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Adenosine receptors control HIV-1 Tat-induced inflammatory responses through protein phosphatase

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Abstract

Recently, adenosine has been proposed to be a "metabolic" switch that may sense and direct immune and inflammatory responses. Inflammation and pro-inflammatory cytokine production are important in development of HIV-1 associated dementia, a devastating consequence of HIV-1 infection of the CNS. The HIV-1 protein Tat induces cell death in the CNS and activates local inflammatory responses partially by inducing calcium release from the endoplasmic reticulum. Because activation of adenosine receptors decreases production of the pro-inflammatory cytokine TNF-α in several experimental paradigms both in vitro and in vivo, we hypothesized that adenosine receptor activation would control both increased intracellular calcium and TNF-α production induced by Tat. Treatment of primary monocytes with Tat significantly increased the levels of intracellular calcium released from IP_3 stores. Activation of adenosine receptors with CGS 21680 inhibited Tat-induced increases of intracellular calcium by 90 ± 8% and was dependent on protein phosphatase activity because okadaic acid blocked the actions of CGS 21680. Tat-induced TNF-α production was inhibited 90 ± 6% by CGS 21680 and concurrent treatment with okadaic acid blocked the inhibitory actions of CGS 21680. Using a model monocytic cell line, CGS 21680 treatment increased cytosolic serine/threonine phosphatase. Together, these data indicate that A_{2A} receptor activation increases protein phosphatase activity, which blocks IP_3 receptor-regulated calcium release and reduction of intracellular calcium inhibits TNF-α production in monocytes.

Keywords: Monocytes/macrophages; Immunodeficiency diseases; Cytokines; Cellular activation; Signal transduction

Introduction

At the end of 2003, an estimated 40 million people worldwide were living with HIV. HIV-1 associated dementia (HAD) is an important manifestation of HIV infection (Nath and Berger, 2004) which include clinical symptoms of ataxia, memory loss, and cognitive, motor and behavioral abnormalities. Before the development of highly active anti-retroviral therapy (HAART), incidence of HIV dementia was 20% in HIV-1-infected individuals (Manji and Miller, 2004). While HAART therapy has lowered the number of patients who develop HAD by 50% (Manji and Miller, 2004), HAD remains an important neurological complication of HIV infection. Pathologically, HAD is associated with astrogliosis, neuronal loss, demyelination, and inflammation (Rappaport et al., 1999; Wiley et al., 1998). Development and progression of HAD is thought to associate with infiltration of peripheral immune cells into

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium levels; FBS, fetal bovine serum; Fura-2/AM, fura-2-acetoxymethyl ester; H89, [*N*-2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide hydrochloride; IP_3, inositol 1,4,5 trisphosphate; PKA, protein kinase A; PLC, phospholipase C; PKC, protein kinase C; TNF-α, tumor necrosis factor alpha.

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the CNS rather than high viral burden indicating that the viral infection results in secondary events causing pathology. Central to these secondary events may be the HIV-1 protein Tat. Tat is produced by HIV-infected cells and assists in viral replication by binding to a transactivation response element in HIV-1 mRNAs and promoting transcriptional initiation and elongation (Rappaport et al., 1999; Saha and Pahan, 2003). Tat can also bind to and activate transcription of host genes whose products may be involved in development of HAD. Tat binds to the genes encoding collagen and fibronectin, two extracellular matrix proteins involved in cell adhesion (Rappaport et al., 1999). Tat also binds to the gene encoding the pro-inflammatory cytokine TNF-α and activates its transcription (Rappaport et al., 1999). TNF-α is postulated to play a central role in HAD development by damaging astrocytes function and potentially reducing their neuroprotective actions (Jones et al., 1998; Nath et al., 1996, 1998; Saha and Pahan, 2003). TNF-α expression is increased in macrophages isolated from HIV-1 patients and Tat injected in the brains of mice increases TNF-α in cerebrospinal fluid (CSF) and increases monocyte/macrophage infiltration into the central nervous system (CNS) (Jones et al., 1998; Philippon et al., 1994; Pu et al., 2003; Rappaport et al., 1999; Saha and Pahan, 2003).

Infiltration of monocytes into the CNS is an early feature of HIV-1 infection and later recruitment of macrophages may be a key step in CNS disease development. Tat is produced and released by infected macrophages and microglia and can then interact with uninfected macrophages (Rappaport et al., 1999). Macrophages respond by producing inflammatory mediators including TNF-α. TNF-α may contribute to HAD by directly inducing neurotoxicity, by synergizing with other viral toxins (gp120) or excitotoxins (glutamate) (Saha and Pahan, 2003), and by increasing leukocyte migration across the blood–brain barrier by increasing chemokine production and adhesion molecule expression on endothelial cells (Nottet et al., 1997; Pu et al., 2003; Rappaport et al., 1999). Tat also induces toxicity within the CNS by causing Ca^{2+} dysregulation, excitotoxicity, and altering cytokine production (Benjouad et al., 1993; Chen et al., 1997; Cheng et al., 1998; Haughey et al., 1999; Johnston et al., 2001; Jones et al., 1998; Rappaport et al., 1999). Because Tat appears to promote an inflammatory response within the CNS that contributes to HAD pathology, understanding endogenous immune modulators will give us insight into mechanisms of disease progression and may identify novel targets for drug intervention.

One such immune modulator is the purine nucleoside adenosine. Adenosine can influence the function of monocytes, macrophages, neutrophils, T cells, and dendritic cells suggesting that adenosine may play a key role in regulating inflammation and immune responses (Bouma et al., 1994; Bowlin et al., 1997; Cronstein, 1994; Eigler et al., 1997; Hasko et al., 1996, 2000; Link et al., 2000; Mayne et al., 2001; McWhinney et al., 1996; Ohta and Sitkovsky, 2001; Revan et al., 1996; Sajjadi et al., 1996; Sitkovsky, 2003). More specifically, numerous studies have identified the ability of adenosine and adenosine receptor agonists to control cytokine production. Adenosine controls TNF-α in monocytes and macrophages activated by several inflammatory stimuli and may also control TNF-α production induced by Tat. As a result, we designed experiments to identify signaling mechanisms involved in adenosine-mediated inhibition of Ca^{2+} and TNF-α production as indicators of the potential protective and anti-inflammatory properties of adenosine.

Results

Adenosine inhibits Tat-induced increases of $[Ca^{2+}]_i$

As previously reported for primary cultures of human neurons, astrocytes (Haughey et al., 1999) and macrophages (Mayne et al., 2000), Tat induced a transient and statistically significant increase of $[Ca^{2+}]_i$ in primary human monocytes (Fig. 1a). Tat-induced increases of $[Ca^{2+}]_i$ appeared to originate from intracellular stores because the increases were similar when Tat was applied in the absence or presence of extracellular calcium (Fig. 1b). Xestospongin C, an inhibitor of IP_3 receptor-dependant calcium release, significantly ($P < 0.001$) reduced ATP-induced (data not shown) and Tat-induced initial calcium transients from 742 ± 44 to 42 ± 9 nM (Fig. 1d) indicating that the source of intracellular calcium was IP_3 receptor-regulated stores in the endoplasmic reticulum. Confirming previous observations (Mayne et al., 2000), the increases of $[Ca^{2+}]_i$ by Tat were not significantly affected by pre-treatment with 10 μM ryanodine (data not shown) indicating that increases in $[Ca^{2+}]_i$ are not from ryanodine receptor-regulated stores.

Adenosine receptor activation inhibited calcium mobilization from IP_3 receptor-regulated stores induced by Tat and ATP

Addition of the adenosine receptor agonist CGS 21680 (100 nM) blocked Tat-induced increases of $[Ca^{2+}]_i$ (Fig. 1a) and significantly inhibited ATP-induced increases of $[Ca^{2+}]_i$ (Fig. 1c). Inhibition of Tat-induced increases of $[Ca^{2+}]_i$ by CGS 21680 were completely blocked by the adenosine A_{2A} receptor antagonist ZM 241385 suggesting that these effects were mediated by adenosine A_{2A} receptors (Fig. 1b). Endogenous adenosine regulated Tat-induced increases of $[Ca^{2+}]_i$ because both ZM 241385 and adenosine deaminase significantly increased $[Ca^{2+}]_i$ (Fig. 1d). ZM 241385 blocked the actions of endogenous adenosine on A_{2A} receptors and adenosine deaminase increased endogenous adenosine levels by inhibiting adenosine metabolism.

Signal transduction systems involved in CGS 21680 regulation of Tat-induced increases of $[Ca^{2+}]_i$

We next determined signaling events downstream of A_{2A} receptor activation responsible for the above observed

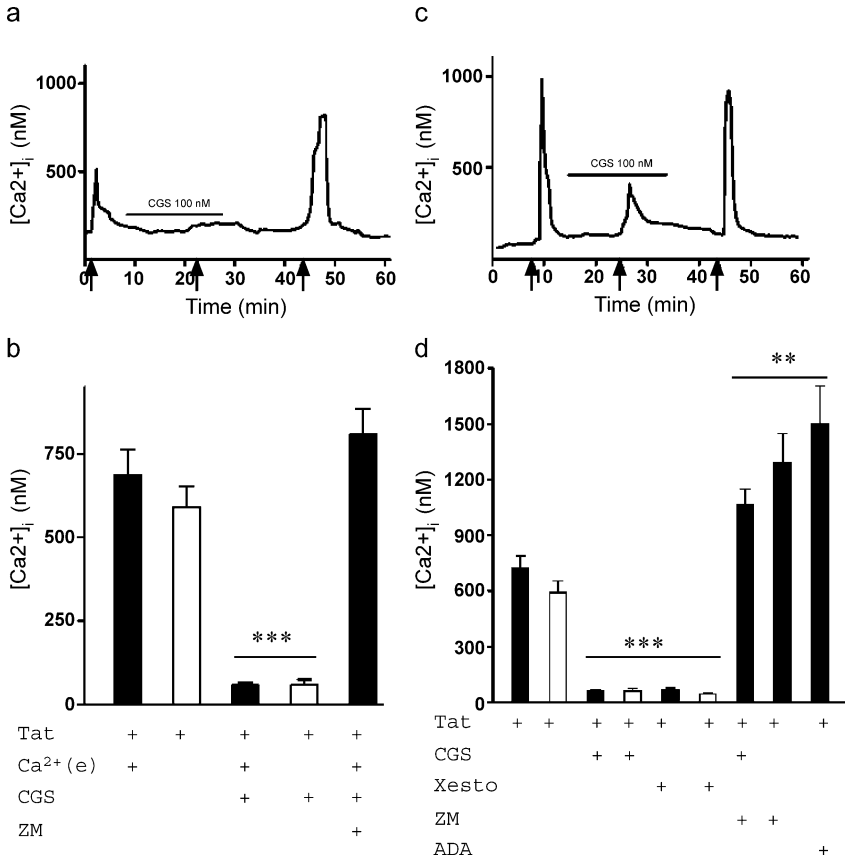

Fig. 1. Activation of A_{2A} adenosine receptors inhibits calcium release from IP_3 receptor-regulated stores in monocytes. (a) Tat-induced increases of $[Ca^{2+}]_i$ were blocked by CGS 21680. (b) Independent of the presence (dark bars) or absence (white bars) of extracellular calcium, CGS 21680 (100 nM) significantly decreased Tat-induced increases of $[Ca^{2+}]_i$, and the actions of CGS 21680 were blocked by the adenosine A_{2A} receptor antagonist ZM 241385 (10 nM). The removal of extracellular calcium did not alter intracellular calcium levels when cells were treated with the A_{2A} agonist and/or antagonist alone (not shown). (c) CGS 21680 decreased significantly ATP-induced increases of $[Ca^{2+}]_i$. (d) Tat-induced increases of $[Ca^{2+}]_i$ originated from IP_3 receptor-regulated pools because xestospongin C (1 μM) blocked Tat-induced increases of $[Ca^{2+}]_i$ irrespective of the absence or presence of extracellular calcium. Blocking A_{2A} receptors with ZM 241385 (10 nM) or decreasing adenosine levels using adenosine deaminase (2 IU/ml) before Tat treatment significantly increased $[Ca^{2+}]_i$. Levels shown are mean ± SEM values from experiments conducted using monocytes purified from six individuals. Arrows indicate additions of Tat or ATP. $^{***}P < 0.001$ vs. Tat and $^{**}P < 0.01$ vs. Tat.

effects on $[Ca^{2+}]_i$. We reported previously that inhibition of phospholipase C with 1 μM U71322 or type 1 protein kinase C with 1 μM H7 attenuated significantly Tat-induced increases in TNF-α production by approximately 90% in primary macrophages (Mayne et al., 2000). Because A_{2A} receptors activate, through G_s protein linked processes, an increase in cAMP levels and PKA activity (Olah and Stiles, 1995), we hypothesized that these signaling pathways were involved in A_{2A} receptor-mediated inhibition of Tat-induced increases of $[Ca^{2+}]_i$ originating from IP_3-regulated stores. However, decreases in Tat-induced increases of $[Ca^{2+}]_i$ by CGS 21680 were not reversed by 1 μM SQ 22536, an inhibitor of adenylyl cyclase or 1 μM H89, a selective inhibitor of PKA (Fig. 2). Because A_{2A} receptors have been linked to activation of protein phosphatases (Revan et al., 1996), we determined whether an inhibitor of serine/threonine protein phosphatase, okadaic acid, would reverse the actions of CGS 21680. Okadaic acid did not inhibit Tat-induced increases of $[Ca^{2+}]_i$, but did reverse the actions of CGS 21680 on Tat-induced increases of $[Ca^{2+}]_i$ (Fig. 2) indicating that the effect of CGS 21680 on $[Ca^{2+}]_i$ was mediating by serine/threonine protein phosphatase.

CGS 21680 inhibits Tat-induced TNF-α production through serine/threonine protein phosphatase

Previously, we reported that Tat induces the release of calcium from IP_3 receptor-regulated pools of intracellular calcium and that this calcium partially regulates Tat-induced TNF-α production (Mayne et al., 2000). Because CGS 21680-mediated inhibition of $[Ca^{2+}]_i$ required protein phosphatase, we tested the hypothesis that the inhibitory actions of CGS 21680 on TNF-α production were similarly regulated by protein phosphatase activation. As expected, TNF-α production was significantly inhibited by CGS 21680 when added 30 min before the addition of Tat (100 nM) or LPS (100 nM) (Figs. 3a and b). The anti-TNF-α effects of CGS 21680 were mediated by A_{2A} receptors because the A_{2A} receptor antagonist ZM 214385

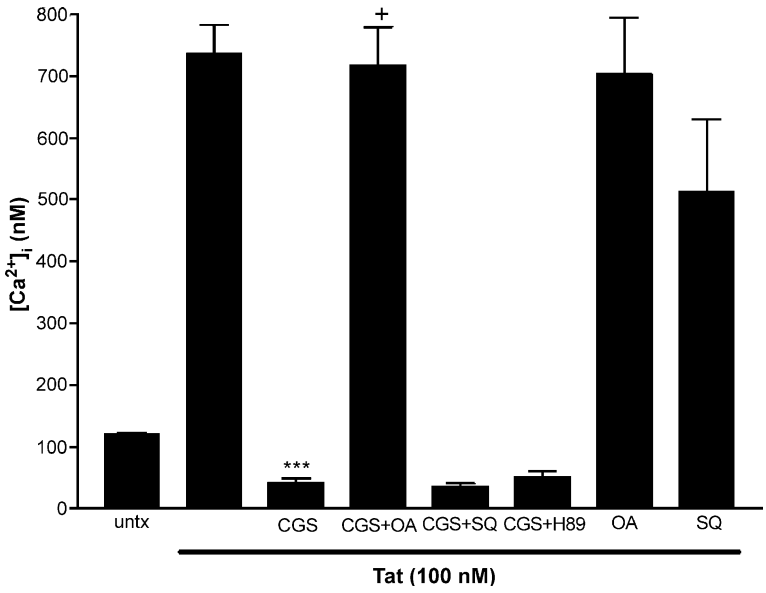

Fig. 2. A_{2A} receptor activation blocked Tat-induced increases of $[Ca^{2+}]_i$ by mechanism(s) dependent on protein phosphatase activation and independent of cAMP and PKA. A_{2A} receptor activation with CGS 21680 decreased Tat-induced increases of $[Ca^{2+}]_i$, and the effects of CGS 21680 were reversed by okadaic acid (100 nM) but not by SQ 22536 (1 μM) or H89 (1 μM). Okadaic acid (1 or 10 nM) did not reverse the actions of CGS 21680 (not shown). Levels shown are mean ± SEM values from experiments conducted using monocytes purified from six individuals. $^{**}P < 0.001$.

completely reversed the actions of CGS 21680 (Figs. 3a and b). Okadaic acid (100 nM) reversed the inhibitory actions of CGS 21680 against Tat- and LPS-induced increases of TNF-α production (Figs. 3a and b) indicating that the anti-TNF-α effects of CGS 21680 required protein phosphatase.

CGS 21680 increases cytosolic serine/threonine phosphatase activity

Because okadaic acid, a serine/threonine phosphatase inhibitor, reversed the anti-TNF-α effects of CGS 21680, we hypothesized that adenosine receptor activation by CGS 21680 increases phosphatase activity. We measured phosphatase activity in cytosolic and membrane protein extracts of pro-monocytic U937 cells as a model of monocytes because our enzyme assay required cell numbers that could not be obtained from primary monocyte cultures. To verify that U937 cells would provide a model similar to human monocytes with respect to signaling pathways activated by CGS 21680, we measured TNF-α production in these cells induced by phorbol esters in the presence and absence of CGS 21680 (Fig. 4a). The anti-TNF-α effects of CGS 21680 and the requirement of protein phosphatase was similar in U937 cells and primary human monocytes (Figs. 3 and 4a). CGS 21680 increased serine/threonine phosphatase activity in cytosolic extracts after 10 min. These increases in enzyme activity decreased to basal levels after 60 min (Fig. 4b). In membrane preparations, we found no significant increases in enzyme activity up to 60 min following CGS 21680 administration.

Discussion

Inflammation is a central feature in the early stages of HIV-1 infection of the CNS and may be involved in later stages of CNS disease development. Stimulatory actions of the HIV-1 protein Tat on macrophages and monocytes infiltrating into the CNS during infection may contribute to inflammation and tissue damage associated with HAD. Tat is thought to cause dysfunction and death of neural cells by inducing dysregulation of cytokines and chemokines produced by infiltrating immune cells within the brain (Rappaport et al., 1999). Tat itself also induces neuronal death by facilitating the excitotoxic actions of glutamate (Haughey et al., 2001; Self et al., 2004). Understanding endogenous mechanisms for controlling neuroinflammation is essential for designing therapies that can target the inflammatory and toxic actions of Tat.

The purine nucleoside adenosine was recently proposed to act as a "metabolic switch" capable of sensing different degrees of inflammation and subsequently triggering an appropriate immune response (Sitkovsky, 2003). This proposal was based on findings that adenosine levels are increased during inflammatory responses in a manner that reflects severity of inflammation. Although adenosine is a well-studied molecule, signaling mechanisms governing its anti-inflammatory actions are not well understood. As an endogenous immune regulator, adenosine may play an important role therapeutically for CNS inflammatory disorders and its signaling mechanisms become important as novel therapeutic targets.

Consistent with other studies, we found that activation of adenosine A_{2A} receptors significantly inhibited TNF-α

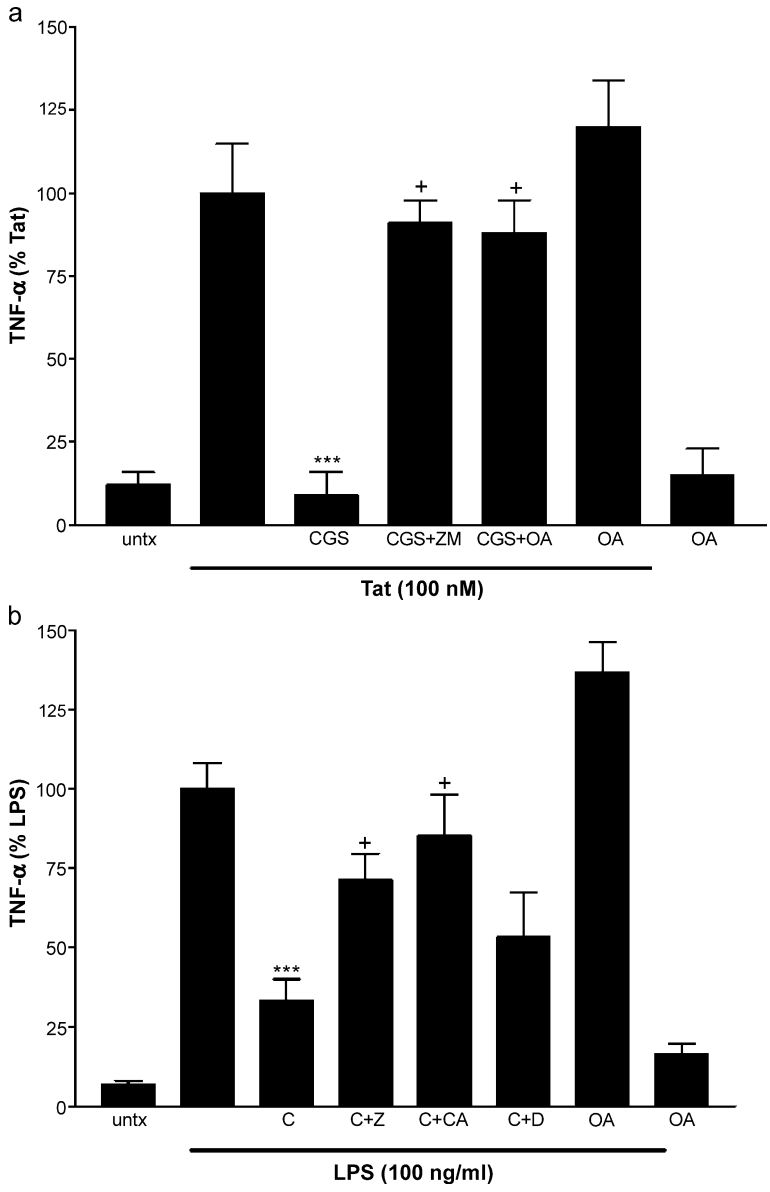

Fig. 3. Adenosine A_{2A} receptor activation inhibits TNF-α production in monocytes treated with pro-inflammatory agents Tat or LPS in a protein phosphatase dependent manner. CGS 21680 (C) significantly decreased the production of TNF-α stimulated by Tat (a) or LPS (b) and the adenosine A_{2A} receptor antagonist ZM 241385 (Z) reversed these effects. The serine/threonine protein phosphatase inhibitor okadaic acid (OA; 100 nM) alone did not affect levels of TNF-α production, but reversed the inhibitory actions of CGS 21680 on TNF-α production. The anti-TNF-α effects of CGS 21680 were unchanged by the tyrosine phosphatase inhibitor dephostatin (D; 1 μM). TNF- α levels shown are mean ± SEM values from experiments conducted using monocytes purified from six individuals. ***$P < 0.001$ vs. Tat or LPS, $^{+}P < 0.001$ vs. CGS+Tat or CGS+LPS.

production (Hasko et al., 2000; Mayne et al., 2001; Sitkovsky, 2003). Because TNF-α may play a pathological role in HAD, this finding suggests that pharmacological manipulations of adenosine systems may be an effective therapeutic strategy against HIV-1 encephalopathy. In addition, endogenous adenosine appears to be involved in regulating TNF-α production and intracellular calcium because the A_{2A} receptor antagonist ZM 241385 alone elevated levels of TNF-α and intracellular calcium (Figs. 1 and 3).

Here, we report that the inhibitory actions of adenosine receptor activation on Tat-induced release of calcium from IP_3 receptor-regulated stores and on Tat-induced TNF-α production were dependent on protein phosphatase. Importantly, decreases in intracellular calcium by CGS 21680 did not involve activation of adenylyl cyclase and PKA. This finding suggests that the signaling mechanisms governing control of calcium levels are independent of classical A_{2A} receptor pathways (Collis and Hourani, 1993; Fredholm et al., 2001). Serine/threonine phosphatases may be an important part of this novel signaling pathway and have also been implicated in regulation of neutrophil function by adenosine A_2 receptors (Revan et al., 1996). By regulating actions of adenosine in neutrophils and monocytes, protein

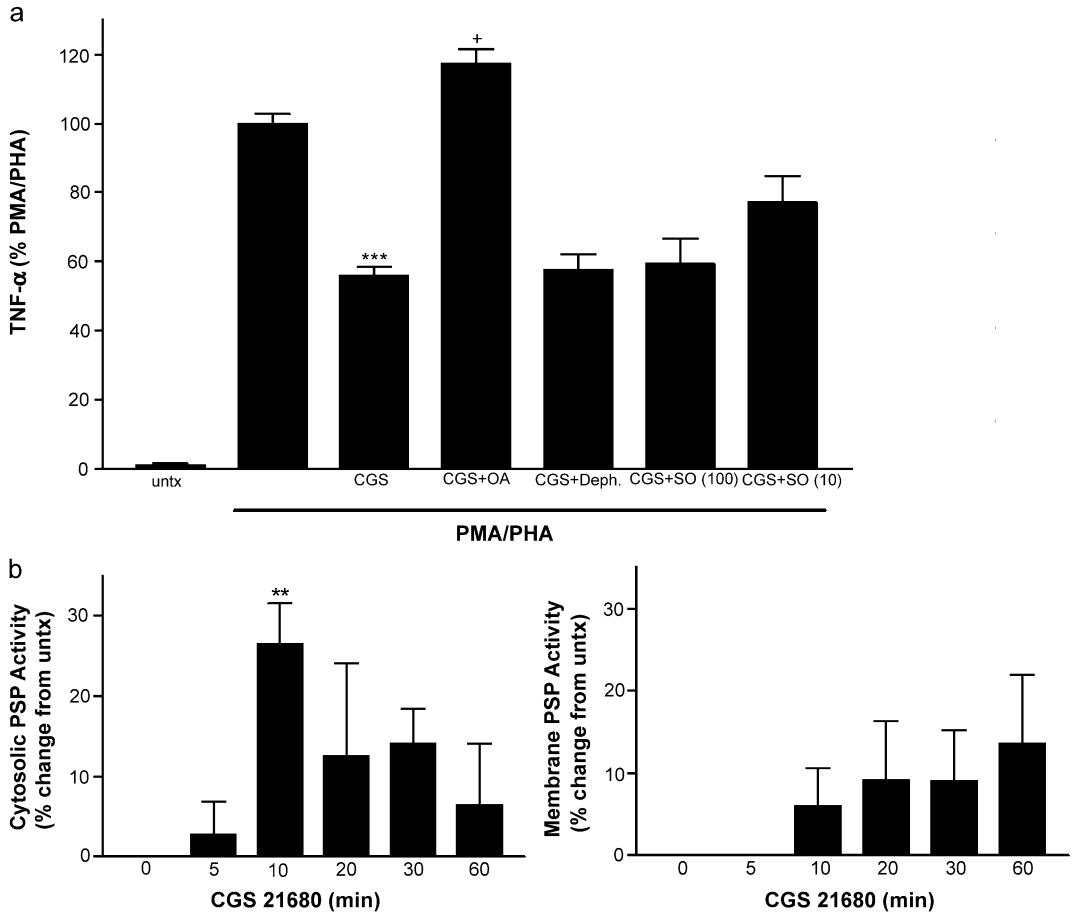

Fig. 4. Adenosine A_{2A} receptor activation increases cytosolic serine/threonine phosphatase activity. (a) U937 cells were stimulated with PMA/PHA for 4 h alone or in combination with CGS 21680, okadaic acid to inhibit serine/threonine phosphatase, dephostatin to inhibit tyrosine phosphatase, or sodium orthovanadate to inhibit tyrosine phosphatase. The anti-TNF-α effects of CGS 21680 were reversed by okadaic acid. (b) Serine/threonine phosphatase activity was measured in cytosolic and membrane extracts from U937 cells treated with CGS 21680 for time intervals ranging from 5 to 60 min. $^{**}P < 0.01$ vs. time 0, $^{***}P < 0.001$ vs. untx, $^{+}P < 0.001$ vs. PMA/PHA + CGS.

phosphatases may mediate acute anti-inflammatory actions of adenosine. Protein phosphatases regulate cellular signaling events and direct diverse and broad cellular functions including neurotransmission, muscle contraction, glycogen synthesis, T cell activation, cellular plasticity, and differentiation (Aggen et al., 2000). Eukaryotic cells contain tyrosine and serine/threonine phosphatase activity (Hunter, 1995), and within the serine/threonine category, there are at least eight major types (Aggen et al., 2000). Of these, four major isoforms have been studied in detail including PP1, PP2A, PP2B (calcineurin), and PP2C. Because our studies used okadaic acid, findings here implicate a role for PP1 and/or PP2A. Doses of okadaic acid used in our studies (nM range) suggested that okadaic acid is acting primarily on PP1 because PP1 is blocked by only high nM concentrations of okadaic acid (Cohen et al., 1989) and we did not observe inhibition of the anti-TNF-α effects of CGS 21680 with okadaic acid at 1 nM concentration (data not shown).

Blocking protein phosphatase activation can reverse several signal transduction events including the release of calcium from IP_3 receptor-regulated stores (Hu et al., 1997; Jimenez et al., 1999). The cellular targets of protein phosphatase may include protein kinase C and PLC, both of which are critical for Tat-induced TNF-α production and are regulated by intracellular calcium (Kirk and Richardson, 1995; Liang and Morley, 1996; Norenberg et al., 1997, 1998; Sexl et al., 1997). Protein phosphatase may also dephosphorylate IP_3 receptors and block their activation (Jayaraman et al., 1996). Our previous results showing that blocking PKC activation reduces both calcium release from IP_3 receptor-regulated pools and TNF-α production is consistent with this latter mechanism of action (Mayne et al., 2000).

Although our previous findings indicated that intracellular calcium release from IP_3R-regulated stores is involved in TNF-α production (Mayne et al., 2000), findings reported here do not prove that A_{2A} mediated inhibition of TNF-α production is solely dependent on release of calcium from IP_3 receptor-regulated stores. Rather, our data indicate that A_{2A} activation and inhibition of TNF-α is dependent on protein phosphatase activity and in part, reduction of high levels of intracellular calcium. The latter statement is supported by our previous observation

that blockade of calcium release from IP_3R stores using xestospongin C (an inhibitor of IP_3 receptors) in macrophages reduced TNF-α production by approximately 30% (Mayne et al., 2000).

Based on these observations, we have proposed an adenosine A_{2A} receptor signaling pathway outlined in Fig. 5. Tat stimulation of human monocytes increases intracellular calcium levels by inducing calcium release from endoplasmic reticulum pools. Increased intracellular calcium induces TNF-α transcription and protein production by activating calcium-dependent signaling pathways including PKC and NF-κB. By activating serine/threonine protein phosphatase activity, adenosine receptor activation efficiently controls intracellular calcium levels and TNF-α production induced by the HIV-1 protein Tat. Protein phosphatase may control calcium release by decreasing phosphorylation of endoplasmic reticulum IP_3. By controlling intracellular calcium, adenosine receptors may control TNF-α production at a pre-transcriptional level. In a human monocyte cell line stimulated with phorbol esters, adenosine receptor activation controlled TNF-α production post-transcriptionally by decreasing mRNA stability (Fotheringham et al., 2004, revised manuscript submitted). Adenosine receptors may then control TNF-α induced by Tat pre-transcriptionally through intracellular calcium and post-transcriptionally through mRNA stability. Activating cell signaling pathways that can control cytokine production both pre- and post-transcriptionally may allow adenosine receptors to efficiently control cytokine production in monocytes and macrophages induced by multiple stimuli. Clearly, TNF-α protein production and regulation involves complex signaling events in monocytes and macrophages and the consistent regulation of TNF-α by A_{2A} receptor activation implicates adenosine as an important endogenous regulator of pro-inflammatory cytokine production.

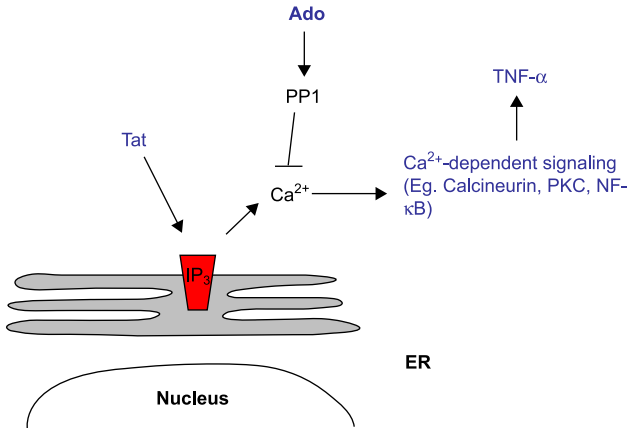

Fig. 5. Schematic of proposed signaling mechanisms mediating the anti-inflammatory actions of adenosine receptors. The HIV-1 protein Tat induces TNF-α production in monocytes in part by increasing release of calcium from IP_3-receptor-regulated stores in the ER. Activation of adenosine receptors increases cytosolic PP1 activity that can control both calcium release and TNF-α production. PP1 may control TNF-α production by blocking calcium release and by controlling Ca^{2+}-dependent signaling such as transcription factor activation. Because phosphorylation of IP_3 receptors increases calcium release, PP1 may control calcium by decreasing receptor phosphorylation. Finally, by controlling both Ca^{2+} and cytokine production, adenosine receptor activation may have significant effects of cellular signaling, particularly pathways dependent on Ca^{2+}-like calcineurin.

Materials and methods

Chemicals and recombinant $Tat_{1–72}$

Fura-2-acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes Inc. (Eugene, OR). EGTA, L-leucine methyl ester and neomycin were purchased from Sigma (St. Louis, MO). Xestospongin C and okadaic acid were purchased from Calbiochem (San Diego, CA). All drugs were dissolved in Krebs buffer except for ryanodine that was dissolved in ethanol and diluted with Krebs (final ethanol concentration was 0.05%). $Tat_{1–72}$ was prepared and purified as described previously (Ma and Nath, 1997) and biological activity was confirmed by activation of β-galactosidase in transfected HeLa cells (AIDS Repository, NIH). Tat protein was lyophilized and stored at −80 °C until taken for assay. The serine/threonine phosphatase assay kit was purchased from New England Biolabs (Pickering, ON).

Primary monocyte culture preparation

Human peripheral blood mononuclear cells (PBMC) were purified from whole blood obtained from healthy volunteers as outlined previously (Mayne et al., 2000). Briefly, peripheral blood monocytes were isolated from whole blood from healthy donors using a Ficoll isolation gradient. The white blood cell layer was plated on T75 flasks over night in RPMI medium containing 10% FBS and 1% antibiotics. Adherent monocytes were scraped and cultured at a density of 2×10^5 cells/ml for 7 days in RPMI supplemented with 10% FBS and antibiotics. All cells were maintained at 37 °C in a humidified growth chamber supplemented with 5% CO_2.

U937 cell culture preparations

Human pro-monocytic U937 cells (ATCC CRL 1593.2; batch F12641) were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (pH 7.2) media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Chen et al., 1997). Cells were maintained at 37 °C in a humidified growth chamber supplemented with 5% CO_2.

Levels of intracellular calcium

($[Ca^{2+}]_i$): $[Ca^{2+}]_i$ were determined using the Ca^{2+}-specific fluorescent probe Fura-2/AM as described previously (Haughey et al., 1999; Mayne et al., 2000). Cover

slips containing Fura-2 loaded monocytes were placed in a LU-CSD Leiden cover slip dish situated in a PDMI-2 open perfusion micro-incubator (Medical Microsystems Corp., Greenvale, NY) and the cells were superfused at the rate of 2 ml/min with Krebs buffer pre-warmed to 37 °C. Cells were excited at 340 and 380 nm, and emission was recorded at 510 nm with a video-based Universal Imaging system (EMPIX, Mississauga, ON). R_{max}/R_{min} ratios were converted to nM $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). All images were acquired by real time averaging of 16 frames of each wavelength that included a background reference subtraction from each of the acquired images. Tat (100 nM) was loaded into glass micropipettes (1.0 mm OD, 0.78 mM ID) pulled to a final outer tip diameter of <1.0 μm. Micropipettes were positioned approximately 3 cell bodies away from target cells and Tat was pressure-applied to cells (3 × 100 ms, 8 psi) using a Picospritzer (General Valve Corp, Fairfield, NJ). Peak increases of $[Ca^{2+}]_i$ were determined by calculating the difference between maximum $[Ca^{2+}]_i$ levels achieved during a 5-min period following Tat applications and baseline $[Ca^{2+}]_i$.

Tat treatment of primary monocytes and TNF-α assays

Primary monocyte cultures (200,000 cells/ml) were maintained in RPMI media supplemented with 10% FBS and antibiotics for 7 days following purification. The cells were subsequently stimulated for 4 h with 100 nM Tat. In some experiments, cells were pretreated for 30 min with pharmacological agents before addition of Tat. In experiments where effects on TNF-α production were determined, cells were incubated for 4 h at 37 °C following the addition of Tat and supernatants were collected, centrifuged at 700 × *g* for 5 min and analyzed for TNF-α by ELISA (Mayne et al., 2000). Positive controls were incubated for 4 h with 100 ng/ml lipopolysaccharide (LPS, *Escherichia coli* type 055:B5, Sigma). For all experiments, Tat was used immediately following thawing and tubes and tips used with Tat were siliconized.

U937 cells were cultured to a density of 800,000 cells/ml in RPMI growth media and were plated in 96 well plates with fresh media at a density of 800,000 cells/ml 1 h before each experiment. TNF-α production was induced using 10 ng/ml phorbol-12-myristate-13-acetate (PMA) and 5 μg/ml phytohemagglutinin (PHA) for 4 h at 37 °C. PMA is a phorbol ester that activates protein kinase C, a well-characterized signaling pathway that transmits signals in activated immune cells. PHA is a mitogen that stimulates cell proliferation and agglutination, both consequences of monocyte and lymphocyte activation. TNF-α levels were determined by ELISA (Chen et al., 1997).

Serine/threonine phosphatase activity assays

Serine/threonine protein phosphatase activity was determined using a commercially available enzyme assay kit (New England Biolabs). Briefly, myelin basic protein (MyBP) was labeled on serine and threonine residues with [γ-^{33}P] ATP by protein kinase A. Experimental protein extracts containing active protein phosphatases were incubated with ^{33}P labeled MyBP. Protein phosphatase activity was determined by measuring ^{33}P released from MyBP during incubation with experimental protein extracts, indicating degree of dephosphorylation of labeled MyBP. Experiments were performed on U937 cells cultured to a density of 1 000 000 cells/ml in RPMI 1640 media supplemented with 10% FBS. Following experimental treatments, cells were collected on ice and pellets were washed once in ice-cold PBS. After washing, cell pellets (approximately 10 × 10^6 cells/pellet) were resuspended and homogenized. Whole cells, nuclei and large debris were removed by centrifugation for 10 min at 300 × *g* at 4 °C. Supernatant was collected and centrifuged for 20 min at 18 000 × *g* at 4 °C. Supernatant was collected (cytosolic fraction) and pellets (membrane fraction) were resuspended in 1× protein phosphatase buffer (NEB kit). Protein content in both fractions was determined by Bradford assay (BioRad Laboratories). 100 ng of protein from membrane or cytosol fractions were used to measure serine/threonine phosphatase activity (New England Biolabs kit) as per manufacturer's instructions.

Statistical analysis

Significant differences between groups were determined by one-way ANOVA with Tukey's post hoc comparisons. For all tests, statistical significance was considered to be at $P < 0.05$ (Instat2, Graphpad Software, San Diego, CA).

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