

# Effect of pteridine derivatives on intracellular calcium concentration in human monocytic cells

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Pteridines are heterocyclic compounds which are synthesized and released by human monocytes/macrophages following stimulation by interferon- $\gamma$ . Their concentration in various body fluids proved to be indicative for the stimulation of the cellular immune system, and determination of pteridines has become an important diagnostic tool. We show that pteridine derivatives, namely neopterin (N), 7,8-dihydroneopterin (NH<sub>2</sub>), and 5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) increase intracellular calcium (Ca<sub>i</sub>) in human monocytic cells. Significant increases of Ca<sub>i</sub> are observed at 10 nmol/l NH<sub>2</sub>, at 100 nmol/l BH<sub>4</sub> and at 1 mol/l N, i.e. at concentrations encountered in vivo. At a concentration of 1  $\mu$ mol/l, Ca<sub>i</sub> is increased (from a control value of  $145 \pm 7$  nmol/l) to  $464 \pm 62$  nmol/l (NH<sub>2</sub>),  $340 \pm 41$  nmol/l (BH<sub>4</sub>) and  $344 \pm 46$  nmol/l (N), respectively. The increase of Ca<sub>i</sub> depends on the presence of extracellular calcium and is likely to be due to activation of a calcium channel. We show that the absence of extracellular calcium or the addition of lanthanum ions to the extracellular fluid fully reverses the pteridine-induced increase of Ca<sub>i</sub>. According to these observations, pteridines may mimic the effects of other inflammatory mediators on monocytic cells and seem to be involved in the crosstalk of immunocompetent cells.

Intracellular calcium; Macrophage; Neopterin; Tetrahydrobiopterin; Lanthanum

## 1. INTRODUCTION

Pteridines are pyrazino-pyrimidine compounds which are synthesized by the cleavage of guanosine triphosphate (GTP) catalyzed by the enzyme GTP-cyclohydrolase I [1]. Depending on the enzyme pattern of the cell the first intermediate of the biosynthetic pathway, 7,8-dihydroneopterin-triphosphate, is either converted to 7,8-dihydroneopterin and neopterin or to 5,6,7,8-tetrahydrobiopterin [2], the latter known to be an essential cofactor for hydroxylation reactions [3] or nitric oxide synthase [4,5]. Neopterin and 7,8-dihydroneopterin are produced in excess by human macrophages/monocytes upon activation by interferon- $\gamma$  [6]. The diagnostic value of neopterin has been established in autoimmune diseases, rejection of transplanted organs, malignant diseases, infectious diseases such as AIDS or hepatitis (reviewed in [7]). While it was evident that stimulation of the cellular immune system leads to an increase of pteridine biosynthesis and release, a possible function of pteridines in the inflammatory process remained elusive. The present study has been performed to identify cellular effects of pteridines in human monocytic cells with characteristics of macrophages (THP-1) [8]. Since an increase of intracellular calcium concen-

tration was discussed to play a role in the activation of macrophages [9,10], the effect of pteridines on intracellular calcium activity in THP-1 cells was tested for. To this end, fura-2 fluorescence was determined in monocytic cells before and during application of neopterin, dihydroneopterin and tetrahydrobiopterin. As a result, all three pteridines were able to enhance intracellular calcium activity at concentrations encountered in vivo.

## 2. MATERIALS AND METHODS

Experiments were performed with the human myelomonocytic cell line THP-1 [8] obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l glutamine, 100 U/ml penicillin and 0.1 ng/ml streptomycin. Cells were seeded at a density of  $1 \times 10^6$ /ml and cultured in serum free medium for 24 h. Then serum was added (final concentration: 10% FCS) and effects of pteridines on the intracellular calcium concentration were investigated between 1–8 h after serum supplementation.

Determinations of intracellular calcium activity were made utilizing fura-2 fluorescence. To this end the cells were incubated for 15 min with 2.5  $\mu$ mol/l fura-2-AM (Molecular Probes, Junction City, OR, USA), thereafter fixed on polylysine-coated cover glasses and mounted in a perfusion chamber, enabling determination of fluorescence signals at a single cell level. Measurements were made under an inverted phase-contrast microscope (IM-35, Zeiss, Germany) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, Germany) [11]. Light from a xenon arc lamp (XB075, Osram, Berlin, Germany) was directed through a grey filter (nominal transmission 3.16% Oriel, Darmstadt, Germany), alternatively through a 340-nm and 380-nm, respectively, interference filter (halfwidth 10 nm, Oriel, Darmstadt, Germany) and a diaphragm and was deflected by

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a dichroic mirror (FT425, Zeiss, Germany) into the objective (Plan-Neofluar 63x oil immersion, Zeiss, Germany). The emitted fluorescence was directed through a 420 nm cutoff filter to a photomultiplier tube (R4829, Hamamatsu, Herrsching, Germany). To reduce the region from which fluorescence was collected a pinhole was placed in the image plane of the phototubus (limitation to a circular area of 60  $\mu\text{m}$  diameter).

Fluorescence values were corrected for cellular autofluorescence; intracellular calcium ( $\text{Ca}_i$ ) was calculated from the ratio ( $r$ ) of the fluorescence intensities at the two different excitation wave lengths (340 nm/380 nm) [12,13]:

$$\text{Ca}_i = K_d S f_2 (R - R_{\text{min}}) / S b_2 (R_{\text{max}} - R)$$

where  $R_{\text{min}}$  and  $R_{\text{max}}$  are the fluorescence ratios corrected for autofluorescence at experimental condition, at minimal and maximal calcium binding.  $K_d$  is the dissociation constant for fura-2 (= 225),  $S f_2$  and  $S b_2$  are the proportionality coefficients for the fluorescence at 380 nm excitation of free and calcium-bound dye, respectively.  $R_{\text{min}}$  and  $S f_2$  were determined by exposure of the cells to 2 mmol/l  $\text{Mn}^{2+}$  + 20  $\mu\text{mol/l}$  A 23187,  $R_{\text{max}}$  and  $S b_2$  after exposure of the cells to either, 20  $\mu\text{mol/l}$  digitonin (Sigma, Munich, Germany) or 20  $\mu\text{mol/l}$  A 23187.

The extracellular perfusate was composed of (all numbers mmol/l): 114 NaCl, 5.4 KCl, 0.8  $\text{MgCl}_2$ , 1.2  $\text{CaCl}_2$ , 0.8  $\text{Na}_2\text{HPO}_4$ , 0.2  $\text{NaH}_2\text{PO}_4$ , 20  $\text{NaHCO}_3$  and 5.5 glucose. The solution was equilibrated with 5% carbon dioxide and 95% air (pH 7.4), and experiments were carried out at 37°C.

Applicable data are expressed as arithmetic means  $\pm$  S.E.M. Statistical analysis was made by paired or unpaired  $t$ -test, where applicable. Statistically significant differences were assumed at  $P < 0.05$ .

### 3. RESULTS AND DISCUSSION

As listed in Table I, intracellular calcium activity approaches 145 nmol/l in the absence of pteridines, i.e. a value within the range reported previously for human monocytic cells [14]. The pteridines employed in this study, i.e. neopterin (N), dihydroneopterin ( $\text{NH}_2$ ), and tetrahydrobiopterin ( $\text{BH}_4$ ) lead to a rapid, transient increase of intracellular calcium (Fig. 1). The most effec-

Table I

Intracellular calcium concentration in nmol/l ( $\text{Ca}_i$ ) in the absence of pteridines (control), in the absence of extracellular calcium (0  $\text{Ca}^{2+}$ ), in the presence of 100  $\mu\text{mol/l}$  lanthanum ions ( $\text{La}^{3+}$ ), and in the presence of various concentrations of neopterin (N), 7,8-dihydroneopterin ( $\text{NH}_2$ ), 5,6,7,8-tetrahydrobiopterin ( $\text{BH}_4$ ) ( $n$  = number of experiments)

Condition	$\text{Ca}_i$	(n)
Control	145 $\pm$ 7	40
10 nmol/l N	210 $\pm$ 86	4
100 nmol/l N	251 $\pm$ 82	4
1 $\mu\text{mol/l}$ N	344 $\pm$ 46	5
10 $\mu\text{mol/l}$ N	425 $\pm$ 90	4
10 nmol/l $\text{NH}_2$	254 $\pm$ 51	6
100 nmol/l $\text{NH}_2$	249 $\pm$ 48	6
1 $\mu\text{mol/l}$ $\text{NH}_2$	464 $\pm$ 62	8
10 $\mu\text{mol/l}$ $\text{NH}_2$	553 $\pm$ 74	6
10 nmol/l $\text{BH}_4$	183 $\pm$ 24	5
100 nmol/l $\text{BH}_4$	249 $\pm$ 35	8
1 $\mu\text{mol/l}$ $\text{BH}_4$	340 $\pm$ 41	9
10 $\mu\text{mol/l}$ $\text{BH}_4$	346 $\pm$ 23	5
0 $\text{Ca}^{2+}$	104 $\pm$ 10	13
0 $\text{Ca}^{2+}$ + 1 $\mu\text{mol/l}$ $\text{NH}_2$	102 $\pm$ 11	13
$\text{La}^{3+}$	144 $\pm$ 15	4
$\text{La}^{3+}$ + 1 $\mu\text{mol/l}$ $\text{NH}_2$	138 $\pm$ 13	4

tive pteridine is  $\text{NH}_2$ , followed by  $\text{BH}_4$  and N. The concentrations required to obtain significant effects are 10 nmol/l for  $\text{NH}_2$ , 100 nmol/l for  $\text{BH}_4$  and 1  $\mu\text{mol/l}$  for N. Such neopterin concentrations are approaches in blood during activation of the immune system [7,15,16], and local concentrations might even be higher. Accordingly, the effects of the pteridines on the intracellular calcium concentration of monocytic cells may also be expected to occur in vivo following activation of the

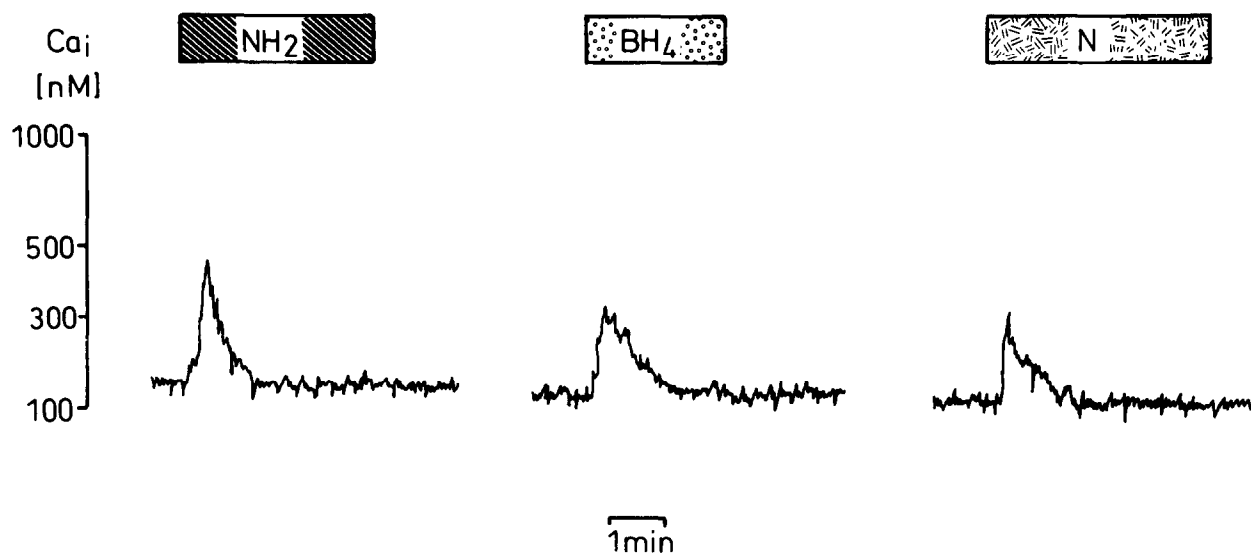


Fig. 1. Effect of pteridine derivatives on intracellular calcium concentration ( $\text{Ca}_i$ ) in human monocytic cells. Original tracings showing the effect of 7,8-dihydroneopterin ( $\text{NH}_2$ ), 5,6,7,8-tetrahydrobiopterin ( $\text{BH}_4$ ), and neopterin (N) (final concentrations 1  $\mu\text{mol/l}$  each).

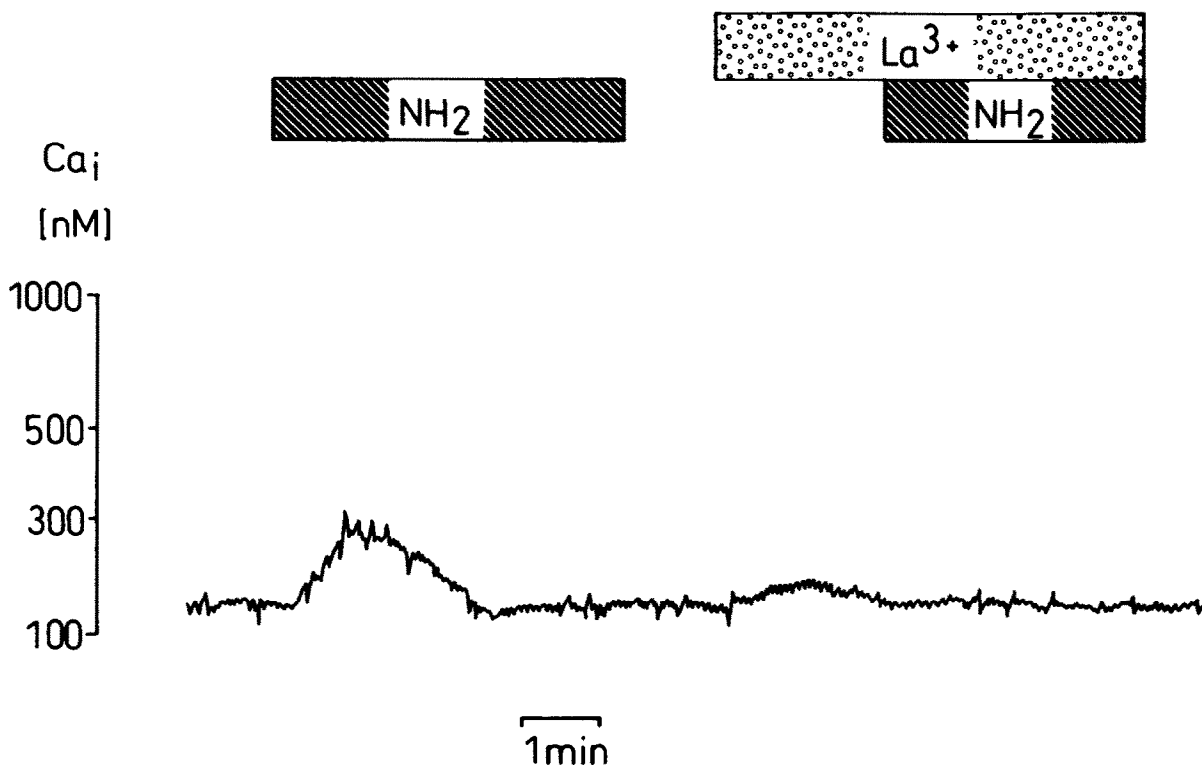


Fig. 2. Effect of lanthanum ions ( $\text{La}^{3+}$ ,  $100 \mu\text{mol/l}$ ) on pteridine induced increase of intracellular calcium concentration ( $\text{Ca}_i$ ). Original tracings, showing the effect of  $1 \mu\text{mol/l}$  7,8-dihydroneopterin ( $\text{NH}_2$ ) on  $\text{Ca}_i$  in both the presence and absence of lanthanum ions.

macrophages by interferon- $\gamma$ . The effect of the pteridines is variable, most probably depending on the cell cycle and/or differentiation stage of the cells. When cells are synchronized by serum deprivation and then stimulated by the addition of FCS, the pteridine effects on  $\text{Ca}_i$  are most pronounced in the  $G_1$  phase when cells are also differentiated badly (not shown). This is in accordance with recent findings demonstrating that calcium effects in THP-1 monocytic cells depend on the differentiation stage of the cell [17]. As shown for  $\text{NH}_2$  (Table I), the pteridine effect on the intracellular calcium concentration requires the presence of extracellular calcium. Deprivation of extracellular calcium by the addition of EGTA ( $1 \text{ mmol/l}$ ) decreases  $\text{Ca}_i$  to some  $104 \text{ nmol/l}$ . In none of the experiments performed  $\text{NH}_2$  ( $1 \mu\text{mol/l}$  final concentration) was able to induce an increase of  $\text{Ca}_i$  under these conditions (Table I). Thus, the presence of a pteridine-inducible calcium channel seems to be evident since the addition of lanthanum ions ( $100 \mu\text{mol/l}$ ), known to block calcium channels [18], abolishes the pteridine-induced increase of  $\text{Ca}_i$  (Table I, Fig. 2). A number of mediators of the immune system have been reported to increase the intracellular calcium concentration, such as interferon- $\gamma$  [14,19], the cytokine-stimulating pteridine biosynthesis in human monocytes/macrophages [6]. An increase of intracellular calcium has in turn been shown to play an important role in the activation of human monocytic cells [9,10,14,19,20]. Accord-

ingly, the ability of pteridine derivatives to increase the intracellular calcium concentration in human monocytic cells indicates that these macrophage-derived substances may be involved in the regulation of calcium-mediated macrophage activation.

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