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Up-regulation of A_{2A} adenosine receptors by proinflammatory cytokines in rat PC12 cells

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Abstract

The purpose of this study was to examine the regulation of A_{2A} adenosine receptor (A_{2A} AR) gene expression induced by proinflammatory cytokines in PC12 cells. The A_{2A} AR mRNA levels were substantially increased following 3–48 hr PC12 cell treatment with interleukin 1 beta (500 unit/mL) or tumor necrosis factor alpha (1000 unit/mL), as revealed by RT-PCR analysis. In parallel, cell cytokine treatment induced an up-regulation of A_{2A} receptor protein. Equilibrium radioligand binding studies on treated-cells showed a significant increase in maximum density of [^{3}H] 2-(carboxyethylphenylethylamino) adenosine-5′-carboxamide binding sites, with no significant changes in the affinity constant value. The increase in A_{2A} receptor density was also demonstrated by Western blot analysis. Interleukin 1 beta and tumor necrosis factor alpha effects on A_{2A} AR mRNA and protein levels were detectable after 3 hr cytokine treatment and reached a maximum within 24 and 48 hr, respectively. These results demonstrated the existence of heterologous regulation of A_{2A} ARs by proinflammatory cytokines. The biological significance of this regulation might be associated with modulating cellular activity in response to tissue damage associated with inflammatory mediator production.

Keywords: A_{2A} adenosine receptors; Proinflammatory cytokines; Rat PC12 cells; Heterologous receptor regulation; mRNA expression levels; Adenylyl cyclase assay; A_{2A} AR radioligand binding

1. Introduction

Adenosine, the final metabolite in the stepwise dephosphorylation of ATP, is produced and released in the central nervous system [1–4] and mediates a protective function in response to ischemia and hypoxia [5,6]. Although early studies suggested that adenosine acts predominantly as a neuroprotectant during cerebral ischemia, the complexity of its role has been increasingly appreciated with the identification of four major AR subtypes (A_1, A_{2A}, A_{2B} and A_3), each having a unique distribution among brain regions and their neuronal, glial, and vascular elements [7]. In particular, the knowledge about A_{2A} AR involvement in generation or prevention of ischemic brain damage is very limited. The relative specific A_{2A} agonist CGS21680 reduces ischemic or excytotoxic hippocampal damage [8,9]. On the other hand, several relative specific A_{2A} antagonists have been found to reduce ischemic damage in animal models of global or permanent ischemia, as well as in excitotoxic neuronal damage [10–14]. Moreover, Chen *et al.* [15] demonstrate that A_{2A} inactivation attenuates brain damage and preserves neurological function after transient middle cerebral arterial occlusion.

Furthermore, a regulation of A_{2A} AR gene expression during hypoxia has been demonstrated [16], suggesting A_{2A} AR gene expression regulation is involved in the pathophysiological role of this receptor in brain damage.

Since hypoxia induce the expression of a number of enzymes, cytokines and growth factors [17–19], we tested the expression and protein level of A_{2A} AR following proinflammatory cytokine treatment. Cytokines are potent multifunctional pleiotropic proteins which are constitutively expressed at a low level in healthy adult brain and increase in

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Abbreviations: GPCRs, G protein-coupled receptors; A_{2A} AR, A_{2A} adenosine receptors; NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS21680, 2-carboxyethylphenylethylamino-adenosine-5′-carboxamide; PC12, pheochromocytoma cells; TRIS, tris(hydroxy-methyl)amino-methane; cAMP, cyclic adenosine monophosphate; TNF-alpha, tumor necrosis factor alpha; IL-1 beta, interleukin 1 beta.

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response to injury or infection, contributing to neurodegeneration [20–23]. Recently, emerging evidence has suggested the role of growth factors and cytokines in heterologous AR control [24]. In particular, it has been demonstrated that IL-6 enhances the expression of A_1 AR mRNA and signalling in cultured rat cortical astrocytes and brain slices [25], nerve growth factor mediates the down-regulation of A_{2A} ARs in PC12 cells [26,27] and inflammatory cytokines regulate the functioning and expression of A_{2A} ARs in human monocytic THP-1 cells [28]. Moreover, IFN-gamma up-regulates A_{2B} AR expression in macrophages [29]. At present no data are available about the cross-talk between proinflammatory cytokines and A_{2A} AR gene expression during hypoxia/ischemia. The aim of this work was to investigate the role of proinflammatory cytokines, IL-1 beta and TNF-alpha in the regulation of A_{2A} AR mRNA expression and protein levels in PC12 cells. Sheding light on the cross-talk between adenosine receptors and cytokines, which are produced in response to cell damage, could be an useful tool to investigate the pathophysiological role of A_{2A} ARs in mediating the cellular function in response to damage.

2. Materials and methods

2.1. Materials

RNeasy Mini Kit was obtained from QIAGEN. Amplification grade Dnase I, AccuTaq™ LA DNA polymerase, 10× polymerase chain reaction (PCR) buffer, dNTPs, anchored oligo $d(T)_{23}$ primers, 25 mM $MgCl_2$ solution, enhanced Avian Reverse Transcriptase (eAMV RT) and Rnase inhibitor were from Sigma–Aldrich, β-actin and A_{2A} primers were synthetized by Sigma–Genosys Ltd. [^{3}H] CGS21680 was from NEN Life Sci. NECA was from RBI/Sigma. Cell culture media and fetal calf serum were from Bio-Whittaker and Boehringer/Roche, respectively. All other chemicals were supplied by standard commercial sources. Goat anti-rat A_{2A} AR antibody was supplied by Santa Cruz.

2.2. Cell culture

PC12 cells were maintained in RPMI 1640 medium supplemented with 5% FCS, 10% horse serum, 2 mM L-glutamine and penicillin/streptomycin in a humidified atmosphere (5% CO_2) at 37°. Cells were treated with IL-1 beta (500 unit/mL) or TNF-alpha (1000 unit/mL) for 3–48 hr and then, harvested for mRNA, radioligand binding and immunoblotting analysis.

2.3. RT-PCR

Total RNA was isolated from PC12 cells using RNeasy RNA isolation kit (QIAGEN). RNA concentration was measured spectrophotometrically and was separated on 1% agarose gel to check integrity. The RNA sample was DNase-treated at 25° for 15 min. cDNA was synthesized by reverse transcription of 1 μg of total RNA in a final volume of 20 μL containing 1 U of eAMV RT, 3.5 μM of oligo $(dT)_{23}$ primers, 0.5 mM of dNTP, 20 U of RNasin ribonuclease inhibitor in the reaction buffer 50 mM TRIS–HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT for 50 min at 43°. For PCR a set of forward and reverse primers specific to rat A_{2A} receptor were: 5′-CGAATTCAACCTG-CAGAACGTCACC-3′, sense and 5′-TCGAATTCGCGG-TC(G/A)ATGGCGAT(A/G)-3′, antisense [30], expected to code for a product of 216 bp. Amplification of A_{2A} AR cDNAs was performed in 50 μL reaction mixtures containing 2.5 U of AccuTaq™ LADNA polymerase, 0.6 μM of DNA primers, 1× PCR-buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, and cDNA template. PCR was performed in a THERMOLYNE TEMP-TRONIC (pbi international) thermal cycler by initial denaturation for 3 min at 94° followed by 30 cycles of sequential 94° for 1 min, 65° for 30 s and 72° for 1 min. The final extension was prolonged by 7 min at 72°, β-actin 5′-CATTGTGATGGACTCCCGGAGACG-3′, sense 5′-GCCACCAGACAGCACTGTGTTG-3′, antisense. The plateau phase of the PCR reaction was not reached under these PCR conditions. Potential contamination by genomic DNA was checked for by running the reactions without reverse transcriptase and using β-actin primers in subsequent PCR amplifications. Only RNA samples which showed no bands after that procedure were used for further investigation.

2.4. Radioligand binding assay

PC12 cells were washed with 50 mM TRIS–HCl buffer (pH 7.4) containing 1 mM EDTA and 150 mM NaCl and were homogenized in 5 mM TRIS–HCl, pH 7.4, containing 1 mM EDTA, using a polytron. The homogenates were centrifuged at 48,000 *g* for 20 min, and the resulting pellet was resuspended in the same buffer and centrifuged. [^{3}H] CGS21680 binding to PC12 cell membranes was tested as previously reported [31]. Saturation binding experiments were performed incubating aliquots of control and cytokine-treated cell membranes (60 μg of proteins) with increasing [^{3}H] CGS21680 concentrations ranging from 5 to 100 nM. The binding reaction was terminated by filtration under reduced pressure through GF/C filters, washing three times with 5 mL incubation buffer. Non-specific [^{3}H] CGS21680 binding was defined in the presence of 50 μM NECA. Protein concentrations were determined with a protein assay kit from Biorad, using bovine serum albumine as the standard.

2.5. Protein analysis by Western blotting

Following cytokine cell incubation, monolayers were directly lysed in 500 μL per well RIPA buffer (150 mM NaCl, 50 mM TRIS–HCl, pH 8, 1% Nonidet P-40, 0.5%

sodium deoxhycolate, 1 mM PMSF, 10 μg/mL aprotinin, 100 μM $NaVO_4$) for 60 min at 4°. After centrifugation at 15,000 *g* for 30 min, soluble fractions were assayed for protein content using the BioRad protein assay. Equivalent amounts of protein (50 μg per sample) were resolved by SDS–PAGE, transferred to nitrocellulose and 0.1 μg/mL goat anti-rat A_{2A} AR antibody was used for immunoblotting. A_{2A} AR antibody is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of A_{2A} AR of rat origin. It specifically reacts with A_{2A} AR of mouse and rat origin and non-cross reacts with A_1, A_{2B} or A_3 AR subtypes. After washing, the nitrocellulose membrane was incubated for 120 min at room temperature. Visualization was accomplished using streptavidin peroxidase and a color development reagent in accordance with manufacturer's instructions (Sigma–Aldrich).

2.6. cAMP assay

PC12 cells were treated for 24 hr with 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha. On the day of the cAMP assay, the cells were placed in fresh medium and incubated with NECA (10 nM–10 μM) for 15 min at 37° in the presence of 1 U/mL adenosine deaminase (ADA). Cells were then harvested, lysed and assayed for cellular cAMP accumulation using the cAMP enzyme immunoassay system kit (Sigma–Aldrich), following the manufacture's instruction.

2.7. Data analysis

For data analysis and graphic presentation we used the non-linear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad). Computer imaging analysis (GS-670 BIO-RAD) was used for semi-quantitative analysis of RT-PCR products.

3. Results

RT-PCR analysis were performed to determine the effects of 3–48 hr proinflammatory cytokines, TNF-alpha (1000 unit/mL) or IL-1 beta (500 unit/mL), treatment on A_{2A} AR constitutive and inducible gene expression in PC12 cells.

As shown in Figs. 1 and 2, the message for A_{2A} AR was significantly altered after treatment of PC12 cells with each of the cytokines used. Cell incubation with IL-1 beta or TNF-alpha led to a time-dependent increase in expression of the A_{2A} message with a similar kinetic profile. Three-hour cell incubation with 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha induced a significant increase in the expression of A_{2A} message that peaked within 24 and 48 hr, respectively (160 and 148% of control, respectively; $P < 0.001$). Amplification of β-actin transcripts did not show a parallel induction of this housekeeping gene under the same conditions; thus, β-actin mRNA was used as an internal control. To compare mRNA levels by RT-PCR, we used the ratio of cytokine mRNA to β-actin mRNA, and the β-actin mRNA level was also monitored for the efficiency of RT-PCR.

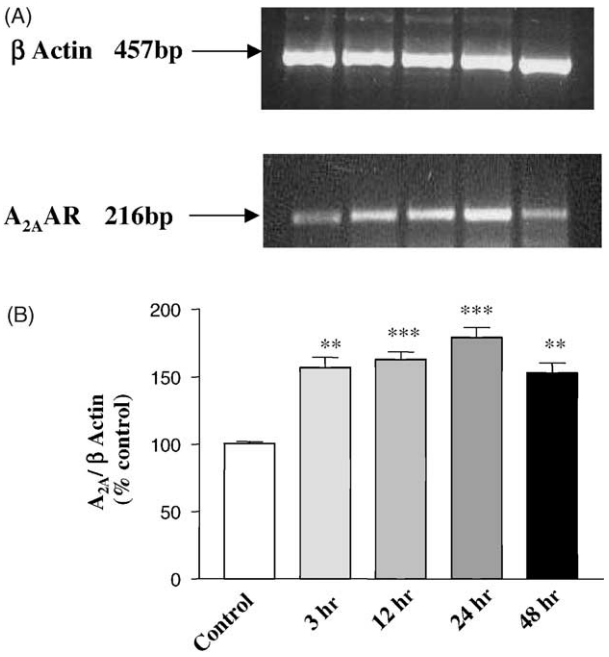

Fig. 1. A_{2A} AR mRNA expression in PC12 cells after treatment with IL-1 beta. Cells, incubated with 500 unit/mL IL-1 beta, were harvested at indicated time points and A_{2A} AR mRNA levels were determined by RT-PCR, as described in Section 2. (A) A_{2A} AR and β-actin RT-PCR products ethidium bromide staining. The PCR product sizes for A_{2A} AR and β-actin mRNA were 216 and 457 bp, respectively. The figure is representative of a typical experiment performed three times with similar results. (B) Averaged data of three separate experiments. Data are represented as the ratio of RT-PCR products of A_{2A} AR to β-actin, determined by densitometric analysis. Each bar shows the mean ± SEM as compared with the control (100%). (∗) $P < 0.05$; (∗∗) $P < 0.01$.

In order to determine whether the change in mRNA levels was accompanied by an alteration in A_{2A} AR protein expression, we quantified receptor density through radioligand binding assay using the selective A_{2A} agonist, [^{3}H] CGS21680. Equilibrium binding parameters, dissociation constant (K_d) values and maximum numbers of binding sites (B_{max}), were obtained from linear Scatchard analysis of [^{3}H] CGS21680 saturation curves. In untreated control cells, Scatchard analysis of [^{3}H] CGS21680 binding data demonstrated the presence of homogeneous population of high-affinity binding sites with a K_d of 21.8 ± 2 nM and a B_{max} value of 219 ± 18 fmol/mg of proteins (Fig. 3), similar to the data previously described [31,32]. The incubation of PC12 cells with 1000 unit/mL TNF-alpha or 500 unit/mL IL-1 beta induced a time-dependent increase in the A_{2A} AR B_{max} value, with a maximum at 24 hr for IL-1 beta (359 ± 33 fmol/mg proteins vs. 219 ± 18 fmol/mg proteins in controls) and at 48 hr for TNF-alpha (360 ± 34 fmol/mg proteins vs. 219 ± 18 fmol/mg proteins in controls) (Fig. 3). The increase in maximum density of [^{3}H] CGS21680 binding sites was not accompanied by a significant change

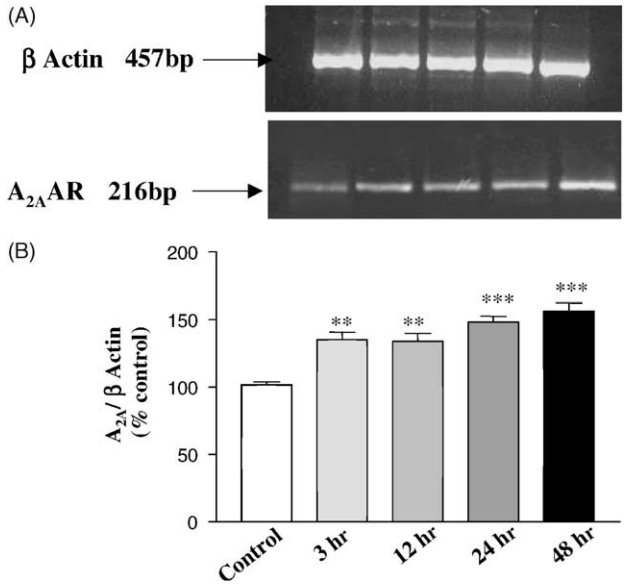

Fig. 2. A_{2A} AR mRNA expression in PC12 cells after treatment with TNF-alpha. Cells, incubated with 1000 unit/mL TNF-alpha, were harvested at indicated time points and A_{2A} AR mRNA levels were determined by RT-PCR, as described in Section 2. (A) A_{2A} AR and β-actin RT-PCR products ethidium bromide staining. The PCR product sizes for A_{2A} AR and β-actin mRNA were 216 and 457 bp, respectively. The figure is representative of a typical experiment performed three times with similar results. (B) Averaged data of three separate experiments. Data are represented as the ratio of RT-PCR products of A_{2A} AR to β-actin, determined by densitometric analysis. Each bar shows the mean ± SEM as compared with the control. (∗) $P < 0.05$; (∗∗) $P < 0.01$.

in the K_d values. [^{3}H] CGS21680 B_{max} and K_d values obtained following cytokine cell treatment were reported in Table 1. These data demonstrated that the increase in [^{3}H] CGS21680 binding sites showed the same kinetic profile of

Table 1
Modulation of [^{3}H] CGS21680 binding parameters by cytokines

Cells treatment	Time (hr)	K_d (nM)	B_{max} (fmol/mg protein)
Control	–	21.8 ± 2.0	219 ± 18
IL-1 beta (500 unit/mL)	3	20.0 ± 1.9	315 ± 28**
	12	17.9 ± 1.7	322 ± 30**
	24	25.3 ± 2.0	359 ± 33**
	48	25.3 ± 2.3	346 ± 32**
TNF-alpha (1000 unit/mL)	3	20.9 ± 1.8	300 ± 28*
	12	22.0 ± 2.0	311 ± 27**
	24	26.0 ± 2.2	320 ± 31**
	48	17.1 ± 1.5	359 ± 33**

PC12 cells were incubated with 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha for 3–48 hr. At each time, cells were harvested and used for membrane preparations as described in Section 2. [^{3}H] CGS21680 saturation curves on membrane preparations were analysed by Scatchard analysis and equilibrium binding parameters determined. Each value represents the mean ± SEM of [^{3}H] CGS21680 B_{max} and K_d values obtained from three separate experiments.

* $P < 0.05$; ** $P < 0.01$.

mRNA expression levels and suggested that cytokine treatment of PC12 cells induced an up-regulation of A_{2A} AR without affect the conphormational state of the receptor. The increase of A_{2A} AR was also confirmed by Western blot analysis performed using an antibody against the A_{2A} AR. The immunoblots of lysed control and cytokine-treated cells revealed a single band of a 42 kDa protein, corresponding to A_{2A} AR. Compared with controls, protein levels of A_{2A} AR were increased by IL-1 beta and TNF-alpha treatment for 24 hr (Fig. 4).

As a first step to clarify the effect of cytokines-mediated A_{2A} AR up-regulation, we investigate the ability of the A_{2A}

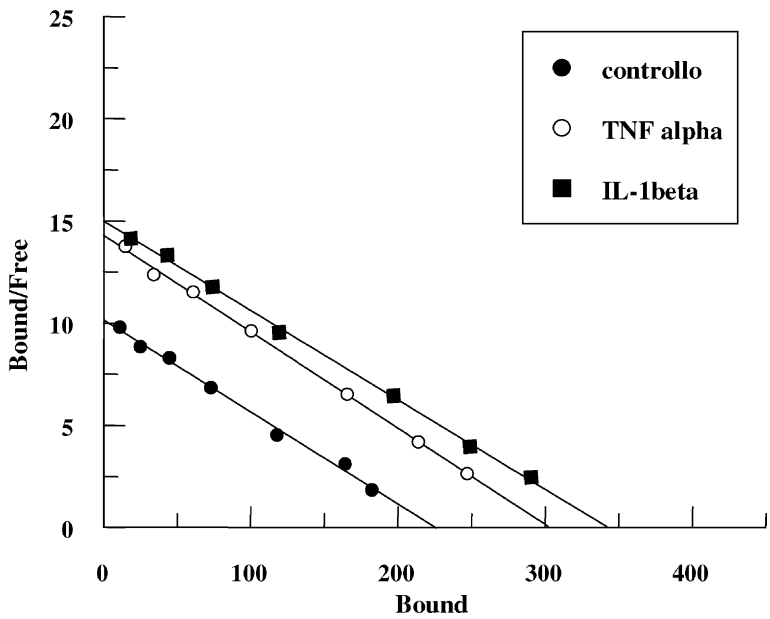

Fig. 3. Cytokine-induced increase of A_{2A} AR expression in PC12 cells. Membranes from cells treated with medium alone (control) or 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha for 24 hr were used in binding saturation experiments as described in Section 2. Graph shows a representative Scatchard plots of [^{3}H] CGS21680 saturation binding performed three times with similar results; (●) control, (■) IL-1 beta-treated cells, (○) TNF-alpha-treated cells.

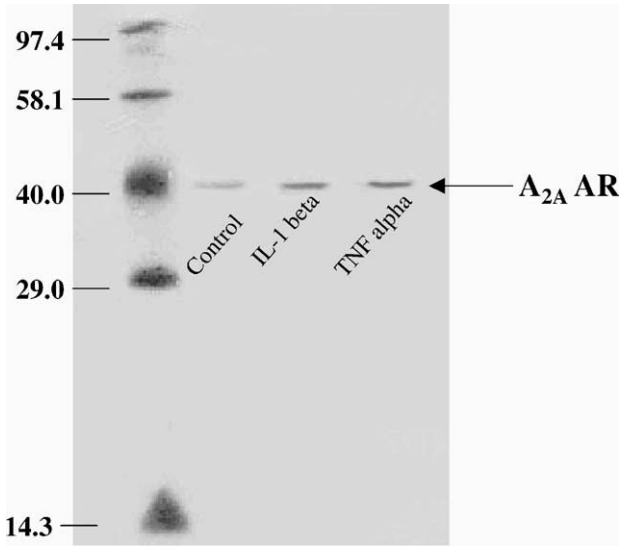

Fig. 4. Immunoblotting of A_{2A} AR from PC12 cell treated with IL-1 beta or TNF-alpha. Cells were incubated at 37° for 24 hr with medium alone (control) or 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha. Equal amounts of proteins were loaded on polyacrylamide gel and immunoblotted with 0.1 μg/mL goat anti-rat A_{2A} AR antibody as described in Section 2.

AR agonist NECA to stimulate adenylyl cyclase activity before and after PC12 cell cytokine treatment. The dose–response curves were shown in Fig. 5. The cAMP basal levels were significantly comparable in both untreated and cytokine-treated cells. Following cytokine cell treatment, no significant differences in the NECA dose–response curve was observed; in control, IL-1 beta- and TNF-alpha-treated cells, NECA-stimulated adenylyl cyclase with an EC_{50} value of 33 ± 2.2, 34.3 ± 2.6, and 49.2 ± 3.4 nM, respectively.

4. Discussion

In this study A_{2A} AR heterologous regulation by proinflammatory cytokines was demonstrated in PC12 cells.

Adenosine, that is released in various tissues in response to a number of physiological and pathological conditions, including hypoxia, mediates a protective function [1,3,6] by its interaction with specific G protein-coupled receptors [33]. Recently, it has been demonstrated that the final cellular responses mediated by GPCRs do not involve the sole stimulation of conventional second-messenger generating systems, but result from the functional integration of an intricate network of intracellular signalling pathways [34] which contribute to the control of receptor functioning [35]. In this view, the changes in adenosine receptor expression and binding properties induced by endongenous mediators, associated with ischemia, may play an important role in the cell responses mediated by adenosine.

There have been some lines of evidence suggesting a regulatory connection between adenosine and its receptors and inflammatory cytokines that are released during ischemia and play an important role in the pathophysiology of brain injury [23,36–38]. In peripheral cells and in the central nervous system, it has been demonstrated that the activation of A_{2A} ARs modulates the production of different cytokines including TNF-alpha, IL-6, IL-12, etc. [39–41]. Furthermore, other lines of evidence have suggested that the expression and function of adenosine receptors may be regulated by numerous endogenous factors involved in inflammation, such as growth factors and cytokines [24,26,29]. In particular, a recent paper demonstrates that inflammatory cytokines regulate the

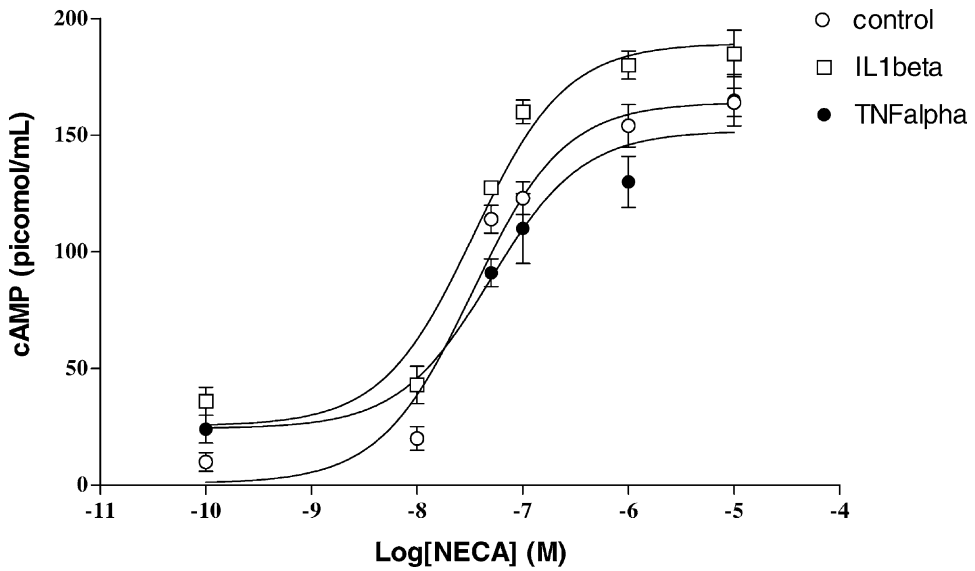

Fig. 5. A_{2A} AR-mediated accumulation of intracellular cAMP in untreated and cytokine-treated PC12 cells. Cells were treated for 24 hr with medium alone (control) or 500 unit/mL IL-1 beta or 1000 unit/mL TNF-alpha, as described in Section 2, and thereafter stimulated with the agonist NECA at different concentrations (10 nM–10 μM). The intracellular cAMP content of the cells was assayed using the enzyme immunoassay kit; (○) control cells; (□) IL-1 beta-treated cells; (●) TNF-alpha-treated cells. Values are expressed as the mean ± SEM of three independent experiments. EC_{50} values were: control, 33 ± 2.2; IL-1 beta, 34.3 ± 2.6; TNF-alpha, 49.2 ± 3.4 nM.

function and expression of A_{2A} ARs at the inflamed site, in human monocytic cells [28].

In our experimental conditions, the proinflammatory cytokines induced an increase in A_{2A} AR mRNA expression and protein levels in PC12 cells, which represent a widely used model to study A_{2A} ARs and the sympathetic neuronal function. RT-PCR studies showed that PC12 cytokine treatment induced an increase in gene expression associated with a significant increase in total receptor density, as evidenced by Western blot analysis and equilibrium binding studies. Scatchard analysis showed that, with respect to control cells ($B_{max} = 219 \pm 18$ fmol/mg of proteins), 24 hr IL-1 beta or TNF-alpha cell treatment caused an up-regulation of A_{2A} receptor density up to 359 ± 33 fmol/mg of proteins and 320 ± 31 fmol/mg of proteins, respectively. The increase in A_{2A} AR expression induced by IL-1 beta or TNF-alpha was significant after 3 hr cell treatment and reached a maximal effect after 24 and 48 hr, respectively. These data showed a kinetic profile similar to that of cytokine production following cerebral ischemia [19]. Moreover, these effects were observed in a range of cytokine concentrations referred to induce cell damage [42,43].

The cytokine-mediated increase of A_{2A} AR density was not accompanied by changes in the NECA-mediated cAMP stimulation. It is noteworthy that maximal efficacy at G protein-coupled receptors does not always correlate with the total receptor number but may be function of receptor G protein-coupling stoichiometry [44]. In this view it may be enough the activation of only a fraction of total available receptors to obtaine the maximal cAMP stimulation. Moreover, the cytokine effects should not be confined to A_{2A}-mediated cAMP stimulation but should also be expanded to the complete range of behaviours of GPCRs, such as internalisation, desensitisation, oligomerization, phosphorylation and association with other membrane proteins. Studies in our laboratory are in progress to investigate whether cytokines modulate intracellular regulatory mechanisms involved in the control of receptor phosphorylation altering the long-term responsiveness to agonists.

At present, in human monocytic THP-1 cells, it has been demonstrated that TNF-alpha and IL-1 beta induce an increase of A_{2A} AR expression and of the ability of these receptors to stimulate adenylyl cyclase activity [28]. The different results obtained in our cellular model with respect to monocytic cells may reflect the existence of distinctive A_{2A} AR regulatory mechanisms that may account the contradictory effects of these receptor subtypes in different (vascular and neuronal) cell systems.

The exact role of A_{2A} AR up-regulation induced by cytokines in PC12 cells is not yet clear. It might represent a defence mechanism of the cell to counteract cytokine induced cell damage, during hypoxia and inflammatory response, or play a synergic effect on cell death. Efforts to clarify the role of A_{2A} AR in ischemic injury have indeed produced mixed results. These receptors can mediate beneficial effects reducing cell death following intracerebral hemorrhage [45], and protecting the hippocampus from excitotoxicity in a model of kainate-induced neuronal cell death [8]; however, data showed A_{2A} AR antagonist protective effects [14], that can be related the well known A_{2A}-mediated vasoldilatation [46], inhibition of platelet aggregation [47] and suppression of neutrophil superoxide generation [48]. It has been also demonstrated that A_{2A} AR deficiency attenuates brain injury induced by transient focal ischemia in mice [15]. The neuroprotective effects of A_{2A} AR antagonists consist, most likely, of several components that involve reduction of neurotrasmitter release (particularly glutamate); on the constrast, the peripheral A_{2A} AR-mediated protective effects, might be achieved indirectly by the known anti-inflammatory effects of these receptors on neutrophils. Therefore, the activation of a same receptor subtype can elicit several responses in different tissues and cellular models. Studies are in progress to investigate whether A_{2A} AR contribute to the control of neuronal cell death mediated by proinflammatory cytokines.

Acknowledgments

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