

# The role of protein kinase C isoenzymes in the regulation of calcineurin activity in human peripheral blood mononuclear cells

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**Abstract.** It is known that PMA (phorbol-12-myristate-13-acetate) can activate the classical and novel protein kinase C isoenzymes (cPKC  $\alpha$ ,  $\beta$ ,  $\gamma$  and nPKC  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), while the calcium ion can induce only the activity of cPKC. Calcineurin binding protein (Cabin 1) belongs to the group of endogenous inhibitors of calcineurin. Cabin 1 becomes hyperphosphorylated in response to PKC activation and may play a negative role in calcineurin signalling. It was observed that both PMA treatment and the increase in intracellular  $\text{Ca}^{2+}$  contributed to the reduction of calcineurin activity in human peripheral blood mononuclear cells without modulating the mRNA and the protein levels of calcineurin. PMA and Ca-ionophore (A23187), the activating agents of PKC, applied alone or in combination, significantly increased the phosphorylation state of Cabin 1 as revealed by immunoprecipitation of Cabin 1 detecting its phospho-Ser content by specific antibodies. GF109203X, an inhibitor of the classic and the novel protein kinase C isoenzymes, and Gö6976, the selective inhibitor of the classical cPKC isoenzymes were able to abolish the effect of PMA or/and Ca-ionophore on the calcineurin activity with concomitant reversal of the hyperphosphorylation of Cabin 1. The calcineurin/Cabin 1 system was not influenced by Rottlerin, an inhibitor of PKC  $\delta$  isoenzyme either in the absence or in the presence of Ca-ionophore and PMA. We presented evidence for the prominent role of cPKC  $\alpha$ ,  $\beta$ ,  $\gamma$  isoenzymes in the inhibition of calcineurin as induced by PMA and Ca-ionophore. We demonstrated also that hyperphosphorylation of Cabin 1 by

PMA/ $\text{Ca}^{2+}$ -activated cPKC isoenzymes resulted in a simultaneous inhibition of calcineurin in peripheral blood mononuclear cells. These results suggest a negative regulatory role for Cabin 1 in calcineurin signalling and provide a possible mechanism of feedback inhibition through cross-talk between PKC and calcineurin.

## Introduction

A consequence of T cell activation is an increase in the level of second messengers inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG). The calcium release induced by  $\text{IP}_3$  influences the activity of protein phosphatase 2B also termed calcineurin. Calcineurin promotes the nuclear translocation of NFAT (nuclear factor of activated T cells) by dephosphorylating the transcription factor. NFAT plays a prominent role in the transcription of several genes of pro-inflammatory cytokines including interleukin-2 (IL2), IL4, IL5, IL8, IL10, tumour necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ), interferon  $\gamma$  ( $\text{IFN}\gamma$ ), cell surface molecules (e.g. CD40 and ICAM), and Fas ligand (1-3). Calcineurin plays essential roles in T cell receptor (TCR)-mediated peripheral T cell activation, cell proliferation, differentiation, and death. Its dependence on calcium and calmodulin for the catalytic function is unique among all known protein phosphatases, thus making it one of the intracellular transducers of calcium signalling pathways. The phosphatase activity of calcineurin depends on the binding of  $\text{Ca}^{2+}$  to its regulatory subunit and the  $\text{Ca}^{2+}$ -dependent binding of calmodulin to the catalytic subunit of the enzyme (4-8). Recently, a growing number of endogenous calcineurin-binding proteins have been discovered which affect enzyme activity. They are classified as dual regulators, anchoring proteins and inhibitors of calcineurin. Calcineurin binding protein (Cabin 1 or Cain) belongs to the group of endogenous inhibitors of calcineurin, which results in the inhibition of enzyme activity by binding to calcineurin through a conserved motif PXIXIT also found in Cabin 1 as (PEITVT). Cabin 1 is hypophosphorylated in non-activated T cells, and in response to protein kinase C (PKC) activation, it becomes hyperphosphorylated exhibiting a higher affinity for

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calcineurin. It has been reported that the interaction between Cabin 1 and calcineurin requires both calcium signalling and activation of PKC (8-10).

The enzymes of the PKC superfamily phosphorylate Ser and Thr residues in many target proteins and play central roles in the regulation of various cellular processes in numerous cell types. To date, at least 11 different PKC isoenzymes have been identified, which can be classified into three major groups that differ in their cofactor requirements. These are known as the conventional PKC (cPKC  $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ), the novel PKC (nPKC  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ), the calcium and phorbol-ester-independent atypical PKC (aPKC  $\zeta$ , and  $\lambda$ / $\iota$ ) isoforms, and the unique PKC $\mu$ . Both cPKCs and nPKCs can be activated with phorbol ester, however, the most important difference is that the nPKCs do not contain a Ca<sup>2+</sup>-binding domain unlike cPKC, i.e. nPKCs are insensitive for Ca<sup>2+</sup> signalling. These isoforms, possessing characteristic tissue and cellular distribution, regulate in an isoform-specific manner various cellular functions such as proliferation, differentiation, cytokine production and release, and receptor-mediated signal transduction (11,12).

Systemic lupus erythematosus (SLE) is an autoimmune disease. In SLE various abnormalities affecting signal transduction pathways have been reported, for example abnormal NF- $\kappa$ B (nuclear factor- $\kappa$ B) activity and intracellular distribution of NFAT1, overproduction of pro-inflammatory cytokines, decreased expression of TCR  $\zeta$  chain and PKC  $\theta$ , and decreased PKC-dependent protein phosphorylation (13-18). Previously, we studied the activity of calcineurin in healthy and lupus T cells treated with activating agents of PKC (5  $\mu$ M of Ca-ionophore, A23187 and 80 nM phorbol-ester, PMA, phorbol-12-myristate-13-acetate) in the presence or the absence of glucocorticosteroid (GCS) mostly used for the treatment of SLE in the active period of the disease. We reported a significant decrease in calcineurin activity in peripheral blood mononuclear cells (PBMC) from patients with SLE. It was demonstrated also that the GCS-treatment of PBMC from healthy controls exhibited lower calcineurin activity in the presence of Ca-ionophore and PMA. Our observation suggested that the PKC enzymes may play a role in the regulation of calcineurin in T cells (19).

In this study we aimed at investigating the possible role of PKC isoenzymes upon stimulation by phorbol-ester and Ca-ionophore in the inhibition of calcineurin in PBMC from healthy controls. Furthermore, we also showed that the inhibition of calcineurin is related to Cabin 1 hyperphosphorylated by PKC.

## Materials and methods

*Preparation of human peripheral blood mononuclear cells (PBMC) and characterizations of cells by flow cytometry.* PBMC, containing 88-95% lymphocytes and 5-12% monocytes, were prepared (20) from the heparinized blood of healthy donors. The averages of various cellular subsets were detected by flow cytometry: CD3<sup>+</sup> 69.4%, CD19<sup>+</sup> 11.5%, CD56<sup>+</sup> 0.8%, and CD14<sup>+</sup> 8.3%. The suspension of mononuclear cells (10<sup>6</sup> cells per sample) was labelled by saturating concentrations of anti-CD3-FITC (T3, Coulter, Hialeah, FL,

USA), anti-CD19-RD1 (B4, Coulter), anti-CD56-PE (Leu-19, Becton Dickinson, Mountain View, CA, USA), and anti-CD14-RD1 (MY4, Coulter). After staining and fixing, the cells were analyzed by a Coulter Epics XL flow cytometer (Coulter).

*Stimulation of PBMC.* The cells (5x10<sup>6</sup> cells/ml) were incubated with 5  $\mu$ M of Ca-ionophore (A23187, Sigma, St. Louis, MO) or/and 80 nM of phorbol-12-myristate 13-acetate (PMA, Sigma) for 4 h in a CO<sub>2</sub> incubator at 37°C. For PKC inhibition studies the cells were preincubated for 1 h with various cell-permeable inhibitors [1  $\mu$ M GF109203X, 200 nM Gö6976, 10  $\mu$ M Rottlerin (Calbiochem, EMD Bioscience Inc., San Diego, CA)] prior to the treatment with Ca-ionophore or/and PMA.

*Viability assay.* After the stimulation of PBMC, 2x10<sup>6</sup> cells/100  $\mu$ l were treated with alamarBlue according to the manufacturer's instructions (BioSource International, Inc., Camarillo, CA). The cells were incubated in a CO<sub>2</sub> incubator at 37°C for 45 min. Following the incubation, the fluorescence of the alamarBlue was determined at 530-nm excitation and at 590-nm emission by using Fluoroskan Acent FI (Thermo Labsystems, Stockholm, Sweden). The stimulating agents of PKC had no significant effects on the arbitrary fluorescence unit (AFU) measured in the samples. The means of the AFU (3-4 independent experiments) with standard deviation values were as follows: 3434 $\pm$ 321 for the control, 3250 $\pm$ 414 for PMA, 3688 $\pm$ 395 for Ca-ionophore, and 3512 $\pm$ 219 for PMA and Ca-ionophore. The treatment of the samples with GF109203X, Gö6976, and Rottlerin did not affect the values of AFU determined in the absence and presence of stimulating agents.

*Preparation of cell extracts.* After the stimulation of PBMC, cells were pelleted and washed thoroughly with PBS (20 mM Na<sub>2</sub>HPO<sub>4</sub> and 115 mM NaCl, pH 7.4), then suspended in 100  $\mu$ l of homogenization buffer containing 50 mM Tris-HCl buffer (pH 7.0), 0.5 mM dithiothreitol, 10  $\mu$ g/ml Gortex, 10  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 5 mM benzamidine, 10  $\mu$ g/ml trypsin inhibitor as protease inhibitors, and 0.5% Triton X-100. After freezing and storing at -70°C, thawed suspensions were sonicated by a pulsing burst four times for 30 sec by 50 cycles (Branson Sonifier, Danbury, CT, USA). The supernatants were used promptly for calcineurin assays after centrifugation at 10,000 x g for 10 min at 4°C. For Western blot analysis, total cell lysates were used. For RT-PCR analysis samples were washed three times with nuclease-free physiological sodium chloride solution and then the cultures were stored at -70°C.

*Assay of calcineurin.* Calcineurin activity was measured by the release of <sup>32</sup>Pi from <sup>32</sup>P-labelled protein phosphatase inhibitor-1 (780 cpm/pmol) (21) with some modifications (22). The assay mixture (30  $\mu$ l) contained 50 mM Tris-HCl buffer (pH 7.0), 0.16 mM dithiothreitol, 3.4  $\mu$ g/ml Gortex, 3.4  $\mu$ g/ml leupeptin, 1 mM PMSF, 1.6 mM benzamidine, 3.4  $\mu$ g/ml trypsin inhibitor as protease inhibitors, 40  $\mu$ g/ml calmodulin, 0.2 mM CaCl<sub>2</sub>, 100 nM okadaic acid (OA), 2 nM protein phosphatase inhibitor-2, an appropriate amount of

cell extract (~80  $\mu$ g protein/assay) and  $^{32}$ P-labelled protein phosphatase inhibitor-1 (20,000-30,000 cpm/reaction mixture). The assay mixtures were incubated at 30°C for 20 min. The reaction was terminated by the addition of 100  $\mu$ l of 20% trichloroacetic acid and 100  $\mu$ l of 6 mg/ml bovine serum albumin (BSA, Sigma). After centrifugation the radioactivity of the supernatant (180  $\mu$ l) was determined in a liquid scintillation counter.

**RT-PCR analysis.** Total RNA was isolated from cells using an RNA isolation kit according to the manufacturer's instruction (Gentra Systems Inc., Minneapolis, MN, USA). The assay mixture for reverse transcriptase reaction contained 2  $\mu$ g RNA, 0.112  $\mu$ M oligo(dT), 0.5 mM dNTP, 200 units M-MLV RT in 1X RT buffer. The sequences of the primer pairs for polymerase chain reaction were as follows: for human calcineurin, 5'-TAC CCT GCA GTT TGT GAA TT-3' and 5'-ATA TGT TGA GCA CAT TTA CCA-3'; for human GAPDH, 5'-CCA GAA GAC TGT GGA TGG CC-3' and 5'-CTG TAG CCA AAT TCG TTG TC-3'. Amplifications were performed in a thermocycler (PCR Express temperature cycling system, Hybaid, UK) as follows: 94°C for 1 min, followed by 30 cycles (94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec) and then at 72°C for 5 min. PCR products were analyzed by electrophoresis in 1.2% agarose gel containing ethidium bromide.

**Immunoprecipitation of Cabin 1.** After sonification of cell suspensions, the samples were centrifuged at 13000 x g for 10 min at 4°C, and the supernatants were used for immunoprecipitation analysis. The immunoprecipitation buffer contained 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1% Triton X-100, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, 5 mM benzamide, and 10  $\mu$ g/ml trypsin inhibitor. Cell lysates containing 200  $\mu$ g of protein were incubated with 0.25  $\mu$ g/ml anti-rabbit IgG antibody (Sigma) and Protein A Sepharose (Sigma) for 2 h at 4°C. After centrifugation at 2000 x g for 1 min at 4°C, the supernatants were incubated with 5  $\mu$ l anti-Cabin 1 antibody (Affinity BioReagent, Golden, CO) for 2 h at 4°C. Then 50  $\mu$ l Protein A Sepharose beads were added to the precleared samples containing antibody-protein complexes and were incubated overnight at 4°C. After collecting the antigen-antibody-protein A complexes by centrifugation at 1500 x g for 5 min at 4°C and discarding the supernatant, pellets were washed three times with immunoprecipitation buffer. For SDS-PAGE, antigen-antibody-protein A samples were prepared by adding 1/2 volume of 2-fold-concentrated electrophoresis sample buffer (124 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 40 mM DTT, 0.004% bromophenol blue) and boiling for 5 min.

**Western blot analysis.** Samples for SDS-PAGE were prepared by adding 1/5 volume of 5-fold-concentrated electrophoresis sample buffer (310 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 100 mM DTT, 0.01% bromophenol blue) to the cell lysates and boiling for 10 min. Approximately 10-50  $\mu$ g of proteins was separated by 10 or 5% SDS-PAGE gel for calcineurin and Cabin 1, respectively. After the gel electrophoresis, proteins were transferred electrophoretically

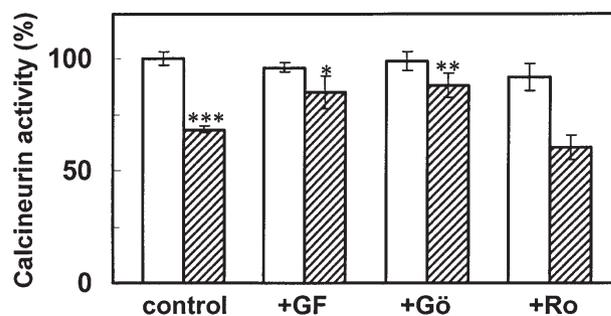


Figure 1. Calcineurin activities in PBMC of healthy donors treated with PMA and various PKC inhibitors. The effect of 80 nM PMA on the activity of calcineurin in the absence (open columns) and presence (hatched columns) of PKC inhibitors (1  $\mu$ M GF, 0.2  $\mu$ M Gö, 10  $\mu$ M Ro). Data represent the average values  $\pm$  SEM of 4-6 independent experiments. Calcineurin activity in PBMC stimulated with PMA was compared to the non-stimulated control, while the effect of PKC inhibitors on the phosphatase activity was compared to the non-stimulated and PMA-treated controls, respectively. Significant differences are shown as \* $p$ <0.05, \*\* $p$ <0.01, and \*\*\* $p$ <0.001. Calcineurin assay and the conditions of stimulation can be found in Materials and methods.

to a nitrocellulose membrane. After blocking with 5% non-fat dry milk in PBST (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 115 mM NaCl, 0.1% Tween-20, pH 7.4), the membranes were washed and exposed to the primary antibodies overnight at 4°C. For detection of phospho-Ser of Cabin 1, 3% BSA in PBST was used for blocking. Monoclonal anti-calcineurin ( $\alpha$ -subunit) primary antibody (Sigma) in 1:300 dilution, polyclonal anti-Cabin 1 antibody (Affinity BioReagent) in 1:500 dilution, and monoclonal anti-phospho-Ser antibody (Calbiochem, EMD Bioscience Inc., San Diego, CA) in 1:50 dilution were used. After washing three times for 10 min with PBST, the membranes were incubated with the second antibody, anti-mouse IgG (Sigma) in 1:2000 dilution for calcineurin and phospho-Ser, and anti-rabbit IgG (Sigma) in 1:2000 dilution for Cabin 1. The dilution was conducted in PBST containing 1% non-fat dry milk for calcineurin and Cabin 1 and 1% BSA for phospho-Ser. The signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

**Statistical analysis.** Statistical means and standard error of the mean (SEM) values were evaluated by the Student's t-test. For the densitometric analysis of mRNA and protein levels, each value was calculated as the mean of the data from 3-6 healthy control subjects. The evaluation and statistics of optical densities are based on 3 independent experiments (RT-PCR and Western blots).

## Results

**Effect of various protein kinase C inhibitors on the calcineurin activity of human PBMC stimulated by phorbol-ester.** PKC inhibitors were used as follows: GF109203X (GF), an inhibitor of the classic and the novel; Gö6976 (Gö), an inhibitor of the classic; and Rottlerin (Ro), an inhibitor of the  $\delta$  type of PKC isoenzymes. None of the three PKC inhibitors were found to have any significant effect on the activity of calcineurin in the absence of PMA (Fig. 1). Stimulation of cells with PMA resulted in a significant

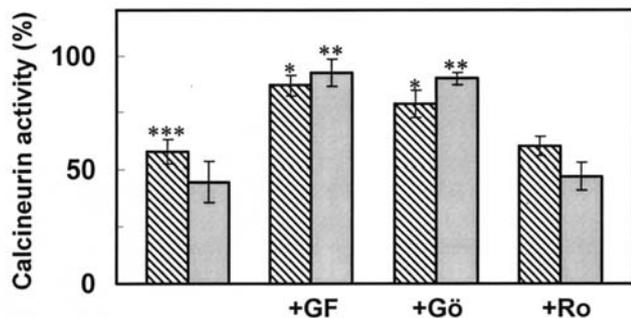


Figure 2. Calcineurin activities in the PBMC of healthy donors treated with Ca-ionophore, PMA, and various PKC inhibitors. Samples were stimulated with 5  $\mu$ M Ca-ionophore in the absence (hatched columns) or in the presence of 80 nM PMA (filled columns) using the same concentrations of PKC inhibitors as given in Fig. 1. Data represent the average values  $\pm$  SEM of 4-6 independent experiments. Calcineurin activity in PBMC stimulated with Ca-ionophore was compared to the non-stimulated control, while samples treated with Ca-ionophore and PMA were compared to the enzyme activity of PBMC stimulated with Ca-ionophore. The effect of PKC inhibitors on the calcineurin activity was compared to that of PBMC treated with Ca-ionophore in the presence or absence of PMA, respectively. Significant differences are shown as \* $p$ <0.05, \*\* $p$ <0.01, and \*\*\* $p$ <0.001. Calcineurin assay and the conditions of stimulation can be found in Materials and methods.

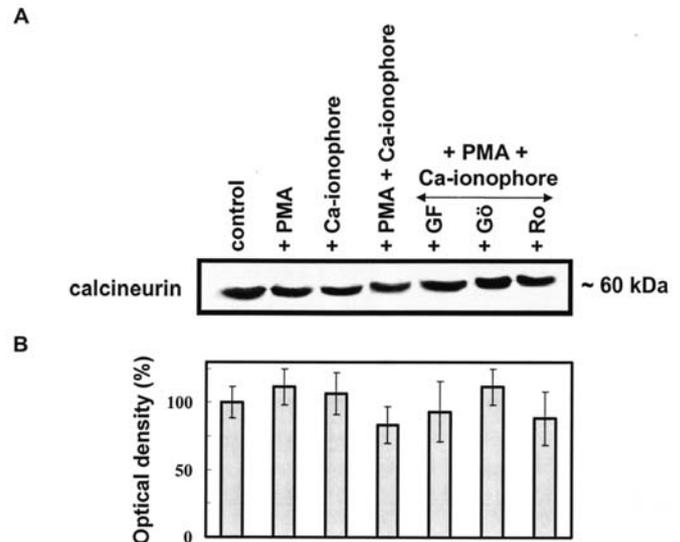


Figure 4. Western blot analyses of calcineurin in the PBMC of healthy patients. (A) Immunoblot of calcineurin in the presence of various effectors. Concentrations of effectors were the same as used in experiments shown in Figs. 1 and 2. (B) Optical density of the protein level of calcineurin showing the average values with standard error of the mean of 3 independent experiments. No significant changes were found in the various samples.

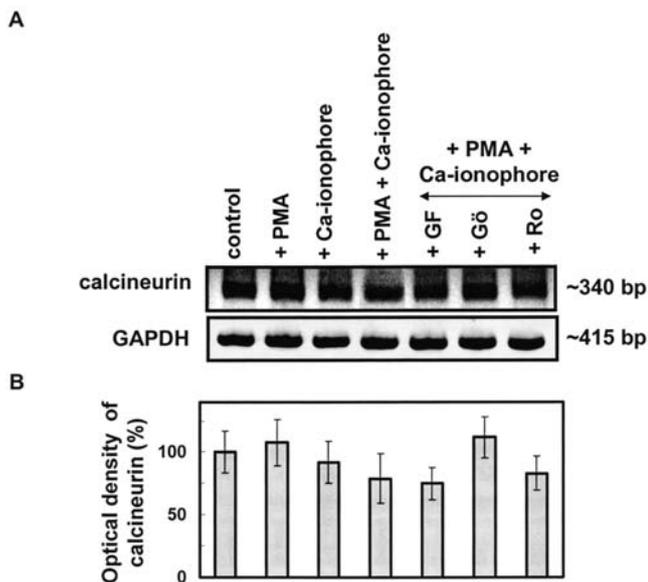


Figure 3. RT-PCR analyses of calcineurin and GAPDH mRNAs from the PBMC of healthy donors. (A) Representative mRNA values for calcineurin in PBMC treated with PMA, Ca-ionophore, and PKC inhibitors. Concentrations were the same as those given in Figs. 1 and 2. (B) Optical density of calcineurin mRNAs representing the average values with standard error of the mean of 3 independent experiments. The RT-PCR assay and the conditions of stimulation are given in Materials and methods.

decrease in calcineurin activity as compared to the non-stimulated control (68 versus 100%). This reduced calcineurin activity was elevated in PMA-treated samples when GF109203X and Gö6976 were also applied. On the other hand, Rottlerin did not restore the reduced enzymatic activity of calcineurin.

*Effect of various PKC inhibitors on the activity of calcineurin in human PBMC stimulated by Ca-ionophore with or without*

*PMA.* Similar to PMA, Ca-ionophore stimulation also resulted in a significant decrease in the activity of calcineurin (58 versus 100%). Moreover, this was a slightly higher reduction than that observed in the presence of PMA. There was no significant change in the calcineurin activity of PBMC simultaneously treated with both activating agents as compared to the enzyme activity of PBMC stimulated with Ca-ionophore alone. PKC inhibitors, GF109203X and Gö6976, reversed the phosphatase activity in the stimulated cells approximately with the same efficacy, while Rottlerin had no effect under these conditions (Fig. 2). It is known that GF109203X is a less selective inhibitor of PKC isoenzymes than Gö6976. Gö6976, a selective inhibitor of cPKC isoenzymes, alone was able to counteract the inhibition of calcineurin suggesting the involvement of cPKC  $\alpha$ ,  $\beta$ ,  $\gamma$  isoenzymes in the PMA- and/or Ca-ionophore-induced changes.

*RT-PCR and Western blot analyses of mRNA and protein levels of calcineurin in human PBMC.* The RT-PCR and Western blot analysis indicated that neither PMA nor Ca-ionophore applied alone modified the mRNA and protein levels of the enzyme (Figs. 3 and 4). The combined application of PMA and Ca-ionophore resulted in a 20% decrease in the mRNA level of calcineurin as compared to the non-stimulated control. However, it was not a significant change and had no appreciable effect on the protein level of calcineurin. Gö6976, an inhibitor of cPKC isoenzymes, slightly increased the mRNA level of calcineurin in the presence of PMA and Ca-ionophore as compared to that of the stimulated PBMC although the statistical analysis failed to show any significant change (Fig. 3). The above data imply that the decrease in the calcineurin activity of PBMC treated with PMA and Ca-ionophore is not due to the inhibition of transcription or translation of calcineurin.

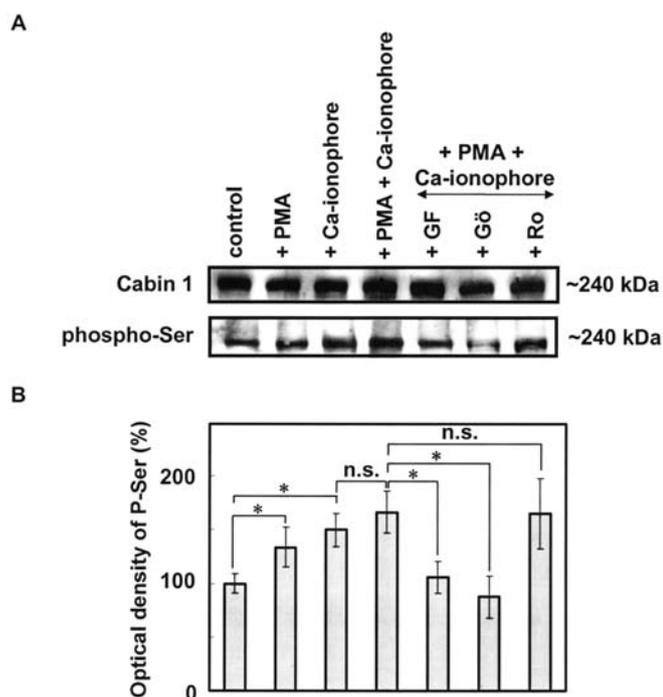


Figure 5. Western blot analysis of Cabin 1 and the phosphorylation level of Ser residues after the immunoprecipitation of Cabin 1. (A) Immunoblot of Cabin 1 and the detection of phospho-Ser from non-stimulated and stimulated PBMC in the presence of various effectors. Concentrations of effectors were the same as used in the experiments shown in Figs. 1 and 2. (B) Optical density of phospho-Ser in Cabin 1 representing the average values with standard error of the mean of 3 independent experiments. Significant differences are shown as \* $p < 0.05$ . Detection of Western blots and the immunoprecipitation of Cabin 1 can be found in Materials and methods.

#### *Cabin 1 as a signal transducer between PKC and calcineurin.*

As the phosphorylation of calcineurin by PKC does not affect its enzymatic activity (23,24), one can suppose that an upstream signal transduction molecule activated by PKC is involved in the reduction of calcineurin activity. It has been reported that in response to PKC activation Cabin 1 becomes hyperphosphorylated exhibiting a higher affinity for calcineurin, and the binding of Cabin 1 to the enzyme inhibits its activity (7,9). To investigate the possible role of Cabin 1 in this signalling pathway, Cabin 1 was immunoprecipitated with a polyclonal antibody. After immunoprecipitation the protein level of Cabin 1 and its phosphorylation state on Ser residues were analyzed by immunoblotting. As shown in Fig. 5 the phosphorylation state of Cabin 1 was remarkably enhanced by activating agents of PKC applied alone or in combination. On the other hand, PKC inhibitors (GF109203X and Gö6976) significantly reduced the hyperphosphorylation of Cabin 1, and Rottlerin had no effect on the hyperphosphorylation of Cabin 1 as compared to the data of stimulated PBMC.

#### Discussion

Our observation suggests that both PMA and  $Ca^{2+}$  treatments contribute to the decrease of calcineurin activity of T cell-enriched PBMC without modulating the mRNA and protein levels of calcineurin. The present data also showed that several PKC isoenzymes play active roles in the inhibition of

the activity of calcineurin in human PBMC stimulated by PMA and Ca-ionophore. The use of cell-permeable PKC inhibitors suggests that the cPKC  $\alpha$ ,  $\beta$ ,  $\gamma$  isoenzymes are involved in the inhibition of the enzyme. Gö6976, the selective inhibitor of cPKC isoenzymes, was able to reverse the inhibitory effect of PMA and Ca-ionophore applied alone or in combination on the calcineurin activity. There are some endogenous protein inhibitors of calcineurin including Cabin 1 which are activated by hyperphosphorylation in response to PKC activation. Hyperphosphorylated Cabin 1 gains higher affinity for calcineurin, thus Cabin 1 may be responsible for dampening calcineurin activity in the course of T cell activation (7,9,10). Our data also confirmed that Cabin 1 may be a transducer which mediates the PKC signalling towards calcineurin, as an increasing phosphorylation state of Cabin 1 was observed in response to the stimulation by Ca-ionophore and PMA. The hyperphosphorylation of Cabin 1 was suppressed in the presence of GF109203x and Gö6976, inhibitors of cPKC and nPKC. It has been shown that the interaction between Cabin 1 and calcineurin requires both calcium signalling and activation of PKC. It has also been demonstrated that PMA treatment of Jurkat cells leads to hyperphosphorylation of Cabin 1, while ionomycin did not change the phosphorylation state of this protein (7,10). However, we failed to detect a decrease in calcineurin activity in Jurkat cells treated with PMA and Ca-ionophore (data not shown). It has also been reported that the autophosphorylated calcium/calmodulin-dependent protein kinase II (CaM kinase II) is able to phosphorylate calcineurin inhibiting its phosphatase activity *in vitro* (23,24). Phospho amino acid sequence analysis confirmed that PKC and CaM kinase II phosphorylated the same site (25). These results may suggest a role of CaM kinase II in the calcineurin and PKC-dependent signalling system. Further experiments are required to elucidate the lack of inhibition of calcineurin in a T cell line and to demonstrate the possible role of other protein kinases in the regulation of calcineurin activity.

A number of signalling molecules in SLE T cells have been reported to malfunction, including transcription factor elf-1, inflammation signal transducer NF- $\kappa$ B and PKC  $\theta$  (18). It has been shown that the treatment of the healthy and lupus T cells with GCS decreased the activity of calcineurin and elevated the expression of most isoforms of PKC close to the normal values (19,26). In this study we provided evidence for the transducer role of hyperphosphorylated Cabin 1 between the increase of PKC activation and the decrease of calcineurin activity in PBMC from healthy patients. It is known that in T cells the NFAT/calcineurin pathway is involved in the induction of pro-inflammatory cytokines, and lupus T cells have a major role in the pathogenesis of SLE via overproduction of cytokines (IL2, IL4, IL5, IL8, IL10). Our data show that the calcineurin/NFAT signal transduction pathway, i.e. the overexpression of pro-inflammatory cytokines, is able to be suppressed through the activation of hyperphosphorylated Cabin 1 via cPKC  $\alpha$ ,  $\beta$ ,  $\gamma$  isoenzymes.

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