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Original Article

Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians

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ABSTRACT. Substance P and substance K (Neurokinin A) are mammalian peptides belonging to the tachykinin family. Both have been studied extensively, are widely distributed in both central and peripheral mammalian nervous systems, and seem to be involved in pain reactions and inflammatory responses. We report here that substance P and substance K, as well as Epidermal Growth Factor (EGF), are potent mitogens, at micro and nanomolar concentrations, for planarian cells. This stimulation is inhibited by the substance P and substance K antagonist spantide, while capsaicin, a pungent agent of capsicum peppers that destroys sensory neurons, stimulates cell division, probably through release of substance P. These results, jointly with the reported stimulation of cell division by naloxone and its inhibition by Met-Enkephalin (Baguña, 1986), both probably acting on tachykinin release, suggest that target cells, the neoblasts, must have in their cell membranes numerous receptors for growth hormones and neuropeptides analogous to their mammalian counterparts.

Introduction

Freshwater planarians (Fig. 1) are widely known for their high power of regeneration. When a planarian is cut (Fig. 2), the wound rapidly contracts and a thin film of epidermal cells from the stretched old epidermis covers it. Below the wound epithelium, groups of undifferentiated cells soon appear forming a few layers of cells that grow by addition of new undifferentiated cells formed by cell division in the underlaying parenchyma. At one day of regeneration, this accumulation of undifferentiated cells is externally visible due to its unpigmented character, and is known as the regenerative blastema. Over the next few days, the blastema grows exponentially, and is clearly visible at 3-5 days of regeneration (Fig. 2) when new structures (e.g. eyes and brain ganglia in anterior (cephalic) regeneration) appear within the blastema and postblastema areas. The lost pattern is thus restored and the initial body proportions finally acquired after 3-4 weeks of regeneration (Fig. 2).

Besides their ample power of regeneration, freshwater planarians are also known for their ability to grow and degrow (to shrink in volume and length) continuously, depending on body size, temperature and food availability. Quantitative cellular studies of both processes have shown them to depend on the daily balance between cells born by cell proliferation and cells lost by cell lysis and death (Baguñà and Romero, 1981; Romero 1987).

Regeneration and growth/degrowth in planarians seem to have a common cellular basis. Planarians can be considered, in cellular terms, to be made of two main compartments: 1) a proliferative one which comprises 20-25% of total cells and is composed of a single, though heterogeneous, class of small undifferentiated cells (the so-called neoblast) which give rise, by differentiation, to all differentiated cell types; and 2) a functional compartment, made of 12-15 terminally differentiated non proliferating cell types (75-80% of total cells) that turn over continuously during the life-time of the organisms. Several lines of evidence indicate that the undifferentiated cells (neoblasts) of the intact organism, which serve as stem cells for daily cell renewal are similar to the undifferentiated cells of the blastema, the latter arising directly from the former (Saló and Baguñà, 1984; Baguñà *et al.*, 1988). Since both processes, especially regeneration, are driven through increased cell proliferation, and since neoblasts are known to be the only proliferative cell type in planarians, one of the main areas of the active research lies in the discovery of how they are stimulated to divide during growth and regeneration, what kind of factors or substances are responsible for such increase, what type of tissues or organs produce them, and what the molecular mechanisms through which neoblasts respond are.

To address such questions, a first and valid approach has been to test in intact and regenerating planarians the action (stimulatory or inhibitory) of some growth factors and neuropeptides isolated and characterized from several vertebrates (mainly mammals) and invertebrates (Saló and Baguñà, 1986). The neuropeptides substance P (SP), substance K (SK), bradykinin, hydra peptide, and the epidermal growth factor (EGF) have been found to be, at concentrations known to enhance cell proliferation in mammalian cell cultures (100 - 0.1 nM), potent mitogens in intact and regenerating planarians (Baguñà, 1986; Baguñà *et al.*, 1988). These results suggest that these substances,

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Fig. 1. External apperance of a live specimen of Dugesia(G)tigrina fissiparons strain. *.x 10*

known to be widely distributed in both central and peripheral nervous systems of mammalians and other organisms (Black *et al.*, 1982; Cuello *et al.*, 1982) may also have an important specific role in controlling cell proliferation in planarians, a group placed in one of the lowest ranks of the phylogenetic ladder.

A specific role for substance P was further supported when Met-enkephalin, an opiate that inhibits the release of SP (Mudge *et al.*, 1979), and naloxone, a potent antagonist of Met- enkephalin by competing to bind to opiate receptors (Simon and Hiller, 1978), were found to inhibit and activate, respectively, cell proliferation in intact planarians (Baguñà, 1986). However, whether the effects of Met-enkephalin and naloxone on SP release in planarians were direct or whether they resulted through interactions with any of the myriad of substances or factors known to be involved in the control of cell proliferation (Zachary *et al.*, 1987), remains uncertain.

A more specific test of the action of SP and SK on cell proliferation in planarians would be to study both the action of specific antagonists and activators of these substances. We address here this problem studying the effects on cell proliferation in intact planarians of spantide, a substance P antagonist acting probably through competitive inhibition of SP and SK receptor binding (Holmdahl *et al.*,1981), and capsaicin, a pungent agent from capscicum pepper known to stimulate the release of SP from sensory nerve terminals (Cuello *et al.*, 1982). If these substances inhibit or activate, respectively SP and SK-mediated action on cell proliferation this would be a further indication of the presence of SP and SK and their receptors in planarians and of their role in controlling cell proliferation.

Results

Effects of neuropeptides and EGF on cell proliferation

The addition of SP, SK and EGF to intact Dugesia(G)tigrina increased significantly (Student's t test) their mitotic indices as compared to intact control organisms (Fig. 3). The effect is more pronounced for both tachykinines, mainly for SK, and less so for EGF, this latter only being significant from 10^{-7}M up. The activation is similar at different times of incubation (8,15 and 48 hrs) but from now on only those for 15 hours will be represented here.

The concentrations found where SP and SK activate cell proliferation are similar to those found by Nilsson *et al.* (1985) in tests to stimulate proliferation of cultured muscle cells and fibroblasts from rats and humans respectively. Similary, a higher effect of SK was also found.

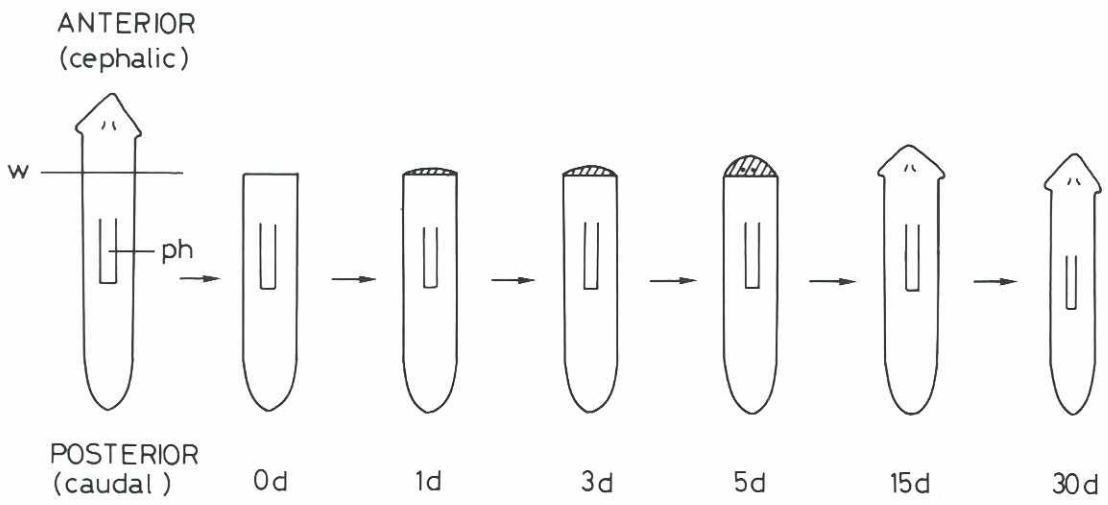

Fig. 2. *Diagramatic representation of anteriorly regenerating* Dugesia(G)tigrina *up to 30 days of regeneration. Hatched: blastema region; ph: pharynx; w: wound.*

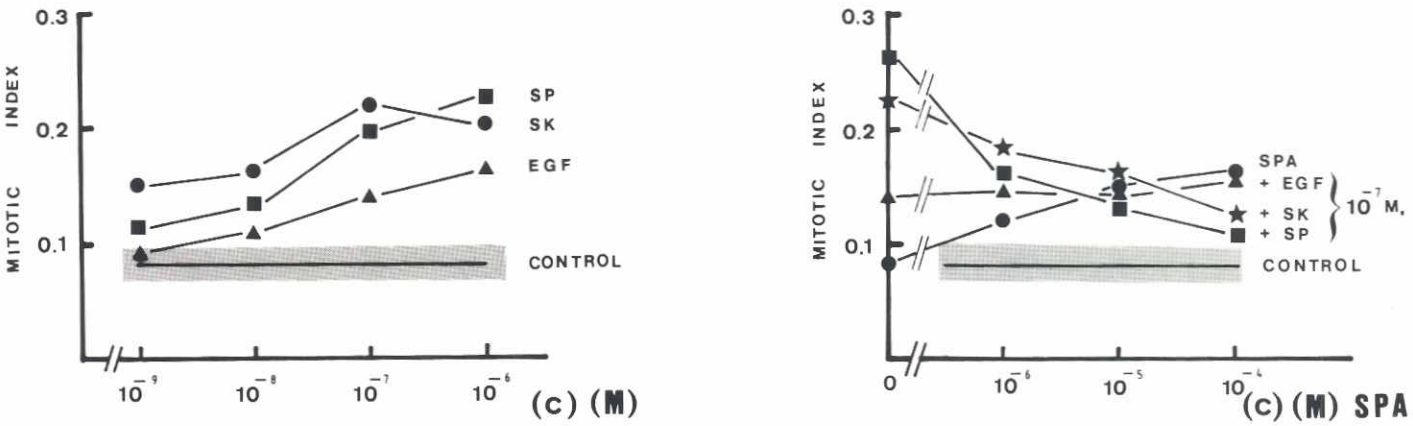

Fig. 3. *Stimulatory effect of substance P (SP), substance K (SK) and epidermal growth factor (EGF) on the mitotic index of 6-7 mm. long intact planarians after 15 hours incubation (10^{-6}-10^{-9} M.). Control: non treated organisms. Ordinate: mitotic index (mitoses/100 nuclei); Abscissa: concentration (in M.) of the substances tested. Each point represents the average of 15 values. For the sake of clarity standard deviations (with ranges not higher than 15% of the average value) have been omitted.*

Fig. 4. *Inhibitory effect of substance P analog Spantide (SPA) on mitotic activation of 6-7 mm long intact organisms co-incubated with substance P (SP), substance K (SK) or Epidermal Growth Factor (EGF) at 10^{-7} M., or spantide alone (10^{-4} to 10^{-6} M.) for 15 hours. Control: non treated organisms. Ordinate: mitotic index (mitoses/100 nuclei); Abscissa: concentration (in M.) of spantide (SPA). Each point represents the average of 15 values. For the sake of clarity, standard deviations (not higher than 15% of the average value) have been omitted.*

Effect of spantide on SP and SK activation of cell proliferation

To determine the specificity of the stimulatory action of SP and SK, experiments were performed with organisms co-incubated with 10^{-7} M SP or SK and the SP-antagonist spantide. Organisms incubated either with spantide alone, with 10^{-7} M EGF and spantide, or in planarian saline (PS) served as controls.

The addition of spantide (10^{-4} to 10^{-6}M) alone caused a limited, though significant, stimulation of cell proliferation, 10^{-4}M doubling the mitotic index of controls without spantide (Fig. 4). Added together with SP or SK (10^{-7}M), spantide produced a significant reduction of SP and SK mediated increases in cell proliferation, whereas co-incubation of spantide and 10^{-7}M EGF did not affect the proliferative increase caused by EGF alone.

Effect of capsaicin on cell proliferation

The effects of 10^{-4} to 10^{-6}M capsaicin on cell proliferation in planarians are summarized in Figure 8. Capsaicin was found to be highly stimulatory at 10^{-6} and 10^{-5}M, and lethal to whole organisms at 10^{-4}M. Surprisingly, the solvent solution used also stimulated cell proliferation, its effect increasing with higher concentrations. Sorting out both effects it becomes clear that at 10^{-6} M capsaicin is clearly stimulatory since the effect of the solvent alone, though slightly stimulatory, is significantly lower. At 10^{-5} M however, the activating effect of capsaicin is blurred by a similar effect of the solvent alone, reducing the former to insignificance. Finally, the lethality of 10^{-4} M capsaicin should be mainly assigned to capsaicin and not to the solvent, as the latter shows by itself a highly stimulatory effect.

Discussion

The results described here are further evidence that mammalian growth factors and neuropeptides, mainly the

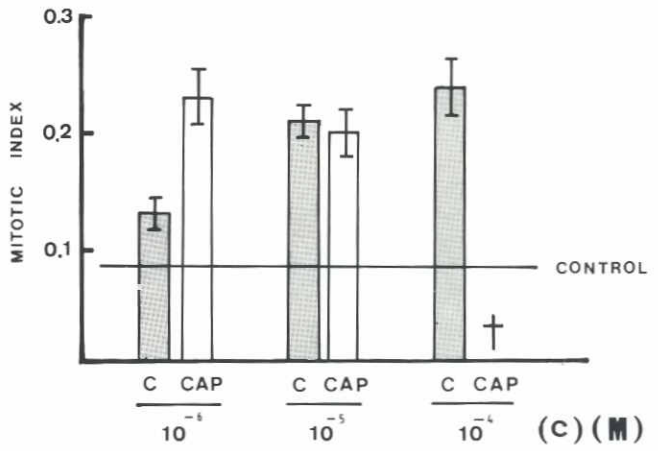

Fig. 5. *Effect of capsaicin (CAP) and its solvent (Tween- methanol)(C) on mitotic index of 6-7 mm. long intact organisms after 15 hours incubation (10^{-4}-10^{-6} M.). Control: non treated organisms. Ordinate: mitotic index (mitoses/100 nuclei); Abscissa: concentration (in M.) of capsaicin (CAP). Data represent means ± S.D. (n=3; 5 individuals per experiment).*

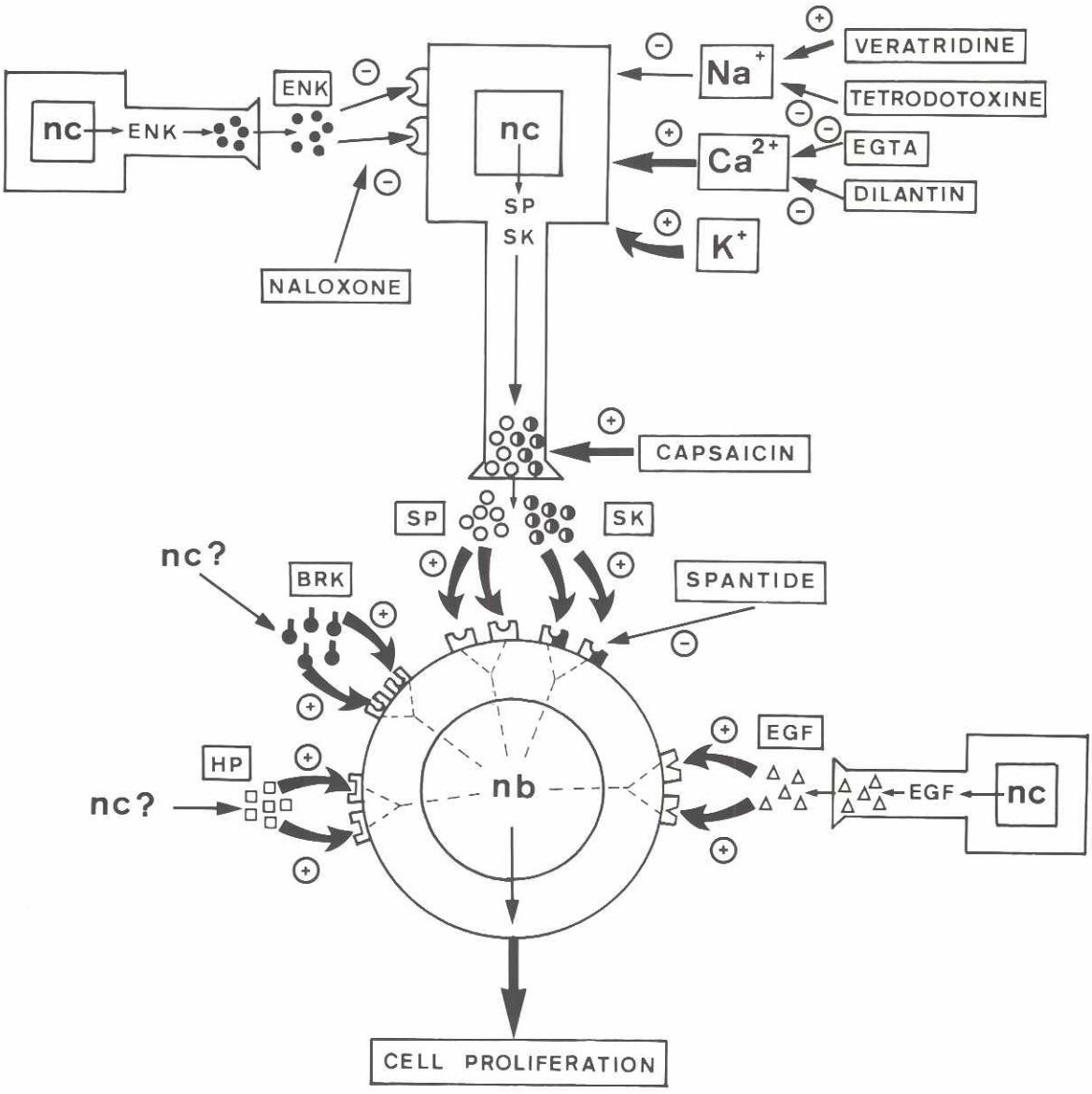

Fig. 6. *Diagram of the presumed effects and interactions between neuropeptides, growth factor and its receptors, target cells (neoblast) and nerve cells in planarians according to data from this work and recent literature. nb: neoblast; nc: nerve cell; BRK: bradykinin; EGF: Epidermal Growth Factor; HP: Hydra Peptide; SK: substance K; SP: substance P. Thick lines represent stimulatory effects; thin lines, inhibitory effects. For more details, see text.*

tachykinines, substance P (SP) and substance K (SK), act as specific potent mitogens for planarian cells.

The specific role of SP and SK clearly stems from the use of the SP analogue, spantide. First, spantide alone had a small, though significant, stimulatory effect, a fact consistent with the reported agonistic activity of substance P analogues (Holmdahl *et al.*, 1981). Second, cell proliferation induced by SP and SK was markedly reduced by co-incubation with increasing concentrations of spantide, suggesting a competitive inhibition of receptor binding. Finally, EGF-mediated increases in cell proliferation were not affected by increasing concentrations of spantide. Moreover it is also interesting to note that bombesin, a neuropeptide that has two C-terminal amino acids in common with SP and SK, failed to increase cell proliferation in planarians (Baguñà, 1986), a result also found for mammalian fibroblasts (Nilsson *et al.*, 1985). We therefore conclude that the stimulatory effect of SP and SK should be mediated via specific tachykinin receptors.

The stimulatory effect of capsaicin, an agent known to enhance, at low concentrations, the release of SP and other neuropeptides and neurotransmitters from sensory neurons in newborn mice and, at higher doses, to destroy selectively small diameter sensory neurons and deplete SP immuno-reactivity (Cuello *et al.*, 1982), represents further evidence of the role of both tachykinines in planarians. As expected, low doses gave highest effects while higher doses, probably through nerve terminal destruction, led to the death of organisms. In the latter case, a brief high spurt of mitotic activity due to the masive release of SP during early phases of sensory cell destruction, can be anticipated (Baguñà, work in progress).

An interesting, though rather unexpected, outcome of capsaicin action was the stimulatory effect shown, even at low doses, by capsaicin solvent made of Tween and methanol (Fig. 5). Given the effects of both substances as enhancers of cell permeability, it is suggestive to think they act enhancing the entrance, into the cells, of specific ions (Na^+, K^+, Ca^{2+}) somehow involved in the transducing path leading to cell proliferation. Indeed, substances like veratridine and tetrodotoxine that stimulate or inhibit Na^+ flux into the cells, are known to block or enhance, respectively, the release of SP from sensory neurons *in vivo* and *in vitro* (Black *et al.*, 1982). In planarians, high concentrations of extracellular Na^+ are known to inhibit cell proliferation, whereas high concentrations of K^+ and Ca^{2+} are stimulatory, the latter effect shown by the absence of mitoses in planarians incubated in calcium-free medium or in media where calcium has been depleted by Ca^{2+}-chelating substances such as EDTA and dilantin (Baguña, 1973; Franquinet, 1981).

A general diagram summarizing all the data here considered is shown in Figure 9. Although some relationships are still highly uncertain and speculative, two main points warrant further consideration. First, it is becoming evident that simple animals like planarians show immunoreactivity to antibodies against a pleyade of neuropeptides and growth factors first detected and isolated in higher groups (mainly in mammals). This suggests very strongly the existence of such factors and substances in planarians, calling for a thorough study of their role in such primitive organisms as a means to understand their further role in more advanced groups. It is, therefore, of the outmost interest to study the tissular and cellular distribution of such factors in intact planarians as well as to look for qualitative and quantitative changes during regeneration. Immunocytochemical studies in planarians (Burgaya, Bueno, Sumoy, Romero and Baguñà, unpublished data) and related *Turbellaria* (Wikgren and Reuter, 1985) have shown them mainly located within nerve cells and processes, occurring in different, but partially overlapping, sets of neurons, though the changes of expression, if any, during regeneration have not been worked out.

Second, undifferentiated cells (neoblasts) of planarians, known to be the only cell type able to divide (Baguña, 1981; Ehlers, 1985), seem the likely target for such factors and, hence, to display in their membranes an array of receptors specific to them. This will make of such cells ideal systems to study, both *in vivo* and *in vitro*, the single and combined action of such factors in stimulating (or inhibiting) cell proliferation and cell differentiation. Moreover, the morphological and functional heterogeneity of neoblasts (Saló and Baguñà, 1984; Auladell and Baguñà, work in progress) can be considered to be due, at least partially, to different sets of receptors displayed in their surfaces. A molecular screening of this presumed heterogeneity using oligonucleotide probes and monoclonal antibodies, may lead to the discovery of subsets of neoblast in different stages of proliferation and/or determination/diferentiation, as well as the confirmation of whether cells other than neoblasts bear such receptors.

Materials and Methods

The planarians used were individuals, 6-7 mm long of an asexual strain of *Dugesia(G)tigrina (Platyhelminthes; Turbellaria; Tricladida)*. They were maintained in Petri dishes in the dark at 17±1°C in planarian saline (PS; Saló, 1984), and fed with Tubifex. In all experiments, one-week-starved organisms were used and the temperature kept at 17±1°C.

Chemicals

Substance P, substance K, epidermal growth factor and capsaicin were all from Sigma (London). Spantide (D-Arg^1, D- Trp^{7,9}, Leu^{11}-SP) was from Bachem (Switzerland).

Stock solutions of SP, SK and EGF were made at 10^{-4}M in PS. Spantide was dissolved in PS at 10^{-3} M, and capsaicin was dissolved in methanol: Tween 80: PS (1:1:8) at 10^{-2} M. All solutions were kept in 100 mcl aliquots at -20° C until used.

Effect of neuropeptides and growth factors on cell proliferation

To test the effects of SP, SK and EGF, intact organisms kept in PS were used. SP, SK and EGF were added, from stock solutions, at 10^{-6} - 10^{-9}M and organisms left for 2 days. To avoid bacterial contamination all solutions were filtered (Millipore, 0.22μm) and

contained 5 µg.ml-1 of kanamycin sulfate (Sigma, London). At 8, 15 and 48 hours of incubation, 5 individuals were set apart, fixed in 1N HCl, stained by a modified Gomori technique, and mounted whole (Saló and Baguñà, 1984).

Mitotic indices (number of mitoses/100 nuclei) were measured as described (Saló and Baguñà, 1986). As controls, intact organisms kept in PS without SP, SK or EGF were used, and their mitotic indices measured similarly.

Effect of spantide on cell proliferation

Spantide was diluted, from stock solutions, to 10^{-4} to 10^{-6} M in PS and used either alone or jointly with SP, SK or EGF at 10^{-7}M; this latter group aimed to determine the specificity of stimulatory action of SP and SK. Incubations were for 15 and 48 hours, and the experimental conditions and measurement of mitotic indices were as described.

Effect of capsaicin on cell proliferation

Capsaicin was tested at 10^{-4} to 10^{-6} M in PS. Incubation periods were for 15 and 48 hours, and experimental conditions and measurement of mitotic indices were as described. Control groups were intact organisms kept in PS alone or in PS with a similar amount of the solvent used (methanol:Tween 80:PS; 1:1:8) as in experimental groups. In terms of methanol and Tween 80 this range between 0.1 and 0.001% of total volume.

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