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Research Article

Protein kinase C-dependent NF-κB activation is altered in T cells by chronic stress

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Abstract. Chronic stress has been associated with impaired immune function. In this work we studied the effect of chronic mild stress (CMS) exposure on the early intracellular pathways involved in T cells after stimulation with mitogen. We found that mitogen stimulation of T lymphocytes from CMS-exposed mice resulted in a reduction of the intracellular [Ca^{2+}] rise, an impairment of growth-promoting protein kinase C (PKC) activation, a lower NF-κB activation and an increase in the inhibitory cAMP-protein kinase A (PKA) pathway activity with respect to those found in control lymphocytes. However, T cell activation with the direct PKC activator phorbol 12-myristate 13-acetate plus calcium ionophore led to a similar proliferative response in both CMS and control lymphocytes, indicating that signals downstream of PKC would not be affected by stress. In summary, our results show that chronic stress induced an alteration in T cell early transduction signals that result in an impairment of the proliferative response.

Key words. T cell; stress; cAMP; protein kinase C (PKC); NF-κB; chronic mild stress (CMS); early signaling.

Accumulating evidence indicates that physiological and psychological stress has profound effects on immune function [1]. The consequences of the physiologic response to stress are generally adaptive in the short run but can be damaging when stress is chronic and long lasting [2]. Thus acute stress enhances whereas chronic stress suppresses immune function [3]. It has been demonstrated that acute stress might activate acute-phase responses that are critical for rapid and effective pathogen clearance in both systemic and local tissue infections [4, 5]. Acute stress has also been shown to enhance antigen-specific cell-mediated immunity [6]. In contrast, chronic stressful experiences have been associated with a diminished ability of the immune system to respond to challenge and, thus, augment the susceptibility to infectious diseases and neoplasia development [7–10]. Accordingly, we have found that chronic stress exposure induces a decrease in T cell proliferative response to concanavalin A (Con A) and to phytohemagglutinin (PHA) [11] in vitro and lower T cell-dependent antibody formation in vivo [12, 13]. However, the effects of stress exposure on the intracellular signaling pathway after triggering of T cells that results in proliferation and immune competency have been poorly studied.

T lymphocyte activation by antigen presentation, monoclonal antibodies or a specific mitogen leading to proliferation is mediated by a complex series of intracellular signaling events [14, 15]. These signal transduction pathways are initiated by the obligatory activation of tyrosine protein kinases (TPKs) which in term activates phospholipase C-γ resulting in a transient rise in the cytosolic free calcium concentration ([Ca^{2+}]) and in the plasmatic membrane content of diacylglycerol (DAG) [16]. These second messengers activate the growth-promoting protein

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kinase C (PKC) [15]. To date, 11 closely related PKC isoenzymes have been described and classified into three subfamilies based on their domain structure and their ability to respond to Ca^{2+} and DAG [17]. For some time, the contribution of the different PKC isoforms to T cell receptor (TCR)-mediated activation was not clearly understood. Recent studies on PKC θ, a 'novel' PKC isoform selectively expressed in T cells and skeletal muscle [18] have uncovered its critical role in T cell activation [19]. Following T cell stimulation, PKC θ translocates to the center of the immunological synapse where it co-localizes with the TCR and this event is essential in the activation of transcription factors such us nuclear factor-kappa B (NF-κB) and the activator protein-1 (AP-1) [19] which are required for cell proliferation and the production of cytokines in response to T cell activation. Recently, PKCα was demonstrated to act upstream of PKC θ to activate IκB kinase and NF-κB in T lymphocytes [20]. The canonical NF-κB is a heterodimer of p65 (RelA) with p50 or p52 [21]. This heterodimer is anchored by a group of proteins named I-κB, which function to retain NF-κB in the cytosol by masking its nuclear localization signal [22]. Following activation of certain signal transduction pathways, a site-specific hyperphosphorylation of IκB renders the inhibitor molecule susceptible to site-specific ubiquitination and subsequent degradation by the proteasome complex [22]. This releases NF-κB, allowing it to undergo nuclear translocation where it activates target genes. On the other hand, the inhibitory cyclic AMP/protein kinase A (cAMP/PKA) pathway is the best characterized early signal implicated in the network of activating-inhibiting processes that follow normal lymphocyte activation [23, 24].

The aim of the present study was to analyze the impact of chronic mild stress (CMS) exposure on the early signals triggered in T cells after mitogen stimulation. For this purpose, BALB/c mice were subjected to CMS for 8 weeks and the intracellular [Ca^{2+}], the growth-promoting PKC activity and the inhibitory cAMP-PKA pathway activity were evaluated. Moreover, the participation of the nuclear transcription factor NF-κB through the activation of the PKC θ pathway was investigated. Our results showed that chronic stress induced an alteration of these early transduction signals that in turn could result in a diminished proliferative response.

Materials and methods

Drugs

Con A, phorbol 12-myristate 13-acetate (PMA), isobutyl methyl xanthine (IBMX), the ionophore ionomycin, fura 2AM and pluronic were purchased from Sigma (St. Louis, Mo.). [γ^{32}P]-ATP (3000 Ci/mmol) and [^{3}H]-thymidine (20 Ci/mmol) were purchased from New England Nuclear (NEN), Life Science Products (Boston, Mass.). Other materials were from standard commercial sources.

Mice

Inbred female BALB/c mice were purchased from the Instituto Nacional de Tecnología Agropecuaria (INTA). All animals were between 60 and 100 days of age. Animal care was in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996.

Stress model

Seventy female BALB/c mice, weighing 29–31 g at the beginning of the experiment were used. All animals were housed singly and maintained on a 12-h light/dark cycle under controlled temperature conditons (18–22 °C). After 2 weeks of habituation, animals were distributed into two groups (n = 33 per group). One group remained under standard conditions (control) and the other was subjected throughout the experiment to a chronic unpredictable mild stress schedule (CMS mice). The stress regime was slightly modified from that previously used in mice [25]. It consisted of: one 16-h period of water deprivation; two periods of continuous overnight illumination; two periods (7 and 17 h) of 45° cage tilt; one 17-h period in a soiled cage (100 ml water in sawdust bedding); one period (8 h) of food deprivation; one 17-h period of paired housing (animals were always housed in the same pairs, but the location alternated between the home cages of each member of the pair). The stressors were scheduled through the week for 8 weeks, in a similar manner to that previously described [25].

Cell suspensions and culture conditions

Spleens were removed and disrupted through a 1-mm metal mesh, and the cell suspension was filtered through a 10-µm nylon mesh. The suspension was depleted of non-lymphoid cells after centrifugation over Ficoll/Hypaque. After three washes in RPMI 1640, cells were resuspended in RPMI 1640 supplemented with 10% batched-tested non-stimulatory fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-mercaptoethanol [26]. Cell viability was estimated according to the Trypan blue exclusion criteria and was higher than 90%. T lymphocyte-rich lymphoid cell fractions were obtained by passage of the cell suspension through a nylon wool column according to the method of Julius et al. [27]. Purification of more than 97% was obtained, as checked by lysis with anti-Thy plus complement or direct immunofluorescence.

Mitogen assay

Proliferation was determined by culturing 2×10^5 cells per well in 96-well plates in a final volume of 0.200 ml of supplemented medium at 37 °C in 5% CO_2 as described

elsewhere [26]. Aliquots of 50 µl of Con A, to yield the optimal mitogen concentration (2 µg/ml) according to previous dose-response curves, or PMA (2 nM) plus ionomycin (2 µM) were added to the microcultures. In control cultures, stimulants were replaced by 50 µl of culture medium. Then, cells were cultured at 37 °C in 5% CO_2 for different periods. Mitogenic activity was measured by adding 1 µCi [^{3}H]-thymidine per well for the last 18-h period of culture. [^{3}H]-thymidine incorporation was determined by scintillation counting after retention over GF/C glass-fiber filters (Whatman) of the acid-insoluble macromolecular fraction. The means of triplicate determinations were calculated for each cultured condition. Con A- and PMA plus ionomycin-stimulated cells displayed the expected kinetics of proliferation, with a peak at the third day of culture.

Determination of the intracellular Ca^{2+} concentration

Calcium concentration was measured in lymphocytes loaded with fura 2AM (final concentration 5 µM) at 37 °C for 10 min and in the presence of 20 µg/ml pluronic (to favor the dispersion of the probe) as previously described [28]. After washing and centrifuging (200 g for 10 min) twice in PBS, cells were transferred to PBS containing 5 mg/ml of fatty acid-free BSA. The fluorescence was recorded at 510 nm in an Amico Bowman Spectronic spectrofluorometer using an excitation source of 340 or 380 nm. Maximal fluorescence was determined at the end of the assay by adding 10 µl of 10% SDS. Minimal fluorescence was determined by adding 15 µl of a solution containing 0.5 M EGTA, pH 9.0. The intracellular [Ca^{2+}] was calculated from the fura 2AM fluorescence intensity according to Cobbold and Rink [29] as: $[Ca^{2+}]_i = Kd\ (F–F_{min})/(F_{max}–F)$, where Kd = 224 nM for the intracellular dye.

Protein kinase purification

After stimulation with Con A, lymphocyte suspensions (10^7 cells/ml) were immediately centrifuged (5000 g for 30 s) and cell pellets were chilled on liquid N_2. The homogenization was performed in 2 ml of an ice-cold medium containing 10 mM β-mercaptoethanol, 2 mM EGTA, 2 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin and 20 mM HEPES, pH 7.4. The soluble (cytosolic) and particulate (pellet) fractions were obtained as described previously [28]. PKC enzyme was purified by filtering through a DE 52 column (3.5 × 0.5 cm). The enzyme was eluted in a buffer containing 120 mM NaCl, 10 mM β-mercaptoethanol, 0.5 mM EGTA and 10 mM HEPES pH 7.4.

PKC assay

The synthetic peptide myelin basic protein (MBP 4–14), a well-known specific PKC substrate [30] (Gibco BRL Life Technology Invitrogen Argentina, Bs As, Argentina), was also used to measure PKC activity purified from subcellular lymphocyte fractions, following the instructions of the PKC assay system of Gibco. PKC specificity was confirmed by using the enzyme pseudosubstrate inhibitor peptide PKC (19–36) provided by Gibco. Efficiency of PKC activity was 85–90% of the total kinase activity.

PKC immunoblot analysis

Cytosol and particulate fractions of unstimulated or stimulated cells from control or CMS exposed mice were obtained representing 5×10^6 cell equivalents/10 µl and supplemented with 50 µl 3 × 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]. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with blocking buffer (5% non-fat dried milk, containing 0.1% Tween-20 in 100 mM Tris-HCl, pH 7.5 and 0.9% NaCl) for 1 h. Membranes were subsequently incubated with protein G-purified anti-peptide antibodies to the PKC α and θ isoforms (Santa Cruz Biotechnology, Santa Cruz, California) for 18 h. Anti-β-actin antibody (Santa Cruz Biotechnology) was used as control of equal loading and transfer efficiency. Negative controls were incubated in the presence of immunogenic peptides (2 µg/ml of antibody to 1 µg/ml of peptide). Then membranes were incubated with a monoclonal anti-rabbit IgG alkaline phosphatase conjugate antibody (Sigma) 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, Amersham Biosciences Argentina, Bs As, Argentina) was used.

Preparation of cytosolic and nuclear extracts

T lymphocytes were stimulated for 30 min with Con A (2 µg/ml). After stimulation, cells were centrifuged and stored at –80 °. Cell pellets were homogenized in 200 µl of buffer A (10 mmol/l HEPES, pH 7.9, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l KCl, 1 mmol/l DTT, 0.5 mmol/l phenylmethyl sulfonyl fluoride, 40 µg/ml leupeptin, 2 µg/ml tosyl-lysyl-chloromethane, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 10 mmol/l Na_2MoO_4), and NP-40 was added to 0.5% (v/v). After 15 min at 4 °C, the tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 8000g for 15 min. The supernatants were stored at –80 °C (cytosolic extracts) and the pellets were resuspended in 50 µl buffer A supplemented with 20% (v/v) glycerol and 0.4 M KCl, and mixed for 30 min at 4 °C. Nuclear proteins were obtained by centrifugation at 13,000 rpm for 15 min, and aliquots of the supernatant (nuclear extracts) were stored at –80 °C [31]. For Western blot analysis, cytosolic samples were boiled in Laemmli sample buffer, and equal amounts of protein (15 µg) were

separated by 10% SDS-PAGE as indicated for PKC immunoblotting. The protein levels of IκB-α and IκB-β were determined using specific antibodies. Anti-β-actin was used as control of equal loading and transfer efficiency. Different exposure times were performed with each blot to ensure the linearity of the band intensities. Band intensities were measured on a densitometric scanner (Amersham) and expressed in arbitrary units.

Nuclear samples were stored at –80 °C until electrophoretic mobility shift assays (EMSAs) were performed.

Electrophoretic mobility shift assays

Protein concentration was determined using the method of Bradford (Bio-Rad, Hercules, California). Oligonucleotide probes containing the consensus sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were end-labeled with a Klenow enzyme fragment in the presence of 50 µCi of [α-^{32}P]dCTP and the other unlabeled dNTPs in a final volume of 50 µl. DNA (5×10^4 dpm) probe was used for each binding assay: 5 µg of nuclear protein was incubated for 15 min at 4 °C with the probe and with 1 µg of poly(dI-dC), 5% glycerol, 1 mmol/l EDTA, 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l DTT and 10 mmol/l Tris-HCl (pH 7.8) in a final volume of 20 µl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer [31]. Gels were dried and exposed to Kodak film at –70 °C.

Cyclic AMP assay

Cells (1×10^7/ml) were incubated alone or with Con A in 1 ml PBS for 30 min. Some samples were pre-treated with IBMX (200 µM) for 20 min to inhibit phosphodiesterase (PDE) activity. At the end of the incubation time, 2 ml chilled ethanol was added. Cells were homogenized, supernatants were evaporated at 55 °C under a nitrogen stream and the residue was dissolved in 0.5 ml assay buffer. Nucleotide determination was carried out by a commercial cAMP enzyme immunoassay (Amersham Pharmacia Biotech).

Flow cytometry

CD3, CD4 T helper/inducer and CD8 T cytotoxic/suppressor lymphocytes were determined in T lymphocyte-rich lymphoid cell fractions by flow cytometry. Briefly, aliquots of cell suspensions were stained with fluorescein-conjugated anti-mouse CD3, fluorescein-conjugated anti-mouse CD4 or with phycoerythrin-conjugated anti-mouse CD8 monoclonal antibodies. Lymphocytes were identified by FACS analysis using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with log amplification and FACScan research software and the percentage of lymphocytes expressing CD3, CD4 and CD8 was determined. Isotype controls (IgG1-FITC/IgG2a-PE) were used for each assay to determine non-specific staining.

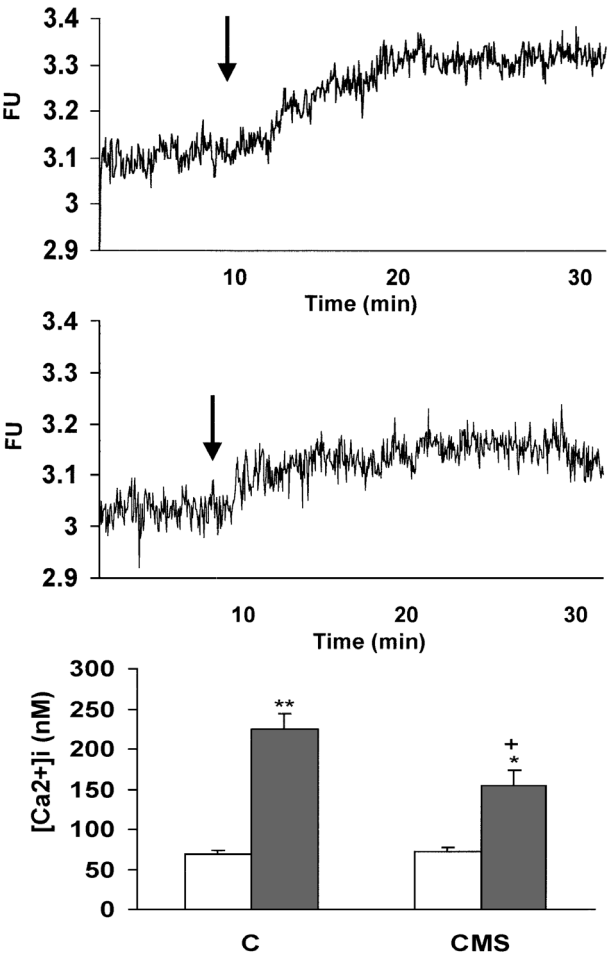

Figure 1. Cytosolic [Ca^{2+}] in mitogen-stimulated T cells of control and CMS-exposed mice. T cells were loaded with 5 µM fura 2AM and the units of fluorescence (FU) recorded at 510 nm after excitation at 340 and 280 nm were used to calculate [Ca^{2+}] according to Cobbold and Rink as described in Materials and methods. The upper (control) and middle (CMS) panel show the FU registered after using an excitation source of 340 nm from a representative experiment. After a 10-min period of stabilization, cells were stimulated with Con A (2 µg/ml) as indicated by the arrow. The lower panel shows the intracellular Ca^{2+} levels in control (C) and CMS T cells (CMS) before (open bars) and after (dark bars) 10 min of Con A stimulation. Results shown are the mean ± SE of six independent experiments using one animal of each group. * $p < 0.05$, ** $p < 0.01$ with respect to basal values; + $p < 0.05$ with respect to control values.

FITC-Con A binding

Scatchard analyses of FITC-Con A binding to T lymphocyte-rich lymphoid cell fractions were performed according to the method described by Gordon [32]. Briefly, reactions were performed using 5×10^6 cells in 1 ml RPMI 1640 containing 2 mg/ml BSA as medium. After 30 min of binding, cells were washed by centrifugation in medium supplemented with 20 mM NaN_3, resuspended in 0.5 ml and kept in an ice bath until passed through a FACScan flow cytometer (Becton Dickinson). Immediately before analysis, cells were dispersed by passage five times through a 26-gauge needle. A minimum of 10,000

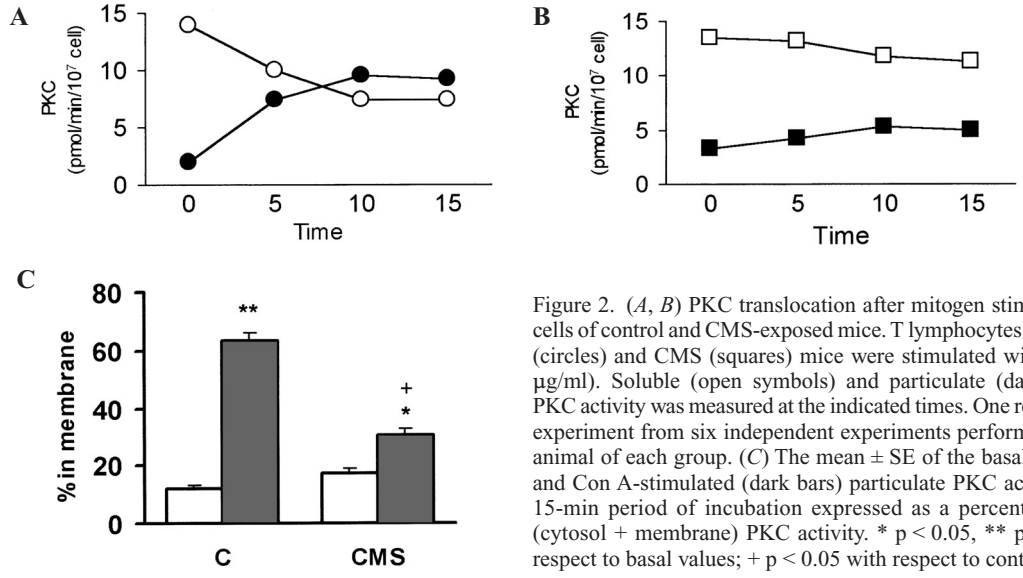

Figure 2. (*A*, *B*) PKC translocation after mitogen stimulation in T cells of control and CMS-exposed mice. T lymphocytes from control (circles) and CMS (squares) mice were stimulated with Con A (2 µg/ml). Soluble (open symbols) and particulate (dark symbols) PKC activity was measured at the indicated times. One representative experiment from six independent experiments performed with one animal of each group. (*C*) The mean ± SE of the basal (open bars) and Con A-stimulated (dark bars) particulate PKC activity after a 15-min period of incubation expressed as a percentage of total (cytosol + membrane) PKC activity. * $p < 0.05$, ** $p < 0.01$ with respect to basal values; + $p < 0.05$ with respect to control values.

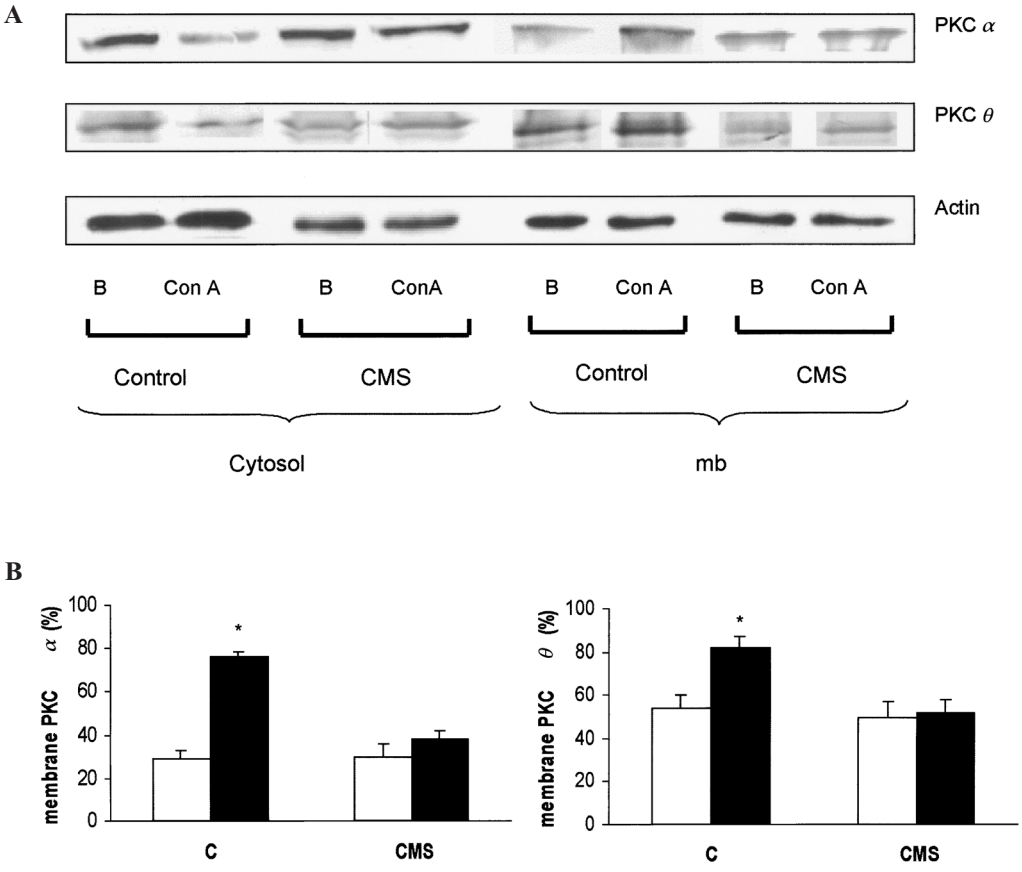

Figure 3. Translocation of PKC α and θ isoforms in normal and CMS lymphocytes after Con A stimulation. (*A*) Cytosolic and membrane fractions from non-stimulated (*B*) or mitogen-stimulated (Con A) lymphocytes from control and CMS-exposed (CMS) mice were analyzed by Western blot using antibodies for α and θ isotypes. β-Actin was used as internal loading control. Data shown correspond to one representative experiment of three performed independently. (*B*) The relative intensity of the different PKC bands was quanfied by densitometry and the percentage of the membrane associated PKC out of the sum of cyotosolic plus membrane-associated and PKC was calculated in each case and represented in the bar graph. White bars correspond to non-stimulated cells and dark bars to Con A-stimulated lymphocytes. Results shown represent the mean ± SE of three independent experiments. * $p < 0.05$ with respect to basal values.

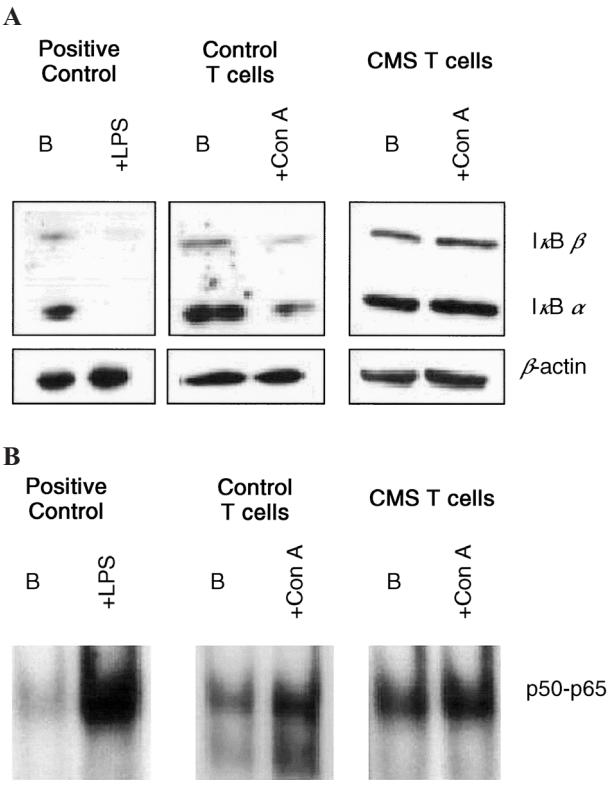

Figure 4. T cells from control and CMS mice were stimulated for 30 min with Con A. The protein levels of IκB-α and IκB-β were determined in cytosolic extracts by Western blot (*A*). β-Actin was used as internal loading control. NF-κB activity was determined by EMSA using nuclear protein extracts (B). Cytosolic and nuclear extracts from the RAW 264.7 cell line were used as positive controls after being stimulated with LPS for 30 min. Each panel shows one representative experiment from four independent experiments performed with one animal of each group.

stained cells were analyzed for each FITC-Con A concentration employed. The specific binding was calculated as the difference between binding in the absence and in the presence of 100 mM α-methyl mannoside.

Statistical analysis

The Student t test for unbalanced values was used to determine the level of significance. When multiple comparisons were necessary after analysis of variance, the Student-Newman-Keuls test was applied. The difference between means was considered significant if $p \leq 0.05$.

Results

Con A-stimulated [Ca^{2+}] rise is reduced by chronic stress

To determine if the [Ca^{2+}] rise was altered by chronic stress exposure, the analysis of short-term changes in cytosolic [Ca^{2+}] after T cell stimulation was performed. As shown in figure 1, when fura 2-loaded control T cells were incubated with Con A, a progressive [Ca^{2+}] rise, that required about 10–15 min to reach a sustained maximum level, was observed. T cells from CMS-exposed mice showed a similar time course of [Ca^{2+}] increase but the intensity of response was significantly lower than that observed for control cells.

PKC activation after Con A stimulation is impaired by chronic stress.

To analyze the effect of chronic stress on mitogen-induced PKC activation, T cells were incubated with Con A for different periods of time. PKC enzymatic activity was determined in both cytosolic and membrane fractions. As shown in figure 2, when T cells were stimulated, a clear time-dependent change in basal subcellular distribution of PKC was observed. PKC activity diminished in the cytosolic fraction and was recovered in the particulate fraction of both control and CMS-exposed T cells but in the latter, the translocation of PKC activity toward the membrane was significantly lower than that observed in controls. Important to note is that a non-significant change in basal subcellular distribution of PKC was observed in untreated T cells at the tested times (data not shown). Since PKC θ and PKC α are the principal isoforms of the PKC family involved in T cell activation [19, 20], we analyzed the translocation of these isoenzymes by Western blot after T cell stimulation. As can be seen in figure 3, a lower increment of PKC θ and PKC α expression in the membrane after stimulation was observed in CMS-exposed cells. Accordingly, a higher percentage of these enzymes is shown in the cytosolic fraction.

NF-κB activation is diminished in activated T cells from CMS mice

To investigate whether the impaired PKC θ activation after T cell activation elicited by chronic stress induced an alteration in NF-κB activation, we measured the presence of NF-κB inhibitor in the cytosolic extract by Western blot and NF-κB DNA binding activity in nuclear extracts after T cell stimulation by EMSA. As expected, T cell activation with Con A induced a decrease in cytosolic NF-κB inhibitor (fig. 4A) and promoted mainly the corresponding p65 (RelA)/p50 heterodimer translocation into the nucleus (fig. 4B). As shown in figure 4, T cells from CMS mice had a lower NF-κB activation after mitogen stimulation.

The cAMP-PKA pathway is involved in the effect of chronic stress exposure on T cell activation

To evaluate the effect of chronic stress exposure on the cAMP-dependent PKA pathway, changes of intracellular cAMP levels in lymphocytes after stimulation were determined. As can be seen in figure 5, Con A significantly diminished cAMP intracellular levels in control lymphocytes. The percentage of change was significantly lower in lymphocytes from CMS-exposed mice. cAMP synthe-

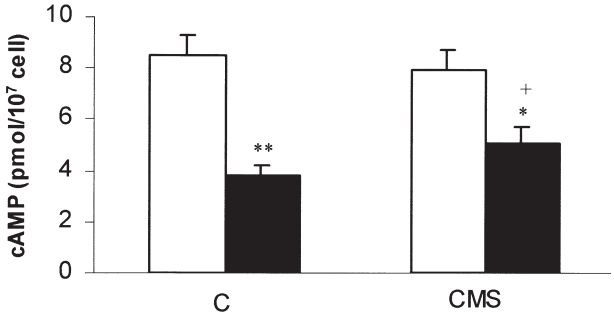

Figure 5. cAMP levels in stimulated T cells from control and CMS mice. Cells from control (C) and CMS mice were incubated alone (open bars) or in the presence of Con A for 30 min (dark bars) and cAMP levels were determined. Results shown represent the mean ± SE of six independent experiments using one animal of each group performed in triplicate. * $p < 0.05$, ** $p < 0.01$ with respect to basal values; + $p < 0.05$ with respect to control values.

sis is well known to depend on adenylate cyclase and cAMP hydrolysis depends on cyclic nucleotide PDE. IBMX, an inhibitor of PDE activity [33] reversed the ability of Con A to reduce cAMP levels in both control and CMS lymphocytes (data not shown).

PMA and Ca^{2+} ionophore treatment resulted in the same proliferative response for lymphocytes from control and CMS-exposed mice

Chemical treatment with the phorbol ester PMA or with 12-O-tetradecanonylphorbol-13-acetate (TPA) combined with the calcium ionophore, ionomycin, is known to stimulate T cells to proliferate. To analyze the involvement of PKC downstream signaling, and considering the previously reported impairment of the T cell proliferative response in mice exposed to chronic stress [11], we examined the effect of PMA and ionomycin on ^{3}[H]-thymidine incorporation in lymphocyte from control and CMS-exposed mice. As shown in figure 6, ^{3}[H]-thymidine incorporation was similar in lymphocytes from both conditions. In contrast, as expected, the mitogen-induced proliferative response in CMS-exposed lymphocytes was significantly lower than that observed in controls.

Table 1. Percentage of lymphocyte populations in T cells from control and CMS-exposed mice.

T cell origin	Lymphocyte populations (percentage of total lymphocytes)		
	CD3+	CD4+	CD8+
Control	93 ± 6	78 ± 6	13 ± 2
CMS-exposed mice	95 ± 5	81 ± 7	15 ± 3

FACS analysis was performed on T cell-enriched fractions obtained from spleen of non-exposed and CMS-exposed animals. Results are expressed as percentage of total lymphocytes and represent the mean ± SD of four independent experiments.

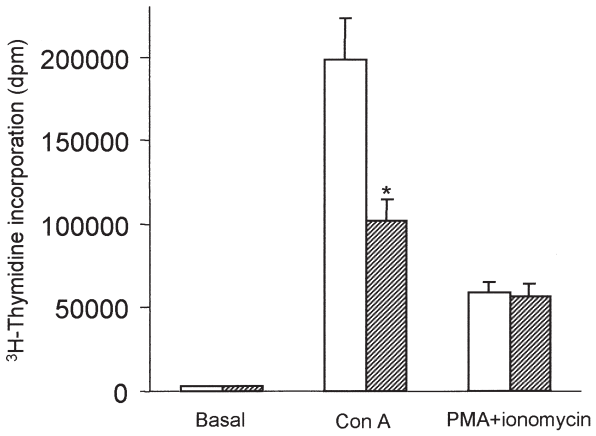

Figure 6: Stimulated T cell proliferation in splenic cells from control and CMS mice. Cells from control (open bars) and CMS (hatched bars) mice were cultured alone (basal) or were stimulated with Con A (2 µg/ml) or with PMA (2 nM) plus ionomycin (2 µM). ^{3}H-thymidine incorporation was determined after 3 days. Results shown represent the mean ± SE of five independent experiments performed in triplicate. * $p<0.01$ with respect to control values.

CMS alteration in signaling pathways triggered after Con A stimulation is not related to redistribution of lymphocyte populations

We have previously shown that changes in proliferative response are not related to changes in the distribution of lymphocyte subsets in lymphoid compartments of CMS-exposed animals [11]. To enure that changes in intracellular signaling observed in CMS-exposed T cells were not due to differences in lymphocyte populations, percentages of CD3+, CD4+ and CD8+ were determined in T cell-enriched fractions obtained from spleens of non-exposed and CMS-exposed animals. As can be seen in table 1, no significant differences were observed in CD3, CD4 and CD8 cell counts between fractions analyzed.

Impairment in CMS signaling pathways triggered after Con A stimulation is not due to a diminishment in lymphocyte ligand binding.

To investigate if one of the possible mechanisms involved in the alteration of the signaling pathway observed in CMS lymphocytes was a modification in Con A receptor expression and/or affinity, a binding assay of FITC-Con A to T cells was performed. Figure 7 shows a representative binding study on T cell-enriched fractions from non-exposed and CMS-exposed animals. As can be seen, FITC-Con A binding was specific and saturable within a concentration range of 2–150 µg/ml of Con A. Scatchard analyses of the binding curves showed that CMS lymphocytes had an increased number of Con A receptors but this augmentation was not significant with respect to the non-exposed cells (B_{max}: non-exposed $7.6 \pm 0.5 \times 10^6$ sites per cell, CMS $8.7 \pm 0.8 \times 10^6$ sites per cell). Both groups of cells showed similar affinity to the ligand (K_d: non-exposed 0.76 ± 0.09 pM, CMS 0.98 ± 0.11 pM).

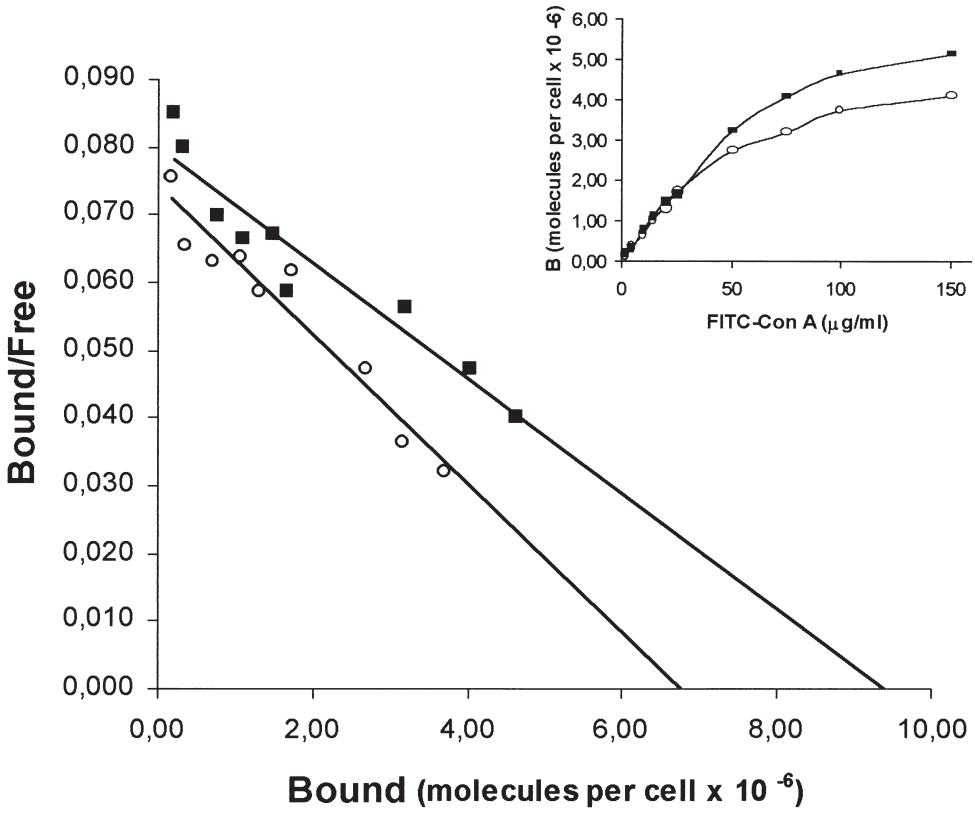

Figure 7. Binding of FITC-Con A to T lymphocyte-rich lymphoid cell fractions. T cells from non-exposed (circles) and CMS-exposed (squares) mice were incubated with increasing concentrations of FITC-Con A. Data show one representative experiment from three separate experiments performed in duplicate. Each experiment was performed with a pool of three animals from each group.

Discussion

We investigated the effects of chronic stress exposure on the early intracellular signal triggered by Con A in T cells in an attempt to correlate them with our previously reported impairment of T cell proliferative response [11]. The current view of T cell activation through the binding of lectin to cell surface receptors involves an increase in cytosolic calcium concentration and the induction of two functionally well-characterized kinases, PKC and PKA, which have opposite functions. PKC activation promotes cell proliferation [15] whereas activation of the cAMP-dependent PKA pathway leads to its inhibition [24]. Our results indicated that chronic stress induced an inhibition of the [Ca^{2+}] rise in T cells after Con A stimulation. While increasing [Ca^{2+}] per se is not sufficient to trigger lymphocyte proliferation [14], reducing [Ca^{2+}] influx markedly attenuates mitogen-stimulated lymphocyte proliferation indicating that the rise in free [Ca^{2+}] concentration would be an early and essential event for mitogen-induced cell proliferation [14, 34].

PKC is known to be normally present in the cytoplasm and translocates to the plasma membrane after lectins bind to cell surface receptors. This rapid PKC activation is critical for T cells to enter into the proliferative phase of the cell cycle, as described by Berry et al. [35]. As expected, after T cell activation with Con A, a subcellular redistribution of the enzyme activity from the cytosol toward the membrane was observed. However, we found that subcellular PKC redistribution was lower for T cells from CMS-exposed mice compared to controls. The reduced increase of calcium concentration observed for T cells from CMS-exposed mice is likely to render a reduced PKC activation. As mentioned above, T cell activation results in a transient increase of cytosolic Ca^{2+} and DAG. In turn, these second messengers activate PKC. Since DAG is as equally important as Ca^{2+} for PKC activation, the possibility that a lower increase in DAG content occurs, in parallel to a reduced increase in Ca^{2+}, cannot be ruled out. The PKC family was first thought to be an important target in the process of T cell activation because phorbol ester treatment of T cells, together with the calcium ionophore ionomycin, was found to bypass lymphocyte surface receptor signaling to cause T cell activation. Over the years, however, it has become evident that there are multiple other cellular receptors (C1 domain-containing proteins) for phorbol esters including important T cell signaling molecules. However, the fact remains that pharmacological downregulation of PKCs impairs T cell activation signaling and effector function, so PKCs

do play an important role in T cell activation signaling [36]. Among the PKC isoforms, PKC α and θ are recruited to the inner leaflet of the plasma membrane within minutes following T cell receptor ligation, an event that is crucial to NF-κB activation [19, 20]. Our results indicated that T lymphocytes from mice exposed to chronic stress have a lower translocation of both PKC α and θ after T cell stimulation. Accordingly, a lower NF-κB activation was observed in these cells.

Genetic studies also support the role of PKC θ as a crucial factor in TCR signaling. Sun et al. [36] generated a PKC θ-deficient mouse strain and demonstrated that mature T cells from these mice show a striking defect in IκBα degradation and NF-κB activation following exposure to crosslinking anti-CD3 and anti-CD28 antibodies. The lack of response correlates with markedly decreased cytokine production and T cell proliferation, both of which depend upon efficient NF-κB signaling. Activation of NF-κB is required for antigen-induced proliferation, cytokine production and survival of T cells, and these processes form the cornerstone of the adaptive immune response [37]. Since NF-κB plays an essential role in the adaptive immune response, our results indicating a lower NF-κB activation after stimulation in T cells from CMS mice could explain the previously reported impaired T cell response induced by chronic stress [8–13].

Regarding the cAMP-PKA pathway, we found that Con A induced a decrease in intracellular cAMP levels. An inverse relationship exists between cAMP levels and lymphocyte proliferation [38, 39]. Not clear is whether a decrease in cAMP levels triggers a proliferative response. In fact, some reports show that cAMP blocks the progression but not the initiation of T cell proliferation. However, in most of the T cell activation models, decreased cAMP levels accompany lymphocyte proliferation [23]. The effect of Con A stimulation on cAMP levels could reflect an effect over the adenylate cyclase activity or on cAMP-PDE activity. Several reports have demonstrated that phorbol esters alter the hormone-responsive adenylate cyclase system, an effect presumably mediated by PKC [40]. Previously, we demonstrated that PKC activation induced by incubation with PMA (2×10^{-9} M) for 5 min leads to a significant decrease in cAMP levels, thus indicating that PKC activity could be involved in diminishing cAMP levels after Con A stimulation [41]. However, the inhibition of PKC activity with staurosporine did not reverse the ability of the mitogen to reduce cAMP production. Nevertheless, the downregulation of PKC by PMA treatment (2×10^{-8} M) for 180 min leads to a significant increase in basal and Con A-treated lymphocyte cAMP levels [41]. Moreover, we found that IBMX, an inhibitor of PDE, impaired the decrease in cAMP levels evoked by Con A. Thus, PKC activation would not be essential to induce a decrease in cAMP levels after Con A stimulation but would play an important role in preventing the augmentation in cAMP levels, which in turn could lead to the inhibition of the process that follows normal lymphocyte proliferation. In the present study we were not able to find a significant effect of chronic stress exposure on basal levels of PKC activity and cAMP levels. Moreover, the inhibition of PDE activity totally impaired the cAMP decrease after Con A stimulation in both control and CMS-exposed lymphocytes. In addition, we did not find significant differences in the crosstalk between PKC and cAMP pathways in lymphocytes from normal and CMS-exposed mice (data not shown).

The alteration in the intracellular pathway after Con A stimulation observed in T cells from CMS-exposed mice could be due to redistribution of lymphocyte populations. In a previous report, we described that CMS exposure was not able to induce significant variations in CD4+ and CD8+ cells in lymphoid compartments [11]. To confirm these data, we determined the percentage of total T cell (CD3+), T helper/inducer (CD4+) and T cytotoxic/suppressor (CD8+) subpopulations in the T cell-enriched fractions used in the present work. Since no alterations in lymphocyte subsets were observed, changes in intracellular signaling induced by chronic stress would not be related to this issue.

In addition, the combination of ionomycin plus PMA bypasses the need for surface receptor interaction in T cells and directly stimulates PKC and Ca^{2+} signaling pathways, which are necessary for cellular proliferation. Our results indicate that the simultaneous treatment with PKC activator and Ca^{2+} ionophore was able to induce the same proliferative response in lymphocytes from both normal and CMS-exposed mice. Thus, pathways involved in cell proliferation downstream of PKC would not be affected by chronic stress. These findings suggest that the surface receptor interaction with its ligand or the subsequent coupling to the early signaling events could be affected by CMS exposure. A FITC-Con A binding assay showed that T cells from CMS animals had no alteration in receptor number or in ligand affinity, allowing us to conclude that the cell surface receptor interaction would not be involved in the impairment of the signaling pathway of CMS lymphocytes after Con A stimulation. Moreover, the involvement of signals from other cell surface receptors, particularly the co-stimulatory receptor CD28, that co-operate with TCR/CD3 complex signaling to produce full T cell activation, should be considered. These multiple events that may be participating in the stress-induced alteration of the required signals for T cell proliferation are under study.

From all these results, on can reasonably conclude that stress induces a decrease in [Ca^{2+}] rise after Con A stimulation that in turn induces a decrease in PKC α and θ translocation and consequently a lower NF-κB activation. Moreover, the lower PKC activation could be influencing the inhibitory cAMP-PKA pathway. This alteration in the

intracellular signals triggered by Con A stimulation could mediate the stress-induced suppression of the lymphocyte proliferative response. However, signals downstream of PKC would not be affected by stress. Identification of the molecular events regulating T cell activation is paramount to understanding the regulation of the immune response. In summary, our results show for the first time that chronic stress induces an alteration in T cell early transduction signals that results in an impairment of the proliferative response.

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