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Differential expression of protein kinase C isoenzymes related to high nitric oxide synthase activity in a T lymphoma cell line

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Abstract

Protein kinase C (PKC) is critical for T lymphocyte activation and proliferation, while nitric oxide synthase (NOS) may function both as an activator or inhibitor of T cell apoptosis. Both enzymatic activities were studied in T lymphoma cells in comparison to normal and activated T lymphocytes. Here we show a higher translocation of PKC in BW5147 lymphoma cells than in mitogen-stimulated T lymphocytes. Tumor cells overexpressed PKC ζ isoform, while high levels of the PKC β isotype were found in mitogen-stimulated T lymphocytes. Moreover, tumoral T cells showed high NOS activity, almost undetectable in normal or stimulated T lymphocytes. PKC and NOS inhibitors or the intracellular delivery of an anti-PKC ζ antibody diminished both NO production and proliferation in tumor cells.

These results suggest that atypical PKC ζ isoform expression and its association with NOS activity regulation would participate in the multistep process leading to BW5147 cell malignant transformation.

Keywords: NOS activity; PKC activity; PKC isoenzyme; T lymphocyte; T lymphoma cell line; Proliferation

1. Introduction

The knowledge of the signaling events that control cellular growth and differentiation is of paramount importance to understand the mechanisms involved in malignant transformation. Particularly, in lymphoid cells, it has been suggested that deregulation of intracellular pathways participates in leukemic disorders [1]. Phosphorylation and dephosphorylation of protein substrates by kinases and phosphatases, respectively, play prominent roles in the transduction of signals from cell membrane receptors to the nucleus. Physiological activation of T lymphocytes leading to differentiation and proliferation involves a complex series of intracellular signaling events that is initiated by protein tyrosine kinases (TPK) activation. The biochemical cascade downstream includes increased hydrolysis of inositol phospholipids, mediated by phospholipase C (PLC) γ1 phosphorylation, which gives rise to inositol triphosphate and diaciylglicerol (DAG). These second messengers, in turn, increase intracellular Ca^{2+} concentrations and activate protein kinase C (PKC), respectively [2,3].

PKC represents a family of serine/threonine kinases that plays a central role in signal transduction as it is involved in the control of numerous cellular processes, such as proliferation and differentiation, as well as in carcinogenesis [4–6]. Although all PKCs require the lipid phosphatidylserine as a cofactor, differences in their structure and substrate requirements allowed isoform classification into three subclasses: the conventional or classical PKCs (cPKC) (α, βI, βII and γ), which are Ca^{2+}-dependent and activated by DAG and phorbol esters; the novel PKCs (nPKC) (δ, ε, η and θ), which can be activated by DAG and phorbol esters but are Ca^{2+}-independent; and the atypical isoforms (aPKC) (ζ and τ/λ), which are unresponsive to both Ca^{2+} and DAG or phorbol esters [5]. The existence of such a large family of PKC isoenzymes, which exhibits different tissue distribution, subcellular localization and biochemical properties, suggests that individual PKC isoforms may play specialized roles in cellular functions. In many cases, PKC isoforms exhibit distinct and even opposing cellular effects [7,8]. A differential expression of PKC isozymes has been described for distinct hemopoietic lineages [9] and their participation in determined cellular functions has also been assigned. In fact, modulation of α and β isoform levels was related to B lymphocyte differentiation [9] while particular activation of atypical PKC ζ was demonstrated to participate

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in interleukin-2-mediated proliferation of T cells [10]. Also, PKC θ, an isoenzyme that was found predominantly in hematopoietic cells and skeletal muscle, was demonstrated to be expressed in T, but not in B lymphocytes, and played an important role in T lymphocyte activation as an essential component of the T-cell synapse [11,12].

On the other hand, nitric oxide (NO) is a physiologically important signal molecule in many cells and tissues, regulating a variety of biological functions, including immune processes [13]. The role of NO in the control of cell growth is controversial since both stimulation and inhibition have been demonstrated [14]. Also, NO exerts contrasting effects on apoptosis, depending on its concentration, flux and cell type, having, in some situations, an apoptotic effect while protecting cells against spontaneous or induced apoptosis in other [15]. The redox state of the cell seemed to be crucial for the ultimate action of NO on cell multiplication and survival. A protective antiapopatotic effect is commonly related to the endogenous NO production through NO synthases (NOS) and is frequently observed in lymphoid cells from B origin [16,17]. Cells from a wide variety of malignancies have a decreased ability to undergo apoptosis in response to some physiological stimuli and inhibition of apoptosis can result in the expression of cells with increased susceptibility to subsequent malignant transformation [1,18]. Furthermore, production of NO, as well as expression of different NOS isoenzymes, both at the protein and mRNA level, were demonstrated to be related to PKC activity in several cell types including tumor cells [19,20]. Production of NO downstream PKC was demonstrated to be induced by immunopotentiators in splenic lymphocytes [21].

The present study was undertaken to analyze PKC activity and isoenzyme expression and their relationship with NO production in a T lymphoma cell line. Also their contribution to cellular proliferation was analyzed. All these studies were performed in comparison to normal unstimulated and mitogen stimulated T cells. Here we report that tumor T cells show significantly higher levels of PKC ζ and increased NOS activity than resting or mitogen-stimulated normal T lymphocytes. Intracellular delivery of PKC ζ isoform-specific antibodies results in a decrease of both proliferation and NOS activity in tumor T cells. The implications of these results in neoplastic transformation are discussed.

2. Materials and methods

2.1. Cell suspensions and culture conditions

The tumor cell line BW5147 (a generous gift from Dr. A. Schimpl, Institute für Virologie und Immunobiologie der Universität Würzburg, Germany) is a T cell lymphoma that expresses H-2^k haplotype, $CD3^+$ and αβ T cell receptor, as tested routinely by flow cytometry with specific antibodies against the corresponding surface markers. These cells were cultured at an optimal concentration of $1-5 \times 10^5$ cells/ml, in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics, with twice-weekly splitting once they have reached exponential growth. Where indicated, synchronized (kept for 24 h in FCS-deprived medium) BW5147 cells were used.

Aseptically prepared lymphoid cell suspensions from C3H (H-2^k) inbred mice lymph nodes were obtained from nylon wool purification of T cells as described before [22,23]. T cell purification was higher than 97% as checked by lysis with anti-Thy plus complement and by indirect immunofluorescence. Cells, at a concentration of 1×10^6 cells/ml, were cultured in the same medium as tumor cells, alone or in the presence of 2 μg/ml of concanavalin A (Con A), as previously described [22].

Cells were settled at a final volume of 0.2 ml in 96-well flat-bottom microtiter plates (Nunc™) for microcultures or were kept in T-25 or T-75 culture flasks (Corning, NY) for macrocultures. Cells were cultured for different times and inhibitors were added at the beginning of the culture where indicated.

2.2. Proliferation assays

Proliferation was evaluated on microcultures and controlled in 0.2-ml aliquots of macrocultures established for enzymatic assays.

The proliferation kinetics of BW5147 cells was determined on BW5147 synchronized cells after re-culturing them in FCS-rich medium. Cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR, NEN, 20 Ci/mmol) for the last 6 h of incubation, as described before [22]. Results are expressed as dpm values in experimental cultures subtracting the dpm control values obtained on BW5147 synchronized cells. The percentage of inhibition (% inh) for experimental cultures performed in the presence of inhibitors was calculated as:

$$\%\ \text{inh} = \left[1 - \frac{\text{dpm BW5147 cells} + \text{inhibitor} - \text{dpm control}}{\text{dpm BW5147 cells} - \text{dpm control}}\right] \times 100$$

For Con A-stimulated T lymphocytes, cultures were pulsed with [^{3}H]TdR as indicated for BW5147 and cultures of unstimulated cells were used as controls. The action of inhibitors upon Con A-stimulated T cells was evaluated as indicated for BW5147 cells.

It is worth noting that inhibitors alone do not significantly modify either the basal control values of BW5147 (synchronized BW5147 cells) or the unstimulated cultures of normal T lymphocytes.

2.3. PKC assay

Synchronized BW51457 cells (0.5×10^7 cells/sample) were re-cultured for different times in FCS-rich medium

alone or in the presence of the indicated drugs and were immediately frozen in liquid N_2. Normal or Con A-stimulated T cells (1×10^7 cells/sample) were incubated alone or with inhibitors for the indicated times and processed as tumor cells. PKC was purified from total cell extracts or from subcellular fractions as previously described [24]. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into histone H_1 [24]. Incubations were conducted in a final volume of 85 μl at 30 °C for 30 min. In the final concentrations, the assay mixture contained 25 μM ATP (0.4 μCi), 10 mM Mg acetate, 5 mM β-mercaptoethanol, 50 μg of histone H_1, 20 mM HEPES, pH = 7.5, 10 μg/ml of phosphatidylserine vesicles and in the presence or absence of 0.2 mM $CaCl_2$. The incorporation of [^{32}P]-phosphate into histone was linear for at least 30 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid with 10 mM H_3PO_4. The radioactivity retained on GF/C glass fiber filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the ^{32}P-incorporation in the absence of Ca^{2+} and phospholipids. Data were expressed as picomoles of phosphate incorporated into the substrate per minute and per 10^7 cells (pmol/min/10^7 cells). The selective PKC substrate peptide, MBP(4–14) [25] (GIBCO BRL), was also used to measure PKC activity purified from subcellular lymphoid fractions, following the instructions of the PKC assay system from GIBCO BRL. PKC specificity was confirmed by the PKC pseudosubstrate inhibitor peptide PKC (19–36) provided by GIBCO BRL.

2.4. NOS activity determination

NOS activity was measured by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine [26]. Briefly, cells were incubated in 500-μl RPMI-1640, in the presence of [U-^{14}C]-arginine (0.5 μCi). After incubation, cells were disrupted by sonication (Vibra-cell, Sonics and Materials) in a medium containing 10 mmol/l EGTA, 0.1 mmol/l citrulline, 0.1 mmol/l dithiothreitol and 20 mmol/l HEPES, pH 7.5. After centrifugation at 20,000 × *g* for 10 min, supernatants were applied to 2-ml columns of Dowex AG 50WX-8 (sodium form, Bio-Rad) and [U-^{14}C]-citrulline was eluted with 3 ml of water and quantified by liquid scintillation counter.

2.5. Immunoblot analysis of PKC isoenzymes

Samples of whole cell lysates from tumor and normal T lymphocytes were prepared by dissolving cellular pellets in SDS-sample buffer (2% SDS, 10% (v/v) glycerol, 62.5 mM Tris–HCl, pH = 6.8, 0.2% bromophenol blue, 1% (v/v) 2-mercaptoethanol) at a final concentration of 10 mg/ml. Equal amounts of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Where indicated, cytosol and particulate fractions of unstimulated or stimulated cells, obtained as described by Meller et al. [27], representing 2×10^6 cell equivalents per 10 μl and supplemented with 2.5-μl 5 × SDS sample buffer, were analyzed by SDS-PAGE and immunoblotted, as above.

Nonspecific binding sites in nitrocellulose membranes were blocked with blocking buffer (5% nonfat dried milk, containing 0.1% Tween-20 in 100 mM Tris–HCl, pH = 7.5 and 0.9% NaCl) for 1 h. The nitrocellulose was subsequently incubated with protein G-purified anti-peptide antibodies to specific PKC isoforms for 18 h. Anti-PKC isoenzyme specific antibodies from GIBCO BRL are polyclonal antibodies raised against specific peptides from each isoenzyme. The α-, β- and γ-specific antibodies were raised against the peptides corresponding to amino acids 313–326, 313–329 and 306–318, respectively, derived from murine cDNA sequences. The δ-, ε- and ζ-specific antibodies were raised against C-terminal peptides corresponding to amino acids 662–673, 726–737 and 577–592, respectively. The anti-PKC θ antibody (C-18, from Santa Cruz Biotechnologies) specific for an epitope mapping at the carboxy terminus of nPKC θ of mouse origin was also used. Negative controls were incubated in the presence of immunogenic peptides at a concentration 2 μg/ml antibody to 1 μg/ml peptide. The membrane was then incubated with a monoclonal anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co.) for 1 h. Immunoreactive bands were visualized using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). As molecular weight markers, a Full Range Rainbow (Amersham Pharmacia) was used. Anti-actin antibody (rabbit polyclonal, against actin of human origin, but cross-reactive to mouse actin, from Santa Cruz Biotechnol.) was used as a control for protein loaded. Densitometric analysis was performed by UN-SCAN-IT (version 5.1, Silk Scientific Corporation) software. Densitometric values of actin (arbitrary units) for each gel were normalized relative to each other and PKC densitometry values (arbitrary units) in each lane were divided by the corresponding normalized actin value.

2.6. Intracellular delivery of anti-PKC ζ antibody in normal and tumor T cells

Anti-PKC ζ antibody against the C-terminal region of PKC (GIBCO BRL) was introduced into normal or BW5147 tumor T cells after permeabilization with lysolecithin as described [28]. Briefly, BW5147 cells were equilibrated in 7% FCS-containing medium for 24 h. Normal cells or tumor cells, cultured as indicated, were washed with serum-free medium and 0.1-ml glycerol in PBS at 37 °C was added. After 6 min on ice, 4 μl of a 1 mg/ml lysolecithin (Sigma) solution in water was added, and the incubation continued for an additional 5 min. Cells were then brought to 37 °C and 0.1 ml of a dilution of antibody (alone or preincubated with 3 × concentration of the corresponding peptide) was added. After 10 additional minutes at 37 °C, during which the cells reseal, 0.1 ml of medium containing 21% FCS was added. Cells were brought back to

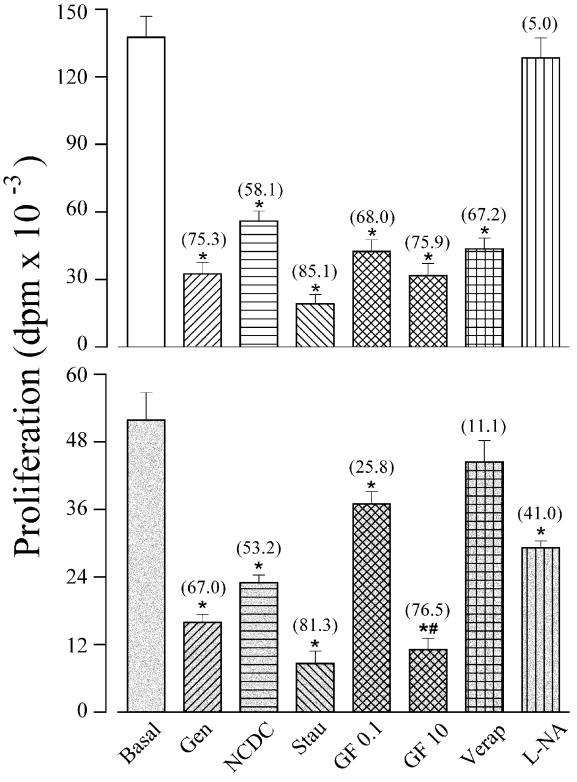


Fig. 1. Effect of different enzymatic and calcium blockers on Con A-stimulated and tumor T cell proliferation. Purified T lymphocytes were prepared aseptically following the indicated procedures and stimulated with Con A (2 μg/ml), for 72 h (▭). Cells were cultured in the absence (basal) or presence of the drugs mentioned above. BW5147 synchronized cells (▬) were incubated for 24 h in FCS-rich medium alone (basal) or with the indicated blockers. The following blockers, added at the beginning of culture, were used: genistein (GEN) (30 μg/ml), NCDC (10 μM), staurosporine (STAU) (0.1 μM), GF109203X (GF) (0.1 or 10 μM), verapamil (VER) (0.1 mM) and L-NAME (L-NA) (5 mM). Proliferation was evaluated by [^{3}H]TdR incorporation. Percentage of inhibition of cellular proliferative activity, calculated as described in Materials and methods, is shown between brackets. Results shown are the mean ± SD of three independent experiments performed in triplicate. * Differs significantly from the corresponding basal value with at least $P<0.01$. # Differs significantly from the other values of BW5147 cells with at least $P<0.05$.

the 7% FCS-containing medium. Incubations were continued for 72 h, in the presence of 2 μg/ml Con A, for normal T cells or 24 h for BW5147 cells. Control untreated cells were submitted to the same permeabilization schedule than intracellular antibody-delivered cells. Proliferation was evaluated by [^{3}H]TdR incorporation by adding the radioligand substrate 6 h prior to culture finalization. NOS enzymatic activity was evaluated immediately upon the ending time of incubation, as indicated before.

2.7. Drugs

The following drugs were used in cultures at the final concentrations indicated in Results. The protein kinase inhibitors staurosporine, the selective PKC inhibitor GF-109203X (bisindolylmaleimide), the PLC blocker 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC) and the TPK inhibitor genistein (all from Sigma) were dissolved in DMSO. N^G-Nitro-L-arginine methyl ester (L-NAME) (RBI, USA) was dissolved in water. Stock solutions were freshly prepared before use. All drugs were further diluted (at least 1:1000 or more) in RPMI 1640 medium to achieve the concentrations indicated in Results.

2.8. Statistical analysis

The Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparison were necessary after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P \leq 0.05$.

3. Results

3.1. Blockers of intracellular pathways involved in T lymphocyte activation displayed different actions on mitogen-stimulated and tumor T cell proliferation

In order to analyze differences in the enzymatic intracellular pathways involved in both mitogen-induced and tumor T cell proliferation, the effect of TPK, PLC, PKC and calcium blockers was analyzed. As shown in Fig. 1, the blockade of TPK by genistein, at a dose that has no effect on cellular viability, PLC by NCDC or PKC by either staurosporine or the more selective GF109203X inhibit both normal and tumor cell proliferation. It is worth noting that

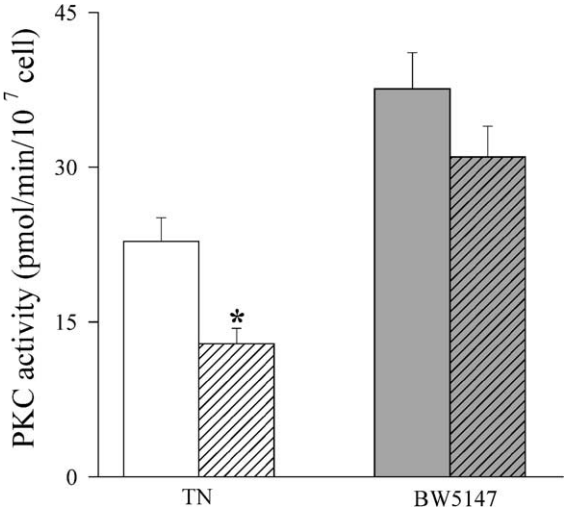


Fig. 2. Calcium dependence of PKC total activity in normal and tumor T cells. PKC total activity was purified from normal T lymphocytes (TN, white bars) or tumor BW5147 cells (gray bars) and was measured, in the presence (open bars) or in the absence of Ca^{2+} (dashed bars), using the substrate MBP (4–14), as described in Materials and methods. Results shown are the mean ± SD from four independent experiments performed in duplicate. * Differs significantly from the corresponding basal value with $P<0.01$.

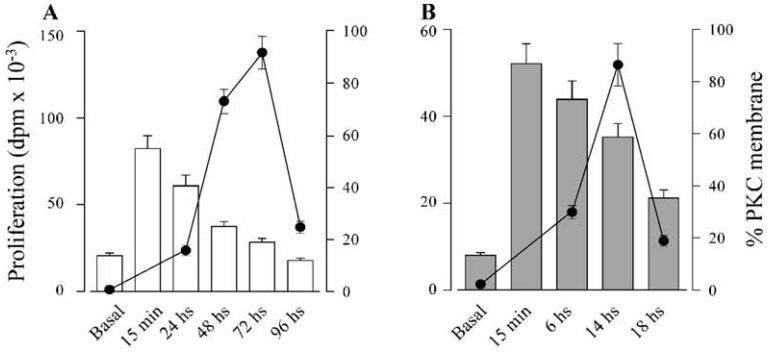
GF109203X is a potent inhibitor of cPKC (IC50 ≅ 0.01–0.02 μM), but a weak inhibitor of nPKC (IC50 ≅ 0.1–0.2 μM) or aPKC (IC50 for PKC ζ ≅ 6.0 μM) [7]. So, the fact that this blocker exerted a significant higher effect at 10 μM than 0.1 μM concentration could be related to the different IC50 s. Furthermore, the calcium blocker verapamil, although efficiently inhibiting Con A-stimulated proliferation, had little effect on T lymphoma cell growth.

To assess the role of cell-specific pathways for NO in normal and tumor cells, the action of the NOS competitive blocker, L-NAME, was also determined. Fig. 1 also shows that L-NAME was able to abrogate tumor, but not normal, T cell proliferation.

3.2. PKC and NOS activities in normal, activated and tumor T cells

To compare PKC activity on both normal and tumor cells and to evaluate the balance between calcium-dependent and independent PKC isoforms, enzymatic activity was measured on whole cell extracts in the presence or absence of Ca^{2+} using the specific PKC substrate, MBP (4–14) peptide. As shown in Fig. 2, total PKC activity was higher in tumor than in normal T cells, and it was unaffected in the absence of Ca^{2+}. In normal T cells a decrease of approximately 43% was observed in the absence of Ca^{2+}.

To analyze differences in PKC translocation to plasma membrane, related to cellular proliferation in both normal and tumor T lymphocytes, PKC enzymatic activity was measured on both soluble and particulate fractions from mitogen-stimulated normal and tumor T cell, at different times during the kinetics of proliferation. For this purpose BW5147 synchronized cells were induced to proliferate by re-culturing them in FCS-rich medium. Resting normal T lymphocytes were activated by Con A in vitro as indicated before. As shown in Fig. 3 and in accordance to Fig. 2, total PKC activity was higher on synchronized tumor (basal) than in normal T cells. Within 15-min exposure to FCS, synchronized BW5147 cells displayed an early and significant increment in membrane PKC activity that was accompanied by the decrease in cytosolic PKC (Table in Fig. 3). The increment in phosphorylated substrate was observed



Cell type	Treatment	PKC[a] (pmol/10^7 cell) Cytosol / Membrane	Proliferation[b] (dpm)
T Normal	---	12.7 ± 0.8 / 2.3 ± 0.3	1034 ± 108
	Con A	6.1 ± 0.7 / 7.4 ± 0.7*	137524 ± 9370*
BW5147	Without FCS	29.4 ± 2.9 / 4.5 ± 0.5	1209 ± 235
	With FCS	4.0 ± 0.4 / 26.7 ± 1.8*#	51875 ± 4612*

Fig. 3. PKC activity and proliferation of normal, mitogen-stimulated and tumor T cells. Panel A: Normal T lymphocytes were kept in culture alone (basal) or in the presence of Con A (2 μg/ml) for the indicated times. Proliferation (curve) was evaluated by [^{3}H]TdR incorporation, in 0.2-ml aliquots of macrocultures established for enzymatic PKC activity determination. Results shown are the mean ± SD of three independent experiments performed in triplicate. PKC activity in membrane fractions was determined using MBP (4–14) as described in Materials and methods and percent of total (cytosol + membrane) PKC activity (bars) from the three experiments performed in duplicate were calculated. Panel B: BW5147 cells, synchronized by 24-h culture in FCS-deprived medium, were recultured in FCS-rich RPMI 1640 medium as indicated before. Proliferation (curve) was evaluated by 6 h lasting pulses of [^{3}H]TdR, as indicated in panel A, on BW5147 synchronized (without FCS, basal) and after resetting them in FCS-rich medium for the indicated times. Results shown are the mean ± SD of three experiments performed in triplicate. Percentages of total PKC activity corresponding to membrane purified fractions determined as in Panel A are shown in the bar graphic.

A summary of both PKC activity (pmol/10^7 cells) and proliferation (dpm) is shown in the adjoining table. (a) PKC activity values from purified cytosol and membrane fractions corresponding to basal or 15 min treatment with Con A (normal T cells) or with FCS-rich medium (BW5147 cells) are shown. Results are the mean ± SD of three experiments performed in duplicate. (b) Mean dpm ± SD of [^{3}H]TdR incorporation corresponding to basal or to the peak of proliferation (72 h for Con A-treated T lymphocytes or 14 h for BW5147 cells). * Differs from basal values with at least $P < 0.01$. *# Differs from basal values and from Con A-stimulated T cells with at least $P < 0.01$.

both when using histone H_1 (data not shown) or the specific PKC peptide MBP (4–14). This membrane PKC activity diminished but stayed higher than basal values after the peak of proliferation. It is worth noting that exponentially growing BW5147 cells displayed similar values for total PKC activity, with most of this activity (80–90%) in membrane fractions.

On the other hand, mitogenic stimulation of resting normal T lymphocytes also induced an early translocation of PKC (within 15 min), but to a lesser extent to that found in tumor cells. Membrane PKC returns to basal levels after the peak of proliferation.

With respect to NOS activity, we found very low levels in normal T cells (Fig. 4), with a slight nonsignificant increase upon physiologic activation. On the contrary, BW5147 cells showed an important basal NOS activity, which was blunted by L-NAME (Fig. 4); an effect that was reverted by L-arginine (data not shown). To analyze the relationship between high PKC and NOS activities in BW5147 cells, the effect of PKC blockers on NOS was also determined. As can be seen in Fig. 4, both staurosporine and GF109203X in high doses were able to inhibit, similarly, NOS activity in tumor cells.

3.3. PKC isoform expression in mitogen-activated and tumor T cells

In order to asses that differences in PKC activity could be related to distinct isoenzyme expression, PKC isoform profile was analyzed on both tumor and resting or mitogen-stimulated T cells by Western blot analysis. The expression of cPKC α and β, nPKC δ, θ and ε, and the atypical PKC ζ isoform in lysates from naive or Con A-stimulated T cells and exponentially growing BW5147 cells was measured. A representative plot of the levels of PKC isoenzymes in the different cell types is shown in Fig. 5 (Panel A). As can be seen, resting T cells express the cPKC α and β and the nPKC θ, but not γ (data not shown) isoform with barely detectable amounts of PKC δ and ζ. As an example of PKC translocation upon cellular activation, within 15 min of mitogenic stimulation, membrane levels of cPKC β isoform were increased in membrane fractions (Fig. 5, Panel B). Also, after 72 h of Con A treatment, a significant increase in levels of cPKC α and β was observed. On the contrary, exponentially growing BW5147 cells showed only vanishing amounts of the β isoform and an increased expression of PKC ζ (Fig. 5, Panel A). Whereas levels of α, γ, δ and ε isoforms were identical to those found in activated normal T lymphocytes of the same H-2 haplotype (Fig. 5A), expression of the θ isotype was not found. It is worth noting that all the bands were detected at the positions corresponding to PKC isoenzymes from T lymphoid cells previously described [29]. PKC ζ translocation to the particulate fraction was obtained upon 15-min stimulation of synchronized BW5147 cells with FCS (Fig. 5, Panel B).

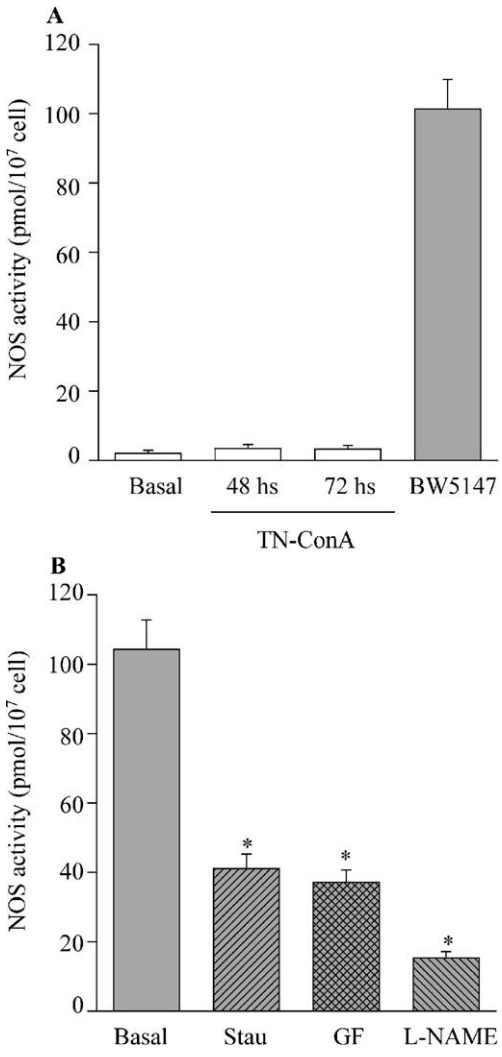


Fig. 4. NOS activity in normal, mitogen-stimulated and tumor T cells. Panel A: NOS activity was measured by the conversion of [^{14}C]-arginine to [^{14}C]-citrulline as indicated in Materials and methods in normal T lymphocytes (▭), basal or Con A-stimulated for 48 or 72 h, or BW5147 (▬) cells. Panel B: Effect of the PKC blockers, staurosporine (Stau, 0.1 μM) or GF109203X (GF, 10 μM) and the NOS blocker, L-NAME (5mM), on BW5147 cells incubated alone (Basal) or in the presence of the indicated blocker concentrations for 24 h is shown. Results are the mean ± SD of $n=4$ experiments. * Differs significantly from basal values with at least $P<0.05$.

3.4. Effect of the intracellular delivery of anti-PKC ζ isoform antibody on BW5147 proliferation and NOS activity

In order to determine the involvement of PKC ζ in the high proliferative rate and NOS activity from tumor T cells, the effect of intracellular delivering of a specific antibody against the C-terminal region of PKC ζ, was assessed. Anti-PKC ζ antibody (Fig. 6) was able to diminish, in the concentrations tested, both proliferation and NOS activity in BW5147 cells, but not in normal activated T lymphocytes. It is worth noting that no differences from basal values with dilutions higher than 1:500 (data not shown)

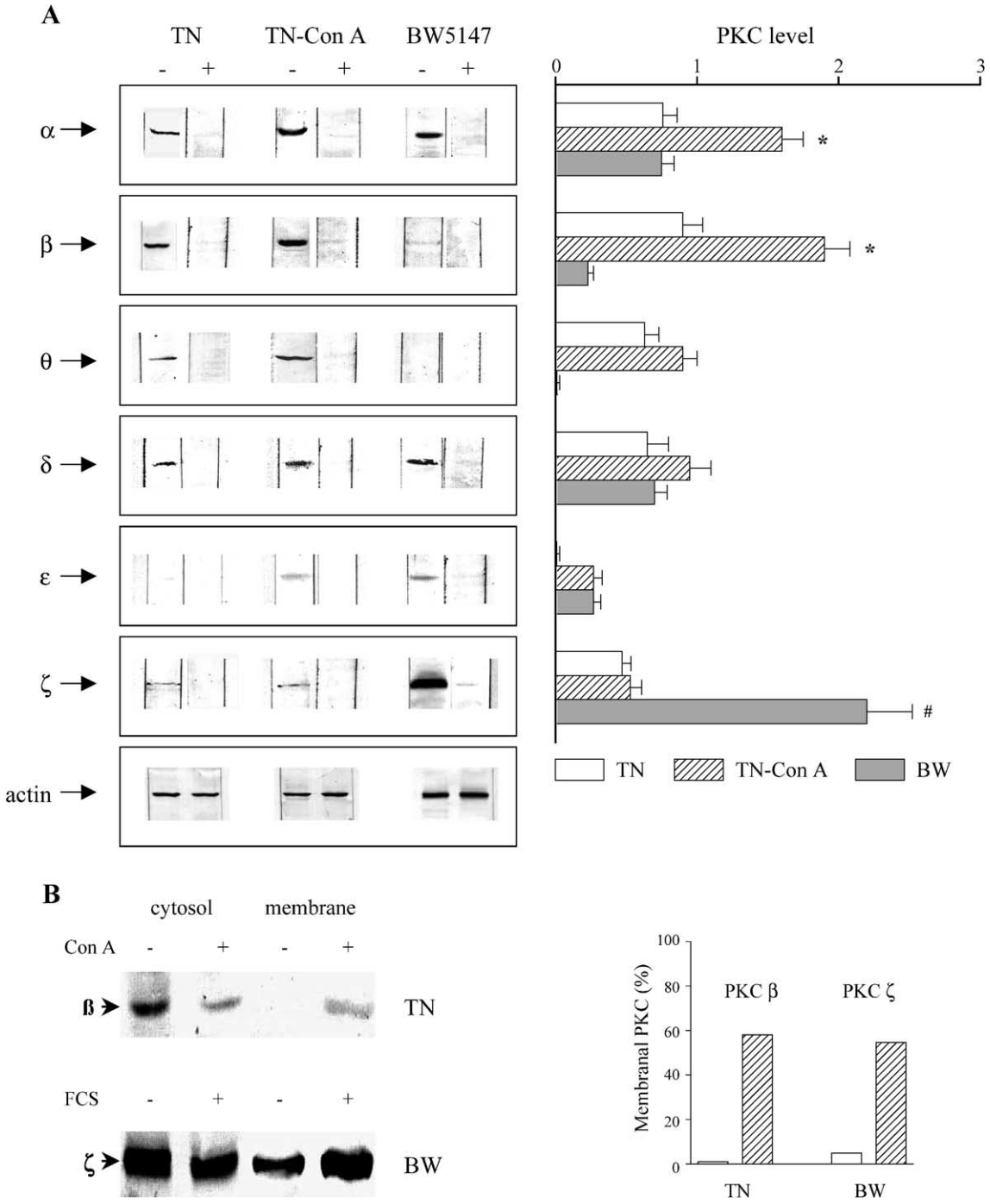
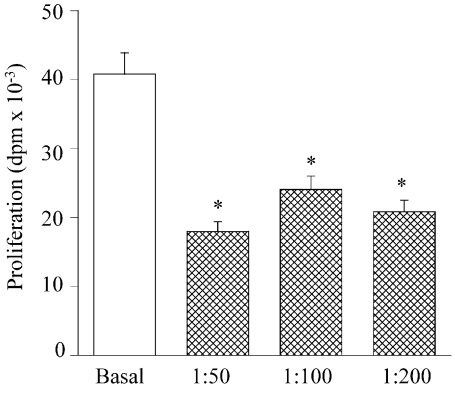


Fig. 5. PKC isotype expression in normal (TN), Con A-stimulated (TN-Con A) or BW5147 T cells. Panel A: The data show the Western blot analysis of PKC α, β, θ, δ, ε and ζ isotypes. Specificity of anti-PKC antibodies was assessed by performing Western blot analyses in the absence (−) or presence (+) of the corresponding peptide against which the antibodies had been raised. Results, corresponding to TN, TN-Con A (culturing time 72 h) and BW5147 total cells, are representative of seven independent experiments. Anti-actin antibodies were tested on same samples used for PKC isoform analysis as control for protein loading in three of these experiments. Also, PKC levels for each isoenzyme (bar graph) were calculated in relation to the expression level of actin in the same sample as described in Materials and methods. Results shown are the mean ± SD of $n=3$ experiments. Statistic analysis was performed within each isoenzyme group. * Differs significantly from TN and BW values with at least $P<0.01$. # Differs significantly from TN and TN-Con A values with at least $P<0.001$. Panel B: Subcellular distribution of PKC β and ζ isoenzymes in normal and tumor T cells, respectively, upon cellular activation. Freshly isolated T lymphocytes or synchronyzed BW5147 cells were either left unstimulated (−) or stimulated (+) with Con A (2 μg/ml) or FCS (10%), respectively, for 15 min. Cytosol and membrane fraction of 2×10^6 cell equivalents were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. Arrows indicate the position of the corresponding PKC protein bands. The relative intensity of the different PKC bands was quantitated by densitometry and the percentage of the membranal PKC out of the sum of cytosolic plus membranal PKC was calculated in each case and represented in the bar graph. It is worth noting that both Western blots and the bar graph are one representative out of three experiments.

A



B

Cell type	Addition	Proliferation (dpm)	NOS activity (pmol / 10^7 cell)
BW5147	none	38752 ± 3410	91.0 ± 7.8
	Anti-PKCζ	19376 ± 1744*	47.5 ± 3.6*
	Anti-PKCζ + peptide	41276 ± 3343	95.3 ± 6.9
TN-ConA	none	98234 ± 8841	5.9 ± 0.6
	Anti-PKCζ	88428 ± 7074	6.1 ± 0.5

Fig. 6. Effect of intracellular delivery of an anti-PKC ζ antibody on cellular proliferation and NOS activity. Panel A: BW5147 cells were permeabilized with lysolecithin and incubated in the absence or presence of different dilutions of the anti-PKC ζ for 10 min and resealed. After 24 h of incubation, cellular proliferation was assessed as described before. Panel B: both normal or tumor cells were permeabilized, as indicated in panel A, and incubated in the absence or presence of anti-PKC ζ antibody (in a concentration 1:200) or anti-PKC ζ plus PKC ζ peptide (1:3). After resealing, BW5147 cells were incubated for 24 h and normal cells were stimulated with Con A (TN-Con A) for 72 h. Proliferation and NOS activity were measured as described. Results shown are the mean ± SD of three independent experiments. *Differs significantly from the corresponding basal (none) values with at least $P < 0.05$.

were found in tumor cells. These effects were blunted by pretreatment of the antibody with the specific immunogenic ζ peptide (Fig. 6).

4. Discussion

Both PKC and NOS activities play important roles in the regulation of cellular functions. In this work the involvement of these enzymatic pathways in the proliferation of a T lymphoma cell line was studied in comparison to normal and mitogen-stimulated T lymphocytes. Tumor BW5147 cells exhibit higher total PKC activity and membrane PKC translocation than normal or mitogen-stimulated T cells. In normal T lymphocytes, PKC activity was diminished in the absence of calcium, while it was almost unmodified in tumor cells, thus pointing to a prevalence of calcium-independent PKC isoform activity in tumor cells in comparison to normal lymphocytes. It is worth noting that the calcium blocker verapamil inhibits Con A-induced proliferation of normal cells, but has not a significative effect in the proliferation of BW5147 cells. PKC inhibitors or blockers of the upstream signaling pathways, namely, TPK and PLC, are capable of inhibiting both mitogen-stimulated and tumor T cell proliferation. GF109203X, a potent inhibitor of cPKC but a weak inhibitor of calcium-independent isoforms, was able to efficiently block tumor cell proliferation at high doses, while equally inhibiting Con A-stimulated proliferation at low and high doses. These results indicate that different PKC isoforms would have differential hierarchical participation in normal and tumor T cell activation. The higher PKC translocation observed in tumor cells with respect to the physiologic translocation induced by the mitogenic stimulus would point to the participation of this intracellular transduction mechanism in the sustained proliferation of BW5147 cells. In fact, a correlation has been proposed between PKC activity and the rate of cellular growth, as well as a parallelism between PKC inhibition and induction of cellular differentiation in lymphoid cells [4].

Several experimental evidences indicate that PKC isoenzymes are localized in specific tissues and that different isoforms are related to lymphoid proliferation or differentiation [4,9,10], so the pattern of PKC isoenzymes was studied on both normal and tumor T cells. We found that normal and activated T lymphocytes display the calcium-dependent α and β PKC isoforms, as well as the nPKC θ, and detectable levels of ε and ζ, with no expression of the γ isoenzyme. Similarly, Mischak et al. [9] found the expression of mRNA encoding for α and β, but not γ isoforms, with low levels of PKC ε and ζ, in resting murine T lymphocytes and a close correlation between protein expression and mRNA levels. With respect to the θ isoform, its expression and role in T cell activation was already demonstrated [27]. The expression of cPKC α and β was progressively up-regulated by Con A stimulation and this correlates with cell proliferation. Several stimuli, including mitogens, were demonstrated to induce changes in T lymphocyte PKC isoenzyme expression, both at protein and mRNA levels [30–32]. These changes were time- and cell-type-dependent. Thus, a decrease in PKC β protein levels within 24 h of mitogen stimulation [30] and an earlier but transient loss of β and α PKC mRNA were described in murine thymocytes [31]. Despite this, Isakov et al. [32] demonstrate that mitogen stimulation of human mature T cells is followed by a four- to fivefold increase in PKC α and β mRNA, peaking at 48 h and correlating with cellular proliferation, which is in agreement with our results. Differences from the data of Strulovici [30] could be related with the distinct T cell populations studied.

BW5147 cells have barely detectable levels of the β isoform, with similar levels of α and δ isoforms, and no detectable levels of the γ and θ isoenzymes. Interestingly, we found an increased expression of the atypical calcium-

independent ζ isoenzyme. These results are in agreement with the fact that cellular proliferation is mainly calcium-independent in BW5147 cells, and PKC activity involves calcium-independent isoforms, as it was not modified in the presence of EGTA. Goodnight et al. [33] have described elevated expression of PKC ζ in B lymphocytic neoplasms at both mRNA and protein levels. Also, a high expression of the β isoform has been related to accelerated leukemic cell differentiation [34], while final differentiation of B lymphocytes into antibody-secreting plasma cells is accompanied by a loss in this isoenzyme [9]. Taken together, these facts and our results, it is possible that the increase in PKC β isoform observed during mitogenic stimulation of T cells would allow an efficient but limited activation, leading to differentiation to immune effector cells, while low levels of this isoenzyme, together with high levels of the ζ isoform, would be responsible for the hyperproliferative pattern of BW5147 cell line.

On the other hand, the NOS blocker L-NAME impaired tumor, but not normal T cell proliferation. Also, BW5147 cells exhibit high NOS activity, while a low basal activity was found in resting normal T cells, which was not increased by mitogenic stimulation. High NOS activity levels in tumor cells were blunted by L-NAME and were partially impaired by PKC blockers, thus indicating that PKC activity is, in part, responsible for NOS activity in BW5147 cells. High levels of endogenous NOS activity would allow the survival of tumor cells, protecting them from apoptotic mechanisms. Furthermore, intracellular delivery of an antibody directed to the most abundant PKC isoform in BW5147 cells, an anti-PKC ζ antibody, not only diminished cellular proliferation, but also lowered NOS activity in tumor cells, with no effect on mitogen-activated T lymphocytes. Other authors have previously demonstrated that intracellular delivery of antibodies directed to the C-terminal region of specific PKC isoforms are able to modify physiological functions of different cell types [28], including T lymphocytes [35]. In support to our results, overexpression of PKC ζ isoform was demonstrated to markedly increase the inducible NOS isoenzyme expression in rat mesangial cells [36]. The fact that anti-PKC ζ antibody was not able to completely abrogate NOS activity or proliferation of BW5147 cells indicates that probably other isoforms could be involved in these functions, but nevertheless, they point to a role for the PKC ζ isoenzyme. Actually, PKC activation by phorbol esters, which activates cPKC and nPKC but not aPKC, was demonstrated to increase NOS expression and/or activity in several cell types [19,20,26,37].

Experimental evidence showed that aPKCs play important roles in controlling cell growth and survival [38]. In fact, it was demonstrated that PKC ζ is critical for mitogenic signal transduction in oocytes [39], while aPKC τ was shown to protect human leukemic cells from drug-induced apoptosis [40]. Furthermore, Tsutsumi et al. [41] demonstrated the down-regulation of PKC ζ RNA following cross-linking of membrane IgM on B lymphoma cells, and growth arrest and apoptosis were induced in a B cell line by cross-linking of surface immunoglobulin [42]. There are also evidences that endothelial NOS autocrine is able to protect T lymphocytes from apoptosis [43].

For the first time, shown here are evidences that strengthened the crosstalk of PKC and NOS enzymatic pathways in T lymphoid cells and their participation in cellular proliferation. Although further studies would be necessary to analyze the role of other isoforms, our results would indicate that hyperproliferation of BW5147 cells is related to PKC hyperactivity, with a major participation of calcium-independent isoenzymes and particularly of PKC ζ. This activity is in part responsible for inducing NOS activation that would protect BW5147 cells from apoptotic signals, contributing in this way to cellular survival. Our findings also suggest that the PKC ζ member of the PKC multigene family participates in the multistep process of BW5147 cell growth, leading to the malignant phenotype.

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References

[1] C.L. Sawyus, C.T. Denny, O.N. Wilte, Cell 64 (1991) 337–350.
[2] A. Weiss, D. Littman, Cell 76 (1994) 263–274.
[3] M. Szamel, A. Appel, R. Schwinzer, K. Resch, J. Immunol. 160 (1998) 2207–2214.
[4] M.J. Clemens, I. Trayner, J. Menaya, J. Cell Sci. 103 (1992) 881–887.
[5] Y. Nishizuka, FASEB J. 9 (1995) 484–496.
[6] G.C. Mayne, A.W. Murray, J. Biol. Chem. 273 (1998) 24115–24121.
[7] K.J. Way, E. Chou, G.L. King, Trends Pharmacol. Sci. 21 (2000) 181–187.
[8] E.C. Dempsey, A.C. Newton, D. Mochly-Rosen, A.P. Fields, M.E. Reylad, P.A. Insel, R.O. Messing, Am. J. Physiol., Lung Cell. Mol. Physiol. 279 (2000) L429–L438.
[9] H. Mischak, W. Kolch, J. Goodnight, W.F. Davidson, U. Rapp, S. Rose-John, J.F. Mushinski, J. Immunol. 147 (1991) 3981–3987.
[10] J. Gomez, C. Pitton, A. Garcia, A. Martinez de Aragon, A. Silva, A. Rebollo, Exp. Cell Res. 218 (1995) 105–113.
[11] A. Altman, N. Isakov, G. Baier, Immunol. Today 21 (2000) 567–573.
[12] N. Meller, A. Altman, N. Isakov, Stem Cells 16 (1998) 178–192.
[13] B. Brune, A. von Knethen, K.B. Sandau, Eur. J. Pharmacol. 351 (1998) 261–272.
[14] C. Sciorati, G. Nisticò, J. Meldolesi, E. Clementi, Br. J. Pharmacol. 122 (1997) 687–697.
[15] J.P. Kolb, Leukemia 14 (2000) 1685–1694.
[16] J.B. Mannick, K. Asano, K. Izumi, E. Kieff, J.S. Sttamler, Cell 79 (1994) 1137–1146.
[17] A.M. Genaro, S. Hortelano, A. Alvarez, A. Martinez, L. Bosca, J. Clin. Invest. 95 (1995) 1884–1890.
[18] C.B. Thompson, Science 267 (1995) 1456–1462.
[19] Y. Hirose, C.V. Rao, B.S. Reddy, Int. J. Oncol. 18 (2001) 141–146.

[20] I. Momose, M. Terashima, Y. Nakashima, M. Sakamoto, H. Ishino, T. Nabika, Y. Hosokawa, Y. Tanigawa, Biochim. Biophys. Acta 1498 (2000) 19–31.
[21] S. Goenka, T. Das, G. Sa, P.K. Ray, Biochem. Biophys. Res. Commun. 250 (1998) 425–429.
[22] C.A. Cazaux, L. Sterin-Borda, G. Gorelik, G.A. Cremaschi, FEBS Lett. 364 (1995) 120–124.
[23] A.M. Genaro, E. Borda, G.A. Cremaschi, L. Sterin-Borda, M. Braun, Immunopharmacology 11 (1986) 129–140.
[24] A.M. Genaro, L. Boscá, J. Immunol. 151 (1993) 1832–1843.
[25] I. Yasuda, A. Kishimoto, S. Tanaka, M. Tominaga, A. Sakurai, Y. Nishizuka, Biochem. Biophys. Res. Commun. 166 (1990) 1220–1227.
[26] S. Hortelano, A.M. Genaro, L. Boscá, FEBS Lett. 320 (1993) 135–139.
[27] N. Meller, Y. Elitzur, N. Isakov, Cell. Immunol. 193 (1999) 185–193.
[28] U. Leli, P.J. Parker, T.B. Shea, FEBS Lett. 297 (1992) 91–94.
[29] C. Keenan, D. Kelleher, A. Long, Eur. J. Immunol. 25 (1995) 13–17.
[30] B. Strulovici, S.D. Isaakani, G. Baxter, J. Knopf, L. Sultzman, H. Cherwinsky, J. Nestor, D.R. Webb, J. Ransom, J. Biol. Chem. 266 (1991) 168–173.
[31] J. Freire-Moar, H. Cherwinsky, F. Hwang, J. Ransom, D. Webb, J. Immunol. 147 (1991) 405–409.
[32] N. Isakov, M.I. Mally, A. Altman, Mol. Immunol. 29 (1991) 927–933.
[33] J. Goodnight, H. Mischak, J.F. Mushinski, Mol. Carcinog. 11 (1994) 131–137.
[34] E. Melloni, S. Pontremoli, B. Sparatore, M. Patrone, F. Grossi, P.A. Marks, R.A. Rifkind, Proc. Natl. Acad. Sci. U. S. A. 87 1990, 4417–4420.
[35] M. Szamel, U. Ebel, P. Uciechowski, V. Kaever, K. Resch, Biochim. Biophys. Acta 1356 (1997) 237–248.
[36] B.W. Miller, L.D. Baier, A.R. Morrison, Am. J. Physiol. 273 (1997) C130–C136.
[37] J. Kang, M. Yang, I. Jou, E. Joe, Neurosci. Lett. 299 (2001) 205–208.
[38] J. Moscat, M.T. Diaz-Meco, EMBO Rep. 11 (2000) 399–403.
[39] E. Berra, M.T. Diaz-Meco, I. Dominguez, M.M. Municio, L. Sanz, J. Lozano, R.S. Chapkin, J. Moscat, Cell 74 (1993) 555–563.
[40] N.R. Murray, A. Fields, J. Biol. Chem. 272 (1997) 27521–27524.
[41] A. Tsutsumi, J. Freire-Moar, J.T. Ransom, Cell. Immunol. 142 (1992) 303–312.
[42] S. An, K.A. Knox, FEBS Lett. 386 (1996) 115–122.
[43] C. Sciorati, P. Rovere, M. Ferrarini, S. Heltai, A.A. Manfredi, E. Clementi, J. Biol. Chem. 272 (1997) 23211–23215.