Relative Quantification of mRNA Levels in Jurkat T Cells with RT-Real Time-PCR (RT-rt-PCR): New Possibilities for the Screening of Anti-Inflammatory and Cytotoxic Compounds

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Received April 4, 2002; accepted April 23, 2002

Purpose. Quantification of the pro-inflammatory action of mitogens on mRNA levels of growth-related genes, transcription factors, and cytokines in T cells as markers for the screening of compounds with immunomodulatory, anti-inflammatory or cytotoxic potential.

Method. A reverse transcription-real time-polymerase chain reaction assay with TaqMan probes was developed. Jurkat T cells were treated with cyclosporin A, hypericin, capsaicin, and catechin before phorbol 12-myristate 13-acetate stimulation, and their effects on the relative mRNA levels were determined. A cell viability assay was performed in parallel.

Results. Cyclosporin A and capsaicin were potent inhibitors of PMA-induced cytokine transcription. Cyclosporin A further targeted cyclin D1 transcription. Capsaicin exhibited no effects on the cell viability at low concentrations, whereas cyclosporin A did. Hypericin down-regulated nearly all investigated mRNAs, resulting in a strong time-dependent cytotoxicity. Catechin showed no effects on mRNA levels and cell viability.

Conclusions. The inhibition of the up-regulation of mRNA levels of cytokines points to a specific anti-inflammatory potential of capsaicin. Hypericin showed no specific effects on the mRNA expression. The overall decrease of mRNA levels is probably an early indication of the strong cytotoxic effect observed after 48 h. Therefore, quantification of mRNA levels by reverse transcription-real time-polymerase chain reaction is, in combination with the monitoring of cell viability, a valuable tool to distinguish between specific immunomodulatory and cytotoxic effects *in vitro*.

KEY WORDS: RT-real time-PCR; cytokines; Jurkat T cells; NF-κB; capsaicin; hypericin.

INTRODUCTION

The search for natural products with anti-inflammatory, immunomodulatory or antineoplastic potential has always been an important issue in drug discovery. There is an increasing interest in drugs involving new targets to treat inflammatory, autoimmune, or cancer-related diseases (1). Because drug action leads to direct or indirect changes in gene transcription, the analysis of relative mRNA levels might be a valid and relatively inexpensive approach for the screening and investigation of bioactive drugs. However, the insight that drugs can specifically modulate gene transcription, e.g., by blocking the activation of transcription factors (TFs) is a relatively new one (2). It has been postulated that the search for compounds that inhibit TFs, such as nuclear factor (NF)-κB or activated protein-1 (AP-1), should be a good approach for the finding of new lead compounds with anti-inflammatory potential (1,2). Here, we show that the natural products capsaicin and hypericin, which inhibit NF-κB at similar concentrations, show different effects on the mRNA levels of pro-inflammatory and regulatory genes. We also demonstrate that the quantification of relevant mRNA levels can have greater predictive power with respect to the specific immunomodulatory potential of a drug than the investigation on its inhibition of TFs. Furthermore, cytotoxic vs. anti-inflammatory effects can be distinguished due to the fact that other mRNAs, which are crucial in cell regulation and proliferation, can be quantified and correlated with the observed cell viability.

Because pro-inflammatory gene transcription events are well understood in T cells, we set up an assay with Jurkat T cells to monitor phorbol 12-myristate 13-acetate (PMA)-induced mRNAs relevant to inflammation, cell proliferation, and apoptosis. Because many compounds are cytotoxic and thereby indirectly inhibit pro-inflammatory gene expression, cell viability and proliferation testing was performed together with the analysis of mRNAs of housekeeping genes. T cells produce a series of cytokines, which are regulated by different families of TFs, such as NF-κB, AP-1, c-myc, and NF-ATc (3). Because TFs are important regulators of gene expression, we also monitored steady-state levels of NF-ATc, p65, and Iκ-Bα mRNAs, factors that are essential for the expression of interleukin (IL)-2, granulocyte macrophage colony-stimulating factor (GM-CSF), and interferon (INF)-γ. PMA is well known to induce cytokine expression in Jurkat T cells by up-regulating cytokine gene transcription (4), but little is known about its impact on the transcription of the TFs, which control this process. It has been shown that p65 and Iκ-Bα expression is important for the viability growth of different cell types (5). In our assay, the impact of PMA on the transcription of these TFs was assessed and used as potential target for cytotoxic drug action. Particularly, the expression of p65 has been shown to be essential for cell viability, protecting cells from different apoptosis inducing stimuli (6).

We used a rapid, sensitive, and highly reproducible 5′-exonuclease-based reverse-transcription–real-time–polymerase chain reaction (RT-rt-PCR) methodology based on TaqMan probes (7) to quantify relative mRNA levels in Jurkat T cells. The TaqMan methodology allows accurate measurement of DNA amplification kinetics and gives a value derived from the most exponential phase of the rt-PCR, which is directly proportional to the amount of starting cDNA and hence mRNAs of specific genes (8). To evaluate, whether the modulation of gene transcription is correlated with an effect on cell viability and proliferation we also measured the effects of the investigated compounds in a colorimetric (WST-1 based) assay using test conditions comparable to the RT-rt-PCR assay (9).

To validate our RT-rt-PCR assay we used clinically relevant concentrations of the immunosuppressive drug cyclosporin A (2.5 μM), a substance known to act on T cells by blocking calcineurin-mediated signal transduction. This event finally leads to a strong inhibition of IL-2, INF-γ, and GM-

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0724-8741/02/0800-1236/0 © 2002 Plenum Publishing Corporation

CSF transcription in T cells (10). The established method was then applied for the screening and characterization of potentially anti-inflammatory and cytotoxic natural products. An interesting anti-inflammatory candidate resulting from these investigations was capsaicin, a major phenolic compound from chili pepper (*Capsicum annum* L.). Although the compound is well known to act on vanilloid receptors in the nervous system (11) and has been shown to induce apoptosis *in vitro* (12), there have only been few studies on its potential immunomodulatory action. However, it has been shown previously that capsaicin inhibited phorbol ester-induced NF-κB and AP-1 activation in human HL-60 leukemia cells (13). The second compound, hypericin, from St. John's Wort (*Hypericum perforatum* L.), has been studied extensively, mainly in the context of its use in the treatment of depression (14). Hypericin is also known to induce apoptosis in different neoplastic cell lines (15). Furthermore, there are some reports on the possible anti-inflammatory potential of hypericin, such as inhibition of phorbol ester-induced NF-κB activation (16). We also tested catechin, a widespread phenolic natural product with recognized antioxidant properties (17). Catechin derivatives have been reported as a source of false-positive *in vitro* screening results of plant extracts as a result of their nonspecific interactions with proteins (18).

MATERIALS AND METHODS

Cell Culture, RNA Isolation, and Reverse Transcription

$CD4^+$ Jurkat human leukemia T cells (ATCC TIB-152) were cultured in RPMI 1640 medium (Gibco, Life Technologies, Switzerland) supplemented with 10% fetal bovine serum, 1 μg/mL fungizone (amphotericin B), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Gibco, Life Technologies) at 37°C and 5% CO_2 in 50-mL culture flasks (TPP). 5×10^5 cells were incubated with 1 mL of fresh medium in a 24-well plate and left to rest for 3 h. The compounds capsaicin ($C_{18}H_{27}NO_3$, Roth, Germany), hypericin ($C_{30}H_{16}O_8$, Roth, Germany), and (±)-catechin ($C_{15}H_{14}O_6$, Fluka, Switzerland) were incubated in the relevant concentration range 1 h before mitogen stimulation. The solvent part never exceeded 0.5% and a solvent only control was run. For mitogen activation, 2 μg of PMA (ICN, Switzerland) was added to each well containing 1 mL of cell suspension and incubated for 2.5 or 20 h, respectively, depending on the experiment. To diminish variability and pipetting errors, three wells were pooled for RNA extraction as one experiment. Total cytoplasmatic RNA was isolated from cells using RLN buffer (Qiagen) and RNeasy® spin columns (Qiagen) according to manufacturers instructions. Total RNA was checked for DNA contamination on an ethidium bromide-stained 2.5% agarose gel. One microgram of total RNA was incubated together with 33 ng/μL random hexamers (Microsynth, Switzerland) and ribonuclease free water at 60°C and then reverse transcribed for 60 min at 37°C with Omniscript® reverse transcriptase (RTase) (Qiagen) in a GeneAmp® 2400 thermocycler (Perkin Elmer). Alternatively, 10 μM of RTase Stratascript® (Stratagene) was used with an initial incubation temperature of 65°C for 5 min prior to reverse transcription at 42°C. Each experiment was performed in duplicate and at least three times.

Primer and Probe Design and rt-PCR

Primers and probes for rt-PCR were designed to the cDNA sequences of the genes investigated, using DNA sequence entries from GenBank™. Primer and probe sequences were chosen to prevent homologies to undesired genes and other coding sequences and checked with BLAST™ software. To exclude amplification from possible DNA contamination, either the probes or the primers (both from Microsynth) were designed to overlap exon junctions in cDNA regions derived from intron-bearing genes. All probes but GAP-DH (purchased as Pre-Developed Factor from PE Biosystems) were labeled with the fluorescent dyes 5′-FAM (6-carboxy-fluorescein) as reporter and 3′-TAMRA (6-carboxy-tetramethyl-rhodamine) as quencher. GAP-DH was 5′-VIC labeled. The sequences employed in our assay together with the corresponding GenBank™ entries are shown in Table I.

Primer and probe concentrations were optimized to 300–1000 nM primers and 100–300 nM probes. The rt-PCR was run with TaqMan PCR master mix (PE Biosystems) using a total reaction volume of 25 μL. The increase in fluorescence signal in each of the 96 wells with optical caps (Biolabo SA, Switzerland) was monitored in real time during PCR amplification by the PRISM 7700 Sequence Detector (PE Biosystems) equipped with a laser and charge-coupled device camera (CCD). Spectral data collected were analyzed by on-line software and the standardized log fluorescent intensity (ΔRn) was plotted against the threshold cycle number (C_T).

Relative Quantification of rt-PCR

According to Gibson *et al.*, a 2-fold dilution series of each factor was run, and the C_T values were plotted against the log cDNA concentration added (19). The obtained linear graphs (correlation coefficients > 0.95) were used to determine differences in C_T values and to express them as relative percentage of mRNA present. As the input target quantity increased, the cycle number at which an increase in fluorescence signal could be detected notably decreased, indicating that the assay was sensitive to the amount of starting target cDNA. The dynamic range of all factors covered about eight C_T values (approximately 20-fold relative concentration difference). For relative quantification, cDNA dilution series were run to set up a standard curve for each factor. Non-stimulated Jurkat cells were used as control and compared to PMA-stimulated cells. Importantly, experiments were not normalized to a standard because cell populations were identical.

For the inhibition measurements, the theoretical zero value (where there is no cDNA) was set to C_T value 31 as in some cases spontaneous amplification curves with genomic DNA template appeared after cycle 31. The relative inhibition was hence determined with respect to C_T value 31. Each PCR experiment was carried out in duplicates and at least two times.

Cell Viability Testing

$CD4^+$ Jurkat human leukemia cells were cultured as described above. A cell suspension of 1.5×10^5 cells / well was incubated with six different concentrations of cyclosporin A, capsaicin, hypericin and catechin in a 96-well plate for 1.5 and 19 h in a humidified atmosphere (37°C, 5% CO_2). The final

Table I. Primer and Probe Sequences Used for Rt-rt-PCR. Forward Primers (FP), Reverse Primers (RP), and TaqMan Probes Are Listed with the Corresponding GenBank™ Accession Number

	Primer and probe sequences
IL-2 (GenBank™ S77834)	FP: 5′-ACCAGGATGCTCACATTTAAGTTTTA-3′ RP: 5′-GAGGTTTGAGTTCTTCTTCTAGACACTG-3′ Probe: 5′-CCCAAGAAGGCCACAGAACTGAAACATC-3′
GM-CSF (GenBank™ M10663)	FP: 5′-TGACCATGATGGCCAGCC-3′ RP: 5′-AGGTGATAGTCTGGGTTGCACA-3′ Probe: 5′-TGCCCTCCAACCCCGGAAACTT-3′
NF-ATc (GenBank™ XM 008694)	FP: 5′-CCCCAGATGGCCACCAT-3′ RP: 5′-TTCGGCTTGCACAGGTCC-3′ Probe: 5′-TCTGGGAGATGGAAGCGAAAACTGACC-3′
I-κBα (GenBank™ M83221)	FP: 5′-AAACGGCGGAAGAAAAAGC-3′ RP: 5′-AGGAACGGGCCTGAGCC-3′ Probe: 5′-CATCCTGGACCACTTCCTGCCCAAC-3′
p65 (GenBank™ M62399)	FP: 5′-AGCACAGATACCACCAAGACCC-3′ RP: 5′-CCAGGGAGATGCGCACTG-3′ Probe: 5′-CATCAAGATCAATGGCTACACAGGACCAGG-3′
INF-γ (GenBank™ XM 006883)	FP: 5′-GTTTTGGGTTCTCTTGGCTGTT-3′ RP: 5′-CATCTGAATGACCTGCATTAAAATATTT-3′ Probe: 5′-CTGCCAGGACCCATATGTAAAAGAAGCAGA-3′
CD95 (Fas) (GenBank™ X89101)	FP: 5′-TGGCCAATTCTGCCATAAGC-3′ RP: 5′-TCATCCCCATTGACTGTGCA-3′ Probe: 5′-CTGTCCTCCAGGTGAAAGGAAAGCTAGGG-3′
Cyclin D1 (GenBank™ XM006138)	FP: 5′-AAGATCGTCGCCACCTGG-3′ RP: 5′-GGAAGACCTCCTCCTCGCAC-3′ Probe: 5′-TGCTGGAGGTCTGCGAGGAACAGA-3′
β-actin (GenBank™ NM001101)	FP: 5′-ACCGAGGCCCCCCTG-3′ RP: 5′-GGTCTCAAACATGATCTGGGTCA-3′ Probe: 5′-ACCCCAAGGCCAACCGCGA-3′

volume was 150 μL/well. Ten microliters of WST-1 (4-[3-(4-Iodophenyl-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche) was added and incubated for 1 h under the same conditions. The absorbance of the samples was measured at 405 nm (reference wavelength 650 nm) against a background control (culture medium with 10 μL WST-1), using a microplate reader (MRX, Dynex Technologies). For determination of the IC_{50}, the cell viability was determined as a percentage of the control response (1.5×10^5 cells without test compounds plus 10 μL WST-1). Every test was performed at least in duplicates, and all experiments were repeated three times. Positive control experiments were performed with helenalin. Values are given as means ± standard deviations. Maximum observed standard deviation was 10% (absolute). Trypan blue staining was performed as reported in (20).

RESULTS

Assay Setup

To develop a functional inflammation-model we had to check the constitutive mRNA expression levels as well as the mitogen-induced mRNA profile of the genes investigated in Jurkat T cells. PMA induced a time-dependent up-regulation of several genes (Fig. 1), most of which take part in the cellular immune response. The NF-κB elements p65 and I-κBα were relatively well expressed in non-stimulated Jurkat T cells (C_T value < 25), a finding that might indicate the overall key importance of NF-κB for the proliferation of this cell line (Fig. 1). The same seems to be true for NF-ATc (C_T value < 20), which however appears to be far less sensitive to PMA signaling than p65 and I-κBα. The increase of p65 and I-κBα mRNA levels observed after PMA stimulation coincides with earlier reports on the overall increased NF-κB activity (21). These TFs are of key importance in the transcriptional regulation of several cytokines and have been recognized to play a role in many disease states (22).

The PMA-induced up-regulation of IL-2, GM-CSF, and INF-γ mRNAs seen in our assay has been described many times (23). We found that PMA alone was sufficient to induce significant expression of these cytokines. The mRNA profiles of IL-2, GM-CSF, and INF-γ were increased more than 12-fold after 20 h incubation with PMA (Fig. 1). The two-stimulus requirement for IL-2 production in Jurkat T cells as reported earlier (24) was not confirmed with our Jurkat clone. Our results further show that the induction of IL-2 and INF-γ mRNAs is less rapid than GM-CSF mRNA induction. The similar transcription kinetics of IL-2 and INF-γ might confirm earlier reports by Wiskocil *et al.* on the coordinate expression of these factors in Jurkat T cells: They found peak mRNA levels after 6 h PMA stimulation with scanning densitometry dot plot analysis (25). With our assay we found the highest mRNA levels after 20 h but with a rapid increase within the first 2.5 h. It is possible that the kinetics reported by Wiskocil *et al.* either represent an artifact resulting from reduced stability of older mRNAs or the mRNAs are more stable with the higher PMA concentration tested in our assay (26).

The 6-fold induction of CD95 (Fas/APO-1) by PMA observed in our assay (Fig. 1) is likely to be mediated through the protein kinase C pathway. In many cellular systems, the

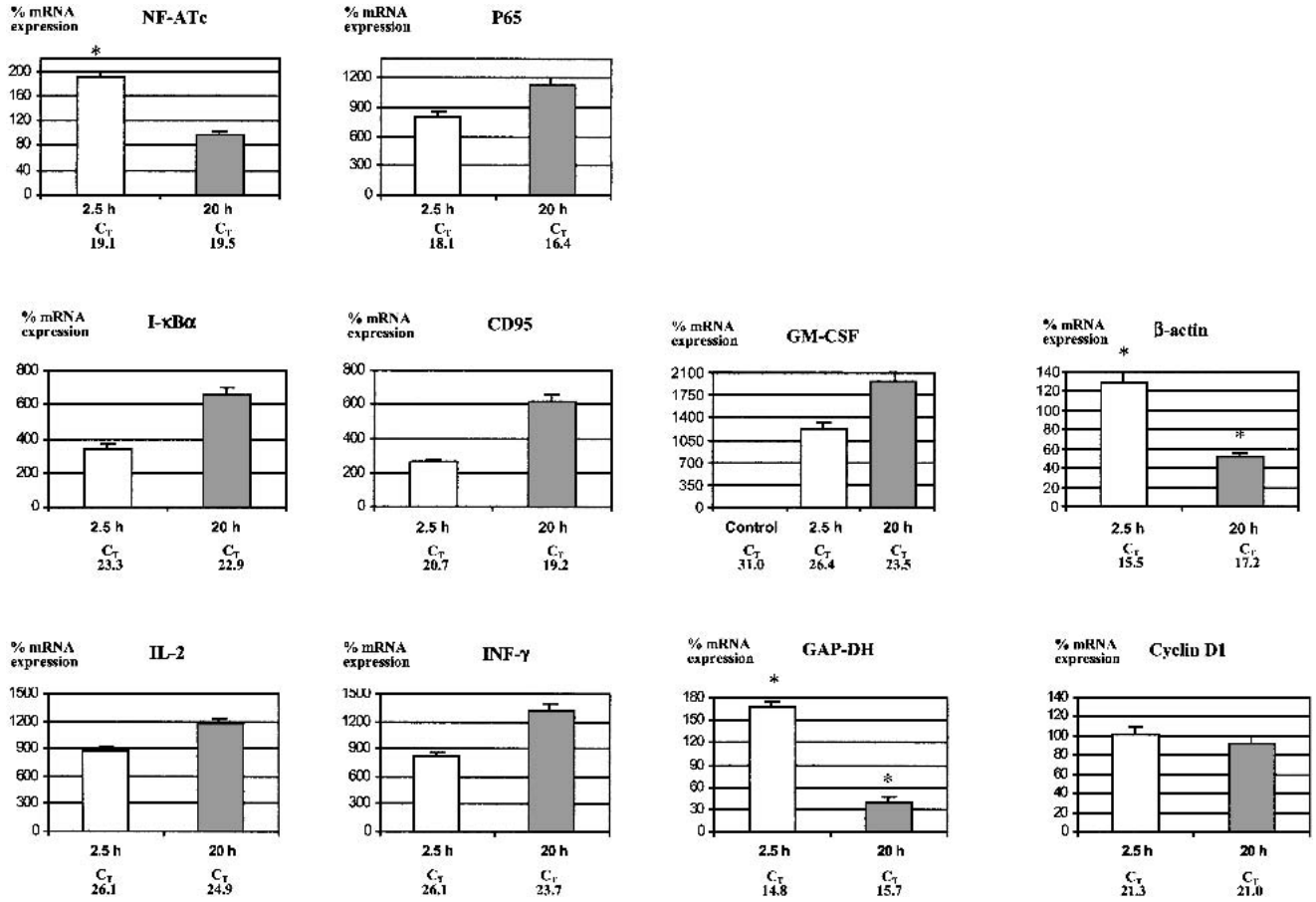

Fig. 1. Relative mRNA expression (%) induced by 2 μg/mL phorbol 12-myristate 13-acetate after 2.5 h (white bars) and 20 h (gray bars). Data shown as mean values (experiments n = 5) ± SEM and corresponding mean threshold cycle numbers (*$p \leq 0.01$). The mRNA expression was calculated relative to nonstimulated cell populuations. Corresponding mean threshold values (in brackets) for nonstimulated controls after 20 h were as follows: NF-Atc (19.6), p65 (22.3), I-κBα (25.2), CD95 (21.8), IL-2 (29.8), INF-γ (30), GM-CSF (31.3), β-actin (15.9), GAP-DH (15.1), cyclin D1 (21.2).

transcription of CD95 is strictly controlled (27). That PMA activation of protein kinase C can mimic TCR signals and thereby induces the expression of CD95 in different cell lines has been described by Wang *et al.* (28). CD95 up-regulation in Jurkat cells is a sign for Fas-mediated apoptosis and its modulation could either prevent or accelerate cell death.

Interestingly, the cyclin D1 mRNA profile is not modulated by PMA in Jurkat T cells over a time-course of 20 h. PMA has been shown to strongly induce cyclin D1 transcription in megakaryocytic cell lines whereas HeLa cells were not affected (29). Based on observations by Ajchenbaum *et al.*, the constitutive high expression of cyclin D1 in T cells seems to be restricted to cancer cells because primary T cell cultures do not express high levels of cyclin D1 (30). Cyclin D1 might therefore represent an important target in cancer treatment. NF-ATc and cyclin D1 mRNAs were not modulated significantly upon PMA treatment. The down-regulation of GAP-DH and β-actin in PMA-treated Jurkat cells observed after 20 h might reflect an early toxicity of PMA (Fig. 1). Cell viability studies with the PMA concentration used in the experiments did not show a decrease in cell viability up to 48 h. However, 2 μg/mL PMA induced a 35% decrease in cell viability after 72 h (data not shown).

Assay Validation

To test whether our inflammation model worked, we incubated the Jurkat cells with 2.5 μM of cyclosporin A. Cyclosporin A completely and specifically inhibited the PMA-induced up-regulation of the cytokines (Fig. 2). This well-known pharmacologic effect showed that our RT-rt-PCR assay was accurate enough to reliably measure steady-state levels of mRNA in T cell populations. No modulation of the transcription factors and housekeeping genes was observed, but cyclosporin A specifically reduced cyclin D1 mRNA levels in a time-dependent manner.

The effect of cyclosporin A on the viability of the T cells was measured in a concentration range between 0.5 and 25 μM (Fig. 3B). Addition of 1–4 μM cyclosporin A increased the cleavage of WST-1 significantly after 2.5 h, pointing to a higher metabolic turn over of the T cells. No decrease in cell viability was observed with concentrations between 4 and 25 μM. Interestingly, the down-regulation of cyclin D1 mRNA observed after cyclosporin A treatment did not lead to a rapid inhibition of cell proliferation. A significant reduction of cell viability caused by cyclosporin A was measured after 20 h only at concentrations higher than 4 μM (Fig. 3B). To test

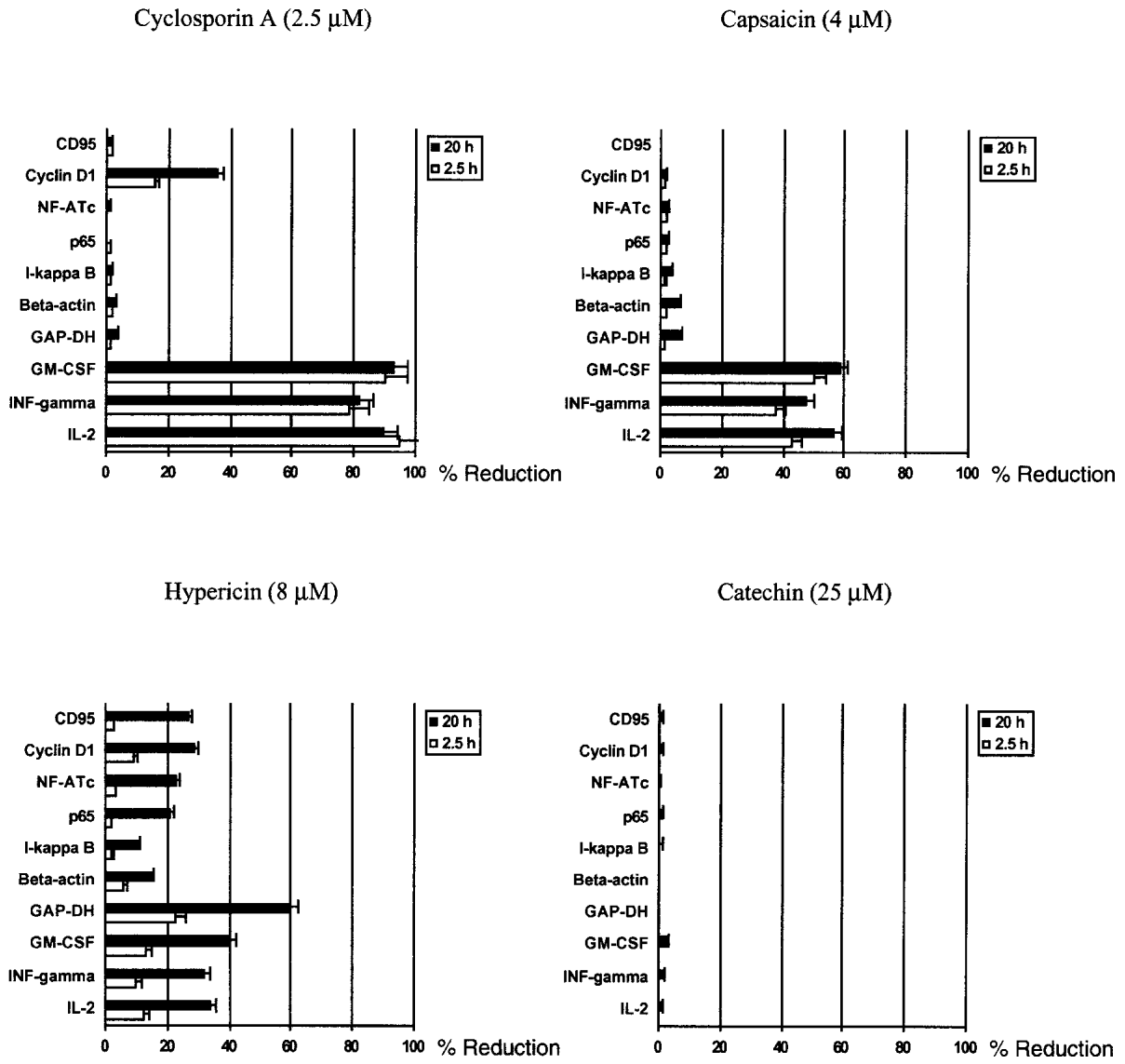

Fig. 2. Relative inhibition (%) of phorbol 12-myristate 13-acetate-induced mRNA levels in Jurkat T cells after 2.5 h and 20 h by 2.5 μM cyclosporin A, 4 μM capsaicin, 8 μM hypericin, and 25 μM catechin. Data are shown as mean values (experiments n = 4) ± SEM. The percent inhibition is calculated assuming a theorhetical zero amound at corresponding mean threshold value 31.

whether cyclosporin A blocks cell division and thereby leads to decreased cell numbers, and hence less WST-1 cleavage, or causes cytotoxic effects, we counted cell numbers with trypan blue exclusion. At 7.0 μM (the IC_{50} concentration obtained after 48 h in the WST-1 assay) cyclosporin A showed little toxicity with trypan blue exclusion but an inhibition of cell proliferation over a time span of 48 h (data not shown). This result correlates well with the almost identical cell viability curves measured with WST-1 after 20 and 48 h (Fig. 3B).

Capsaicin

The influence of capsaicin on the viability of the Jurkat cells was tested in a concentration range of 0.5–65 μM. After 2.5 h cell viability was not affected at all capsaicin concentrations tested. The 20 h measurements showed a significant reduction only at concentrations higher than 30 μM (Fig. 3D). In accordance, 4 μM capsaicin showed no modulation of TFs and house-keeping genes in the RT-rt-PCR. Inhibition of mRNA expression by 4 μM capsaicin was observed only for the PMA-induced up-regulation of the cytokines, showing immunosuppressive effects comparable in quality with cyclosporin A, but significantly weaker (Fig. 2).

Hypericin

Hypericin induced an overall down-regulation of mRNAs and did not show a specific anti-inflammatory modulation of mRNA levels. After 2.5 h, the investigated mRNA levels were only weakly affected by a hypericin concentration of 8 μM. 17.5 h later, the effects of hypericin became significantly stronger and most of the mRNA levels were down-regulated between 20% and 60%. GAP-DH, GM-CSF, and IL-2 mRNA levels decreased most significantly (Fig. 2). The influence of hypericin on the viability of the T cells was tested in a concentration range of 0.5–41 μM. Measurements with WST-1 showed decreased cell viability after 2.5 h only for concentrations above 30 μM, but the effects of hypericin on

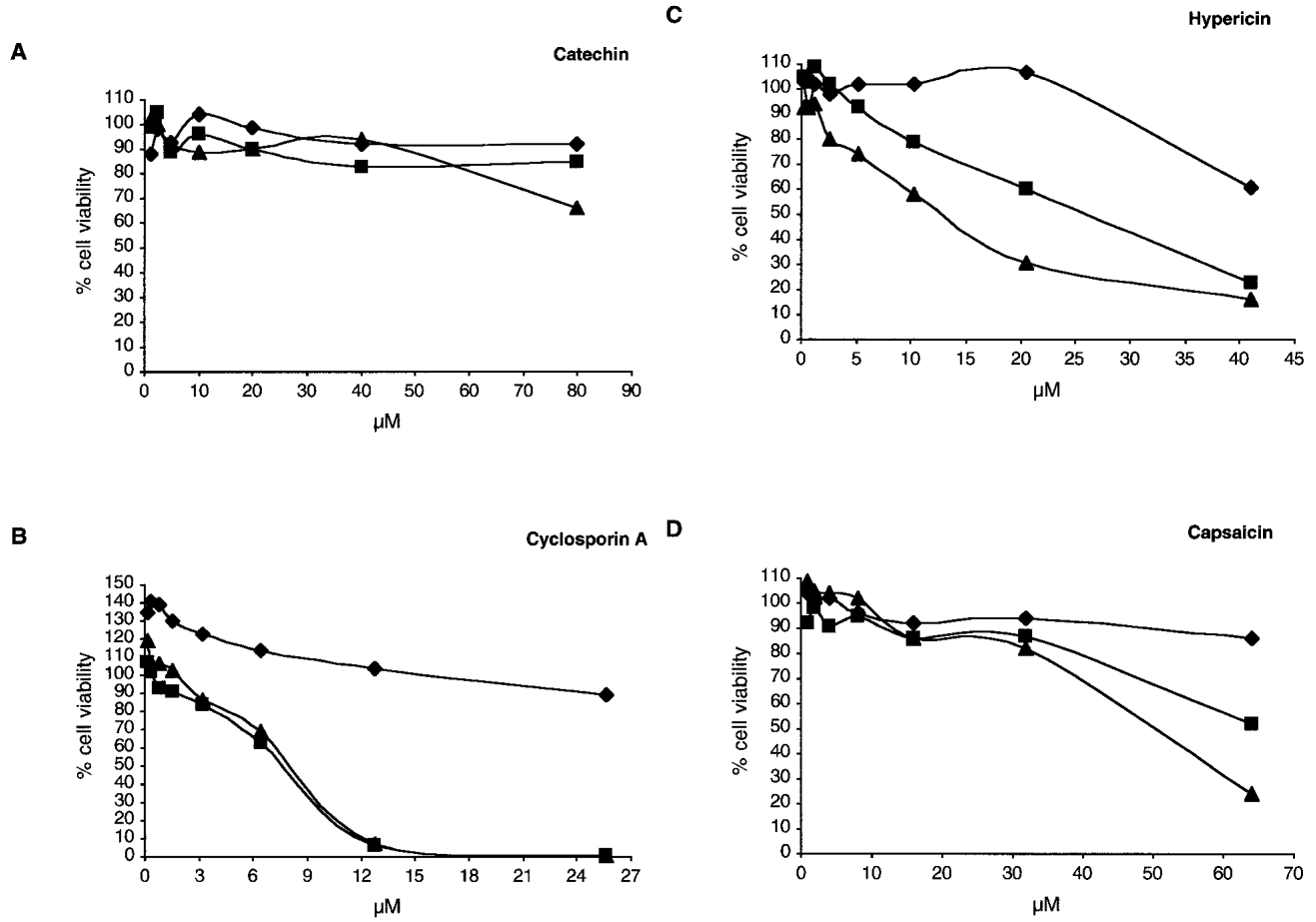

Fig. 3. Time-dependent cytotoxicity of catechin (A), cyclosporin A (B), hypericin (C), and capsaicin (D) against Jurkat T cells measured in the WST-1 assay. Symbols: rhombus, cytotoxicity after 2.5 h; square, cytotoxicity after 20 h; triangle, cytotoxicity after 48 h. Standard deviations are omitted for clarity (see Experimental section).

cell viability increased time-dependently, and after 48 h a significantly reduced cell viability was detected at concentrations as low as 10 μM (Fig. 3C). Trypan blue exclusion counting performed after 2.5, 20, and 48 h with 10 μM, the IC_{50} concentration obtained after 48 h in the WST-1 assay, showed that hypericin inhibited cell proliferation within the first 20 h and then led to cell death (data not shown).

Catechin

Catechin did not affect mRNA levels after 2.5 and 20 h incubation even at concentrations as high as 25 μM (Fig. 2). This compound did not affect cell viability up to a concentration of 80 μM (Fig. 3A).

DISCUSSION

In the present study, a T-cell–based assay was developed that successfully describes the modulation of mRNA levels after mitogen activation and is stable enough for the screening of natural compounds that target gene expression. The genes employed in our study are suitable as markers for immunomodulation, anti-inflammatory effects, as well as cytotoxicity. Cyclin D1, NF-ATc, I-κBα, and p65 are presented as possible marker genes, the down-regulation of which result in inhibition of proliferation or cell death. For the first time we show that p65 and I-κBα mRNA levels are strongly increased in PMA stimulated Jurkat cells after 2.5 h and 20 h, whereas NF-ATc and cyclin D1 mRNA levels were comparable to those in non-stimulated controls. The pro-inflammatory transcripts IL-2, GM-CSF, and INF-γ were up-regulated up to 20-fold after PMA stimulation in a time-dependent manner. It was our aim to investigate possible anti-inflammatory effects in a broader context, looking at both, factors that are up-regulated during inflammation and factors that are important for cell growth. We show that the RT-rt-PCR system presented is sensitive enough to distinguish between specifically anti-inflammatory and cytotoxic compounds, if it is connected to systems for quantification of cell viability. So far, it was not known, whether the anti-inflammatory effects reported for the natural products capsaicin and hypericin could be correlated to specific immunomodulation, i.e. through their specific inhibition of TFs or were caused by their cytotoxic potential and hence non-specific down-regulation of pro-inflammatory genes. The results obtained in our assay showed a specific immunomodulatory and anti-inflammatory effect with capsaicin but a non-specific and finally cytotoxic effect of hypericin in Jurkat cells. Capsaicin showed a specific inhibitory effect on the pro-inflammatory mRNA levels. The continuous up-regulation of cytokines over a time-span of 20 h was specifically inhibited at low and nontoxic concentrations (4 μM), none of TF or housekeeping mRNAs were modulated, and no decrease in cell viability was observed.

Capsaicin, therefore, provides an interesting lead structure for drugs used in the treatment of chronic inflammatory diseases. In contrast, down-regulation of NF-ATc, p65, I-κBα, and cyclin D1 in Jurkat T cells, by hypericin is coupled to cell death. The time-dependent and continuously growing down-regulation of the housekeeping genes seemed to be a further indication for a possible toxic action. This assumption is well supported by the time-dependent decrease in cell viability over a time span of 48 h.

The fact that cyclosporin A did not target any of the TF mRNAs tested, together with the observation that cell proliferation and viability was not inhibited at 2.5 μM over a time scale of 48 h, further confirms our assumption that constitutive NF-κB and NF-ATc mRNA levels are essential for the survival of Jurkat T cells. The decreased cell viability with higher concentrations of cyclosporin A is likely to be correlated to the down-regulation of cyclin D1. This was also shown by the fact that cyclosporin A did not kill the cells over a time span of 48 h but inhibited cell proliferation. The decreased cell number therefore led to a lower WST-1 cleavage. Whether the specific down-regulation of cyclin D1 observed after cyclosporin A incubation is the cause of the cell cycle inhibition in Jurkat cells would need further investigation. Our results are in concordance with previously published data on the antiproliferative nature of cyclosporin A (31). Cyclosporin A has also been shown to induce the cyclin inhibitor p21 in lymphocytes (32).

Despite the reported antioxidative properties of catechin (17), no effects on mRNA levels were observed. The data obtained in the RT-rt-PCR and WST-1 assays suggest that catechin has no anti-inflammatory (up to 25 μM tested) or cytotoxic (up to 80 μM tested) activity in the chosen test assays.

The RT-rt-PCR methodology used in our assay has several advantages over conventional competitive RT-PCR: 1) According to Luthra *et al.,* it is 100 times more sensitive than conventional PCR (8); 2) it has a higher specificity because of three sequence-specific oligonucleotides involved in the amplification process; 3) quantification is always performed in the log phase of PCR; 4) the linear range of quantification is wider; 5) there is almost no risk of cross-contamination with amplicons; 6) the detection of faulty amplifications is easy by software-based on-line verification of the overall quality of the PCR run.

We further showed that the cytotoxic and unspecific potency of a compound could be detected in RT-rt-PCR very early and in low concentrations. With hypericin a non-specific down-regulated mRNA profile was obtained between 2.5 and 20 h at concentrations, where the WST-1 assay did not show any reduction in cell viability. As shown in Fig. 3, the reduced cell viability with 8 μM hypericin only became visible after 48 h in theWST-1 assay. Further testing of other cytotoxic compounds have to answer the question, whether the time-dependent overall down regulation of mRNA level profile obtained for hypericin is a characteristic marker for unspecific cytotoxicity.

In conclusion, quantification of gene induction with RT-rt-PCR is not only a convenient assay in studying signaling events but opens up new possibilities for pharmacological research. Gene transcription studies can serve as functional broad-spectrum assays for lead discovery and assessment.

ACKNOWLEDGMENTS

The authors thank Dr. Reto Schwendener, Paul Scherrer Institute, Villigen, for providing the Jurkat cell line and Dr. Detlev Boison, Pharmacology and Toxicology, University of Zurich for having introduced the ABI PRISM 7700 system. They also thank Dr. Conwitha Lapke for proofreading the manuscript.

REFERENCES

1. A. J. Lewis and A. M. Manning. New targets for anti-inflammatory drugs. *Curr. Opin. Chem. Biol.* **3**:489–494 (1999).
2. M. J. Suto and L. Ransone. Novel approaches for the treatment of inflammatory diseases: inhibitors of NF-κB and AP-1. *Current Pharm. Design* **3**:515–528 (1997).
3. A. O'Garra and N. Arai. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Rev. Trends Cell. Biol.* **10**:542–550 (2000).
4. A. Tsuboi, M. Muramatsu, A. Tsusumi, K. Arai, and N. Arai. Calcineurin activates transcription from the GM-CSF promoter in synergy with either protein kinase C or NF-kappa B/AP-1 in T cells. *Biochem. Biophys. Res. Commun.* **199**:1064–1072 (1994).
5. S. Gerondakis, M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. *Oncogene* **18**:6888–6895 (1999).
6. K. K. Mann, S. Doerre, J. J. Schlezinger, D. H. Sherr, and S. Quadri. The role of NF-kappaB as a survival factor in environmental chemical-induced pre-B cell apoptosis. *Mol. Pharmacol.* **59**:302–309 (2001).
7. C. A. Heid, J. Stevens, K. J. Livak, and P. M. Williams. Real time quantitative PCR. *Genome Res.* **6**:986–994 (1996).
8. R. Luthra, J. A. McBridge, F. Cabanillas, and A. Sarris. Novel 5′ exonuclease-based real-time PCR assay for the detection of t(14;18)(q32;q21) in patients with follicular lymphoma. *Am. J. Pathol.* **153**:63–68 (1998).
9. T. Takenouchi and E. Munekata. Trophic effects of substance P and beta amyloid peptide on dibutyryl cyclic AMP-differentiated human leukemic (HL60) cells. *Life Sci.* **56**:479–484 (1995).
10. V. F. Quesniaux. Immunosuppressants; tools to investigate the physiological role of cytokines. *Bioassays* **15**:731–739 (1993).
11. D. Piomelli. The ligand that came from within. *Trends Pharmacol. Sci.* **22**:17–19 (2001).
12. Y. Surh. Molecular mechanism of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutat. Res.* **428**:305–327 (1999).
13. Y. J. Surh, S. S. Han, Y. S. Keum, H. J. Seo, and S. S. Lee. Inhibitory effects of curcumin and capsaicin on phorbol ester-induced activation of eukaryotic transcription factors, NF-kappaB and AP-1. *Biofactors* **12**:107–112 (2000).
14. J. Barnes, L. A. Anderson, and J. D. Phillipson. St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *J. Pharm. Pharmacol.* **53**:583–600 (2001).
15. C. M. Schempp, B. Simon-Haarhaus, C. C. Termeer, and J. C. Simon. Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. *FEBS Lett.* **493**:26–30 (2001).
16. P. M. Bork, S. Bacher, M. L. Schmitz, U. Kaspers, and M. Heinrich. Hypericin as a non-antioxidant inhibitor of NF-kappa B. *Planta Med.* **65**:297–300 (1999).
17. R. F. Anderson, L. J. Fisher, Y. Hara, T. Harris, W. B. Mak, L. D. Melton, and J. E. Packer. Green tea catechins partially protect DNA from OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. *Carcinogenesis* **22**:1189–1193 (2001).
18. K. M. Riedl and A. E. Hagerman. Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **49**: 4917–4923 (2001).
19. U. E. Gibson, C. A. Heid, and P. M. Williams. A novel method for real time quantitative RT-PCR. *Genome Res.* **6**:995–1001 (1996).

20. T. Lindl. *Zell- und Gewebekultur*, 4. Edition, Spektrum Akademischer Verlag, Berlin, 2000.
21. C. S. Sun, P. A. Ganchi, D. W. Ballard, and W. C. Greene. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science* **259**:1912–1915 (1993).
22. O. Silvennoinen. Immunodeficiencies and haematological disorders, direct connections to cellular signalling pathways. *Rev. Ann. Med.* **29**:519–529 (1997).
23. K. J. Hardy, B. Manger, M. Newton, and J. D. Stobo. Molecular events involved in regulating human interferon-gamma gene expression during T cell activation. *J. Immunol.* **138**:2353–2358 (1987).
24. A. Weiss, R. L. Wiskocil, and J. D. Stobo. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pretranslational level. *J. Immunol.* **133**:123–128 (1984).
25. R. L. Wiskocil, A. Weiss, J. Imbodden, R. Kamin-Lewis, and J. D. Stobo. Activation of a human T cell line: a two-stimulus requirement in the pretranslational events involved in the coordinate expression of interleukin 2 and gamma-interferon genes. *J. Immunol.* **134**:1599–1603 (1985).
26. O. Bill, C. G. Garlisi, D. S. Grove, G. E. Holt, and A. M. Maestro. IL-2 mRNA levels and degradation rates change with mode of stimulation and phorbol ester treatment of lymphocytes. *Cytokine* **6**:102–110 (1994).
27. V. Depraetere and P. Goldstein. Fas and other cell death signaling pathways. Review. *Semin. Immunol.* **9**:93–107 (1997).
28. R. Wang, L. Zhang, D. Yin, R. A. Mufson, and Y. Shi. Protein kinase C regulates Fas (CD95/APO-1) expression. *J. Immunol.* **161**:2201–2207 (1998).
29. C. C. Wilhide, C. van Dang, J. Dispersio, A. A. Kenedy, and P. F. Bray. Overexpression of cyclin D1 in the Dami megakaryocytic cell line causes growth arrest. *Blood* **86**:294–304 (1995).
30. F. Ajchenbaum, K. Ando, J. A. De Caprio, and J. D. Griffin. Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. *J. Biol. Chem.* **268**:4113–4119 (1993).
31. A. K. Khanna and J. D. Hosenpud. In vitro and in vivo transfection of p21 gene enhances cyclosporin A-mediated inhibition of lymphocyte proliferation. *J. Immunol.* **165**:1882–1888 (2000).
32. A. K. Khanna and J. D. Hosenpud. Cyclosporine induces the expression of the cyclin inhibitor p21. *Transplantation* **67**:1262–1268 (1999).