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Short-Term TNF-Alpha Treatment Induced A_{2B} Adenosine Receptor Desensitization in Human Astroglial Cells

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Abstract Long-term glial cell treatment with the proinflammatory cytokine TNF-alpha has been demonstrated to increase the functional responsiveness of A_{2B} adenosine receptors (A_{2B} ARs), which in turn synergize with the cytokine inducing chronic astrogliosis. In the present study, we investigated the short-term effects of TNF-alpha on A_{2B} AR functional responses in human astroglial cells (ADF), thus simulating the acute phase of cerebral damage which is characterized by both cytokine and adenosine high level release. Short-term TNF-alpha cell treatment caused A_{2B} AR phosphorylation inducing, in turn, impairment in A_{2B} AR-G protein coupling and cAMP production. These effects occurred in a time-dependent manner with a maximum following 3-h cell exposure. Moreover, we showed PKC intracellular kinase is mainly involved in the TNF-alpha-mediated regulation of A_{2B} AR functional responses. The results may indicate the A_{2B} AR functional impairment as a cell defense mechanism to counteract the A_{2B} receptor-mediated effects during the acute phase of brain damage, underlying A_{2B} AR as a target to modulate early inflammatory responses. J. Cell. Biochem. 104: 150–161, 2008.

Key words: ADF cells; TNF-alpha; A_{2B} adenosine receptor desensitization; intracellular kinases

Astrocytes, which are involved both in physiological brain functions and in pathological events, are the most important source of extracellular adenine-based purines in the brain [Ciccarelli et al., 1999] and moreover express all the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor (AR) subtypes [Peakman and Hill, 1996; Trincavelli et al., 2002a, 2004]. Among all these receptors, A_{2B} ARs are quite unique, since they exhibit a relatively low affinity for adenosine and seem to be activated only under hypoxic or ischemic conditions, when large amounts of adenosine are released (micromolar range) [Ribeiro et al., 2003]. Under these pathological conditions proinflammatory cytokines, a group of potent multifunctional pleiotropic proteins, are released in the brain at high levels, contributing to neurodegeneration and inflammation processes [Szelenyi, 2001; Zaremba et al., 2001]. Evidence suggests a regulatory connection between inflammatory cytokines and the adenosine system: the former are known to be involved in ARs response regulation mechanisms [for A_1 AR see Biber et al., 2001; for A_{2A} AR see Khoa et al., 2001; Trincavelli et al., 2002b; for A_{2B} see Rosi et al., 2003; Trincavelli et al., 2004], while ARs are involved in the regulation of the cytokine production [for A_{2A} AR see Hasko et al., 2000; for A_{2B} AR see Zhang et al., 2005]. This functional cross-talk between ARs and cytokines plays an important role in the regulation of

Abbreviation used: CNS, central nervous system; GPCRs, G protein-coupled receptors; ARs, adenosine receptors; ADF, human astrocytoma cells; NECA, 5′-*N*-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3 dipropyl xanthine; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine; MRS 1220, 9-chloro-2-(2-furyl)-5-phenylacetamino[1,2,4]triazolo[1,5-c]quinazoline; TNF-alpha, tumor necrosis factor alpha; ADA, adenosine deaminase.

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AR responses during pathological conditions. In particular, data from our group have demonstrated that chronic exposure of human astroglial cell to the cytokine, tumor necrosis factor alpha (TNF-alpha), induces a delay in the A_{2B} AR desensitization process, causing the up-regulation of the receptor functioning. Moreover, A_{2B} AR has contributed, in association with TNF-alpha, to mediate chronic astrogliosis, suggesting a role for this AR subtype in the long-term control of astrocytic functions [Trincavelli et al., 2004].

Following ischemia and trauma, TNF-alpha is quickly synthesized (2–6 h) in the central nervous system (CNS) by resident macrophages, astrocytes, and microglia [Yu and Lau, 2000; Tehranian et al., 2002]. Converging lines of evidence support a pivotal role for cytokines in driving the short-term inflammatory response [Wang and Shuaib, 2002]. In this context, to understand how the cytokines regulate the functional responsiveness of adenosine neuromodulator system in the acute phase of cerebral damage represents an important goal to develop new strategic therapeutic intervention.

The aim of the present work was to investigate the A_{2B} AR modulation in ADF cells following short-term TNF-alpha exposure and to dissect the intracellular kinase pathways involved in TNF-alpha-mediated regulation of A_{2B} AR functioning.

MATERIALS AND METHODS

Materials

[^{35}S]GTPγS (specific activity 1,250 Ci/mmol) and protein-A sepharose^{TM} were purchased from Amersham Biosciences (Freiburg, Germany). NECA and DPCPX were obtained commercially from Sigma-RBI (St. Louis, MO) while MRS 1220 was from Tocris Cookson (Bristol, United Kingdom). SCH 58261 was a gift from Schoering-Plough. TNF-alpha was purchased from Li StarFish (Milan, Italy) and H89 dihydrochloride, Bisindolylmaleimide I GFX109203 and wortmannin were from Calbiochem (EMD Biosciences, affiliate of Merck KgaA, Darmstadt, Germany) while PKI (6–22 peptide) and chelerythrine were from Sigma-RBI, adenosine deaminase (ADA) was from Roche Diagnostics GmbH (Mannheim, Germany). Cell culture media and fetal bovine serum were from Cambrex Bio-Science (Verviers, Belgium). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA). Human A_{2B} AR antibody was supplied by Alpha Diagnostic (San Antonio, TX), while anti-phosphothreonine antibody was from Chemicon International (Temecula, CA) and secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The Bio-Rad Protein assay based on Bradford method was from Bio-Rad (Munich, Germany). All other chemicals were supplied from standard commercial sources.

Cell Culture and Treatments

Human astrocytoma cells (ADF) were kindly supplied by Prof. Maria Pia Abbracchio (Department of Pharmacological Sciences, University of Milan) [Malorni et al., 1994]. ADF cells were grown adherently using RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids as previously described [Trincavelli et al., 2002a]. Aliquots of cells were treated without (control) or with increasing TNF-alpha concentrations (10–1,000 U/ml) for different times (5 min–12 h) and then, harvested for [^{35}S]GTPγS binding, cAMP assay, and immunoblotting analysis. Aliquots of the cells were pre-incubated for 15 min with different kinase inhibitors before being treated with TNF-alpha (1,000 U/ml) for 3 h: in particular, PKA inhibitors, H89 dihydrochloride (1 μM) and PKI (50 nM); PKC inhibitors, Bisindolylmaleimide I GFX109203 (1 μM) and chelerythrine (6 μM); and PI3K inhibitor, wortmannin (500 nM), were used [Glass et al., 1989; Herbert et al., 1990; Toullec et al., 1991; Fantozzi et al., 1992; Geilen et al., 1992; Nakanishi et al., 1992; Davies et al., 2000; Schulte and Fredholm, 2003].

[^{35}S]GTPγS Binding Assay

After different treatments, ADF cell membrane fractions were prepared and assayed as previously described [Trincavelli et al., 2004]. A_{2B} AR coupling to G proteins was evaluated assessing the ability of the AR agonist NECA to stimulate [^{35}S]GTPγS binding. Briefly, after pre-incubation for 15′ at 30°C with ADA (2 U/ml), aliquots of membrane fractions (10 μg proteins) were incubated for 60 min at 25°C in binding buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4) containing GDP 5 μM and [^{35}S]GTPγS 0.3 nM. Cell membranes were

stimulated with different NECA concentrations (10 nM–100 μM), in presence of the selective AR antagonists DPCPX, SCH 58261, and MRS 1220 (all used at 100 nM concentration), to block A_1, A_{2A}, and A_3 receptors, respectively. Non-specific binding was determined in presence of 10 μM GTPγS and it resulted less than 10% of total binding.

cAMP Assay

cAMP assay was performed as previously described [Trincavelli et al., 2004]. Briefly, cells were incubated for 15 min at 37° in fresh DMEM medium containing 2 U/ml ADA and 10 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 201724) as phosphodiesterases inhibitor. Then, 1 nM–10 μM NECA-mediated cAMP production was evaluated by the cAMP enzyme immunoassay system kit (Sigma–Aldrich, St. Louis, MO), following the manufacture's instruction. These experiment were carried out in the presence of the A_{2A} AR antagonist SCH 58261 (100 nM) to exclude A_{2A} AR component.

A_{2B} AR Immunoprecipitation and Immunoblotting

ADF cells were processed by immunoblot following the method previously described [Trincavelli et al., 2004]: cell lysates (1 mg of proteins) were immunoprecipitated with an anti-A_{2B} AR antibody and Protein A Sepharose™. Immune-complexes were resolved by SDS–PAGE (12%), transferred to nitrocellulose membranes and treated overnight at 4°C with primary antibody against A_{2B} AR (1 μg/ml) or against phosphothreonine residues (0.5 μg/ml). Blots were developed using the ECL Western blotting detection reagents (Amersham Biosciences). Phosphothreonine immunoreactive bands were quantified by densitometric scanning of films, using an image analysis system and normalized by corresponding A_{2B} ARs immunoreactive bands.

Data Analysis

Data were statistically analyzed with One-Way ANOVA (Tukey's multiple comparison test) by the GraphPad PRISM Version 4.00 program (GraphPad Software, San Diego, CA). Data have been reported as mean ± SEM of three different experiments (performed in duplicate). Significance refers to results where $P < 0.05$ was obtained.

RESULTS

A_{2B} AR-G Protein Coupling in Control and Short-Term TNF-Alpha Treated Cells

As a first step, we investigated the effect of short-term TNF-alpha cell exposure on A_{2B} AR-G protein coupling efficacy. TNF-alpha cell treatment induced a time-dependent decrease in A_{2B} AR-G protein coupling (Fig. 1A), with a maximal effect after 3-h incubation ($P < 0.01$ TNF-alpha 3 h vs. control). Moreover, following 3-h cell exposure, TNF-alpha-mediated impairment of receptor-G protein activation occurred in a concentration dependent manner (Fig. 1B). TNF-alpha cell treatment did not induce any significant change in the basal [^{35}S]GTPγS binding (data not shown), demonstrating the cytokine did not affect G protein functional state.

Therefore, the effect of different kinase inhibitors on TNF-alpha-mediated A_{2B} AR-G protein uncoupling was assessed. All inhibitors alone were not able to modulate NECA-mediated response ($P > 0.05$ vs. control, Fig. 2). Cell pre-exposure to 1 μM H89 or 1 μM GFX109203, before TNF-alpha treatment, were able to counteract TNF-alpha (1,000 U/ml for 3 h) effects, inducing a partial recovery both in the agonist effectiveness in stimulating GTPγS binding ($P < 0.01$ TNF-alpha + H89 or TNF-alpha+ GFX109203 vs. TNF-alpha alone). Moreover, the simultaneous cell pre-incubation with the two inhibitors completely restored the A_{2B} AR-G protein coupling efficacy up to control level ($P < 0.01$ vs. TNF-alpha alone, Fig. 2). PKI inhibitor, at 50 nM concentration, induced a weak and no significant recovery of A_{2B} AR-G protein coupling ($P > 0.05$ vs. TNF-alpha alone). Chelerythrine, at 6 μM concentration, is the most effective in restoring A_{2B} AR-G protein coupling ($P < 0.001$). On the contrary, the PI3K inhibitor, wortmannin, did not counteract TNF-mediated effects ($P > 0.05$ vs. TNF-alpha alone). These data may suggest PKC is primary involved in TNF-alpha-mediated regulation of A_{2B} AR-G protein coupling.

Therefore, we evaluated the effects of TNF-alpha cell treatment (1,000 U/ml for 3 h) on NECA concentration-response curves. In control cells, the agonist NECA induced a significant stimulation of [^{35}S]GTPγS binding with an EC_{50} value of 281 ± 17.3 nM and a maximal efficacy of 178.5 ± 10.25%, according to data previously reported [Trincavelli et al., 2004]. TNF-alpha cell treatment induced a significant

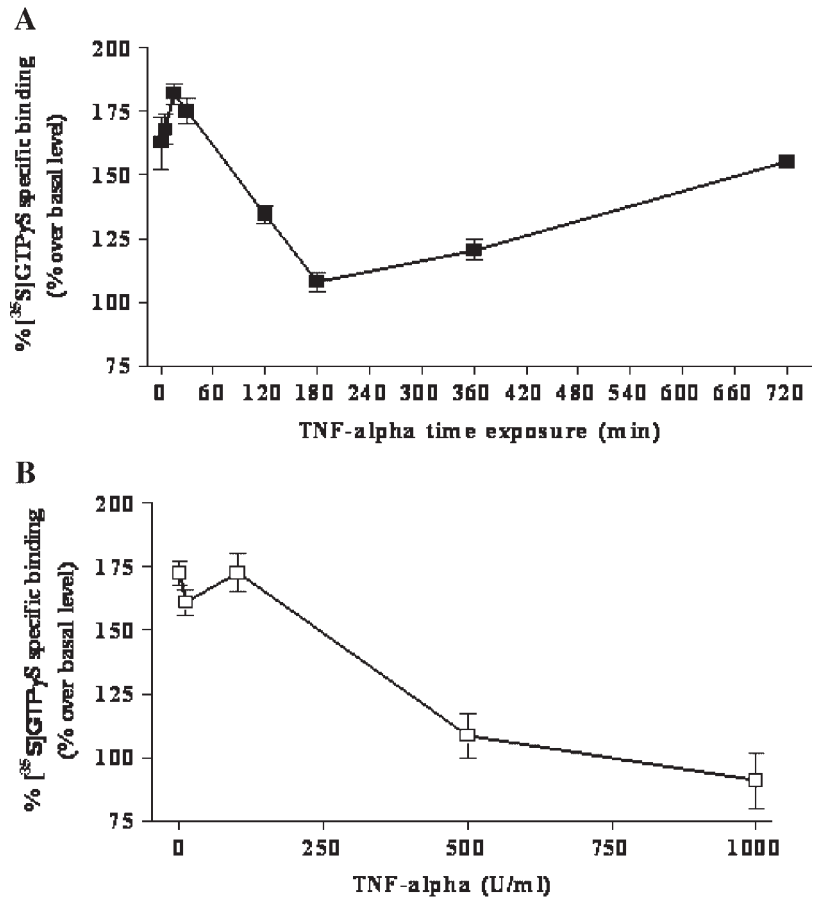

Fig. 1. TNF-alpha-mediated regulation of the A_{2B} AR-G protein coupling: time (**A**) and concentration (**B**) dependence. The ability of 1 μM NECA (in the presence of A_1, A_{2A}, and A_3 antagonists) to stimulate [^{35}S]GTPγS binding was evaluated in ADF cell membranes following cell treatment without or with 1,000 U/ml TNF-alpha for different times (5 min–12 h, A) or following cell treatment with different TNF-alpha concentrations (10–1,000 U/ml) for 3 h (B). Data, reported as mean ± SEM (from five different experiments), are expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%.

right shift of agonist response curve with an EC_{50} value of 616 ± 11.54 nM ($P < 0.001$ TNF-alpha vs. control cells) suggesting the cytokine induced A_{2B} AR-G protein uncoupling (Fig. 3). Moreover, a significant decrease in agonist maximal response was detected (123.6 ± 1.35%). The kinase inhibitors H89, GFX109203, and chelerythrine were able to counteract TNF-alpha effects partially restoring A_{2B} AR agonist potency and efficacy. Concentration-response curves and the relative EC_{50} values, which were obtained following cell incubation with TNF-alpha in the presence of kinase inhibitors, are reported in Figure 3.

A_{2B} AR Functional Responsiveness in Control and Short-Term TNF-Alpha Treated Cells

As a second step, we evaluated the time and concentration dependence of TNF-alpha effects on the A_{2B} AR functional responsiveness. The A_{2B} AR responsiveness was quantified evaluating the ability of the agonist NECA, in the presence of the A_{2A} AR antagonist SCH 58261 (100 nM), to stimulate intracellular cAMP production in control and treated cells [Trincavelli et al., 2004]. Cell pre-incubation with TNF-alpha (1,000 U/ml) for different times induced a significant decrease in the A_{2B} AR functional responsiveness (Fig. 4A) with a maximal effect following 3-h incubation ($P < 0.01$ TNF-alpha 3 h vs. control). The TNF-alpha-mediated impairment of A_{2B} AR response occurred in a concentration dependent manner, with a maximal effect at 1,000 U/ml cytokine following 3-h treatment (Fig. 4B). These data suggested TNF-alpha was able to induce short-term A_{2B} AR desensitization.

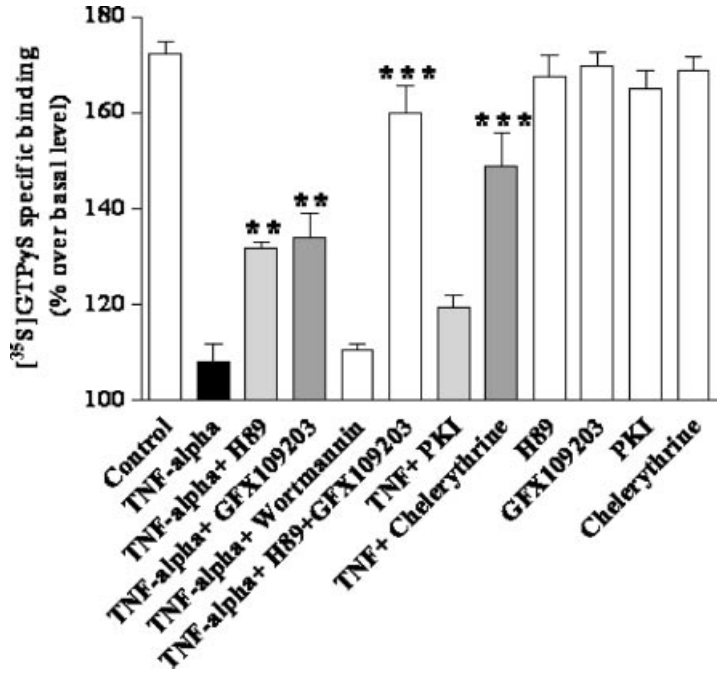

Fig. 2. Effects of intracellular kinase inhibitors on the TNF-alpha-mediated A_{2B} AR-G protein uncoupling. ADF cells were pre-incubated without or with kinases inhibitors (1 μM H89; 1 μM GFX109203; 50 nM PKI; 6 μM chelerythrine; 50 nM wortmannin) for 15 min. Cells were then treated with or without TNF-alpha 1,000 U/ml for 3 h and then the ability of 1 μM NECA (in the presence of A_1, A_{2A}, and A_3 antagonists) to stimulate [^{35}S]GTPγS binding was assessed. Data, reported as mean ± SEM (from five different experiments), are expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus TNF-alpha.

Cell pre-incubation with the kinase inhibitors H89, GFX109203, or chelerythrine counteracted the TNF-alpha effects, inducing a recovery of the agonist-mediated cAMP accumulation ($P < 0.01$ vs. TNF-alpha alone, Fig. 5). On the contrary, wortmannin and PKI were not able to restore the A_{2B} AR functional responsiveness at the level of second messenger system

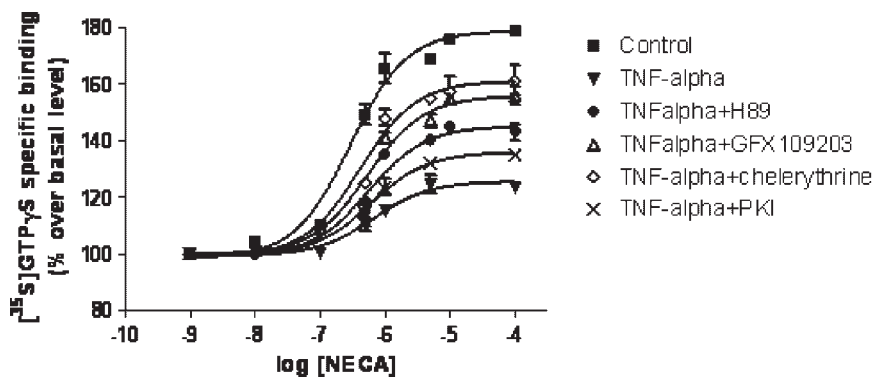

Fig. 3. NECA-mediated activation of A_{2B} AR-G protein coupling: concentration-response curves. ADF cells were pre-incubated without or with kinase inhibitors (1 μM H89; 1 μM GFX109203; 50 nM PKI; 6 μM chelerythrine) for 15 min and then treated with or without TNF-alpha 1,000 U/ml for 3 h. The ability of different NECA concentrations (10 nM–100 μM) to stimulate [^{35}S]GTPγS binding was then assessed. Data, reported as mean ± SEM (from five different experiments), are expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%. Control cells: $EC_{50} = 281 \pm 17.3$ nM; TNF-alpha: $EC_{50} = 616 \pm 11.54$ nM, $P < 0.001$ versus control; TNF-alpha+ H89: $EC_{50} = 550 \pm 5.77$, $P < 0.05$ versus TNF-alpha; TNF-alpha+ GFX109203: $EC_{50} = 536 \pm 11.54$, $P < 0.05$ versus TNF-alpha; TNF-alpha+ PKI: $EC_{50} = 603 \pm 12$, $P > 0.05$ versus. TNF-alpha; TNF-alpha+ chelerythrine: $EC_{50} = 418 \pm 19.6$, $P < 0.001$ versus. TNF-alpha. Control cells: $E_{max} = 178.5\% \pm 10.25$; TNF-alpha: $E_{max} = 123.6 \pm 1.35\%$, $P < 0.05$ versus control; TNF-alpha+ GFX109203: $E_{max} = 155.7 \pm 3.2\%$, $P < 0.05$ versus TNF-alpha; TNF-alpha+ chelerythrine: $E_{max} = 161.25 \pm 5.2\%$, $P < 0.05$ versus TNF-alpha.

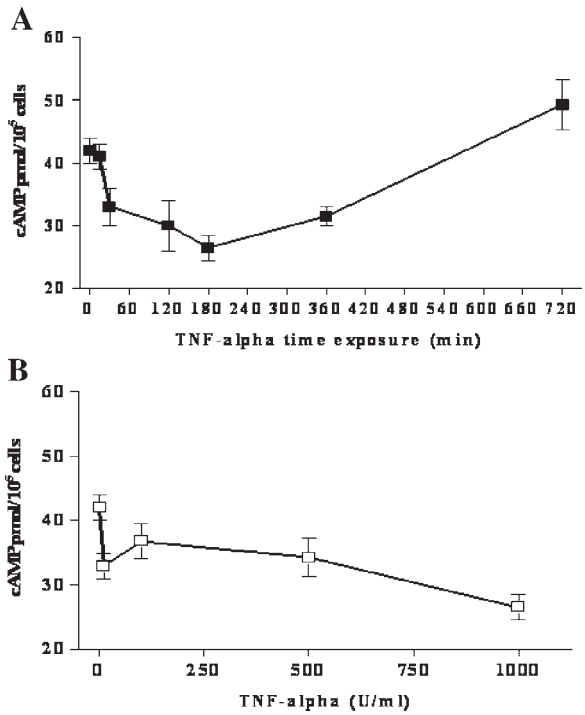

Fig. 4. TNF-alpha effect on the A_{2B} AR-mediated cAMP accumulation: time (**A**) and concentration dependence (**B**). The ability of 1 μM NECA (in the presence of 100 nM SCH 58261) to stimulate cAMP accumulation was evaluated in ADF cells following cell treatment without or with 1,000 U/ml TNF-alpha for different times (15 min–12 h, A) or following cell treatment with different TNF-alpha concentrations (10–1,000 U/ml) for 3 h (B). Data, reported as mean ± SEM (from three different experiments), are expressed as cAMP levels (pmol/10^5 cells).

($P > 0.05$ vs. TNF-alpha alone). The cell pre-exposure to H89 and GFX109203 together, induced a nearly complete recovery of the A_{2B} AR functional responsiveness ($P > 0.05$ vs. control). These data, according to those obtained in GTPγS binding, suggested TNF-alpha induced A_{2B} AR desensitization through a mechanism mainly involving PKC pathway.

Therefore, we investigated the effects of TNF-alpha on the time and concentration-response curves of agonist NECA. In control cells, the agonist NECA induced a time-dependent accumulation of intracellular cAMP with a maximal effects following 15 min (Fig. 6). TNF-alpha treatment caused a decrease in the maximal agonist response without any changes in kinetic agonist response. This effect appeared to be counteracted by cell pre-incubation with kinase inhibitors, H89, GFX109203, and chelerythrine. Wortmannin and PKI inhibitors did not affect TNF-alpha-mediated effects on agonist response.

Therefore, we evaluated the effects of TNF-alpha on A_{2B} AR agonist concentration-response curves. The agonist concentration-response curves and the relative EC_{50} values, obtained following cell incubation with TNF-alpha in the absence or in the presence of kinase inhibitors, are reported in Figure 7. As observed in GTP binding, TNF-alpha reduced A_{2B} AR agonist potency and efficacy in stimulating cAMP accumulation. This effect appears to be counteracted by GFX109203 and chelerythrine which induce a partial recovery in agonist potency and completely restore in agonist maximal efficacy.

A_{2B} AR Expression and Phosphorylation Levels in Control and Short-Term TNF-Alpha Treated Cells

To investigate the molecular mechanisms involved in the TNF-alpha-mediated A_{2B} AR desensitization, A_{2B} AR expression and phosphorylation levels were evaluated following cell treatment with TNF-alpha (1,000 U/ml) for different times.

As a first step, we investigated the TNF-alpha time-dependent effects on A_{2B} AR protein expression (Fig. 8A) and threonine-phosphorylation levels (Fig. 8B,C). Results showed in Figure 8B,C demonstrated TNF-alpha induced a time-dependent increase in the basal A_{2B} AR phosphorylation levels, with a maximal effect following 3-h cell exposure ($P < 0.01$ all columns vs. TNF-alpha 3 h; Fig. 8B). On the contrary, no significant changes in A_{2B} AR expression levels were detected (Fig. 8A). The kinetic of A_{2B} AR phosphorylation induced by the cytokine correlated with data obtained in functional studies suggesting receptor phosphorylation may account for A_{2B} AR desensitization observed following 3-h incubation.

As shown in Figure 9, the TNF-alpha-mediated A_{2B} AR phosphorylation was significantly prevented by PKA (optical density = 0.4 ± 0.19; $P < 0.01$ vs. TNF-alpha alone) and PKC (optical density = 0.52 ± 0.11; $P < 0.01$ vs. TNF-alpha alone) inhibitors. Cell pre-incubation with PKA and PKC inhibitors together induced a nearly complete inhibition of the TNF-alpha-mediated A_{2B} AR phosphorylation (optical density = 0.46 ± 0.08; $P > 0.05$ vs. control and $P < 0.01$ vs. TNF-alpha alone). On the contrary, PI3K inhibitor was not able to modulate TNF-alpha-mediated A2B AR phosphorylation levels (optical density = 1.21 ± 0.11; $P > 0.05$ vs. TNF-alpha alone).

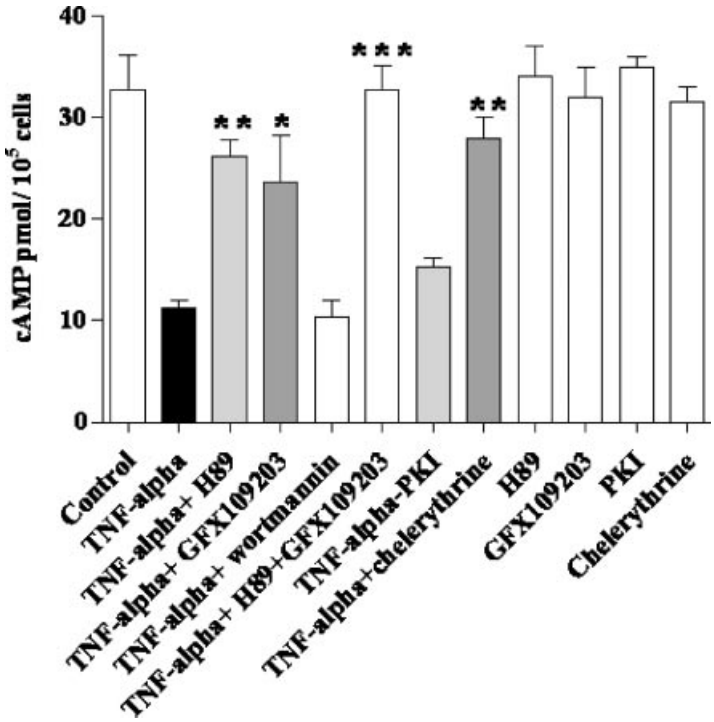

Fig. 5. Effects of intracellular kinase inhibitors on the TNF-alpha-mediated A_{2B} AR desensitization. ADF cells were pre-incubated without or with kinase inhibitors (1 μM H89; 1 μM GFX109203; 500 nM wortmannin; 50 nM PKI; 6 μM chelerythrine) for 15 min. Cells were then treated with or without TNF-alpha 1,000 U/ml for 3 h and the ability of 1 μM NECA (in the presence of 100 nM SCH 58261) to stimulate cAMP accumulation was evaluated. Data, reported as mean ± SEM (from three different experiments), are expressed as cAMP (pmol/10^5 cells). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus TNF-alpha.

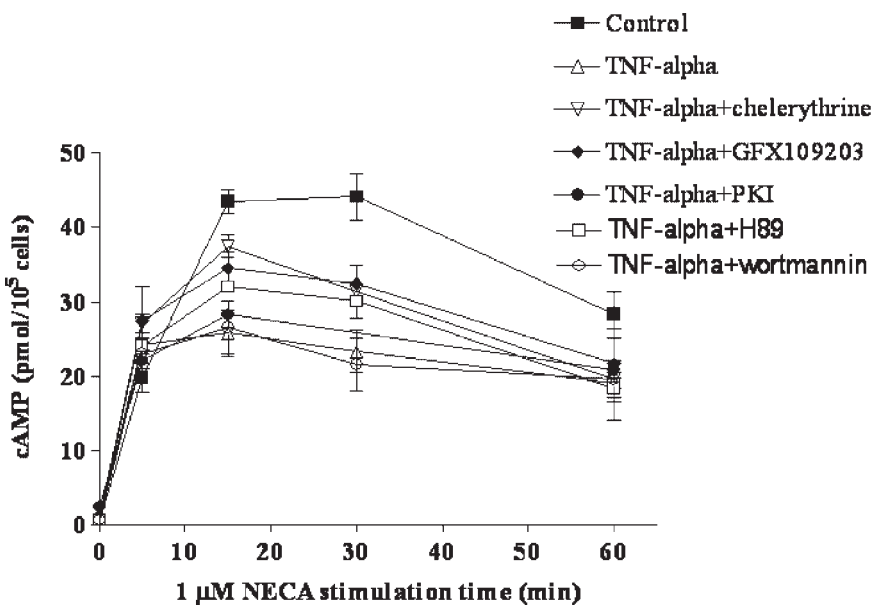

Fig. 6. TNF-alpha effects of NECA-mediated cAMP accumulation: time-dependence. ADF cells were pre-incubated without or with kinase inhibitors (1 μM H89; 1 μM GFX109203; 50 nM PKI; 6 μM chelerythrine; 500 nM wortmannin) for 15 min and then treated with or without TNF-alpha 1,000 U/ml for 3 h. The ability of 1 μM NECA (in the presence of 100 nM SCH 58261) to stimulate cAMP accumulation was evaluated at different incubation times (5–60 min). Data, reported as mean ± SEM (from three different experiments), are expressed as cAMP (pmol/10^5 cells).

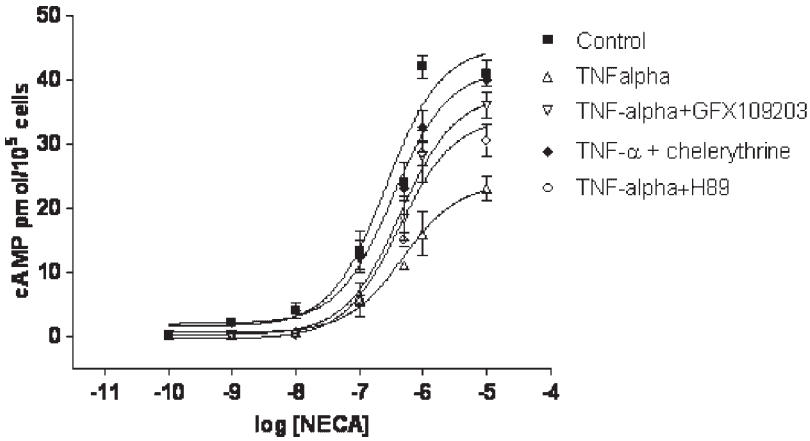

Fig. 7. NECA-mediated cAMP accumulation: concentration-response curves. ADF cells were pre-incubated without or with kinase inhibitors (1 μM H89; 1 μM GFX109203; 60 μM chelerythrine) for 15 min and then treated with or without TNF-alpha 1,000 U/ml for 3 h. The ability of different NECA concentrations (1 nM–10 μM) to stimulate cAMP accumulation was then assessed. Data, reported as mean ± SEM (from three different experiments), are expressed as cAMP (pmol/10^5 cells). Control cells: $EC_{50} = 263 \pm 8.7$ nM; TNF-alpha: $EC_{50} = 479 \pm 17$ nM, $P < 0.001$ versus control; TNF-alpha + H89: $EC_{50} = 419 \pm 13$ nM, $P < 0.05$ versus TNF-alpha; TNF-alpha+ GFX109203: $EC_{50} = 437 \pm 7.5$, $P < 0.05$ versus TNF-alpha; TNF-alpha+ chelerythrine: $EC_{50} = 337 \pm 11$ nM, $P < 0.01$ versus TNF-alpha. Control cells: $E_{max} = 41 \pm 2.0$; TNF-alpha: $E_{max} = 23 \pm 4.2$, $P < 0.01$ versus control; TNF-alpha+ GFX109203: $E_{max} = 36 \pm 1.73$, $P < 0.05$ versus TNF-alpha; TNF-alpha+ chelerythrine: $E_{max} = 40 \pm 2.9$, $P < 0.05$ versus TNF-alpha.

DISCUSSION

In the present work, we demonstrated ADF short-term cell exposure to the proinflammatory cytokine TNF-alpha induced impairment of the A_{2B} AR responsiveness causing, in particular, a reduction of the agonist effectiveness in promoting receptor-G protein coupling and in stimulating adenylate cyclase pathway. These effects appeared to be also associated with an increase in the receptor basal phosphorylation levels, which may be responsible for the observed functional effects. These data suggested TNF-alpha was able to induce a short-term A_{2B} AR desensitization.

G protein-coupled receptor (GPCR) heterologous regulation is mediated by different second messenger activated kinases including PKA, PKC, and PI3K which might affect receptor signaling acting at level of the receptor itself and/or affecting G protein and second messenger activity [Casas-Gonzalez et al., 2000; Jo et al., 2002; Tan et al., 2003]. Since PKA, PKC, and PI3K have been demonstrated to be activated both by the cytokine TNF-alpha and by A_{2B} ARs [Linden et al., 1999; MacEwan, 2002; Zhang et al., 2002], and to represent a converging point for the signaling pathways activated by the two receptors, we investigated the involvement of these protein kinases in the TNF-alpha-mediated regulation of A_{2B} functional responses. The obtained results demonstrated that: (i) wortmannin, a selective PI3K inhibitor, did not affect TNF-alpha-mediated A_{2B} ARs regulation; (ii) PKA inhibitor, PKI, did not affect heterologous A_{2B} ARs desensitization whereas significant effects were obtained using the less selective PKA inhibitor, H89. This difference can be explained by the fact that H89, even if marketed as selective for PKA, is able to partially inhibit other kinases [Davies et al., 2000]; (iii) both PKC inhibitors, GFX109203 and chelerythrine, were able to counteract TNF-alpha-mediated effects. Both in GTP and cAMP assay we demonstrated these inhibitors were able to induce a partial recovery in agonist potency and to completely restore agonist maximal efficacy. All together these results suggest that PKC, but not PKA and PI3K, is the protein kinase mainly involved in TNF-alpha-mediated A_{2B} AR desensitization. This is not surprising since these receptors do no appear to contain consensus sequences for phosphorylation by PKA [Mundell and Kelly, 1998]. In addition, PKC signaling was proposed as a contribution to A_{2B} ARs-modulated interleukin synthesis suggesting this kinases as converging signaling between cytokines and A_{2B} ARs system [Feoktistov and Biaggioni, 1995].

The effects of TNF-alpha on A_{2B} AR functional responsiveness were not accompanied by alteration in the receptor expression levels,

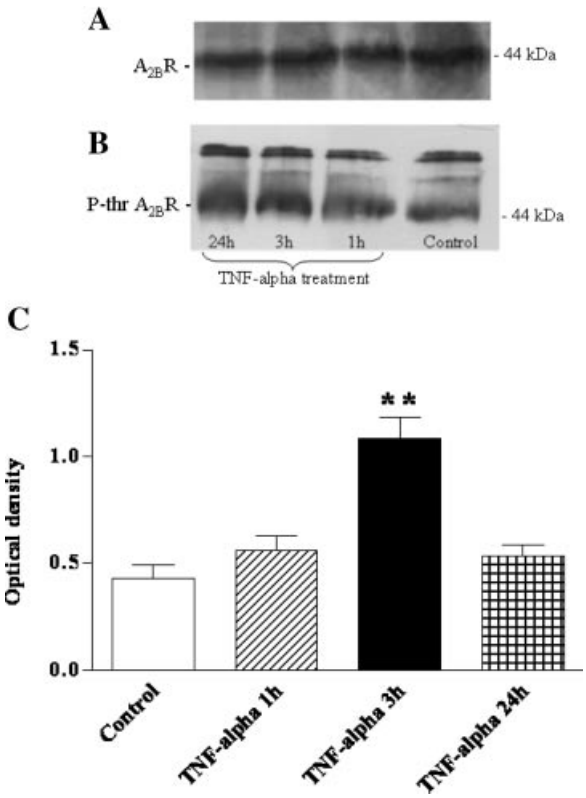

Fig. 8. TNF-alpha time-dependent effects on the A_{2B} AR threonine-phosphorylation levels. ADF cells were treated with medium alone (control) or with TNF-alpha (1,000 U/ml) for different time (1, 3, 24 h). Cells were then lysed and an amount of 1 mg of proteins was immunoprecipitated using a polyclonal antibody against A_{2B} AR. The immunoprecipitates were probed with an anti-A_{2B} AR antibody (**A**) and then with an anti-phosphothreonine antibody (**B**) to detect the threonine receptor phosphorylation levels. Phosphothreonine immunoreactive bands (at around 44 kDa) were quantified by densitometric scanning and normalized by the corresponding A_{2B} AR immunoreactive bands. **C**: Graph bar represents the normalized data (mean ± SEM; n = 3) obtained by the densitometric scanning of phosphor-threonine immunoreactive bands, normalized by corresponding A_{2B} AR immunoreactive bands. $^{**}P < 0.01$ all columns versus TNF-alpha 3 h.

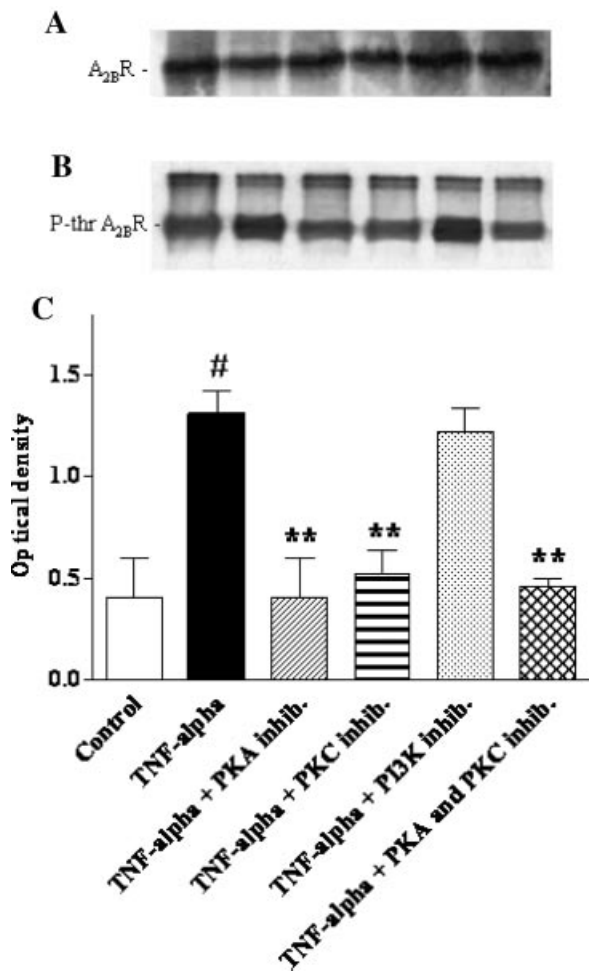

Fig. 9. Effects of intracellular kinase inhibitors on the TNF-alpha-mediated A_{2B} AR threonine-phosphorylation levels. Cells were pretreated without or with PKA inhibitor (H89, 1 μM), PKC inhibitor (GF109203X, 1 μM) or PI3K inhibitor (wortmannin, 500 nM) or PKA and PKC inhibitors (1 μM) together for 15 min and then stimulated with TNF-alpha (1,000 U/ml) for 3 h. The immunoprecipitate samples, after being probed with the antibody specific for A_{2B} AR, were subjected to analysis with the anti-A_{2B} AR antibody (**A**) and then with the anti-phosphothreonine antibody (**B**) to detect the extent of the threonine receptor phosphorylation levels. **C**: Graph bar represents the normalized data (mean ± SEM; n = 3) of the phosphothreonine immunoreactive bands quantified by densitometric scanning and normalized by the corresponding A_{2B} AR immunoreactive bands. $^{**}P < 0.01$ versus TNF-alpha.

such as already demonstrated in our previous work [Trincavelli et al., 2004] and also reported for IFN-gamma [Kolachala et al., 2005]. Anyway, other works described that different inflammatory signals (i.e., LPS) regulate A_{2B}R signaling also increasing A_{2B} receptor expression level [Nemeth et al., 2003]. These differences could be ascribed to the use of different cell systems and to different cytokine stimulation times.

The A_{2B} AR responsiveness to chronic TNF-alpha exposure in ADF cells had been previously characterized: a significant up-regulation of A_{2B} AR functioning was detected when TNF-alpha cell treatment was prolonged up to 24 h [Trincavelli et al., 2004], inducing a reduced agonist-mediated receptor phosphorylation and, in turn, a delay in receptor desensitization processes, an increase in agonist-mediated receptor-G protein coupling and in adenylyl cyclase activation.

Comparing the present data with those previously described [Trincavelli et al., 2004], we might suggest a dualistic and opposite effect of TNF-alpha on the A_{2B} AR functioning after short- and long-term cytokine cell exposure. These dualistic time-dependent effects induced by TNF-alpha parallel with those obtained in astroglioma [Fredholm and Altiok, 1994] and endothelial cells [Nguyen et al., 2003], demonstrating the A_{2B} AR-mediated effects are differently modified by inflammatory mediators in a time-dependent manner, and might suggest the AR system as a promising target to selectively modulate cerebral damage progression in the acute and chronic phases.

In response to injuries, resident CNS cells generate proinflammatory cytokines, which may contribute to acute and chronic brain disease pathogenesis through the recruitment of immune cells and the activation of glial cells [for review see Lucas et al., 2006]. However, cytokines may display a dual role with detrimental acute effects but also beneficial effects in long-term repair and recovery. In particular, TNF-alpha, synthesized by macrophages, astrocytes, and microglia with a rapid kinetic following cerebral trauma or ischemia (up to 2 h) [Yu and Lau, 2000; Yin et al., 2003; Vitarbo et al., 2004], has been demonstrated to be proinflammatory during the acute phase of the inflammatory response and immunosuppressive during the chronic phase [Wang and Shuaib, 2002]. Zhong et al. [2005] have showed a synergic effect between A_{2B} ARs and hypoxia in activating human lung fibroblasts, suggesting the adenosine system/inflammatory mediator interaction as a common mechanism in both central and peripheral tissues. In particular in astroglial cells, a bi-directional functional cross-talk between cytokines and A_{2B} AR adenosine system has been described as an important mechanism to regulate the cerebral damage progression [Fredholm and Altiok, 1994; Rosi et al., 2003; Trincavelli et al., 2004]. The A_{2B} AR has been implicated both in the stimulation [Feoktistov et al., 2002; Zhong et al., 2004; Zhang et al., 2005] and inhibition [Kreckler et al., 2006; Yang et al., 2006] of cytokines release, which in turn can exacerbate or reduce inflammation processes.

Since A_{2B} ARs have been involved in inflammatory processes [Linden, 2006], we can speculate that the A_{2B} AR activity impairment induced by the cytokine may represent a potential feed-back mechanism to control the inflammatory effects induced by neurotoxic compounds and proinflammatory cytokines, released in the acute phase of brain damage. On the contrary, in the chronic phase of brain injury, TNF-alpha, by a significant up-regulation of A_{2B} AR responsiveness, contributes to the reactive astrogliosis, suggesting A_{2B} ARs as a novel target for neuroinflammatory and neurodegenerative diseases.

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