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In vitro effects of methionine-enkephalin, somatostatin and insulin on cultured gonadal cells of the snail *Helix aspersa*

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Abstract. Isolated snail gonadal cells were cultured in the presence of synthetic neuropeptides in order to determine the subsequent effect of these substances on gonadal synthetic activities. Gonadal cells were incubated for 24 h in concentrations of methionine-enkephalin, somatostatin and insulin ranging from 10^{-4} M to 10^{-9} M, in medium 199 supplemented with 6% Ultroser G. Synthesis of DNA and protein by the cultured cells were simultaneously estimated by measuring incorporation of ^{3}H thymidine and ^{35}S methionine. The rate of labelled precursor incorporation was measured using the liquid scintillation technique. All substances tested exerted a dose-dependent effect. The synthetic activity of the cultured cells was highest when the concentration of the peptides added to the medium approximated the physiological levels. Methionine-enkephalin, somatostatin and insulin at 2×10^{-8} M significantly increased ^{3}H thymidine incorporation, by 62%, 69% and 69% respectively, and protein synthesis by 42%, 57% and 57%, respectively. In the case of juvenile gonadal cultured cells, a similar increase in ^{3}H and ^{35}S incorporation was registered for a 10^{-7} M peptide concentration. Both lower and higher peptide concentrations inhibited ^{3}H thymidine and ^{35}S methionine incorporation. Pharmacological studies suggest the existence of methionine-enkephalin and somatostatin-like receptors on snail gonadal cells. These results indicate that our gonadal cell culture model provides a useful tool for the study of the neuroendocrinological control of the activity of snail gonadal cells.
Key words. *Helix aspersa*; gonadal cells; estimation of the synthetic activity; methionine-enkephalin; somatostatin; insulin.

Our laboratory has intensively investigated the complex mechanisms that trigger and control gametogenesis in the hermaphroditic gland of the snail *Helix aspersa*. First of all, the influence of the cephalic complex on snail gametogenesis was demonstrated using the organ culture method[1-3]. Next, the control of snail growth and reproduction by the neuroendocrine system was studied[4]. Recently, we reported an influence of nerve-ganglion extracts on the synthetic activity of snail gonad cells grown in primary culture[5].

Our present article concerns the influence of three vertebrate neuropeptides on the gonadal synthetic activity of snails. Several neuropeptides have been detected and localized by immunochemistry in the brain and various organs of snails[6-8]. From among these substances, we chose to test three synthetic neuropeptides: somatostatin, methionine-enkephalin and insulin.

A somatostatin-like substance has been characterized by immunocytochemical methods in the brain[6] and hepatopancreas[9] of the snail *Helix aspersa aspersa*, as well as in cerebral, pedal, visceral and left parietal ganglia of *Helix aspersa maxima*[10]. High levels were especially evident in young animals[9-11]. The in situ hybridization method confirmed that the mRNAs encoding somatostatin-like substances were synthesized in the brain of snails[12].

A methionine-enkephalin-like substance has also been detected in various tissues of *Helix aspersa*[7]. Since all but the oldest cells of the different spermatogenetic and oogenetic stages are methionine-enkephalin immunopositive[13] the role of this substance in sexual maturation may be associated with paracrine or autocrine effects. Moreover, quantitative variations of a methionine-enkephalin-like substance in the dorsal bodies during different physiological stages would suggest that it is involved in the regulation of reproductive processes.

Very little is known about the role of insulin in regulating neurologically mediated events in snails. Recently an insulin-like substance was detected in the central nervous system, particularly in the cerebral Green cells, visceral and pleural ganglia. The removal of the cerebral Green cells halts growth and increases the amount of glycogen present in the mantle edge[14].

Several studies have demonstrated the influence of peptides on vertebrate gonadal cells. However, little information is available concerning their effects on invertebrate ones. Insulin is known for its trophic effect on vertebrate gonadal cells. It stimulates progesterone production by pig granulosa cells, and its effect can be enhanced by ovarian steroid hormones[15]. Furthermore, in rat ovarian or thecal cells grown in primary culture, it stimulates androsterone biosynthesis[16]. Insulin, and

somatomedin C/insulin-like growth factor I, have limited mitogenic effects on pig Leydig and Sertoli cells, while amplifying the mitogenic action of Fibroblast Growth Factor[17]. In the case of *Helix aspersa*, the central nervous system plays a role in gonad development and function. However, the mode of action of this cephalic complex, and the nature of the substances which intervene in this regulation, remain enigmatic to this day.

By the use of a bioassay performed on juvenile gonads kept in organ culture, the stimulatory effects of the brain, dorsal bodies and methionine-enkephalin on the synthetic activity of young gonads have been demonstrated[18]. However, the results obtained in various experimental series were heterogeneous. Subsequently, we have developed a culture technique for snail gonadal cells which maintains their structural and immunological characteristics[19], and thus provides a homogeneous material suitable for rapid bioassays[5]. With this technique, we investigated the influence of three selected neuropeptides on the synthetic activity of snail gonadal cells.

Materials and methods

Cell preparation and culture

Adult *Helix aspersa aspersa* (Haa) and both adult and juvenile *Helix aspersa maxima* (Ham) were used as sources for cell cultures. Adult snails were obtained from a population that had been hibernating at 6 °C for 12 months prior to their use in the study. Normal activity of snails following hibernation was restored by raising the temperature to 20 °C and providing high relative humidity (RH 95%). Both adults and juveniles were maintained under controlled environmental conditions [LD 18:6 (18 h light followed by 6 h darkness); 95% RH, 20 °C] for one week before the experiment.

Methods used to prepare suspensions of snail gonadal cells were as previously described[19,5]. Briefly, gonads were dissociated in a PBS (Phosphate Buffer Saline) solution containing 0.1% collagenase-dispase (Boehringer-Mannheim) at 37 °C for 10 min. The mixture was passed through a nylon filter (80 µm), and the cells collected by centrifugation (at 500 *g* for 5 min at 4 °C), washed three times with medium 199 (Serva) and seeded in Falcon 24-well plates (Primaria) at a concentration of $25 \pm 5 \times 10^4$ cells per milliliter. Medium 199 with Hanks' salts (Serva) was supplemented with 20% fetal calf serum, 0.2 M $NaHCO_3$, penicillin (100 $IU \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), Epidermal Growth Factor (EGF) 20 $ng \cdot ml^{-1}$, and 0.1% methyl cellulose (Sigma).

After an incubation period of 24 h in primary culture, gonadal cells were exposed to ^3H thymidine (10 $mCi \cdot ml^{-1}$, specific activity 1 $Ci \cdot mmol^{-1}$) and ^{35}S methionine (10 $mCi \cdot ml^{-1}$, specific activity 1140 $Ci \cdot mmol^{-1}$) in fresh medium 199 containing 6% substitute of serum ULTROSER G (IBF) 0.2 M $NaHCO_3$, penicillin (100 $UI \cdot ml^{-1}$), streptomycin (100 $mg \cdot ml^{-1}$) and the various synthetic neuropeptides which were to be tested. To compensate for spontaneous degradation, more neuropeptide was added every 3 h without changing the medium. Incubation lasted for 24 h. At the end of this period, the cells were transferred to fresh medium containing nonradioactive thymidine and methionine ($\times 100$ concentration), incubated for 1 h, collected, and stored at −20 °C. After 24 hours' storage, DNA and proteins were solubilized in liquid soluene (Tissue solubilizer Packard) at 50 °C for 2 h. This procedure was chosen because it permitted us to obtain values that were directly comparable from one experimental series to the next. The process avoids the possible loss of information due to specific extractions of both DNA and protein; in addition, it gives a better approximation to the total radioactivity than can be obtained with preparations on filters. Radioactivity was measured in a Beckman L8-70 liquid scintillation counter using a double labelling program. The effects of the three neuropeptides on the gonadal synthetic activity were determined by measuring the rate of ^3H and ^{35}S incorporation. Fifteen experimental series were conducted with each of the three neuropeptides using gonadal cells from adult or juvenile snails. Pooled data from all replicate experiments are expressed as a percentage of inhibition or stimulation. This percentage was calculated in comparison to the control samples.

Concentrations of synthetic substances

To determine the optimal conditions for cellular synthetic activities, methionine-enkephalin, somatostatin-14, and insulin were added to the incubation medium in concentrations ranging from 10^{-4} to 10^{-9} M. Stock solutions of all three synthetic peptides were prepared in 0.1 N HCl. Agonistic and antagonistic substances were utilized for the study of the membrane receptors. Naloxone (opiate antagonist) was added to the cultures in concentrations ranging from 10^{-6} to 10^{-8} M, 30 min before methionine-enkephalin. CCP1 (somatostatin-cyclic analog) was added to the cultures in concentrations ranging from 10^{-6} to 10^{-8} M, alone or at the same time as somatostatin. Student's test was used to determine the statistical significance of the results[20].

Results

Influence of somatostatin, methionine-enkephalin and insulin on the synthetic activity of adult *Helix aspersa maxima* cell cultures

All three peptides influenced ^3H thymidine and ^{35}S methionine incorporation in a dose-dependent manner (table 1). No matter which neuropeptides were used, the effect on the cell's synthetic activity varied with different concentrations. For inhibition (negative percentages)

Table 1. Effect of methionine-enkephalin, insulin and somatostatin on the synthetic activity of adult *Helix aspersa maxima* gonadal cells after 48 h in culture.

	Incorporation of ^{3}H thymidine (dpm)	% inhibition (−) or stimulation	Incorporation of ^{35}S methionine (dpm)	% inhibition (−) or stimulation
Control	128973 ± 103		149166 ± 204	
Methionine-enkephalin				
2×10^{-5} M	50171 ± 141	−61	74519 ± 124	−50
2×10^{-7} M	83833 ± 194	−35	106002 ± 188	−28
2×10^{-8} M	202361 ± 144	56	209004 ± 170	40
2×10^{-9} M	99214 ± 191	−23	125351 ± 141	−15
Insulin				
2×10^{-5} M	39975 ± 138	−69	62652 ± 123	−57
2×10^{-6} M	76034 ± 140	−41	107449 ± 142	−27
2×10^{-7} M	97967 ± 137	−24	128443 ± 290	−13
2×10^{-8} M	217821 ± 175	68	232713 ± 176	56
2×10^{-9} M	184365 ± 135	42	185069 ± 132	24
Somatostatin				
2×10^{-5} M	41240 ± 96	−68	70051 ± 204	−53
2×10^{-6} M	81206 ± 104	−37	119347 ± 139	−19
2×10^{-7} M	165014 ± 173	27	176072 ± 134	18
2×10^{-8} M	214098 ± 163	66	229709 ± 193	53
2×10^{-9} M	103131 ± 92	−20	126888 ± 177	−14

Cells of 150 gonads were cultured in medium enriched with 20% fetal calf serum for 24 h. After this period, the cells were exposed to ^{3}H thymidine and ^{35}S methionine in fresh medium containing 6% Ultroser G in the absence (control) or presence of 3 neuropeptides. Each value for incorporation of ^{3}H, ^{35}S represents the mean ± standard deviation (SD) of 10 wells. The percentage of inhibition or stimulation is calculated in comparison to the control samples.

the range was between −13% and −69%, and for stimulation (positive percentages) between +18% and +68% (table 1). Incorporation rates of ^{3}H thymidine and ^{35}S methionine are not different in all the wells of the same series (i.e. standard deviation is insignificant) (table 1). For all three neuropeptides, a concentration of 10^{-8} M significantly elevated ^{3}H thymidine and ^{35}S methionine incorporation. The change in ^{3}H thymidine incorporation in cell cultures under the influence of peptides was greater than that of ^{35}S methionine (table 1). Methionine-enkephalin showed a lower stimulation effect for both ^{3}H thymidine and ^{35}S methionine incorporations (respectively 56%, 40%) than did the two other neuropeptides (somatostatin 66% and 53%, in-

Table 2. Comparison of inhibition (−) and stimulation percentages of the synthetic activity of two species of adult snail gonadal cultured cells in the presence of 3 neuropeptides.

		Methionine-enkephalin		Insulin		SST-14	
		Haa	Ham	Haa	Ham	Haa	Ham
2×10^{-5} M	Incorporation of thymidine	−64.63	−61.09	−69.51	−69.72	−70.65	−68.02
	Incorporation of methionine	−51.37	−50.04	−58.16	−57.99	−57.13	−53.03
2×10^{-6} M	Incorporation of thymidine	-	-	−40.31	−41.04	−39.15	−37.03
	Incorporation of methionine	-	-	−27.84	−27.96	−27.96	−19.99
2×10^{-7} M	Incorporation of thymidine	−39.85	−35.00	−24.52	29.65	27.94	
	Incorporation of methionine	−33.95	−28.93	−14.50	−13.89	20.11	18.03
2×10^{-8} M	Incorporation of thymidine	61.71	56.90	69.09	68.89	69.14	66.00
	Incorporation of methionine	42.16	40.11	57.43	56.00	57.60	53.00
2×10^{-9} M	Incorporation of thymidine	−29.70	−23.33	43.36	42.94	−19.17	−20.03
	Incorporation of methionine	−18.72	−15.96	25.42	24.06	−13.61	−14.93

SST-14: synthetic 14 somatostatin; Haa: *Helix aspersa aspersa*; Ham: *Helix aspersa maxima*.

sulin 68%, 56%). Methionine-enkephalin had a stimulating effect only at one concentration (10^{-8} M), whereas 10^{-7} M somatostatin and 10^{-9} M insulin were effective in inducing a stimulating response. For all three neuropeptides, both lower and higher concentrations inhibited the synthetic activity of cultured cells (table 1).

Comparisons of the effect of 3 peptides on the synthetic activity of cultured cells from 2 developmental stages in 2 snail species

In both species (*Helix aspersa maxima*, *Helix aspersa aspersa*), all three neuropeptides produced similar effects on the synthetic activity of adult gonadal cells (table 2). In juvenile gonadal cells of *Helix aspersa maxima*, concentrations 10 times higher than those used with adults of the same species were required to achieve the same results (fig. 1). The maximum stimulation of both ^{3}H thymidine and ^{35}S methionine incorporation was obtained when the juvenile cells were exposed to a neuropeptide concentration of 10^{-7} M (fig. 1).

Pharmacological studies

Naloxone. Naloxone is considered to be an opiate antagonist which binds to μ receptors. At 10^{-7} M it inhibited the incorporation of ^{3}H thymidine and ^{35}S methionine (−20%) in adult snail gonadal cells (fig. 2). The incorporation of both ^{3}H thymidine and ^{35}S methionine was higher in cultures exposed to 10^{-8} M methionine-enkephalin. However, the combined presence of naloxone and methionine-enkephalin appeared to prevent the stimulation observed when only methionine-enkephalin is added (fig. 2). The simultaneous presence of naloxone (10^{-7} M) and methionin-enkephalin (10^{-8} M) induced an inhibitory effect on the synthetic activity of gonadal cells. This inhibition was less marked (−16%) than that observed when naloxone alone was used (−20%).

With juvenile gonadal cells, we also observed a dose-dependent inhibitory action. The usual stimulating effect of methionine-enkephalin (10^{-7} M) was replaced by a decrease in synthetic activity (−17%) in the presence of 10^{-7} M naloxone. The simultaneous presence of naloxone (10^{-6}–10^{-8} M) and methionine-enkephalin at the same concentration accentuated the inhibitory action of methionine-enkephalin (fig. 2).

CPP1: cyclo (7-amino heptanoyl-Phe-D-Trp-Lys-Thr). The presence of CPP1 (somatostatin cyclic analog) showed a stimulating dose-dependent effect on the synthetic activity of adult *Helix aspersa aspersa* gonadal cells (fig. 3). The maximum incorporation of ^{3}H thymidine (65%) and ^{35}S methionine (55%) was obtained at 10^{-6} M. This stimulation was decreased by 10% for each 10-fold reduction of the CPP1 concentration (fig. 3). The maximum response of gonadal cells to somatostatin occurred at a concentration of 10^{-8} M (+58%); the response decreased by one-half when the concentration was increased to 10^{-7} M (+25%). The simultaneous presence of CPP1 and somatostatin (3 concentrations were tested 10^{-6}, 10^{-7}, 10^{-8} M) induced a dose-dependent inhibitory effect; maximum inhibition (−41%) occurred at 10^{-6} M (fig. 3). This inhibition was higher than when somatostatin was used alone at the same concentration (−22%).

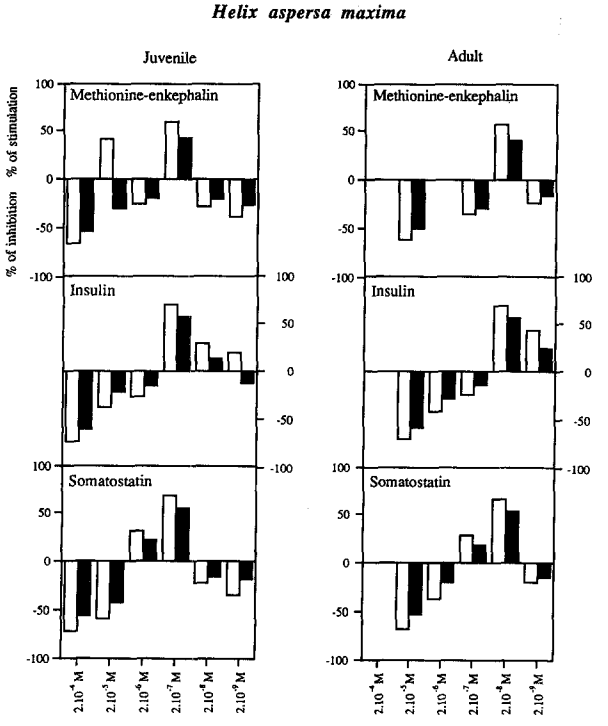

Figure 1. Comparison of methionine-enkephalin, insulin and somatostatin effects on the synthetic activity of juvenile and adult gonadal cells after 48 h in culture. Cells were cultured in medium enriched with 20% fetal calf serum for 24 h. After this period, the cells were exposed to ^{3}H thymidine and ^{35}S methionine in fresh medium containing 6% Ultroser G in the absence (control) or presence of tested substances. Each value represents the mean of 10 wells. The percentage of inhibition of stimulation is calculated in comparison to the control samples.
□ = ^{3}H thymidine incorporation; ■ = ^{35}S methionine incorporation.

Discussion

To the best of our knowledge, this is the first report on the in vitro influence of neuropeptides on invertebrate gonadal cell cultures. Our conclusions are based on the comparison of assays with controls, that is they are relative values. While it is not possible to take into account a probable release of cellular proteins into the medium, nor a possible influence of endogenous peptides, the identical experimental conditions used allow us to assume that the background 'noise' is constant. Our results with snail gonadal cells in culture demonstrate that neuropeptides influence the incorporation of precursors of DNA and protein synthesis. These neuropeptides probably act as hormones, as a dose effect has been demonstrated (table 2). Their influence on

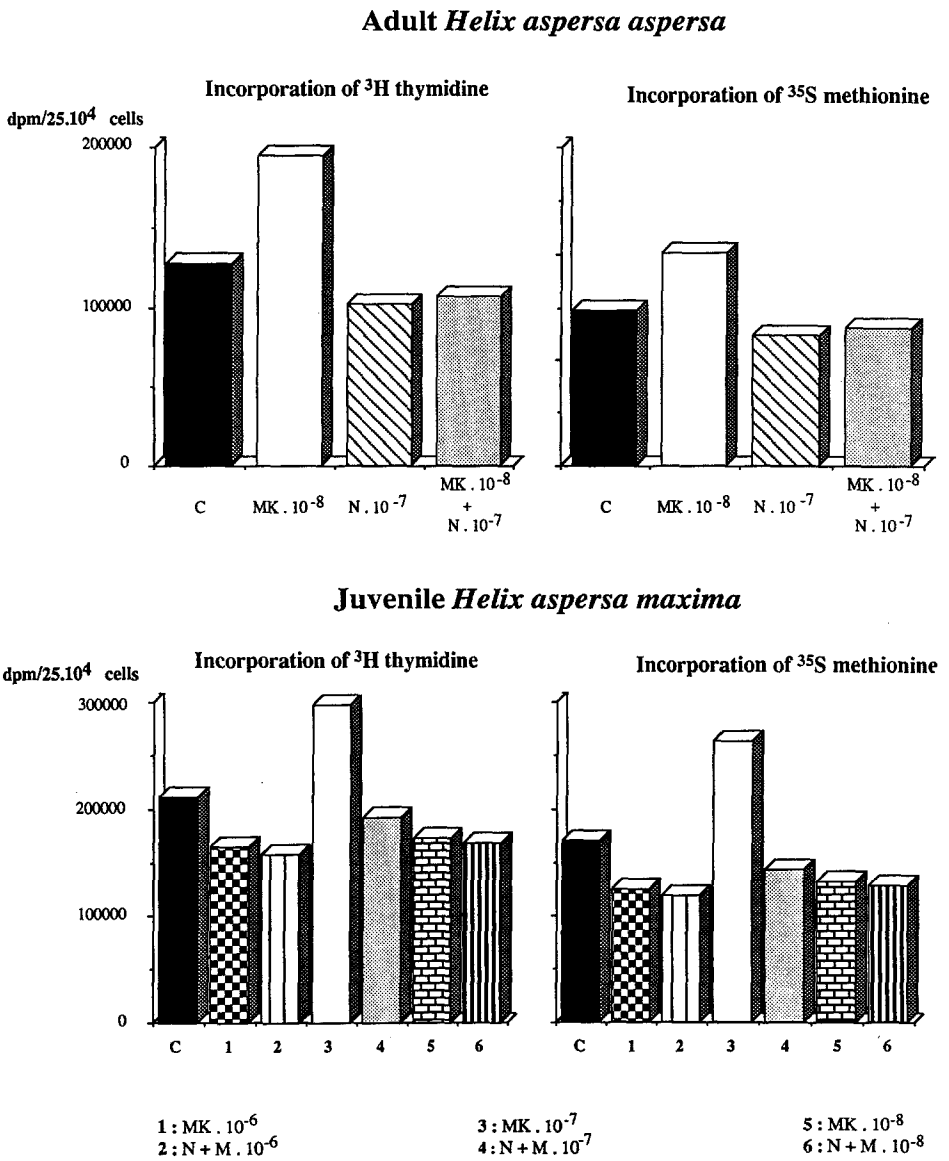

Figure 2. Effect of methionine-enkephalin (MK) and naloxone (N) on the synthetic activity of the gonadal cells of adult and juvenile snails after 48 h in culture. Cells were cultured in medium enriched with 20% fetal calf serum for 24 h. After this period, the cells were exposed to ^{3}H thymidine and ^{35}S methionine in fresh medium containing 6% Ultroser G in the absence (control) or presence of tested substances. The values are the mean of 10 wells.

DNA synthesis seems to be greater than on protein synthesis. Our previous results showed that cells which survive two days in culture are predominantly spermatogonia. Such cells divide rapidly and have a high rate of DNA synthesis[21].

Results obtained with both species of snails were similar (table 2) and they can therefore be used alternatively as cell culture material in a bioassay for neuroendocrinological studies. This had been previously observed in studies of brain extract influence on the synthetic activity of gonadal cells[5]. The advantage of using *Helix aspersa maxima* is that its gonad is much larger and therefore more easily dissected.

The developmental stage of the snail seems to influence the receptivity of the gonadal cells to neuropeptide, independently of which of the three neuropeptides was used; juvenile cells appeared to be less receptive than adult cells, and required a 10 times higher concentration of neuropeptide. It is interesting to observe that the level of somatostatin-like material is higher in the hemolymph and hepatopancreas of young snails than in those of adults[10,11,22]. Due to the fact that juvenile cells aggregate in cell culture[21], the number of accessible receptors is probably reduced.

In vertebrates, the stimulating role of insulin on the synthesis of DNA, RNA and proteins is well known; for example the internalization of insulin entails protein synthesis in Xenopus oocytes[23].

The presence of neuropeptides in snail gonad and hepatopancreas[13,22] suggests a paracrine or autocrine role for gonadal peptides, in addition to their role in endocrine control. In mammals a number of in vitro or

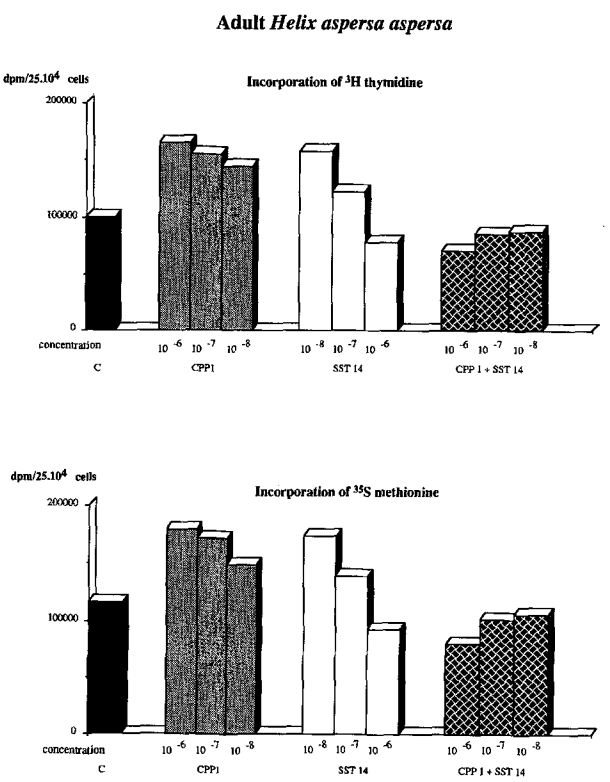

Figure 3. Effect of 14-somatostatin (SSt-14) and its cyclic analogue (7-amino heptanoyl-Phe-D-Trp-Lys-Thr or CPP1) on the synthetic activity of the gonadal cells of adult *Helix aspersa aspersa* after 48 h in culture. Cells were cultured in medium enriched with 20% fetal calf serum for 24 h. After this period, the cells were exposed to ^{3}H thymidine and ^{35}S methionine in fresh medium containing 6% Ultroser G in the absence (control) or presence of tested substances. The values are the mean of 10 wells.

in vivo studies have been conducted that demonstrate the paracrine or autocrine role of gonadal peptides[24].

With regard to two of the neuropeptides tested (somatostatin and methionine-enkephalin), only the concentrations which may be considered as physiological (10^{-8} M) increased the synthetic activity of the gonadal cells. The higher as well as the lower concentrations used were shown to be inhibitory. The two peptides are known to produce a decrease of the cAMP level and to provoke an early inhibiting effect. Recently it was shown that an 'acute' activation of opiate receptors often induces an effect diametrically opposite to that of a 'chronic' one. The binding of opiates to their receptors for a short period of time (acute treatment) inhibits the adenylyl cyclase activity, while a chronic treatment (beyond 12 h) produces a stimulating effect[25]. In our experiments, low doses produce an effect comparable to that obtained with acute treatment. Such results had already been observed in studies with snail brain extracts[5]. The stimulating effect of physiological doses (10^{-8} M) is probably caused by a dissociation of receptors from G proteins, a hyperproduction of cAMP, and a cascade of metabolic stimulation. At high peptide doses, there is a saturation of receptors, and internalization, which results in their down-regulation; this occurs after the disassociation of the peptides from their receptors inside the cell. In this manner the diminution of the number of receptors produces a situation closely ressembling that obtained by an acute activation.

While the best-known intracellular action of somatostatin is connected with the cAMP pathway, another pathway of message transduction exists: IP3 (inositol-1,4,5-triphosphate). It has been demonstrated on various areas of the rat brain in vitro that somatostatin increases IP3 concentration in a dose- and time-dependent manner[26]. Perhaps, by acting through the IP3 pathway, somatostatin modulates or compensates the inhibiting effect via the cAMP pathway. In rats, CPP1 (somatostatin analog) affects the release of growth hormones and both low and high doses give similar results[27].

Insulin acts by a quite different intracellular mechanism (tyrosine-kinase activation). The synthetic activities of gonadal cells were stimulated by insulin concentrations of 10^{-8} M and 10^{-9} M. With the latter the stimulation was less evident. With concentrations above 10^{-8} M, the internalization of receptors probably leads to a global inhibition. The formation of receptosomes and their intracellular degradation presumably do take place during the 24 hours' bioassay[28].

All three of the neuropeptides used in our bioassays have the same effect on the synthetic activity of gonadal cells (see fig. 1); if we admit that in the cells the end product of metabolic pathways is phosphorylation, our results are not astonishing. The identification of the mechanism responsible for the similarity in our results is, however, difficult. The phosphorylation of proteins (replication/transcription factors and of ribosomal S6 proteins) regulate the synthesis of DNA, RNAs and proteins[28,29]. The diversity of the signalling molecules implicated in the message transduction mechanism permits each cell a high degree of modulation; it is probable that the mechanism responsible for the similarity observed in our results operates at this level.

Hormones, particularly insulin, can intervene indirectly by increasing the number of receptors for other hormones, thus acting synergistically with the growth factors. In pig Leydig cells, insulin increases the number of hCG receptors and their coupling with adenylate cyclase. At the same time, insulin enhances the activity of several enzymes of the steroidogenic pathway. Insulin generally has a small but significant mitogenic action on both Leydig and Sertoli cells; however, in cells treated by FGF, insulin potentiates its mitogenic action[17]. In snail gonadal cell cultures, insulin may interact with other hormones or growth factors present in the culture medium or produced by the cells themselves. In vitro metabolism studies have shown that snail gonads contain endogenous steroids[30].

If all the available literature on neuropeptides is taken into consideration, it appears that at the cellular level, neuropeptides play a role in invertebrates which is not always the same as that in vertebrates. For example, in vertebrates somatostatin has an inhibiting effect on the proliferation of cells[26], whereas in snails we have demonstrated that it has a stimulating effect.

A final question needs to be discussed: do neuropeptide receptors exist in snails? The response of target cells to neuropeptides would lead us to presume their existence. Insulin receptors have been detected in ganglia of *Helix aspersa*[31]. Our pharmacological experiment with naloxone, whose binding to opiate μ receptors is considered as specific, suggests the presence in snail gonadal cells of receptors that are similar to those found in vertebrates. The results described in this paper show that the gonadal cell culture method is more reliable than the organotypic culture method; our results were similar for all experimental series. Our method appears to be ideal for bioassays that evaluate the effect of neuropeptides on DNA and total protein synthesis; this method could also be used to investigate molecular mechanisms that regulate the effect of these peptides. Understanding the physiological mechanisms by which snail neuropeptides influence the functioning of the gonad might be the goal of our future studies.

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