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The role of cyclic-AMP on arginase activity by a murine macrophage cell line (RAW264.7) stimulated with lipopolysaccharide from *Actinobacillus actinomycetemcomitans*

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Aims: The aim of the present study was to determine the role of cyclic adenosine monophosphate (cAMP) on arginase activity in a murine macrophage cell line (RAW264.7 cells) stimulated with lipopolysaccharide (LPS) from *Actinobacillus actinomycetemcomitans*.

Materials and methods: The cells were treated with *A. actinomycetemcomitans* LPS for 24 h. The effects of SQ22536 (an adenylyl cyclase inhibitor), ODQ (a guanylyl cyclase inhibitor), dibutyryl cAMP (a cAMP analog), 8-bromo cyclic guanosine monophosphate (a cGMP analog), forskolin (an adenylyl cylase activator), and cycloheximide (a protein synthesis inhibitor) on arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells were also determined. Arginase activity was assessed in LPS-stimulated cells in the presence of 3-isobutyl-1-methylxanthine (IBMX), siguazodan and rolipram [phosphodiesterase (PDE) inhibitors] as well as KT5720 [a protein kinase A (PKA) inhibitor].

Results: Arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells was suppressed by SQ22536 but not ODQ. Enhancement of arginase activity was observed in the presence of cAMP analog or forskolin but not cGMP analog. Cycloheximide blocked arginase activity in the cells in the presence of cAMP analog or forskolin with or without *A. actinomycetemcomitans* LPS. IBMX augmented arginase activity in *A. actinomycetemcomitans* LPS-stimulated cells. Rolipram (a PDE4 inhibitor) increased the levels of arginase activity higher than siguazodan (a PDE3 inhibitor) in the

Key words: *Actinobacillus actinomycetemcomitans*; Arginase; cyclic adenosine monophosphate; lipopolysaccharide; RAW264

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antigen-stimulated cells. The effect of cAMP analog or forskolin on arginase activity in the presence or absence of *A. actinomycetemcomitans* LPS was blocked by the PKA inhibitor (KT5720).

Conclusion: The results of the present study suggest that *A. actinomycetemcomitans* LPS may stimulate arginase activity in murine macrophages (RAW264.7 cells) in a cAMP-PKA-dependent pathway.

Actinobacillus actinomycetemcomitans, a gram-negative oral bacterium, is believed to play a crucial role in the development of chronic inflammatory periodontal diseases, such as localized juvenile periodontitis and adult periodontitis (11, 17, 23). It has been reported recently that lipopolysaccharide (LPS) from periodontopathogens such as *A. actinomycetemcomitans*, *Prevotella intermedia*, and *Porphyromonas gingivalis* stimulated murine macrophages to produce nitric oxide (NO) (2, 9, 18, 19, 20). Interestingly, NO production by these cells when stimulated with *A. actinomycetemcomitans* LPS was up-regulated by interferon-γ (IFN-γ) and interleukin-2 (IL-2) but down-regulated by IL-4 (18, 19). It was speculated that IL-4 might increase arginase activities in *A. actinomycetemcomitans* LPS-activated murine macrophages (18, 19). Indeed, our previous study showed that *A. actinomycetemcomitans* LPS stimulated arginase activity in RAW264.7 cells via a CD14–Toll-like receptor 4 molecule complex (21). In that study, arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells was enhanced by IL-4 but suppressed by IFN-γ, suggesting that arginase activity and nitric oxide synthase (NOS) in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells may be reciprocally regulated by T helper type1 (Th1) and Th2-derived cytokines.

Both arginase and NOS metabolize L-arginine; arginase and NOS catalyze this amino acid to produce NO and either L-ornithine or urea, respectively (12). Two isoforms of arginase are known to exist, the hepatic isoform, arginase I, which is thought to play a role in ureagenesis, and the extrahepatic isoform, arginase II, which is thought to play a role in biosynthesis of polyamines and inflammation (3). The induction of arginase activity is dependent on the cyclic adenosine monophosphate–protein kinase A (cAMP–PKA) pathway (3, 12) because the activity of this enzyme in macrophages was up-regulated in the presence of the cAMP analog forskolin, an adenylate cyclase activator, or phosphodiesterase (PDE) inhibitors, but was inhibited in the presence of a PKA inhibitor (4, 7, 8, 13, 22). Therefore, the aim of the present study was to determine whether or not the induction of arginase activity in a murine macrophage cell line (RAW264.7 cells) stimulated with *A. actinomycetemcomitans* LPS was under the control of the cAMP–PKA pathway.

Materials and methods

Preparation of *A. actinomycetemcomitans* LPS

The *A. actinomycetemcomitans* Y4 (serotype b) was grown in Todd–Hewitt broth supplemented with 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI) under anaerobic conditions as described elsewhere (24). LPS from this periodontopathic bacterium was isolated using a hot phenol–water extract, treated with nuclease (Sigma, St Louis, MO), and then lyophilized as previously described (18).

Cell cultures

A murine macrophage cell line (RAW264.7), a kind gift from Dr T. Sizfizul (School of Biology, Universiti Sains Malaysia), was grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 1% penicillin–streptomycin (Sigma). After they reached confluence, the cells were harvested and washed three times, and then viable cells were counted. Two hundred microliters of culture medium containing 2×10^5 cells were cultured in an incubator in 5% CO_2 at 37°C for 24 h. Untreated cells were used as a negative control. SQ22536 (an adenylyl cyclase inhibitor), ODQ, (a guanylyl cyclase inhibitor), dibutyryl cAMP, 8-bromo cyclic guanosine monophosphate (cGMP) or forskolin (Sigma) were added to the cell cultures at various concentrations with or without the presence of 10 μg *A. actinomycetemcomitans* LPS. In other experiments, various concentrations of cycloheximide (Sigma) were added to the cell cultures in the presence of *A. actinomycetemcomitans* LPS (10 μg) and/or dibutyryl cAMP (10 μM) or forskolin (10 μM). The involvement of PDE was determined by adding various concentrations of 3-isobutyl-1-methylxanthine (IBMX) (a non-specific PDE inhibitor), siguazodan (a PDE3 inhibitor) or rolipram (a PDE4 inhibitor) in *A. actinomycetemcomitans* LPS-stimulated cells. All PDE inhibitors were purchased from Sigma. A PKA inhibitor (KT5720) (Sigma) at various concentrations was added in the cultures stimulated with or without 10 μg of *A. actinomycetemcomitans* LPS in the presence of dibutyryl cAMP (10 μM) or forskolin (10 μM). All experiments were repeated three times, each consisting of triplicate cultures.

Measurement of arginase activity

Arginase activity in cell lysate was determined by a colorimetric assay as previously described (14). Briefly, cells were lysed with 50 μl 0.1% Triton X-100 and shaken for 30 min. The cell lysate was then added with 50 μl 25 mM Tris–HCl (pH 7.5). To 25 μl of this lysate, 5 μl 10 mM $MnCl_2$ was added and the mixture was heated for 10 min at 56°C. After adding 50 μl 0.5 M L-arginine (pH 9.7), the mixture was incubated for 1 h at 37°C. The reaction was stopped with 450 μl acid solution mixture (1 : 3 : 7; H_2SO_4 : H_3PO_4 : H_2O). The color was developed by adding 20 μl 9% α-isonitropropiophenone (dissolved in 100% ethanol), heated for 45 min at 100°C, and then read at 540 nm by MicroQuant spectrophotometer (Biotek-Instrument Inc., Winooski, VT). Arginase activity was calculated as the arginase activity index (AAI) using the following formula (1): AAI = (test arginase activity/media-only arginase activity) × 100

Arginase activity in cultured cells alone (the negative control) was assigned an activity index value of 100.

Statistical analysis

The data were analyzed by a one-way analysis of variance followed by Fischer least squares differences using the SPSS statistical package (SPSS Co., Chicago, IL).

Results

Effect of adenylyl cyclase and guanylyl cyclase inhibitor

SQ22536, an adenylyl cyclase inhibitor, decreased arginase activity in *A. actinomycetemcomitans* LPS-stimulated

RAW264.7 cells in a dose-dependent fashion ($P < 0.05$) (Fig. 1A). In sharp contrast, the levels of arginase activity in the same cell cultures were not altered by the presence of ODQ, a guanylyl cyclase inhibitor ($P > 0.05$) (Fig. 1B). These results indicate that arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells was an adenylyl cyclase-dependent mechanism.

Effect of cAMP and cGMP analogs

When cells were stimulated with *A. actinomycetemcomitans* LPS in the presence of dibutyryl cAMP, arginase activity was increased significantly compared with that in cells stimulated with *A. actinomycetemcomitans* LPS alone ($P < 0.05$) (Fig. 2A). Dibutyryl cAMP alone augmented arginase activity in RAW264.7 cells three-fold, albeit the activity levels were lower when compared with cells stimulated with *A. actinomycetemcomitans* LPS and this cAMP analog ($P < 0.05$). In contrast, arginase activity in cells stimulated with *A. actinomycetemcomitans* LPS in the presence of a cGMP analog was unaltered ($P > 0.05$) (Fig. 2B). The cGMP analog alone failed to induce arginase activity in RAW264.7 cells ($P > 0.05$).

Effect of forskolin

Forskolin, an adenylate cyclase activator, is known to increase the levels of cAMP (16). The present study showed that the addition of increasing concentrations of forskolin was paralleled with increased arginase activity in RAW264.7 cells with or without the presence of *A. actinomycetemcomitans* LPS ($P < 0.05$) (Fig. 3), suggesting that increased arginase activity may be associated with increased cAMP activity in the LPS-stimulated RAW264.7 cells.

Effect of cycloheximide

Cycloheximide reduced arginase activity in cell cultures that were stimulated with *A. actinomycetemcomitans* LPS in the presence of dibutyryl cAMP or forskolin ($P < 0.05$) (Fig. 4A,B). Similarly, arginase activity in RAW264.7 cells in the presence of cAMP analog or forskolin alone was reduced by cycloheximide ($P < 0.05$).

Effect of PDE inhibitors

In the presence of IBMX, siguazodan or rolipram, arginase activity was significantly enhanced in the LPS-stimulated RAW264.7 cells as compared with that in cells stimulated with *A. actinomycetemcomitans* LPS alone ($P < 0.05$) (Fig. 5). The increased levels of arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells in the presence of IBMX were significantly higher than those in the presence of either siguazodan or rolipram ($P < 0.05$). Comparison between the effects of siguazodan and rolipram showed that the levels of arginase activity in the LPS-stimulated RAW264.7 cells were higher in the presence of rolipram, particularly after adding the drugs at concentrations of 10 and 100 μM ($P < 0.05$).

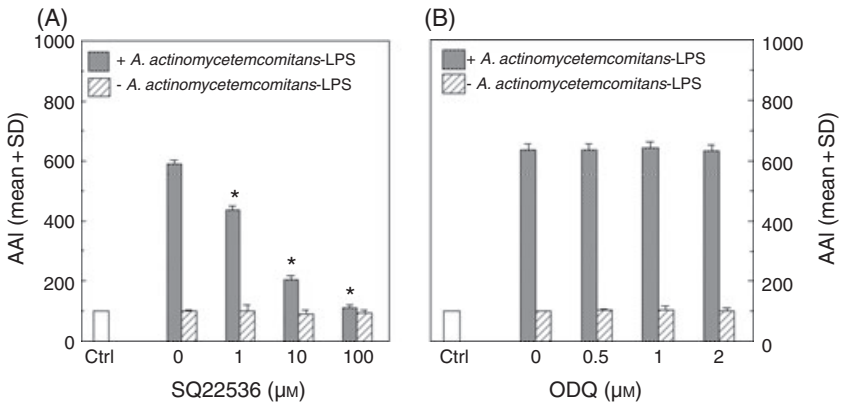

Fig. 1. Effect of SQ22536, an adenylyl cyclase inhibitor, (A) and ODQ, a guanylyl cyclase inhibitor, (B) on the levels of arginase activity in *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *$P < 0.05$ between arginase activity in RAW264.7 cells in the presence of SQ22536 or ODQ with and without *A. actinomycetemcomitans* LPS stimulation. AAI, arginase activity index; SD, standard deviation.

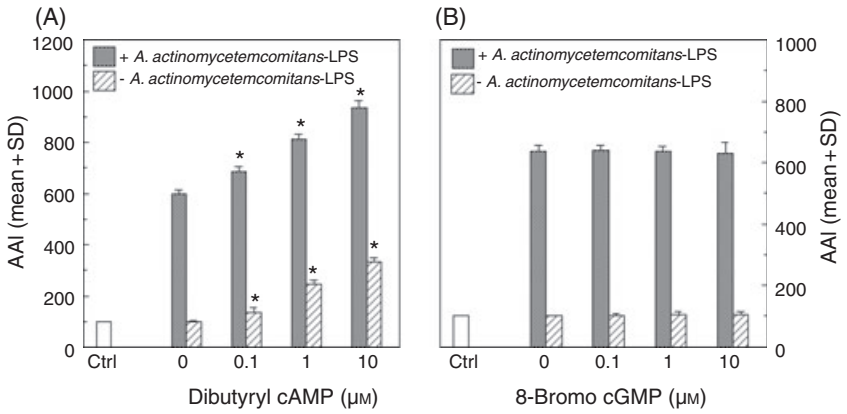

Fig. 2. Effect of dibutyryl cAMP, a cAMP analog, (A) and 8-bromo cGMP, a cGMP analog, (B) on the levels of arginase activity in *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *$P < 0.05$ between arginase activity in RAW264.7 cells in the presence of dibutyryl cAMP or 8-bromo cGMP with and without *A. actinomycetemcomitans* LPS stimulation. AAI, arginase activity index; SD, standard deviation.

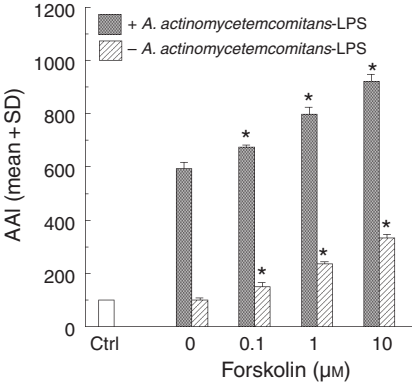

Fig. 3. Effect of forskolin, an adenylyl cyclase activator, on the levels of arginase activity in *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *$P < 0.05$ between arginase activity in RAW264.7 cells in the presence of forskolin with and without *A. actinomycetemcomitans* LPS stimulation. AAI, arginase activity index; SD, standard deviation.

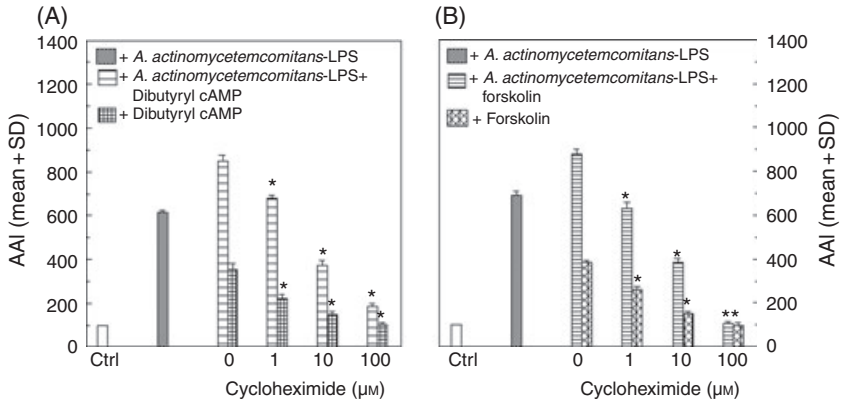

Fig. 4. Effect of cycloheximide on the levels of arginase activity in RAW264.7 cells in the presence of cAMP analog (A) or forskolin (B) with or without *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS). *$P < 0.05$ between arginase activity in RAW264.7 cells in the presence of cycloheximide and dibutyryl cAMP or forskolin with and without *A. actinomycetemcomitans* LPS stimulation. AAI, arginase activity index; SD, standard deviation.

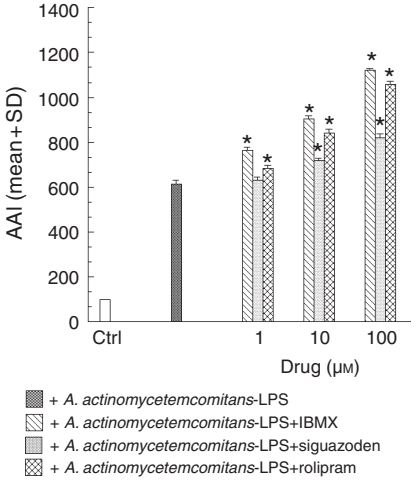

Fig. 5. Effect of IBMX, a non-specific phosphodiesterase (PDE) inhibitor, siguazodan, a PDE3 inhibitor, and rolipram, a PDE4 inhibitor, on the levels of arginase activity in *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *$P < 0.05$ between arginase activity in RAW264.7 cells stimulated with *A. actinomycetemcomitans* LPS with or without the presence of PDE inhibitors. AAI, arginase activity index; SD, standard deviation.

Effect of a PKA inhibitor

Arginase activity in RAW264.7 cells in the presence of *A. actinomycetemcomitans* LPS and dibutyryl cAMP or forskolin could be significantly reduced by KT5720, a PKA inhibitor, in a dose-dependent fashion ($P < 0.05$) (Fig. 6A,B). Similarly, increased concentrations of PKA inhibitor added in cAMP analog-activated or forskolin-activated RAW264.7 cell cultures were associated with steadily decreased arginase activity ($P < 0.05$).

Discussion

Our previous study indicated that *A. actinomycetemcomitans* LPS stimulates arginase activity in a murine macrophage cell line (RAW264.7) (21). However, the exact intracellular signal(s) involved in the activation of this enzyme activity by *A. actinomycetemcomitans*-LPS-stimulated RAW264.7 cells was not established. The results of the present study showing that the level of arginase activity stimulated by *A. actinomycetemcomitans* LPS was decreased by an adenylyl cyclase inhibitor but increased by a cAMP analog suggest that *A. actinomycetemcomitans* LPS may induce a cAMP pathway, but not a cGMP pathway, to activate arginase activity in RAW264.7 cells. These results are in accordance with previous reports showing that LPS from enteric bacteria stimulates arginase activity in murine macrophages and human colon carcinoma cells in a cAMP-dependent mechanism (7, 8, 13, 22). Interestingly, cAMP analog alone was able to stimulate arginase activity in RAW264.7 cells. Taken together, these findings seem to suggest that *A. actinomycetemcomitans* LPS may amplify the arginase activity induced by cAMP analog in RAW264.7 cells. The exact mechanisms by which *A. actinomycetemcomitans* LPS promoted cAMP-induced arginase activity in RAW264.7 cells are unclear. Morris et al. (13) demonstrated that cAMP alone induces high expression of arginase I, whereas bacterial LPS stimulates the expression of arginase II in RAW264.7 cells. In this study, increased expression of both arginase I and arginase II could be observed when the cells were stimulated with both LPS and cAMP analog. If so, one may assume that the stimulatory effect of both *A. actinomycetemcomitans* LPS and cAMP on arginase activity in RAW264.7 cells, as seen in the present study, may be the result of increased expression of arginases I and II. However, this notion remains to be investigated further because the present study did not assess the expression of arginase isoforms.

The involvement of the cAMP pathway in generating arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells was also supported by the fact that forskolin with or without the presence of LPS-increased arginase activity in these cells. Arginase activity was up-regulated by forskolin and LPS from enteric bacteria in rabbit alveolar macrophages (8). In this study, forskolin alone was able to activate the arginase activity. If so, it would seem to suggest, yet again, that *A. actinomycetemcomitans* LPS may amplify the stimulatory effect of forskolin on arginase activity. Forskolin-mediated adenylate cyclase activation leads to activation of the G protein $G_s\alpha$-subunit, which may in turn alter intracellular cAMP levels (16) thereby enhancing arginase activity. However, whether or not the ability of both forskolin and *A. actinomycetemcomitans* LPS to up-regulate arginase activity in RAW264.7 cells involves G protein activation needs to be further clarified.

The fact that cycloheximide prevented arginase activity in RAW264.7 cells stimulated with *A. actinomycetemcomitans* LPS in the presence of cAMP analog or forskolin indicates that up-regulation of arginase activity by the cAMP pathways requires intact protein synthesis such that this pathway may regulate the *de novo* synthesis of arginase proteins. These results are in line with a previous report (8).

PDE, a family of proteins consisting of 11 isoforms, catalyzes the hydrolysis of cAMP to inactive 5′-AMP, thereby reducing the levels of intracellular cAMP (5). Therefore, increased levels of cAMP may be achieved by reducing the activation of PDE by using specific PDE isoform inhibitors. Indeed, the present study showed that inhibition of PDE function may result in increased levels of cAMP which may in turn enhance arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells, as also seen in previous reports using rabbit (8) and rat (10) alveolar macrophages. Interestingly, the results of the present study revealed that PDE4 might play a more important role than PDE3 in regulating cAMP levels following activation of

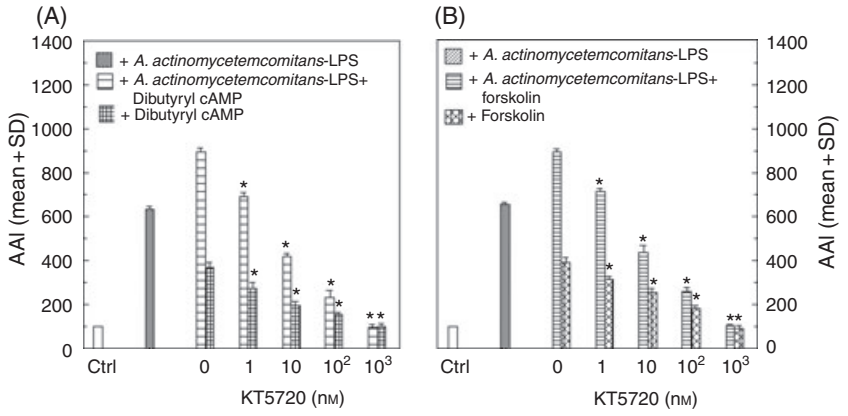

Fig. 6. Effect of KT5720, a protein kinase A (PKA) inhibitor, on the levels of arginase activity in RAW264.7 cells in the presence of cAMP analog (A) or forskolin (B) with or without *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS). $*P < 0.05$ between arginase activity in RAW264.7 cells in the presence of KT5720 and dibutyryl cAMP or forskolin with and without *A. actinomycetemcomitans* LPS stimulation. AAI, arginase activity index; SD, standard deviation.

RAW264.7 cells by *A. actinomycetemcomitans* LPS. Similarly, Hammermann et al. (8) demonstrated that inhibition of PDE4 by rolipram resulted in higher levels of arginase activity in rabbit alveolar macrophages than inhibition of PDE3 by siguazodan. However, the exact explanation of the results in the present study is unclear. One possibility is that differential levels of PDE3 and PDE4 in RAW264.7 cells may account for the different levels of arginase activity in the cells in the presence of *A. actinomycetemcomitans* LPS and either PDE3 or PDE4 inhibitor as seen in the present study. Despite the fact that PDE1, PDE3, and PDE4 are all expressed by macrophages, the latter is the major PDE isoform in these cells (5, 15). That the levels of cAMP after PDE4 inhibition may be higher than those after PDE3 inhibition in activated macrophages is therefore obvious.

Activation of adenylyl cyclase leads to increased levels of intracellular cAMP, which in turn binds to regulatory (R) subunits of PKA, thereby inducing dissociation of the holoenzymes and subsequent phosphorylation of key substrates (6). Hence, PKA is believed to be the most important effector of the cAMP pathway. Indeed, the results of the present study demonstrated the involvement of PKA in the arginase activity of *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells. The present results are in accordance with previous reports using RAW264.7 cells (4, 8) and human Caco-2 tumor cells (22). Therefore, one may speculate that PKA is part of a downstream signal that mediates the cAMP-dependent up-regulation of arginase activity in RAW264.7 cells stimulated with *A. actinomycetemcomitans* LPS.

In conclusion, the present study shows that arginase activity in *A. actinomycetemcomitans* LPS-stimulated RAW264.7 cells was up-regulated by cAMP and forskolin, but not by a cGMP analog. Inhibition of PDE4 activation induced higher levels of arginase activity than inhibition of PDE3 in RAW264.7 cells in the presence of LPS and/or cAMP analog and forskolin. Arginase activity in these cells stimulated with *A. actinomycetemcomitans* LPS in the presence of cAMP analog or forskolin was reduced by KT5720, a PKA inhibitor. These results suggest, therefore, that the *A. actinomycetemcomitans* LPS may stimulate arginase activity in murine macrophage-like cells (RAW264.7 cells) via a cAMP–PKA-dependent pathway.

Acknowledgments

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