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Stimulation of Epithelial Cell Growth by the Neuropeptide Substance P

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Abstract The neuropeptide substance P (SP) was found to stimulate DNA synthesis and cell growth for epithelial cells (cornea and lens) in a serum-free environment. The length of treatment time was shown to be important since longer times shifted the dose-response curve to the left. In short-term DNA synthesis studies (40 h) the stimulation with SP (or synergism with insulin) was not apparent until close to 10 μM, however, when DNA synthesis assays were carried out over a long period of time (5 days) stimulation with SP was seen at 1 pM. The stimulation of DNA synthesis by SP was synergistic with insulin for lens epithelial cells, but little synergism was seen with corneal epithelial cells. It cell growth studies on lens epithelial cells SP also showed growth stimulation by itself and synergism with insulin at concentrations of 1–2 pM. The neuropeptide calcitonin gene related peptide (CGRP) showed no DNA synthesis stimulating ability on epithelial cells by itself at concentrations as high as 2.5 μM; however, it was synergistic with SP at a concentration of 0.025 μM. SP pretreatment of epithelial cells for 2 h causes an increase in cellular sensitivity to subsequent addition of either SP or insulin. This increase is consistent with the hypothesis that either the signal from SP persists after its removal from the cell or the dissociation time for SP from its receptor is longer than the wash time.

Key words: substance P, neuropeptide, calcitonin gene related peptide, CGRP, cell growth, DNA synthesis, insulin, epithelial cells, cornea, lens

Substance P (SP) is a constituent of sensory nerve fibers and has been postulated to mediate several different axonal reflexes such as plasma extravasation, vessel vasodilation, and mast cell histamine release [Burnstock, 1977; Henry, 1977; Hagermark et al., 1978; Lembeck and Holzer, 1979; Lembeck and Donnerer, 1981a]. In the eye, many nerve fibers containing SP immunoreactivity are found in the cornea and iris, and are assumed to play a key role in the ocular neurogenic responses to noxious stimuli [Tervo et al., 1982]. Immunofluorescence of SP in embryonic and newborn rats suggest that SP might play a role in developing ocular tissue in addition to its neurotransmitter or neuromodulator roles, because SP appears before establishment of synaptogenesis during fetal development [Sakiyama et al., 1984].

Dense networks of SP positive nerve fibers are found in most layers of the mammalian eye. Denervation of the trigeminal nerve to the eye results in a complete disappearance of all SP immunoreactive axons in the rabbit iris [Unger et al., 1981]. The SP level in the cornea of the adult mouse is reduced 40% by a similar procedure [Keen et al., 1982]. It has been shown that in cases where there is complete loss of SP from the cornea there is still no change in corneal sensitivity [Bynke et al., 1984]. Yet, it has been known since Magendie [1824] that sectioning of the trigeminal nerve causes trophic or degenerative changes in the cornea. Thus, while SP is not necessary for nociception, it may mediate maintenance of the epithelial barrier.

Recently, it was also found that several of the neuropeptides can act as mitogens for cells in culture. A direct growth promoting effect of SP and substance K has been reported in smooth muscle cells and human skin fibroblasts [Nils-

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son et al., 1985, 1986; Payan, 1985]. SP also enhances the proliferation of human blood T-lymphocytes [Payan et al., 1983], an effect apparently mediated by specific receptors for this peptide [Payan et al., 1984]. Recently, it was also reported that SP stimulates release of PGE_2 and proliferation in rheumatoid synoviocytes [Lotz et al., 1987]. These findings are in accord with other evidence which indirectly suggests that the release of tachykinins from sensory nerves in the skin, joints, and other peripheral tissues might function as mediators of local inflammatory and wound healing responses [Lotz et al., 1987; Zachary et al., 1987]. However, recent work on the possible mitogenic role of SP with a transformed mouse epidermal cell line [Toshihiro et al., 1988], and rat tongue epithelial cells in culture [Ivanov et al., 1987], showed that it was inactive as a mitogen unless fetal calf serum was present. Since the epithelial cells of the cornea and the lens are the only epithelial cells of the body that grow in a serum free environment, a series of experiments were carried out to see if SP could stimulate DNA synthesis in these cells under serum free conditions.

MATERIALS AND METHODS

Chemicals

Synthetic substance P (SP) was obtained from Multiple Peptide Systems, San Diego, CA, and calcitonin gene related peptide (CGRP) was obtained from Sigma Chemical Co., St. Louis, MO, and Peninsula Labs., Belmont, CA. It was found that aqueous solutions of SP were unstable if they were frozen and thawed; however they could be kept at 4°C for 1 week with only a loss of half of their activity. All solutions that were used in these experiments were made up fresh daily. Enkephalinase was obtained from Genentech, Inc. Enkephalinase is stable as a solution in 0.15 M NaCl in 0.01 M Hepes (pH 7.2) at 4°C for over a year. MTT (3,(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide was obtained from Sigma Chemical Co., St. Louis, MO.

Cell Culture

The Nakano mouse lens epithelial cells were obtained from Paul Russell (National Eye Institute, NIH, Bethesda, MD) and were described previously [Jones and Reid, 1984]. The cells used in these experiments were a clone obtained from these cells (NK-11). The rabbit cornea epithelial cells (SIRC) were obtained from the American Type Culture Collection (ATCC CCL-60). The NK-11 cells are grown in RPMI 1640 supplemented with 10% calf serum (JRH). The SIRC cells were grown in MEM supplemented with 5% new born calf serum (JRH) and 5% fetal bovine serum (JRH).

DNA Synthesis Assay

This assay is similar to that reported previously [Reid and Reid, 1987], with minor modifications. The NK-11 cells were plated in 96 well plates at a density of 1×10^4 per well in RPMI 1640 plus 10% calf serum. The cells were fed and grown to near confluence (2–5 days) with or without a refeeding on day 3 with fresh RPMI 1640 containing 10% calf serum. The cells were then fed with RPMI 1640 (0% serum) and allowed to reach quiescence. After 2 to 3 days the cells were then used for the assay. The SIRC cells were plated in 96 well plates at a density of 2×10^4 per well in MEM plus 5% new born serum and 5% calf serum. After 3 days the cells were fed with MEM plus 5% new born serum and 5% calf serum. After 3–4 days the cells were then fed with MEM and allowed to become quiescent. After 3 days the cells were ready to use in the assay. In order to start the assay the cells (NK-11 or SIRC) were re-fed fresh media (RPMI 1640 or MEM depending on the cell type) and then the desired hormones and peptides were added. After 16 h 0.1 μCi of ^{3}H-thymidine was added to each well. After a specified time period (2–5 days) the cells were washed with 0.15 M NaCl fixed in 0.3 M HCl for 20 min at 4°C, washed with 0.3 M HCl, and then with 95% ethanol. The cells were then solubilized in 0.5 M NaOH and counted in a scintillation counter. For one experiment the cells (NK-11) were stepped down for three days (RPMI 1640, no serum) and then allowed to grow for 5 days in the presence of SP and insulin. The cells were given a second dose of ^{3}H-thymidine after 3 days. All error bars represent standard errors of the mean.

Cell Growth Assays

For the cell growth assay as measured by MTT reduction, 500 cells were plated per well in a 96 well plate in 10% serum, grown in 0% serum for 1 day, and allowed to grow for 1 week in the presence or absence of added factors. At the end of this time the cells were worked up using the MTT assay for cell growth, where the formation of formazan by the reduction of MTT,

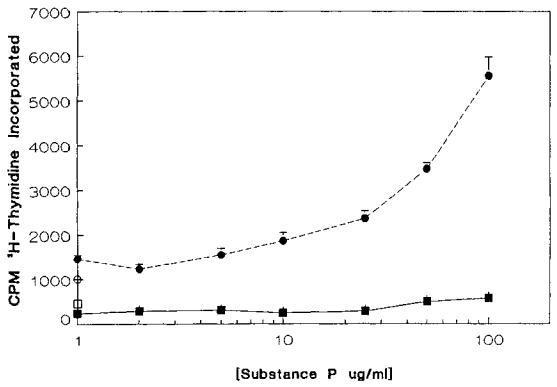

Fig. 1. Dose response of DNA synthesis to substance P in mouse lens epithelial cells incubated for 40 h with substance P in the presence or absence of insulin. Substance P (■); substance P plus insulin at 3 μg/ml (●); control (□); insulin only (○).

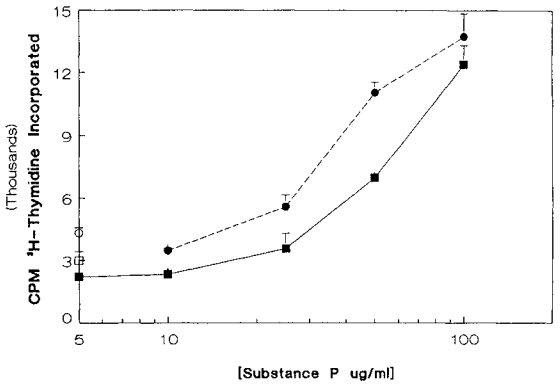

Fig. 2. Dose response of DNA synthesis to substance P in rabbit corneal epithelial cells incubated for 40 h with substance P in the presence or absence of insulin. Substance P (■); substance P plus insulin at 3 μg/ml (●); control (□); insulin only (○).

is thought to take place at two points in the respiratory chain of intact mitochondria [Slater et al., 1963]. The procedure used was based upon a modification [Hansen et al., 1989] of the original assay [Mosmann, 1983]. In this procedure the wells are emptied and 100 μl of MTT solution (1 mg/ml) is added. This is then incubated for 2 h at 37°C. To each well is then added 100 μl of the extraction buffer [20% sodium dodecylsulfate (SDS), 50% dimethyl formamide (DMF), pH 4.7] and this is allowed to stand overnight at 37°C. The absorbance of the solution is then read at 600 nm. All error bars represent standard errors of the mean.

For cell growth assays where cell numbers were determined, cells were plated at 2,000 cells/well of a 96 well plate. The cells were grown in 10% serum for 4 days; they were then fed with media containing no serum. At the end of 1 day the cells were fed with media containing SP, or SP plus insulin. After 4 days the cells were removed with trypsin. The cells from three wells were pooled, suspended in 0.5 ml, and counted. All error bars represent standard errors of the mean.

RESULTS

Effects of SP, With or Without Insulin, on DNA Synthesis in NK-11 (Lens Epithelial) Cells (40 h)

As can be seen in Figure 1, SP by itself only shows a small stimulation of DNA synthesis in the lens epithelial cells at about 100 μg/ml, while in the presence of 3 μg/ml insulin it shows synergistic effects at as low as 10 μg/ml. At higher concentrations a synergistic response of about 4-fold is seen.

Effect of SP, With or Without Insulin, on DNA Synthesis in SIRC (Corneal Epithelial) Cells (40 h)

These results are seen in Figure 2 and are quite different from those seen for the lens epithelial cells. In this case SP by itself shows an effect on DNA synthesis at as low as 25 μg/ml with a 5-fold increase over background at 100 μg/ml. When 3 μg/ml insulin was added only a slight increase in DNA synthesis was seen. Thus, SP shows little synergism with insulin on corneal epithelial cells.

Effect of Enkephalinase on the Ability of SP to Stimulate DNA Synthesis in NK-11 Cells (40 h)

In order to test whether the stimulation of DNA synthesis was due to SP or an impurity in the solution, the enzyme enkephalinase was added. The enzyme will specifically cleave SP but will not effect insulin under the conditions of the experiment [Turner, 1987]. In Figure 3 it can be seen that the effect of SP, both alone and in the presence of insulin, is abolished by treatment with enkephalinase. Enkephalinase had no effect on insulin alone. The same effect was seen if the factors were preincubated with enkephalinase before addition to the cells (data not shown). In order to rule out the possibility that enkephalinase could be acting directly on the cells to block the SP effect, the cells were pretreated with enkephalinase for 1 h, removed, and then SP or SP plus insulin was added. Enkephalinase pretreatment did not block the SP effect (data not shown).

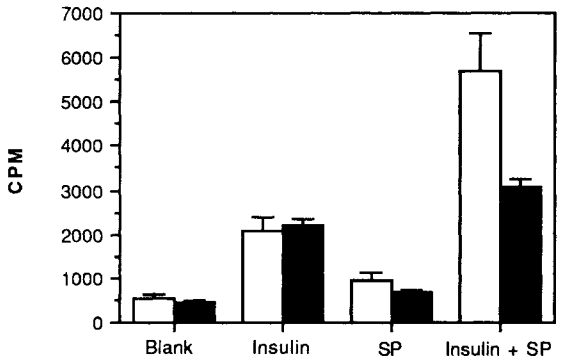

Fig. 3. Effects of enkephalinase on the ability of SP to stimulate DNA synthesis in lens epithelial cells in the presence or absence of insulin. Substance P (200 μg/ml) ± insulin (1.2 μg/ml) in the presence (■) or absence (□) of enkephalinase (1.25×10^{-8} M).

Effect of Calcitonin Gene Related Peptide (CGRP) Alone and in the Presence of SP on DNA Synthesis in SIRC Cells (40 h)

CGRP had no effect on DNA synthesis by corneal epithelial cells when added at 2.5 μM. However, when CGRP at 0.025 μM was added in the presence of SP 1 nM it was synergistic in its stimulatory ability (Fig. 4). It should also be noted that the SP concentration 1 nM was well below the concentration that had an effect by itself (25 μg/ml: 18.6 μM) on short term (40 h) DNA synthesis. This synergistic effect was also seen when CGRP and SP were added in the presence of insulin (data not shown). Similar results were also obtained with lens epithelial cells (data not shown).

Effect of the Pretreatment of NK-11 and SIRC Cells With SP, on Their Ability to Synthesize DNA After Further Stimulation With Insulin and SP (40 h)

Cells were pretreated with SP for various periods of time (30 min to 3 h) and then the SP washed out and either more SP or insulin was then added. It was found that a 2 h pretreatment with SP made the cells more responsive to the subsequent addition of SP or insulin (Fig. 5). Although the effect is not large, it was seen in three separate experiments. Pretreatment for longer periods of time showed the same effect as the 2 h treatment, while shorter periods of time showed less effect (data not shown). If the initial pretreatment with SP was not followed by more SP or insulin, no stimulation was observed. Similar results were obtained for both the lens and the corneal epithelial cells.

Effect of Hydroxyurea on the Ability of SP to Stimulate DNA Synthesis in SIRC Cells (40 h and 5 days)

Hydroxyurea is known to block the transition between G_1 and S phase in the cell cycle [Walters et al., 1973, 1976]. Thus if the increase in DNA synthesis caused by SP was due to DNA repair rather than an increase in scheduled DNA synthesis, hydroxyurea would have no effect [Clarkson, 1978]. It was found that hydroxyurea (1 mM) blocked the effect of SP or SP plus insulin (data not shown).

Effect of Low Concentrations of SP on DNA Synthesis in NK-11 Cells (5 Days)

In order to test whether lower concentrations of SP will stimulate DNA synthesis if left on the NK-11 cells for a longer period of time, cells were treated with SP in the concentration range of 0.3 pM to 1 μM, for 5 days. Significant stimulation was seen at 0.3 pM. A companion study carried out for 40 h did not show any stimulation at these low SP concentrations consistent with previous results. These results can be seen in Figure 6.

Effect of SP and Insulin on the Stimulation of SIRC and NK-11 Cell Growth (4 Days and 7 Days)

In order to test whether SP was actually stimulating cell growth and not just DNA synthesis, long-term growth studies were carried out with SIRC and NK-11 cells in the presence of SP. The results in Figure 7 shows that SIRC cells left in the presence of SP for 7 days showed growth stimulation, as measured by the MTT assay at low (10 ng/ml) concentrations of SP. This same result was found both with or without insulin, in a serum free environment. In other studies with NK-11 and SIRC cells where actual cell number was measured (after 4 days), it was found that SP at 1–2 pg/ml stimulated cell growth and was synergistic with insulin (2 μg/ml) for lens cells (Fig. 8a) but additive for the cornea cells (Fig. 8b).

DISCUSSION

In order to study the ability of SP to stimulate epithelial cells, experiments were carried out with clonal populations of cultured cells in a serum free environment. In this manner the effect could be studied without the interference of other factors. In addition, it was necessary to

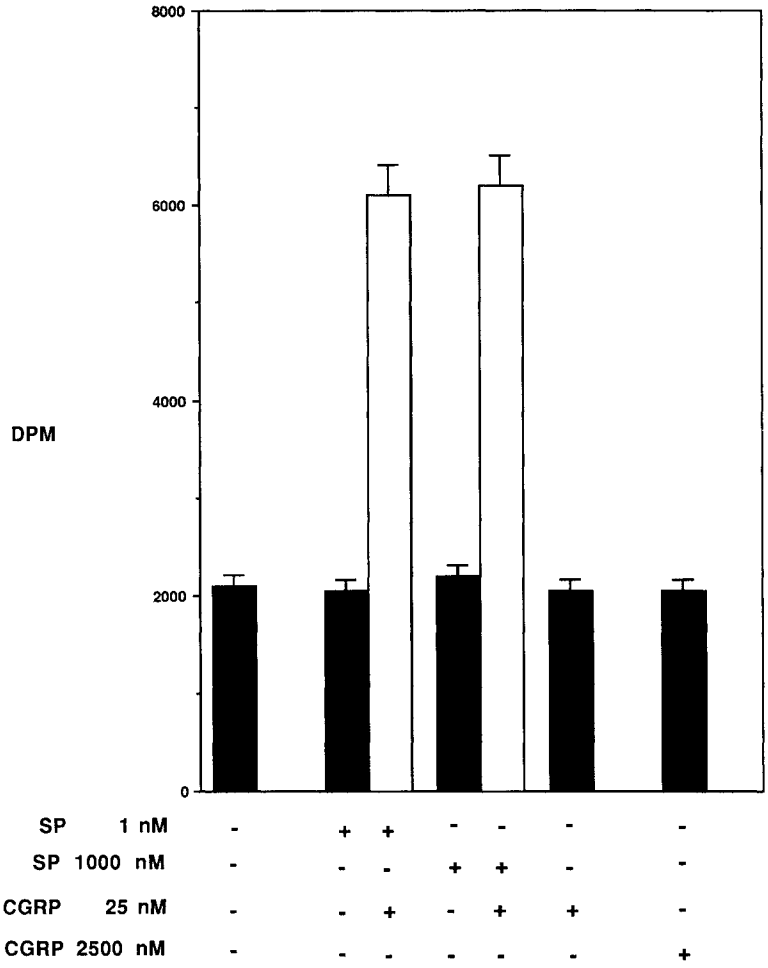

Fig. 4. The effect of CGRP and SP on DNA synthesis in corneal epithelial cells. SP or CGRP alone (■); SP plus CGRP, 25 nM (□).

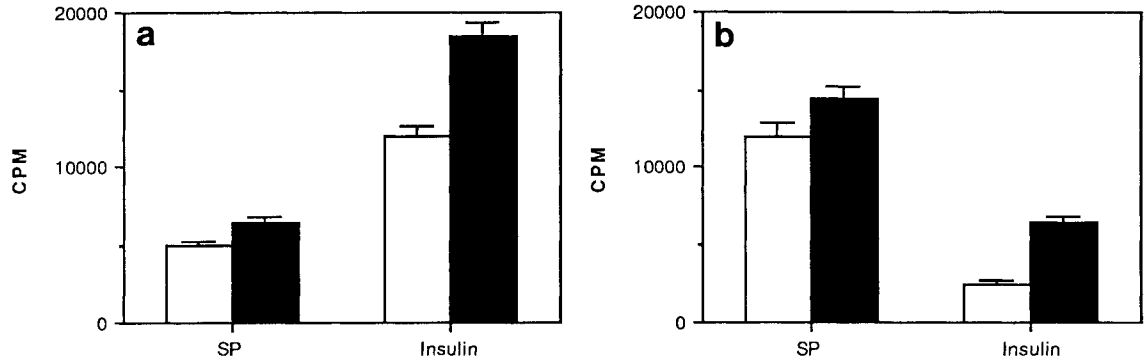

Fig. 5. Effect of a 2 h pretreatment of epithelial cells with SP (100 μg/ml) on their synthesis of DNA after further stimulation with insulin, SP (100 μg/ml) and SP + Insulin (3 μg/ml) for 40 h. **a**: Lens epithelial cells; with SP pretreatment (■); without SP pretreatment (□). **b**: Corneal epithelial cells; with SP pretreatment (■); without SP pretreatment (□).

study whether SP required serum in order to stimulate ocular epithelial cells, since it had been reported for both skin and tongue epithelial cells, that serum was required [Toshihiro et al., 1988; Ivanov et al., 1987]. In short-term studies (40 h) it was found that for lens and cornea epithelial cells SP could stimulate DNA synthesis in a serum free environment; how-

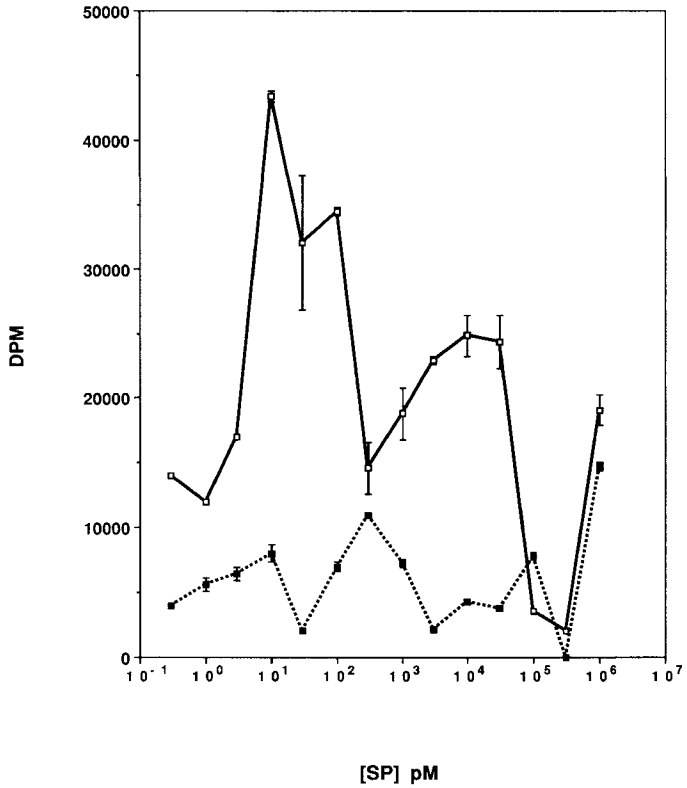

Fig. 6. Dose response of DNA synthesis to substance P in mouse lens epithelial cells incubated for 2 or 5 days with substance P (□), 2 days (■).

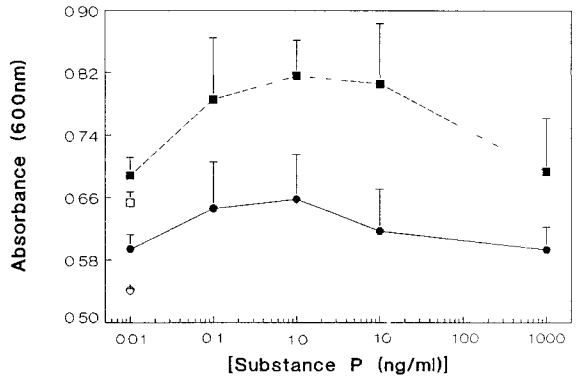

Fig. 7. Dose of substance P in rabbit corneal epithelial cells incubated for 7 days with substance P in the presence or absence of insulin as measured by the MTT assay Control (○), insulin 2 μg/ml only (□); SP only (●); SP plus insulin 2 μg/ml (■)

ever, the lens cells were less responsive to SP unless insulin was present (Fig. 1), while the cornea epithelial cells were sensitive to SP alone but showed little synergism in the presence of insulin (Fig. 2). In cell growth experiments where cell number was determined, however, the lens cells showed synergism between SP and insulin (Fig. 8a), while the cornea cells showed an additive effect between SP and insulin (Fig. 8b). In each case the stimulation was seen at concentrations of SP as low as 1–2 pM.

In order to test whether the SP effect was due to an impurity in the SP solution or an SP fragment, the enzyme enkephalinase was used. Enkephalinase will catalyze the hydrolysis of SP in a specific manner [Malfroy et al., 1987]. The finding that the addition of enkephalinase could abolish the effect of SP was consistent with the intact SP molecule being required for the stimulation. Enkephalinase under these same conditions had no effect on the stimulatory ability of insulin (Fig. 3). The effect of enkephalinase on SP was not due to its interaction with the cells, since prior exposure of the cells to enkephalinase had no effect.

Calcitonin gene related peptide (CGRP) is a 37-residue peptide hormone, with a disulfide between cysteines at position 2 and 7 and a carboxy-terminal phenylalanine amide. The existence of this peptide was first predicted from studies of alternate processing of RNA from the calcitonin gene [Rosenfeld, 1983]. It has been found that virtually all substance P positive neurons are immunoreactive for CGRP, while

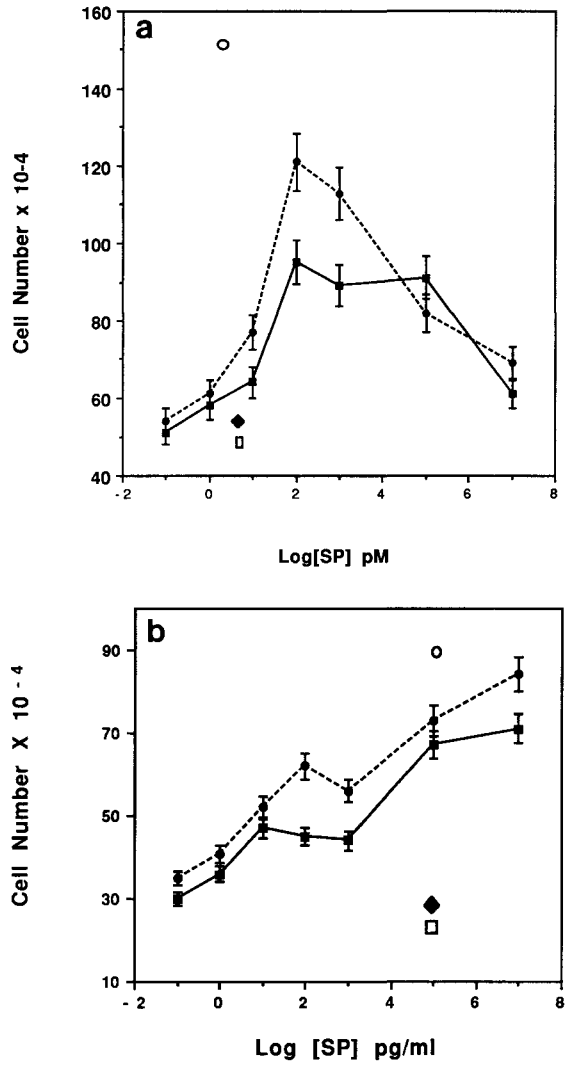

Fig. 8. Effect of SP and insulin on the growth (after 4 days) as measured by cell counts for **a)** lens epithelial cells and **b)** cornea epithelial cells. Blank (□); SP alone (■); insulin (2 μg/ml) alone (◆); insulin + SP (●); 10% FBS alone (○).

some neurons are found in the cornea that contain only CGRP [Kuwayama and Stone, 1987; Terenghi et al., 1985]. SP and CGRP are even co-localized within the same secretory vesicles [Gulbenkian et al., 1986]. CGRP is also diminished in sensory and sympathetic nerves by capsaicin treatment [Terenghi et al., 1986], as is SP. Thus, many of the in vivo effects that have been attributed to substance P may really reflect the action of CGRP. Although some studies on these two compounds show that they seem to have similar activities [Brown and Morice, 1987], there are several which show they are quite different [Andersson, 1987; Oksala, 1988; Krootila, 1988]. We carried out experiments to see if CGRP would also have an effect on epithelial cell growth. As seen in Figure 4, there is very little effect of CGRP by itself on the stimulation of DNA synthesis by the epithelial cells even when added as high at 2.5 μM. However, when CGRP (0.025 μM) was added in the presence of substance P (1 nM) it was synergistic in its stimulation of DNA synthesis. The concentration of SP (1 nM) is also well below that which stimulates DNA synthesis by itself (25 μg/ml; 18.6 μM) in a 40 h assay. This synergistic effect was also seen when CGRP and SP were added together with insulin (data not shown). Thus, CGRP and SP may function together to stimulate DNA synthesis in vivo, in epithelial cells.

The results of pretreating cells with SP, followed by the addition of either insulin or more SP, showed that addition of SP for a short time (2 h) can have an effect on the stimulation caused by subsequent addition of a second hormone. This would seem to indicate that either the dissociation rate for SP from its receptor is slow, or that the internal signals for DNA synthesis persist for quite some time. A lower limit on the speed of the dissociation rate can be obtained since if no factors were added after the SP pretreatment, there is no effect of SP on DNA synthesis. This would say that the effect does not last until the cells go into S phase after 16 h. It is known that the insulin signal is quickly lost after insulin is removed [Reid and Reid, 1987]. But, if other factors are present such as vanadate [Reid and Reid, 1987], or RDGF [Tarsio et al., 1983], this effect is retained. Since it is known that insulin dissociates from its receptor with a half time of approximately 15 min, these results are consistent with vanadate or RDGF protecting an internal cell growth signal [Reid and Reid, 1987]. A possible mechanism for the above results would be that insulin is able to synergize with an SP signal that persists after SP removal, or that the dissociation rate for SP is slow enough to yield a signal with which insulin can interact. These results are also interesting in light of the fact that exposure of parotid acinar cells to SP results in inositol lipid metabolism (inositol 1,4,5-triphosphate, formation); however, subsequent additions of SP to the cells yields a diminished response, termed desensitization [Sugiya et al., 1988]. Since subsequent addition of SP to the cells resulted in increased DNA synthesis it is doubtful that desensitization is related to the enhanced signal

seen from pretreatment in the DNA synthesis assay.

In studying the effect of SP on cell growth it is interesting that the SP effect was seen at as low as 1 pM (Figs. 7 and 8). This was in contrast with the results obtained with the short term DNA synthesis studies (40 h) where the lowest concentration that showed stimulation was about 10 μg/ml. However, when the SP is left on the cells for 5 days in a DNA synthesis experiment, stimulation is seen at low concentrations (Fig. 6). Thus, it would appear that the increased sensitivity to SP is due to the fact that the SP was present longer on the cell in the cell growth studies (4 to 7 days) and the 5 day DNA synthesis study vs the short term DNA synthesis studies (40 h). This would imply that a low level of SP in vivo, if continuously present, would be sufficient to stimulate cell growth, especially in the presence of other factors such as insulin or CGRP. It is also interesting to note that the inhibition of DNA synthesis seen at high concentrations of SP is shifted to the left in the case of the longer incubation times for the lens cells. Possible explanations for the need for the longer times for SP to stimulate cells at low concentrations could be 1) SP as used to treat the cells in vitro is in a different conformation than that found in vivo where it is released in a neurosecretory vessicle and might be held in the proper conformation for interaction with the receptor by a chaperone protein or the hydrophobicity of the environment; 2) it requires time for SP to cause the release of a secondary growth factor which is required to stimulate the cells to grow in an autocrine manner similar to the effect of TGFb on PDGF release [Leof et al., 1986]; and 3) in vivo another protein or peptide, such a CGRP, is present which allows SP to work more effectively at low concentrations.

Other evidence for a role for neuropeptides in cell growth comes from studies with the compound capsaicin. In 1978, Jessell and co-workers reported that capsaicin depletes SP, from the dorsal horn of the rat spinal cord apparently without a general deleterious effect on spinal neurons and without destruction of primary afferent neuron terminals in the cord. The depletion action of capsaicin seems to be limited to primary afferent neurons and tissues innervated by these neurons such as the cornea. The subject of capsaicin and its relation to SP has been reviewed [Duke-Elder, 1965; De Haas, 1962; Davies, 1970; Lewis et al., 1982]. Moreover, the content of SP in the cornea and trigeminal ganglion is reduced with capsaicin treatment [Gamse, 1981]. However, other workers find that the primary effect of capsaicin in the cornea is that of a neurotoxic substance that prevents SP release [Tervo, 1981; Lembeck and Donnerer, 1981b; Gamse, 1986]. In either event, all authors agree that systemic capsaicin functionally blocks the biologic activity of SP in all animal models studied. Of more interest is the fact that one of the few side effects of systemic capsaicin administration is the occurrence of corneal ulcers which have been reported in several species [Shimizu et al., 1984]. These ulcers appear to result from the inability of the epithelial cells on the cornea to respond properly to corneal injury, possibly due to improper or lack of release of extracellular matrix material [Tanaka and Nishida, 1985]. It will be of interest to see if the SP effect that we see for our cornea epithelial cells in culture is verified in vivo. Preliminary studies in vivo have shown that neuropeptide depletion impairs corneal wound healing [Murphy et al., 1990].

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