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Barbiturates Directly Inhibit the Calmodulin/Calcineurin Complex: a Novel Mechanism of Inhibition of Nuclear Factor of Activated T Cells

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ABSTRACT

Barbiturates are frequently used for the treatment of intracranial hypertension after brain injury but their application is associated with a profound increase in the infection rate. The mechanism of barbiturate-induced failure of protective immunity is still unknown. We provide evidence that nuclear factor of activated T cells (NFAT), an essential transcription factor in T cell activation, is a target of barbiturate-mediated immunosuppression in human T lymphocytes. Treatment of primary $CD3^+$ lymphocytes with barbiturates inhibited the PMA and ionomycin induced increase in DNA binding of NFAT, whereas the activity of other transcription factors, such as Oct-1, SP-1, or the cAMP response element-binding protein, remained unaffected. Moreover, barbiturates suppressed the expression of a luciferase reporter gene under control of NFAT (stably transfected Jurkat T cells), and of the cytokine genes interleukin-2 and interferon-γ that contain functional binding motifs for NFAT within their regulatory promotor domains (human peripheral blood $CD3^+$ lymphocytes). Neither GABA receptor-initiated signaling nor direct interactions of barbiturates with nuclear proteins affected the activity of NFAT. In contrast, barbiturates suppressed the calcineurin-dependent dephosphorylation of NFAT in intact T cells and also inhibited the enzymatic activity of calcineurin in a cell-free system, excluding upstream regulation. Thus, our results demonstrate a novel mechanism of direct inhibition of the calcineurin/calmodulin complex that may explain some of the known immunosuppressive effects associated with barbiturate treatment.

Barbiturates are frequently used for the treatment of intracranial hypertension after severe head injury (Wilberger and Cantella, 1995). Under these conditions, they can decrease cerebral metabolic demands, oxygen need, and intracranial pressure (Roberts, 2000). However, accumulating evidence suggests that the barbiturate-mediated neuroprotection is associated with increased incidence of infections, which may contribute to the high mortality rate in these patients (Eberhardt et al., 1992).

Despite the fact that several inhibitory effects of barbiturates on lymphocyte and leukocyte functions have been reported (Correa-Sales et al., 1997; Nishina et al., 1998; Salman et al., 1998), the molecular mechanism of barbiturate-induced immunosuppression remains elusive. In this regard, we have recently demonstrated that barbiturates can inhibit the activation of nuclear transcription factor κB (NF-κB) in human T cells upon TNF receptor stimulation (Loop et al., 2002). NF-κB plays a central role in the regulation of the immune system and several previously reported effects of barbiturates on the function of immune cells could be explained by inhibition of NF-κB. However, barbiturate-mediated immunosuppression, such as impeded proliferation (Correa-Sales et al., 1997), cytokine production (Salo et al., 1997), and CD69 expression of T lymphocytes (Loop et al., 2002) implies that, in addition to TNF-induced cascades, T cell receptor (TCR)-mediated signal transduction pathways could also be affected.

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M.H. and S.E.P. contributed equally to this work.

ABBREVIATIONS: NF-κB, nuclear factor κB; TCR, T cell receptor; NFAT, nuclear factor of activated T cells; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; FK 506, tacrolimus; CGP 52432, 3-*N*-(3,4-dichlorobenzyl)aminopropyl-*p*-diethoxymethylphosphinic acid; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; CREB, cAMP response element-binding protein; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; PBMC, peripheral blood mononuclear cells; CsA, cyclosporin A; RII, regulatory subunit (type II) of the cAMP-dependent protein kinase.

T lymphocytes direct specific immune functions by antigen recognition via the T cell receptor complex. Pharmacological and cell biological evidence support the notion that the serine/threonine phosphatase calcineurin is a rate-limiting intracellular signaling enzyme involved in TCR-mediated signal transduction (Batiuk et al., 1997). Calcineurin activity is regulated by the intracellular concentration of calcium and calmodulin. The influx of calcium after TCR cross-linking leads to the binding of the calcium sensor calmodulin to calcineurin, promoting the displacement of the autoinhibitory domain (Sagoo et al., 1996) and leading to enzyme activation (Cardenas and Heitman, 1995). Among the substrates of calcineurin are members of the nuclear factors of activated T cells (NFAT) family (Rao et al., 1997). These transcription factors are phosphorylated in the cytosol of resting T cells. Activated calcineurin dephosphorylates NFAT, allowing it to translocate to the nucleus and to bind response elements of genes critical for lymphocyte activation and regulation of immune function. Target genes include cytokines, such as interleukin (IL)-2, IL-3, IL-4, IL-8, IL-13, the antiviral and antiproliferative glycoprotein IFN-γ, inflammatory mediators (e.g., TNFα), growth factors (e.g., granulocyte macrophage–colony-stimulating factor), and pro-apoptotic genes (e.g., *FAS-L*). Further studies have implicated NFAT/calcineurin in the regulation of cell surface receptors, such as the activation antigen CD69 (Rao et al., 1997).

Multiple reports describe calcineurin/NFAT-dependent immunosuppression (Castigli et al., 1993; Feske et al., 2000; Ortega-Perez and Redondo, 2000). The calcium-dependent activation of antigen-reactive and cytokine-dependent T cells can be blocked by repression of calcineurin/NFAT activity. Thus, the inhibition of IL-2 synthesis might lead to anergy of T lymphocytes (Johnson and Jenkins, 1994), and the inactivation of other cytokine genes can result in severe immunodeficiency syndromes (Castigli et al., 1993). Today, a number of immunosuppressive drugs that specifically inhibit the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin, such as Cyclosporin A or FK 506, are used in transplantation medicine or for the treatment of chronic autoimmune inflammatory diseases (Bierer et al., 1993). The aim of the present study was to determine whether barbiturates might interfere with the activation of the nuclear factor of activated T cells.

Materials and Methods

Materials. Barbiturates were obtained as sodium salts: thiopental from Byk Gulden (Konstanz, Germany), pentobarbital from Alvetra (Neumünster, Germany), thiamylal from Pharmacia and Upjohn (Erlangen, Germany), and secobarbital from Sigma-Aldrich (Saint Louis, MO). γ-Aminobutyric acid, (+)-bicuculline, and CGP 52432 were from Tocris (Köln, Germany). The IntraPrep permeabilization and fixing reagent was purchased from Beckman Coulter (Fullerton, CA). All other reagents were obtained from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Isolation and Activation of CD3-Positive T Cells. Extracts from human T lymphocytes were used for electrophoretic mobility gel shift assays (EMSA) and immunoblot experiments. Peripheral blood mononuclear cells were purified by Ficoll-Hypaque (Pharmacia, Freiburg, Germany) centrifugation from whole blood and subsequently enriched by immunomagnetic cell sorting with anti-CD3 microbeads according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Lymphocytes were suspended in RPMI 1640 medium supplemented with 10 mM HEPES, pH 7.3, 50 μM β-mercaptoethanol, and 2 mM glutamine. T cells were incubated with 1 to 1000 μg/ml barbiturates alone or in combination with 1 to 10 mM (+)-bicuculline or 100 to 500 nM CGP 52432 for 2 h before 15 ng/ml PMA plus 1 μg/ml ionomycin were added for the last 4 h of the incubation period unless indicated otherwise. For GABA receptor activation, thiopental was replaced by 3 or 10 mM γ-aminobutyric acid.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assays. For the detection of activated NFAT, Oct-1, SP-1, or CREB, nuclear cell extracts were prepared from isolated $CD3^+$ lymphocytes as described previously (Schreiber et al., 1989). Briefly, cells were incubated for 15 min at 4°C in 400 μl of extraction buffer (10 mM HEPES, pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, and 2.5 μl of Nonidet P40). Nuclear pellets were solubilized in 50 μl of suspension buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiotreitol) and centrifuged at 15,000*g* at 4°C for 15 min. Supernatants were used for EMSA. Inhibitors of proteinases and phosphatases were added at the following concentrations to the extraction and suspension buffer: 10 μg/ml aprotinin, 25 μM leupeptin, 2 mM PMSF, 2 mM iodoacetamide, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. Protein concentrations were measured by Bradford assay (Bradford, 1976). The binding reactions were carried out in a volume of 20 μl containing 20 μg of nuclear extract in a solution consisting of 22 mM HEPES, pH 7.9, 70 mM KCl, 50 μM EDTA, 2.2 mM dithiotreitol, 2% glycerol, 4% Ficoll 400, 0.025% Nonidet P40, 0.03% PMSF, 20 μg of BSA and 2 μg of poly(dI-dC). The protein solutions were incubated at room temperature for 30 min with 1.75 pmol of ^{32}P-end-labeled double-stranded oligonucleotides (5 μCi/pmol of DNA) corresponding to the NFAT motif of the human granulocyte macrophage–colony-stimulating factor enhancer region (5′-TTTCTCATGGAAAGATGACATA-3′). The respective oligonucleotide sequences were 5′-TGTCGAATGCAAATCACTAGAA-3′ for Oct-1; 5′-ATTCGATCGGGGCGGGGCGAGC-3′ for SP-1; and 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ for CREB. The resulting DNA-protein complexes were separated by electrophoresis on a 4% nondenaturating polyacrylamide gel.

NFAT-Dependent Luciferase Reporter Gene Expression. Assays were performed using C4-NFAT Jurkat cells (generous gift from Prof. C. T. Baldari, Sienna, Italy). This T cell leukemia cell line has been stably transfected with a reporter construct containing the gene for the firefly luciferase under the control of three tandem repeat units of the NFAT binding site from the IL-2 promoter (Baldari et al., 1998). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37°C in the presence of 5% CO_2. Incubations with PMA, ionomycin, and barbiturates were performed in serum-free medium. Before stimulation, cells were starved for 15 h. Harvested cells were washed with PBS and lysed in 100 μl of luciferase reporter lysis buffer (Promega, Madison, WI). Luciferase reporter gene expression was measured in lysates that had been obtained from treated or untreated cells using a luminometer (Microluminat Plus LB 96P; Berthold Technologies, Bad Wilbach, Germany) and normalized to protein levels.

Labeling of RII Peptide and Calcineurin Assay. In these experiments, the effect of barbiturates on calcineurin activity was analyzed using an in vitro assay system that did not contain any cellular extracts. The peptide DLDVPIPGRFDRRVSVAAE, corresponding to a sequence in the RII subunit of the cAMP-dependent kinase (Blumenthal et al., 1986), was phosphorylated at the serine residue with unlabeled dATP by the catalytic subunit of the cAMP-dependent protein kinase as described previously (Fruman et al., 1992). The resultant RII phosphopeptide was loaded onto Sephadex G-25 spin columns (Amersham Biosciences, Freiburg, Germany) to remove free phosphates, and the eluate was used as a substrate. Calcineurin activity was measured by a serine and threonine phosphatase assay system from Promega (Madison, WI), according to the instructions of the manufacturer with minor modifications. Briefly, 50 U of recombinant calcineurin (Calbiochem, San Diego, CA) was

preincubated with 1 to 10 mM barbiturate at 37°C for 30 min in the reaction buffer containing 50 mM Tris, pH 7.4, 1 mM $NiCl_2$, 0.5 mg/ml BSA, and 10 μg/ml calmodulin. Dephosphorylation of the synthetic phosphopeptide was performed after addition of 10 μg of labeled RII peptide per reaction for an additional 20 min. At the end of the experiment, the molybdate dye was added and the absorbance of the molybdate-malachite green-phosphate complex was determined at 600 nm with a plate reader (SPECTRAmax PLUS^{384}; Molecular Devices, München, Germany). Readings were adjusted for $NiCl_2$- and calmodulin-independent background activity. This in vitro assay system was used because it does not contain any cellular extracts.

Analysis of NFAT Dephosphorylation by Immunoblot Analysis. For detection of NFAT, peripheral $CD3^+$ T-lymphocytes were incubated with 1 to 10 mM barbiturate for 6 h and stimulated with 1 μg/ml ionomycin during the 30 min before harvesting. Total cell lysates were prepared by incubation of the cells in high-salt detergent lysis buffer [20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) Nonidet P40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol] for 15 min at 4°C. Inhibitors of proteinases and phosphatases were added at the following concentrations: 10 μg/ml aprotinin, 25 μM leupeptin, 2 mM PMSF, 2 mM iodoacetamide, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. For immunoblot analysis, total cellular extract (20 μg of protein) was separated on 7.5% SDS polyacrylamide gels, blotted to polyvinylidene difluoride membrane and analyzed by chemiluminescence with an NFATc2-specific antibody (clone 4G6-G5, 1:1000 dilution; BD Biosciences, Heidelberg, Germany).

Immunocytochemistry. T cells used for immunocytochemistry were grown on 0.2% bovine gelatin (Sigma-Aldrich)-coated coverslips, incubated with 2.5 mM barbiturate for 6 h, and stimulated with 1 μg/ml ionomycin for the final 20 min. Nuclear export was inhibited with 20 nM leptomycin B (Sigma-Aldrich). Immediately after stimulation, cells were fixed in 4% paraformaldehyde/PBS for 20 min and permeabilized in 0.2% Triton X-100/PBS for 10 min. Nonspecific binding was blocked in PBS and 0.2% bovine gelatin for 1 h at room temperature. T lymphocytes were stained with the NFATp-specific monoclonal antibody G1-D10 [1:50 in PBS and 0.2% bovine gelatin (BD Biosciences)] for 1 h followed by anti-mouse-biotin (human absorbed; 1:500; Dianova, Hamburg, Germany) and streptavidin-Alexa 488 (1:1000; Molecular Probes, Eugene, ON, Canada). Nuclear counter-staining was performed with 20 μM DRAQ5 (Biostatus, Leicestershire, UK) for 5 min. Cover slides were analyzed by confocal fluorescence microscopy (LSM410; Zeiss, Jena, Germany).

Measurement of IL-2 and IFN-γ Secretion by Flow Cytometry. Cytokine production of activated T lymphocytes was measured in whole blood by the FastImmune Cytokine System (BD Biosciences). Aliquots (100 μl) of heparinized whole blood were incubated with or without barbiturates in a humidified incubator at 37°C with 5% CO_2 for 15 h. Stimulation was performed with 15 ng/ml PMA and 1 μg/ml ionomycin during the final 13 h of the experiments. Cytokine secretion was inhibited by 10 μg/ml brefeldin A. After cell surface staining of activated whole blood for CD3 (2.5 μg/ml; clone SK7-PerCP) and CD8 (2.5 μg/ml; clone SK1-FITC), red blood cells were lysed by addition of 900 μl of 1× FACS lysing solution to each reaction according to the manufacturer's instructions. The remaining PBMCs were suspended in 100 μl of IntraPrep fixation reagent for 15 min and then treated with 100 μl of IntraPrep permeabilization reagent containing 0.3 μg/ml FastImmune Anti-Hu-IL2PE (clone 5344.11) or 1.5 μg/ml FastImmune Anti-Hu-IFN-γPE (clone 25723.11) antibody for 20 min. Stained PBMCs were washed with PBS and prepared in 500 μl of 1× CellFIX fixing solution for flow cytometry analysis. The cells were analyzed using a FACSCalibur (BD Biosciences).

Statistical Analysis. Data are shown as mean ± S.E.M. Statistic analysis was performed using a one-way analysis of variance on ranks followed by a nonparametric Student-Newman-Keuls test for multiple comparisons. P values less than 0.05 were considered significant.

Results

Barbiturates Inhibit Nuclear Factor of Activated T Cells in Human T Lymphocytes. Inactivation of the transcription factor NFAT is associated with severe immunodeficiency syndromes and results in increased susceptibility to infections (Feske et al., 2000). To study the effect of barbiturates on the activation of NFAT, EMSAs were performed using nuclear extracts from peripheral human T lymphocytes. Compared with nuclear extracts from untreated cells (Fig. 1, A and B, lane 1) treatment of lymphocytes with PMA and ionomycin induced strong NFAT binding (Fig. 1, A and B, lane 2). The thiobarbiturates thiopental and thiamylal inhibited the NFAT DNA binding activity in stimulated cells at concentrations that have been used for treatment of patients with intracranial hypertension (Fig. 1, A and B, lanes 3–7, left) (Neuwelt et al., 1982). The inhibition of the binding activity was dose-dependent. The inhibitory potency of thiobarbiturates was compared with the respective structural oxybarbiturate analogs pentobarbital and secobarbital (Fig. 1, A and B, lanes 3–7, right). The replacement of the C2 sulfur atom of thiobarbiturates with oxygen diminishes the inhibitory effect on the DNA binding activity of NFAT.

The specificity of the DNA binding activity induced by PMA and ionomycin was confirmed by competition experiments. Incubation with an excess of unrelated oligonucleotides spanning an activator protein-1 binding site did not antagonize NFAT binding, whereas competition with a 100-fold excess of unlabeled NFAT oligonucleotides abolished the specific band (data not shown). Moreover, inhibition of the DNA binding activity of other transcription factors, such as Oct-1, SP-1, or CREB, in response to barbiturates was variable and only marginal compared with the binding of nuclear proteins to the NFAT probe (Fig. 2), thus indicating a preferential effect of these agents on signal transduction pathways leading to the activation of NFAT.

Barbiturates Inhibit NFAT-Dependent Luciferase Reporter Gene Expression. To determine whether barbiturate-mediated inhibitory effects on the DNA binding activity of NFAT are associated with a respective inhibition of NFAT dependent gene expression, we used C4-NFAT Jurkat cells that had been stably transfected with a reporter construct containing the firefly luciferase gene under the control of NFAT. These experiments revealed that barbiturates inhibit NFAT-dependent gene transcription in a dose-dependent manner (Fig. 3). In accordance with the results shown in Fig. 1, the relative potency of the different agents was influenced by the substitutions at the carbon atoms 2 and 5 of the barbiturate molecule. 2-Thiobarbiturates were 3 to 4 times more potent inhibitors of NFAT dependent reporter gene expression than their structural oxy analogs (Fig. 3). Substitutions at the C5 atom of barbituric acid also reduced luciferase expression upon stimulation. Thiobarbiturates as well as oxybarbiturates showed increasing reporter gene repression from 5-ethyl-5-(1-methyl-buthyl) substitutions (Fig. 3A) to 5-allyl-5-(1-methyl-buthyl) (Fig. 3B) and 5-allyl-1-methyl-5-(1-methyl-pent-2-ynyl) substitutions (data not shown for methohexital). Inhibition of NFAT seemed to be specific for barbiturates because other anesthetics or analgesics, such as

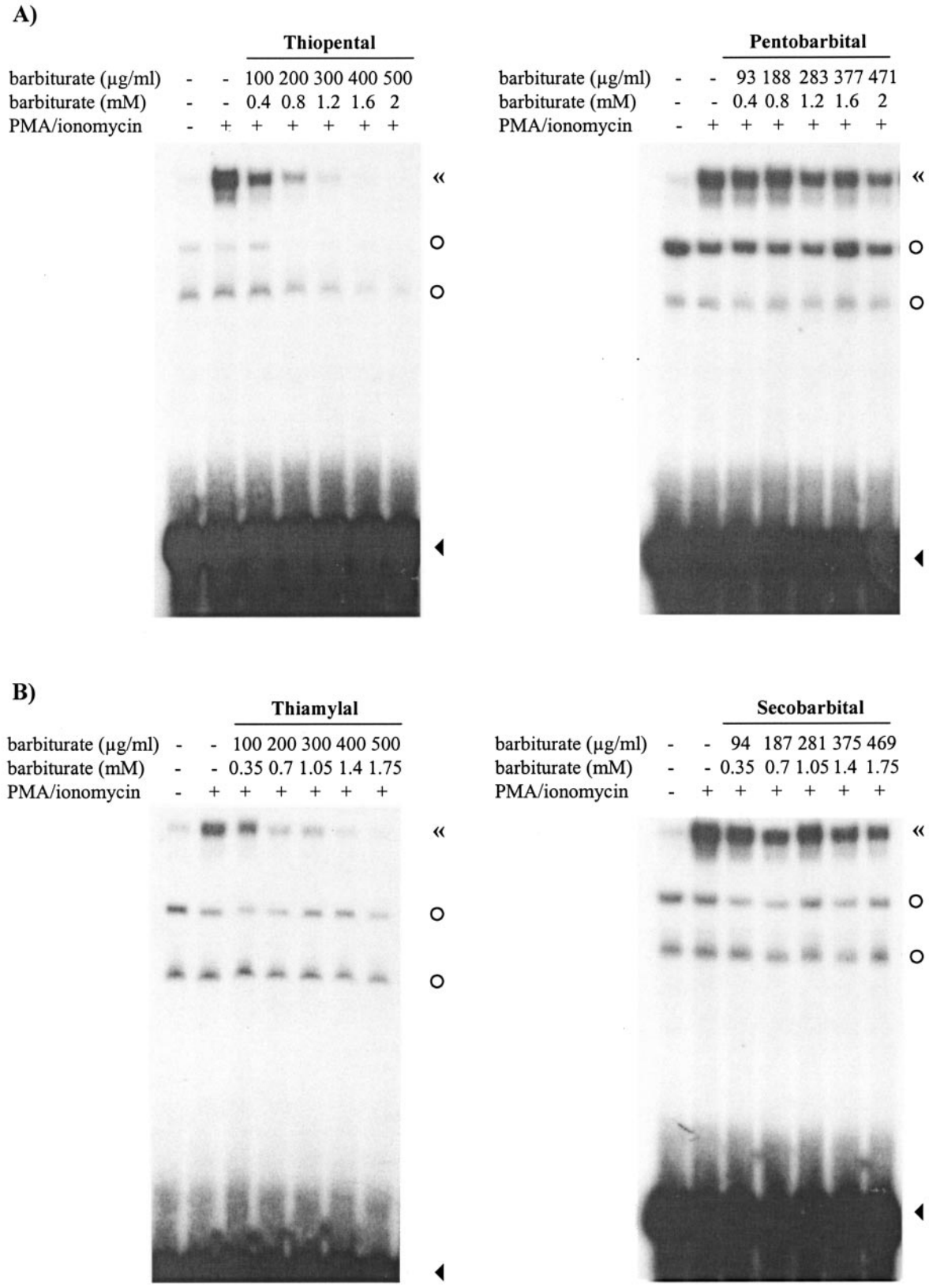

Fig. 1. Barbiturates inhibit NFAT nuclear activity in electrophoretic mobility gel shift experiments. Peripheral human blood lymphocytes were isolated from healthy donors and incubated with barbiturates for 6 h. Cells were stimulated with 15 ng/ml PMA and 1 μg/ml ionomycin for the last 4 h. Nuclear extracts were prepared and incubated with an end ^{32}P-labeled NFAT oligonucleotide probe containing the recognition site of NFAT. Samples were separated on 4% nondenaturating acrylamide gels. Untreated (lane 1) and stimulated control cells (lane 2) in the absence of barbiturates. The thiobarbiturates thiopental (A) and thiamylal (B) were used at concentrations of 100 to 500 μg/ml, their structural analogs pentobarbital (A) and secobarbital (B) in equimolar concentrations (lanes 3–7). ≪, position of NFAT DNA complexes; ○, unspecific binding activity to the probe; ◀, free probe.

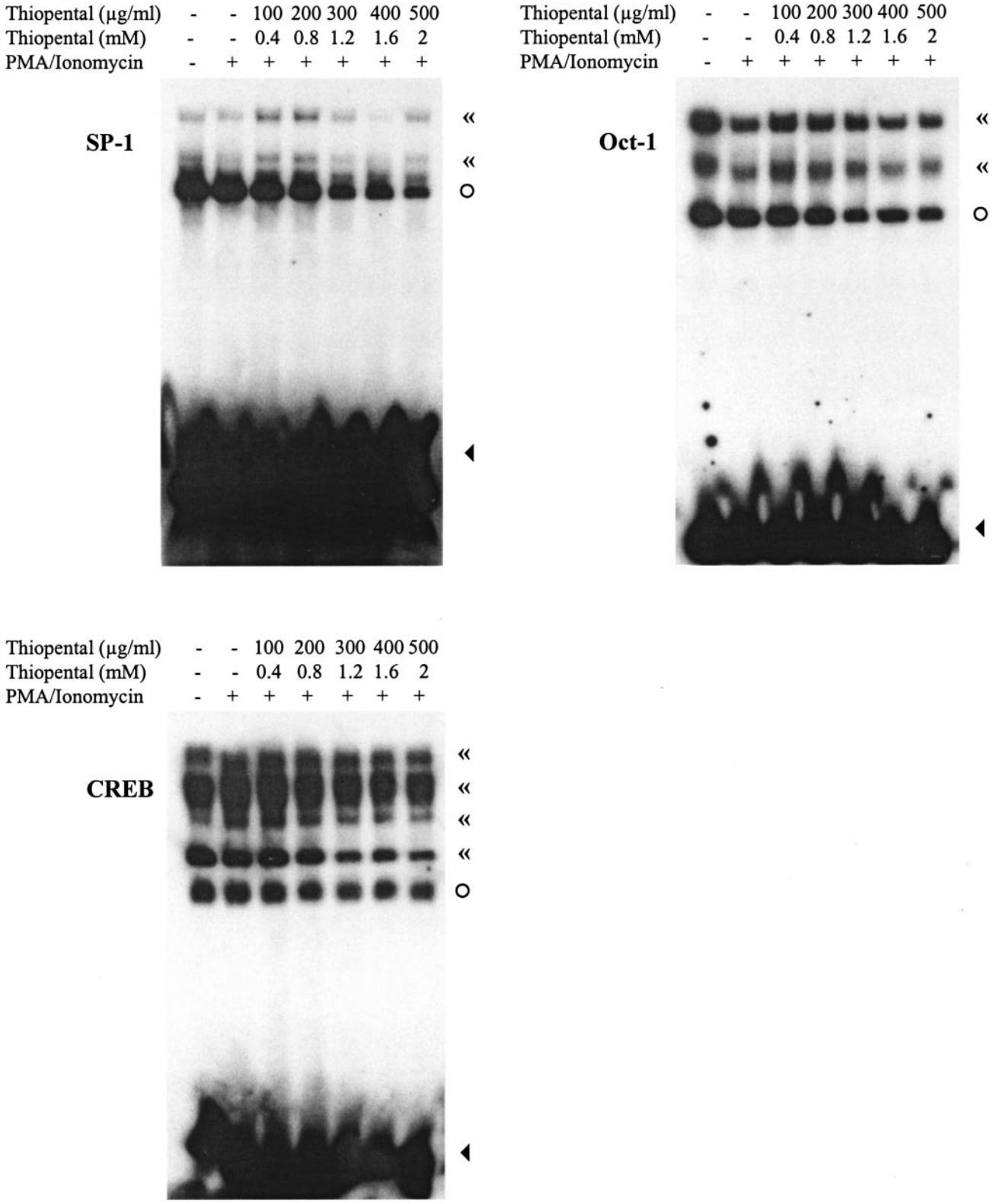

Fig. 2. Thiopental does not influence the transcription factors Oct-1, SP-1, or CREB. Peripheral human blood lymphocytes were isolated from healthy donors and incubated with 100 to 500 μg/ml thiopental for 6 h. Cells were stimulated with 15 ng/ml PMA and 1 μg/ml ionomycin for the last 4 h. Nuclear extracts were prepared and incubated with an end ^{32}P-labeled oligonucleotide probe containing the recognition site of SP-1 (top left), Oct-1 (top right), or CREB (bottom left). Electrophoretic mobility gel shift assays were performed on 4% nondenaturating acrylamide gels. Untreated (lane 1), stimulated control cells (lane 2) in the absence of thiopental. ≪, position of Oct-1, SP-1, or CREB DNA complexes; ○, nonspecific binding activity to the probe; ◀, free probe.

benzodiazepines (midazolam), opioids (morphine, fentanyl, remifentanil), and hypnotics (ketamine, propofol, etomidate), did not repress NFAT-dependent reporter gene activity (data not shown).

GABA Receptors Do Not Mediate the Effect of Barbiturates on NFAT. Most biological effects of barbiturates are mediated through the GABA receptor complex (Fruman et al., 1992). Moreover, GABA receptor-mediated inhibition of T cell responses has been described previously (Tian et al., 1999). We therefore tested whether GABA receptors were involved in the suppression of NFAT activation by barbiturates. However, 1 to 10 mM GABA failed to alter NFAT DNA binding in gel shift experiments (Fig. 4) and NFAT-dependent reporter gene expression (data not shown). In addition, the $GABA_A$ and $GABA_B$ receptor antagonists (+)-bicuculline and CGP 52432 did not diminish the barbiturate-mediated inhibitory effect on NFAT (Fig. 4). These data strongly suggest that inhibition of NFAT activation by barbiturates is not caused by a GABA receptor-initiated signal transduction pathway.

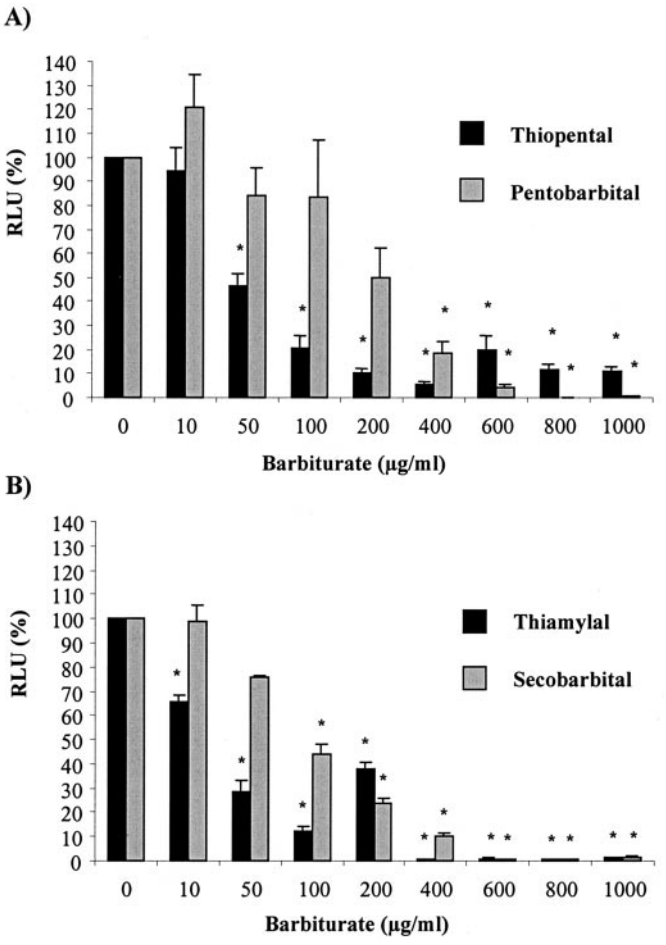

Fig. 3. Barbiturates inhibit NFAT-dependent reporter gene expression. Jurkat cells were stably transfected with an NFAT-dependent reporter gene construct and preincubated with barbiturates at the indicated concentrations for 8 h. NFAT activation was induced by addition of 15 ng/ml PMA and 1 μg/ml ionomycin for the last 6 h. Cell lysates were prepared and luciferase activity was measured in a photospectrometer and normalized to protein content. Maximal reporter gene expression was found in PMA and ionomycin-stimulated cells in the absence of barbituric acid derivatives (100%; positive controls). IC_{50} is 47.4 μg/ml (0.18 mM) for thiopental; 172.6 μg/ml (0.7 mM) for pentobarbital; 29 μg/ml (0.105 mM) for thiamylal; and 88.4 μg/ml (0.34 mM) for secobarbital. Values are expressed as the means of six independent experiments ± S.E.M. *, $P <$ 0.05 versus positive controls.

Inhibition of NFAT by Barbiturates Is Not Mediated through a Direct Interaction with the Transcription Factor or with Components of the Nuclear Compartment. To further evaluate the mechanism of the barbiturate-mediated inhibition of NFAT, we analyzed the effect of barbiturates on nuclear extracts of activated T lymphocytes. In contrast to the experiments described above, in which barbiturates were added to cultured T lymphocytes, in these studies, activated nuclear extracts were directly incubated with thiopental (Fig. 5A) or thiamylal (Fig. 5B). The experiments revealed that the coincubation of nuclear extracts from activated T cells with increasing concentrations of thiobarbiturates showed no inhibitory effects on the DNA binding activity of NFAT (Fig. 5, lanes 2–7). Although concentrations >200 μg/ml of thiopental or thiamylal clearly inhibited NFAT activation in whole-cell experiments (Figs. 1 and 3), similar concentrations were unable to do so in nuclear extracts. Even concentrations >500 μg/ml, which reduced NFAT activity to basal levels in intact cells, did not significantly alter DNA binding (Fig. 5, lane 7). These results indicate that the inhibition of DNA binding and consequently the inhibition of gene transcription is not based on a direct interaction of barbiturates with NFAT or other components of the nuclear compartment.

Barbiturates Directly Inhibit Calmodulin/Calcineurin Activation. Activation of NFAT is associated with its translocation into the nucleus. This process is tightly regulated by dephosphorylation through calcineurin, unmasking the nuclear translocation signal of NFAT (Rao et al., 1997). We therefore investigated whether the inhibition of NFAT by barbiturates might be attributable to a specific regulation of calcineurin/calmodulin activity. We performed a colorimetric assay to measure phosphatase activity in an in vitro system lacking cellular extracts. Reactions were performed with recombinant human calcineurin in a reaction buffer containing 50 mM Tris, pH 7.4, 1 mM $NiCl_2$, 0.5 mg/ml BSA, 10 μg/ml calmodulin, and 10 μg synthetic RII phosphopeptide as a calcineurin-specific phosphatase substrate. Absence of calmodulin and nickel or 10 mM EGTA reduced the phosphatase activity to <7%, which is in agreement with the importance of 2-valent metal ions, such as Ca^{2+} or Ni^{2+} in the catalytic process, and confirms that the release of phosphate is the result of the enzymatic reaction. Okadaic acid (500 nM), a specific inhibitor of PP-1 and PP-2A, had no effect (data not shown). Pilot experiments demonstrated a linear increase in calcineurin-dependent phosphate release for at least 45 min after addition of the phosphorylated RII peptide substrate (data not shown). For this reason, the level of relative total phosphatase activity was calculated at 20 min after addition of the phosphosubstrate.

The coincubation of calcineurin/calmodulin complexes with barbiturates resulted in a dose-dependent inhibition of phosphatase activity (Fig. 6, A and B). Thiopental suppressed calcineurin activity by 96.6 ± 1.3, 97.1 ± 1.8, and 4.3 ± 5.9% at 10, 5, and 1 mM, respectively. Similar concentrations of the thiobarbiturate thiamylal caused 97.3 ± 1.1, 95.2 ± 2.7, and 14.5 ± 3.2% inhibition. The oxybarbiturates pentobarbital and secobarbital also inhibited calcineurin activity. However, at 5 mM, the inhibition was only about half of maximal (42.7 ± 8.2%, pentobarbital; 47.1 ± 8.9%, secobarbital).

These results indicate that barbiturates directly inhibit the calcineurin/calmodulin-dependent phosphatase activity. In contrast, the immunosuppressive drugs FK-506 (10 μM) and cyclosporin A (CsA; 100 μM) were found not to affect RII dephosphorylation (data not shown). This observation can be explained by the fact that these substances indirectly inhibit the calcineurin-dependent dephosphorylation of NFAT via complexation to their respective immunophilins, which are not included in this assay (Kiani et al., 2000).

To investigate whether barbiturates primarily affect calcineurin or calmodulin, competition experiments were performed (Fig. 6C). A 100-fold excess of calmodulin reverted the inhibitory potential of thiopental, indicating that calmodulin, but not calcineurin, is the primary target of the barbiturate-mediated inhibition.

Calcineurin-Dependent Dephosphorylation and Nuclear Translocation of NFAT Is Inhibited by Barbiturates in Human T Cells. NFAT is a natural substrate of calcineurin. Functionally relevant inhibition of the enzymatic activity of calcineurin should therefore affect the dephosphorylation and nuclear translocation of NFAT upon stimulation. Moreover, even a partial inhibition of these processes seems to be sufficient for mediating biological effects (Neilson et al., 2001). We performed immunoblotting experiments using a primary antibody that detects both, phosphorylated and dephosphorylated NFATc2. Stimulation of human $CD3^+$ lymphocytes led to a complete dephosphorylation of NFATc2; i.e., the slower migrating band, which could be detected under control conditions (Fig. 7, A and B, lane 1), disappeared, and a protein of smaller molecular size was detected that corresponds to dephosphorylated NFATc2 (Fig. 7, A and B, lane 2). In accordance with previous reports, this dephosphorylation could be inhibited by CsA (Fig. 7A, lane 9) (Loh et al., 1996). Interestingly, preincubation of the cells with barbiturates resulted also in a dose-dependent suppression of the phosphatase activity. At the highest concentrations tested, the oxybarbiturates pentobarbital and secobarbital partially inhibited the dephosphorylation of NFAT, whereas the thiobarbiturates thiopental and thiamylal were able to prevent dephosphorylation almost completely under these experimental conditions (Fig. 7, A and B, lanes 3–8).

The movement of NFAT from the cytosol to the nucleus is dependent upon the dephosphorylation of NFAT by calcium- and calcineurin-dependent mechanisms. Consequently, we determined whether the inhibition of NFAT dephosphorylation was accompanied by a barbiturate-dependent blockade in the nuclear translocation of NFAT using confocal fluorescence microscopy. Cells were stimulated with 1 μg/ml ionomycin for 30 min in medium containing 20 nM leptomycin B followed by fixation and staining with an NFAT-specific monoclonal antibody (clone G1-D10). Under these conditions, nuclear translocation of NFAT was observed in 100% of all cells (Fig. 8B) compared with complete cytoplasmic staining in unstimulated lymphocytes (Fig. 8A). In contrast, the nuclear translocation of NFATc2 was blocked in cells that had been pretreated with thiobarbiturates (Fig. 8, C and E). Oxybarbiturates did not significantly influence the nuclear translocation at similar concentrations (Fig. 8, D and F). These results suggest that thiobarbiturates severely compromise the dephosphorylation of NFAT and its nuclear translocation.

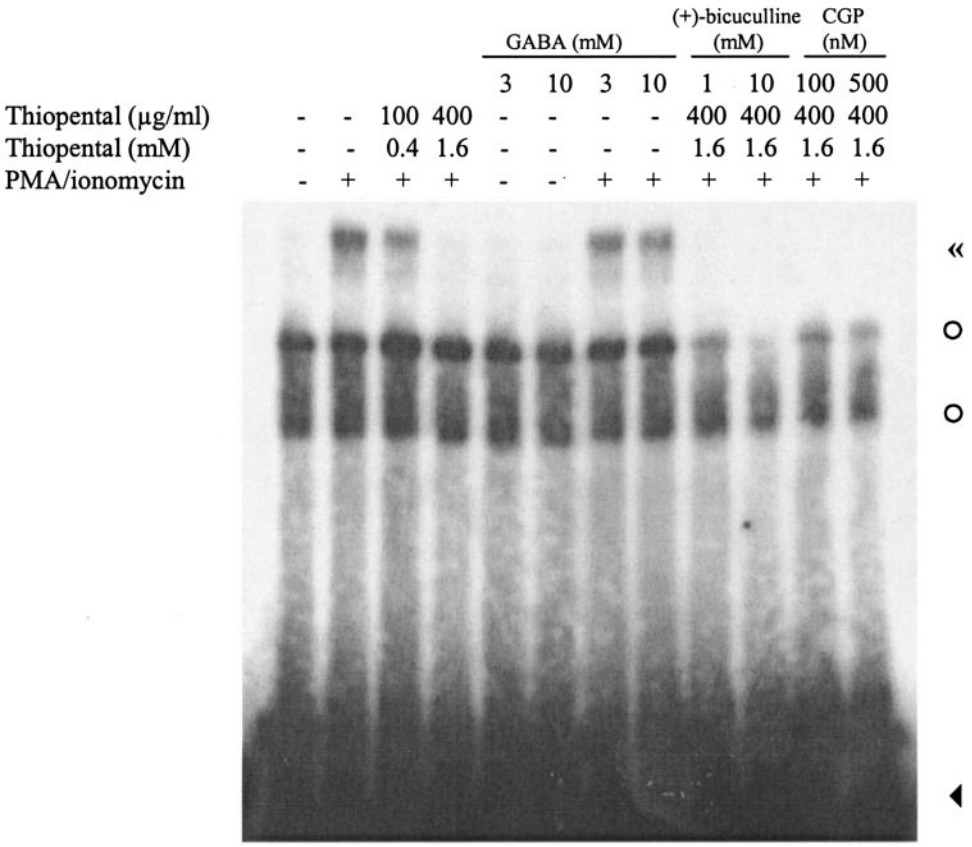

Fig. 4. NFAT DNA binding after exposure to $GABA_{A/B}$ agonist and antagonists. Primary T cells were preincubated with thiopental, γ-aminobutyric acid, (+)-bicuculline, or CGP 52432 for 6 h at the indicated concentrations. NFAT activation was induced by 15 ng/ml PMA plus 1 μg/ml ionomycin for the last 4 h. Nuclear extracts were prepared and reacted with an end ^{32}P-labeled oligonucleotide probe containing the recognition sequence of NFAT. Untreated (lane 1) and stimulated control cells (lane 2) in the absence of thiopental. Thiopental (100 μg/ml; lane 3) and 400 μg/ml thiopental (lane 4) plus PMA and ionomycin. γ-Aminobutyric acid on untreated (lanes 5 and 6) or stimulated (lanes 7 and 8) lymphocytes. Representative electrophoretic mobility shift assay. ≪, position of NFAT DNA complexes; ○, nonspecifc binding activity to the probe; ◀, free probe.

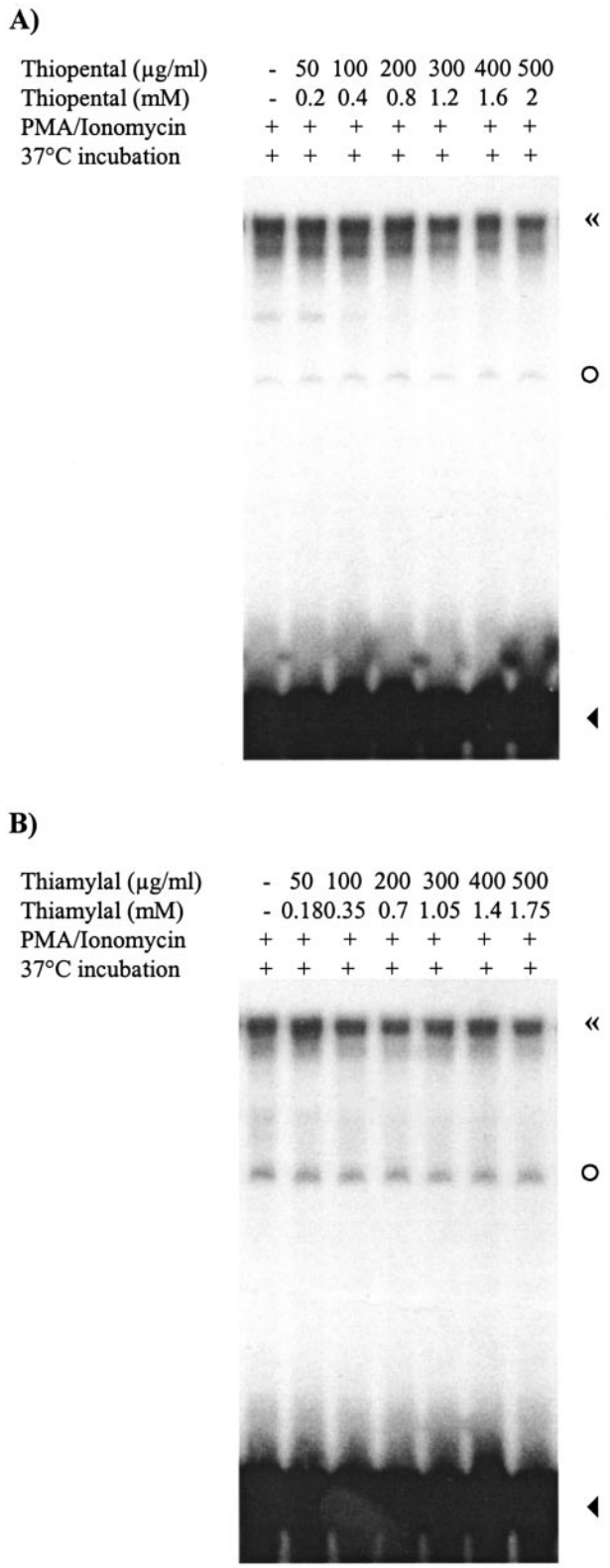

Fig. 5. Inhibition of NFAT binding to DNA consensus sequences is not influenced by direct interaction of NFAT with barbiturates. $CD3^+$ lymphocytes were stimulated with 15 ng/ml PMA and 1 μg/ml ionomycin for 4 h and nuclear extracts were prepared. Nuclear extracts from activated T cells were used for coincubation with thiopental (A) and thiamylal (B) at the indicated concentrations. Coincubation was performed for 2 h at 37°C, and DNA binding was analyzed by electrophoretic mobility gel shift assays. ≪, position of NFAT DNA complexes; ○, nonspecific binding activity to the probe; ◀, free probe.

Barbiturates Inhibit NFAT-Dependent Cytokine Gene Expression. Multiparameter flow cytometry was used to characterize the effects of barbiturates on cytokine expression in whole blood. $CD3^+$ lymphocytes were gated and analyzed for protein expression of IL-2 and IFN-γ. Regulatory promotor domains that contain functional binding motifs for NFAT characterize these two cytokines, both of which have been shown to be induced in response to the activation of this transcription factor (Jain et al., 1995; Kiani et al., 2000). All barbiturates tested caused a dose-dependent suppression of the stimulated synthesis of IL-2 (Fig. 9, A–C and G) and IFN-γ (Fig. 9, D–F and H). In contrast to the other experimental observations, there were no major differences in the inhibitory potency between thiobarbiturates and oxybarbiturates.

Discussion

Barbiturates are frequently used to treat patients with intracranial hypertension (Wilberger and Cantella, 1995). High-dose administration is necessary to achieve a neuroprotective effect but is associated with a profound increase in the incidence of nosocomial infections that may contribute to the high mortality of these patients (Eberhardt et al., 1992). The molecular mechanisms responsible for barbiturate-induced immunosuppression are basically unknown. It is important, however, to identify and understand the basis of the neuroprotective and the immunosuppressive actions of barbiturates. We therefore investigated the barbiturate-mediated effects on the activation of NFAT, a transcription factor that plays a central role in the regulation of T cell functions (Rao et al., 1997).

The regulation of T cell functions is a critical component of the host defense response, and its impairment leads to the failure of protective immunity. The present results show that barbiturates suppress the stimulation-induced activation of NFAT and the expression of its target genes that are critically involved in the human T cell response. Previous reports about a barbiturate-mediated suppression of T lymphocytes involving their activation, differentiation, and proliferation (Correa-Sales et al., 1997; Salo et al., 1997; Loop et al., 2002) can now be explained by their effect on NFAT.

Our observations raise the question of how barbiturates may inhibit the activation of NFAT. Most biological effects of barbiturates are mediated through GABA receptors, and GABA receptor-mediated immunosuppression in T lymphocytes has been described previously (Tian et al., 1999). Thus, we speculated that the inhibitory effect of barbiturates might involve GABA receptor stimulation. However, our experiments suggest that inhibition of NFAT in primary T lymphocytes is not related to a GABA-mediated pathway. First, the naturally occurring agonist GABA was unable to mimic barbiturate-induced repression of NFAT. Second, the GABA antagonists (+)-bicuculline and CGP 52432 failed to prevent the barbiturate-mediated suppression of NFAT. Finally, other hypnotics, such as benzodiazepines, also acting via the GABA-receptor complex (Mohler and Richards, 1988), showed no inhibitory potential on NFAT activation. Thus, the effect of barbiturates on NFAT does not seem to be primarily mediated via a GABA receptor-initiated signal transduction pathway.

Alternatively, inhibition of the DNA binding activity of

NFAT by barbiturates could be the result of a direct molecular interaction. The DNA binding domain of NFAT is highly complex and functional interaction with its specific *cis*-acting elements requires an intact secondary and tertiary structure of the molecule (Chen et al., 1998). In addition, barbiturates do have a strong antioxidant capacity (Wilson and Gelb, 2002) that might allow them to alter the sensitive three-dimensional structure of NFAT. However, the results of our coincubation experiments using activated nuclear extracts strongly suggest that inhibition of NFAT by barbiturates is not mediated through a direct interaction with the transcription factor itself or with other components of the nucleic compartment required for DNA binding but might rather affect the cytoplasmic signal transduction cascade leading to the activation of NFAT.

The hypothesis mentioned above is supported by the results of the present study. Changes in the phosphorylation status of NFAT play a key role in the control of its activation process. In resting cells, NFAT is located within the cytoplasm in an inactive, hyperphosphorylated state, whereas

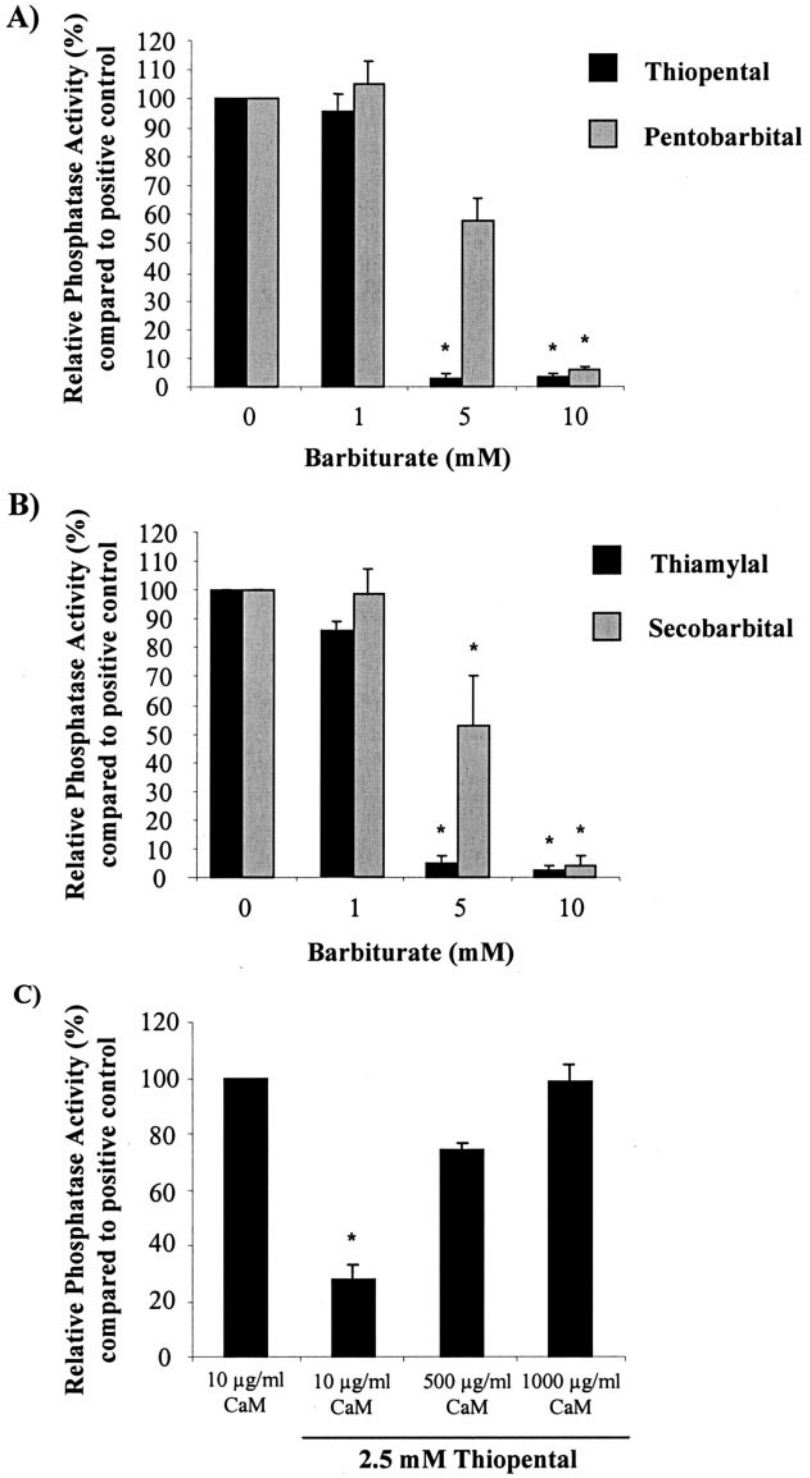

Fig. 6. Inhibition of recombinant human calcineurin by barbiturates as measured by an in vitro assay. Assays were performed at 37°C and included 50 U of the phosphatase calcineurin in 50 mM Tris/HCl, pH 7.4, 1 mM $NiCl_2$, 0.5 mg/ml BSA, 10 μg/ml calmodulin, and 1 to 10 mM of thiopental and pentobarbital (A), 1 to 10 mM thiamylal and secobarbital (B), or 10 to 1000 μg/ml calmodulin and 2.5 mM thiopental (C). Reactions were incubated for 30 min before addition of 10 μg of phosphorylated RII-peptide. Dephosphorylation of the RII peptide was measured at 600 nm by a photospectrometer after 20 min by addition of a molybdate dye staining solution. Bar 1, control reaction, performed without barbiturates, maximal phosphate activity (100%). Bars 2 to 4, incubation with 1 to 10 mM barbiturate or 10 to 1000 μg/ml calmodulin. Values are expressed as the means of six independent experiments ± S.E.M. *, $P < 0.05$ versus maximal phosphatase activity without barbiturates.

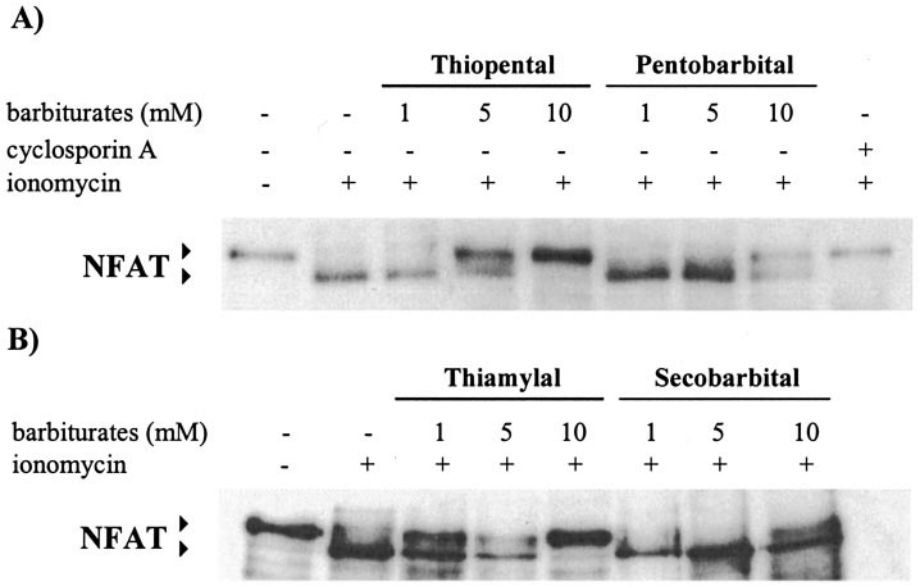

Fig. 7. Calcineurin dependent activation and dephosphorylation of NFAT in primary human T cells is inhibited by barbiturates. Human $CD3^+$ lymphocytes were exposed to various barbiturates (A and B, lanes 3–8) or 1 μM CsA (A, lane 9) for 6 h at the indicated concentrations. Lane 1, untreated lymphocytes. Dephosphorylation of NFAT was induced by 1 μg/ml ionomycin for 30 min (A and B, lanes 2–9). Cell lysates were prepared and loaded on 7.5% SDS-PAGE gels. Immunoblot experiments were performed to analyze dephosphorylation of NFATc2.

dephosphorylation is required to allow its translocation into the nucleus (Rao et al., 1997). Our experiments revealed that barbiturates cause a dose-dependent inhibition of the dephosphorylation of NFAT in human T cells and consequently compromise its nuclear translocation. NFAT is the substrate of the phosphatase calcineurin, which could therefore serve as a target for barbiturates. Thus, we evaluated the effect of barbiturates on calcineurin using an in vitro enzymatic assay system. Strikingly, our experiments showed that barbiturates directly suppress the enzymatic activity of calcineurin in contrast to CsA or FK506. Barbiturate-mediated effects must be caused by a direct inhibition of the calmodulin/calcineurin complex, because our in vitro enzymatic assay system lacks all upstream regulatory proteins, including calmodulin kinases or FKBPs. In conclusion, our results provide evidence for a novel mechanism of NFAT inhibition by calcineurin that does not require the binding to intracellular receptors.

Different types of interactions could be responsible for inhibition of calcineurin. Barbiturates could interfere with calmodulin and its binding to calcineurin. Thus, it is of particular interest that the competition experiments indicated that calmodulin is a target of thiobarbiturates. Barbiturates might also alter protein function by changing the active conformation or by binding to the enzymatic groove. The sulfur of thiobarbiturates might influence the formation of disulfide bonds and thereby inhibit calmodulin/calcineurin activity. However, thio substitutions are not exclusively responsible for inhibition of calcineurin, because oxybarbiturates also had an inhibitory but weaker effect on calcineurin function.

The barbiturates tested inhibited not only the enzymatic activity of calcineurin, the dephosphorylation and DNA-binding of NFAT, and NFAT-dependent reporter gene expression but also the synthesis of cytokines that have been shown to be regulated by NFAT (Jain et al., 1995; Kiani et al., 2000). However, in contrast to all other upstream experimental endpoints studied, the potency of thiobarbiturates to inhibit the synthesis of IL-2 or IFN-γ was not higher than that of their respective oxy-analogs. This observation underscores the results of previous reports showing that the expression of these cytokine genes does not exclusively depend on NFAT. In fact, multiple transcription factors have been shown to be involved in the control of their expression (Jain et al., 1995; Penix et al., 1996). Our results strongly suggest that in addition to the inhibition of NFAT, barbiturates may exert differential effects on other immunomodulatory transcription factors that are able to partially overcome their actions on NFAT. Indeed, preliminary evidence suggests that activator protein-1, a transcription factor that can cooperatively bind with NFAT to the IL-2 promoter, is similarly inhibited by thio- and oxybarbiturates (M. Humar, unpublished observations).

Understanding the mechanisms of barbiturate-mediated immunosuppression could form the basis of new strategies for the therapy of intracranial hypertension. For example, aspiration frequently occurs in the early period after traumatic brain injury and predisposes to the subsequent devel-

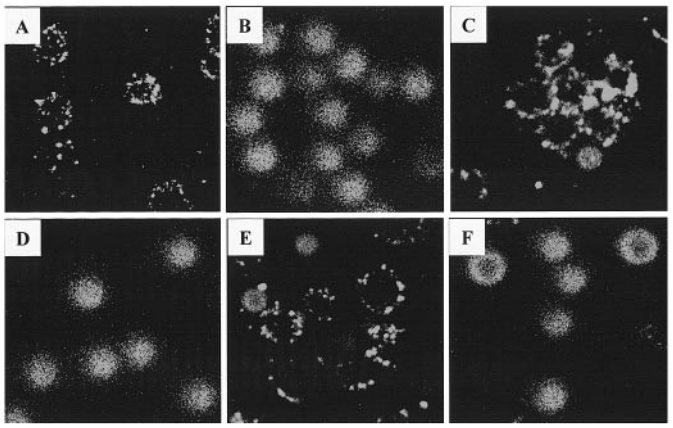

Fig. 8. Ionomycin-induced nuclear translocation of NFAT is compromised by thiobarbiturates in primary T lymphocytes. T cells were left unstimulated (A) or stimulated with 1 μg/ml ionomycin for 20 min (B-F) in the presence of 20 nM leptomycin B to inhibit nuclear export. Barbiturates (2.5 mM) were added for 6 h: thiopental (C) or its oxy-analog pentobarbital (D), thiamylal (E) or its oxy-analog secobarbital (F). Cells were fixed and stained with an NFATc2 specific antibody (clone G1-D10). Representative confocal fluorescence microscopy.

opment of pneumonia (Morgan and Mackay, 1999). Thus, resolution of the neuroprotective action of barbiturates from the immunomodulating effects may be beneficial in patients whose survival depends on lowering the intracranial pressure but who are simultaneously at a high risk to develop lower respiratory tract infections. The results of the present study provide a molecular rationale for future investigations that systematically examine the structural requirements for the effect of barbiturates on neuronal cell integrity and immune function after brain injury. Immunosuppressive drugs, such as cyclosporin A or FK506, that are currently used to target NFAT in patients have numerous side effects (Kiani et al., 2000). Our observation of a direct inhibitory effect of barbiturates on NFAT might therefore yield an additional avenue for suppressing deleterious immune responses in other clinical settings.

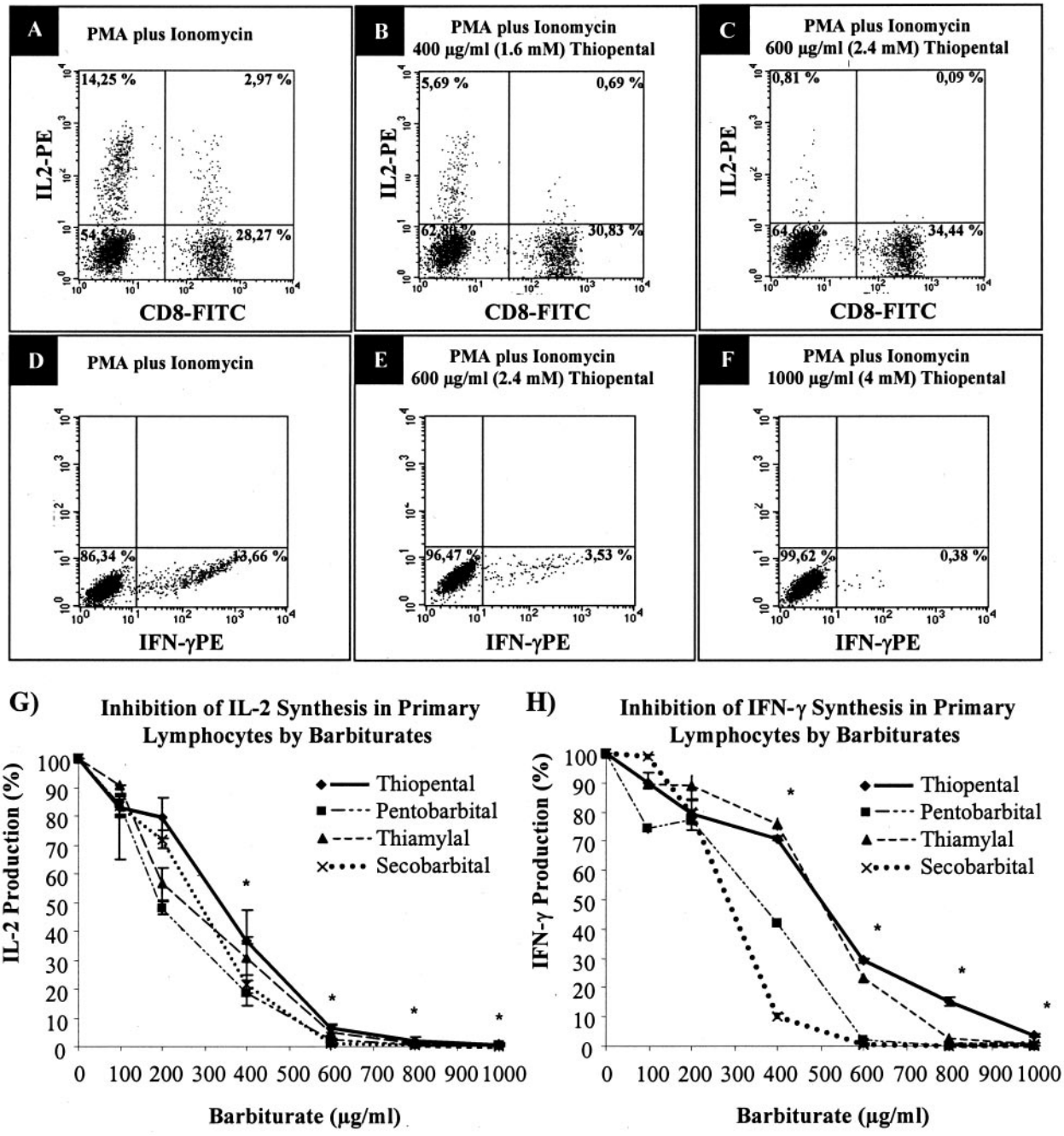

Fig. 9. NFAT-transcription factor regulated cytokine expression is inhibited by barbiturates. Heparinized blood was preincubated with different barbiturates at the indicated concentrations. After 2 h, IL-2 and IFN-γ expression was induced by addition of 15 ng/ml PMA and 1 µg/ml ionomycin. Secretion of cytokines was inhibited by 10 µg/ml brefeldin A. After 15 h, erythrocytes were lysed. Remaining PBMCs were fixed, permeabilized, and stained with a CD3/CD8/IL2 (A–C, G) or a CD3/IFN-γ (D–F, H) antibody cocktail. CD3-positive lymphocytes were gated and analyzed for cytokine expression by FACS. Maximal cytokine expression was found in PMA- and ionomycin-stimulated cells in the absence of barbiturates (positive controls). IC_{50} for inhibition of IL-2 production was 340 µg/ml for thiopental (1.29 mM); 195 µg/ml for pentobarbital (0.78 mM); 293 µg/ml for thiamylal (1.06 mM); and 253 µg/ml for secobarbital (0.97 mM). IC_{50} for inhibition of IFN-γ expression was 503 µg/ml for thiopental (1.9 mM); 359 µg/ml for pentobarbital (1.45 mM); 506 µg/ml for thiamylal (1.83 mM); and 291 µg/ml for secobarbital (1.12 mM). $n = 3$. *, $P < 0.05$ versus positive controls.

In conclusion, our data indicate that barbiturates directly inhibit the calmodulin/calcineurin complex, inhibit dephosphorylation and DNA-binding activity of NFAT, and attenuate NFAT-dependent reporter gene expression and cytokine synthesis in human T cells. These results provide a novel molecular mechanism for several immunosuppressive effects associated with barbiturate treatment.

Acknowledgments

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