 15, 30 and 120 s again showed no significant change in the rate of production of the main components of the inositol phosphate signalling pathway (data not shown).

Stimulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production is dependent on the duration of FMRFamide incubation

Information on the time course of the stimulating effects of FMRFamide on the inositol phosphate pathway would allow more detailed comparisons to be made with the pharmacological responses of the peptide on the whole heart. This was examined by incubating homogenized heart tissue with 1 μmol l^{-1} FMRFamide for varying periods and then measuring the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Batches of homogenized heart tissue were incubated for specific periods of time (3, 5, 10, 15, 30, 45 and 120 s) and rate of production of the inositol phosphates measured (Fig. 4A,B).

The rate of production of Ins(1,4,5)P_3 (Fig. 4A) and Ins(1,3,4,5)P_4 (Fig. 4B) rose rapidly between 3 and 5 s following the application of 1 μmol l^{-1} FMRFamide. At 5 s after FMRFamide incubation the production of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 was significantly greater than in control tissue (P<0.001, N=11). The time of incubation required to produce maximum Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production (5–10 s) coincides with the onset of the maximum excitatory effects of FMRFamide in the isolated whole heart preparation (Fig. 4C). During longer exposures to FMRFamide (10–120 s), production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 slowly returned to control levels. As predicted, the rate of production of the metabolites of these two secondary messengers (e.g. $InsP_1$ or $InsP_2$) increased after the longer periods of incubation (30, 45 and 120 s) (data not shown).

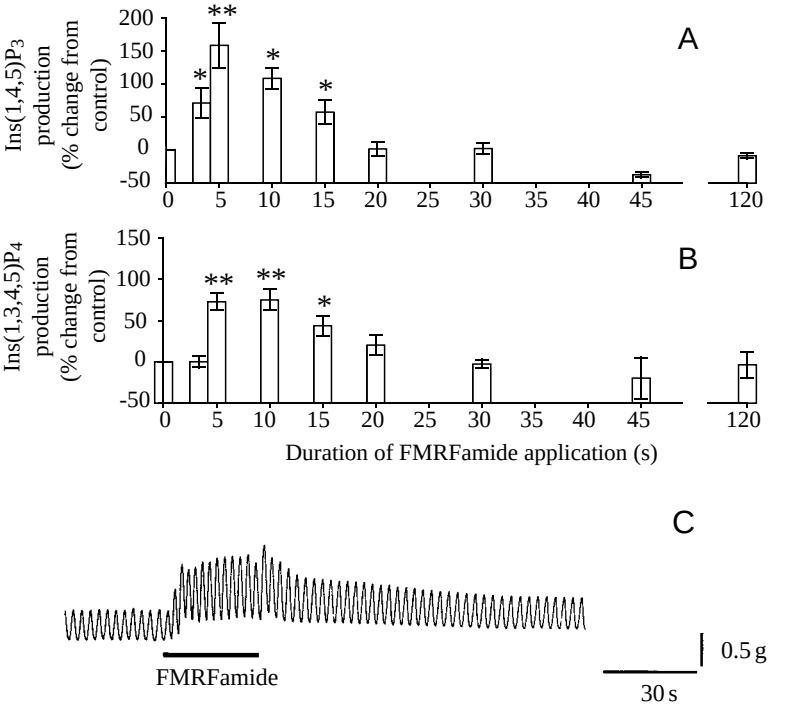

Fig. 4. Time course of increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production following the application of 1 μmol l^{-1} FMRFamide measured over a 2 min period. (A) Ins(1,4,5)P_3 production following application of 1 μmol l^{-1} FMRFamide, calculated as a percentage increase over control production. Values were taken at 0, 3, 5, 10, 15, 20, 30, 45 and 120 s of FMRFamide incubation. (B) Production of Ins(1,3,4,5)P_4 in the same samples, calculated as a percentage of controls. Values are mean ± S.E.M. (N=11). Asterisks indicate values significantly different from control; *P<0.05, **P<0.001. (C) An example of the physiological effects of 1 μmol l^{-1} FMRFamide when perfused through the isolated whole heart. 1 μmol l^{-1} FMRFamide was applied for the duration indicated. The vertical scale bar calibrates the increase in underlying tonus of the heart muscle.

These data give an indication of the rate of Ins(1,4,5)P_3 production during the perfusion of FMRFamide through the isolated heart. It is possible that the peak rate of Ins(1,4,5)P_3 production under more physiological conditions might be limited by the access of the peptide to its receptor. Nevertheless, it would appear that FMRFamide has the potential to stimulate inositol phosphate production significantly within 3–5 s.

The data so far described were obtained from experiments on homogenate prepared from the whole heart. However, a smaller number of experiments compared FMRFamide-stimulated increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production in homogenates of separated auricle and ventricle tissue from the heart (*N*=2). Tissue from both parts of the heart responded to FMRFamide stimulation with similar potency following a 5 s FMRFamide incubation. A second tetrapeptide, FLRFamide, which is also encoded by exon II of the FMRFamide gene in *L. stagnalis*, was tested for its stimulating effects on whole hearts in a small number of experiments (*N*=2), and produced equipotent effects to those mediated by FMRFamide (data not shown).

FMRFamide and Ins(1,4,5)P_3 produce comparable excitatory effects in the isolated heart of L. stagnalis

In pharmacological experiments, FMRFamide produced increases in beat frequency, force of contraction (beat amplitude) and underlying tonus on the isolated heart of *L. stagnalis* (Fig. 5Ai). The threshold dose of FMRFamide required to excite the heart was found to be approximately 100 pmol l^{-1} (Fig. 2D). This concentration was comparable to that required to stimulate the inositol phosphate pathway in the heart (Fig. 2C), suggesting that the inositol pathway might be important for mediating the excitatory effects of FMRFamide on the whole heart. More direct evidence for this hypothesis would be to show that Ins(1,4,5)P_3 could produce similar effects to FMRFamide when applied to the isolated heart. At the time of these experiments, no permeable analogues of Ins(1,4,5)P_3 were commercially available, so 0.1–1.0 % dimethylsuphoxide (DMSO) was used to permeabilize the heart tissue prior to Ins(1,4,5)P_3 application (5 mmol l^{-1}). DMSO applied alone did not produce any obvious stimulatory effects on the resting heart beat (Fig. 5Aiii), nor did it interfere with the normal beating pattern of the heart.

The excitatory effects of a 30 s application of 1 μmol l^{-1} FMRFamide in the heart were similar both in normal saline and after DMSO treatment (compare Fig. 5Ai with Bi). 5 mmol l^{-1} Ins(1,4,5)P_3, perfused directly through a permeabilized isolated heart preparation, was able to produce cardioexcitatory responses with an increase in the frequency and amplitude of heart beat (Fig. 5Bii, *N*=3). An increase in tonus was also seen but this was weaker than that occurring during the application of FMRFamide (compare Fig. 5Bii with Bi). No response to Ins(1,4,5)P_3 was seen when it was perfused through a non-permeabilized heart (Fig. 5Aii). Similar results to this were obtained in two other separate experiments. Statistical analysis of these data show that Ins(1,4,5)P_3 produced significant increases in beat frequency (35±5 % increase from controls, $P<0.05$) and in tonus of the

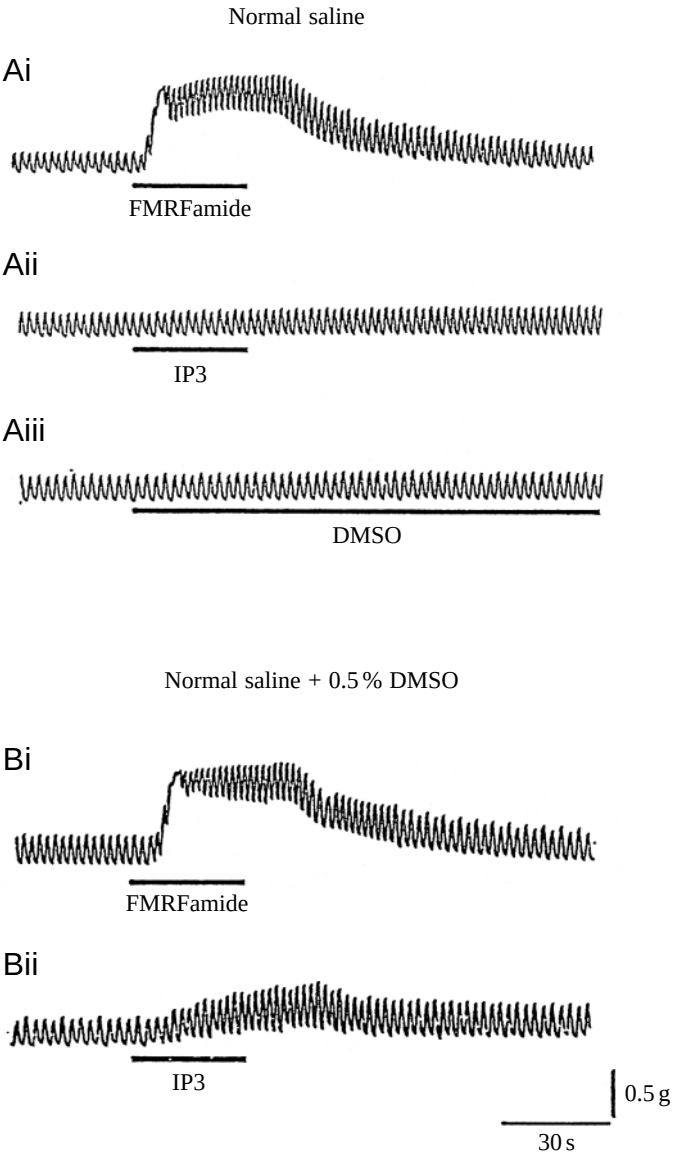

Fig. 5. Recordings to show the similarity between the effects of 1 μmol l^{-1} FMRFamide and 5 mmol l^{-1} Ins(1,4,5)P_3 in a permeabilized whole heart preparation. (Ai) 1 μmol l^{-1} FMRFamide produces increases in beat frequency, beat amplitude and tonus of the heart when perfused in normal saline. (Aii) 5 mmol l^{-1} Ins(1,4,5)P_3 had no effect on heart beat when perfused in normal saline. (Aiii) shows maintenance of a regular beat rate when perfusion saline is switched from normal saline to saline containing 0.5 % DMSO to permeabilize the heart tissue to Ins(1,4,5)P_3. (Bi) The excitatory response to 1 μmol l^{-1} FMRFamide in the permeabilized heart is comparable to the control FMRFamide response shown in (Ai). (Bii) Cardioexcitatory effects of 5 mmol l^{-1} Ins(1,4,5)P_3 when applied to the heart following permeabilization with 0.5 % DMSO include increases in beat frequency, beat amplitude and a small increase in tonus of the heart. The horizontal bar indicates the duration of each application. The vertical scale bar calibrates the increases in underlying tonus of the heart.

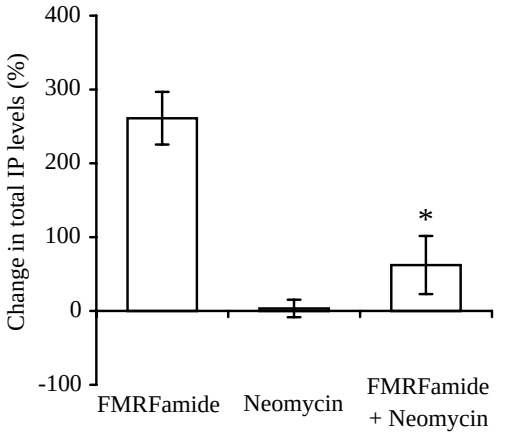

Fig. 6. Summary of the inhibitory effects of neomycin on FMRFamide-stimulated inositol phosphate (IP) production in homogenized heart muscle tissue. Data are plotted as percentage change of total inositol phosphate production in experimental *versus* control tissue. The stimulatory effects of a 5 s application of 1 μmol l^{-1} FMRFamide were reduced following a 10 min pretreatment of the tissue with 2.5 mmol l^{-1} neomycin. A 10 min incubation of the heart tissue with 2.5 mmol l^{-1} neomycin alone had no significant effect on the total inositol phosphate production, which remained at control levels. Each value is plotted as mean ± S.E.M. (*P<0.05, N=5).

permeabilized hearts (0.23±0.02 g increase from controls, P<0.05). The increases in beat amplitude were not statistically significant (57±23 % increase from controls). These data were consistent with the hypothesis that Ins(1,4,5)P_3 was at least partly responsible for the three main types of effects that FMRFamide can produce on the heart.

Neomycin inhibits the effects of FMRFamide in the heart

The ability of the phospholipase C inhibitor, neomycin, to block stimulation of the Ins(1,4,5)P_3 pathway by FMRFamide was ascertained. Neomycin has been shown to inhibit agonist-induced Ins(1,4,5)P_3 production in a number of cell and tissue types (Phillippe, 1994; Christ et al., 1995; Abdulghani et al., 1996; Stanimirovic et al., 1996) and would be expected to have similar effects in the snail heart. Pretreatment of heart muscle extracts from *L. stagnalis* with 2.5 mmol l^{-1} neomycin for 10 min significantly reduced the ability of FMRFamide (1 μmol l^{-1}) to stimulate Ins(1,4,5)P_3 production (Fig. 6). Neomycin reduced FMRFamide-stimulated inositol phosphate production by approximately 65 % on average (P<0.05, N=5). Application of 2.5 mmol l^{-1} neomycin alone produced no significant change in the rate of production of the inositol phosphates compared with control rates.

These biochemical data indicated that neomycin could significantly block the excitatory effects of FMRFamide on Ins(1,4,5)P_3 production, and were consistent with the hypothesis that Ins(1,4,5)P_3 is an important mediator of the excitatory effects of FMRFamide in *L. stagnalis*. Also supportive of this hypothesis were experiments on the whole heart showing that a 10 min pre-treatment with 2.5 mmol l^{-1} neomycin was able to significantly reduce the cardioexcitatory effects of a 30 s application of 1 μmol l^{-1} FMRFamide (Fig. 7Aii, N=9) compared with a control application of FMRFamide in normal saline (Fig. 7Ai). The overall excitatory response to FMRFamide recovered after 85 min wash in normal saline (Fig. 7Aiii). Statistical analysis of data from nine experiments showed significant reductions in the ability of FMRFamide to increase all three parameters of heart beat: beat frequency (Fig. 7B) (P<0.05), beat amplitude (Fig. 7C) and tonus (Fig. 7D) (P<0.01). Statistical analysis also showed that the effects of neomycin on beat amplitude (Fig. 7C) and tonus (Fig. 7D) were partially reversed by washing in normal saline, but that beat frequency remained significantly reduced (P<0.01) when compared with normal saline controls (Fig. 7B).

Effects of exon II peptide mixtures on inositol phosphate production

FMRFamide and the 'isoleucines'

Despite having no effects when applied alone, it was still possible that the 'isoleucine' peptides could modulate the FMRFamide-stimulated increase in inositol phosphate production as both peptides may be coreleased on neuronal stimulation (Benjamin and Burke, 1994). However, when a mixture of 1 μmol l^{-1} FMRFamide and 1 μmol l^{-1} pQFYRIamide was applied to a heart homogenate for 5 s, the rate of inositol phosphate production was similar to that following a 5 s application of 1 μmol l^{-1} FMRFamide alone (N=3, data not shown). This implied that the signal transduction pathways involved in the physiological actions of the 'isoleucine' peptide, pQFYRIamide (and presumably EFLRIamide and pQFLRIamide also), do not interact with the FMRFamide-mediated pathway responsible for stimulating the rate of production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4.

FMRFamide and SEEPLY

Unlike pQFYRIamide, SEEPLY had a significant influence on FMRFamide-stimulated inositol phosphate production. In initial experiments a 5 s co-application of FMRFamide with SEEPLY (Fig. 8A) appeared to reduce the rate of total inositol phosphate production compared with the application of FMRFamide alone. A threshold dose of approximately 1 nmol l^{-1} SEEPLY was required before any reduction in 1 μmol l^{-1} FMRFamide-induced stimulation of the inositol phosphate pathway was seen. This reduction was significant at SEEPLY doses of 10 nmol l^{-1} and above (P<0.05, N=8), whilst complete elimination of the 5 s response of the Ins(1,4,5)P_3 pathway to FMRFamide stimulation was seen at concentrations of 100 nmol l^{-1} and above. The IC_{50} dose for the inhibitory effects of this peptide on FMRFamide-stimulated inositol phosphate production was approximately 10 nmol l^{-1}.

The high negative charge of the SEEPLY molecule led to concern that it might be electrostatically combining with positively charged FMRFamide molecules and consequently reducing the effective concentration of FMRFamide. Synthesis of a SEEPLY-like molecule ('RSEEPLY', see Materials and

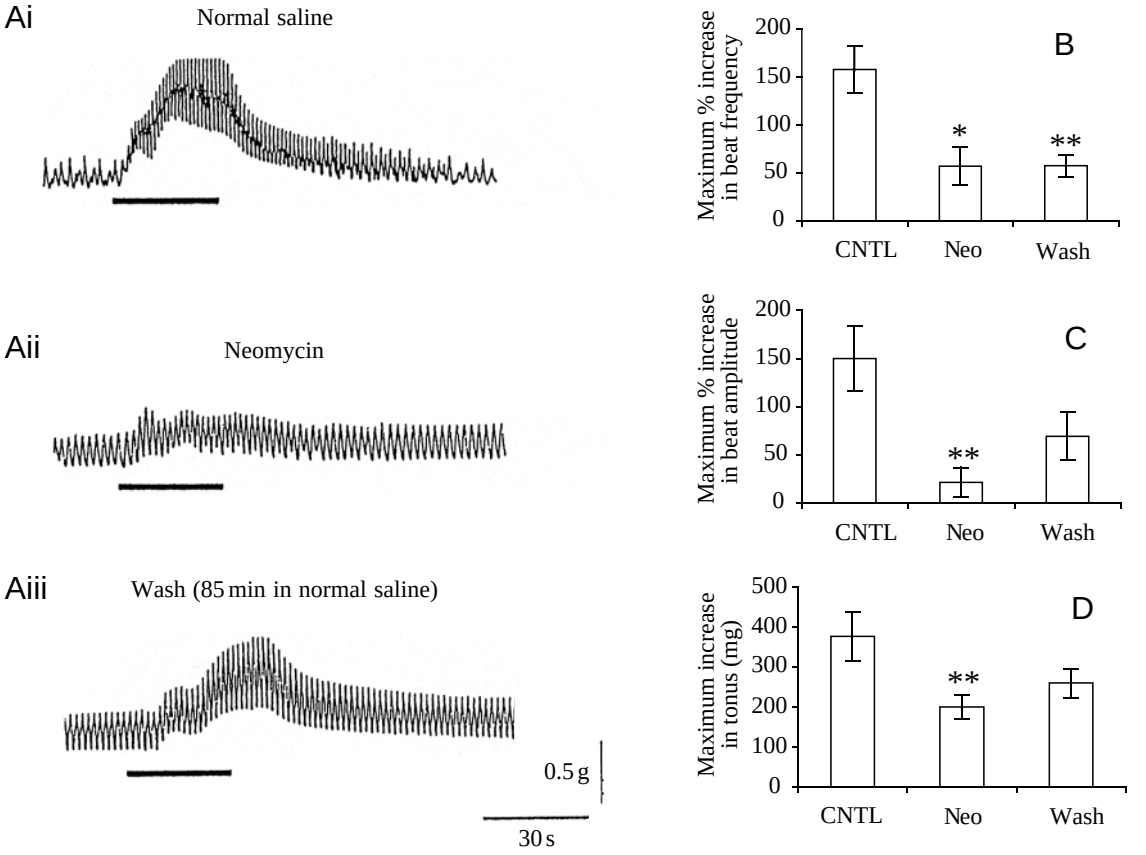

Fig. 7. Blocking effects of the PLC inhibitor, neomycin, on the FMRFamide-induced excitatory response in the isolated heart bioassay. (Ai) Excitatory effects of perfusing the heart with 1 μmol l^{-1} FMRFamide in normal saline. (Aii) Response of the isolated heart to 1 μmol l^{-1} FMRFamide following pre-treatment with 2.5 mmol l^{-1} neomycin for 10 min. (Aiii) Reversal of the blocking effects of 2.5 mmol l^{-1} neomycin after an 85 min wash of the heart in normal saline. FMRFamide was applied for the duration indicated. The vertical scale bar calibrates the tonus increase. (B) A statistical analysis of the percentage maximum increase in beat frequency during 1 μmol l^{-1} FMRFamide application in normal saline (CNTL), following a 10 min incubation of the heart in 2.5 mmol l^{-1} neomycin (Neo), and after a period of washing in normal saline (Wash). (C) Maximum increase (%) in beat amplitude during 1 μmol l^{-1} FMRFamide application and (D) maximum tonus change (mg) during 1 μmol l^{-1} FMRFamide application under the same three conditions (CNTL, Neo and Wash). All values are plotted as mean ± S.E.M. (N=9). Asterisks indicate values significantly different from those obtained by the application of 1 μmol l^{-1} FMRFamide under control conditions; *P<0.05, **P<0.01.

methods), which had the same amino acid composition as SEEPLY but with the amino acids arranged in a random order, enabled this hypothesis to be tested. Results from HPLC inositol phosphate analysis suggested that, unlike authentic SEEPLY, the 'RSEEPLY' molecule was unable to produce significant inhibitory effects on the response of the inositol phosphate pathway to FMRFamide (Fig. 8B). In the same experiments SEEPLY produced significant inhibition of FMRFamide-induced inositol phosphate production (P<0.005, N=4).

Further experiments (N=3) to examine the effects of SEEPLY over longer peptide incubation periods suggested that SEEPLY was not actually inhibiting the ability of FMRFamides to increase Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production, but only delaying the onset and time-to-peak response (Fig. 9). On its own, 1 μmol l^{-1} FMRFamide caused maximum increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production at 5 s. This production declined progressively with longer incubation periods, until at 30 s there was no significant difference between control and FMRFamide-stimulated levels of either Ins(1,4,5)P_3 (Fig. 9A) or Ins(1,3,4,5)P_4 (Fig. 9B). The peak percentage increases in response at 5 s for FMRFamide were similar to those at 30 s for FMRFamide + SEEPLY (P>0.05), supporting the hypothesis that the main effects of SEEPLY were on the time course of the FMRFamide-induced response rather than a direct regulation of the rate of inositol phosphate production. InsP_1 production was also monitored following a 2 min application of FMRFamide + SEEPLY (Fig. 9C). This inositol phosphate is only slowly metabolized, hence any overall inhibitory effect of SEEPLY on the inositol phosphate pathway should be apparent from monitoring the rate of production of this metabolite. The delay in the production of the metabolite was similar to that seen for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. However, no inhibitory effect of SEEPLY on FMRFamide-stimulated InsP_1 production was found at 2 min.

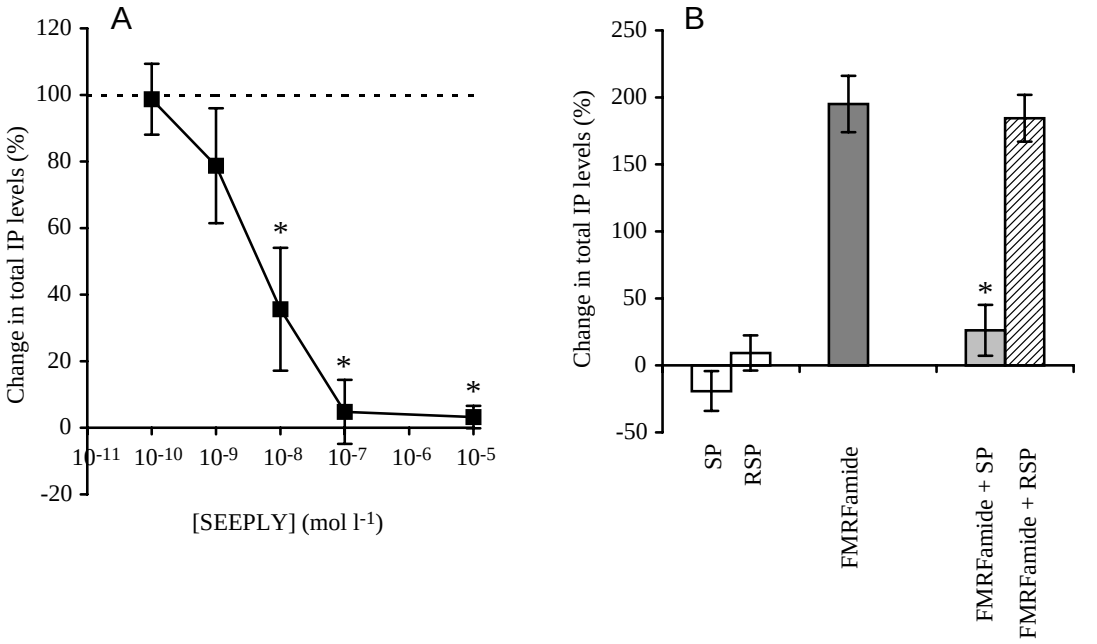

Fig. 8. (A) FMRFamide-induced inositol phosphate (IP) production is inhibited by SEEPLY in a concentration-dependant manner. The dashed line (100 %) represents the maximum stimulatory effects of 1 μmol l^{-1} FMRFamide on inositol phosphate production following a 5 s application. Increasing doses of SEEPLY (100 pmol l^{-1} to 10 μmol l^{-1}) produced a progressive increase in the short-term inhibition of the stimulatory effects of FMRFamide. Data are plotted as mean ± S.E.M. (N=8), *P<0.05. (B). Comparison of the effects of SEEPLY (SP) and the modified peptide 'RSEEPLY' (RSP) on FMRFamide-stimulated inositol phosphate production in *L. stagnalis* heart tissue. Data are presented as the percentage change in total inositol phosphate levels compared to controls. Peptide applications were for 5 s. Values are mean ± S.E.M. (N=4), *P<0.005.

Discussion

In this study we have demonstrated the excitatory effects of FMRFamide as a pharmacological agent on the isolated whole heart of *L. stagnalis* at doses greater than 10 pmol l^{-1}. This data was comparable to that previously described by Buckett et al. (1990a) for the isolated auricle of *L. stagnalis*. Perfusion of isolated whole heart preparations with FMRFamide stimulated the frequency and force of contraction of individual heart beats and also enhanced the underlying tonus of the heart muscle. Results from HPLC experiments measuring inositol phosphate production illustrated a high sensitivity of this calcium-mobilizing second messenger pathway to the tetrapeptides FMRFamide (threshold 100 pmol l^{-1}) and FLRFamide. The threshold and maximal doses of FMRFamide that stimulated the production of Ins(1,4,5)P_3 were highly comparable to those mediating cardioexcitation. Similar increases in Ins(1,3,4,5)P_4 production were also seen in response to FMRFamide.

The possibility of a cardioregulatory role for Ins(1,4,5)P_3 in *L. stagnalis* was strengthened by the observation that exogenous application of the messenger produced excitatory effects in permeabilized preparations of the isolated heart, similar to those mediated by FMRFamide. Neomycin caused a dramatic reduction in the stimulatory effects of FMRFamide. The inhibitory effects of neomycin, a PLC inhibitor, were significant at both the biochemical (Ins(1,4,5)P_3-producing) level and at the whole heart pharmacological level, suggesting that neomycin mediated its effects *via* an inhibition of InsP_3 production. However, the inhibitory effects of neomycin presumably also affected DAG levels *via* the second PLC-mediated pathway shown in Fig. 2A. DAG has been shown to activate a variety of membrane channels in numerous systems *via* the activation of protein kinase C (PKC), but the inhibitory effects of neomycin on DAG in the heart of *L. stagnalis* are currently unknown. A proportion of the excitatory response of the heart to FMRFamide did not appear to be blocked by neomycin. This might have been due to either an insufficient incubation time with the inhibitor and/or the actions of another second messenger whose production did not depend upon the activation of PLC. The 'isoleucine' peptides did not significantly effect inositol phosphate production in the heart and are thought to mediate their physiological effects *via* an alternative signalling pathway.

A functional role for the 22 amino acid peptide SEQPDVDDYLRDVVLQSEEPLY ('SEEPLY') in the heart of *L. stagnalis* is not fully established. Previous patch-clamp investigations by Brezden et al. (1999) on dissociated heart muscle cells have suggested that SEEPLY is able to both up- and downregulate the effects of FMRFamide to maintain a particular ('set-point') level of calcium channel activity in heart muscle cells from *L. stagnalis*, while having no effect when applied alone. In the present experiments analysis of the actions of SEEPLY at the biochemical level indicated a complex transient inhibitory effect of SEEPLY on FMRFamide-stimulated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production in the heart. The main effect of SEEPLY was to delay the peak inositol phosphate response to FMRFamide. At

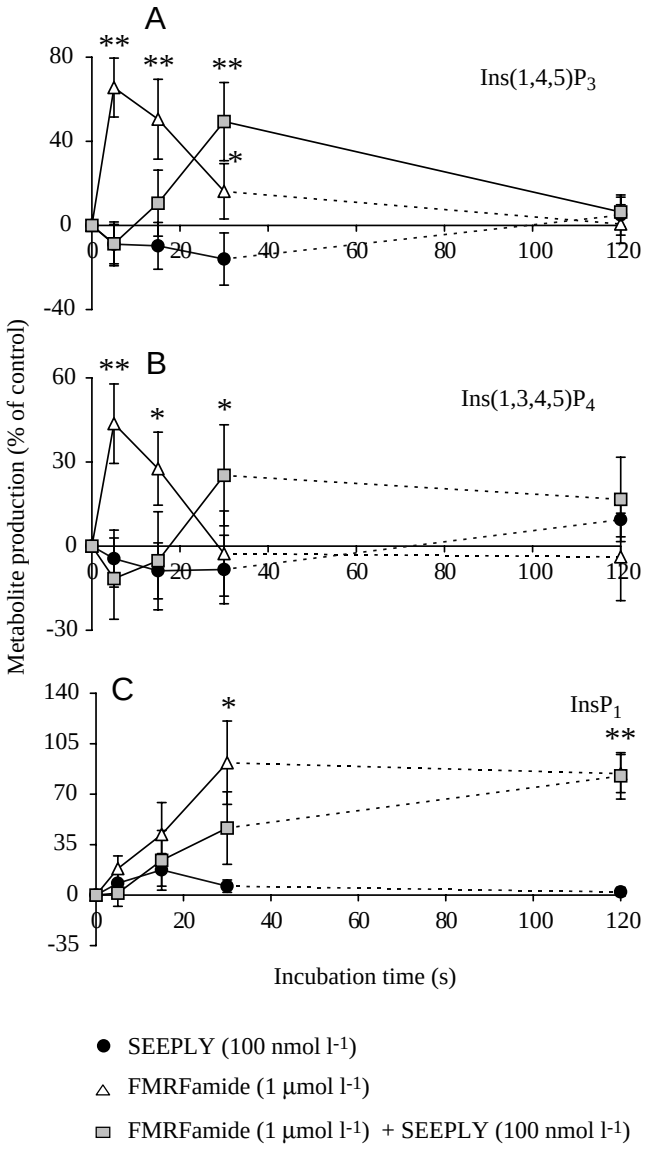

Fig. 9. SEEPLY delays the onset and time of peak Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 response to FMRFamide rather than inhibiting the effects of peptide stimulation. Inositol phosphate production was analysed at 5, 15, 30 and 120 s and is plotted as a percentage increase from control levels (100 %). (A) Changes in Ins(1,4,5)P_3 levels. 100 nmol l^{-1} SEEPLY applied alone has no effect on Ins(1,4,5)P_3 levels throughout the 2 min time course. 1 μmol l^{-1} FMRFamide produces a peak increase at 5 s, after which production of Ins(1,4,5)P_3 gradually decreases back to control levels. In contrast, when 1 μmol l^{-1} FMRFamide + 100 nmol l^{-1} SEEPLY are coapplied Ins(1,4,5)P_3 production begins to rise above that of controls at 15 s of peptide incubation, and peaks at approximately 30 s incubation. (B) Comparable results are seen for the three peptide conditions when Ins(1,3,4,5)P_4 levels in the heart tissue are analysed. (C) InsP_1 production was monitored throughout the 2 min time course, and shows similar values for FMRFamide-stimulated InsP_1 production in the presence and absence of SEEPLY.

present, no pharmacological effects of FMRFamide and SEEPLY co-application to the whole heart have been described that can be attributed to the delaying effects of SEEPLY on the inositol phosphate pathway. The apparent ability of SEEPLY to modulate FMRFamide-induced excitation at the second messenger level (described here) and at the level of ion channel activation (Brezden et al., 1999) is an important finding, as evidence from our laboratory suggests that FMRFamide and SEEPLY are probably coreleased. More detailed pharmacological experiments are necessary to investigate the modulatory effects of SEEPLY on the FMRFamide response in the whole heart.

In summary, the results obtained by this study provide the first direct evidence for activation of the inositol phosphate pathway by FMRFamide in the molluscan heart. Previous studies have linked this signalling pathway to peptide action in several invertebrate muscle types. For example, the application of cardioacceleratory peptides (CAPs) to the myogenic heart of the tobacco hawkmoth *Manduca sexta* has been shown to increase intracellular levels of Ins(1,4,5)P_3 (Tublitz, 1988). Increases in InsP_3 levels were also seen following the application of proctolin to the mandibular closer muscle of *Locusta migratoria* (Baines et al., 1990), and during the application of FMRFamide to the tentacle retractor muscle of *Helix aspersa* (Falconer et al., 1993). Falconer and colleagues suggest that stimulation of Ins(1,4,5)P_3 levels by FMRFamide causes a release of calcium from intracellular stores that is responsible for mediating the delayed rise in tension and phasic contractions of the isolated tentacle retractor muscle. In contrast, a sustained decrease in inositol phosphate levels was caused by the cardioexcitatory peptide, FMRFamide, in the ventricle of the bivalve clam *Geukensia demissa* (Bayakly and Deaton, 1992).

In other molluscan heart preparations it has been proposed that FMRFamide acts *via* the stimulation of cyclic AMP production (Higgins et al., 1978; Painter, 1982). In both *Aplysia californica* and *Helisoma* spp. FMRFamide has also been shown to mediate some of its neuronal effects *via* the production of lipoxygenase metabolites of arachidonic acid (Piomelli et al., 1987; Bahls et al., 1992). In preliminary whole heart pharmacological experiments with *L. stagnalis*, arachidonic acid produced increases in tonus that were reduced in the presence of a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). However, NDGA was unable to reduce the tonus increase seen during perfusion of the heart with FMRFamide. These preliminary results suggest that lipoxygenase metabolites are not responsible for the effects of FMRFamide in the heart of *L. stagnalis*, but these data need to be confirmed before any definite conclusions can be drawn.

In vertebrate studies noradrenaline has been shown to act at α-adrenergic receptors on the heart to stimulate the production of both Ins(1,4,5)P_3 and diacylglycerol, suggesting that these messengers have an important role in regulation of the vertebrate heart (Irisawa et al., 1993). In addition, muscarinic and endothelin receptors have also been linked with Ins(1,4,5)P_3 production and calcium homeostasis in the vertebrate heart (Irisawa et al., 1993). A role for the stimulation

of Ins(1,4,5)P_3 levels in the invertebrate heart has not previously been shown. In the vertebrate heart, noradrenaline also mediates excitatory effects *via* β-adrenergic receptors. These receptors are linked to cyclic AMP production, which enhances both an inward calcium current (I_{Ca}) (Hartzell and Fischmeister, 1986) and the hyperpolarization-activated current to increase the frequency of heart beat (Difrancesco and Tortora, 1991). Data will be presented in the following paper (Willoughby et al., 1999) to suggest that FMRFamide might act *via* the cyclic AMP pathway, as well as *via* the inositol phosphate signalling pathway described here.

We are grateful to the BBSRC for funding. We would also like to thank Professor Robin Irvine and Dr Pete Cullen at the Babraham Institute, Cambridge, UK for their help in the early stages of this project.

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