Journal of Poultry Science, 42 : 56–63, 2005

Effect of Spermidine on Intracellular Calcium Ion Mobilization in Chicken Phagocytes Treated with Leukotriene B_4 (LTB_4)

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The effect of spermidine, a type of polyamine present in exudates from inflamed tissues and bacteria-infected sites in humans, on calcium mobilization of leukotriene B_4 (LTB_4)-stimulated chicken abdominal macrophages and heterophils was examined *in vitro*. The intracellular calcium ion concentration $[Ca^{2+}]_i$ was elevated immediately and reached a maximum a few seconds after the addition of LTB_4. The elevated $[Ca^{2+}]_i$ returned to the basal (resting) level approximately 2 min after LTB_4 addition. Pretreatment of cells with spermidine significantly prolonged the duration of $[Ca^{2+}]_i$ elevation in LTB_4-treated chicken phagocytes. However, no change was induced in the level of $[Ca^{2+}]_i$ elevation by pretreating the LTB_4-treated cells with spermidine. These results suggest that polyamines would enhance the activities of chicken macrophages and heterophils in inflammation sites and bacteria-infected sites through delaying the return to resting levels of stimulated cells, as in the case of human polymorphonuclear leukocytes.

Key words : spermidine, LTB_4, calcium ion, chicken phagocytes

Introduction

Phagocytes such as polymorphonuclear leukocytes (PMN) and macrophages play important roles in the elimination of invading microorganisms by phagocytosing and killing them through production of active oxygens, and in the case of macrophages, through the production of cytokines such as tumor necrosis factor (TNF) and interleukin-12 (Powell, 1987 ; Medzhitov and Janeway, 2000). Leukotriene B_4 (LTB_4), a type of Leukotriene (Lindbom *et al.,* 1982), as well as formyl-methionyl-leucyl-phenylalanine (fMLP) (Lee *et al.,* 2002 ; Wu *et al.,* 2004), phorbol myristate acetate (PMA) (Wolfson *et al.,* 1985) and lipopolysaccharide (LPS) (Taramelli and Varesio, 1981) have been reported to activate these phagocyte functions. Calcium ion in the cytoplasm is known to be an important factor in cytoplasmic signal transduction pathways (Samelson, 2002 ; Winslow *et al.,* 2003). In mammalian phagocytes, several types of stimulation, including application of activating agents such as LTB_4 (Kobayashi *et al.,* 1994), fMLP (Hsu *et al.,* 1997), PMA (Kong *et al.,* 1993) and LPS (Hotchkiss *et al.,* 1997) have been reported to elevate the calcium ion concentration $[Ca^{2+}]_i$ in the cytoplasm. Recently, we observed that these activating agents other than fMLP elevated $[Ca^{2+}]_i$ in the cytoplasm of chicken phagocytes such as abodominal

Received : September 9, 2004, Accepted : October 20, 2004
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macrophages and heterophils (Yong *et al.,* 2005).

Calcium mobilization in fMLP-stimulated human polymorphonuclear leukocytes (PMN) was reported to be enhanced by application of spermidine and putrescine, two types of polyamines (Walter *et al.,* 1992), which are widely distributed organic cations that support the growth and differentiation of cells (Tabor and Tabor, 1984).

In the present study, we examined the effect of exogenous spermidine in vitro on the calcium mobilization of leukotriene B_4 (LTB_4)-stimulated chicken abdominal macrophages and heterophils.

Materials and Methods

Animals and cell preparation

Male chicks of the White Leghorn Shaver strain (6–12 weeks old) were used. The care of chicks and conduct of experiments were in accordance with the Guidelines for Animal Experiments of Okayama University Advanced Science Research Center. Chicks were injected intraperitoneally with 5% gelatin solution as a phagocyte-inducing factor. Sixteen hours after the injection, phagocyte-rich exudates were collected from the abdominal cavities of the chicks as described previously (Cahyaningsih *et al.,* 1990). Heterophils and macrophages in the exudates were isolated by gradient centrifugation ($280\times g$, 15℃, 60 min) using Histopaque (specific gravity : 1.077, Sigma Chemical Co., MP, USA). The purity of the cells obtained, determined using Natt & Herrick staining solution (Natt and Herrick, 1952), was $>90\%$.

Agents

Leukotriene B_4 (LTB_4 ; Sigma) was diluted with DMSO to 10^{-5} M. The solution was stored at −20℃ until use. Spermidine (Sigma) was dissolved in DMSO to 20 mM and stored at −20℃ until use.

Measurement of $[Ca^{2+}]_i$

The cells obtained (1×10^7 cells/m*l*) were suspended in Hanks' balanced salt solution (HBSS) and loaded with 5μM Fura2-AM (Sigma) at 37℃ for 45 min. After the cells were washed twice, they were resuspended in HBSS. Ten micro- liters of LTB_4 solution was added to a cuvette containing 1 m*l* of Fura-2 AM-loaded cell suspension (2×10^6/m*l*) that had been preincubated with or without 20μl of spermidine solution for 4 min at 37℃. Fluorescence with stimulation at 340 and 380 nm and emission at 510 nm was continuously monitored by using an Intracellular Ion Analyzer (CAF-110, JASCO Corporation, Tokyo, Japan). $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows : $Kd\cdot[(R-R_{min})/(R_{max}-R)]\cdot A/B$, where R is the observed fluorescence intensity and Kd is the dissociation constant of Fura-2 (224 nM) (Merritt *et al.,* 1990). The Rmax and Rmin values were obtained at the end of each measurement by sequentially adding Triton X-100 and 5 mM EGTA (Fig. 1). The A and B values were the fluorescence intensity of Fura-2 bound to Ca^{2+} and the fluorescence intensity of free Fura-2, respectively.

Statistical analysis

Five to 6 chicks were used in each experimental group. Student's T test was used to determine the significance of differences between macrophages and heterophils, and

between spermidine-treated and sprmidine-nontreated (control) cells. Significance was taken at the 5% probability level. All results are expressed as means and standard errors.

Results

$[Ca^{2+}]_i$ elevation by LTB_4 in chicken phagocytes pretreated with or without spermidine

In the resting condition (LTB_4-nonstimulating condition), $[Ca^{2+}]_i$ levels were 100 to 200 nM in macrophages (198.6±8.2 nM ; mean±SE) and heterophils (196.7±21.8 nM ; mean±SE). These were about the same as the levels in rat neutrophils and macrophages (Al-Mohannna and Hallet, 1988). The $[Ca^{2+}]_i$ levels in phagocytes of chickens was not changed by addition of sperimidine (Fig. 1). The $[Ca^{2+}]_i$ levels were elevated immediately and reached a maximum a few seconds after the addition of LTB_4, and then decreased to the basal (resting) levels after approximately 2 min (Fig. 1). The levels of $[Ca^{2+}]_i$ elevation induced by LTB_4 treatment in spermidine-pretreated macrophages and heterophils were 61.5±4.7 nM and 400.1±37.9 nM, respectively. These values did not differ from the values of spermidine-non-pretreated macrophages (59.2±3.5 nM) and heterophils (405.7±41.4 nM), respectively (Fig. 2). The levels of $[Ca^{2+}]_i$ elevation in spermidine-treated and -nontreated heterophils were significantly higher ($p<0.01$) than those in the corresponding macrophages (Fig. 2), as detected previously (Yong *et al.,* 2005).

Duration time of $[Ca^{2+}]_i$ elevation induced by LTB_4 in chicken phagocytes pretreated with or without spermidine

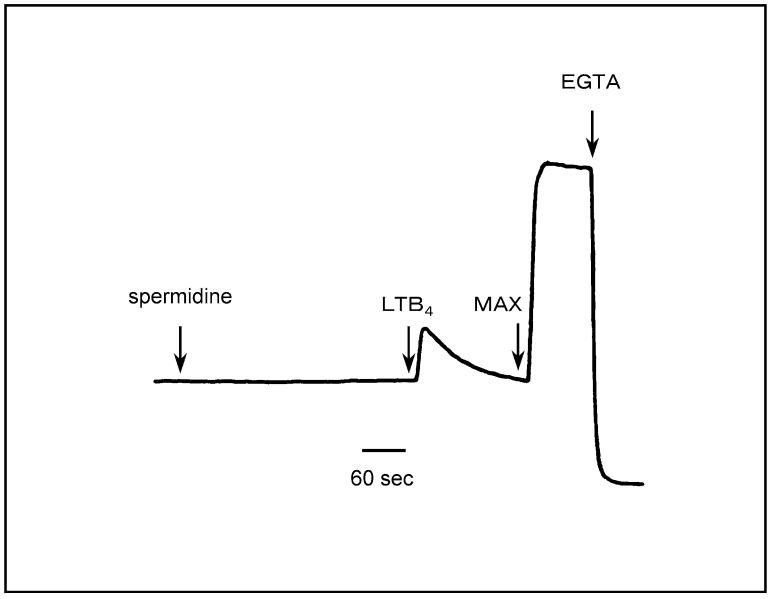

Fig. 1. Representative time course of intracellular $[Ca^{2+}]_i$ elevation in spermidine-pretreated chicken macrophages. Arrows represent addition of spermidine, LTB4, Triton X-100 and EGTA.

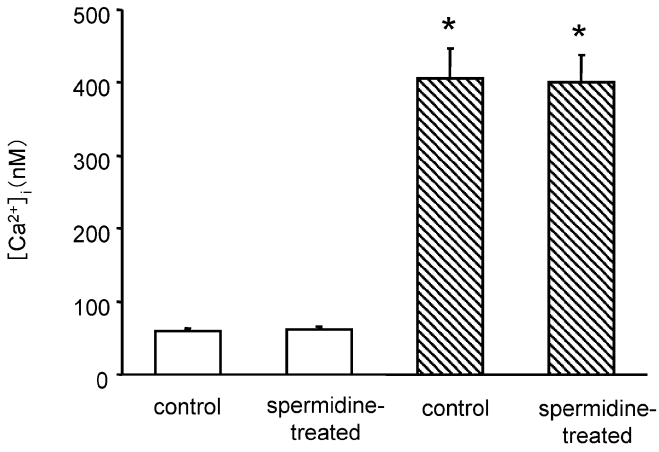

Fig. 2. Levels of elevation of $[Ca^{2+}]_i$ in LTB_4-stimulated chicken phagocytes pretreated with or without spermidine. Open columns and shaded columns represent abdominal macrophages and abdominal heterophils, respectively. Asterisks represent significant differences between the values for abdominal macrophages and heterophils ($p < 0.05$).

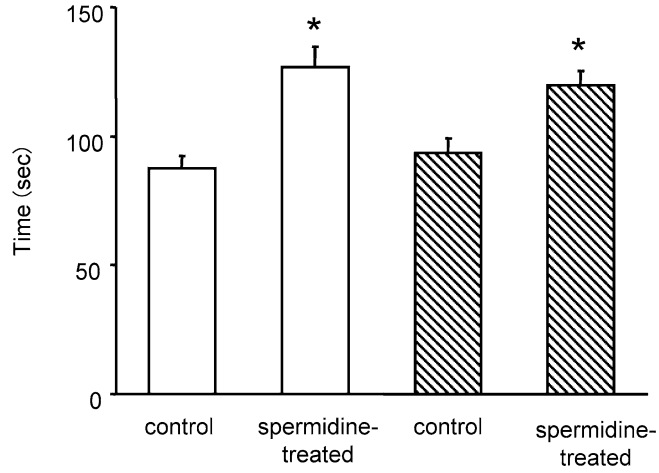

Fig. 3. Duration of $[Ca^{2+}]_i$ elevation of LTB_4-stimulated chicken phagocytes pretreated with or without spermidine. Open columns and shaded columns represent abdominal macrophages and abdominal heterophils, respectively. Asterisks represent significant differences between the values for spermidine-treated cells and controls ($p < 0.05$).

The elevated $[Ca^{2+}]_i$ level returned to the basal (resting) level approximately 2 min after LTB_4 addition to cells. Figure 3 shows that duration of the $[Ca^{2+}]_i$ elevation induced by LTB_4 addition in heterophils (119.8 ± 5.4 sec) and macrophages (126.7 ± 7.8 sec) pretreated with spermidine was significantly ($p < 0.05$) longer than that in control

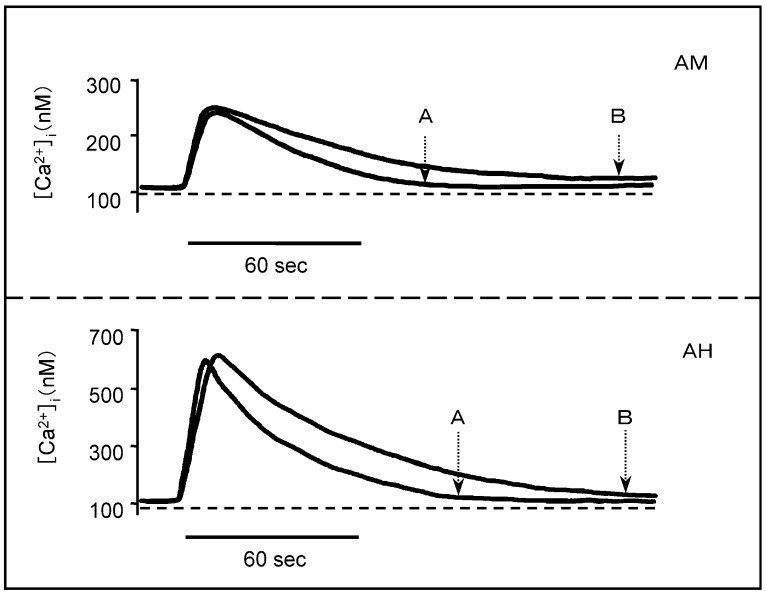

Fig. 4. Representative time course of intracellular $[Ca^{2+}]_i$ elevation in spermidine-nontreated controls (A) and spermidine-treated chicken phagocytes (B). AM=abdominal macrophages and AH=abdominal heterophils.

cells not pretreated with sperimdine (heterophils : 93.6±5.7 sec and macrophages : 87.7±4.7 sec). The time course of the elevation of $[Ca^{2+}]_i$ induced by LTB_4 in macrophages and heterophils shows that the prolongation of the elevation of $[Ca^{2+}]_i$ by spermidine in LTB_4-treated cells was due to prolongation of the decreasing phase after attainment of maximal $[Ca^{2+}]_i$ in LTB_4-treated phagocytes (Fig.4).

Discussion

The results of the present study revealed that spermidine, a type of polyamine, enhanced calcium mobilization by prolongation of the duration of $[Ca^{2+}]_i$ elevation induced in LTB_4-treated chicken phagocytes without changing the level of $[Ca^{2+}]_i$ elevation induced by LTB_4. In human PMN, polyamines such as spermidine and putrescine were reported to significantly enhance fMLP-induced Ca^{2+} mobilization by delaying the return to basal cytosolic $[Ca^{2+}]_i$ levels, although the polyamines used did not change the level of $[Ca^{2+}]_i$ elevation induced by treatment with fMLP (Walters *et al.,* 1992). These results suggest that spermidine amplifies the LTB_4-induced activation of chicken phagocytes by enhancing calcium mobilization through delaying the return to basal cytosolic $[Ca^{2+}]_i$ levels after the treatment as in the case of the prolongation of the duration of $[Ca^{2+}]_i$ elevation polyamines in fMLP-treated human PMN.

LTB_4 has been reported to bind to G protein-coupled receptors on the surface of mice PMN (kavelaars *et al.,* 2003), and then to induce intracellular signaling pathways such as those involving cytoplasmic $[Ca^{2+}]_i$ elevation (Tarlowe *et al.,* 2003). The

elevation of cytoplasmic $[Ca^{2+}]_i$ in LTB_4-stimulated cells was reported to be caused by the influx of Ca ions into the cytoplasm from the extracellular fluid and the intracellular Ca ion pool (Winslow *et al.,* 2003). The results of the present study indicate that spermidine does not enhance Ca ion influx from the extracellular fluid or the intracellular calcium pool in LTB_4-stimulated chicken phagocytes. In LTB_4-stimulated rat phagocytes (Kobayashi *et al.,* 1994), the elevated $[Ca^{2+}]_i$ returned to basal levels within a few minutes due to efflux to the extracellular fluid and return to the intracellular calcium pool. In a human PMN-like cell line stimulated by fMLP, spermidine and putrescine have been reported to inhibit the plasma membrane Ca^{2+}-ATPase and thereby delay the return to resting cytoplasmic Ca^{2+}levels (Walters *et al.,* 1992). It may be suggested that spermidine enhances calcium mobilization by delaying the return to resting levels of LTB_4-stimulated chicken phagocytes through inhibition of plasma membrane Ca^{2+}-ATPase, as in the case of fMLP-stimulated human cells.

Polyamines such as spermidine, putrescine and cadaverine were reported to be present at high concentrations in exudates from inflamed tissues and bacteria-infected tissues of humans (Lamster *et al.,* 1987). Superoxide production by fMLP-stimulated human PMN (Guamieri *et al.,* 1987 ; Walters and Chapman, 1995) and respiratory burst activity of LPS-stimulated human macrophages (Messina *et al.,* 1992) were reported to be elevated by application of polyamines. These results suggest that endogenous polyamines contribute to the enhancement of chicken phagocyte functions through increasing the duration of Ca^{2+}elevation in the cytoplasm by delaying the return to resting levels of the activities of activated phagocytes, although high concentration of polyamines have not yet been demonstrated in inflammation sites or bacteria-infected sites of chickens.

On the other hand, recent study revealed that polyamines suppress the activation of human neutrophil NADPH oxidase (Ogata *et al.,* 1996), the production of pro-inflammatory cytokines such as interferonγ and interleukin 12 by mouse macrophages (Hasko *et al.,* 2000), nitric oxide production by rat alveolar macrophages (Mossner *et al.,* 2001) and tumor necrosis factor and monocyte chemoattractant protein-1 (Prez-Cano *et al.,* 2003) in mammalian phagocytes. These results indicate the importance of significance of polyamines as multi-potent and multi-direction immunomodulatory agents.

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