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Research Articles

A new biological activity for the neuropeptide FMRFamide: experimental evidence for a secretagogue effect on amylase secretion in the scallop *Pecten maximus*

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Abstract. FMRFamide immunoreactivity in the digestive tract of the bivalve mollusc *Pecten maximus* was investigated by immunocytochemistry. Positive FMRFamide-like immunoreactivity was detected in nerve fibres in close contact with exocrine α amylase secreting cells. Physiological studies on enzymatically dissociated cells of the stomach-digestive gland complex demonstrated the involvement of FMRFamide and analogues in the control of α amylase release from the cells. The FMRFamide-induced secretion was shown to be time- and dose-dependent. In contrast to most naturally occurring vertebrate secretagogues which are hormones, FMRFamide appears to work in our in vitro system as a paracrine factor.
Key words. Molluscs; scallop; digestive cells; cell suspension; enzyme secretion; FMRFamide.

The neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) was first isolated from the nervous system of the bivalve mollusc *Macrocallista nimbosa*[1]. Following the structural analysis of this tetrapeptide, FMRFamide as well as a variety of related peptides (FaRPs) were identified in several molluscs[2-4] and in all the other major animal phyla ranging from cnidaria to mammals[5]. Throughout the animal kingdom, FaRPs are mainly found within the central and peripheral nervous systems[6].

These peptides, which all share the C-terminal sequence Arg-Phe-NH_2 required for both bio- and immuno-reactivity[7,8], appear to function as neuromodulators, neurotransmitters and/or neurohormones. Moreover they modulate the activity of various target tissues and cells[9,10].

In molluscs, FMRFamide was first identified as a cardioregulator[1]. FaRPs are currently known to display a wide range of activities including myotropic effects[11,12], and the control of activity of glands[13-15] and neurons[16]. In the scallop *Pecten maximus*, recent immunocytochemical investigations have shown the presence of FMRFamide immunoreactivity in the central nervous system and in the nerve fibres connected to peripheral organs such as the gonad wall[16a] and the gut[17]. In the present study, FMRFamide immunoreactivity in the vicinity of amylase secreting cells is examined. The hypothesis that these cells may release amylase in response to FMRFamide and related peptides is investigated via in vitro incubation of scallop digestive cell suspensions.

Materials and methods

Animals. Adult *Pecten maximus* were dredged from the bay of Seine (Normandy, France) and maintained in aquaria with constant water flow and temperature (15 °C). Animals were fed daily with marine liquifry (Liquifry Co., Dorking, UK) but starved 3 days prior to being sacrificed.

Immunofluorescence. The stomach-digestive gland complex was fixed in 4% paraformaldehyde in Sörensen phosphate buffer (0.2 M, pH 7.4) overnight. After washing in the same buffer (1 vol.) and 30% sucrose (3 vol.) for 20 min at +4 °C, the samples were immersed for 24 h in a solution containing 15% sucrose in phosphate buffer (0.1 M, pH 7.4), and for a further 24 h in 25% sucrose and 2% glycerol in the same buffer at +4 °C. Finally, tissues were embedded in Tissue-Tek, frozen in liquid nitrogen and freeze-sectioned at −35 °C. 10 µm fresh-frozen sections were placed on slides coated with 0.5% gelatine and treated with 0.1 M PBS containing 1/30 nonimmune goat serum and 0.2% Triton X100 for 1 h at room temperature to reduce nonspecific labelling. After a quick wash in 0.1 M PBS, the sections were incubated overnight at +4 °C with FMRFamide antiserum (kind gift of Dr. van Minnen, Amsterdam). The characteristics of the

FMRFamide antiserum have been previously described[6]. The antiserum was diluted 1/1000 in 0.15 M PBS containing 1% nonimmune goat serum and 0.2% Triton X100. Sections were washed (3 × 20 min) in 0.1 M PBS, and then incubated with fluoresceine isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1/200 in 1% nonimmune goat anitserum in 0.1 M PBS for 1 h at room temperature. Finally, sections were washed in 0.1 M PBS and mounted with a mixture of glycerol and mowiol (Calbiochem) containing 1.4 diazobicyclo (2.2) octane (Sigma) to reduce fading. These samples were then examined and photographed with a Leitz Aristoplan fluorescent microscope. Controls were used in which the primary antiserum was either omitted, diluted or adsorbed to its homologous antigen.

Transmission electron microscopy. The fragments of tissue were rapidly dissected and fixed by immersion in 2% glutaraldehyde in Sörensen phosphate buffer (0.1 M, pH 7.35), washed in phosphate buffer (1 vol.) sucrose 0.33 M (3 vol.) and post-fixed in 2% osmium tetroxide in phosphate buffer (0.1 M) for 30 min at +4 °C. The tissues were then ethanol–dehydrated and embedded in Epon. Ultrathin sections were double-stained with uranyl acetate and lead citrate. Sections were examined and photographed at 80 kV on a Philips 400 transmission electron microscope.

Cell dissociation. Dissociation was carried out in 20 mM Hepes pH: 7.4; 436 mM NaCl, 53 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, 11 mM glucose. Scallop digestive gland and stomach were dissociated enzymatically over 30 min by two incubations with 0.1% pronase at room temperature as previously described[18]. Cell suspension was strained through a 100 µm mesh nylon screen, washed twice with fresh medium by centrifugation (75 *g*, 7 min), and adjusted to a final concentration of 20 million cells per ml.

The viability of the cells was estimated by using the trypan blue dye exclusion test[19]. Viability ranged from 90 to 95%.

Bioassay. The substances to be tested (10 µl) were added to 490 µl of cell suspension and incubated with gentle agitation for 30 min at 15 °C in the dissociation medium in which calcium was either present or omitted. After this incubation, cells were separated from the medium by centrifugation. Amylase content in the incubation medium was measured using a commercial kit (Sigma 577-20). In addition, the remaining amylase activity in the dissociated cells was determined after the 30 min incubation period. Total amylase was calculated after summation of these 2 values. Results are expressed as percentage of amylase released from total amylase.

Statistical analysis. Results were analysed using the Student's t-test. Results were expressed as mean ± SE. Each experiment was repeated at least three times; the number of samples is indicated in the figure legends.

Results

Immunocytochemistry. In the stomach-digestive gland-complex, numerous nerve fibres were observed within the connective and muscular wall (fig. 1 A and B). The nerve fibres deeply penetrated the stomach epithelium and ended at amylase-secreting cells (fig. 1 A). The amylase-secreting cells were previously characterised by immunocytochemical studies[20]. Nerve fibres were easily identified by the presence of numerous clear and dense vesicles classified into three categories: the small clear vesicles (40–60 nm), the dense-core vesicles (50–80 nm) and the large granular vesicles (60–100 nm) (fig. 1 A and C). In the stomach, numerous nerve fibres were observed within the connective-muscular wall. Axon terminals containing the different vesicles were often seen in close apposition with the plasma membrane of the amylase-secreting cells but synaptic differentiations were never found (fig. 1 A and C).

A fairly intense FMRFamide immunoreaction was found both in the fibres running into the connective and muscular stomach wall and at the basis of the stomach epithelium. (fig. 1 D). This high immunoreactivity exactly corresponded to the localisation of the nerve endings.

Time-dependent stimulation release of α amylase by FMRFamide. FMRFamide stimulated time-dependent release of α amylase from the scallop digestive cell suspensions in both calcium and calcium-omitted media (fig. 2). FMRFamide at a final concentration of 10^{-6} M elicited a maximum enhancement of secretion which was reached after 30 min and remained constant for at least two hours. No significant difference in the potency of FMRFamide to induce secretion was observed in the presence or absence of calcium in the medium.

Effect of some FaRPs and non-related peptides. After 30 min FMRFamide induced a twofold increase in α amylase secretion with respect to control. The related peptide FLRF-amide evoked approximately the same reaction. Similarly, Met-enkephalin-RF (Met-Enk-RF: YGGFMRF) was almost equally effective. By contrast, Met-Enkephalin (Met-Enk: YGGFM), Leu-Enkephalin (Leu-Enk: YGGFL) and Met-Enkephalin-RGL (YGGFMRGL) did not show any effect versus control in this bioassay (fig. 3).

Dose-dependent responses. The amylase–releasing effect of FMRFamide was shown to be dose-dependent. A maximum activity was observed for a concentration of 10^{-6} M, with half maximum response between 10^{-8} M and 10^{-7} M. FLRF-amide exhibited a less potent activity; the dose-response curve was shifted to 10 times higher concentrations, the maximum activity being reached at a concentration of 10^{-5} M (fig. 4). A secretory response of the same order of magnitude with respect to control was obtained in the calcium–omitted medium but the percentage of α amylase released was somewhat lower (fig. 5).

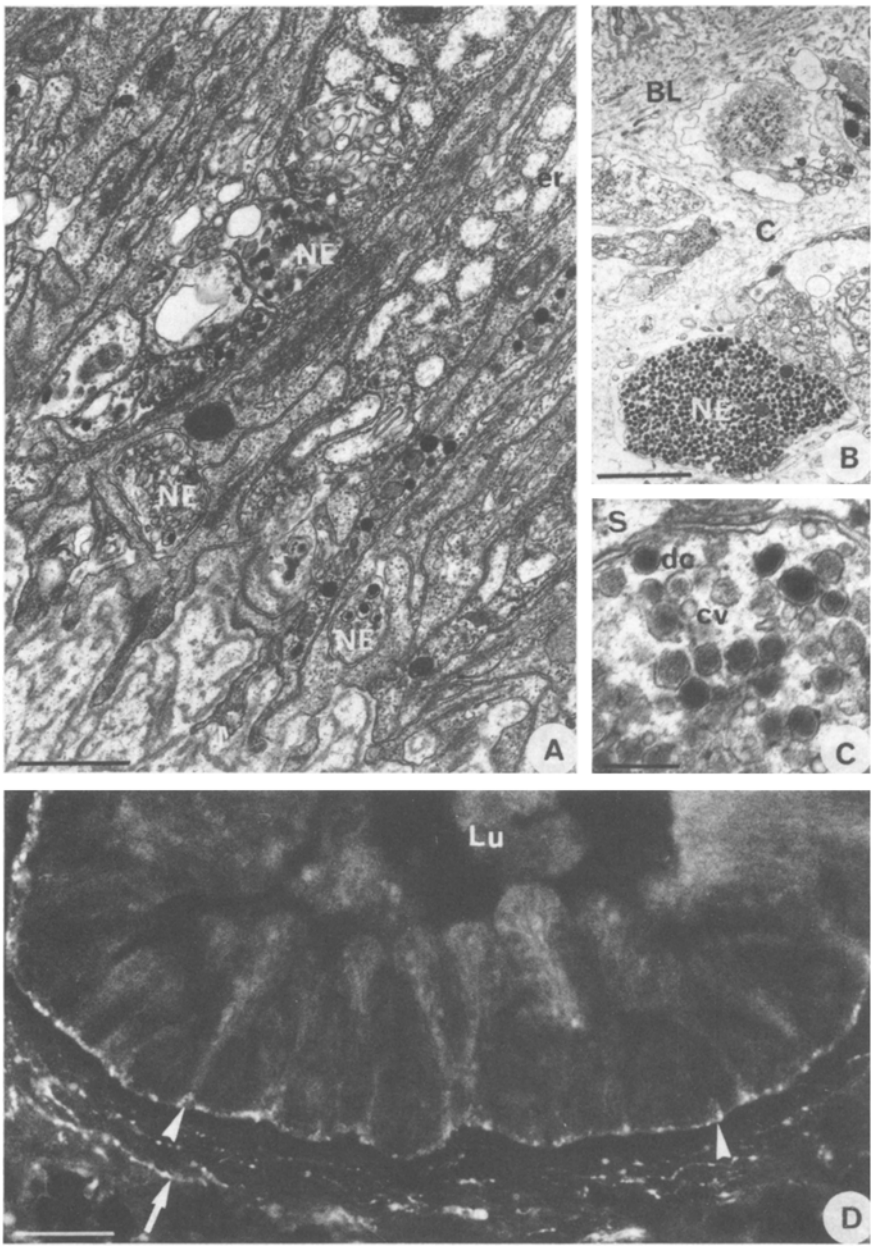

Figure 1. *A* Nerve endings (NE) in contact with secreting cells (S) characterised by their developed rough endoplasmic reticulum (er), scale bar = 1 μm.
B Nerve endings (NE) containing numerous dense vesicles in the connective tissue (C) lying under the basal lamina (BL) of the stomach wall. Scale bar = 2 μm.
C The three types of nerve vesicles, clear (cv), dense core (dc) and some granular, occur in a nerve ending in close contact to a secreting cell (S). Scale bar = 1.5 μm.
D Nerve fibres reacting with anti-FMRFamide in the connective and muscular wall of the stomach (arrow) and in the basal part of the stomach epithelium (arrow head), Lu: lumen of the stomach. Scale bar = 50 μm.

Discussion

The present study provides evidence for the involvement of FMRFamide-related peptides to regulate enzyme release in the bivalve mollusc *Pecten maximus*.

The demonstration of this new biological activity has been favoured by our first findings of intense FMRFamide-like immunoreactivity in nerve terminals connected to the gut of the scallop. These observations support similar immunocytochemical studies on the digestive system of other animal groups such as insects[21] and echinoderms[22]. In other groups, including protochordates[23] and vertebrates[24], the gut FMRFamide-like immunoreactivity is mainly detected in endocrine-like cells. Because of this localisation it has been argued that FMRFamide may regulate important biological processes in the digestive system. Indeed, in molluscs, FMR-

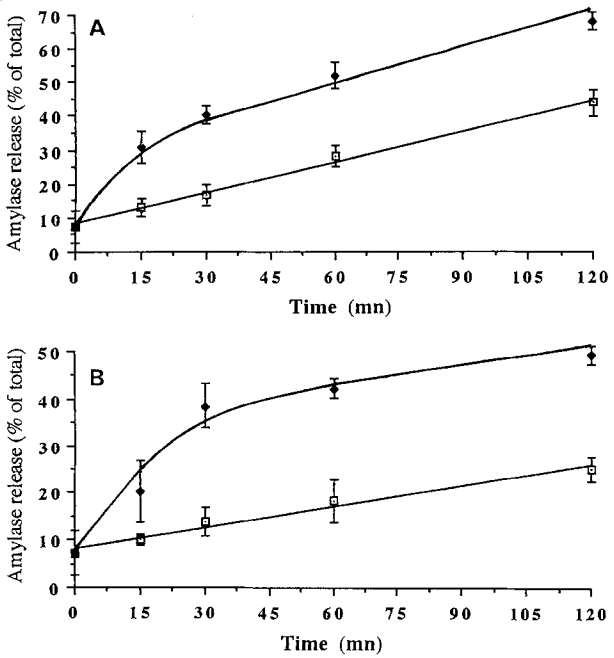

Figure 2. Time-dependent release of α amylase from dissociated cells after stimulation with 10^{-6} M FMRFamide in a calcium-containing (*A*) and calcium-omitted medium (*B*).
Results are expressed as mean percentage ± SE, n = 6, of basal release (without peptide: □) and stimulated release (with peptide: ◆).

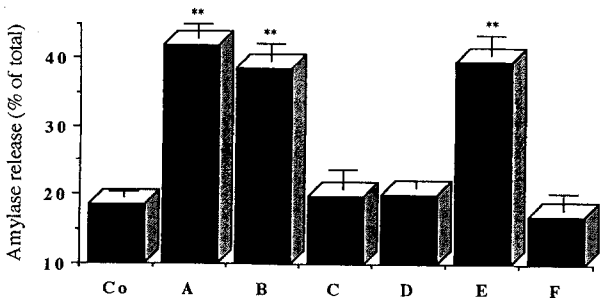

Figure 3. Effect of different peptides upon the release of α amylase from dissociated cells. Each peptide was tested at a final concentration of 10^{-6} M; incubations were done for 30 min. Co: control; A: FMRFamide; B: FLRFamide; C: Leu-Enk; D: Met-Enk; E: Met-Enk-RF; F: Met-Enk-RGL. Results are expressed as mean percentage ± SE, n = 5. Significance of difference versus control is $^{**}p < 0.001$.

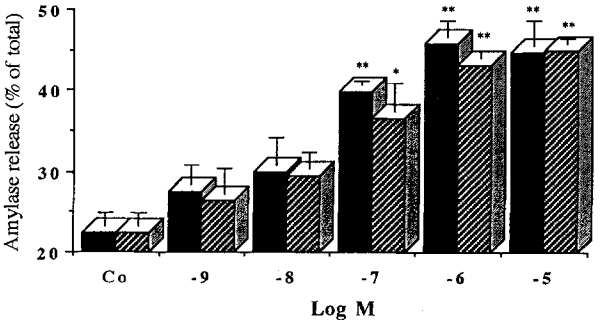

Figure 4. Dose-response of α amylase release by FMRFamide (black) and FLRFamide (hatched) from dissociated cells incubated for 30 min in a calcium-containing medium. Mean percentage ± SE of experiments repeated 6 times. Significance of difference versus control is $^{*}p < 0.01$, $^{**}p < 0.001$.

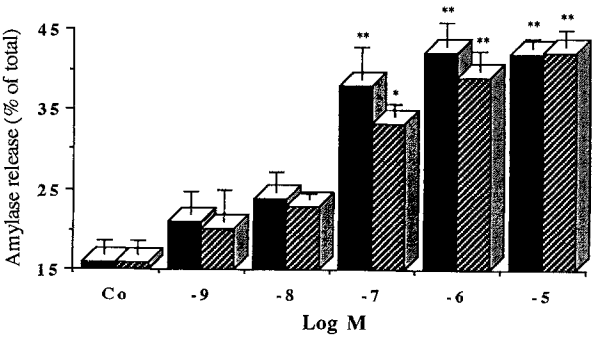

Figure 5. Dose-response of α amylase release by FMRFamide (black histograms) and FLRFamide (hatched) from dissociated cells incubated in a calcium-omitted medium. Mean percentage ± SE of experiments repeated 6 times. Significance of difference versus control is $^{*}p < 0.01$, $^{**}p < 0.001$.

Famide was shown to affect motility of the anterior gizzard in *Aplysia*[25] and to increase the tone activity of the rectum in the mollusc *Merceneria*[26]. Since a relationship between gastrointestinal activity and secretion has been clearly shown in vertebrates[27], it was reasonable to suggest an involvement of FMRFamide in secretion.
The use of the present in vitro bioassay allowed the assessment of the secretagogue potency of FMRFamide and related peptides. The FMRFamide-induced change in α amylase release shared some features with the response achieved with the well-known vertebrate secretagogue cholecystokinin (CKK): 1) the kinetic pattern of FMRFamide-induced secretion is similar to the one observed in vertebrates with secretagogues that, like CCK, cause a mobilisation of intracellular calcium, e.g. the rate of secretion is greater during the initial 30 min than at later times[28]; 2) a comparable magnitude of secretion is obtained with optimal concentration of peptide[29]. By contrast, FMRFamide and related peptides need to be present at far higher concentrations to evoke a biological activity. Most naturally occurring vertebrate secretagogues are hormones and work at concentrations close to 10^{-9} M–10^{-8} M[28]. The discrepancy observed in this study for FMRFamide suggests that this family of peptides behave in our system more as a paracrine effector than as a genuine hormone. Such a hypothesis is in keeping with our findings of significant immunoreactivity near scallop exocrine cells. In such a situation, a rather high concentration of peptide can be expected in vivo at the vicinity of the receptors. Therefore, the optimal concentration required to trigger a biological response which is in the same molar range as cholinergic agents in vertebrates, appears consistent. The N-terminal sequence of the endogenous FMRFamide immunoreactive peptides is not known. It has been shown that although the C-terminus sequence is crucial for activity and for immunoreactivity, the

N-terminal regions of FMRFamide modulate the biological potency of the peptide[30]. We cannot rule out the possibility that a potent endogenous peptide is actually present in the end terminals of the nerves projecting to the stomach-digestive gland complex. From the present data, it appears that the sequence RF represents the active core. Both FLRFamide and the opioid derived FMRF are active in the bioassay. Other enkephalins, which have been identified in bivalve molluscs[31] and are known to be abundant in the scallop digestive tract[31a], are totally inactive although they have some sequences in common with FaRPs. Surprisingly, the amide group does not appear to be essential. Such a result is in agreement with previous data on the induction of fluid secretion from salivary glands of *C. vomitoria* by some amidated or non-amidated calliFMRFamide peptides[30,32]. At least two receptor subtypes are possibly present on the amylase-secreting target cells.

In most secretory processes, calcium plays a crucial role. In scallop amylase secreting cells, calcium appears to be important. We have shown that artificially increased concentration of intracellular calcium induces secretion[32a]. It is interesting to notice that the stimulatory effect of FMRFamide is not rigorously dependent on extracellular calcium, but very likely involves as a first step a mobilisation of intracellular calcium stores as is the case in numerous vertebrate secretory tissues[33,34]. Although we cannot conclude that the ability of the FMRFamide to regulate enzyme secretion is physiologically relevant in vivo since our experiments were performed in vitro, there is growing evidence implicating this family of peptides in the regulation of digestive processes in other animal groups[35].

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1 Price, D. A., and Greenberg, M. J., Science *197* (1977) 670.
2 Cottrel, G. A., Schot, L. P. C., and Dockray, G. J., Nature *304* (1983) 638.
3 Lehman, H. K., Price, D. A., and Greenberg, H. J., Biol. Bull. *167* (1984) 460.
4 Price, D. A., Davies, N. W., Doble, K. E., and Greenberg, M. J., Zool. Sci. *4* (1987) 395.
5 Price, D. A., and Greenberg, M. J., Zool. Sci. *4* (1989) 395.
6 Boer, H. H., Schot, L. P. C., Veenstra, J. A., and Reichelt, D., Cell Tissue Res. *213* (1980) 21.
7 Cotrell, G. A., and Davies, M. W., J. Physiol., Lond. *382* (1987) 51.
8 Price, D. A., Am. Zool. *26* (1987) 1007.
9 Raffa, R. B., Peptides *9* (1988) 915.
10 Walker, R. J., Comp. Biochem. Physiol. *102* (1992) 213.
11 Raffa, R. B., and Bianchi, C. P., Biochem. Physiol. *84C* (1986) 23.
12 Muneoka, Y., Fujisawa, Y., Matsuura, M., and Ikeda, T., Comp. Biochem. Physiol. *98C* (1991) 105.
13 Brulloch, A. G. M., Price, D. A., Murphy, A. D., Lee, T. D., and Borves, H. N., J. Neurosci. *8* (1988) 3459.
14 Griffon, B., and Mounzih, K., Tissue and Cell *223* (1990) 741.
15 Saleuddin, A. S. M., Khan, M. R., Ashton, M-L., and Griffond, B., Tissue and Cell. *24* (1992) 179.
16 Brussaard, A. B., Kit, K. S., Ter Maat, A., Van Minnen, J., and Moed, P. J., Brain Res. *447* (1988) 35.
16a Henry, M., Benlimane, N., Belhsen, Y., Jule, Y., and Mathieu, M., Neuropeptides (1994) in press.
17 Benlimame, N., Thèse de Doct., Univ. Aix-Marseille III (1993).
18 Lenoir, F., and Mathieu, M., C. Acad. Sci. Paris, *303* (1986) 223.
19 Philips, H. J., in: Tissue Culture Methods and Applications, pp. 104, 406. Eds P. P. Kruse Jr and M. K. Patterson, Academic Press, New York 1971.
20 Henry, M., Benlimane, N., Boucaud-Camou, E., Mathieu, M., Donval, A., Van Wormhoudt, A., Tissue and Cell *25* (1993) 537.
21 Sivasubramanian, P., Comp. Biochem. Physiol. *103C* (1992) 333.
22 Garcia-Arraras, J. E., Enamorado-Ayala, I., Torres-Avilan, I., and Rivera, V., Neurosci. Lett. *132* (1991) 199.
23 Pestarino, M., Lucaroni, B., and Distefano, S, Eur. J. Histochem. *37* (1993) 225.
24 Kubben, F. J. G. M., Van Assche, C. L. M. V. J., and Bosman, F. T., Histochemistry, *84* (1986) 439.
25 Austin, T., Weis, S., and Lukowiak, K. Can. J. Physiol. Pharmac. *61* (1982) 949.
26 Doble, K. E., and Greenberg, M. J., Neuropeptides *2* (1982) 157.
27 Greenwood, B., and Davidson, J. S., Am. J. Physiol. *252* (1987) G1.
28 Peikin, S. R., Rottman, A. J., Batzri, S., and Gardner, J. D., Am. J. Physiol. *235* (1978) E-743.
29 Gardner, J. D., and Tensen, R. T., Recent Prog. Horm. Res *39* (1983) 211.
30 Duve, H., Johnsena, H., Sewell, J. C., Scott, A. G., Orchard, I., Rehfeld, F. J., and Thorpe, A., Proc. natl Acad. Sci. USA *89* (1992) 2326.
31 Leung, M. K., and Stefano, G. B., Proc. natl Acad. Sci. USA *81* (1984) 955.
31a Henry, M., Benlimane, N., Baki, C., Jule, Y., Cupo, A., Herbrecht, F., and Mathieu, M., J. comp. Neurol. (1994) in press.
32 Duve, H., Sewell, J. C., Scott, A. G., and Thorpe, A., Reg. Pept. *35* (1991) 145.
32a Giard, W., Favrel, P., and Boucaud-Camou, E., J. comp. Physiol. (1994) in press.
33 Schulz, I., and Stolze, H. H., A. Rev. Physiol. *42* (1980) 127.
34 Cheek, T. R., and Barry, V., J. expl Biol. *184* (1993) 183.
35 Elia, A. J., Terruge, V. A., and Orchard, I., J. Insect Physiol. *39* (1993) 459.