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Dopaminergic D_1-like receptor-dependent inhibition of tyrosine hydroxylase mRNA expression and catecholamine production in human lymphocytes

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Abstract

Activation of human peripheral blood mononuclear cells (PBMC) triggers endogenous production of catecholamines (CA) through protein kinase (PK) C-dependent induction of tyrosine hydroxylase (TH; EC 1.14.16.2), the first and rate-limiting enzyme in the synthesis of CA. Since CA themselves are major mediators of the neural input to the immune system, we have examined their ability to affect PKC-induced TH mRNA expression and CA production in human isolated PBMC. In T- and B-lymphocytes (but not in monocytes) the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (but not its inactive analogue 4α-phorbol-12,13-didecanoate) induced TH mRNA expression which was followed by an increase in the amount of intracellular CA. Coincubation of human PBMC with dopamine (DA) (but not with norepinephrine or epinephrine) inhibited TPA-induced TH mRNA expression. The effect of DA was concentration-dependent and was mimicked by the dopaminergic D_1-like receptor agonist SKF-38393 but not by the D_2-like receptor agonist bromocriptine. The D_1-like antagonist SCH-23390 shifted to the right the concentration–response curves of both DA and SKF-38393, while neither the D_2-like antagonist domperidone, nor the α_1-adrenoceptor antagonist prazosin, the α_2-adrenoceptor antagonist yohimbine, or the β-adrenoceptor antagonist propranolol affected to any significant extent the inhibitory effect of DA. SKF-38393 also significantly reduced TPA-induced increase of intracellular CA, an effect which was antagonized by SCH-23390. It is thus suggested that in human T- and B-lymphocytes PKC activation leads to TH mRNA expression and subsequent increase of intracellular CA, which can be inhibited by D_1-like receptor activation. Inhibition of intracellular CA production in human PBMC promotes cell survival through reduction of activation-induced apoptosis, and dopaminergic modulation of TH expression and intracellular CA content may thus represent a novel mechanism in the cross-talk between the nervous and the immune system as well as among immune system cells.

Keywords: T- and B-lymphocytes; Tyrosine hydroxylase; Protein kinase C; Catecholamines; Dopamine; Dopaminergic D_1-like receptors

1. Introduction

It is now accepted that endogenous production of the CA, DA, NE and E occurs not only in neurons and chromaffin cells but also in cells of the immune system, including murine lymphocytes [1], peritoneal macrophages [2], bone marrow derived mast cells [3], human PBMC [4–6], lymphocytes [7], granulocytes [8], and hematopoietic cell lines [9]. CA are therefore likely to act not only as transmitters from the nervous and endocrine systems to the immune system (reviewed in [10,11]), but also as autocrine/paracrine mediators in immune system cells and as transmitters between these cells and the nerves.

Over the last decade, evidence has been accumulating about the functional relevance of endogenous production of CA in immune system cells. Endogenous NE regulates tumor necrosis factor α production by murine peritoneal macrophages [2], while incubation with the DA antagonist haloperidol decreases cell proliferation in hybridoma T cells, and growth of these cells correlates with the expres-

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Abbreviations: PBMC, peripheral blood mononuclear cells; CA, catecholamines; DA, dopamine; NE, norepinephrine; E, epinephrine; PKC, protein kinase C; TH, tyrosine hydroxylase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; PCR, polymerase chain reaction.

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sion of TH (EC 1.14.16.2), the first and rate-limiting enzyme in the synthesis of CA [12]. We have recently shown that stimulation of human PBMC with the polyclonal mitogen phytohaemagglutinin induces the synthesis of CA through a PKC-dependent mechanism which triggers the *de novo* expression of TH mRNA and the subsequent activation of the enzyme [13]. Pharmacological inhibition of TH results in decreased activation-induced apoptosis [14], supporting a role of endogenous CA in the functional modulation of PBMC activation. Studying the role of endogenous CA in immune cells is therefore likely to provide new insights into the regulation of the neuroimmune network, eventually leading to the identification of novel targets for pharmacological intervention.

CA themselves are presently acknowledged as major mediators of the neural input to the immune system [10,11], however at present no information is available concerning their possible modulatory effects on endogenous CA production in immune cells. The present investigation was therefore undertaken in order to examine the ability of CA to affect PKC-induced TH mRNA expression and CA production in human isolated PBMC, and characterization of the receptors involved was attempted by use of selective pharmacological agents. PKC activation was chosen as a reference standard since in previous experiments we found that activation-induced production of CA in human PBMC is entirely dependent upon activation of this enzyme. This is in agreement with the notion that PKC is increasingly acknowledged as a key enzyme in the activation sequence of both T- [15] and B-lymphocytes [16]. Some experiments were also performed to assess the occurrence of TH mRNA expression and its regulation following PKC activation in highly purified PBMC subsets obtained by immunomagnetic sorting.

2. Materials and methods

2.1. Chemicals

Dopamine hydrochloride, norepinephrine bitartrate, epinephrine hydrochloride, 1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF-38393), bromocriptine hydrochloride, TPA, 4α-PDD, calphostin C, cycloheximide, domperidone, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH-23390), prazosin hydrochloride, yohimbine hydrochloride, propranolol hydrochloride, bovine serum albumin and HEPES were purchased from Sigma Chemical Co. RPMI 1640, heath-inactivated fetal calf serum, glutamine, penicillin/streptomycin were from Celbio. Dextran and Ficoll-Paque Plus were from Pharmacia Biotech. All other reagents and solvents were of HPLC grade and were obtained from Merck. Highly purified, distilled, deionized water was used to prepare buffers and solutions.

2.2. Cells

Human PBMC were isolated from venous blood obtained from healthy volunteers using heparinized tubes. Whole blood was allowed to sediment on dextran at 37° for 30 min. Supernatant was recovered and PBMC were separated by Ficoll-Paque Plus density-gradient centrifugation. Cells were then washed two times in NaCl 0.15 M and resuspended in RPMI 1640 medium supplemented with 10% heath-inactivated fetal calf serum, 2 mM glutamine and 100 U/mL penicillin/streptomycin, at the final concentration of 1×10^6 cells/mL for subsequent culture at 37° in a moist atmosphere of 5% CO_2. Typical PBMC preparations contained about 80% lymphocytes and 16% monocytes, as assessed by flow cytometry. Cell viability, assessed by the trypan blue exclusion test, was always >99%.

2.3. Immunomagnetic isolation of mononuclear cell subsets

In some experiments, at the end of the culture period PBMC were collected and highly purified $CD2^+$ (T-lymphocytes), $CD19^+$ (B-lymphocytes), and $CD14^+$ (monocytes) subpopulations were isolated by immunomagnetic cell sorting. To this end, CD2, CD14 or CD19 Dynabeads (Dynal A.S.) were added to the cell suspensions using a target-to-bead ratio of 1:4. After a 20-min incubation at 4°, rosetted cells were isolated from the mixture by placing the test tube in a magnetic particle concentrator. By means of the magnet, rosetted cells were retained on the wall of the test tube, while the medium was removed. Cells were then washed three times and finally resuspended for subsequent RT–PCR analysis (see below). A counting chamber was used to enumerate cell number and the purity of the preparations, as assessed by flow cytometry, was usually 98–99%. The viability was 95–98% by the trypan blue exclusion test.

2.4. RNA isolation and RT–PCR analysis of TH mRNA

RNA isolation and RT–PCR analysis of TH mRNA were performed as described previously [13]. Briefly, total RNA was extracted from 1×10^6 PBMC by Perfect RNA Eukaryotic Mini kit (Eppendorf) and the amount estimated by spectrophotometry at 260 nm. Total RNA was then reverse transcribed and cDNA was amplified by one-step RT–PCR reaction kit (Finnzymes). PCR was performed by thermocycler (GeneAmp PCR System 2400, Perkin-Elmer Instruments), according to the following steps: 45 min at 48°, then 1 min at 94°, 1 min at 55°, 2.0 min at 72°, followed by a 5 min-extension period at 72° for 30 cycles. Samples were finally chilled at 4° until analysis, which was performed by placing 10 μL of the RT–PCR product on a 2% agarose gel in the presence of ethidium bromide (BioRad). Quantification was carried out by computer-assisted den-

sitometric analysis of photographic negatives of the agarose gel (Multi-Analyst, BioRad). Rat pheochromocytoma cells (PC12 cells, used as positive controls) were obtained from the Interlab Cell Line Collection of the National Institute for Cancer Research/Advanced Biotechnology Center. For selection of the primers, we referred to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database. The sequences of the primers for TH mRNA were as follows: 5′-primer, sense (exon 5) 5′-TGTCAGAGCTGGACAAGTGT-3′, and 3′-primer, antisense (exon 8) 5′-GATATTGTCTTCCCGGTAGC-3′. The quality of the RT–PCR reaction was assessed by amplifying hypoxantine phosphoribosyltransferase (HPRT) mRNA using the following oligonucleotides as primers: 5′-primer, sense 5′-CTCCGCCTCCTCCTCTGCT-3′, and 3′-primer, antisense 5′-GCCTGACCAAGGAAAGCAAAG-3′. Data were finally expressed as TH mRNA/HPRT mRNA optical density (OD) ratio in arbitrary units.

2.5. HPLC-ED assay of CA

CA in PBMC were assayed by high performance liquid chromatography with electrochemical detection according to a previously described method [17]. Briefly, cells were centrifuged for 5 min at 1400 *g* and 4°, the medium was removed and the cell pellet was resuspended in 0.2 mL of $HClO_4$ 0.4 N and disrupted by sonication. The mixture was then centrifuged for 5 min at 15,000 *g* and 20° and the supernatant was recovered, filtered and 30 μL were injected into the chromatographic system. CA in the samples were quantified by using the peak areas of a standard curve and values were finally normalized for cell number.

2.6. Statistical analysis

Data are presented as means ± SD. Statistical significance of the differences among groups was assessed by two-tailed Student's *t*-test or by ANOVA followed by Dunnett or Bonferroni post test, as appropriate. Concentration–response relationships were analyzed by nonlinear regression using a commercial software (Prism 2.0, GraphPad Software Inc.) and a sigmoidal Concentration–response curve was fitted to find the mean value of the EC_{50} (i.e. the concentration which elicited 50% of the maximal response) together with its 95% confidence interval (CI).

3. Results

3.1. Effect of PKC activation on TH mRNA levels in PBMC and in highly purified PBMC subpopulations

Unstimulated PBMC did not express detectable levels of TH mRNA. Incubation with the PKC activator TPA

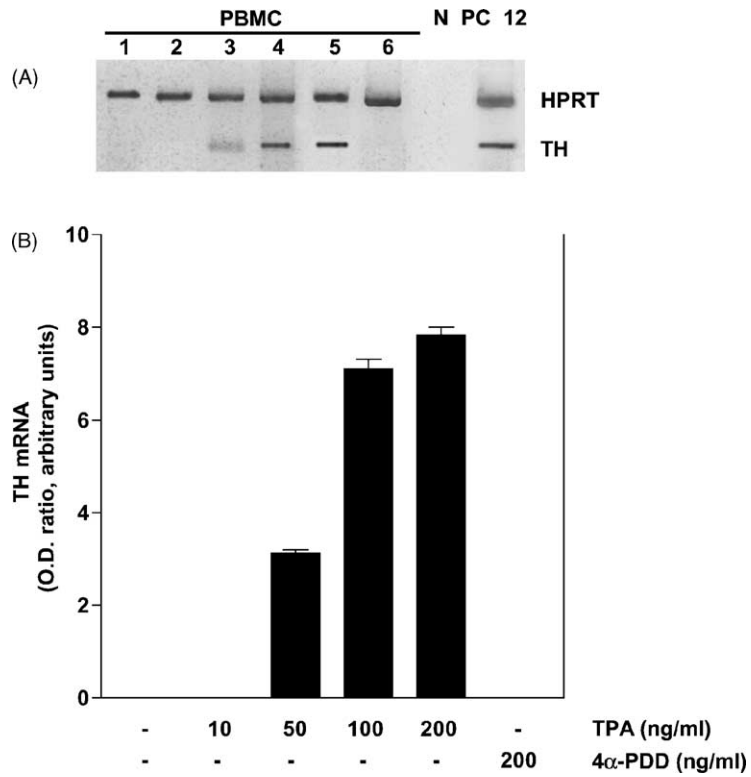

Fig. 1. Effect of 24-hr incubation with TPA on TH mRNA expression in human PBMC. (A) Data from one representative of three independent experiments. Lane 1, control (untreated PBMC); lanes 2–5, TPA 10 ng/mL (lane 2), 50 ng/mL (lane 3), 100 ng/mL (lane 4), 200 ng/mL (lane 5); lane 6, 4α-PDD 200 ng/mL; N, negative control (no mRNA); PC12, positive control. (B) Pooled data from three separate experiments. Each column is the mean ± SD. For further details, see Section 2.

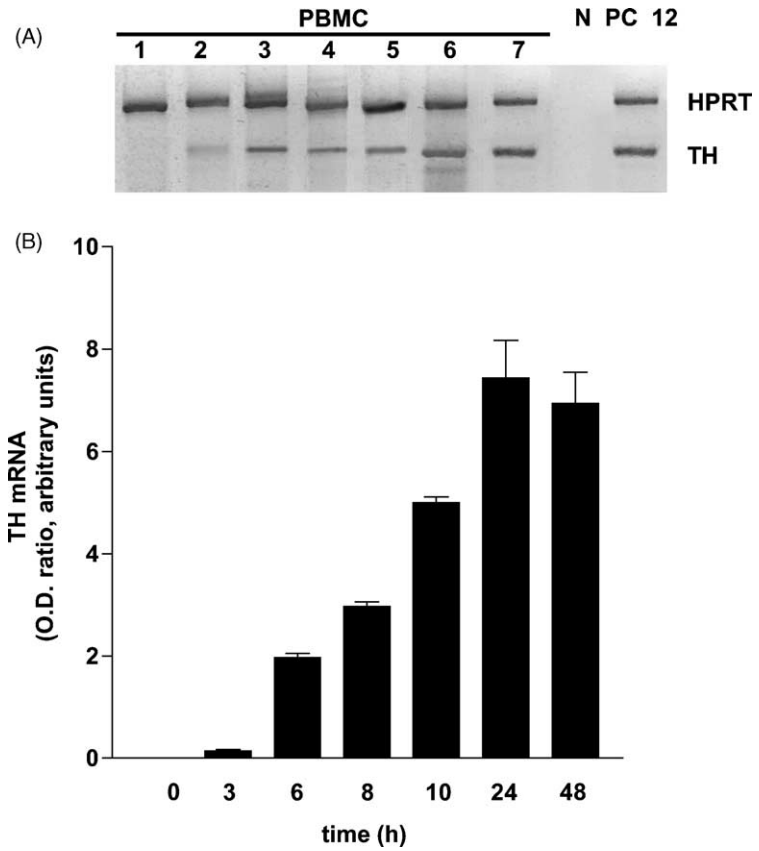

Fig. 2. Time-dependency of the effect of TPA 100 ng/mL on TH mRNA expression in human PBMC. (A) Data from one representative of three independent experiments. Lane 1, 0 hr; lane 2, 3 hr; lane 3, 6 hr; lane 4, 8 hr; lane 5, 10 hr; lane 6, 24 hr; lane 7, 48 hr; N, negative control (no mRNA), PC12, positive control. (B) Pooled data from three separate experiments. Each column is the mean ± SD. For further details, see Section 2.

concentration-dependently induced the expression of TH mRNA, while the TPA inactive analogue 4α-PDD was completely inactive (Fig. 1). TPA-induced TH mRNA expression was already evident at 3 hr, however, maximal values were reached at 24 hr and remained elevated thereafter (Fig. 2). After treatment with TPA, TH mRNA could be detected both in whole PBMC as well as in highly purified T- and B-lymphocytes, but not in monocytes (Fig. 3). Preincubation with the PKC inhibitor calphostin C or with the protein synthesis inhibitor cycloheximide added 30 min before TPA completely supressed TPA-induced TH mRNA expression: under these conditions TH mRNA could not be detected in three out of three separate experiments (Fig. 4).

3.2. Effect of DA, NE and E and pharmacological analysis of the receptors involved

Coincubation with DA, but not with NE or E, inhibited TPA-induced expression of TH mRNA (Fig. 5). The effect of DA was concentration-dependent, was mimicked by the dopaminergic D_1-like receptor agonist SKF-38393 but not by the D_2-like receptor agonist bromocriptine, and the D_1-like receptor antagonist SCH-23390 shifted to the right the concentration–response curves of both DA and SKF-38393 (Fig. 6). The EC_{50} values (with 95% CI) of DA were 11.04 (6.19–17.70) nM (alone) and 514.00 (338.20–780.60) nM (in the presence of SCH-23390 1 μM), and those of SKF-38393 were 0.32 (0.27–0.38) nM (alone) and 9.17 (5.48–15.34) nM (in the presence of SCH-23390 1 μM). In separate experiments, the inhibitory effect of DA was found to be unchanged in the presence of antagonists acting preferentially at D_2-like receptors (domperidon, 1 μM), α_1-adrenoceptors (prazosin, 1 μM), α_2-adrenoceptors (yohimbine, 1 μM) or β-adrenoceptors (propranolol, 10 μM) (data not shown).

3.3. Effect of dopaminergic agents on CA production

In agreement with previous observations [13], in PBMC incubated for 72 hr with TPA intracellular CA levels increased by 14–20-fold with respect to untreated cells. Coincubation with SKF-38393 1 μM, a concentration which completely prevented TPA-induced TH mRNA expression, significantly reduced CA levels and this effect was reverted in the presence of SCH-23390 (Fig. 7).

4. Discussion

The main finding of the present study regards the existence in human lymphocytes of dopaminergic D_1-like

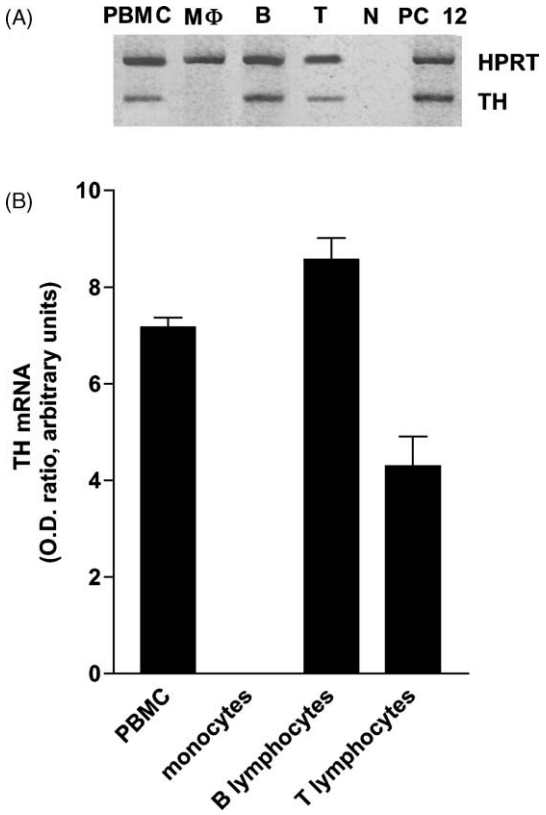

Fig. 3. TPA-induced expression of TH mRNA in human PBMC and in highly purified PBMC subsets. (A) Data from one representative of three independent experiments. PBMC: peripheral blood mononuclear cells; T: T-lymphocytes; B: B-lymphocytes; MΦ: monocytes; N, negative control (no mRNA); PC12, positive control. (B) Pooled data from three separate experiments. Each column is the mean ± SD. For further details, see Section 2.

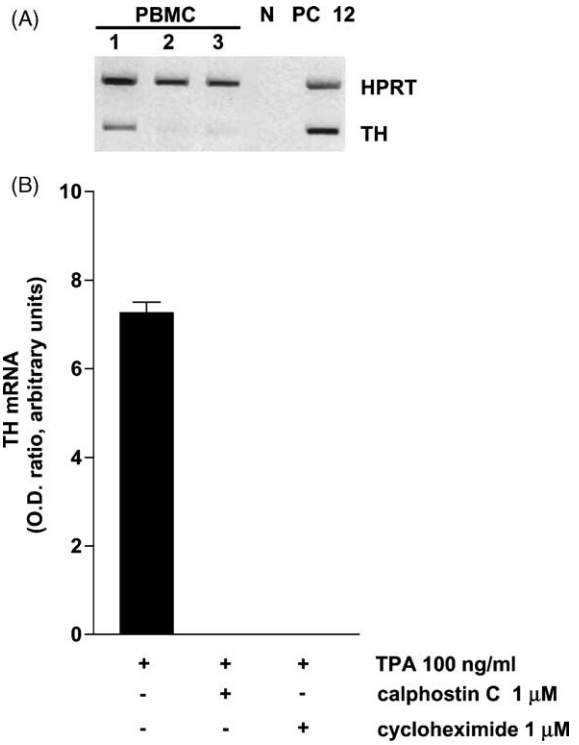

Fig. 4. Effect of calphostin C and cycloheximide on TPA-induced expression of TH mRNA in human PBMC. (A) Data from one representative of three separate experiments. Lane 1, TPA 100 ng/mL; lane 2, TPA + calphostin C 1 μM; lane 3, TPA + cycloheximide 1 μM; N, negative control (no mRNA), PC12, positive control. (B) Pooled data from three separate experiments. Each column is the mean ± SD. For further details, see Section 2.

receptor-operated mechanisms which inhibit PKC-induced TH mRNA expression resulting in reduced intracellular CA levels. In addition, it was also shown that PKC-induced TH mRNA expression in PBMC is specific for lymphocytes. In previous experiments [10], we showed that in human PBMC activation of PKC was the key step in mitogen-induced TH mRNA expression and subsequent intracellular synthesis of CA. TH protein expression in human lymphocytes occurs even in the absence of activating stimuli and mitogen-stimulated cells are TH immunoreactive to the same extent as resting cells [18]. By contrast, TH mRNA cannot be detected in resting cells and sustained CA production is triggered only upon activation, being preceded by TH mRNA expression [13]. Altogether, such observations led us to hypotesize that increased TH protein synthesis might by necessary to sustain the enhanced turnover of the activated enzyme occurring in stimulated cells. The present data clearly indicate that TPA-induced TH mRNA expression occurs through PKC activation (inasmuch as the effect of TPA was prevented by the PKC inhibitor calphostin C and was not mimicked by the TPA inactive analogue 4α-PDD) and it is specific for T- and B-lymphocytes (but not for monocytes). TPA-induced TH mRNA expression was time- and concentration-dependent and was prevented not only by the PKC inhibitor calphostin C but also by the protein synthesis inhibitor cycloheximide, suggesting that synthesis of one or more additional intracellular protein mediators, possibly transcription factors, is required. A possible contribution to increased TH mRNA levels by enhanced mRNA stability cannot be excluded. However, it is unlikely to play a major role since in the absence of PKC activation TH mRNA is undetectable in human lymphocytes even in the presence of cycloheximide and/or RNAse inhibitors (unpublished observations). Extensive studies indeed support the notion that in human lymphocytes PKC activation regulates the expression and the activity of several transcription factors, including c-fos, c-jun and cAMP-responsive element binding protein [19–21], which in turn can act to increase TH transcription by binding to the AP-1 and CRE sites of the promoter region of the TH gene [22]. PKC is a family of serine/threonine kinases and it is increasingly evident that distinct PKCs regulate T- [15] and B- lymphocytes [16]. Assessment of the specific PKCs involved in the regulation of TH mRNA expression in different cell subsets was beyond our present aims, however this issue deserves further investigation as it may represent an approach to exert distinct effects on intracellular production of CA in T- and B-lymphocytes.

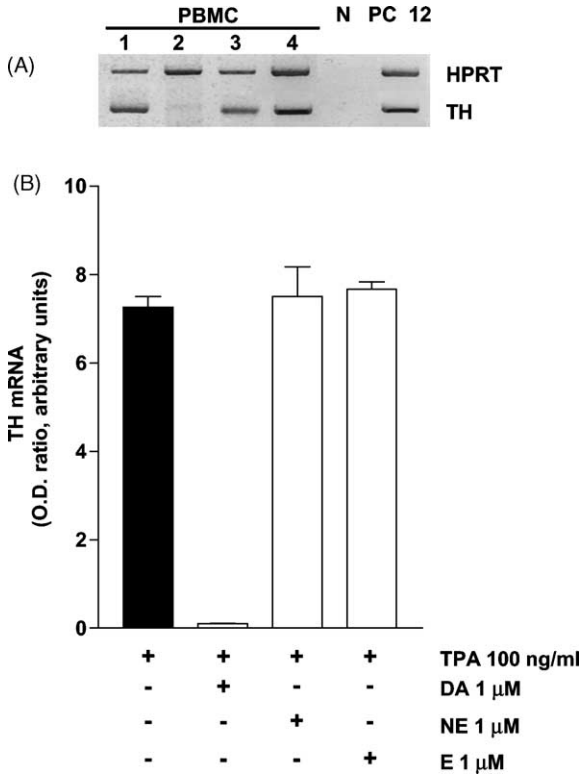

Fig. 5. Effect of DA, NE and E on TPA-induced TH mRNA expression in human PBMC. (A) Data from one representative of three separate experiments. Lane 1, TPA 100 ng/mL; lane 2, TPA + DA 1 μM; lane 3, TPA + NE 1 μM; lane 4, TPA + E 1 μM; N, negative control (no mRNA); PC12, positive control. (B) Pooled data from three separate experiments. Each column is the mean ± SD. For further details, see Section 2.

The most interesting results of our study however regard the existence in human lymphocytes of a dopaminergic mechanism regulating TH mRNA expression and CA production. In our experiments, coincubation of human PBMC with DA (but not with NE or E) inhibited TPA-induced TH mRNA expression. The effect of DA was concentration-dependent and was mimicked by the D_1-like receptor agonist SKF-38393 but not by the D_2-like receptor agonist bromocriptine. Further support to the involvement of D_1-like receptors comes from data obtained using receptor antagonists: in these experiments, only the D_1-like antagonist SCH-23390 shifted to the right the concentration–response curves of both DA and SKF-38393, while neither the D_2-like antagonist domperidone, nor the α_1-adrenoceptor antagonist prazosin, the α_2-adrenoceptor antagonist yohimbine, or the β-adrenoceptor antagonist propranolol affected to any significant extent the inhibitory effect of DA. In human lymphocytes the presence of dopaminergic D_1-like and D_2-like receptors is supported by both molecular biology [23,24] and ligand binding studies [25,26]. Recently, the expression of dopaminergic receptor subtypes has been investigated in leukocyte subpopulations by use of flow cytometry, showing that D_1-like receptors belong exclusively to the D_5 receptor subtype, while D_2-like receptors include the D_2, D_3 and D_4 subtypes, and that B-lymphocytes and NK cells have the higher and more consistent receptor expression [27]. It should be nevertheless considered that available data have been obtained in resting cells, and it cannot be excluded that changes of the expression pattern of these receptors

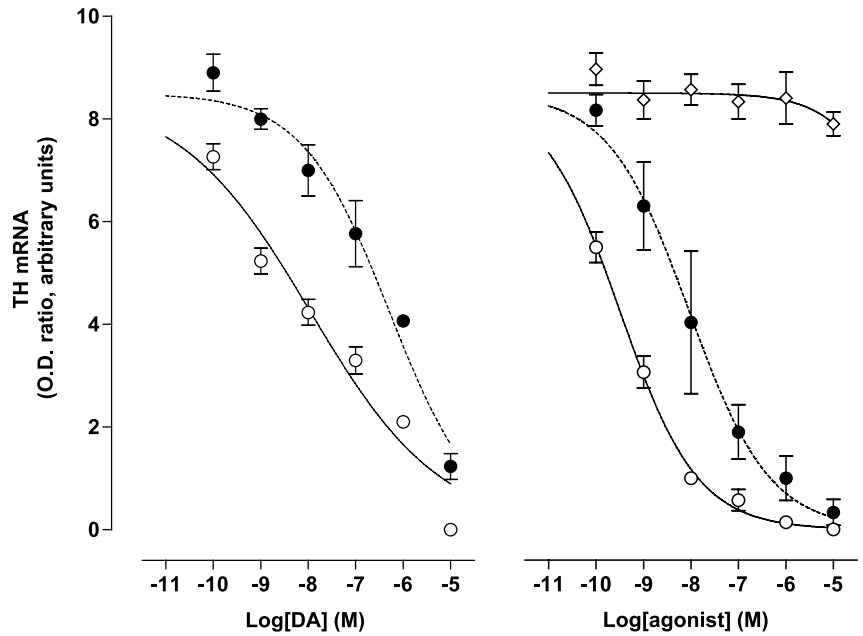

Fig. 6. Effect of DA and of dopaminergic receptor agonists on TH mRNA expression induced by TPA 100 ng/mL in human PBMC. *Left*: Concentration–response curve of DA alone (open symbols) and in the presence of SCH-23390 1 μM (closed symbols). *Right*: Concentration–response curve of SKF-38393 alone (○) and in the presence of SCH-23390 1 μM (●), and lack of effect of bromocriptine (◇). Each point is the mean ± SD of three separate experiments. For further details, see text.

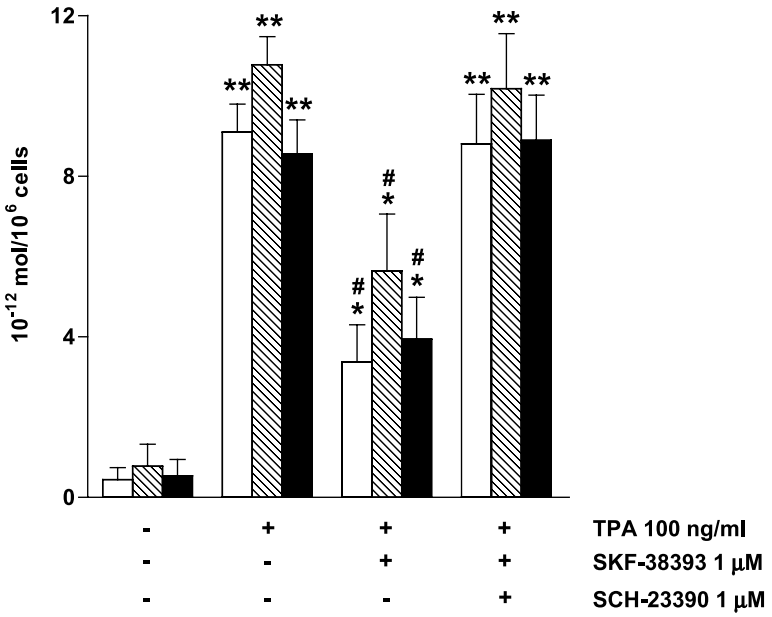

Fig. 7. Effect of dopaminergic agents on TPA-induced production of DA (empty bars), NE (hatched bars), and E (filled bars) in human PBMC. Each column is the mean ± SD of three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. untreated cells, $^{\#}P < 0.05$ vs. TPA-treated cells.

may occur upon cell activation, as shown for instance in the case of α- and β-adrenoceptors [28,29]. Interestingly, in T-lymphocytes up regulation of β-adrenoceptor occurs through the activation of PKC [30].

At present, the intracellular mechanisms involved in the observed D_1-like receptor-mediated suppression of PKC-induced TH mRNA expression and CA production remain a matter of speculation. The inhibition might be ascribed either to a direct interference with PKC activation, or to a downstream effect on the activation of transcription factors involved in the regulation of TH gene transcription. In this regard however, existing data obtained in the central nervous system and in peripheral tissues point to a stimulatory rather than inhibitory effect of D_1-like receptors on the activity of PKC [31–33] as well as on the phosphorylation of transcription factors such as c-fos and cAMP-responsive element binding protein [34,35], although, at least in the case of PKC, inhibitory effects have also been reported, e.g. in rat vascular smooth muscle cells [36]. Intracellular signal trasduction pathways coupled to D_1-like receptors in human lymphocytes need therefore to be investigated in *ad hoc* studies.

Dopaminergic regulation of TH expression has been previously reported in the central nervous system, in midbrain dopaminergic neurons [37,38]. In particular, it was shown that systemic administration of the D_2-like antagonist haloperidol can produce downregulation ot TH immunoreactivity in substantia nigra but not in ventral tegmental area in the rat, thus supporting the existence of a tonic inhibition of TH gene expression by DA released at this level, possibly exerted through D_2-like receptor activation [37,38]. This would be at variance with the functional arrangement in human lymphocytes where, according to our pharmacological data, DA-induced inhibition of TH mRNA expression depends upon activation of D_1-like receptors. Nevertheless, both D_1- and D_2-like receptor activation may result in functional modulation of human lymphocytes. Indeed, although up to a few years ago the immune effects of DA were usually ascribed to the ability of this catechol compound to undergo auto-oxidation followed by generation of reactive oxygen radicals [39], recent reports have shown that physiological concentrations of DA may inhibit the proliferation and cytotoxicity of human $CD4^+$ and $CD8^+$ T cells through the activation of D_1-like receptors in both healthy individuals [40] and lung carcinoma patients [41]. In addition, DA may also interact with D_2-like receptors on human T-lymphocytes to activate β1 integrin function [42] and to inhibit cytokine release and expression of tyrosine kinases [43].

The functional relevance of DA-induced inhibition of TH mRNA expression in human lymphocytes is indicated by the concurrent reduction of intracellular CA following D_1-like receptor activation. The effect of the D_1-like agonist SKF-38393 can be reasonably ascribed to the ability of this compound to prevent TH gene transcription. Interestingly however, even in the presence of agonist concentrations that completely suppressed TH mRNA expression, CA levels were still significantly higher than those in resting cells. As a working hypothesis, we propose therefore that residual CA production may occur through direct, PKC-dependent phosphorylation of existing TH protein, which, as above discussed, can be found even in resting cells [18].

Evidence obtained in rat spleen has shown that DA can be taken up by noradrenergic axon terminals at this level, converted in part into NE and released as both DA and NE in response to neural activity [44]. DA might therefore act

either as a plasma neuroendocrine mediator, as an immune cell-derived signaling molecule and even as a transmitter released by nerve terminals laying in close contact with immune cells in limphoyd organs. We previously showed that TH mRNA expression and CA production in human PBMC occur upon activation [13] and that inhibition of intracellular CA production promotes cell survival through reduction of activation-induced apoptosis [14]. In view of the role of DA as a transmitter in sympathetic terminals, dopaminergic regulation of CA production in lymphocytes, possibly acting through the modulation of cell death and survival, may thus contribute to the immune effects of sympathetic nervous system activity. For instance, it has been shown that, at least in rats, acute stress administered immediately before antigenic challenge results in a significant enhancement of a skin delayed type hypersensitivity response [45]. It cannot be excluded that reduced susceptibility of lymphocytes to activation-induced apoptosis, due to stress-induced sympathetic nervous system activation and subsequent inhibition of endogenuos CA production in lymphocytes, may play a role in this regard.

Increasing evidence suggests that PBMC-derived CA are involved in infiammatory autoimmune diseases such as rheumatoid arthritis [46,47] and multiple sclerosis [14,48], studying the mechanisms regulating endogenous CA production in PBMC is therefore likely to provide relevant knowledge for the better comprehension of the neuroimmune network in health and disease, as well as for the in-depth understanding of the mechanism(s) of action of immunomodulating agents.

Acknowledgments

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