Interleukin-1β stimulates cyclic GMP efflux in brain astrocytes

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Abstract In rat brain astroglia-enriched cultures long-term treatment with interleukin-1β induces NO release and stimulation of soluble guanylyl cyclase. The cGMP formed is recovered in the extracellular medium but not in the cell monolayer. The interleukin-1β effect is mediated by type I receptor and potentiated by interferon-γ. In cells treated with bacterial endotoxin a larger NO-dependent cGMP accumulation occurs only intracellularly, however a significant cGMP egression is observed when cells are co-treated with interleukin-1β. The non-selective anion transport inhibitors probenecid and verapamil block cGMP efflux, indicating that interleukin-1β stimulates a cGMP transporter.

Key words: cGMP efflux; Nitric oxide; Interleukin-1β; Astroglia

1. Introduction

The presence of cyclic nucleotides in extracellular fluids including plasma, urine and cerebrospinal fluid has been known for a long time. cGMP, first discovered in urine [1], is released to the extracellular milieu in several tissues including liver [2], brain [3], pancreas [4], smooth muscle [5], platelets [6] and erythrocytes [7]. In these later cells an ATP-dependent transporter appears to be involved in cGMP extrusion [7,8]. In most studies, efflux of cGMP was related to the intracellular concentration of the nucleotide and interpreted as a mechanism that in conjunction with the action of phosphodiesterases would return intracellular cGMP to basal levels. However, recent studies in neural cells suggest that extracellular cGMP may exert physiological actions by regulating the activity of membrane proteins through interaction with extracellular sites. For instance, in cerebellar granule neurons extracellular cGMP has been shown to inhibit cellular responses mediated by kainate receptors [8] and to protect against glutamate-induced toxicity [9]. In cortical astrocytes, cGMP released after stimulation of particulate guanylyl cyclase by natriuretic peptides inhibits a Na^+/H^+ exchanger leading to a decrease in intracellular pH, an effect that could modulate important astroglial functions such as K^+ conductance or cell proliferation [10]. In this work, we show for the first time that the cGMP formed in rat brain astrocyte cultures after induction of NO release and stimulation of soluble guanylyl cyclase (sGC) by the pro-inflammatory cytokine interleukin-1β (IL-1β) accumulates in the extracellular but not in the intracellular medium. IL-1β also stimulates the release of cGMP formed after induction of NO synthase (NOS) by the bacterial endotoxin, lipopolysaccharide (LPS) and the effect appears to involve stimulation of a transporter.

2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories and fetal calf serum (FCS) from Biological Industries; [^{3}H]cGMP (34.5 Ci/mmol) was from New England Nuclear; IL-1β, interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) from PeproTechec; LPS (*Salmonella typhimurium*), N^G-monomethyl-L-arginine (L-NMMA), 1H-[1,2,4]oxodiazolo-[4,3-a]quinoxalin-1-one (ODQ), probenecid, verapamil, IL-1 receptor antagonist protein (IL-1Ra) from Sigma; anti-cGMP antiserum was prepared in our laboratory as described by Brooker et al. [11].

Astrocyte-enriched primary cultures were prepared from newborn (cortex and hippocampus) and 7-day-old (cerebellum) Sprague–Dawley rats as described ([12]). Dissociated cells suspended in DMEM–10% FCS, were seeded in 35 mm diameter plastic Petri dishes (1.25×10^5 cells/cm^2). Medium was changed once a week and cells were used after 14 days in culture. Cells were incubated for the indicated times with IL-1β or other agents in serum-free media. Cell viability of cytokine-treated astrocytes was determined by measuring release of lactate dehydrogenase (LDH) to the medium [13].

cGMP levels were measured in cell monolayers and in the incubation media after the indicated treatments. Media was aspirated and cell monolayers were washed with HEPES-buffered saline (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose and 20 mM HEPES, pH 7.4) at 37°C and extracted with ice-cold ethanol. Media and ethanolic cell extracts were evaporated and residues dissolved in 5 mM sodium acetate pH 4.8. cGMP was quantified by RIA using acetylated [^{3}H]cGMP as described [14]. Nitrites accumulated in cell incubation media were determined by the Greiss reaction [15]. Protein concentration was determined by the method of Lowry et al. using BSA as standard.

Experiments were always performed in triplicates. Results are expressed as mean ± S.E.M. of the indicated number of experiments performed in different culture preparations.

3. Results and discussion

As shown in Fig. 1, treatment of rat cerebellar astrocyte-enriched cultures with the pro-inflammatory cytokine IL-1β induced a time- (Fig. 1A) and concentration-dependent (Fig. 1C) accumulation of cGMP in the extracellular medium but

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IL-1β, interleukin-1β; IL-1Ra, IL-1 receptor antagonist; INF-γ, interferon-γ; LDH, lactate dehydrogenase; L-NMMA N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; NOS, NO synthase; sGC, soluble guanylyl cyclase; TNF-α, tumor necrosis factor-α; ODQ, 1H-[1,2,4]oxodiazolo-[4,3-a]quinoxalin-1-one

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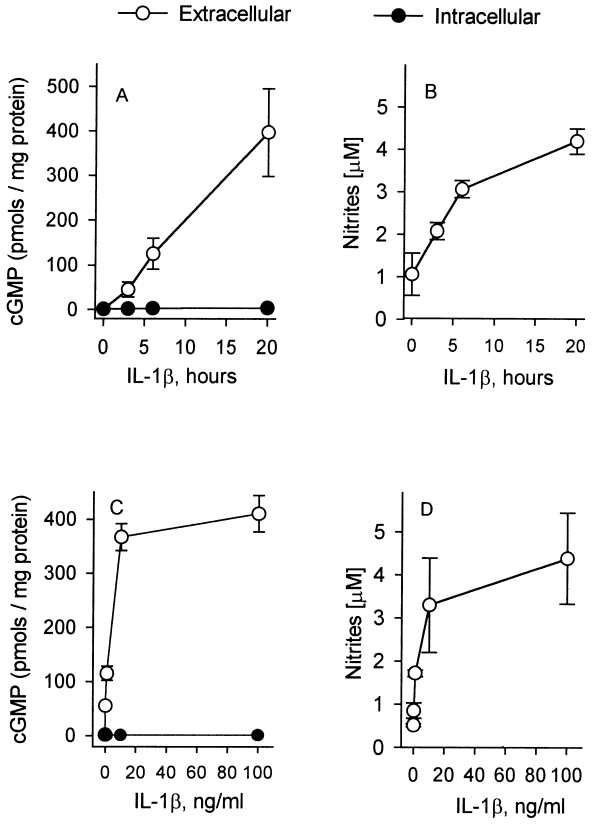

Fig. 1. Effects of IL-1β on NO and cGMP formation in cerebellar astroglia-enriched cultures. Cells were treated with 10 ng/ml IL-1β for the indicated times (A, B) or with increasing IL-1β concentrations for 20 h (C, D). Extracellular cGMP (○) and nitrites were determined in the incubation media. Intracellular cGMP (●) was determined in cells after washing the monolayers. Data are means ± S.E.M. of three (A, B) and two (C, D) experiments performed in different culture preparations.

not in the cell monolayer. Extracellular cGMP in cells treated with a maximally effective concentration of IL-1β (10 ng/ml) was higher than in control cells after 3 h and increased linearly for at least 20 h, while intracellular cGMP remained at control levels. IL-1β also produced an increase in nitrites in the media with a similar time and concentration dependency (Fig. 1B,D), suggesting that accumulated cGMP results from IL-1β induction of NOS and NO stimulation of sGC. In fact, extracellular cGMP accumulation was totally blocked by the NOS inhibitor L-NMMA (1 mM) that also inhibited nitrite accumulation, and by the sGC specific inhibitor ODQ (1 mM) (Table 1). Furthermore, both effects were blocked by the IL-1Ra (2 μg/ml) indicating that they are mediated by IL-1 receptor-I (Table 1). Release of cGMP is not a result of cell death or membrane leakage since LDH released to the medium in cells treated with 10 ng/ml IL-1β for 20 h was no different than in untreated cells (5.7 ± 2.7 and 7.1 ± 1.8% of total, respectively). Accumulation of cGMP in the medium but not in the cells was also observed in cortical and hippocampal astrocyte-enriched cultures treated with 10 ng/ml IL-1β for 20 h (not shown), indicating that the IL-1β effect is not region specific.

As previously shown by other authors in murine astroglial cultures [16,17], nitrite accumulation induced by IL-1β was potentiated 2–3-fold when cells were co-treated with INF-γ (10 ng/ml) but not with TNF-α (10 ng/ml) (Fig. 2A,B). These cytokines alone have little or no effect on NO formation and accordingly produce non-significant extra- and intracellular cGMP accumulations. However, the combination of IL-1β with INF-γ produced a larger accumulation of extracellular cGMP than IL-1β alone in agreement with the higher NO formation (Fig. 2A,B) but again intracellular cGMP was negligible (not shown). Thus stimulation of cGMP efflux appears to be an IL-1β specific effect. This is further supported in studies with LPS. Treatment of the cerebellar cultures with 10 ng/ml LPS for 20 h, that results in a much larger accumulation of nitrites (IL-1β: 3.0 ± 0.6 μM; LPS: 17.5 ± 2.4 μM, $n = 6$), induced accumulation of cGMP inside the cells with no significant levels detected in the extracellular medium (Fig. 3). However, when cells were treated with LPS together with IL-1β there was no potentiation of nitrite formation (17.3 ± 2.9 μM, $n = 6$) but extracellular cGMP was 5-fold higher than with IL-1β alone (Fig. 3), and intracellular cGMP tended to decrease. These results indicate that IL-1β stimulates efflux of cGMP generated in response to LPS. Probenecid (1 mM), a non-selective inhibitor of organic anion transporters diminished the intracellular response to LPS by 54.2 ± 15.9% ($n = 3$), probably by inhibiting sGC activity as reported to occur in other cell types [16], but completely blocked the IL-1β-induced extrusion of cGMP (Fig. 3). Another inhibitor of anion transport, verapamil (10 μM), produced the same effect (Fig. 3). A probenecid-sensitive transporter for cGMP has been reported to mediate extrusion of cGMP formed in response to C-type natriuretic peptide in cortical astrocytes [10], and in other cell types [5,7,18,19]. Results shown here agree with the presence of such a transporter in astrocytes and indicate that its capacity is increased by IL-1β. This is to our knowledge the first report on the stimulation of cGMP extrusion from cells by an inflammatory agent. In the CNS, IL-1β

Table 1
Effect of different agents on IL-1β-stimulated cGMP efflux and nitrite formation in cerebellar astroglia-enriched cultures

	IL-1β effect (%)			
	Extracellular cGMP		Nitrites	
	Untreated	IL-1β	Untreated	IL-1β
Control	1.1 ± 0.3	100	43 ± 6	100
L-NMMA, 1 mM	0.3 ± 0.1	2 ± 1.2	36 ± 13	40 ± 13
ODQ, 1 mM	0.4 ± 0.1	0.2 ± 0.1	ND	ND
IL-1Ra, 2 μg/ml	1.0 ± 0.3	16 ± 10	39 ± 6	43 ± 8

Cells were treated with IL-1β (10 ng/ml) for 20 h in the absence (untreated) or presence of the indicated compounds added 1 h before. Results, expressed as percent of the effect of IL-1β, are means ± S.E.M. of three to five experiments performed in triplicate in different culture preparations; ND, non-determined.

is elevated during infection, trauma and neurodegenerative diseases such as multiple sclerosis or Alzheimer's disease, by increased release from invading inflammatory cells or from reactive glial cells [20]. In these same cells and also in some neuronal populations inflammatory agents induce expression of NOS and relatively high levels of NO can be generated for prolonged periods of time [21,22]. NO and more reactive NO derivatives have been repeatedly implicated in neurodegeneration [23] although recent reports suggest a neuroprotective role of NO acting as an antioxidant [24]. cGMP, the well recognized physiological second messenger of NO can be formed in neurons and in astrocytes [25,26] and has also been implicated in neuroprotective effects [9]. However, there is very little known about the regulation of cGMP formation under inflammatory conditions. We have recently shown that sGC is down-regulated in astroglial cells by LPS treatment [15]. This could be a mechanism to control cGMP formation under conditions of high NO output. The stimulation by IL-1β of cGMP efflux reported here may be an additional mechanism to keep intracellular cGMP levels low. Alternatively, egressed cGMP may play a role during neuroinflammation by regulating the function of membrane proteins as shown for the Na^+/H^+ exchanger in astrocytes [10] and for kainate receptors in neurons [8]. Whether these extracellular actions of cGMP contribute to neurodegeneration or otherwise prevent it, as suggested by the demonstration that extracellular cGMP protects neurons against glutamate-induced toxicity [9], deserves investigation.

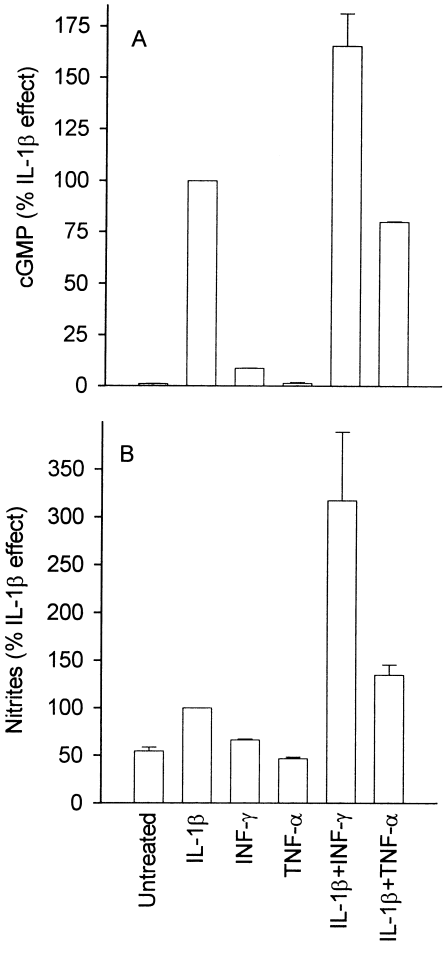

Fig. 2. Extracellular cGMP (A) and nitrites (B) accumulation in cerebellar astroglia-enriched cultures treated with pro-inflammatory cytokines. Cultures were treated with 10 ng/ml IL-1β, INF-γ or TNF-α alone or in combination, for 20 h and cGMP and nitrites determined in the incubation media. Intracellular cGMP was negligible in all cases. Data are means ± S.E.M. of three independent experiments.

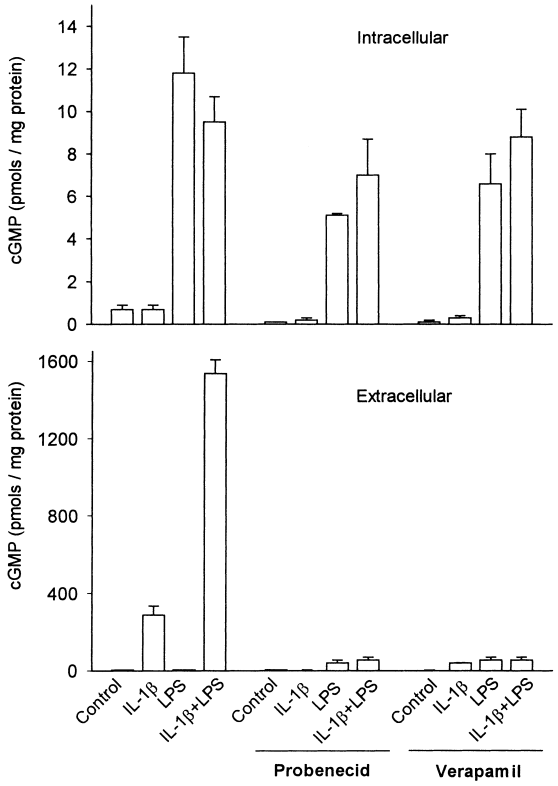

Fig. 3. Effect of probenecid and verapamil on intracellular and extracellular cGMP accumulation in astrocyte-enriched cultures treated with IL-1β and LPS. Cells were incubated with 10 ng/ml LPS, IL-1β or both for 20 h, in the absence or presence of probenecid (1 mM) or verapamil (10 μM) that were added 1 h before. Extracellular cGMP and nitrites were measured in the extracellular media. Monolayers were washed and collected for intracellular cGMP determination. Data are means ± S.D. of triplicate determinations in a representative experiment that was repeated six times without inhibitors, three with probenecid and two with verapamil in different culture preparations.

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