

# Production of nitric oxide by carp (*Cyprinus carpio* L.) kidney leukocytes is regulated by cyclic 3',5'-adenosine monophosphate

Constanze Pietsch<sup>a,\*</sup>, Reinhard Vogt<sup>a</sup>, Nadja Neumann<sup>a</sup>, Werner Kloas<sup>a,b</sup>

<sup>a</sup> Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, D-12587 Berlin, Germany

<sup>b</sup> Department of Endocrinology, Institute of Biology, Humboldt University Berlin, Invalidenstrasse 48, D-10099 Berlin, Germany

Received 18 October 2007; received in revised form 4 March 2008; accepted 6 March 2008

Available online 12 March 2008

## Abstract

The inducible nitric oxide synthase (iNOS) plays a central role in the inflammatory reactions that follow infection or tissue damage. Induction of nitric oxide (NO) synthesis by bacterial lipopolysaccharide (LPS) depends on activation of G protein-coupled receptors in mammals. Thus, it was our intention to evaluate whether similar mechanisms are involved in iNOS activation in fish leukocytes. Therefore, the participation of membrane-bound receptors which activate effectors via G proteins has been confirmed using the G protein inhibitor suramin. Furthermore, the NO produced by iNOS performs both beneficial and detrimental actions. It is thus conceivable that regulatory mechanisms exist which control the timing and intensity of NO production by iNOS in order to outweigh protective effects against detrimental ones. The second messenger cAMP produced by adenylyl cyclases (ACs) plays a key role in the regulation of many cellular functions. Since cAMP signaling inhibits numerous immunological reactions, studies have been carried out to determine whether cAMP-dependent pathways could inhibit NO production by carp leukocytes as well. To measure cellular responses such as NO production by carp leukocytes derived from head and trunk kidneys treatments were performed with the cAMP elevating agents forskolin and dibutyryl-cAMP (db-cAMP) prior to stimulation with *Aeromonas hydrophila*. Pharmacological studies in stimulated kidney leukocytes showed that increased intracellular cAMP levels lead to reduced NO formation. This reduction of NO production was not due to decreased cell numbers, since a tetrazolium dye-based assay revealed no reduction of cell viability by cyclic nucleotide elevating agents. Thus, our data provide evidence that the AC/cAMP signaling pathway is well established in carp leukocytes. Cyclic AMP leads to type II immune response. We provide evidence that the predominant AC in fish leukocytes is a particulate enzyme due to its sensitivity to forskolin. Treatment of leukocytes with agents increasing intracellular cAMP gave clear evidence for participation of this cyclic nucleotide in immune signaling.

© 2008 Elsevier Inc. All rights reserved.

**Keywords:** Nitric oxide (NO); Inducible NO synthase (iNOS); Adenylyl cyclase (AC); Cyclic 3',5'-adenosine monophosphate (cAMP); Forskolin; Dibutyryl-cAMP (db-cAMP)

## 1. Introduction

Innate immunity is critical in defense for the host against microbial infections, in which macrophages play central roles in the initiation of inflammation by producing proinflammatory cytokines and bactericidal radical effector molecules, such as NO and reactive oxygen species (Miller & Hunt, 1996). The production of NO is mediated by the inducible nitric oxide synthase (iNOS) which

converts L-arginine to L-citrulline. NO is important for immune function, vasculature tone, cell proliferation and apoptosis (Kubes et al., 1991; Beauvais et al., 1995; Papapetropoulos et al., 1996; Saeij et al., 2000; Ferret et al., 2002; Dash et al., 2003).

Cyclic AMP is one of the most important secondary messengers that mediates diverse physiological responses (Park-Sarge and Mayo, 1994). Intracellular cAMP levels are tightly regulated by two enzyme families, adenylyl cyclases (ACs) and phosphodiesterases (PDEs) (Defer et al., 2000; Bender & Beavo, 2006). Primarily, the activity of ACs, which produces cAMP, is regulated by various G-protein-coupled receptors (GPCR) by interaction of two distinct GTP-binding proteins  $G_{\alpha s}$  and  $G_{\alpha i}$ . These G proteins are heterotrimeric molecules which

\* Corresponding author. Department of Inland Fisheries, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, D-12587 Berlin, Germany. Tel.: +49 30 64181 631; fax: +49 30 64181 799.

E-mail address: [pietsch@igb-berlin.de](mailto:pietsch@igb-berlin.de) (C. Pietsch).

modulate enzyme activities and ion channels. Regulation of AC activity is generally mediated by a guanine nucleotide regulatory site that is distinct from the guanine regulatory site which mediates stimulation by hormones (Sadler & Maller, 1985). In fish, ACs are present in different tissues, e.g. gills, melanophores, ovaries, retina and olfactory cells (Guibolini & Lahlou, 1992; Morishita et al., 1993; Benninghoff & Thomas, 2003; Osborne, 1990; Gdovskii & Ruzhinskaya, 2001).

It is known that PDEs degrade cyclic nucleotides and subsequently regulate downstream targets such as protein kinase A (PKA) depending on the tissue (Pahan et al., 1997; Shakur et al., 2002). Cyclic AMP is mainly hydrolysed by the PDE isoforms PDE III and PDE IV on immune cells (Jin et al., 2005). The PDE activity determines the intracellular cAMP levels and activation of subsequent cellular targets such as PKA. PDE activity is important to prevent chronically elevated intracellular cAMP levels that would permanently activate cAMP-dependent signaling pathways (Bender & Beavo, 2006). A reduction of the proinflammatory cytokine TNF  $\alpha$  by cAMP was reported to be a PKA-dependent pathway regulating the balance between proinflammatory and antiinflammatory cascades (Bailly et al., 1990; Pahan et al., 1997; Jin et al., 2005).

Cyclic AMP transduces the action of many hormones, neurotransmitters, and other cellular effectors. The concentration of intracellular cyclic nucleotides influences kinases, PDEs and activates cyclic nucleotide-gated ion channels. Cyclic AMP signaling pathways have been also reported to regulate steroidogenesis in gonads of fish (Nagahama, 1997; Chang et al., 2001; Benninghoff & Thomas, 2003). Moreover, NO regulates AC activity in some tissues depending on the redox potential of the cells (McVey et al., 1999; Hudson et al., 2001; Cochrane et al., 2003).

Ca<sup>2+</sup> and cAMP signaling pathways are strictly interconnected both at the level of the second messengers and at the level of their intracellular targets. Ca<sup>2+</sup> and cAMP are capable of influencing gene expression and protein synthesis. Some ACs and PDEs are known to influence intracellular cAMP levels in a Ca<sup>2+</sup> dependent manner (Landa et al., 2005). Ca<sup>2+</sup> and cAMP show typical oscillations in intact cells and rising cAMP levels are restricted to subcellular compartments by distinct distribution of ACs and PDEs (Zaccolo et al., 2006). Most ACs are particulate enzymes so that changes in the intracellular cAMP levels are suggested to take place in compartments near the membranes, but in mammals also a soluble AC isoform has been described (Buck et al., 1999).

In most mammalian inflammatory cells, cAMP signaling down-regulates immune responses, including proinflammatory cytokines and iNOS expression (Pahan et al., 1997; Jin et al., 2005). In contrast, increased intracellular cAMP levels have been reported to upregulate iNOS stability in cardiac myocytes, and NO production was increased in rat macrophages (Oddis et al., 1995; Alonso et al., 1995). Thus, results from different reports investigating different tissues have not been consistent. It is essential to understand the influence of cAMP on immune function in fish since it has already been reported that environmental compounds can influence intracellular cAMP levels (Whalen & Odman-Ghazi, 2006; Fan et al., 2007). To clarify the precise function of intracellular cAMP in the regulation

of NO production, we utilized carp leukocytes derived from head and trunk kidneys. The aim of this study was to access the capacity of agents influencing the intracellular levels of cAMP to modulate the innate immune response in carp leukocytes.

## 2. Materials and methods

### 2.1. Fish

Carp (approximately 250 g wet mass) from the same offspring were reared in 80 L glass tanks in a recirculation system. Water temperature was adjusted to 25 °C and fish were fed with commercial pellets (DanEx 1344, Danafeed, Denmark) at a daily feeding rate of 1% of body weight.

### 2.2. Chemicals

All chemicals were obtained from Sigma (Germany) unless indicated otherwise. Forskolin and milrinone were solubilized in dimethyl sulfoxide (DMSO; Roth, Karlsruhe, Germany), whereas the G protein inhibitor suramin and the synthetic adenosine analogue dibutyryl-cAMP (db-cAMP) were diluted in sterile distilled water. All agents were added at a 1:1000 dilution to culture media to give the required concentration, 4 h before addition of *Aeromonas hydrophila* as recommended by Mustafa & Olson (1998). Solvent controls were included in the experimental design.

### 2.3. Cell culture

Fish were sacrificed and bled from the caudal vein using heparinized syringes ( $n$ =number of individual fish used for leukocyte preparations). Head and trunk kidneys were passed through 50  $\mu$ m sterile nylon meshes and thereby suspended in cold washing medium (RPMI 1640 containing phenol red, bicarbonate and Hepes supplemented with 10% sterile distilled water, penicillin (100 U mL<sup>-1</sup>), streptomycin (100  $\mu$ g mL<sup>-1</sup>) and 2 mM L-glutamine. The resulting cell suspensions were washed twice, and diluted in culture medium (washing medium without phenol red) to  $1 \times 10^7$  cells mL<sup>-1</sup>. Cells were seeded into 96 well microtitre plates (Nunclon Surface, Nunc, Germany) at a concentration of  $1.5 \times 10^6$  cells per well and were allowed to adhere over night at 5% CO<sub>2</sub> and 25 °C. Thereafter, the supernatant was aspirated using sterile pasteur pipets, and cells were treated with freshly prepared culture medium supplemented with 4% stripped and heat inactivated fetal calf serum according to Heringa et al. (2004) containing cAMP-modulating agents for 4 h. In control incubations only vehicle was applied to the leukocytes.

For stimulation of the immune cells the *A. hydrophila* strain Uni38 (isolated by the University of Leipzig; kind gift from G. Kotterba, Friedrich-Löffler-Institute, Institute of Infectology, Greifswald—Insel Riems, Germany) was used. The ubiquitous bacterium *A. hydrophila*, is known to cause septicemia mainly in cyprinids (Camus et al., 1998). Prior to use bacteria were cultured for 24 h on tryptone soy broth (TSB), harvested, and resuspended in physiological saline. *A. hydrophila* were inactivated by addition of formalin (Roth, Germany) to a final concentration of 1% (v/v) and incubated for 1 h at room temperature. Washed

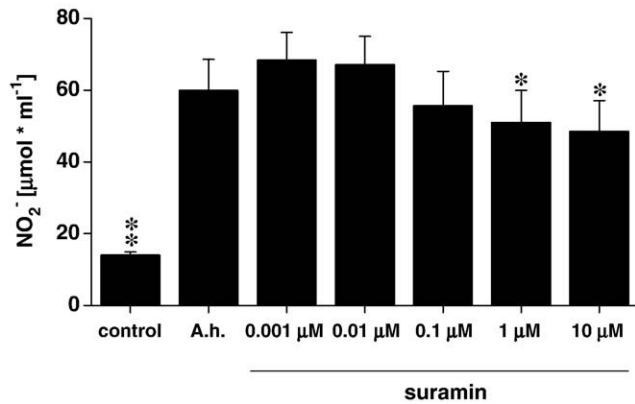


Fig. 1. Nitrite formation in the supernatant of *A. hydrophila*-stimulated head kidney-derived leukocytes after exposure to suramin for 96 h. Values are compared to cells treated with *A. hydrophila* (A.h.). Control incubations remained untreated;  $n=7$ , mean $\pm$ SEM, \* $=p<0.05$ ; \*\* $=p<0.01$ .

bacteria were aliquoted ( $5 \times 10^8$  bacteria mL<sup>-1</sup>) and stored at  $-20^\circ\text{C}$ . Before use bacteria were resuspended in freshly prepared culture medium. The described treatment of leukocytes with agents that modulate intracellular cyclic nucleotide contents was followed by addition of 50  $\mu\text{L}$  bacterial suspension ( $10^7$  cells mL<sup>-1</sup>) to each well whereas unstimulated control incubations received the same amount of medium containing the corresponding solvent only. Subsequently the cell cultures were incubated for further 92 h at 5% CO<sub>2</sub> and 25  $^\circ\text{C}$  in 3 replicates for each mixture.

#### 2.4. Measurement of reactive nitrogen production

The concentration of nitrites, stable products of cellular NO breakdown which accumulate in the cell supernatant, were determined as described by Green et al. (1982) with the following modifications. Nitrite accumulation was determined after cell incubation for 96 h in 50  $\mu\text{L}$  cell-free supernatant by addition of 50  $\mu\text{L}$  Griess reagent (1% sulfanilamide and 0.1% *N*-naphthylethylenediamine dihydrochloride in 2.5% H<sub>3</sub>PO<sub>4</sub>). After incubation for 10 min at room temperature, optical densities were measured using a spectrophotometer (GENios, Tecan, Austria) at 570 nm. Nitrite levels were calculated using a standard curve prepared with sodium nitrite (Roth, Germany).

#### 2.5. Measurement of cell viability (MTT assay)

The assay relies on the ability of living but not dead leukocytes to reduce the water-soluble yellow dye, methylthiazolyldiphenyl-

Table 1  
Cell viability of stimulated head kidney leukocytes after incubation with different suramin concentrations for 96 h as determined by the MTT assay relative to solvent controls in stimulated head kidney cell cultures ( $n=8$ ), mean $\pm$ SEM

Tissue	Suramin	Cell viability [%]
Head kidney	0.001 $\mu\text{M}$	103.1 $\pm$ 1.8
	0.01 $\mu\text{M}$	100.6 $\pm$ 3.1
	0.1 $\mu\text{M}$	104.1 $\pm$ 3.1
	1 $\mu\text{M}$	103.8 $\pm$ 2.0
	10 $\mu\text{M}$	105.1 $\pm$ 2.8

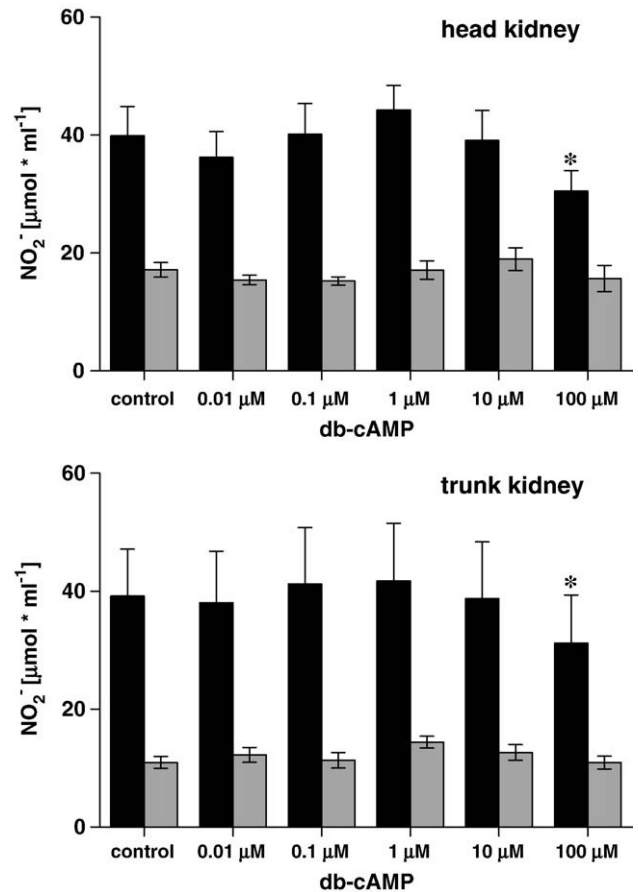


Fig. 2. Nitrite accumulation in the supernatant of head and trunk kidney-derived leukocytes after 4 h incubation with db-cAMP followed by stimulation with *A. hydrophila* (black column) for further 92 h compared to unstimulated cells (grey columns);  $n=7$ , mean $\pm$ SEM, \* $=p<0.05$  compared to the corresponding stimulate or unstimulated controls.

tetrazolium bromide (MTT), to a water-insoluble purple formazan product (Mosmann, 1983). MTT working solution ( $0.5 \text{ mg mL}^{-1}$ ) was prepared in culture medium. To examine the effects of agents on cell viability, they were applied to unstimulated or stimulated cells for 96 h which were incubated for further 60 min with 100  $\mu\text{L}$  MTT solution per well at 25  $^\circ\text{C}$ . Subsequently, the supernatant was discarded and the plates were dried shortly. Formazan was solubilized in isopropyl alcohol containing 0.1% HCl (Roth, Germany), and optical densities were read at 540 nm.

#### 2.6. Statistics

Data are presented as the mean $\pm$ standard error of the mean (SEM) from 6 to 11 cultures corresponding to leukocyte preparations of individual fish. Effects of treatments were determined by comparison of controls with treatment groups using Friedman analysis followed by Wilcoxon test (SPSS 9.0 for Windows). Significance was accepted when  $p<0.05$ .

### 3. Results

Stimulation of head kidney-derived leukocytes with *A. hydrophila* increased NO production 3.5-fold compared to

Table 2

Cell population distribution of freshly isolated leukocytes derived from head kidneys and trunk kidneys;  $n=12$ , mean $\pm$ SEM

Leukocytes	Head kidney [%]	Trunk kidney [%]
Lymphocytes/progenitor cells	40.7 $\pm$ 2.1	41.3 $\pm$ 1.5
Macrophages	18.9 $\pm$ 1.5	15.9 $\pm$ 0.7
Granulocytes	40.4 $\pm$ 1.9	42.9 $\pm$ 1.6

basal NO levels in the supernatant of these cells. To evaluate the participation of G proteins in NO signaling their function was inhibited by treatment of head kidney leukocytes with suramin. Therefore, NO production was measured after 96 h of incubation with different concentrations of this substance (Fig. 1). Control incubation of unstimulated head kidney leukocytes showed significantly lower NO production compared to stimulated kidney leukocytes ( $p<0.01$ ). Suramin in higher concentrations (1  $\mu$ M and 10  $\mu$ M, respectively) significantly inhibited NO production by head kidney leukocytes compared to stimulated controls ( $p<0.05$ ). Furthermore, exposure to suramin had no significant influence on cell viability as assessed by the MTT assay (Table 1).

Stimulation of leukocytes with *A. hydrophila* in further experiments showed a 2-fold and 3.5-fold increase of NO production compared to basal NO levels in the supernatant of

Table 3

MTT assay relative to solvent controls in unstimulated head kidney and trunk kidney cell cultures ( $n=6$ ), mean $\pm$ SEM; n.p.=not performed

Tissue	Concentration	Forskolin [%]	db-cAMP [%]
Head kidney	0.01 $\mu$ M	103.9 $\pm$ 8.9	99.3 $\pm$ 3.8
	0.1 $\mu$ M	104.0 $\pm$ 6.5	103.1 $\pm$ 3.4
	1 $\mu$ M	114.3 $\pm$ 7.3	102.3 $\pm$ 9.9
	10 $\mu$ M	110.3 $\pm$ 5.5	103.5 $\pm$ 6.0
	100 $\mu$ M	n.p.	100.5 $\pm$ 4.4
Trunk kidney	0.01 $\mu$ M	102.9 $\pm$ 0.8	113.7 $\pm$ 6.9
	0.1 $\mu$ M	103.6 $\pm$ 4.4	103.3 $\pm$ 5.0
	1 $\mu$ M	100.9 $\pm$ 4.4	107.7 $\pm$ 2.4
	10 $\mu$ M	87.9 $\pm$ 6.5	88.9 $\pm$ 7.8
	100 $\mu$ M	n.p.	93.2 $\pm$ 6.5

leukocytes derived from head and trunk kidneys, respectively (Fig. 2). Interestingly, trunk kidney leukocytes displayed higher NO levels upon stimulation than leukocytes derived from head kidneys. When isolating head and trunk kidney leukocytes, granulocytes and lymphocytes are co-purified with the macrophages (Table 2). Microscopic examination of slides prepared from freshly isolated head and trunk kidney leukocytes yielded normal cell populations (i.e. macrophages, hematopoietic progenitor cells, small and large lymphocytes and granulocytes). No significant differences were observed between cultures from both organs.

To clarify the regulatory role of cyclic nucleotides in carp we investigated the effect of increased intracellular cAMP on carp kidney leukocytes. The investigation of effects of cAMP elevating agents on NO production in our study yielded similar results for head and trunk kidney leukocyte cultures. The synthetic cAMP analogue db-cAMP at 100  $\mu$ M showed significant inhibitory effects on NO production (Fig. 2) in stimulated head kidney and trunk kidney leukocytes indicating the inhibitory effect of elevated intracellular cAMP levels. Forskolin, a known activator of adenylyl cyclase, reduced the NO release of leukocytes in a dose-dependent manner (Fig. 3). At the highest forskolin concentration used, the NO production by the immune cells was reduced to the basal levels of unstimulated leukocytes.

In addition, unstimulated cells derived from both organs treated with forskolin showed also attenuated basal NO formation which was not observed upon treatment with the synthetic cAMP analogue db-cAMP. However, as investigated

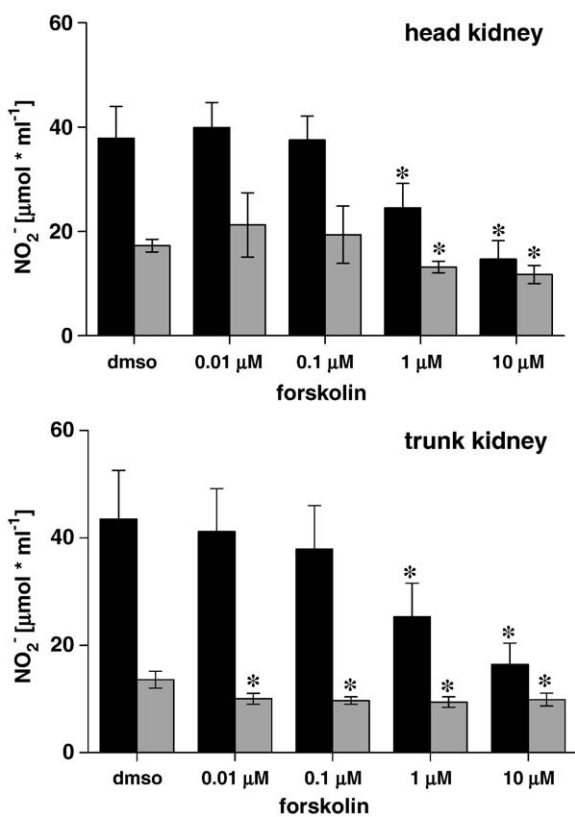


Fig. 3. Nitrite accumulation in the supernatant of head kidney and trunk kidney-derived leukocytes after 4 h incubation with the adenylyl cyclase activator forskolin followed by stimulation for further 92 h with *A. hydrophila* (black columns) compared to unstimulated cells (grey columns); control incubations contained dmsol;  $n=6$ , mean $\pm$ SEM,  $*=p<0.05$  compared to the corresponding stimulate or unstimulated controls.

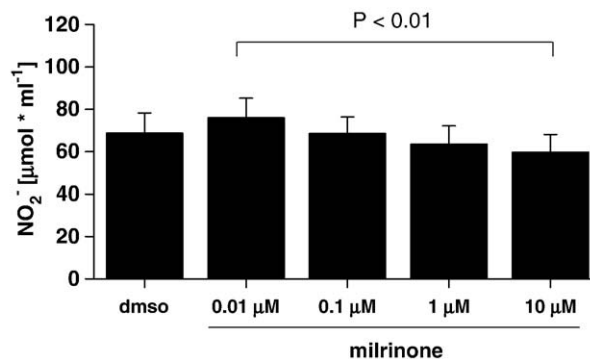


Fig. 4. Nitrite accumulation in the supernatant of trunk kidney-derived leukocytes after 4 h incubation with different concentrations of the PDE III inhibitor milrinone followed by stimulation with  $1 \times 10^7$  *A. hydrophila* mL $^{-1}$  for further 92 h;  $n=11$ ; mean $\pm$ SEM.

Table 4  
Cell viability after incubation with the PDE III inhibitor milrinone as determined by the MTT assay relative to solvent controls in stimulated trunk kidney cell cultures ( $n=7$ ), mean $\pm$ SEM

Tissue	Milrinone	Cell viability [%]
Trunk kidney	0.01 $\mu$ M	93.6 $\pm$ 4.5
	0.1 $\mu$ M	93.0 $\pm$ 4.9
	1 $\mu$ M	95.8 $\pm$ 7.2
	10 $\mu$ M	95.9 $\pm$ 5.6

by the MTT assay, elevation of intracellular cAMP alone did not induce apoptosis-like events as indicated by the small, not-significant changes in formazan production (Table 3). However, a slight decrease of MTT conversion by cells at 10  $\mu$ M forskolin in trunk kidney cells was observed.

The intracellular levels of cyclic nucleotides such as cAMP are regulated by degrading enzymes known as phosphodiesterases. Especially the cGMP-inhibited, cAMP-dependent PDE III seems to be important because it enables a crosstalk between cGMP and cAMP. Studies on cAMP-dependent NO regulation using the PDE III inhibitor milrinone demonstrated that the NO level of stimulated carp leukocytes derived from trunk kidneys exhibited a decrease of the level of NO production after 96 h incubation with increasing concentrations of milrinone although this difference was not significant ( $p=0.062$  at 10  $\mu$ M) compared to control incubations containing vehicle only (Fig. 4). Nevertheless a significant difference between 0.01  $\mu$ M and 10  $\mu$ M milrinone was observed ( $p=0.004$ ). The determination of nitrite levels after leukocyte incubation was followed by evaluation of the number of viable cells to reveal possible cytotoxic effects of the substance milrinone. Congruently, the observed changes in NO production due to incubation of trunk kidney leukocytes with milrinone were not accompanied by differences in cell viability after 96 h of incubation (Table 4).

#### 4. Discussion

In accordance with other studies (Miller & Hunt, 1996), stimulated carp leukocytes produce more NO compared to untreated controls. Furthermore, it has been shown that induction of NO synthesis by LPS depends on activation of G protein-coupled receptors in mammals (Kuo et al., 1997; Hudson et al., 2001). However, the mechanisms underlying endotoxin recognition in fish have not been clarified (Iliev et al., 2005) and it is not known whether different mechanisms underlie receptor specificity in different organs. Because the signal transduction mechanisms are not necessarily the same, G proteins may be more specific to each receptor and to each signaling system than currently assumed. In other words, the question of how information specifically passes from a membrane receptor to the effector *via* G proteins has not yet been fully answered. Despite the roles of G proteins in many different signaling mechanisms, NO signaling is thought to be mediated by direct activation of G proteins. Accordingly, hydrolyzation of GTP at membranes of mammalian macrophages was shown to be increased by LPS (Schroeder et al., 1997), but the LPS-induced activation of macrophages is thought to depend on a variety of cell surface receptors. Similarly, the impairment by

suramin was found to be responsible for reduced bactericidal activity of mammalian leukocytes after *in vitro* culture with this substance (Roilides et al., 1993). The participation of heterotrimeric G proteins in NO signaling in carp leukocytes was confirmed using suramin which showed no effects on cell viability. However, few studies on mammals have examined the site on G proteins where suramin might act. One suggestion is that suramin may interact with the binding on  $G_{\alpha}$  for its downstream effectors, such as ACs. However, the interaction with an effector binding domain has not been addressed explicitly (Freissmuth et al., 1996). Furthermore, it was suggested that suramin uncouples the G protein from its associated receptor. Thus, it favours the retention in an inactive binary complex with the agonist alone. Although it reduces the rate of GDP release from the G protein, this may be an indirect effect (Freissmuth et al., 1996). From other studies, it was concluded that suramin inhibits the binding of guanosine nucleotides and does not release GTP that was already bound (Chung & Kermodé, 2005). It was concluded that although it does not obstruct the receptor docking site directly, suramin might block the access to the binding pocket by any guanosine nucleotide. Therefore, it is not to be considered as a competitive inhibitor.

Since, it was also suggested that suramin suppresses the association of  $G_{\alpha}$  and  $G_{\beta\gamma}$  subunits by blocking the interface between these subunits further downstream effectors of this substance can be considered. Emphasizing the importance of  $\beta\gamma$  subunits in receptor signaling, it has been shown that suramin is much less effective at preventing the cross-linking between the receptor and  $G_{\alpha}$  than between  $G_{\alpha}$  and  $\beta\gamma$  subunits (Chung & Kermodé, 2005). One of the primary functions of the  $\beta\gamma$  subunits is the ability to associate with the  $G_{\alpha}$  subunit by which it anchors the latter to the membrane (Sternweis, 1986; Kurstjens et al., 1991). Consequently, the  $\beta\gamma$  subunit is required for receptor-G protein interaction (Sprang, 1997). For instance, estrogen signaling via G protein-dependent pathways and direct binding of estrogen receptor  $\alpha$  (ER  $\alpha$ ) to  $G_{\alpha i}$  and  $G_{\beta\gamma}$  has been shown in mammals (Kumar et al., 2007). Moreover, testosterone and progesterone attenuate constitutive  $G_{\beta\gamma}$  activity and thus, play an important role in oocyte maturation of *Xenopus laevis* oocytes (Evaul et al., 2007). Consequently, actions of progesterone were reported to be rather due to modulated  $G_{\beta\gamma}$  levels than effects by  $G_{\alpha}$  subunits (Lutz et al., 2000). Therefore, an influence of  $\beta\gamma$  subunits on G protein-coupled receptors regulating NO production by carp leukocytes should also be considered. Nevertheless, the present experiments provide support for the concept that G proteins also participate in the NO signaling in carp leukocytes.

Previous reports have described the effects of cAMP on NO synthesis in mammals, but these reports have provided controversial results depending on the tissue and species under investigation. In carp kidney leukocytes, db-cAMP, an analogue of natural cAMP, significantly decreased NO production at a concentration of 100  $\mu$ M. Joerink et al. (2006) used 1 mM db-cAMP to inhibit NO production by carp leukocytes and cAMP was reported to induce type II immune response in fish which is known to display antiinflammatory properties. The mammalian iNOS promotor contains responsive elements for nuclear factor kappa B (NF- $\kappa$ B) and the binding of the cAMP responsive element-binding protein (Coughlan et al., 2005). Thus, in Jurkat

cells db-cAMP inhibited IFN  $\gamma$  and induced IL10 which is characteristic for antiinflammatory processes during immune responses (Benbernou et al., 1997; Mantovani et al., 2003). Increased levels of intracellular cAMP were also reported to inhibit NF- $\kappa$ B and reduce the proinflammatory cytokine IL-1 $\beta$  in human macrophages (Kamthong & Wu, 2001).

The increase of intracellular cAMP levels activates arginases which also utilize L-arginine as a substrate (Joerink et al., 2006). Thus, iNOS lacks arginine and the proinflammatory NO production can be impaired. In our experiments, we used 10 times lower concentrations of db-cAMP to reduce proinflammatory NO production of kidney leukocytes.

Consistent to many mammalian tissues, treatment with the diterpene forskolin from *Coleus forskohlii* known as a direct activator of adenylate cyclase presumably resulted in an increase of intracellular cAMP levels, and acted as a very potent inhibitor of iNOS protein and NO production in resident macrophages, especially when applied before stimulation (Mustafa & Olson, 1998; Beshay & Prud'homme, 2001; Jin et al., 2005). The transcription factor NF- $\kappa$ B is also inhibited by forskolin (Aizawa et al., 2003). However, also increased NO production by mammalian macrophages has been reported after treatment with cAMP (Alonso et al., 1995). In our study, 1  $\mu$ M forskolin significantly reduced NO production by kidney leukocytes. Forskolin decreased the *A. hydrophila* induced NO formation by carp kidney leukocytes to basal levels in higher concentrations, whereas the membrane-permeable cAMP analogue db-cAMP reduced the nitrite production by leukocytes only at a concentration of 100  $\mu$ M. The lower effects of the synthetic analogue db-cAMP may be due to its lower cell permeability. The degradation by PDEs may also be less efficient for the synthetic cAMP analogue compared to the increase of intracellular cAMP levels by activation of adenylyl cyclases by forskolin. It was proposed that downstream activation of PDEs plays also a role in immune cascades (Pahan et al., 1997; Jin et al., 2005).

In our studies, a concentration-dependent reduction of NO formation was obvious from the treatment of carp leukocytes with forskolin prior to stimulation. Several ACs have been described in mammals, and the forskolin-stimulated forms are inhibited by NO and NO regulates AC activity in rats. Forskolin-sensitivity is typical for membrane-bound ACs. Therefore it can be concluded that the AC in carp leukocytes is most probably not a soluble, forskolin-insensitive enzyme as found in rat testis (Buck et al., 1999). Forskolin-sensitive AC activity was also involved in the signaling pathways of olfactory cells and retina tissue in teleosts (Gdovskii & Ruzhinskaya, 2001; Osborne, 1990).

Our data clearly indicate that the intracellular cAMP level is an important regulator of NO production upon stimulation with *A. hydrophila*. The regulation of macrophage activity in response to inflammatory stimuli must be finely tuned to promote an effective immune response. Basal AC activity remained unchanged by cAMP in mammalian tissues (Oddis et al., 1995). However, the cAMP elevating agent also changed the basal rate of NO production in the kidney leukocytes in the absence of *A. hydrophila*.

Forskolin was reported to interfere with translocation of NF- $\kappa$ B to the nucleus and consequently prevents gene transactivation. When this strategy affecting overall cAMP levels was used

it is most likely that several redundant pathways were activated, rendering it difficult to evaluate hierarchies and intersections among the different regulatory loops. The recorded decrease of NO release by kidney leukocytes treated with the cAMP elevating agents was not due to reduced cell viability of leukocytes as determined by the MTT assay. However, a slight decrease in numbers of viable cells in head and trunk kidney leukocyte cultures treated with forskolin was observed. This might be due to the fact that cAMP affects the cell adhesion in high concentrations (Bryant & Sutcliffe, 1974). In our leukocyte cultures, cells might have been lost during exchange of media on the microtitre plates, which made it difficult to determine the exact numbers of viable cells. However, we found that none of the used agents significantly influenced cell viability.

Cyclic AMP is a potent activator of protein kinase A (PKA). The cAMP-dependent PKA has profound protective actions down-regulating excessive proinflammatory responses, such as high NO production (Bryant & Sutcliffe, 1974). Apparently, PKA regulates the balance between immune response types I and II. Despite the abundant reports documenting cAMP/PKA signaling in inhibition of NO, the specific mechanisms by which this occurs remain unclear. Nevertheless, growing evidence exists that cAMP activates both PKA-dependent and -independent actions (Seino & Shibasaki, 2005). Accordingly, elevation of intracellular levels of cAMP has been shown to reduce the abundance of the soluble guanylyl cyclase providing a crosstalk with cGMP-dependent signaling pathways (Shimouchi et al., 1993). Induction of PDE activity by cAMP is a positive feedback mechanism necessary to overcome the negative modulation of cAMP and to finely control proinflammatory cytokines, such as TNF  $\alpha$ , in time and in response to different extracellular environments. This positive feedback may be operating to oppose the inhibitory effects of prostaglandins and deactivating cytokines and chemokines released at the site of inflammation. Especially, the cGMP-inhibited PDE III seems to be involved in these interactions between cAMP and cGMP (Shakur et al., 2002; Aizawa et al., 2003). In mammals, milrinone has been reported to increase plasma cAMP levels by inhibition of PDE III (Hayashida et al., 1999) and elevation of cAMP levels in mouse skin by application of forskolin or milrinone has been shown to interfere with the LPS-induced increase of the proinflammatory cytokine TNF  $\alpha$  (Irie et al., 2001). This further indicates a connection of cAMP and NO signaling pathways. Congruently, the *A. hydrophila*-stimulated NO synthesis by isolated carp trunk kidney leukocytes was also found to be decreased by 10  $\mu$ M milrinone compared to the lowest milrinone concentration (0.01  $\mu$ M), which was not due to influence of this inhibitor on cell viability. Therefore, it may be considered that PDEs with sensitivity to milrinone are also present in carp leukocytes. Furthermore, this indicates that the regulatory mechanism via PDE III is also present in carp leukocytes and the subsequent increase of intracellular cAMP levels resulted in the suppression of NO production by these cells treated with milrinone at high concentration. Thus, complex regulatory mechanisms prevent chronic activation of inflammatory reactions because the cGMP induced by NO synthesis exerts its inhibitory effects on PDE III, so that cAMP accumulates intracellularly presumably resulting in direct iNOS inhibition in carp leukocytes.

In conclusion, the bacteria-stimulated NO synthesis is dependent on heterotrimeric G protein signal transduction in carp leukocytes. However, the contribution of individual G protein subclasses needs further investigations in these cells. The elevation of cAMP by db-cAMP or by activation of cAMP production by forskolin resulted in a marked decrease of induced NO production by carp leukocytes derived from head and trunk kidneys. The duration of cAMP inhibits both *A. hydrophila*-stimulated and basal NO production suggesting the importance of cAMP-dependent regulation in leukocytes for a fine tuning of immune response type I and type II. Furthermore, the inhibition of PDE activity by milrinone suggests a central role of PDE III in immune regulation in carp leukocytes.

## Acknowledgment

The authors like to thank Eric Eckmann for maintaining the fish.

## References

- Alonso, A., Carvalho, J., Alonso-Torre, S.R., Nunez, L., Bosca, L., Sanchez Crespo, M., 1995. NO synthesis in rat macrophages is induced by IgE/DNP complexes and cAMP analogues. Evidence in favor of a common signaling mechanism. *J. Immunol.* 154, 6475–6483.
- Aizawa, T., We, H., Miano, J.M., Abe, J., Berk, B.C., Yan, C., 2003. Role of phosphodiesterase 3 in NO/cGMP-mediated antiinflammatory effects in vascular smooth muscle cells. *Circ. Res.* 93, 406–413.
- Bailly, S., Ferrua, B., Fay, M., Gougerot-Pocidalo, M.A., 1990. Differential regulation of IL 6, IL 1 A, IL 1 $\beta$  and TNF $\alpha$  production in LPS-stimulated human monocytes: role of cyclic AMP. *Cytokine* 2, 205–210.
- Beauvais, F., Michel, L., Dubertret, L., 1995. Exogenous nitric oxide elicits chemotaxis of neutrophils in vitro. *J. Cell. Physiol.* 165, 610–614.
- Benbernou, N., Esnault, S., Shin, H.C., Fekkar, H., Guenounou, M., 1997. Differential regulation of IFN $\gamma$ , IL-10 and inducing nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway. *Immunology* 91, 361–368.
- Bender, A.T., Beavo, J.A., 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol. Rev.* 58, 488–520.
- Benninghoff, A.D., Thomas, P., 2003. Interactions of calcium and cyclic AMP signaling pathways regulating steroidogenesis in primary cultured theca and granulosa cells of Atlantic croaker. *Fish Physiol. Biochem.* 28, 327–328.
- Beshay, E., Prud'homme, G.J., 2001. Inhibitors of phosphodiesterase isoforms III or IV suppress islet-cell nitric oxide production. *Lab. Invest.* 81, 1109–1117.
- Bryant, R.E., Sutcliffe, M.C., 1974. The effect of 3',5'-adenosine monophosphate on granulocyte adhesion. *J. Clin. Invest.* 54, 1241–1244.
- Buck, J., Sinclair, M.L., Schapal, L., Cann, M.J., Levin, L.R., 1999. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc. Natl. Acad. Sci. U. S. A.* 96, 79–84.
- Camus, A.C., Durborow, R.M., Hemstreet, W.G., Thune, R.L., Hawke, J.P., 1998. *Aeromonas* Bacterial Infections—Motile *Aeromonas* Septicemia, vol. 478. Southern Regional Aquacult Center Publication, pp. 1–4.
- Chang, J.P., Wirachowsky, N.R., Kwong, P., Johnson, J.D., 2001. PACAP stimulation of gonadotropin-II secretion in goldfish pituitary cells: mechanisms of action and interaction with gonadotropin releasing hormone signalling. *J. Neuroendocrinol.* 13, 540–550.
- Chung, W.-C., Kermode, J.C., 2005. Suramin disrupts receptor-G protein coupling by blocking association of G protein  $\alpha$  and  $\beta\gamma$  subunits. *J. Pharmacol. Exp. Ther.* 313, 191–198.
- Cochrane, R., Clark, R.B., Maulik, N., Cordis, G., Cone, R.E., 2003. cAMP-mediated suppression of a Th1 clone associated with an alteration of the intracellular redox environment. *Cell. Mol. Biol.* 49, 301–306.
- Coughlan, T., Gibson, C., Murphy, S., 2005. Modulatory effects of progesterone on inducible nitric oxide synthase expression in vivo and in vitro. *J. Neurochem.* 93, 932–942.
- Dash, P.R., Cartwright, J.E., Baker, P.N., Johnstone, A.P., Whitley, G.S., 2003. Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation. *Exp. Cell Res.* 287, 314–324.
- Defer, N., Best-Belpomme, M., Hanoune, J., 2000. Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am. J. Physiol. Renal Physiol.* 279, F400–F416.
- Evaul, K., Jamnongjit, M., Bhagavath, B., Hammes, S.R., 2007. Testosterone and progesterone rapidly attenuate plasma membrane G $\beta\gamma$ -mediated signaling in *Xenopus laevis* oocytes by signaling through classical receptors. *Mol. Endocrinol.* 21, 186–196.
- Fan, W.Q., Yanase, T., Morinaga, H., Gondo, S., Okabe, T., Nomura, M., Komatsu, T., Morohashi, K.-I., Hayes, T.B., Takayanagi, R., Nawata, H., 2007. Atrazine-induced aromatase expression is SF 1-dependent: implications for endocrine disruption in wildlife and reproductive cancers in humans. *Environ. Health Perspect.* 115, 720–727.
- Ferret, P.-J., Soum, E., Negre, O., Fradelizi, D., 2002. Auto-protective redox buffering systems in stimulated macrophages. *BMC Immunol.* 3, 3–15.
- Freissmuth, M., Boehm, S., Beindl, W., Nickel, P., Ijzerman, A.P., Hohenegger, M., Nanoff, C., 1996. Suramin analogues as subtype-selective G protein inhibitors. *Mol. Pharmacol.* 49, 602–611.
- Gdovskii, P.A., Ruzhinskaya, N.N., 2001. Cyclic-nucleotide-dependent pathway is one of mechanisms of olfactory transduction of amino acid signal in the carp *Cyprinus carpio*. *J. Evol. Biochem. Physiol.* 37, 148–153.
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, J.S., 1982. Analysis of nitrate, nitrite and 15 nitrate in biological fluids. *Anal. Biochem.* 126, 131–138.
- Guibbolini, M.E., Lahlou, B., 1992. Gi protein mediates adenylyl-cyclase inhibition by neurophyseal hormones in fish gill. *Peptides* 13, 865–871.
- Hayashida, N., Tomoeda, H., Oda, T., Tayama, E., Chihara, S., Kawara, T., Aoyagi, S., 1999. Inhibitory effect of milrinone on cytokine production after cardiopulmonary bypass. *Ann. Thorac. Surg.* 68, 1661–1667.
- Heringa, M.B., van der Burg, B., van Eijkeren, J.C.H., Hermens, J.L.M., 2004. Xenoestrogenicity in in vitro assays is not caused by displacement of endogenous estradiol from serum proteins. *Toxicol. Sci.* 82, 154–163.
- Hudson, T.Y., Corbett, J.A., Howlett, A.C., Klein, C., 2001. Nitric oxide regulates adenylyl cyclase activity in rat striatal membranes. *J. Neurochem.* 77, 1279–1284.
- Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V., Goetz, F.W., 2005. Endotoxin recognition: in fish or not in fish? *FEBS Letters* 579, 6519–6528.
- Irie, K., Fujii, E., Ishida, H., Wada, K., Suganuma, T., Nishikori, T., Yoshioka, T., Muraki, T., 2001. Inhibitory effects of cyclic AMP elevating agents on lipopolysaccharide (LPS)-induced microvascular permeability change in mouse skin. *Br. J. Pharmacol.* 133, 237–242.
- Jin, S.-L.C., Lan, L., Zoudilova, M., Conti, M., 2005. Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J. Immunol.* 175, 1523–1531.
- Joerink, M., Savelkoul, H.F.J., Wiegertjes, G.F., 2006. Evolutionary conservation of alternative activation of macrophages: structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.). *Mol. Immunol.* 43, 1116–1128.
- Kamthong, P.J., Wu, M.-C., 2001. Inhibitor of nuclear factor- $\kappa$ B induction by cAMP antagonizes interleukin-1-induced human macrophage-colony-stimulating-factor expression. *Biochem. J.* 356, 525–530.
- Kubes, P., Suzuki, M., Granger, D.N., 1991. Nitric-oxide—an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4651–4655.
- Kumar, P., Wu, Q., Chambliss, K.L., Yuhanna, I.S., Mumby, S.M., Mineo, C., Tall, G.G., Shaul, P.W., 2007. Direct interaction with G $\alpha$ I and G $\beta\gamma$  mediate nongenomic signaling by ER $\alpha$ . *Mol. Endocrinol.* 21, 1370–1380.
- Kuo, P.C., Schroeder, R.A., Bartlett, S.T., 1997. Endotoxin-mediated synthesis of nitric oxide is dependent on Gq protein signal transduction. *Surgery* 122, 394–403.
- Kurstjens, N.P., Fröhlich, M., Dees, C., Cantrill, R.C., Hekman, M., Helmreich, E.J.M., 1991. Binding of  $\alpha$ - and  $\beta\gamma$ -subunits of Go to  $\beta$ 1-adrenoceptor in sealed unilamellar lipid vesicles. *Eur. J. Biochem.* 197, 167–176.
- Landa, L.R., Harbeck Jr., M., Kaihara, K., Chepurny, O., Kitiphongspattana, K., Graf, O., Nikolaev, V.O., Lohse, M.J., Holz, G.G., Roe, M.W., 2005. Interplay of Ca<sup>2+</sup> and cAMP signaling in the insulin-secreting MIN6  $\beta$ -cell line. *J. Biol. Chem.* 280, 31294–31302.

- Lutz, L.B., Kim, B., Jahani, D., Hammes, S.R., 2000. G protein  $\beta\gamma$  subunits inhibit nongenomic progesterone-induced signaling and maturation in *Xenopus laevis* oocytes: evidence for a release of inhibition mechanism for cell cycle progression. *J. Biol. Chem.* 275, 41512–41520.
- Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2003. The chemokines system in diverse forms of macrophage activation and polarization. *Trend Immunol.* 25, 677–686.
- McVey, M., Hill, J., Howlett, A., Klein, C., 1999. Adenylyl cyclase, a coincidence detector for nitric oxide. *J. Biol. Chem.* 274, 18887–18892.
- Miller, L., Hunt, J., 1996. Sex steroid hormones and macrophage function. *Life Sci.* 59, 1–14.
- Morishita, F., Shimada, A., Fujimoto, M., Katayama, H., Yamada, K., 1993. Inhibition of adenylyl-cyclase activity in the goldfish melanophore is mediated by  $\alpha$ -2-adrenoreceptors and a pertussis-toxin-sensitive GTP-binding protein. *J. Comp. Biochem., B. Biochem. Syst. Environ. Physiol.* 163, 533–540.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.
- Mustafa, S.B., Olson, M.S., 1998. Expression on nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J. Biol. Chem.* 273, 5073–5080.
- Nagahama, Y., 1997.  $17\alpha,20\beta$ -Dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: Mechanisms of synthesis and action. *Steroids* 62, 190–196.
- Oddis, C.V., Simmons, R.L., Hattler, B.G., Finkel, M.S., 1995. cAMP enhances inducible nitric oxide synthase mRNA stability in cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 269, H2044–H2050.
- Osborne, N.N., 1990. Effects of GTP, forskolin, sodium fluoride, serotonin, dopamine, and carbachol on adenylyl cyclase in teleost retina. *Neurochem. Res.* 15, 523–528.
- Pahan, K., Nambodiri, A.M.S., Sheikh, F.G., Smith, B.T., Singh, I., 1997. Increasing cAMP attenuates induction of inducible nitric-oxide synthase in rat primary astrocytes. *J. Biol. Chem.* 272, 7786–7791.
- Papapetropoulos, A., AbouMohamed, G., Marczin, N., Murad, F., Caldwell, R.W., Catravas, J.D., 1996. Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. *Br. J. Pharmacol.* 118, 1359–1366.
- Park-Sarge, O.K., Mayo, K.E., 1994. Regulation of the progesterone-receptor gene by gonadotropins and cyclic adenosine-3',5'-monophosphate in rat granulosa-cells. *Endocrinology* 134, 709–718.
- Roilides, E., Paschalides, P., Freifeld, A., Pizzo, P.A., 1993. Suppression of polymorphonuclear leukocyte bactericidal activity by suramin. *Antimicrob. Agents Chemother.* 37, 495–500.
- Sadler, S.E., Maller, J.L., 1985. Inhibition of *Xenopus* oocyte adenylyl cyclase by progesterone: a novel mechanism of action. *Adv. Cyclic Nucleot. Prot. Phosphoryl. Res.* 19, 179–194.
- Saeij, J.P.J., Stet, R.J.M., Groeneveld, A., Verburg-van Kemenade, L.B.M., van Muiswinkel, W.B., Wiegertjes, G.F., 2000. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. *Immunogenetics* 51, 339–346.
- Schroeder, R.A., DelaTorre, A., Kuo, P.C., 1997. CD14-dependent mechanism for endotoxin-mediated nitric oxide synthesis in murine macrophages. *Am. J. Physiol.* 273 (42), C1030–C1039.
- Seino, S., Shibasaki, T., 2005. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol. Rev.* 85, 1303–1342.
- Shakur, Y., Fong, M., Hensley, J., Cone, J., Movsesian, M.A., Kambayashi, J., Yoshitake, M., Liu, Y., 2002. Comparison of the effects of cilostazol and milrinone on cAMP-PDE-activity, intracellular cAMP and calcium in the heart. *Cardiovasc. Drugs Ther.* 16, 417–427.
- Shimouchi, A., Janssens, S.P., Bloch, D.B., Zapol, W.M., Bloch, K.D., 1993. cAMP regulates soluble guanylate-cyclase beta-1-subunit gene expression in Rfl-6 rat fetal lung fibroblasts. *Am. J. Physiol.* 265, L456–L461.
- Sprang, S.R., 1997. G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.* 66, 639–678.
- Sternweis, P.C., 1986. The purified a subunits of G<sub>0</sub> and G<sub>i</sub> from bovine brain require  $\beta\gamma$  for association with phospholipid vesicle. *J. Biol. Chem.* 261, 631–637.
- Whalen, M.M., Odman-Ghazi, S.O., 2006. Effects of adenylyl cyclase and protein kinase A inhibition on signaling enzymes in natural killer cells: comparison to tributyltin. *Human Exp.Toxicol.* 25, 333–340.
- Zaccolo, M., Di Benedetto, G., Lissandron, V., Mancuso, L., Terrin, A., Zamparo, I., 2006. Restricted diffusion of a freely diffusible second messenger: mechanisms underlying compartmentalized cAMP signalling. *Biochem. Soc. Trans.* 34, 495–497.