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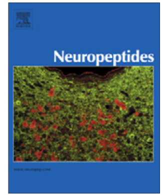

Immunomodulatory role of substance P in the wall lizard *Hemidactylus flaviviridis*: An *in vitro* study

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ABSTRACT

Present *in vitro* investigation for the first time in ectotherms demonstrated the immunomodulatory role of substance P in the wall lizard *Hemidactylus flaviviridis*. Substance P inhibited the percentage phagocytosis and phagocytic index of lizard splenic phagocytes. Inhibitory effect of substance P was completely blocked by NK-1 receptor antagonist spantide I, indicating the NK-1 receptor mediated action. Further, NK-1 receptor-coupled downstream signaling cascade involved in controlling phagocytosis was explored using inhibitors of adenylate cyclase (SQ 22536) and protein kinase A (H-89). Both the inhibitors, in a concentration-related manner decreased the suppressive effect of substance P on phagocytosis. In addition, substance P treatment caused an increase in intracellular cAMP level in splenic phagocytes. Taken together, it can be suggested that substance P via NK-1 receptor-coupled AC-cAMP-PKA pathway modulated the phagocytic activity of splenic phagocytes in wall lizards.

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1. Introduction

Substance P (SP), a member of neurokinin/tachykinin peptide family is an eleven amino acid long peptide (Chang et al., 1971). It is widely expressed in central and peripheral nervous system of vertebrates, and thus regulates number of physiological functions (Hökfelt et al., 1977; Severini et al., 2002). The SP immunoreactive nerve endings are demonstrated in the close proximity of immune cells in lymphoid organs of mammals (Bellinger et al., 1990; Felten and Felten, 1991; Lorton et al., 1991; Romano et al., 1994), indicating the involvement of this peptide in immunomodulation. Although direct role of SP in controlling non-specific (Bar-Shavit et al., 1980; Jeon et al., 1999; Ahmed et al., 2001; Rogers et al., 2006; Sun et al., 2008) and specific (Stanisz et al., 1986) immune responses is reported in mammals, these studies are totally lacking in non-mammalian vertebrates including reptiles. In the present *in vitro* investigation we tried to explore the immunoregulatory role of SP in a phylogenetically important group, reptile, the common ancestors to both birds and mammals. Due to easy availability and maintenance, the Indian wall lizard *Hemidactylus flaviviridis* that belongs to class reptilia was used in the present *in vitro* study.

Members of tachykinin peptide family mediate their effect through tachykinin/neurokinin receptors which are classified into three types, neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3) receptors (Patacchini and Maggi, 1995; Pennefather et al., 2004). While SP shows higher binding affinity for NK-1, the NK-2 and NK-3 receptors preferentially bind with other tachykinins, neurokinin A and neurokinin B, respectively (Liu and Burcher, 2005). Neurokinin receptors are demonstrated on different cell types of vertebrates by cloning (Yokota et al., 1989; Takahashi et al., 1992; Liu et al., 2004), radioligand binding assay (Too and Hanley, 1988; Weld et al., 1994; Liu and Burcher, 2001) and pharmacological (Akasu et al., 1996) techniques. These receptors are the G-protein-coupled receptors (GPCRs) (Nakanishi, 1991; Maggi, 1995; Pennefather et al., 2004) that on ligand binding commence various downstream signaling cascades (Nakajima et al., 1992). Nonetheless, studies pertaining to neurokinin receptor-coupled downstream signaling cascades are confined to mammals, and so far no report is available in non-mammalian vertebrates including reptiles. Thus, in the present *in vitro* study, an attempt was made to pharmacologically demonstrate the existence of functional neurokinin receptor on lizard splenic phagocytes. Also, neurokinin receptor-coupled downstream signaling cascade involved in mediating SP action was investigated.

In ectothermic vertebrates, the adaptive immune responses take time to respond against invading pathogens (Neumann et al., 2001), under the circumstances, the innate immunity plays important role to combat the pathogenic attack. Phagocytes including macrophages and neutrophils are the effector cells of innate immune responses and form the first line of host defense against microbial infections. These cells limit the initial

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dissemination and growth of pathogens by phagocytosis and releasing cytotoxic substances. Considering phagocytosis as an important function of phagocytes in ectotherms, role of SP in controlling phagocytic activity of splenic phagocytes was investigated in the wall lizard *H. flaviviridis*.

2. Materials and methods

2.1. Animals

Common Indian wall lizard *H. flaviviridis* of adult age (8–10 g body weight and 5–6 cm snout–vent length) were collected from suburbs of Delhi, India (Delhi: latitude: 28°12′–28°53′ N, longitude: 76°50′–77°23) and housed in wooden cages with wire mesh at top and sides. Lizards were acclimated to the laboratory conditions (room temperature and 12L:12D with lights on at 07:00 h) for 1 week prior to experiments. They were provided with live house flies as feed and water *ad libitum*. Females were used in this study owing to their greater immune responses as compared to males (Mondal and Rai, 1999). The Institutional Animal Ethics Committee guidelines for the sacrifice and experiments on animals were followed.

2.2. Reagents and culture medium

Cell culture medium RPMI 1640, inhibitors of adenylate cyclase (SQ 22536) and protein kinase A (H-89), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], substance P (SP), neurokinin 1 receptor antagonist spantide I, and cAMP immunoassay kit were purchased from Sigma–Aldrich Co., (St. Louis, MO, USA). Molecular biology grade routine chemicals were purchased from Merck Specialties Pvt., Ltd., (Navi Mumbai, India), Sisco Research Laboratories Pvt., Ltd., (Mumbai, India), Qualigens Fine Chemicals (Mumbai, India), and Central Drug House (P) Ltd., (New Delhi, India).

The cell culture medium was supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, 40 μg/ml gentamicin, 5.94 mg/ml HEPES buffer {N-(2-hydroxyethyl) piperazine-N′-(2-ehtanesulphonic acid)} and 0.2% sodium bicarbonate. Prior to use, 2% heat-inactivated fetal calf serum (FCS) (Biological Industries, Kibbutz Beit, Haemek, Israel) was added to cell culture medium and referred as complete culture medium. The stock solutions (1 mg/ml) of SP and NK-1 receptor antagonist spantide I were made in acidic deionized water (0.05 M acetic acid) and phosphate buffered-saline (PBS, pH 7.2), respectively. Both the stock solutions were serially diluted in PBS, and the final required concentration(s) of SP and spantide I were prepared in complete culture medium prior to use.

2.3. Preparation of splenic phagocyte monolayer

The method of Mondal and Rai (1999) was followed to prepare the splenic phagocyte monolayer. In brief, wall lizards were sacrificed by cervical dislocation, pinned, and dissected in aseptic conditions. The spleens were excised and pooled in chilled PBS. To prepare the single cell suspension of splenocytes, spleens were forced to pass through nylon mesh (pore size 90 μm) in ice-cold complete culture medium. After adjusting the cell number to 10^6 cells/ml, 200 μl of splenocyte suspension was flooded on each pre-washed slide. Following 90 min of incubation, non-adherent cells were washed off with PBS. The viability of adhered phagocytes was above 98% as assessed by trypan blue exclusion method. All the incubations were carried out at 25 °C (±1) in humidified chamber/incubator maintained with 5% CO_2.

2.4. Preparation of yeast cell suspension

The yeast cell suspension was prepared by warming the commercially available Baker's yeast (*Saccharomyces cerevisiae*) (1.5 mg/ml PBS) for 20 min at 80 °C. Afterwards, heat-killed yeast cell suspension was washed and resuspended in the complete culture medium.

2.5. Phagocytic assay

For phagocytic assay, splenic phagocyte monolayer on each slide was incubated with 400 μl yeast cell suspension. After 90 min, non-phagocytosed yeast cells were washed off with PBS. The monolayer was fixed in methanol, stained with Giemsa and mounted in DPX. The cells were observed under the microscope (Nikon Eclipse E400) at 400× magnification. Phagocytes engulfing one or more than one yeast cell were considered as positive phagocytes. These cells characteristically show the extended pseudopodia forming the phagocytic cup(s) around the yeast cell(s). Approximately, 200 phagocytes per slide were counted without any predetermined sequence or scheme. The experimenter was blind to the technical details of slides while counting. Percentage phagocytosis and phagocytic index were calculated using following formulae (Campbell et al., 2003): (a) percentage phagocytosis = number of positive phagocytes/100 phagocytes (b) phagocytic index = average number of yeast cells engulfed by each positive phagocytes × percentage phagocytosis.

2.6. In vitro experiments

2.6.1. Effect of substance P

Splenic phagocytes were treated with different concentrations of SP ranging from 10^{-11} M to 10^{-7} M for 30 min. Cells incubated in medium alone for the same time duration were considered as control. After incubation, phagocyte monolayer was washed with PBS and processed for phagocytic assay. As reports are lacking in reptiles, the literature available in other vertebrates was consulted to determine the range of SP concentrations to be used in this study (Jeon et al., 1999; Ahmed et al., 2001; Rogers et al., 2006; Sun et al., 2008). Pilot experiments were performed using lizard splenic phagocytes to decide the optimum *in vitro* incubation time for SP action.

2.6.2. Effect of neurokinin 1(NK-1) receptor antagonist

In accordance to concentration-related effect of SP on phagocytic activity, splenic phagocytes were incubated with the most effective concentration of SP (10^{-8} M) and 10 times higher concentration of NK-1 receptor antagonist, spantide I (10^{-7} M), simultaneously, for 30 min. Spantide I is the competitive NK-1 receptor antagonist, therefore, splenic phagocytes were treated with spantide I and SP for the time period (30 min) which is optimum for the SP action on phagocytosis. For control, phagocytes were incubated in medium alone/10^{-8} M SP/10^{-7} M spantide I for the same duration. After incubation, cells were washed and processed for phagocytic assay.

2.6.3. Effect of inhibitors for adenylate cyclase and protein kinase A

To delineate the receptor-coupled downstream signaling mechanism for SP action, splenic phagocytes were pre-incubated with different concentrations of SQ 22536 (2.5, 5.0, 7.5, and 10.0 nM)/H-89 (25, 50, 75, and 100 nM) for 30 min. Afterward, cells were treated with 10^{-8} M SP and varying concentrations of SQ 22536/H-89 for 30 min. Several control groups were made in which phagocytes were: (a) incubated in medium alone for 60 min (b) pre-incubated in medium alone for 30 min and then with 10^{-8} M SP for 30 min (c) incubated with 10 nM SQ 22536/100 nM H-89

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