The Journal of Experimental Biology 214, 4217-4222

doi:10.1242/jeb.062935

RESEARCH ARTICLE

Dynorphin regulates the phagocytic activity of splenic phagocytes in wall lizards: involvement of a κ-opioid receptor-coupled adenylate-cyclase–cAMP–PKA pathway

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Accepted 19 September 2011

SUMMARY

This *in vitro* study of the wall lizard *Hemidactylus flaviviridis* demonstrates the role of the opioid peptide dynorphin $A_{(1–17)}$ [dyn $A_{(1–17)}$] in the regulation of the phagocytic activity of splenic phagocytes. Dyn $A_{(1–17)}$ in a concentration-dependent manner inhibited the phagocytic activity, and the maximum inhibition was recorded at a concentration of 10^{-9} mol l^{-1}. To explore the receptor-mediated effect of dyn $A_{(1–17)}$, cells were treated simultaneously with the non-selective opioid receptor blocker naltrexone and dyn $A_{(1–17)}$. Naltrexone completely blocked the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis. Moreover, the involvement of selective opioid receptors was investigated using selective opioid receptor antagonists. CTAP and naltrindole, selective μ- and δ-opioid receptor blockers, respectively, failed to block the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis. However, the selective κ-opioid receptor blocker NorBNI completely antagonized the inhibitory effect of dyn $A_{(1–17)}$. Regarding the κ-opioid receptor-coupled downstream signaling cascade, the adenylate cyclase (AC) inhibitor SQ 22536 and protein kinase A (PKA) inhibitor H-89 decreased the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis. Furthermore, treatment with dyn $A_{(1–17)}$ caused an increase in intracellular cAMP content in splenic phagocytes. Thus, it can be concluded that, in *H. flaviviridis*, dyn $A_{(1–17)}$ negatively regulates the phagocytic activity of splenic phagocytes by acting through κ-opioid receptors that are coupled with the AC–cAMP–PKA signal transduction mechanism.

Key words: dynorphin, phagocytosis, opioid receptor, signal transduction.

INTRODUCTION

Dynorphins are endogenous opioid peptides encoded by a common proenkaphalin-B gene (hereafter referred to as preprodynorphin, *PDYN*) (Civelli et al., 1985) that is widely expressed in the central and peripheral nervous systems of vertebrates (Cone et al., 1982; Goldsmith et al., 1992; Dores et al., 2004; Shirayama et al., 2004; Alrubaian et al., 2006). As for various peptide hormones, dynorphins are synthesized as large, biologically inert precursor proteins that undergo post-translational modifications to produce biologically active molecules. In mammals, these active products include big-dynorphin, dynorphin $A_{(1–17)}$, dynorphin $A_{(1–13)}$, dynorphin $A_{(1–8)}$, dynorphin $B_{(1–29)}$, dynorphin $B_{(1–13)}$ and α-neo-endorphin (Schwarzer, 2009). Cloning and sequence analysis of the preprodynorphin cDNA revealed that it is a highly conserved gene, although some discrepancies have been described in lower ectotherms. In fish and amphibians, the gene encoding PDYN contains an additional opioid sequence for leucine-enkephalin or isoleucine-enkephalin (Dores et al., 2004; Alrubaian et al., 2006) and for methionine-enkephalin (Danielson et al., 2002), respectively. Although the gene encoding PDYN has not been cloned to date in reptiles, different dynorphins such as α-neo-endorphin, dynorphin $A_{(1–17)}$, dynorphin $A_{(1–8)}$ and dynorphin $B_{(1–13)}$ in the lizard *Anolis carolinensis* and α-neo-endorphin and dynorphin $B_{(1–13)}$ in turtle and alligator have been demonstrated by Goldsmith and colleagues (Goldsmith et al., 1992) using an heterologous radioimmunoassay. Owing to its higher potency (James et al., 1984) and conserved nature, dynorphin $A_{(1–17)}$ [dyn $A_{(1–17)}$] was used here to examine its immunomodulatory role in the wall lizard *H. flaviviridis*.

In addition to an analgesic effect (Knoll and Carlezon, 2010), a role for dynorphins in the regulation of immune responses has emerged in recent years (Casellas et al., 1991; Ichinose et al., 1995; Singh and Rai, 2010). Furthermore, functional μ-, δ- and κ-opioid receptors belonging to the G-protein-coupled receptor (GPCR) superfamily (Law et al., 2000) have been demonstrated to be expressed by immune cells (Martin-Kleiner and Gabrilovac, 1997). Interestingly, the same intracellular signaling cascade for different opioid receptors (Lawrence and Bidlack, 1993; Sharp et al., 1996; Wang et al., 2003) and multiple downstream signaling mechanisms for the same opioid receptor (Bohn et al., 2000; Kam et al., 2004) have been reported, depending on which mammalian cell lines were studied. With regard to ectotherms, currently there is only one report exploring the intracellular signaling cascade for β-endorphin action on immune cells (Kumar et al., 2011). In the present *in vitro* study, we aimed to understand the mechanism of action of receptor-coupled downstream signaling for dyn $A_{(1–17)}$ in lizard splenic phagocytes.

The adaptive immune system in ectotherms, unlike that of endothermic vertebrates, is poorly developed (Flajnik, 1996). Therefore, innate immunity, the first line of host defense plays a crucial role in protecting ectothermic vertebrates from microbial infections. Phagocytes are an important constituent of the innate immune system. These cells possess a remarkable property to phagocytose and kill microbes. Furthermore, the phagocytic process initiates an array of events, including antigen presentation and secretion of various cytokines, which, in turn, activate the adaptive immune system. Thus, phagocytosis can be considered as a central phenomenon for the activation of the immune system during

pathogenic attack. Bearing this in mind, the phagocytic activity of splenic phagocytes was selected as the parameter of study in the present investigation.

MATERIALS AND METHODS

Animals

Specimens of the Indian wall lizard, *Hemidactylus flaviviridis* Rüppell 1835, possessing a body mass of 8–10 g (adults) were procured from suburbs of Delhi, India (28°12′ to 28°53′N, 76°50′ to 77°23′E). They were maintained in wooden cages fitted with mesh wire from the top, sides and front for proper air circulation and light. Lizards were acclimated to the laboratory conditions (12 h:12 h light:dark photoperiod, lights on at 07:00 h and room temperature) for one week before experiments. Animals were provided with live house flies as food, and water *ad libitum*. Only female lizards were used in the present *in vitro* study, as they show better immune responses than males (Mondal and Rai, 1999). The Institutional Animal Ethics Committee guidelines of the University of Delhi were followed with regard to the maintenance and killing of wall lizards. In order to minimize the effects related to diurnal variation with respect to the expression of opioid receptors, and their affinity to ligand or antagonists, lizards were vivisected between 10:00 and 11:00 h for all experiments.

Reagents and culture medium

All regular-use chemicals were of molecular biology grade. They were purchased from Qualigens Fine Chemicals (Mumbai, India), Central Drug House (New Delhi, India), Sisco Research Laboratories (Mumbai, India) and Merck Specialities (Navi Mumbai, India). Dyn $A_{(1–17)}$, μ-receptor antagonist CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2), $\delta_{1,2}$-receptor antagonist naltrindole hydrochloride (NTI) and κ-receptor antagonist norbinaltorphimine dihydrochloride (NorBNI) were the generous gifts from the National Institute of Drug Abuse, Bethesda, MA, USA. The cell culture medium RPMI 1640, non-selective opioid receptor blocker naltrexone, adenylate cyclase inhibitor SQ 22536, protein kinase A (PKA) inhibitor H-89, phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX), cAMP enzyme immunoassay kit and MTT [3-(4,5-dimethyl thiazoyl-2)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma-Aldrich (St Louis, MO, USA). The culture medium was supplemented with 100 μg ml^{-1} streptomycin, 100 IU ml^{-1} penicillin, 40 μg ml^{-1} gentamicin, 5.94 mg ml^{-1} HEPES buffer [*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulphonic acid)] and 0.2% sodium bicarbonate. Before use, complete culture medium was made by supplementing 2% heat-inactivated fetal calf serum (FCS, Biological Industries, Kibbutz Beit, Haemek, Israel).

Preparation of lizard splenic phagocyte monolayer

The wall lizards were vivisected by cervical dislocation, their spleens were dissected out, pooled in chilled phosphate-buffered saline (PBS, pH 7.2) and passed through nylon mesh (pore size 90 μm) in ice-cold complete culture medium to prepare the single-cell suspension. The total cell number was adjusted to 10^6 cells ml^{-1}. For the preparation of the phagocyte monolayer, 200 μl of splenocyte suspension was flooded on each pre-washed slide. Following incubation for 90 min, the non-adhered cells were washed off with PBS. The viability of the adhered cell population was >98% as assessed by a Trypan Blue exclusion test. All the incubations were performed at 25°C in a humidified chamber or incubator maintained with 5% CO_2.

Preparation of yeast cell suspension

Yeast (*Saccharomyces cerevisiae*) cell suspension was prepared by warming commercially available baker's yeast (1.5 mg ml^{-1} PBS) at 80°C for 20 min. The heat-killed yeast cell suspension was washed and resuspended in the complete culture medium.

Phagocytic assay

For the phagocytic assay, 400 μl heat-killed yeast cell suspension was flooded on a phagocyte monolayer of each slide. After incubation for 90 min, non-phagocytosed yeast cells were washed off with PBS. Thereafter, the phagocyte monolayer was fixed in methanol, stained with Giemsa and mounted in DPX. Cells were observed under the microscope (Nikon E400, Japan) at a magnification of ×400. Phagocytes engulfing one or more than one yeast cell were considered as 'positive phagocytes'. These cells possess the extending pseudopodia forming the phagocytic cup(s) to phagocytose the yeast cells (Fig. 1). Phagocytes that were not phagocytosing the yeast cell(s) were regarded as 'negative phagocytes'. Without any predetermined sequence or scheme, ~200

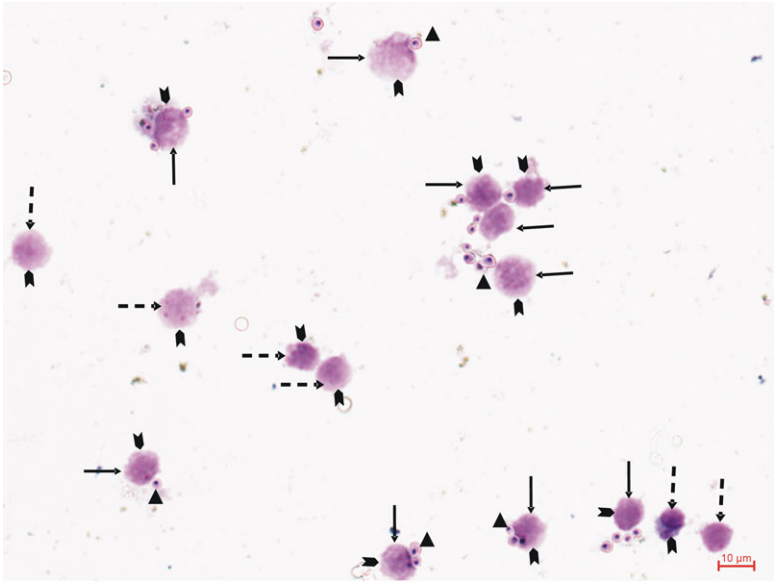

Fig. 1. Bright-field photomicrograph of lizard splenic phagocytes showing phagocytosis of yeast cell(s). In the image are highlighted splenic phagocytes (thick arrowheads) and yeast cells (triangles). Phagocytes showing phagocytic activity are indicated by thin unbroken arrows, whereas those not involved in phagocytosis are indicated by broken arrows. The photomicrograph was acquired using a Nikon Eclipse E400 microscope fitted with a Nikon Digital Sight-Fi1 camera.

phagocytes were counted per slide. The experimenter was blind to the technical details of the slides. The percentage phagocytosis and phagocytic index were calculated using established formulae (Campbell et al., 2003): (1) percentage phagocytosis equals the number of positive phagocytes per 100 phagocytes counted; and (2) the phagocytic index is the average number of yeast cells engulfed by each positive phagocyte multiplied by the percentage phagocytosis.

In vitro experiments

Effect of dyn $A_{(1–17)}$

Splenic phagocytes were treated with varying concentrations of dyn $A_{(1–17)}$ (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} mol l^{-1}) for 30 min. Cells were washed with PBS and processed for the phagocytic assay. A phagocyte monolayer incubated in medium alone for the same duration was considered to function as a control. The concentration range of dyn $A_{(1–17)}$ used in the present *in vitro* study was determined after reviewing the literature (Casellas et al., 1991; Szabo et al., 1993), whereas the optimum time required for incubation of phagocytes with dyn $A_{(1–17)}$ was determined based on pilot experiments.

Effect of the non-selective opioid receptor antagonist

In order to explore the involvement of opioid and non-opioid receptors in mediating the effect of dyn $A_{(1–17)}$ on phagocytosis, the non-selective opioid receptor antagonist naltrexone was used along with the most effective concentration of dyn $A_{(1–17)}$. Splenic phagocytes were incubated with 10^{-8} mol l^{-1} naltrexone and 10^{-9} mol l^{-1} dyn $A_{(1–17)}$, simultaneously, for 30 min. In order to compare the results between different treatment groups, phagocytes were incubated in medium, naltrexone or dyn $A_{(1–17)}$ alone for 30 min as controls. After incubation, cells were washed and processed for the phagocytic assay.

Effect of selective opioid receptor antagonists

Splenic phagocytes were incubated with a 10^{-8} mol l^{-1} concentration of selective μ-, δ- or κ-opioid receptor blocker (CTAP, NTI or NorBNI, respectively) and 10^{-9} mol l^{-1} dyn $A_{(1–17)}$, simultaneously, for 30 min. For controls, phagocytes were incubated in medium, dyn $A_{(1–17)}$ or μ-, δ- or κ-opioid receptor blocker alone for 30 min. Thereafter, phagocytes were washed and processed for the phagocytic assay.

Effect of inhibitors of adenylate cyclase and protein kinase A

Inhibitors of AC and PKA, SQ 22536 and H-89, respectively, were used to examine the opioid-receptor-coupled downstream signaling mechanism for dyn $A_{(1–17)}$ action on phagocytosis. Splenic phagocytes were pre-incubated with different concentrations of SQ 22536 [(2.5, 5.0, 7.5 and 10.0)$\times10^{-9}$ mol l^{-1}] or H-89 [(25, 50, 75 and 100)$\times10^{-9}$ mol l^{-1}] for 30 min. Thereafter, the monolayer was incubated with 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ and the above concentrations of SQ 22536 or H-89, simultaneously, for 30 min. Control groups were made in which phagocytes were incubated: (a) for 60 min in medium alone, (b) for 30 min in medium alone, and then for another 30 min with 10^{-9} mol l^{-1} dyn $A_{(1–17)}$, (c) for 60 min with 10×10^{-9} mol l^{-1} SQ 22536 in the case of the AC inhibitor experiment or 100×10^{-9} mol l^{-1} H-89 for the PKA inhibitor experiment. After incubations, the phagocyte monolayer was washed with PBS and processed for the phagocytic assay. The concentration range of SQ 22536 or H-89 was determined based on the available literature (Roy et al., 2008; Roy and Rai, 2009; Kumar et al., 2011) and pilot experiments.

cAMP assay

The intracellular cAMP content in the dyn $A_{(1–17)}$-treated splenic phagocytes was estimated following the manufacturer's (Sigma-Aldrich) protocol. Briefly, 100 μl of splenocyte suspension was added to each well of a 96-well tissue-culture plate. After incubation for 90 min, non-adhered cells were washed off, and adhered cells were incubated with 10^{-4} mol l^{-1} IBMX for 30 min. Furthermore, phagocytes were incubated in PBS (control) or 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ for 30 min. The cells were washed, lysed with 0.1 mol l^{-1} HCl and centrifuged at 600 ***g*** for 10 min at room temperature. The cAMP in the supernatant was estimated using a commercially available enzyme immunoassay cAMP kit.

MTT assay

The method of Mosmann (Mosmann, 1983) for MTT assay was followed to verify the viability of splenic phagocytes after treatment with dyn $A_{(1–17)}$. In brief, a 100 μl splenocyte suspension was added to a 96-well tissue-culture plate for the preparation of a phagocyte monolayer. The non-adhered cells were washed off with PBS. Adhered phagocytes were incubated with medium alone (control) and different concentrations of dyn $A_{(1–17)}$ for 30 min. Afterwards, splenic phagocytes were washed and incubated in 100 μl of medium containing 0.5 mg ml^{-1} MTT for 2 h. Next, the cells were washed with PBS and permeabilized by adding 20 μl of 0.1% Triton X-100. The reduced product formazan was solubilized in 150 μl of DMSO. Following incubation for 15 min at room temperature, absorbance was measured at 570 nm using an ELISA plate reader (MS 5608A, ECIL, New Delhi, India).

The MTT assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase in live cells to reduce MTT into a membrane-impermeable and insoluble purple crystal called formazan. The number of live cells is directly related to the amount of formazan produced.

Statistical analysis

Each treatment was performed in triplicate. All the experiments were repeated three times. The data of three independent experiments were pooled, analysed by one-way analysis of variance (ANOVA) followed by a Newman–Keuls multiple range test, and represented as means ± s.e.m. A Student's *t*-test was used to compare the means of two groups in the case of cAMP estimation.

RESULTS

Effect of dyn $A_{(1–17)}$

Dyn $A_{(1–17)}$ in a concentration-dependent manner inhibited the percentage phagocytosis and phagocytic index (ANOVA, $P<0.001$; Fig. 2). In comparison with a control, a marked ($P<0.05$) decrease in phagocytic activity was observed at a 10^{-11} mol l^{-1} concentration of dyn $A_{(1–17)}$ [control *vs* 10^{-11} mol l^{-1} dyn $A_{(1–17)}$, $P<0.05$]. The inhibition of phagocytosis induced by dyn $A_{(1–17)}$ was further pronounced with an increase of concentration to 10^{-9} mol l^{-1}. The maximum inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis was noticed at a concentration of 10^{-9} mol l^{-1}. With a further increase in the concentration of dyn $A_{(1–17)}$ (10^{-8} mol l^{-1}), the inhibition of phagocytic activity was not increased [10^{-8} mol l^{-1} dyn $A_{(1–17)}$ *vs* 10^{-9} mol l^{-1} dyn $A_{(1–17)}$, not significant]. Furthermore, a MTT assay indicated that dyn $A_{(1–17)}$ itself, in the range of the concentrations used, does not influence the cell viability (data not shown).

Effect of non-selective opioid receptor blocker

The non-selective opioid receptor antagonist naltrexone alone did not affect the phagocytic activity of splenic phagocytes (control *vs* naltrexone 10^{-8} mol l^{-1}, not significant). However, it completely

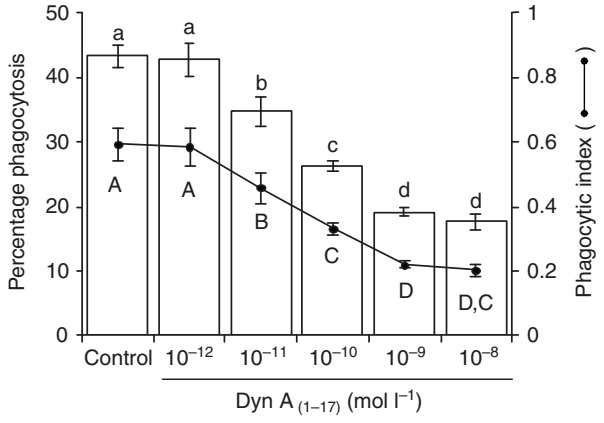

Fig. 2. Effect of dynorphin $A_{(1–17)}$ [dyn $A_{(1–17)}$] on the percentage phagocytosis and phagocytic index of splenic phagocytes. For each experiment, the spleens of 10 lizards were pooled to prepare the phagocyte monolayer. Bar and line graphs represent the means ± s.e.m. of the percentage phagocytosis and phagocytic index, respectively. Error bars bearing different letters (a–d and A–D) differ significantly (Newman–Keuls multiple range test, $P<0.01$ and $P<0.05$).

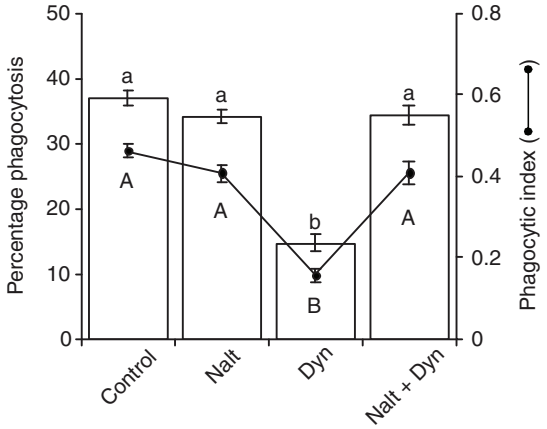

Fig. 3. Antagonistic effect of non-selective opioid receptor antagonist naltrexone (nalt) on dyn-$A_{(1–17)}$-induced inhibition of the phagocytic activity of splenic phagocytes. Each treatment was performed in triplicate, and the experiment was repeated three times using different spleen samples (10 lizards per spleen sample). Values are represented as the means ± s.e.m. Error bars bearing different letters (a, b and A, B) differ significantly (Newman–Keuls multiple range test, $P<0.01$).

antagonized the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis [dyn $A_{(1–17)}$ 10^{-9} mol l^{-1} *vs* dyn $A_{(1–17)}$ 10^{-9} mol l^{-1} plus naltrexone 10^{-8} mol l^{-1}, $P<0.01$; Fig. 3].

Effect of selective opioid receptor antagonists

The μ- and δ-opioid receptor antagonists CTAP (10^{-8} mol l^{-1}) and NTI (10^{-8} mol l^{-1}), respectively, failed to antagonize the inhibitory effect of dyn $A_{(1–17)}$ (10^{-9} mol l^{-1}) on the percentage phagocytosis and phagocytic index [10^{-9} mol l^{-1} dyn $A_{(1–17)}$ *vs* 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 10^{-8} mol l^{-1} CTAP or NTI, not significant, Fig. 4]. By contrast, the selective κ-opioid receptor antagonist NorBNI (10^{-8} mol l^{-1}) completely antagonized the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis [10^{-9} mol l^{-1} dyn $A_{(1–17)}$ *vs* 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 10^{-8} mol l^{-1} NorBNI, $P<0.01$], and the results were comparable to that of a control [10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 10^{-8} mol l^{-1} NorBNI *vs* control, not significant]. However, these antagonists alone did not have any effect on phagocytosis (control *vs* 10^{-8} mol l^{-1} CTAP or NTI or NorBNI, not significant).

Effect of inhibitors of AC and PKA

Inhibitors of AC (SQ22536) and PKA (H-89) decreased the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis in a concentration-dependent manner. A significant decrease in the inhibitory effect of 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ was observed when cells were pre-treated with 5×10^{-9} mol l^{-1} SQ22536 or 50×10^{-9} mol l^{-1} H-89 and then with 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 5×10^{-9} mol l^{-1} SQ22536 or 50×10^{-9} mol l^{-1} H-89 [10^{-9} mol l^{-1} dyn $A_{(1–17)}$ *vs* 10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 5×10^{-9} mol l^{-1} SQ22536 or 50×10^{-9} mol l^{-1} H-89, $P<0.05$; Fig. 5]. Dyn $A_{(1–17)}$-induced inhibition of phagocytosis was completely abolished at higher concentrations of SQ22536 or H-89 (Fig. 5). At 7.5×10^{-9} and 10×10^{-9} mol l^{-1} concentrations of SQ22536 or 75×10^{-9} and 100×10^{-9} mol l^{-1} H-89, the percentage phagocytosis and phagocytic index were found to be comparable to that of a control [10^{-9} mol l^{-1} dyn $A_{(1–17)}$ plus 7.5 or 10×10^{-9} mol l^{-1} SQ22536, or 75 or 100×10^{-9} mol l^{-1} H-89 *vs* control, not-significant].

Intracellular cAMP

In comparison with a control, a marked ($P<0.01$) increase in intracellular cAMP content was recorded in splenic phagocytes incubated with dyn $A_{(1–17)}$ for 30 min (Fig. 6).

DISCUSSION

Our *in vitro* study, for the first time in reptiles, has demonstrated the role of dyn $A_{(1–17)}$ and its downstream signaling cascade in the

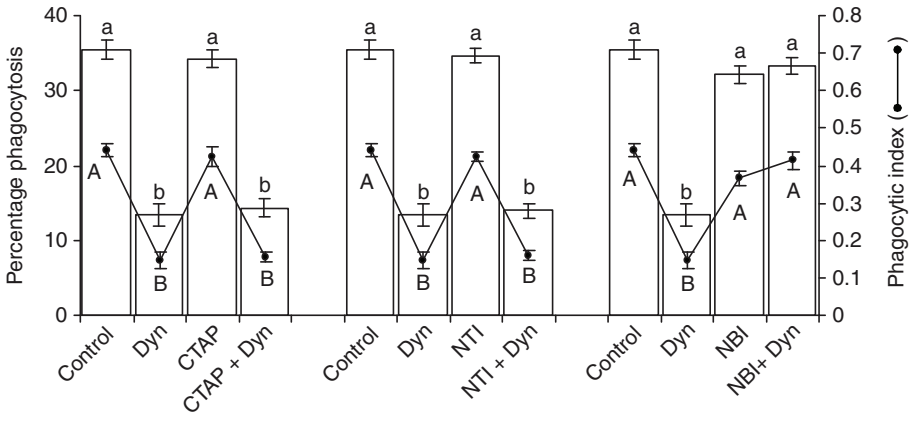

Fig. 4. Effect of the selective μ-, δ- and κ-receptor antagonists CTAP, NTI and NorBNI (NBI), respectively, on dyn $A_{(1–17)}$-induced inhibition of phagocytosis. The experiment was repeated three times using different spleen samples. Error bars bearing the same letters (a, b and A, B) do not differ significantly (Newman–Keuls multiple range test, $P<0.01$).

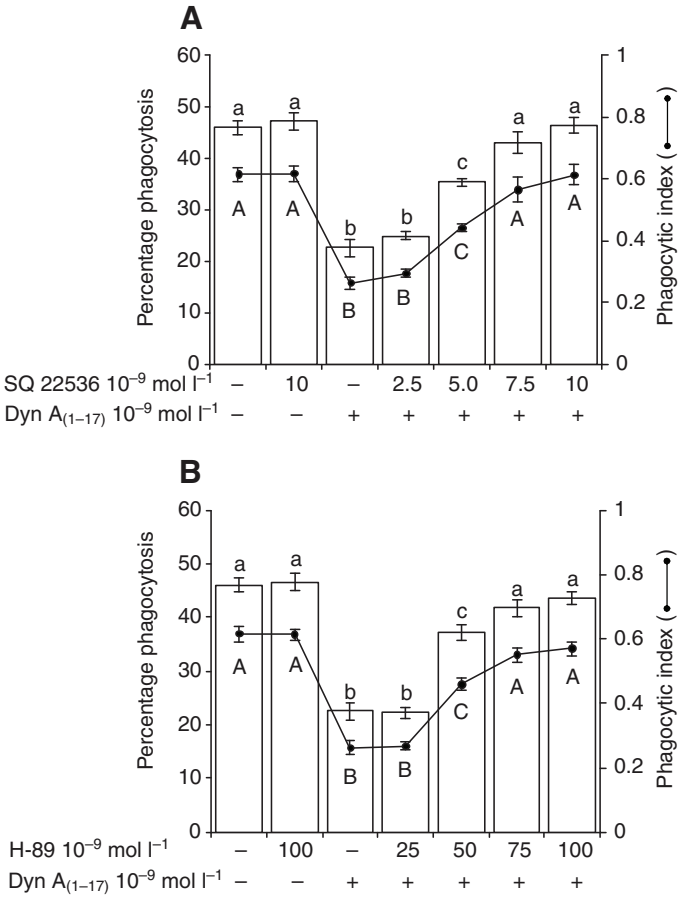

Fig. 5. Concentration-related effect of the adenylate cyclase inhibitor SQ 22536 (A) and the protein kinase A inhibitor H-89 (B), respectively, on dyn-$A_{(1–17)}$-induced inhibition of phagocytic activity. Data are presented as means ± s.e.m. Error bars bearing the same letters (a–c and A–C) do not differ significantly [for panel A, $P<0.01$ for both percentage phagocytosis and phagocytic index; for panel B, $P<0.05$ for percentage phagocytosis and $P<0.01$ for phagocytic index].

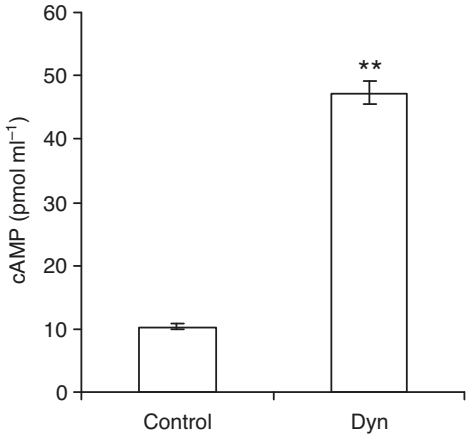

Fig. 6. Effect of dyn $A_{(1–17)}$ on intracellular cAMP production in splenic phagocytes of wall lizards. The intracellular cAMP content was estimated using an enzyme immunoassay. Data were analyzed by a Student's *t*-test and are represented as means ± s.d. (**$P<0.01$).

regulation of an innate immune function – phagocytosis. In the wall lizard *H. flaviviridis*, dyn $A_{(1–17)}$ by acting through the κ-opioid receptor inhibited the phagocytosis of yeast cells by splenic phagocytes. This is in contrast to data reported in fish (Singh and Rai, 2010) and mammals (Ichinose et al., 1995), wherein, dyn $A_{(1–17)}$ stimulated the phagocytosis of yeast cells by fish splenic phagocytes (Singh and Rai, 2010) and latex particles by murine peritoneal macrophages (Ichinose et al., 1995). With respect to receptor-mediated action, dyn $A_{(1–17)}$ stimulated the phagocytosis through a κ-opioid receptor in fish only. Interestingly, in mammals, the stimulatory effect of dyn $A_{(1–17)}$ was mediated through a non-opioid receptor as the non-selective opioid receptor blocker naltrexone failed to block the effect of dyn $A_{(1–17)}$ on phagocytosis (Ichinose et al., 1995). Nevertheless, a specific opioid-receptor-mediated inhibitory effect of dynorphin(s) and its receptor agonist on phagocytosis has been reported in mammals. Dyn $A_{(1–13)}$ and a κ-opioid receptor agonist were shown to inhibit the phagocytosis of sheep erythrocytes (Casellas et al., 1991) and *Candida albicans* (Szabo et al., 1993) by murine peritoneal macrophages. In both the cases, the effect was mediated through a κ-opioid receptor. The reason for the diverging results pertaining to the effect of dynorphin(s) on phagocytosis might be ascribed to the involvement of different receptors (opioid or non-opioid) and also to the specificity of target cells to be phagocytosed by the immune cells. Thus, it seems that, despite the species-specific differential effect, dynorphin(s) modulate the phagocytosis largely through opioid receptors – and particularly through κ-opioid receptors.

It is noteworthy that cDNA for opioid receptors have not been cloned to date from any reptile tissue, although they have been cloned from immune as well as non-immune cells of fish (Darlison et al., 1997; Barrallo et al., 1998; Barrallo et al., 2000; Rodriguez et al., 2000; Alvarez et al., 2006; Chadzinska et al., 2009) and mammals (Chuang et al., 1994; Graveriaux et al., 1995; Sedqi et al., 1995; Sedqi et al., 1996; Sharp et al., 1997; Sharp et al., 2000). The sequence analysis of cDNA revealed that these receptors belong to the superfamily of G-protein-coupled receptors (Sharp et al., 1997; Law et al., 2000). Generally, opioid receptors are coupled with the alpha inhibitory subunit ($G\alpha_i$) of the G-protein that, upon activation, downregulates the adenylate cyclase activity and, consequently, cAMP production (Lawrence and Bidlack, 1993; Sharp et al., 1996; Wang et al., 2003). However, an accumulation of intracellular cAMP upon the activation of opioid receptors is well established in mammalian immune cells (Aymerich et al., 1998) and cell lines (Martin-Kleiner and Gabrilovac, 1997; Heagy et al., 1999). This suggests the coupling of an opioid receptor with a stimulatory alpha subunit (α_s) of the G-protein. In our earlier *in vitro* study in the wall lizard, dibutyryl cAMP was shown to inhibit the percentage phagocytosis and phagocytic index at higher concentrations (Roy and Rai, 2004). Also, an increased intracellular cAMP level in cortisol-treated (Roy and Rai, 2009) and melatonin-treated (Roy et al., 2008) splenic phagocytes is reported to be directly correlated with inhibition of phagocytosis in the freshwater fish *Channa punctatus*. In light of these studies, we speculated that dyn $A_{(1–17)}$ would have inhibited the phagocytic activity of lizard splenic phagocytes by activating the α_s subunit of the G-protein coupled to κ-opioid receptors and, consequently, by increasing the intracellular cAMP level. This assumption was verified using the inhibitor of adenylate cyclase SQ22536, which completely averted the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis. Also, an increase in the intracellular cAMP level in lizard splenic phagocytes in response to dyn $A_{(1–17)}$ reconfirmed the assumption. Furthermore, the cAMP-dependent PKA pathway was shown to be involved in mediating dyn-$A_{(1–17)}$-induced inhibition of phagocytosis as the effect was fully reversed by the PKA inhibitor

H-89. However, an altogether different downstream signaling cascade for dynorphins has been reported by Ichinose and colleagues (Ichinose et al., 1995) in murine peritoneal macrophages. In these cells, dynorphin stimulated the phagocytic activity by means of a non-opioid receptor-mediated calcium signaling cascade.

Although further studies are required across the vertebrates to develop a deeper insight into the evolutionary aspects of the immunomodulatory role of opioids, the dynorphin effect on phagocytosis in reptiles seem to be closer to that in mammals than in fish. The present study in wall lizards reveals the involvement of the AC–cAMP–PKA pathway in transducing the inhibitory effect of dyn $A_{(1–17)}$ on phagocytosis.

LIST OF ABBREVIATIONS

AC	adenylate cyclase
cAMP	cyclic adenosine monophosphate
CTAP	H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
dyn $A_{(1–17)}$	dynorphin $A_{(1–17)}$
IBMX	3-isobutyl-1-methylxanthine
MTT	3-(4,5-dimethyl thiazoyl-2)-2,5-diphenyl tetrazolium bromide
NorBNI	norbinaltorphimine dihydrochloride
NTI	naltrindole hydrochloride
PKA	protein kinase A

ACKNOWLEDGEMENTS

The authors are grateful to the National Institute of Drug Abuse, Bethesda, MD, USA, for the generous gifts of dyn $A_{(1–17)}$, CTAP, NTI and NorBNI.

FUNDING

S.K. is indebted to University Grants Commission, India, for providing the fellowship. We thank the Department of Science Technology, India, and University of Delhi, India for financial assistance.

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