

Calcium-Dependent Nitric Oxide Synthase Activity in Rat Thymocytes

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We examined the conversion of L-[³H]arginine to L-[³H]citrulline in lysate from rat thymocytes, which was dependent on Ca²⁺ and cofactors (FAD, BH₄, NADPH). Removal of Ca²⁺ of the medium, reduced the total L-[³H]citrulline formation by about 97%. The L-[³H]citrulline formation was completely inhibited by the NO synthase inhibitors, N^G-nitro-L-arginine and N^G-monomethyl-L-arginine, with values for IC₅₀ of 1.2 μM and 19.4 μM, respectively. In intact thymocytes, the L-[³H]citrulline formation was dependent on the intracellular Ca²⁺ ([Ca²⁺]_i) concentration. Increasing the extracellular free-Ca²⁺ concentration up to 1.5 mM, was accompanied by an increase in [Ca²⁺]_i inside the thymocytes and there was a parallel increase in the intracellular L-[³H]citrulline formation, which reached a maximal value of 371.2 nM of [Ca²⁺]_i. Addition of N^G-nitro-L-arginine to the medium, completely inhibited the formation of L-[³H]citrulline. The immunolabeling study revealed that 15% of the thymocytes isolated from rat thymus constitutively expressed the endothelial isoform of NO synthase. © 1998 Academic Press

In mammalian cells, nitric oxide (NO) and citrulline are stoichiometrically generated from L-arginine (L-Arg) in an enzymatic reaction catalysed by the nitric oxide synthase (NOS). To date, two constitutive isoforms of NO synthase, which are regulated by physiological changes in the intracellular calcium concentra-

tion ([Ca²⁺]_i), have been identified, the neuronal and the endothelial NOS isoforms (1, 2).

In contrast to the neuronal and endothelial cells, in which the presence and the functional importance of the constitutive enzymes have been demonstrated, in lymphocytes neither the presence nor the biological role of the NO synthase isoforms is known. There is some question as to whether the ability of lymphocytes synthesise NO; thus it has been reported either that T lymphocytes can be triggered to produce NO (3-9), or that lymphocytes produce a very low level of NO (10, 11) even in the presence of lipopolysaccharide (LPS) (12) and cytokines (13), which induce the expression of the inducible isoform of NO synthase in many cell types (1).

The thymus is the organ where the T cell development occurs (14). Despite the extensive production of lymphocytes in the thymus, relatively few of those cells mature to the single-positive (CD4⁺ or CD8⁺) stage and migrate to the peripheral blood, and little is known about the selection process which result in the transition of certain immature T lymphocytes (CD4⁺ CD8⁺, double-positive cells) to mature and viable single-positive cells (15). Recent studies, suggest that depending on the concentration of NO, it may be either toxic or protective for signalling in thymocyte development (16-20).

Previous studies provided evidence for the presence of the constitutive endothelial isoform of NO synthase in T lymphocytes from peripheral blood (5). However, there are no literature reports addressing whether the thymocytes contain the constitutive isoform of NO synthase. Therefore, we investigated whether thymocytes express the constitutive isoform of the NO synthase, and studied the relationship between the rate of citrulline (or NO) formation and the [Ca²⁺]_i in intact thymocytes.

MATERIAL AND METHODS

Isolation of thymocytes from rat thymus. Thymocytes were isolated from five-week male Wistar rats, as previously described (21), in a sodium medium containing 132 mM NaCl, 4 mM KCl, 6 mM

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Abbreviations: [Ca²⁺]_{out}, extracellular Ca²⁺ concentration; [Ca²⁺]_i, intracellular Ca²⁺ concentration; BH₄, 6-(R,S)-5,6,7,8,-tetrahydrobiopterin; eNOS, endothelial constitutive nitric oxide synthase; FAD, flavine adenine dinucleotide; FITC, fluorescein isothiocyanate; IC₅₀, half-maximal inhibitory concentration; L-Arg, L-arginine; L-MetArg, N^G-monomethyl-L-arginine; L-NOArg, N^G-nitro-L-arginine; LPS, lipopolysaccharide; NADPH, β-nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffer saline.

glucose, 1.4 mM MgCl₂, 10 mM HEPES, pH 7.4 and 0.1% bovine serum albumin fatty acid free (Sigma Chemical Co., St. Louis, MO, U.S.A.). The cells in suspension were counted in a T540 Coulter Counter, and the cell viability was evaluated by the trypan blue exclusion test, after the isolation procedure. A total of 96-98% of the freshly isolated thymocytes routinely excluded trypan blue.

To obtain the lysate of freshly isolated thymocytes, the cells isolated from each thymus (approximately 400×10⁶ cells) were resuspended in 2 ml of cold water and then lysed, at 4°C, using an ultrasonic cell disrupter (sonifier cell disrupter, Model W140).

Identification of CD2 positive cells by flow cytometric analysis. Thymocytes in suspension (10⁶ cells) were incubated with 10 μl of mouse anti-rat CD2:FITC (isotype IgG2a) (Serotec, Oxford, England), or mouse IgG2a negative control:FITC (Serotec, Oxford, England), during 15 min, in a total volume of 0.1 ml PBS. Then, the cells were washed and resuspended in 0.8 ml PBS. The fluorescence was analysed by flow cytometry using a EPICS XL (Coulter) with a 550 nm DL and a 525 nm BP filters.

Measurement of the NO synthase activity by the L-[³H]citrulline formation. The enzyme activity of NO synthase was determined in

the lysate of thymocytes, under the following conditions: 400 μg protein of the lysate, 10 nM L-[³H]arginine (69 Ci/mmol) (Amersham Laboratories, Buckinghamshire, England), cofactors (1 mM NADPH, 5 μM FAD or 20 μM BH₄) (Sigma Chemical Co., St. Louis, MO, U.S.A.), and free Ca²⁺ (100 μM), in a total volume of 200 μl. In some experiments varying concentrations (from 0.001 to 500 μM) of N^G-monomethyl-L-arginine (L-MetArg) or N^G-nitro-L-arginine (L-NOArg) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added to the reaction mixture (Sigma Chemical Co., St. Louis, MO, U.S.A.). After incubating for 20 min, the reactions were terminated by the addition of 1.8 ml of ice cold stop buffer. L-[³H]citrulline formation was measured in 2 ml applied on the top of 0.8 ml Dowex AG50WX-8 (Na⁺ form) (Serva, Heidelberg, Germany) columns, previously equilibrated with 20 mM sodium acetate, pH 5.5 containing 1 mM L-citrulline (Sigma Chemical Co., St. Louis, MO, U.S.A.), 2 mM EDTA and 0.2 mM EGTA (stop solution), as previously described (22). The counts were corrected by a blank assay running in the absence of calcium and in the presence of 0.1 mM EGTA.

To determine the NO synthase activity in intact thymocytes, the cells in suspension (11×10⁶) were incubated at 37°C, for 20 min, in the sodium medium containing 10 nM L-[³H]arginine and different

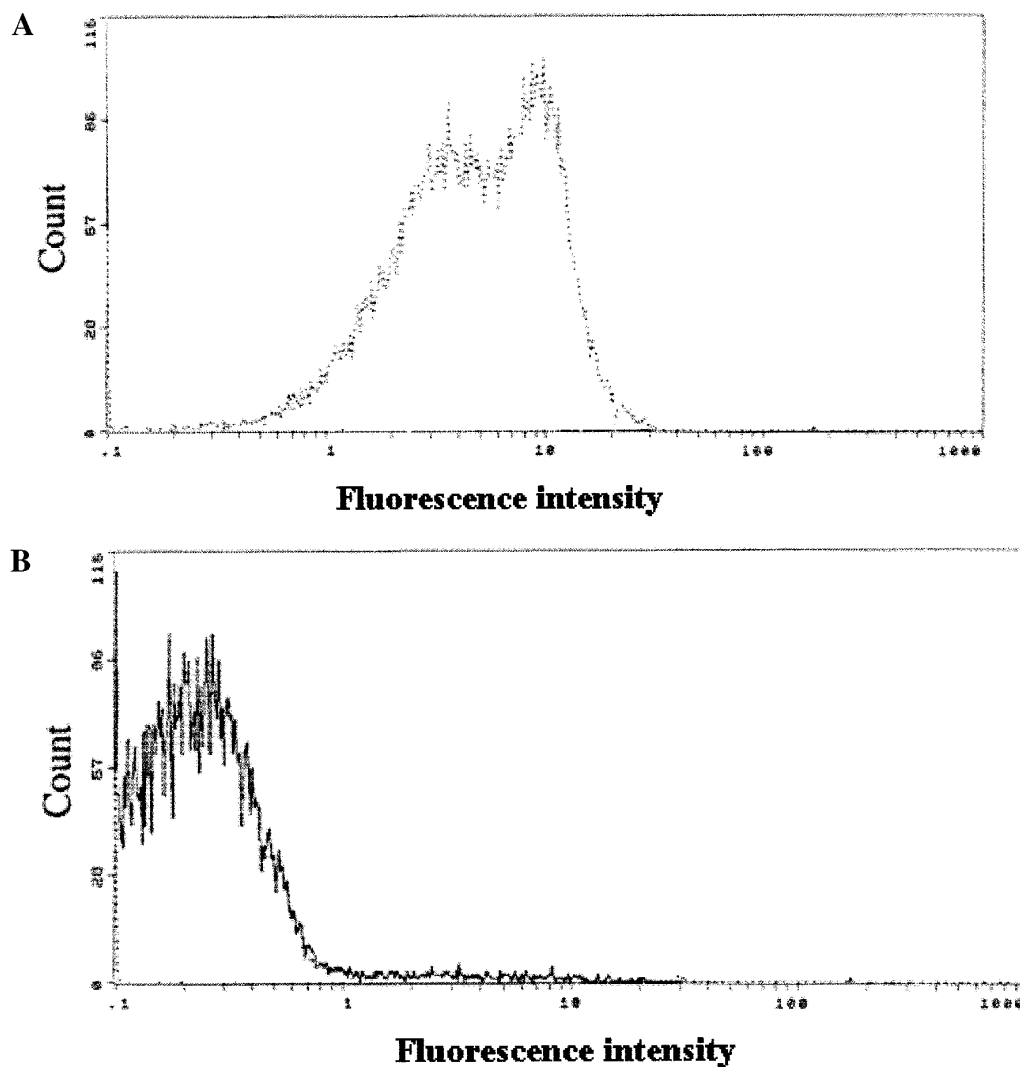


FIG. 1. Percentage of CD2 positive cells isolated from rat thymus. Thymocytes in suspension (10⁶ cells) were incubated with the mouse anti-rat CD2:FITC (A), or mouse IgG2a negative control:FITC (B), and the fluorescence was analysed by flow cytometry, as described in materials and methods. The figure is representative of three separated experiments.

concentrations of Ca^{2+} or L-NOArg, as specified in the legends of the figures. Incubations were terminated by rapid centrifugation (200g/8 min) in eppendorf microcentrifuge (Eppendorf 5402), at 4°C, and by rinsing of the pellet twice with 1 ml of cold sodium medium. Then, the cells were disrupted with 1.5 ml of water and centrifuged at 15800g/5 min. Aliquots of 50 μl of the supernatant were used to measure the radioactivity incorporated into the cells in a liquid scintillation counter. L-[^3H]citrulline formation by the cells was measured in 1 ml of the supernatant, which was applied on the top of 0.8 ml Dowex AG50WX-8 (Na^+ form) columns, previously equilibrated as mentioned above. The counts were corrected for the blank assay in the absence of calcium and in the presence of 1 mM EGTA.

The concentration of free Ca^{2+} in the incubation medium was quantified as previously described (23).

Measurement of cytosolic free Ca^{2+} concentration. Thymocytes (3×10^7 cells/ml) were incubated in Hepes-buffered RPMI-1640 (Sigma, USA) with 1 μM Indo-1-AM (Molecular Probes, Leiden, The Netherlands), for 30 min, at 37°C. The cells were sedimented and washed once with fresh culture medium. Aliquots of 4×10^6 cells were resuspended in sodium medium containing different concentrations of Ca^{2+} , and incubated, for 15 min, at 37°C. Measurements of cytosolic Ca^{2+} concentrations were performed at 37°C with a spectrofluorimeter (λ_{ex} 335 nm, λ_{em} 410 nm). Calibration was performed with 1 mM ionomycin (Calbiochem-Boehringer, San Diego, U.S.A.) and MnCl_2 (1M) (Calbiochem-Boehringer, San Diego, U.S.A.), and intracellular Ca^{2+} concentrations were calculated as previously described (24).

Identification of the endothelial isoform of NO synthase by immunocytochemistry. Cell smears were fixed in acetone for 5 min. Non-specific binding was blocked by incubation in normal rabbit serum in PBS (1:20) for 30 min at room temperature. Cells were then incubated 2h at room temperature, with a rabbit polyclonal antibody directed against human endothelial constitutive NO synthase (ecNOS) and human neuronal constitutive NO synthase (1:75) (Transduction Laboratories, Lexington, U.S.A.). After rinsing with PBS the cells were incubated with biotinylated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) at a 1:200 solution for 30 min. Following a second rinsing in PBS, cells were further incubated with the avidin-biotinylated complex for 30 min. After a final rinsing, peroxidase activity was visualized using diaminobenzidine (6mg per 10ml of PBS) and H_2O_2 (1 μl per 10ml of solution). Cell smears were counterstained with hematoxylin and mounted. All experiments included omission of primary antibodies as negative controls.

RESULTS

Before measuring the NO synthase activity in rat thymocytes, we characterised the cell population isolated from juvenile rat thymus in terms of the CD2 positive cells. Assuming that thymocytes are the only cells in the thymus that express the CD2 surface marker (25), our results obtained by labelling the cells with monoclonal mouse anti-rat CD2:FITC antibody (Fig. 1A), permit concluding that 96% of the cells isolated from rat thymus were thymocytes.

The results of the experiments with the lysate of freshly isolated thymocytes indicate that thymocytes contained the Ca^{2+} -dependent NO synthase (Fig. 2A). The enzyme activity required the presence of Ca^{2+} and cofactors (NADPH, FAD and BH_4) for the maximal activity, which corresponds to the formation of 446 ± 0.4 fmol citrulline/mg protein/20 min (Fig. 2A). Removal of Ca^{2+} , by omission of external CaCl_2 and addition of EGTA (0.1 mM) to the incubation medium, reduced the total NO synthase activity from $100 \pm 3.5\%$ to $3 \pm 1.2\%$ (Fig. 2A).

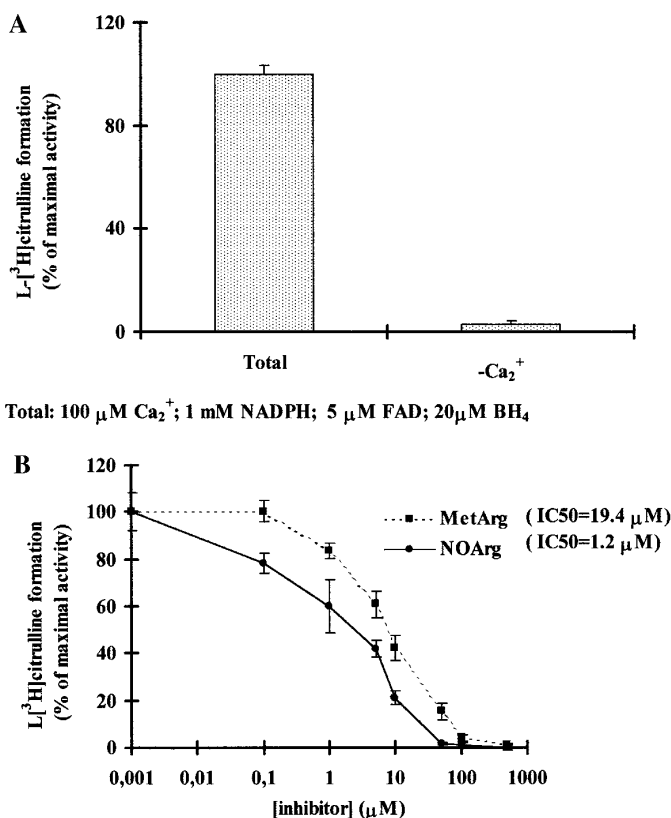


FIG. 2. The Ca^{2+} -dependent NO synthase activity in lysate of thymocytes. The NO synthase activity was measured in the lysate of thymocytes (400 μg of protein), in a sodium medium containing 10 nM of L-[^3H]Arg, cofactors (1 mM NADPH, 5 μM FAD or 20 μM BH_4) and 100 μM free Ca^{2+} concentration (total enzyme activity). The effect of Ca^{2+} (Fig. 2A) and of different concentrations of unlabeled L-NOArg or of L-MetArg (Fig. 2B) was evaluated as described in material and methods. The maximal activity (100%) corresponds to 446 ± 0.4 fmol of L-[^3H]citrulline /mg protein/20 min. Values are mean \pm SEM for three independent experiments, each done in triplicate.

As shown in Fig. 2B, both L-MetArg and L-NOArg, which inhibit the NO synthase activity by competing with L-Arg (2), are potent inhibitors of citrulline formation in the lysate of rat thymocytes. However, L-NOArg was a more potent inhibitor than L-MetArg. The half-maximal inhibitory concentration (IC_{50}) values for L-NOArg and L-MetArg are 1.2 μM and 19.4 μM , respectively (Fig. 2B), as calculated by the Hill plots.

In a previous study, we determined the time-course of the L-[^3H]arginine uptake by intact thymocytes (data not shown), and we found that the maximal incorporation of L-[^3H]arginine into the thymocytes occurs within the first 20 min of time incubation. To study the relation between the L-[^3H]arginine uptake and NO synthase activity in intact thymocytes, we determined the effect of both L-NOArg and L-MetArg on the L-[^3H]arginine uptake and on the conversion of L-[^3H]arginine to L-[^3H]citrulline. The results indicated that 0.1 mM L-MetArg inhibited the L-[^3H]arginine uptake

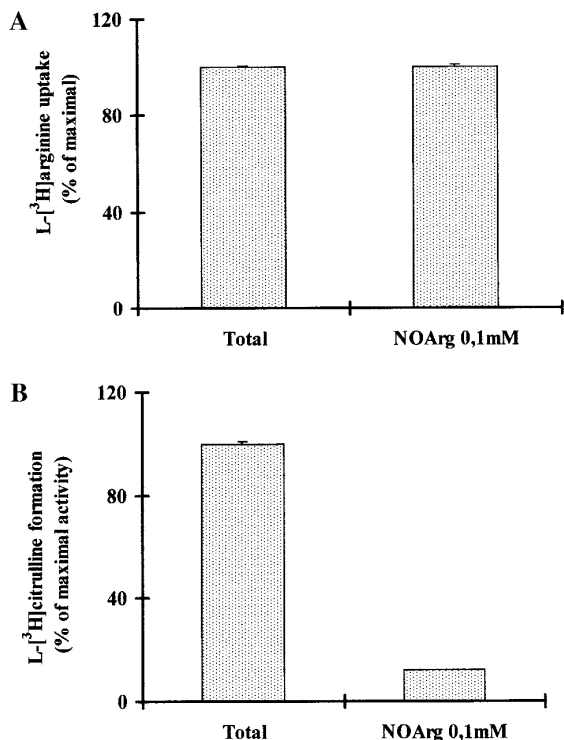


FIG. 3. Effect of the NO synthase inhibitor, L-NOArg, on the L-[³H]arginine uptake and on the Ca²⁺-dependent formation of L-[³H]citrulline in rat thymocytes. Cells (11x10⁶) were incubated during 20 min in the presence of 10 nM of L-[³H]arginine, 1 mM of free calcium and 0.1 mM of L-NOArg. The data on the L-[³H]arginine transport (A), and on the Ca²⁺-dependent L-[³H]citrulline formation (B), were obtained from the same samples, as described in material and methods. Values are mean ± SEM for three independent experiments, each done in triplicate.

by 52.3±1.5% (data not shown) after cell incubation with radiolabelled L-Arg during 20 min, whereas 0.1 mM of L-NOArg had no detectable influence on L-[³H]arginine uptake, but it inhibited NO synthase activity inside the cells (Fig. 3A and Fig. 3B). When thymocytes were incubated in the absence of L-NOArg, the maximal NO synthase activity (100%) was 55±0.69 fmol citrulline/11×10⁶ cells/20 min. The addition of 0.1 mM L-NOArg to the incubation medium reduced by 87.9±0.35 % (6.7±0.35 fmol citrulline/11×10⁶ cells/20 min) the enzyme activity (Fig. 3B).

We also analysed the relation between the extracellular Ca²⁺-concentration and both the [Ca²⁺]_i and the NO synthase activity in thymocytes. Freshly isolated thymocytes in Ca²⁺-free medium had low [Ca²⁺]_i, usually between 60-80 nM, and no NO synthase activity was detected in this experimental condition (Fig. 4B). Addition of 0.1 μM Ca²⁺ to thymocytes increased the [Ca²⁺]_i to 88.3±7.8 nM, and the L-[³H]citrulline formation increased to 5.8±0.148 fmol L-[³H]citrulline/11×10⁶ cells/20 min. When the value of the [Ca²⁺]_{out} in the reaction mixture was 1500 μM, the value of [Ca²⁺]_i became

371.2±28.7 nM, and a maximal value of L-[³H]citrulline formation, 55±0.69 fmol L-[³H]citrulline /11×10⁶ cells/20 min was observed (Fig. 4). These results show that thymocytes constitutively expressed the Ca²⁺-dependent NO synthase.

Immunocytochemical staining of thymocytes was used to identify the NO synthase isoform in these cells. The results obtained by light microscopy of thymocytes labelled with antibodies against the two Ca²⁺-dependent NOS (neuronal and endothelial) revealed that about 15%±0.94 of the thymocytes isolated from rat thymus constitutively express the endothelial isoform of NO synthase (Fig. 5A), and negative controls always showed negative staining (Fig. 5B). No labelling was found in thymocytes stained with polyclonal antibody against the neuronal (type I) isoform of NO synthase, which labelled neuronal cells in culture used as positive control (data not shown).

DISCUSSION

In this work, we show that rat thymocytes isolated from thymus of juvenile rats contained the endothelial

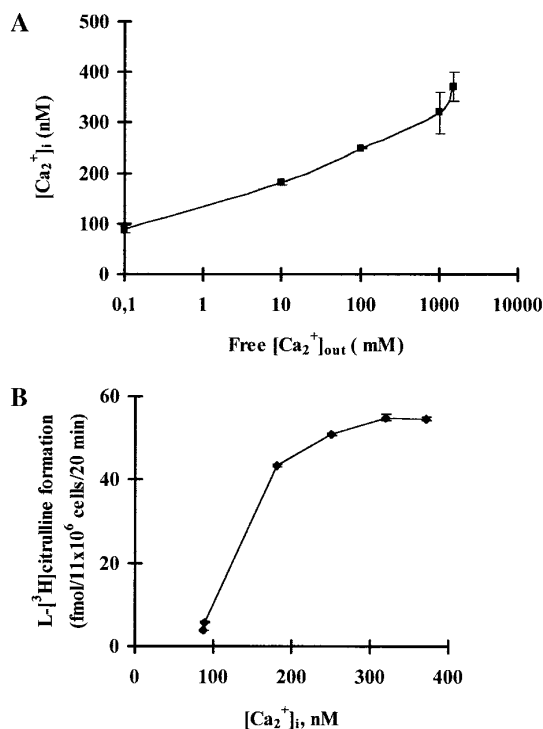


FIG. 4. Effect of [Ca²⁺]_{out} on the [Ca²⁺]_i and on the NO synthase activity in rat thymocytes. Intracellular Ca²⁺ concentration was monitored using 1 μM Indo-1-AM, after cells incubation with the Ca²⁺ indicator dye during 30 min (A). L-[³H]citrulline formation (B) was determined after cells incubation, during 20 min, with 10 nM of L-[³H]arginine and varying concentrations of extracellular free Ca²⁺ (0-1500 μM). The results are expressed as fmol of citrulline/11×10⁶ cells/20 min. Values are mean ± SEM for three independent experiments, each done in triplicate.

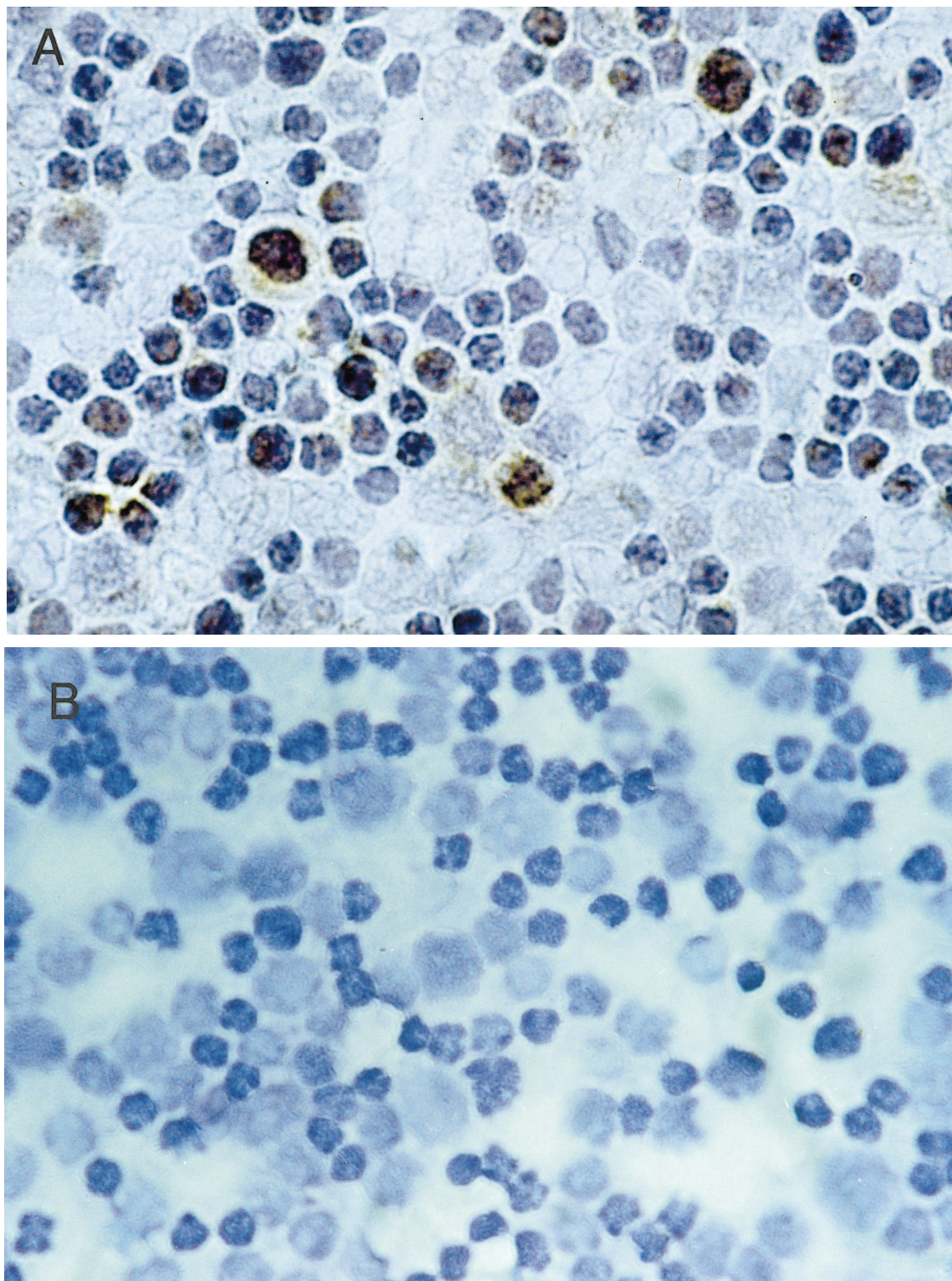


FIG. 5. Immunocytochemistry for the endothelial NO synthase isoform. Immunocytochemistry for NO synthase was performed with anti-ecNOS, as described in materials and methods. These figures are the representative results of four independent experiments. (A), Light microscopy of thymocytes labelled for ecNOS; (B), Control IgG shows no labelling. Original magnification 200 \times .

constitutive isoform of NO synthase. The citrulline formation in the lysate of thymocytes required Ca^{2+} and the specific cofactors for maximal NO synthase activity.

This activity was completely blocked by the two L-Arg analogous, L-MetArg and L-NOArg (Fig. 2B), which competitively inhibit the NO synthase activity (2). In

intact thymocytes, L-NOArg inhibited the citrulline formation, without affecting the uptake of tritiated L-Arg (Fig. 3), whereas L-MetArg inhibited both the NOS activity and the L-Arg transport (data not shown). Similar pattern of inhibition was previously observed in neuronal cells (26, 27). These results, together with the finding that increasing external free Ca^{2+} -concentration up to 1.5 mM was accompanied by an increase in citrulline formation (Fig. 4), demonstrate that thymocytes constitutively express the Ca^{2+} -dependent NO synthase.

Previous studies revealed that nitrite production was not detected in either thymocytes or T helper cell clones (13). These results correlate with our previous observations showing that the nanomolar concentrations of NO, produced in the supernatant of several cell cultures, was not detected when a spectrophotometric assay, based on the Griess reaction (28), was used.

Immunocytochemical staining of thymocytes, with polyclonal antibody against eNOS, revealed the presence of the endothelial NO synthase isoform in these cells (Fig. 5). It was recently reported that lymphocytes from peripheral blood contain the enzyme eNOS (5, 8), although the functional role of this enzyme in lymphocytes is unknown. In this work, we demonstrated the presence of eNOS in 15% of the total population of thymocytes isolated from rat thymus (Fig. 5). From the studies on T cell selection in thymus, it was concluded that 10-15% of the intrathymic thymocytes are single positive ($\text{CD}_4^+\text{CD}_8^-$ or $\text{CD}_4^-\text{CD}_8^+$) thymocytes and migrate to the periphery (29, 30). This opens the question as to whether the eNOS positive thymocytes are the cells released into the blood circulation. Indeed, the development of immunocompetent T-lymphocytes from immature precursor cells take place in the thymus (14), and NO generated by eNOS may play an important role in modulating the process of maturation of thymocytes within the microenvironment of the thymus. Although the role of NO produced by eNOS in the regulation of thymocytes development and maturation is not known, the small amount of NO produced by thymocytes can act as an auto-regulatory molecule during thymocyte development. To clarify this point it is important to have information about the regulation of eNOS expression in different subsets of thymocytes at different stages of development in the thymus.

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