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RECEPTOR-MEDIATED MITOGENIC EFFECTS OF SUBSTANCE P ON CULTURED SMOOTH MUSCLE CELLS

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The neuropeptide substance P, a known mitogen for human blood T-lymphocytes, now is shown to stimulate proliferation of embryonic rat aortic smooth muscle cells of the A7r5 line, at concentrations of 10^{-9}M to 10^{-6}M. Neurotensin (NT), that has vascular and smooth muscle activity similar to SP, failed to induce proliferation of A7r5 cells. At proliferation-enhancing concentrations, SP increased the concentration of cytosolic Ca^{2+} in A7r5 cells, suggesting activation of the phosphatidylinositol pathway. Binding of [^{125}I]-substance P to A7r5 cells reached equilibrium rapidly at 4°C, and was saturable, implying that the activation of smooth muscle cells by SP is a receptor-mediated process.

The undecapeptide substance P (SP), of amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, is a neuropeptide that is widely distributed in the central and peripheral nervous system (PNS) in cells derived from the neural crest (1,2). In the PNS, SP is localized to a subpopulation of primary afferent neurons (1,3), from which it is released during inflammatory responses (4). That SP may play an important role in the regulation of local immunologic responses has been suggested by its capacity to enhance the proliferation of human blood T-lymphocytes (5,6). This effect of SP is a function of stereospecific receptors on a subset of 15-20% of the T-lymphocytes, as defined by the binding of a fluorescent conjugate of SP, SP* (7). The T-lymphocytes in this subset and cultured B-lymphoblasts have

Abbreviations used: SP, substance P; [^{125}I]-SP, [^{125}I]-substance P; SP*, fluorescein-labeled SP; NT, neurotensin; PNS, peripheral nervous system; K_D, dissociation constant; DME-HPS, Dulbecco's modified Eagle's medium with 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, pH 7.4; DME-HPS/FCS, DME-HPS with 10% fetal calf serum; HEPES, 4-2-(hydroxy-ethyl)-1-piperzine ethanesulfonic acid; and FACS, fluorescent-activated cell sorter.

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20,000-30,000 receptors for SP, that have a dissociation constant (K_D) of 10^{-7}M and 0.3 x 10^{-9}M, respectively (7,8).

As SP is a potent stimulus for smooth muscle contractions (2), the specificity of the interactions of SP with cultured smooth muscle cells, and the mitogenic consequences were assessed in the present study.

MATERIAL AND METHODS

A7r5 smooth muscle cells from embryonic rat thoracic aortas (9), BC_3H1 smooth muscle-like cells from a mouse brain tumor (10) (American Type Culture Collection, Rockville, MD), fetal bovine serum, Dulbecco's modified Eagle's medium with 4.5 g of glucose/L, 4-2-(hydroxy-ethyl)-1-piperazine ethane-sulfonic acid (HEPES), penicillin (1,000 U/ml), streptomycin (1,000 µg/ml) (Cell Culture Facility, UCSF), substance P, neurotensin (Peninsula Laboratories, Inc., Belmont, CA), 75 cm^2 plastic tissue culture flasks and 96-well flat bottom tissue culture plates (Corning, Corning, NY), [^{3}H]-thymidine (22-55 Ci/mmol) (New England Nuclear, Boston, MA), [^{125}I]-substance P (2000 Ci/mmol), Quin 2AM (Amersham Corp., Arlington Heights, IL), 1.5 ml conical polypropylene tubes (Sarstedt, Inc., Princeton, NJ), n-butyl phthalate (Fisher Scientific Co., Pittsburg, PA), and dinonyl phthalate (ICN Pharmaceuticals, Inc., Plainview, NY) were obtained from the designated suppliers.

Maintenance of A7r5 and BC_3H1 cell lines: Both cell lines were grown in 75 cm^2 plastic tissue culture flasks in Dulbecco's modified Eagle's medium with 4.5 g of glucose/L containing 25 mM HEPES (pH 7.4), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10% fetal bovine serum (DME-HPS/FCS) at 37°C in 5% CO_2:95% air. The cells were subcultured twice weekly at a ratio of 1:2. Prior to the proliferation experiments, cells were removed with a rubber spatula, washed three times in serum-free culture media (DME-HPS), and replated into 96-well flat-bottomed tissue culture plates at a density of 1 x 10^6/ml of DME-HPS with 0.2 ml/well. After 24 hr of incubation the DME-HPS was decanted and replaced with fresh DME-HPS with or without neuropeptide.

Assessment of proliferation: Smooth muscle cells at the above densities were cultured in DME-HPS in the presence and absence of a specific neuropeptide. After 36 hr of incubation, 1 µCi of [^{3}H]-thymidine was added to each well, and the incubation continued for a further 12 hr. The incorporation of [^{3}H]-thymidine quantified by first replacing the medium with 0.25 % trypsin for 1-2 min, and then trapping and washing the cells on glass fiber filters in a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), and then counting the radioactivity as described (11,12).

Measurement of Quin 2 fluorescence: Smooth muscle cells were loaded with Quin 2 by using a modification of the method described by Tsien et al. (13). Smooth muscle cells growing in DME-HPS/FCS were resuspended with a rubber spatula, washed three times in DME-HPS, replated without subculturing in DME-HPS and incubated for 24 hr at 37°C in 5% CO_2:95% air. At the end of this period of incubation, 1 x 10^8 cells in 10 ml of DME-HPS were incubated with and without 40 µM diacetomethoxyester of Quin 2 for 20 min in the dark at 37°C in 5% CO_2:95% air and then at room temperature for a further 40 min. Following this incubation the cells were washed twice in DME-HPS to remove extracellular Quin 2, and resuspended in DME-HPS at a concentration of 1 x 10^7/ml. Quin 2 fluorescence was analyzed in a Becton-Dickinson fluorescence-activated cell sorter (FACS IV) equipped with an 18 W argon ion laser (Spectra Physics, Mountain View, CA), operating at 400 mW that excited

in the ultraviolet range from 333 nm to 364 nm with a long pass filter at 389 nm. Fluorescence intensity was measured as previously described (7).

Measurement of the binding of [^{125}I]-SP to smooth muscle cells: In each experiment, 1 x 10^7 smooth muscle cells in DME-HPS were incubated with a known concentration of [^{125}I]-SP in the presence and absence of 10^{-6}M unlabeled SP in a final volume of 0.3 ml at 4°C for a specified length of time. The amount of bound radioactivity was determined by sedimenting the smooth muscle cells in each suspension through 0.3 ml layer of phthalate oils in a 1.5 ml conical polypropylene tube that was centrifuged for 60 sec at 8000 x g in a Beckman microfuge B (Beckman Instruments, Inc., Mountain View, CA) (7). The tip of the polypropylene tube containing the cell pellet was cut off with a razor blade, and the radioactivity quantified in a gamma-counter. The amount of radioactivity bound in the presence of 10^{-6}M unlabeled SP determined the level of non-specific binding, as previously described (7). The amount of specifically bound [^{125}I]-SP was calculated by subtracting the non-specific binding from the total binding.

RESULTS AND DISCUSSION

The binding of SP to cultured smooth muscle cells was examined initially with the embryonic rat aortic line of A7r5 cells that had been loaded with Quin 2 dye, which reacts with a fluorescent signal to changes in the concentration of cytosolic Ca^{2+} evoked by the introduction of a specific stimulus. In two separate experiments 10^{-8}M and 10^{-6}M SP elicited an increase in the peak relative fluorescence intensity detected by flow cytometry of 6 ± 2 (mean ± S.D.) and 18 ± 3 (mean ± S.D.) channels, equivalent to approximately 1.5- and 3-fold increases in fluorescence, respectively (Fig. 1). In contrast, concentrations of 10^{-6}M neurotensin (NT) failed to elicit an increase in Quin 2 fluorescence in A7r5 cells (data not shown). Moreover, neither SP nor NT at concentrations as high as 10^{-6}M caused an increase in the fluorescence of BC_3H1 smooth muscle-like cells loaded with Quin 2 (data not shown).

In order to examine the possibility that the interaction of SP with smooth muscle cells is a receptor-mediated process, the binding of [^{125}I]-SP to A7r5 and BC_3H1 was assessed. The time course of specific binding of [^{125}I]-SP by A7r5 and BC_3H1 cells was examined by incubating duplicate suspensions of 1 x 10^7 cells with 0.3 nM [^{125}I]-SP for 5-90 min at 4°C. The specific binding of [^{125}I]-SP to A7r5 cells increased significantly within 5-10 min, and reached a plateau after approximately 35-40 min. In contrast, there was no detectable specific binding of [^{125}I]-SP to BC_3H1

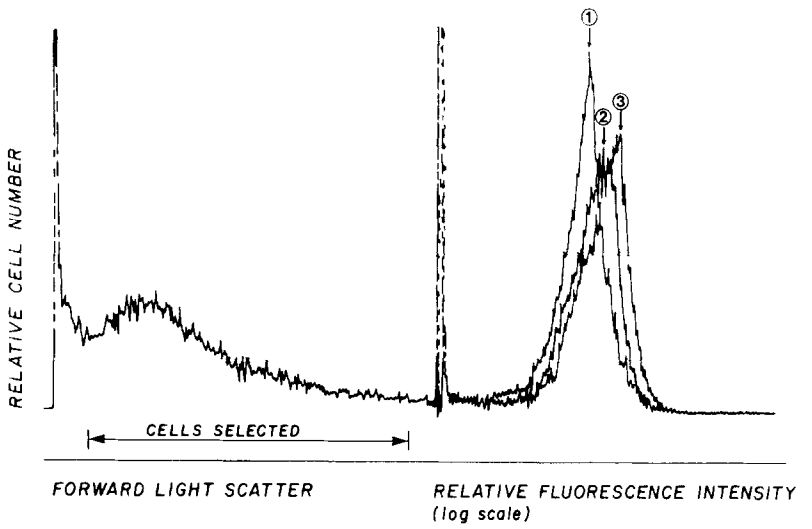

Fig. 1 Detection of Quin 2 fluorescence in rat aorta smooth muscle A7r5 cells stimulated with substance P analyzed in the fluorescence activated cell sorter (FACS). Cells were loaded with Quin 2 and analyzed in a FACS as described in the Methods. Fluorescence intensity is depicted on a log amplitude scale (a change in 56 channels = 10-fold increase in fluorescence). Substance P was introduced into the cell suspension immediately before FACS analysis. Curve 1 represents control (auto fluorescence in the absence of Quin 2 loading). Curve 2 and 3, the maximal fluorescence of A7r5 cells at 10^{-8} and 10^{-6}M SP after 5 min respectively. Identical experiments with BC_3H1 cells failed to show an increase in peak fluorescence, as did experiments where NT was added to A7r5 and BC_3H1 cells (data not shown).

cells. Saturability of [^{125}I]-SP binding by duplicate suspensions of 1 x 10^7 A7r5 cells under equilibrium conditions was demonstrated in the concentration range of 0.01 - 30 nM SP, in the presence and absence of 10^{-6} M unlabeled SP (Fig. 2). Under these conditions, specific binding of [^{125}I]-SP reached saturation at 2-4 nM. Under the same conditions, the level of non-specific binding of [^{125}I]-SP to A7r5 cells was 30% $\pm$ 10% (mean $\pm$ S.D.) of the total binding. An estimate of the dissociation constant (K_D) and SP receptor density derived from the data in Fig. 2 is approximately 0.4 - 0.6 nM, and 40-50,000 receptors/smooth muscle cell, that are similar to the K_D and receptor density for SP binding to the B-lymphoblast cell line (8).

The functional consequence of the interactions of SP with smooth muscle cells was studied by quantifying the incorporation of [^{3}H]-thymidine after 48 hrs to assess alterations in DNA synthesis. SP, but not NT, significantly

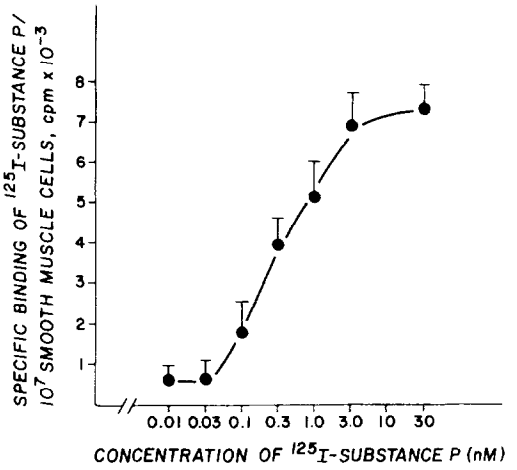

Fig. 2 Specific binding of [^{125}I]-substance P ([^{125}I]-SP) to rat aorta smooth muscle cells (A7r5, -o-, n = 2). Duplicate suspensions of 1 x 10^7 cells in DME-HPS wre incubated with [^{125}I]-SP at concentrations from 0.01 - 30 nM with and without 1 μM unlabeled SP at 4°C for 60-90 min. Specific binding was measured as described in the Methods.

enhanced the incorporation of [^{3}H]-thymidine by A7r5 cells in a concentration-dependent manner in the range 10^{-9}M to 10^{-6}M SP (Fig. 3).

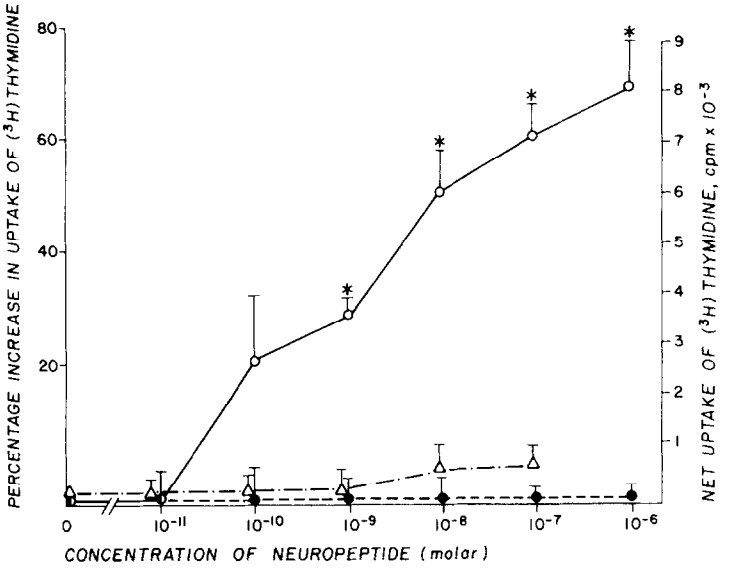

Fig. 3 Smooth muscle cell proliferative response to substance P (SP) and neurotensin (NT). Smooth muscle cell cultures and proliferative response were measured as described in the Methods. The rat smooth muscle cell line A7r5 in the presence of SP (-o-) (n = 3) and NT (-•Δ•-) (n = 2) and the smooth muscle-like mouse brain tumor cell line BC_3H1 with SP (--•--) (n = 3) are represented. The number of cpm incorporated in the absence of neuropeptide was 11,677 + 3,211 (mean + S.D., n = 3) for A7r5 cells and 20,220 + 4,400 (mean + S.D., n = 2) for BC_3H1 cells. * indicates those values which are significantly different (p less than 0.05) from control (no neuropeptide).

A maximal stimulation of 70-80% was achieved at 10^{-6}M SP. This degree of proliferative response to SP is comparable to that which is observed in T-lymphocytes (5) and connective tissue cells (14).

The present work suggests that neuropeptides that are known to exhibit vasoactive properties, may also act as mitogens for smooth muscle cells by specific receptor mechanisms. The affinity of the putative receptors for SP in A7r5 cells correlated with the potency of SP in inducing proliferation. These findings further support the hypothesis that cellular mitogenesis and vasoactivity may have common mechanisms of activation, as was recently described for the smooth muscle contractile properties of epidermal growth factor (15).

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