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Human immunodeficiency virus type 1 Tat protein induces an intracellular calcium increase in human monocytes that requires DHP receptors: involvement in TNF-α production

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Abstract

HIV-1 Tat protein, acting at the cell membrane, stimulates the production by human monocytes of TNF-α, a cytokine implicated in both HIV-1 replication and pathogenesis. Here, we analyze, in primary human monocytes, the mechanisms involved in Tat-stimulated calcium mobilization and its relationship with TNF-α production. We show that the Tat protein induces a calcium signal by mobilizing calcium from extracellular stores. This calcium signal is totally blocked when cells are stimulated in the presence of DHP receptor inhibitors such as nimodipine or calcicludine, thus suggesting the implication of this L-type calcium channel. By using RT-PCR amplification, Western blot with antibodies directed against the α1D subunit, binding assays with specific agonists or antagonists, and inhibition with specific antisense oligonucleotides, we show that DHP receptors are expressed and functional in primary human monocytes. Interestingly, we demonstrate that Tat-induced calcium mobilization is tightly linked to TNF-α production, thus indicating that Tat-induced mobilization and TNF-α production are entirely mediated by DHP receptors, as shown by their total inhibition by nimodipine, calcicludine, or anti-α1D antisense oligonucleotides.

Keywords: HIV-1; Tat; Calcium; Monocyte; L-type calcium channel; TNF-alpha; Dihydropyridine; Antisense oligonucleotide

Introduction

HIV-1 principally infects CD4+ lymphocytes, monocytes/macrophages, and dendritic cells (Cameron et al., 1996; Fauci, 1988). This infection results in profound and selective depletion of CD4+ T cells during progression to AIDS. However, before CD4+ T-lymphocyte depletion in HIV-1-infected patients, a generalized immune dysfunction is observed, which impairs a variety of immune functions, including both specific and innate immunity. This immune disorder is associated mainly with the loss of the T-cell proliferative response to stimulation with recall antigens and with the alteration of the Th1/Th2 balance of cytokines, including TNF-α, IL-1, IL-4, IL-6, IL-10, and IL-12 (Clerici and Shearer, 1993; Clerici et al., 1994; Emilie et al., 1994, Graziosi et al., 1994; Poli, 2002). Several studies have suggested that, during infection, HIV-1 patients develop a progressive loss of the Th1 cellular immune response, the most potent effector against viruses. In parallel to the decrease in Th1 response, an increase in the Th2 immune response was observed, but this seems to be inefficient against HIV-1 infection.

In agreement with this finding, HIV-1-infected patients have been reported to have high levels of TNF-α in sera, brain (Wesselingh et al., 1993), and in vitro in supernatants of monocytes, PBMC, and alveolar macrophages from

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AIDS patients (Wright et al., 1988). Moreover, several studies have demonstrated that TNF-α plays a major role in progression to AIDS: it does so by acting directly on HIV-1 replication through activation of NF-κB (Duh et al., 1989) and indirectly through cytotoxicity mediated by alteration of the cellular redox state (Folks et al., 1989; Westendorp et al., 1995a). Our group and others have shown that the HIV-1 Tat protein induces TNF-α production in monocytes and macrophages (Bennasser and Bahraoni, 2002; Bennasser et al., 2002; Chen et al., 1997; Mayne et al., 2000).

Tat, a 14-kDa protein containing 86–101 amino acids encoded by two exons, is known for its transactivating activity. Its absence can be complemented in trans and is essential for efficient HIV-1 viral replication. Tat binds to the secondary structure sequence Tat activation region (TAR) at the 5′ terminus of viral RNA during transcription. It thereby enables the recruitment of cellular factors, thus constituting the complex cyclin T1/cdk 9, known as Tat-associated kinase (TAK), that phosphorylates the C-terminal domain of RNA polymerase 2, thereby activating transcription elongation. Tat also participates in the pathogenesis of HIV-1 infection by its ability to interact with different cell types and alters the complex network of cytokines involved in the regulation of the immune response. Tat is found in nanomolar concentrations in the serum of HIV-1-infected patients. It is secreted by infected cells and can act on other cells, infected or not. Thus, Tat contributes to immune system disorders, induces apoptosis of T lymphocytes (Westendorp et al., 1995b), inhibits MHC class I expression (Weissman et al., 1998), alters NK cell activity (Zocchi et al., 1998), and inhibits IL-12 production by dendritic cells and monocytes (Poggi et al., 1998).

We have previously shown that TNF-α and IL-10 production by human monocytes stimulated by Tat is, respectively, totally and partially dependent on the presence of calcium in the extracellular medium. A variety of cell functions, including gene expression, cell proliferation, cell death, and muscle contraction, are regulated by calcium (Carlsen and Villarin, 2002; Kuklina and Shirshev, 2001; Lewis, 2001; Orrenius et al., 2003), one of the most important second messengers of the cell. Mobilized calcium in immune cells may have different origins: the extracellular medium, intracellular stores (endoplasmic reticulum, mitochondria, etc.), or both, via various mechanisms. Different types of calcium channels are implicated in calcium mobilization from the extracellular medium: (i) voltage-operated calcium channels (VOCCs), type L, N, P/Q, or T, largely present on excitable and neuronal cells; their activation is mediated by depolarization of the plasma membrane; (ii) receptor-operated calcium channels (ROCCs), expressed on secretory cells and activated at nerve terminals following the binding of specific ligands (ATP, serotonin, glutamate, acetylcholine) to the extracellular domain of the channels; (iii) store-operated calcium channels (SOCCs), which are ubiquitous plasma membrane calcium channels activated in response to the release of intracellular calcium stores (Bootman et al., 2001). Therefore, the study of the involvement of HIV-1 Tat protein in calcium signaling in monocytes may provide some clue to understanding the effect of this protein on immune disorders.

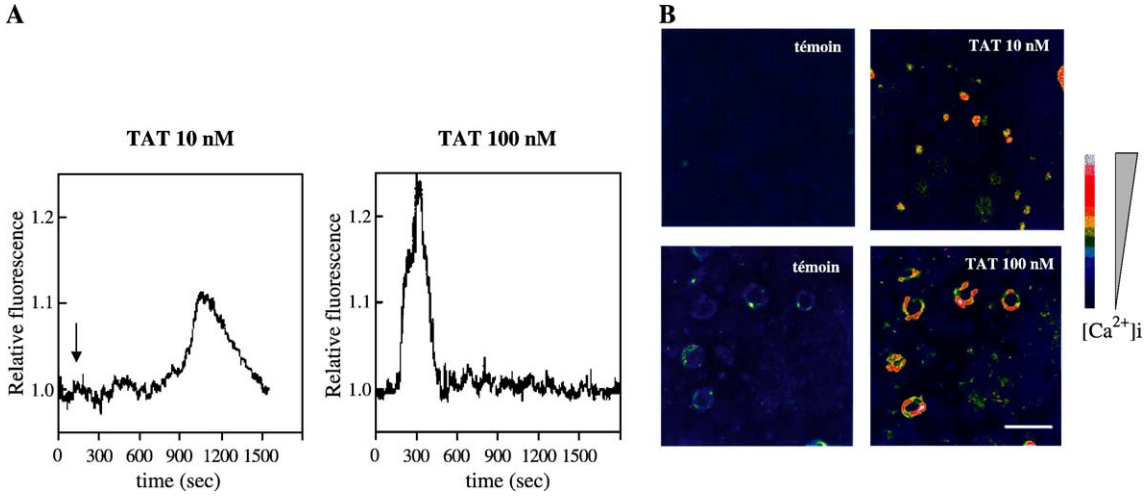

Fig. 1. Analysis of the intracellular variation of calcium concentration by microspectrofluorimetry (A) or confocal microscopy (B). (A) Monocytes preincubated with fluo-3-AM (5 μM) for 30 min before microscopic observation. After two washes, monocytes were stimulated by Tat 1–86 from HIV-1 Lai (10 and 100 nM) at the time indicated by the arrows. Relative fluorescence corresponds to the ratio of fluorescence emitted by stimulated cells to fluorescence emitted by resting cells. Each panel is a representative result of three independent experiments, in which 34 ± 8% of stimulated cells were positive. (B) Monocytes preincubated for 30 min with fluo-3-AM were observed with a confocal microscope (Zeiss, LSM 410) and stimulated by Tat (10 and 100 nM). Images with pseudocolors represent fluorescence variations in resting monocytes (control) and after stimulation by Tat. The images presented here correspond to the maximum response (t = 6 min for 10 nM Tat and t = 4 min for 100 nM Tat). On the color ladder, blue represents low levels of Ca^{2+} and red high levels. Similar results were obtained using HIV-1 SF-2 Tat.

In this study, we have investigated the origin of the calcium increase triggered by the HIV-1 Tat protein in human monocytes, the nature, the expression, and the functional state of the activated calcium channel, and the involvement of calcium in Tat-induced TNF-α production.

Results

Tat protein induces calcium increase in human monocytes

Monocytes from healthy blood donors were stimulated with recombinant Tat protein from HIV-1 Lai and SF2 isolates to determine whether these proteins induced a calcium increase. The calcium transients were investigated by following the variations in cytoplasmic-free calcium concentrations ([Ca2+]i) at the cellular level by using two complementary approaches, microspectrofluorimetry and confocal microscopy, with the cell-permeant calcium fluorescent probe fluo3-AM. Data presented in Fig. 1 show that the Tat protein elicited an increase in $[Ca^{2+}]i$. This increase was dose dependent, reaching 12% and 25% for Tat at concentrations of 10 and 100 nM, respectively (Fig. 1A). Interestingly, in addition to the similar dose-response effect observed by confocal microscopy, the most intense fluorescence appeared to be localized to the cell membrane (Fig. 1B). As a positive control, ionomycin (1 μM), a calcium ionophore, was used in all our experiments. Under these conditions, we observed a strong increase in $[Ca^{2+}]i$ that returned to baseline after 15-min stimulation (data not shown). These results show that Tat induces a transient calcium increase in primary monocytes.

Region 20–45 of HIV-1 Tat protein is involved in calcium mobilization in monocytes

The Tat protein contains several identified functional regions, including a cysteine-rich region (aa 20–31) involved in the transactivating activity, a core region (aa 31–47) responsible for binding to RNA, a basic region implicated in the nuclear localization of Tat (aa 48–57), glutamic acid-rich region involved in virus replication (aa 57–76), and an RGD peptide (aa 78–80) that can interact with integrins αvβ3 and α1β5.

To further characterize the implication of Tat protein in calcium increase, we next performed experiments to identify the domain of Tat that was involved. We thus used different Tat deletion mutants, produced as glutathione-S-transferase (GST) fusion proteins, including Tat 1–45 (RGD, glutamic, and basic domains deleted), Tat 20–72 (N-terminal domain deleted), and Tat 30–72 (cysteine-rich region deleted). The results depicted in Fig. 2 show that Tat 1–45 and Tat 20–72 were still able to induce calcium increase in monocytes, while no signal was induced by Tat 30–72 or the control GST at the same concentrations. Taken together, these data indicate that the active domain of Tat seems to be localized to region 20–45 of Tat.

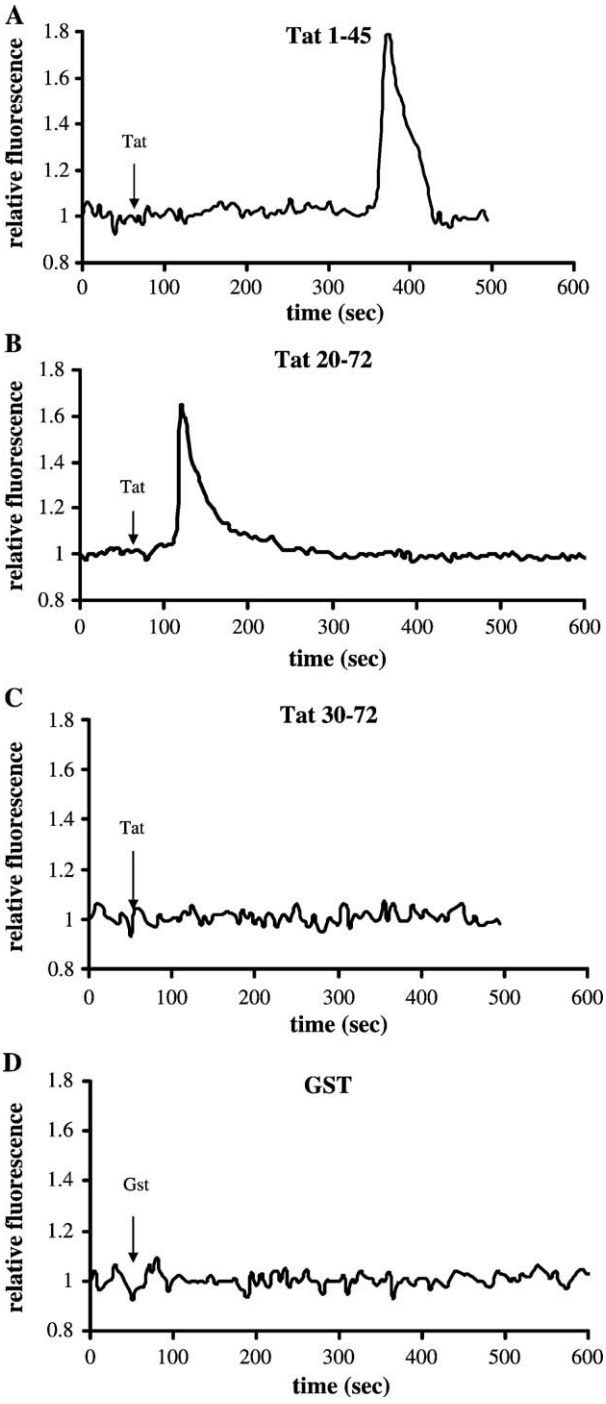

Fig. 2. HIV-1 Tat domain involved in the calcium signal triggered in human monocytes. Monocytes were loaded with fluo-3-AM as described in the legend of Fig. 1 and were then stimulated by different deleted HIV-1 SF-2 Tat mutants (A, Tat 1–45; B, Tat 20–72; C, Tat 30–72; D, GST) at the times indicated by the arrows. Time course of fluorescence was then analyzed by microspectrofluorimetry. Relative fluorescence corresponds to the ratio of fluorescence emitted by stimulated cells to fluorescence emitted by resting cells. Each panel is a representative result of three independent experiments, in which 31 ± 6% of stimulated cells were positive.

The calcium increase in Tat-stimulated monocytes has an extracellular origin

Calcium increase in human monocytes can have two origins: (i) intracellular stocks (cellular organelles) that can be activated mainly through IP_3 or ryanodine receptors and (ii) calcium from the extracellular medium that can enter cells following the activation of membrane channels, such as voltage-operated calcium channels (VOCCs), store-operated calcium channels (SOCCs), and receptor-operated calcium channels (ROCCs).

We thus sought to determine the origin of the calcium increase observed in Tat-stimulated monocytes. To this aim, we first used xestospongin C and TMB-8, inhibitors of intracellular calcium stores stimulated, respectively, by IP_3 or ryanodine. These two inhibitors had no effect on Tat-induced calcium increase in human monocytes (Figs. 3B and C). These results suggest that the calcium mobilized in Tat-stimulated monocytes was not released from intracellular stores. This conclusion cannot be explained by the oligomerization status of Tat in the presence or absence of calcium, since similar filtration chromatography profiles on sephacryl S-100 column were obtained when Tat was eluted in the presence or absence of calcium (data not shown). Moreover, when primary monocytes were stimulated by Tat in a calcium-free medium, no increase in $[Ca^{2+}]i$ was observed (Fig. 3D), whereas caffeine (10 mM), an activator of ryanodine receptors, continued to stimulate the mobilization of calcium (Fig. 3E) under these conditions.

Taken together, these results indicate that exposure of primary monocytes to Tat protein allows calcium entry from the extracellular medium, while calcium from inositol 1,4,5-triphosphate(IP_3)-regulated stores or caffeine-sensitive ryanodine receptor-regulated intracellular stores does not seem to be triggered by Tat. We thus investigated the channels involved at the cytoplasmic membrane of monocytes responsible for this entry of calcium from the extracellular medium.

DHP receptors are expressed in human monocytes

One putative candidate is the L-type calcium channel (voltage-sensitive DHP receptors), a channel known to be involved in the release of cytokines. L-type calcium channels are composed of five subunits: α1 (190 kDa), α2 (143 kDa), β (55 kDa), γ (30 kDa), and δ (24–27 kDa) (Campbell et al., 1988; Caterall et al., 1988, 1990; Taka-

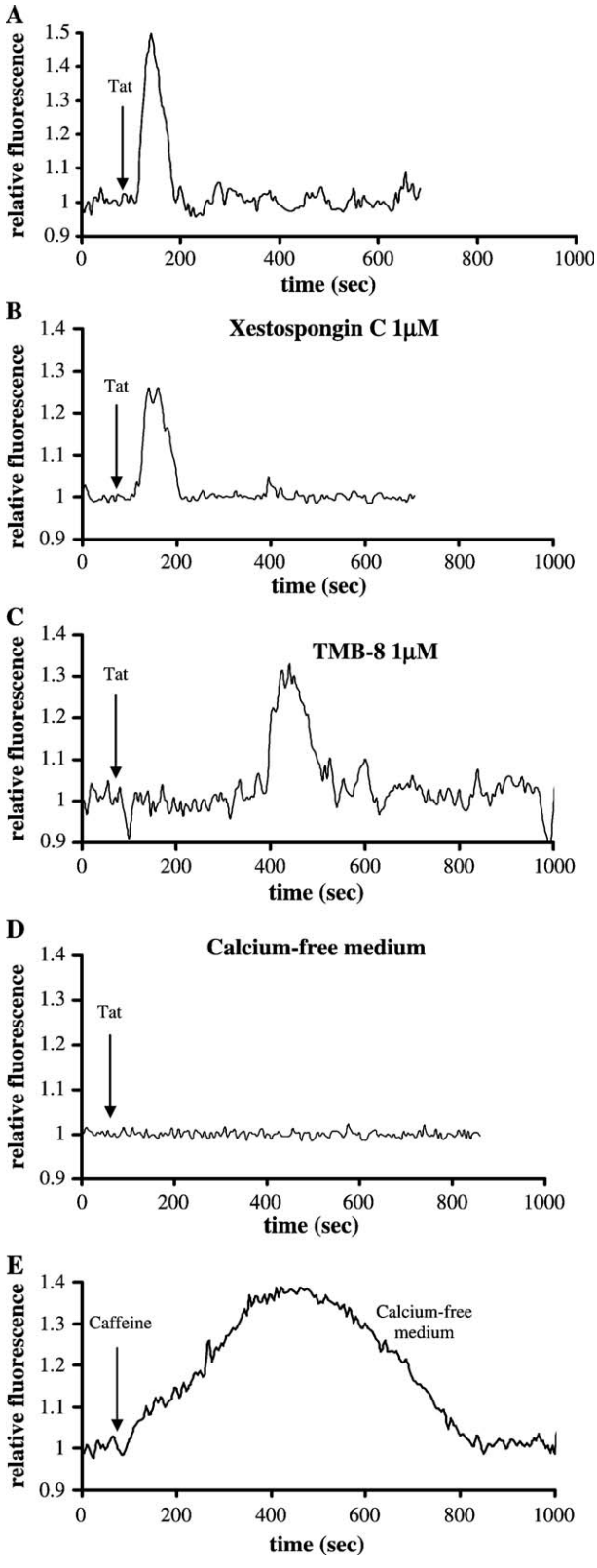

Fig. 3. Origin of the calcium influx stimulated by Tat in monocytes (see Fig. 1). (A) Calcium increase following stimulation by HIV-1 SF2 Tat. (B and C) Microspectrofluorimetric analysis of the involvement of intracellular stores of calcium using Xestospongin C (1 μM, B) and TMB-8 (1 μM, C), inhibitors of intracellular calcium stores stimulated, respectively, by IP_3 and ryanodine. To assess the role of the extracellular calcium, cells were preincubated with calcium-free medium prior to stimulation by Tat (D) or caffeine (E), an activation of intracellular calcium stores. Relative fluorescence corresponds to the ratio of fluorescence emitted by stimulated cells to fluorescence emitted by resting cells. Each panel is a representative result of three independent experiments, in which 36 ± 9% of stimulated cells were positive. Similar results were obtained using HIV-1 Lai Tat.

hashi et al., 1987). While each subunit is encoded by an individual gene, the α2 and δ subunits are the processed products of the same protein precursor (De Jongh et al., 1990; Jay et al., 1991). Different isoforms of the α1 subunit have been described: α1S (present in skeletal muscle), α1C (in heart and smooth muscle), and α1D (in neurons and neuroendocrine tissue).

We thus attempted to determine if these DHP receptors were present. To this end, we analyzed their expression at the transcriptional level by RT-PCR with oligonucleotides from a conserved region of the α1 subunit. Fig. 4A shows the amplification of a fragment of 700 nucleotides that corresponds to the expected size. The specificity and relevance of this result were further investigated by subsequent cloning and sequencing of the amplified 700-nucleotide fragment, which revealed a strong homology (>98%) with α1D (data not shown), thus indicating that the gene coding for the α1D subunit of DHP receptor was at least transcribed.

To confirm the expression of DHP receptors at the protein level, immunoblotting was performed with a membrane protein extract from primary human monocytes using an anti-α1D antibody raised against a synthetic peptide corresponding to the 805–825 region of the α1D subunit. A band was detected with an apparent molecular weight of 200 kDa compatible with the α1 expected molecular weight (Fig. 4B); moreover, when the same experiments were performed with Cos33 cells, this band was not detected. The specificity of this immunodetection was further confirmed by competition with the soluble synthetic peptide aa 805–825 (5 μg/ml). After preincubation of the cell protein extract with the peptide, the 200-kDa band was totally absent (Fig. 4B, lane).

Finally, expression of DHP receptors at the monocyte surface membrane was visualized by confocal microscopy. We did this first by showing that dihydropyridine labeled with a fluorescent probe (DHP-bodipy, 25 nM), a specific ligand for DHP receptors, could bind to monocytes (Fig. 5). This binding was specific since it was strongly inhibited by prior incubation of cells with the nonfluorescent agonist of DHP receptors Bay K- (100 μM) (Fig. 5). Secondly, we further demonstrated the relationship between the synthesis, expression, and DHP-bodipy labeling in human monocytes by targeting α1C and α1D mRNA with specific antisense oligonucleotides. Primary human monocytes were preincubated with α1C or α1D antisense oligonucleotides (1, 10, and 20 μM), or with the corresponding sense

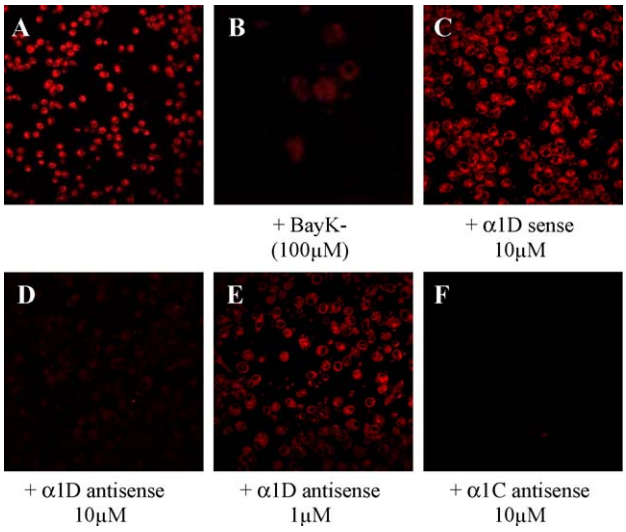

Fig. 5. Identification of DHP receptors on the surface of human monocytes. After preincubation of monocytes with ST-bodipy-DHP fluorescent dye (used to stain the α1 subunit of DHP receptors), fluorescence was observed with 546/590 nm excitation emission dichroic filters under a confocal microscope (Zeiss, LSM 410). Cells were untreated (A) or preincubated with BayK8644- (B), sense oligonucleotide for the α1D subunit of DHP receptors (C), antisense oligonucleotides for α1D 10 μM (D), 1 μM (E), and α1C 10 μM (F).

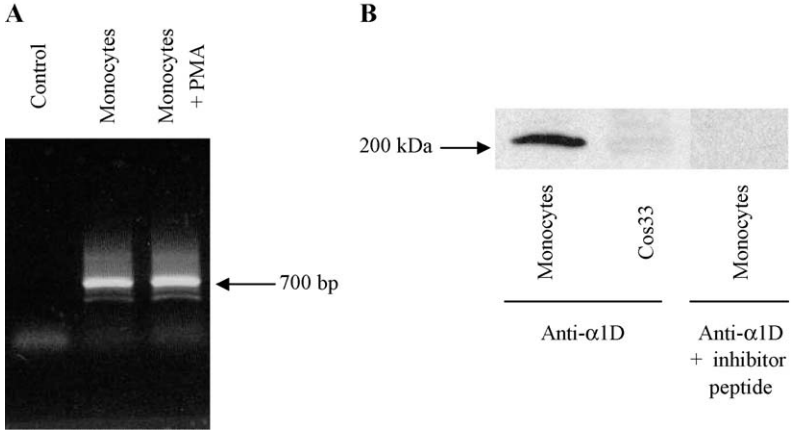

Fig. 4. Presence of DHP receptors in human monocytes. (A) RT-PCR using oligonucleotides 5′-catgtagaagctgatgaa-3′ and 5′-ttcttcatgatgaacatctt-3′ on mRNA from human monocytes. (B) Western blot: 100 μg of membrane proteins from human monocytes and Cos33 cells was electrophoresed on an acrylamide gel, then an antibody raised against the α1D subunit of DHP receptors was used for revelation. As a control for specificity, the same antibody previously incubated with its specific peptide (5 μg/ml) was used.

sequences as negative control, and DHP receptor expression was evaluated by DHP-bodipy labeling. As shown in Figs. 5C–F, treatment of monocytes with α1C or α1D antisense oligonucleotides strongly inhibited DHP-bodipy binding, even at 1 μM, with α1C oligonucleotides, and, in a dose-dependent manner, with those related to α1D (Figs. 5D–E). Preincubation of monocytes with the control sense sequence under the same conditions had no effect on the binding of DHP-bodipy (Fig. 5C). We also used a panel of antisense oligonucleotides directed against α1C and α1D and found no inhibitory effect with some antisense oligonucleotides corresponding to other regions of α1C and α1D (Table 1).

These results show that DHP receptors are present on human primary monocytes. That of these receptors were functional as shown by the capacity of Bay K-, a DHP receptor agonist, to increase intracellular calcium levels in primary human monocytes (Fig. 6).

Taken together, these results strongly indicate the specific expression of functional DHP receptors on human monocytes.

HIV-1 Tat protein mediates calcium entry via DHP receptors

In order to evaluate the implication of DHP receptors in the Tat-mediated signal, calcium signal was measured by microspectrofluorimetry in monocytes loaded with fluo3-AM previously incubated with two DHP receptor inhibitors, nimodipine (1 μM) and calcicludine (0.1 μM), or for more specific and selective effect with L-type calcium channel antisense α1D oligonucleotides. Under these conditions, Tat was no longer able to induce calcium increase (Figs. 7A–D), thus suggesting the involvement of DHP receptors in the mobilization of calcium induced by Tat.

Table 1
Effect of anti-alpha1 antisense oligonucleotides on DHP receptor expression

Human primary monocytes preincubated with	DHP-Bodipy labeling of primary human monocytes
–	+
α1D antisense oligonucleotide no. 1 (cat cat cat cat cat cat cat (355–375 D38101))	–
α1D antisense oligonucleotide no. 2 (tta gcc ttc tct ctt tcc ttt gag aat tct cca cta agg aca cc (1561–1604 D38101))	–
α1D antisense oligonucleotide no. 3 (atc tgg ttg tta tct ctc at (4615–4632 D38101))	–
α1C antisense oligonucleotide no. 1 (cct cgt gtt ttc att gac cat (1167–1187 L01776)	–
α1C antisense oligonucleotide no. 2 (cct tcc gtg ctg ttg ctg ggc tca (5471–5494 L01776))	+
α1C antisense oligonucleotide no. 3 (act ctg ggg cac act tct tg (5450–5469 L01776))	+
α1D sense oligonucleotide (cca cag gaa tca cct ctt aag agt ttc ctt tct ctc ttc cga tt)	+
BayK- (100 μM)	–

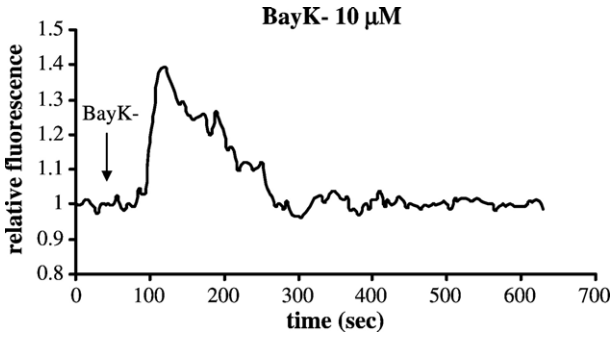

Fig. 6. Functional DHP receptors on primary human monocytes. Monocytes preincubated with fluo-3-AM as described in Fig. 1 were stimulated with the DHP receptor agonist BayK8644- at the time indicated by the arrow and relative fluorescence was analyzed by microspectrofluorimetry. This result is a representative result of three independent experiments in which 42 ± 3% of stimulated cells were positive.

Calcium increase through DHP receptors is involved in the induction of production of TNF-α but not that of IL-6 resulting from HIV-1 Tat stimulation of human monocytes

The analysis by FACS of cytokine production showed that 25.7% and 42.52% of the Tat-stimulated monocytes produced TNF-α and IL-6 (Fig. 8B). More interestingly, blockade of DHP receptor expression with antisense α1D oligonucleotides or function with nimodipine resulted in a considerable inhibition (92.6% and 77.1%, respectively) of TNF-α-positive cells, whereas the population of IL-6-producing monocytes remained comparable to the positive control, performed in the absence of inhibitors (Figs. 8C and D). No inhibition of TNF-α was observed when the same experiments were performed with the corresponding sense oligonucleotides (Fig. 8E). In agreement with these results, a significant inhibition of TNF-α, but not IL-10, production in cell supernatants of Tat-stimulated monocytes in the presence of nimodipine was obtained (Fig. 8G and data not shown). On the other hand, we have shown that blockade of DHP-R by nimodipine does not interfere with CD4 or HLA-DR expression nor with the susceptibility of monocytes to be infected by the macrophage tropic HIV-1 BaL (data not shown).

Overall, these results show the presence in primary human monocytes of DHP receptors that are composed of the α1D subunit, which can be specifically activated by HIV-1 Tat protein to induce TNF-α, a pro-inflammatory cytokine that plays an important role in the pathogenesis of HIV-1 infection.

Discussion

A variety of functions have been associated with HIV-1 Tat protein, including induction of apoptosis (Westendorp et al., 1995b), immunosuppressions (Badou et al., 2000; Shearer and Clerici, 1993), stimulation or inhibition of cytokine production (Brady et al., 1995; Nath et al., 1999;

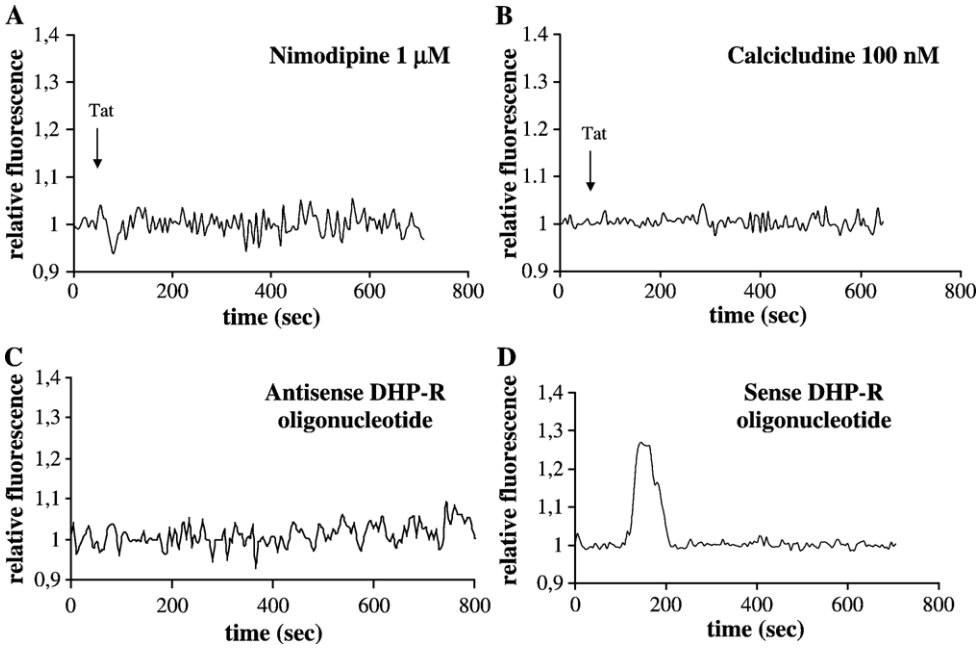

Fig. 7. Microspectrofluorimetric demonstration of the involvement of DHP receptors in the calcium increase in monocytes following stimulation by Tat (see Fig. 1). Monocytes were preincubated with DHP receptor inhibitors (A: nimodipine, B: calcicludine) or antisense oligonucleotides directed against the α1D subunit of DHP Receptors (C) prior to stimulation by HIV-1 SF-2 Tat. As a negative control, the corresponding sense oligonucleotide was used (D). Relative fluorescence corresponds to the ratio of fluorescence emitted by stimulated cells to fluorescence emitted by resting cells. Each panel is a representative result of three independent experiments in which 33 ± 7% of stimulated cells were positive. Similar results were obtained using HIV-1 Lai Tat.

Rautonen et al., 1994; Sharma et al., 1995; Zocchi et al., 1998), vaccinating properties (Fanales-Belasio et al., 2002a, 2002b), and mobilization calcium (Albini et al., 1998; Mayne et al., 2000). The aim of this study was to characterize the origin of mobilized calcium, the nature of the calcium channel activated, and the role of calcium in Tat-induced TNF-α production in human primary monocytes.

Recently, we investigated the signaling pathways triggered by Tat in human monocytes that lead to TNF-α production (Bennasser et al., 2002). We showed that the PKC and calcium pathways are required for TNF-α production, whereas the PKA pathway does not seem to be implicated. Downstream of PKC, we showed that activation of NF-κB is essential, whereas ERK1/2 MAP kinases, even though they are activated by Tat, are not directly involved in the signaling leading to TNF-α production.

In this work, we showed, as reported by Albini et al. (1998) for macrophages, astrocytes, and neurones, and by Canani et al. (2003) for human intestinal epithelial cells, that the Tat protein induces an increase in the concentration of cytoplasmic calcium in primary human monocytes and that this may then be used as a second messenger for activating signaling pathways leading to cytokine gene expression and the production of TNF-α. However, the findings of Albini et al., Canani et al., and ourselves differ from the data of Poggi et al. (1998, 2002) in two respects: the opposite effect of Tat on calcium mobilization and the proposed active domain of Tat (aa 65–80). This latter result is in disagreement with the common active domain (Tat 24–51 and Tat 20–45) determined, respectively, by Albini et al. and by us and with data showing that the Tat deletion mutant Δ31–61 can no longer trigger a rise in intracellular calcium (Self et al., 2004). Moreover, the recent data of Fanales-Belasio et al. (2002a, 2002b), who found that Tat can stimulate IL-12 production and MHC antigen upregulation, do not support either the hypothesis advanced by Poggi et al. either.

Another more fundamental question concerns the origin of the mobilized calcium. Our results indicate that it seems to have an extracellular origin, since (i) no signal was observed when monocytes were stimulated with Tat in a calcium-free medium, (ii) the calcium increase induced by Tat was not affected when stimulation was performed in the presence of Xestospongin C or TMB8, two inhibitors of intracellular stores, mediated, respectively, by IP_3 and ryanodine receptors. The latter conclusion disagrees with the reports of Mayne et al. showing the implication of IP_3-sensitive stores in the calcium increase generated by Tat in macrophages (Mayne et al., 2000) and astrocytes (Mayne et al., 1998). On the other hand, our results do agree with those of Albini et al. (1998), who showed a strong implication of extracellular calcium in the signal induced by Tat. This contradiction could be due in part to the different cell types and sources of Tat used in each study.

At the structural and functional levels, molecular, biochemical, and immunochemical studies have shown that DHP receptors consist of complexes formed by five protein subunits. The α subunit (200 kDa) represents the main part of the channel. At least six isoforms of the α subunit have

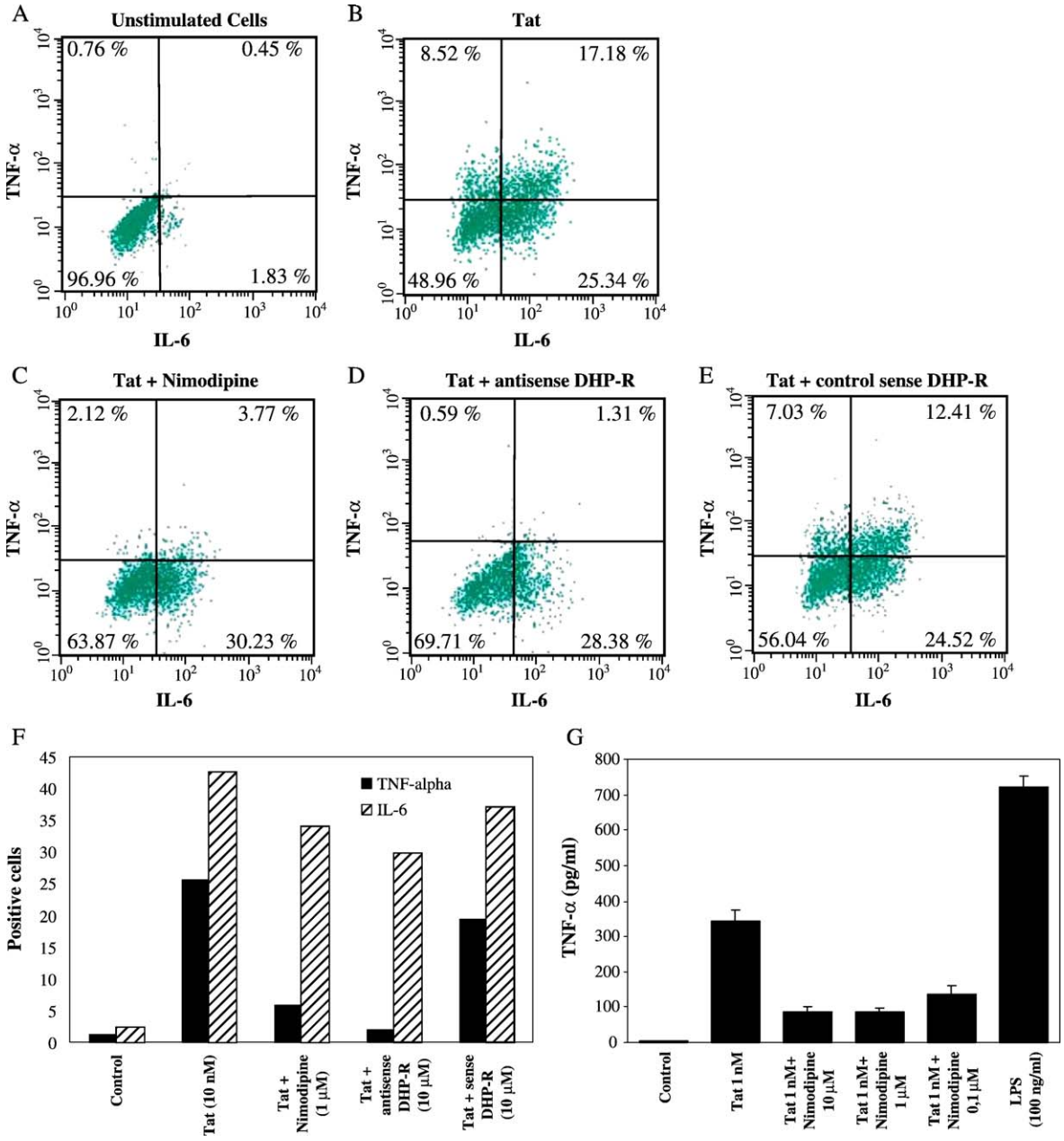

Fig. 8. Cytokine expression in HIV-1 SF-2 Tat-stimulated monocytes. Monocytes (10^6 cells), stimulated or not, were treated with monensin (6 h) in order to block cytokine secretion prior to their analysis by FACS as described in Materials and methods. Briefly, cells were stained with anti-CD14 monoclonal antibody (APC), fixed with formaldehyde, and then stained with anti-TNF-α and anti-IL-6 monoclonal antibodies. Only CD14+ cells (monocytes) were analyzed for the presence of these cytokines. (A) Control unstimulated cells; (B–D) HIV-1 Tat stimulated cells (10 nM) pretreated with nimodipine, 1 μM; (C) antisense oligonucleotides for DHP receptors, 10 μM; (D) or control sense oligonucleotides (E). Results are quantified in the histogram F. In G, monocytes (10^6 cells), pretreated or not with nimodipine (10, 1, and 0.1 μM), were stimulated by Tat (1 nM). Supernatants were collected 6 h later and TNF-α concentration was assessed by ELISA. Data from one donor are shown, representative of three different experiments carried out on cells from three different donors. Similar results were obtained using HIV-1 Lai Tat.

been described, including α1S and α1C, the most abundant DHP receptor isoforms in skeletal and cardiac muscle, respectively. This variety underlies the characteristic electrophysiological parameters of voltage-dependent calcium channels (VDCCs) (Jones, 1998). In addition to the α1 subunit, the DHP receptor channel consists of the β subunit (60 kDa) and a disulfide-connecting complex of α_2 and δ subunits (170 kDa). The α1 subunit is the receptor for calcium antagonist and can function as a voltage-gated channel. DHP receptor channel activity is in part regulated by phosphorylation via protein kinase. In addition to the modulation of DHP receptor channels by PKC (Savignac et al., 2001), other protein kinases were also implied, including cyclic GMP-dependent protein kinase, which seems to act by decreasing L-type calcium channel current (Jiang et al., 2000).

Our results indicate the expression of DHP receptors (L-type calcium channels), mostly α1D, on primary human monocytes as shown by RT-PCR, SDS-PAGE, and Western blot analysis, and inhibition of DHP receptor expression by specific antisense oligonucleotides.

In addition to their presence in primary human monocytes, several lines of evidence support that these channels are also functional. Firstly, the stimulation of human monocytes by BayK8644-, a DHP receptor agonist, induced an intracytoplasmic increase of calcium level similar to that obtained with Tat. Secondly, preincubation of monocytes with nimodipine or calcicludine, two antagonists of DHP receptors, completely blocked the ability of Tat to stimulate calcium entry into cells. It is interesting to note that these channels seem to be voltage independent since no calcium increase was induced following stimulation of monocytes with 50 mM KCl (data not shown). This statement is in agreement with previous results showing that L-type calcium channels (DHP receptors) in immune cells, including dendritic and NK cells, are also independent of voltage (Poggi et al., 1998; Zocchi et al., 1998). More recently, it was shown that DHP receptors can be selectively expressed on Th2 but not Th1 T lymphocytes (Savignac et al., 2001), thus suggesting that DHP receptors are directly implicated in the production of IL-4 cytokine and in the modulation of Th2 effector function. Interestingly, the same group using the Norway rat model described a direct relationship between DHP-sensitive calcium channels and the autoimmune disease induced by chronic injection of gold salts. Indeed, treatment of rats with calcium channel blockers appeared to prevent development of the disease (Fournie et al., 2002).

Finally, we have shown that Tat-induced calcium mobilization and TNF-α production are entirely mediated via DHP receptors, since there was a total inhibition of its release in the presence of nimodipine or calcicludine, inhibitors of DHP receptors, or in the presence of specific α1D antisense oligonucleotides. The capacity of Tat 1–45 to stimulate DHP receptor is also in agreement with its ability to stimulate TNF-α production (data not shown).

Taken together, our results show that DHP receptors are expressed at the membrane of human monocytes, are apparently functional, and are recruited for the mediation of a calcium increase following stimulation by HIV-1 Tat protein.

We show in the present study that TNF-α is produced by human monocytes as a consequence of the mobilization of calcium triggered by Tat by recruiting DHP receptors. This production is observed with as low a concentration as 1–10 nM of Tat protein. Soluble HIV-1 Tat protein has been detected in sera of HIV-1-infected patients at the nanomolar level (Westendorp et al., 1995b; Xiao et al., 2000), but this concentration may be underestimated, since Tat may be trapped by its potential receptors, particularly the heparan sulfates, molecules widely expressed on cell surfaces (Albini et al., 1996). Furthermore, this concentration would be higher near productively infected cells (Ensoli et al., 1993) and can act on other cells, whether they are infected or not (Mann and Frankel, 1991).

TNF-α is believed to contribute to the development of neurological diseases, known as HIV-associated dementia (HAD), associated with HIV (Talley et al., 1995). About 20% and 80%, respectively, of HIV-1 infected adults and children develop some level of HAD (Tardieu et al., 2000). The extracellular Tat released in the central nervous system by infected infiltrating monocytes/macrophages and microglial cells could induce the toxic cytokine TNF-α, which can act directly on adjacent neurons, oligodendrocytes, and astrocytes to cause neurotoxicity and neurodegeneration (Talley et al., 1995). By inducing TNF-α, Tat appears to contribute actively as a pathogenic and immunosuppressive factor to disease progression. As Tat protein is necessary for HIV-1 replication and is involved in a number of physiopathological effects, therapeutic approaches targeting Tat could be particularly effective in reducing lower the physiopathological effects of HIV-1 infection.

Materials and methods

Cells and reagents

PBMCs were isolated from buffy coats of healthy HIV-negative donors in a Ficoll density gradient (Pharmacia, Piscataway, NJ). PBMCs were resuspended in 60/30 complete medium (60% AIMV and 30% Iscove [GIBCO-BRL, Grand Island, NY]) containing penicillin (100 IU/ml), streptomycin (100μg/ml), and 10% FCS. PBMCs were plated at a density of 10^6cells/well in 24-well Primaria (Becton Dickinson, Rutherford, NJ) tissue culture plates. After 24-h culture at 37 °C in 5% CO_2, nonadherent cells were removed, the remaining cells were washed twice, and then incubated with the different compounds tested. Monocytes were 94% pure as tested by FACs with anti-CD14 antibody.

Recombinant HIV-1 Tat protein was obtained from the Agence Nationale de la Recherche sur le SIDA (Paris, France). The level of endotoxin contamination in purified HIV-1 Tat was assessed using the limulus amebocyte lysate assay (Bio-Sepra, Villeneuve-la-Garenne, France).

HIV-1 Tat mutants were produced as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli*. Wild-type GST-Tat 1–101 and Tat-deleted mutants GST-Tat 1–45, GST-Tat 20–72, and GST-Tat 30–72 were purified as described (Benkirane et al., 1998). As control, GST was purified under the same conditions and used in the same experiments. All these constructions were LPS free (<0.3 EU/μg) and biologically active, as described (Sabatier et al., 1991).

The calcium signaling inhibitors Nimodipine, Calcicludine, Xestospongin C, TMB-8 are from Calbiochem. A

putative cytotoxic effect of the different inhibitors was tested by the trypan blue dye exclusion assay and none was found to be cytotoxic (viability was >90%) at the concentrations used.

Calcium signaling

Microspectrofluorimetry

Intracellular calcium (Ca^{2+}) concentrations were determined by emission microspectrofluorimetry as previously described (Badou et al., 2000). Monocytes, untreated or preincubated for 1 h with the inhibitors of calcium signaling or in calcium-free medium, were incubated with 5 μM 3-fluo-acetoxymethylester (AM; Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C. Intracellular Ca^{2+} concentrations were measured in cells stimulated by Tat (10 or 100 nM) or in different Tat mutants (50 nM); cells treated with 1 μM ionomycin (Sigma) were used as positive control in HBSS medium supplemented with 1% FCS. Ionomycin was initially dissolved in DMSO at a concentration of 2 mM. Cell preparations were placed on the stage of an inverted Diaphot microscope (Nikon) and observed with a 40× objective. The excitation wavelength was 490 nm with a 525-nm barrier filter. Fluorescence was detected by an intensified charge-coupled device fitted with a 2400-80 camera (Hamamatsu photonics, Hamamatsu, Japan). At the magnification used (40×), a field of 200 × 200 μm was recorded by the camera. Three to five fields were observed for each type of experiment, and for each field 12 windows (9 μm) were selected on different cells and analyzed for fluorescence. Images were captured at intervals of 5 s and processed with the Argus 50 image processing system (Hamamatsu photonics) or simple PCI software (Compix USA). Time courses of Ca^{2+} mobilization in cells were analyzed with argus 50 or simple PCI software. Data are presented as the ratio of fluorescence (F) in stimulated cells to fluorescence (F0) at the baseline level. Cells were scored as positive if the variation in fluorescence intensity was more than 2% above the baseline level to erase the background signal.

Confocal microscopy

Monocytes loaded with fluo-3-AM as described above were placed under a confocal microscope in HBSS medium containing 1% FCS. The change in calcium concentration was measured in cells stimulated by Tat (10 or 100 nM) using a confocal microscope (Zeiss, LSM 410). The fluorescent probe was excited using an Argon laser with a wavelength of 488 nm. The images were taken every 5 s from the equatorial plane of cells.

Treatment of monocytes with oligonucleotides

Oligonucleotides were synthesized by MWG (MWG, Germany). The sequences of antisense and sense oligonucleotides are listed in Table 1. Sequences specific for α1C and α1D subunits of DHP receptors were selected from sequences of mRNA that are unique and specific using Blast. Monocytes were plated at a density of 10^6 cells/ml in Iscove/AIMV supplemented with 1% FCS and treated with antisense or sense oligonucleotides for 12 h. Then 10% FCS was added to the medium and the monocytes were cultured for a further period of 12 h.

mRNA expression

Total mRNA was extracted and reverse transcribed as described elsewhere (Jiang et al., 2000). LTCCs were sought by using the sense 5′-TTCTTCATGATGAACATCTT-3′ and antisense 5′-CATGTAGAAGCTGATGAA-3′ primers (Catterall et al., 1988), which correspond to conserved regions IIIS6 and IVS6 of each of the α1 isoforms. cDNAs were amplified in a 25-μl reaction volume containing 1 μM of each dNTP (dATP, dTTP, dGTP, and dCTP) (Pharmacia, Uppsala, Sweden), 0.5 μM of each oligonucleotide primer, 2 mM $MgCl_2$, 1 U of Taq-DNA polymerase (GIBCO), and 2.5 μl of PCR buffer 10× (GIBCO). Reactions were performed in a DNA thermal cycler (Perkin-Elmer) for 40 cycles: 45 s at 95 °C, 45 s at 51 °C, and 1 min at 72 °C, preceded by an initial denaturation step (1 min at 93 °C). Ten percent of the products were then subjected to a nested PCR using the sense 5′-AAGAACTGTGAGCTGGACAA-3′ and the antisense 5′GATCTCCTGCCAGGCCTC-3′ primers. Reactions were performed in a DNA thermal cycler (Perkin-Elmer) for 30 cycles: 45 s at 95 °C, 45 s at 62 °C, and 1 min at 72 °C, preceded by an initial denaturation step (1 min at 93 °C). The products were analyzed by electrophoresis on a 1.5% agarose gel. The identity of the PCR products with LTCC sequences was confirmed by sequencing.

Staining of DHP receptors

ST-BODIPY dihydropyridine (Molecular Probes) was used to detect LTCC (De Jongh et al., 1990) at the surface of primary human monocytes by fluorescent confocal microscopy. Monocytes were incubated with 250 nM ST-BODIPY dihydropyridine for 5 min at 37 °C in the dark. To determine nonspecific ST-BODIPY dihydropyridine binding, cells were preincubated for 30 min at 37 °C with 100 μM Bay K- before adding the fluorescent probe (Leclerc et al., 1995). Controls included omission of the fluorescent dye, with or without preincubation with Bay K-. Cells were washed, placed on the stage of an inverted microscope (Nikon, Diaphot) of a laser confocal microscope (LSM, 410, Zeiss), and observed with a Zeiss 63 X/1.4 numerical aperture objective, illuminated by a 100-W xenon lamp. Excitation was provided by a helium-neon laser at 543 nm and barrier filter at 580 nm. Images were processed, integrated, and expressed on a 256-pseudocolor scale.

Extraction of proteins and Western blot analysis

Isolation of cytoplasmic and membrane protein extracts

Monocytes were harvested and rapidly lysed at 4 °C in 100 μl of hypotonic buffer A (20 mM Tris–HCl, 2 mM EDTA, 1 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF; pH 7.5) by repeated aspiration through a syringe fitted with a 21-gauge needle. After addition of 300-μl ice-cold sample buffer B (20 mM Tris–HCl, 2 mM EDTA, 1 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, 0.33 M sucrose; pH 7.5), the lysate was centrifuged at 100,000 × *g* at 4 °C for 40 min. The supernatant corresponding to the cytoplasm was collected; proteins were quantified with the Bradford assay and stored at −20 °C. The membrane pellets were solubilized in 50 μl of cold sample buffer B containing 1% Triton X-100, sonicated (1 min, power 2.5), and stored at −20 °C.

Western blot analysis of α1D subunit of DHP receptors

Equal amounts of protein (100 μg) were subjected to 10% SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. Immunoblotting was conducted with anti-α1D antibody (1:1000) (Calbiochem). The membrane was blocked with 5% milk in Tris-buffered saline with 0.05% Tween 20 (TTBS) for 1 h, washed four times with TTBS, and incubated with the primary antibody for 2 h. Immunoreactive bands were detected by incubation for 2 h with anti-rabbit immunoglobulins conjugated with horseradish peroxidase (1:1000) (DAKO A/S, Roskilde, Denmark). Membranes were visualized using a chemiluminescent substrate (Pierce, Rockford, IL).

Immunostaining and flow cytometric analysis of intracellular cytokines

Monocytes (10^6) isolated from buffy coats cultured in AIMV-Iscove medium supplemented with 1% FCS were preincubated with the appropriate inhibitors prior to stimulation with 10 nM of Tat protein. Monensin was then added to block protein secretion and cells were collected after 6 h of stimulation for the assessment of the presence of TNF-α and IL-6. Cells were then collected, washed in CellWASH (optimized phosphate-buffered saline for cell preparation and cell washing containing NaCl, Na2HPO4, NaH2PO4, NaN3, pH 7.2 ± 0.1 at 25 °C; BDIS). Subsequently, they were resuspended in 100 μl CellWASH and stained at 4 °C for 20 min with an anti-CD14 monoclonal antibody conjugated to allophycocyanin (APC). After two further washes with CellWASH, the cells were fixed for 10 min in 200 μl CellWASH containing 1.5% formaldehyde. Finally, the cells were washed twice and resuspended in CellWASH with anti-TNF-α and anti-IL-6 antibodies conjugated to phycoerythrin (PE) and fluorescein isothiocyanate (FITC), respectively, to stain intracytoplasmic cytokines. This mixture was incubated for 30 min at 4 °C at appropriate concentrations. The cells were then washed twice in CellWASH and were then ready for analysis.

ELISA TNF-α

Monocytes (10^6) isolated from buffy coats cultured in AIMV-Iscove medium supplemented with 1% FCS were preincubated with the appropriate inhibitors prior to stimulation with 1 nM of Tat protein. Supernatants were collected 6 h later and TNF-α was quantified using ELISA TNF-α kit (ibioscience).

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