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Volvariella volvacea Lectin Activates Mouse T Lymphocytes by a Calcium Dependent Pathway

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Abstract The immunomodulatory lectin, *Volvariella volvacea* lectin (VVL), isolated from the edible mushroom, *Volvariella volvacea*, has been shown to stimulate the expression of Th1 cytokines and the proliferative activity of mouse splenocytes (She et al. [1998]: Biochem Biophys Res Comm 247:106–111). In order to elucidate the mechanisms underlying these activities, we conducted a kinetic analysis of the early and late activation markers in mouse T lymphocytes: (1) flow cytometric analysis of calcium influx, (2) induction of activation molecules (CD25 and CD69), (3) expression and DNA-binding activity of the nuclear factor of activated T cells (NFAT), NFκB, and activation protein-1 (AP-1), (4) translational production of cytokines (interleukin-2 (IL-2) and interferon-γ (IFNγ)), and (5) cell proliferation by expression of proliferating cell nuclear antigen (PCNA) and tritiated thymidine incorporation. All results showed that VVL induced a rapid expression of CD69, CD25, NFAT, IL-2, and PCNA in a dose- and time-dependent manner, leading to lymphocyte proliferation. These effects brought about by VVL were more potent than those stimulated by equimolar concentrations of mitogenic lectin, concanavalin A (Con A). Cell activation and proliferation were mediated through a calcium-dependent pathway as demonstrated by a VVL-induced increase of intracellular calcium influx, and a proliferation inhibition by the Ca-dependent phosphatase calcineurin blocker—cyclosporin A (CsA). Taken all data together, VVL is a lectin which activates lymphocyte through successive calcium influx, nuclear localization of NFAT transcription factor, induction of activation markers, CD25 and CD69, intracellular cytokine production, and cell proliferation. J. Cell. Biochem. 92: 1193–1202, 2004.

Key words: *Volvariella volvacea* lectin; lymphocyte activation; calcium influx; CD25, CD69, and IL-2; NFAT transcription factor; flow cytometry

T lymphocytes play an important role in the immune system and can be activated through the binding of antigens to T cell receptors (TCR) or stimulated with mitogens [Pahlavani et al., 1998]. The binding of mitogens to cell surface glycoproteins and/or other molecules triggers multiple intracellular signal pathways, including an elevation of intracellular calcium [Ca^{++}], activation of Ras/mitogen-activated protein kinases (MAPK), up-regulation of down-stream activation markers (CD25 and CD69), production of lymphocyte proliferation factor (interleukin-2 (IL-2)), ultimately resulting in the proliferation of lymphocytes. Since this activation pathway depends on the concentration of intracellular calcium, activation of calcineurin, dephosphorylation, and nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT), and is inhibited by cyclosporin A (CsA) [Patel and Gelfand, 1996; Wu et al., 1999; Mascarell et al., 2000], it is called the calcium–calcineurin–NFAT pathway. A number of lectins, e.g., concanavalin A (Con A) and phytohemagglutinin [Lafont et al., 1998], activates lymphocytes through this pathway.

Abbreviations used: Con A, concanavalin A; CsA, cyclosporin A; VVL, *Volvariella volvacea* lectin; NFAT, nuclear factor of activated T cell; AP-1, activation protein-1; IL-2, interleukin-2; IFNγ, interferon-γ; PCNA, proliferating cell nuclear antigen.

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Lectins form a group of special proteins which agglutinate cells through sugar-specific binding sites and precipitate glycoconjugates [Goldstein et al., 1980]. Some lectins have the ability to stimulate proliferation of lymphocytes through the Ras/MEK1/ERK2-Fos pathway, such as jacalin [Lafont et al., 1998]; or MAPK/JNK pathway, such as Con A [Lafont et al., 1998; Pahlavani et al., 1998], while other lectins do not possess mitogenic activity, such as *Agaricus bisporus* lectin (ABL) [Ho et al., 2004]. Our previous study has demonstrated that the mitogenic activity of *Volvariella volvacea* lectin (VVL), a lectin isolated from an edible Chinese mushroom, was 10 times more potent than that of the Con A [She et al., 1998; Ho et al., 2004]. In order to elucidate the molecular mechanisms underlying the mitogenic activity of VVL, we have taken a series of studies to examine the mobilization of cellular calcium, the expression of activation markers, CD25 and CD69, expression and DNA-binding activity of NFAT, NFκB, and activation protein-1 (AP-1), and the production of IL-2 and interferon-γ (IFNγ) in both mouse splenocytes and T lymphocytes. The results demonstrated that VVL is a lectin with mitogenic activity mediated through the calcium–calcineurin pathway, and can be inhibited by CsA.

MATERIALS AND METHODS

Lectins

Volvariella volvacea agglutinin (VVL) was isolated from the edible mushroom, *Volvariella volvacea*, as described previously with minor modification [She et al., 1998]. Briefly, the homogenate of fresh fruiting bodies of *V. volvacea* was subjected to a combination of an ion-exchange chromatography on DEAE-Sepharose (17-0709-01) and SOURCE 30S (17-1273-01) with an AKTAprime (Amersham Biosciences, Uppsalla, Sweden), and on Mono S (17-0541-07) with an $AKTA_{FPLC}$ (Amersham Biosciences) for the isolation and preparation a purified VVL. The molecular size was around 12 kDa as determined by gel filtration and SDS–polyacrylamide gel electrophoresis (PAGE), and its hemagglutinating activity was inhibited by thyroglobulin. Con A (C2631) was purchased from Sigma Co. (St. Louis, MO). VVL and Con A were dissolved in RPMI culture medium at appropriate concentrations for bioassays.

Cell Culture

Mouse splenocytes were obtained from male inbred BALB/c mice as described in a previous article [She et al., 1998]. T lymphocytes were enriched by removing B lymphocytes using a T cell recovery column kit (CL101, Cedarlane, Hornby, Canada) according to the protocol supplied by the manufacturer. About 80–85% CD3+ cells were demonstrated by our flow cytometric analysis, and all cells were non-B lymphocytes (i.e., CD19−). The cells were maintained in RPMI supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin and 100 IU/ml penicillin in a humidified incubator with 5% CO_2 in air at 37°C before use.

Cell Viability and Proliferation Assay

Both splenocytes and T cells were cultured at 4×10^5 cells/well/0.1 ml in 96-well culture plates (Nunc, Rockilde, Denmark) in the presence of serial concentrations of VVL and Con A in triplicates for 48 h at 37°C in a 5% CO_2 incubator. In order to study if the activation of lymphocytes by VVL is mediated by calcium–calcineurin pathway, cells were incubated with CsA (1 μg/ml, C3662 Sigma) for 30 min before cell stimulation. No cell death was demonstrated by Trypan Blue exclusion method. The proliferative activity of lymphocytes in response to lectins was analyzed by incorporation of ^{3}H-thymidine (0.5 μCi/ well, Amersham, Buckinghamshire, UK) into DNA during the last 6 h culture using a Beckman LS 6000SC liquid scintillation counter [She et al., 1998].

Calcium Influx Assay

The method used for the Ca^{++} influx assay was as described by June and Rabinovitch [1994] except for some modifications. The T lymphocytes were washed twice with HBSS buffer and once with Ca^{++}-free HBSS buffer (CFB) before resuspension in CFB at a concentration of 10^6 cells/ml. The cells were loaded with 4 μM Fluo-3 (Molecular Probes, Eugene, OR) and 0.02% of plutonic F-127 (Sigma), incubated at 37°C for 30 min in the dark. After the incubation, the cells were washed once with CFB and resuspended in assay buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, and 5 mM glucose (pH 7.4), and protected from light at 4°C prior to analysis. For each experiment, the basal fluorescence of the

Fluo-3 loaded cells was measured for 30 s before administration of lectins, and changes in relative fluorescence intensity were recorded at every 10-s interval for 300 s using an Altra EPICS flow cytometer (Beckman-Coulter, Miami, FL). All flow cytometric experiments were repeated at least three times.

Flow Cytometric Analysis of CD25 and CD69

Flow cytometric analysis of CD25 and CD69 expression was performed on both Con A- and VVL-treated T cells. Cell suspensions (5×10^6 cells/ml RPMI supplemented with 2.5% FBS) were incubated with serial concentrations of VVL or Con A at 37°C over 24 h. At different incubation intervals, an aliquot (100 μl) of cell samples was collected, centrifuged, washed once with PBS, and stained with 100 μl predilued PE-conjugated monoclonal antibodies against mouse CD25 or CD69 (553866 and 553237, BD PharMingen, San Diego, CA) in PBS in the dark at room temperature for 10 min. The staining was stopped by adding 400 μl PBS prior to flow cytometric measurement (Altra EPICS Flow Cytometer, Beckman-Coulter), and the results were analyzed using an Expo 32 software.

Cytometric Analysis of Intracellular Cytokines

The flow cytometric analysis of intracellular cytokines was performed according to procedures supplied by the manufacturer. Briefly, the cells were suspended at 1×10^6 cells/ml RPMI medium supplemented with 2.5% FBS and incubated with lectins at 37°C for 8, 16, and 24 h. Four hours after lectin treatment, 1 μl of GolgiPlug (2301KZ, PharMingen) was added to the culture (10^6 cells/ ml) to block the intracellular cytokine transport and secretion, before the cells were centrifuged at 2,000 rpm for 4 min at 4°C, fixed and permeabilized with Intra-Prep™ Permeabilization Reagent #1 (Immunotech, Marseille, France) for 15 min at room temperature. The cells were then washed with PBS, centrifuged, resuspended in Reagent #2 with gentle mixing, and incubated at room temperature for 5 more minutes. The directly conjugated antibodies for IL-2 and IFN-γ (18115A and 18714A, PharMingen) and the corresponding isotype controls were added at a concentration of 4 μg/ml and incubated at room temperature for 30 min in the dark, washed once with PBS, and resuspended in 0.5% formaldehyde/PBS. Fifty thousand cells for each cytokine at each time point were acquired using an Altra flow cytometer and analysis was performed using Expo II software (Beckman Coulter).

Two-Dimensional Gel Electrophoresis–Immunoblotting Analysis of Transcription Factor NFAT

VVL- and Con A-treated lymphocytes (5×10^6 cells) were washed with PBS twice, resuspended in lysis buffer (9 M urea, 4% CHAPS, 40 mM Tris-Cl, and 0.5 mM PMSF) and centrifuged at 10,000 rpm at 4°C for 10 min. Protein samples were separated on a pH 3–10 IPG immobiline DryStrip (17-6001-11, Amersham Biosciences) in the first dimension followed by separation on a 11% SDS–PAGE gel in the second dimension before they were transferred onto a PVDF membrane using a Semi-dry transfer cell (Bio-Rad, Hercules, CA). The blot was then rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBS/T), pH 7.6 and soaked in blocking reagent to prevent nonspecific binding before it was reacted overnight at room temperature with monoclonal antibodies against mouse nuclear factor for activated T cells (NFATc1, 68251A, PharMingen), proliferating cell nuclear antigen (PCNA, sc-56, Santa Cruz Biotechnology, Santa Cruz, CA), and actin (Pan Ab-5, MS-1295-P, Neomarker, Fremont, CA), respectively. The blot was rinsed with TBS/T, linked with HRP-conjugated antibody and detected using enhanced chemiluminescence blotting reagents (RPN2108, Amersham, USA) for 1 min and exposed to a Lumi film (1666657, Roche, Indianapolis, IN). The amount of translational NFAT and PCNA was analyzed using an AGFA scanner and Topspot software (ver. 2.0, Germany).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was used to detect specific DNA-binding of transcription factors (NFAT, NFκB, and AP-1) in lectin-treated T cells, and it was performed according to the protocol provided by the manufacture (Pierce Biotechnology, Inc., Rockford, IL). Briefly, nuclear extracts (5×10^7 cells) were prepared from T lymphocytes treated with Con A and VVL using the NE-PER Nuclear Extraction Reagent (78833 Pierce Biotechnology, Inc., USA) and determined by a Bio-Rad Protein Assay Reagent (500-00006, Bio-Rad). Nuclear protein–DNA binding reactions were carried out with 4 μg nuclear proteins

for 20 min at room temperature in a 20-μl reaction volume containing 1× binding buffer, 0.05% NP-40, 2.5% glycerol, 5 mM $MgCl_2$, 1 μg poly(dI-dC), and 50 fmol biotin-labeled target DNA. Oligonucleotides (Syngen, Inc., San Carlos, CA) used in this assay included.

IL-2 promoter site for NFAT, 5′-GGAGGAAAAACTGTTTCATACAGAAGGCGT-3′; NF-κB consensus, 5′-GATCGAGGGGACTTTCCCTAGC-3′; and IL-2 promoter site for AP-1, 5′-ATTCCAGAGAGTCATCAGAAGA-3′.

The DNA bound transcription factor complexes were separated on a 6% nondenaturing polyacrylamide gel, transferred to a nylon membrane (1417240, Roche, USA) and the biotin-labeled DNA were detected and analyzed with a LightShift™ Chemiluminescent EMSA Kit (20148, Pierce Biotechnology, Inc., USA). For competition assay, 200-fold molar excess of non-labeled DNA was added as competitor and coincubated with the biotin-labeled probes during the assay (Cp). Cells treated with a combination of 1 μM ionomycin and 50 ng/ml PMA (I + P) for 4 h was used as a positive control.

RESULTS

Cell Viability and Proliferation Assay

Con A is an extensively studied mitogenic plant lectin which activates lymphocytes through the calcium–calcineurin pathway [Liu et al., 1991; Clipstone and Crabtree, 1992; Wu et al., 1999]. Treatment of splenocytes (data not shown) and T cells with Con A in a dose-dependent manner stimulated proliferation which peaked at 120 nM (=12.714 μg/ml) after 48 h (Fig. 1). On the other hand, T cells started to proliferate after treatment for 48 h with 2 nM VVL, reaching a peak at 8 nM (=0.102 μg/ml), and then declined to the basal level at 50 nM. This activation was inhibited by a pre-incubation of 1 μg/ml CsA for 2 h prior to VVL stimulation (Fig. 1), indicating that the proliferative activity of VVL is likely mediated by the calcium signal pathway. In order to elucidate the involvement of calcium mobilization in VVL-mediated lymphocyte proliferation, T cells were loaded with fluorochrome Fluo-3 for flow cytometric analysis of calcium influx.

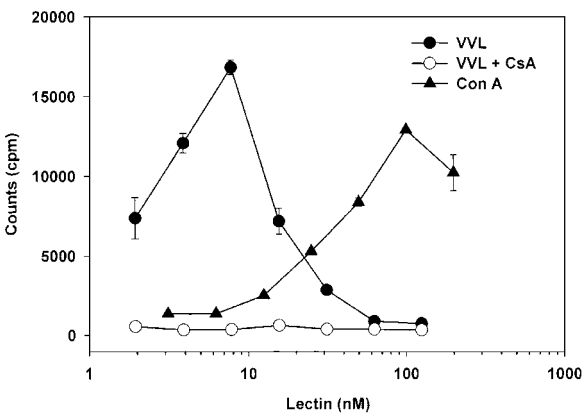

Fig. 1. Tritiated thymidine uptake by mouse T lymphocytes after treatment with serial concentrations of *Volvariella volvacea* lectin (VVL) and concanavalin A (Con A) for 48 h (n = 3). Lymphocyte growth was completely inhibited by pre-incubation with 1 μg/ml cyclosporin A (CsA) for 2 h.

Calcium Influx Assay

The increase in intracellular calcium plays an important role in the activation of T lymphocytes and production of IL-2. Figure 2 shows the activation of VVL on Ca^{++} influx in T cells. The intracellular Ca^{++} ions in normal T cells were maintained at a very low level but increased gradually in a dose- and time-dependent manner in the presence of 100 and 200 nM Con A. In contrast, a sustained rise of Ca^{++} was observed at 60 s and lasted for at least 5 min during the whole tested period when the cells were exposed to 8 nM VVL, with a 2-fold increase in intensity at a concentration of 16 nM VVL. The results demonstrated that VVL stimulated Ca^{++} influx in T cells, which was stronger and faster than that induced by Con A. The calcium influx was completely inhibited by 50 μM nifedipine or partially inhibited by 100 μM verapamil (Fig. 2b), both of them are L-type calcium channel blockers.

Cytometric Analysis of Intracellular Cytokines

Binding of antigens or lectins, such as Con A or antibodies against TCR on T lymphocytes initiates multiple signaling pathways and triggers expression of activation markers (CD25 and CD69) and lymphocyte growth factor IL-2, both of which are involved in lymphocyte activation and proliferation. The expression of CD69 remained undetectable after treatment with 200 nM Con A for 2 h, but the expression increased in a dose- and time-dependent manner from 4 to 16 h, and reached a constant level thereafter (Fig. 3). In contrast to the expression of Con A, a significant expression of CD69 (about 10%) was detected in lymphocytes exposed to a concentration of VVL as low as 2 nM VVL for 2 h and reached 50% at 16 nM VVL which was equivalent to the activity of 100–200 Con A at

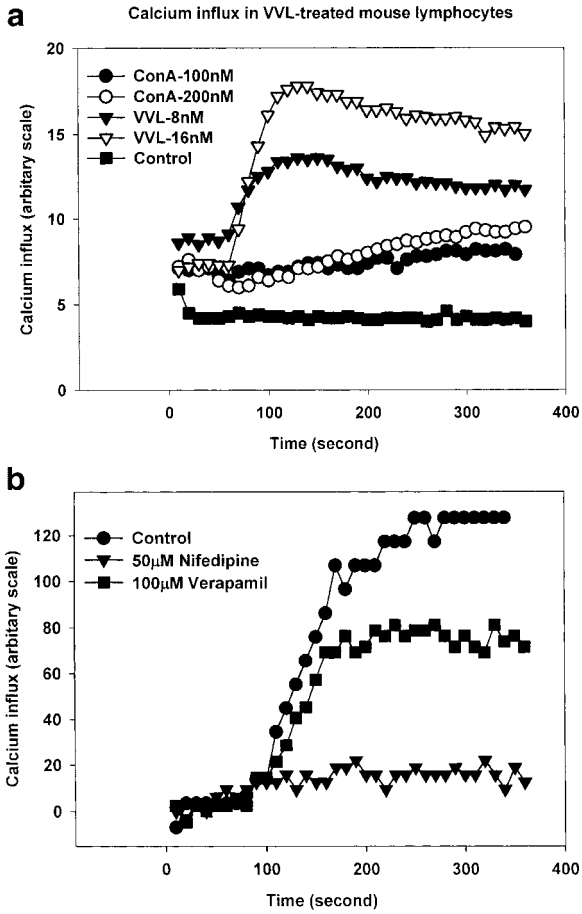

Fig. 2. Effect of lectins on calcium influx in T cells. T cells were loaded with Fluo 3 and stimulated with 100 and 200 nM Con A, or 8 and 16 nM VVL, respectively. A dose-dependent increase of calcium influx was measured by flow cytometry (**a**). The calcium signal was completely inhibited by 50 nM nifedipine but only partially inhibited by 100 nM verapamil (**b**). No intracellular calcium change was measured in lymphocytes in the absence of lectin stimulation (control).

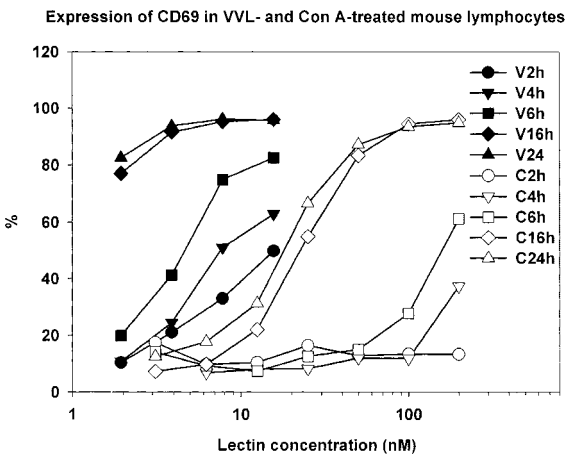

Fig. 3. Flow cytometric analysis of CD69 expression in T cells stimulated with VVL and Con A, respectively.

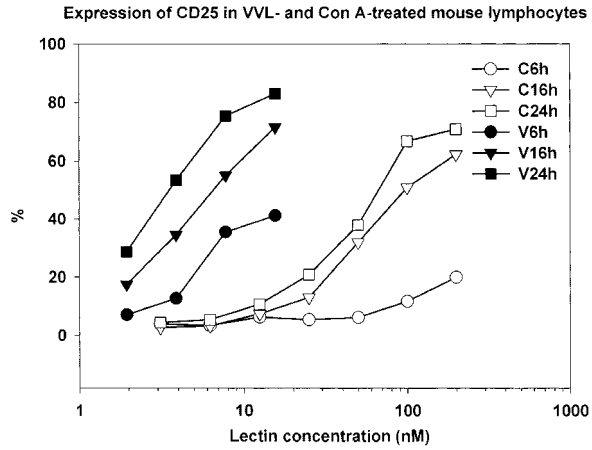

Fig. 4. Flow cytometric analysis of CD25 expression in T cells stimulated with VVL and Con A, respectively.

6 h. The results demonstrated that lower doses of VVL, even at an order of magnitude, intensified the expression of CD69 in lymphocytes.

A similar pattern was also observed in the expression of CD25 in T lymphocytes (Fig. 4). A slow and gradual increase of CD25 (from 0 to 15%) was measured in T lymphocytes after exposure of Con A for 6 h and it rose to 70% after 24 h (Fig. 4). A similar level of expression was, however, observed in lymphocytes exposed to 4–16 nM VVL for 16 h. The CD25 fluorescence increased to 80% at 24 h.

In agreement with proliferation assay and expression of activation markers, both Con A and VVL up-regulated the production of IL-2 and IFNγ proteins in lymphocytes in a dose- and time-dependent manner, but VVL was more potent than Con A (Figs. 5 and 6).

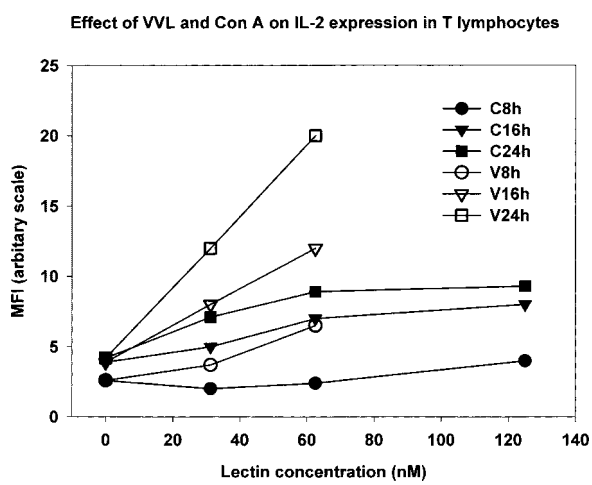

Fig. 5. Flow cytometric analysis of intracellular expression of interleukin-2 (IL-2) in T cells stimulated with VVL and Con A, respectively.

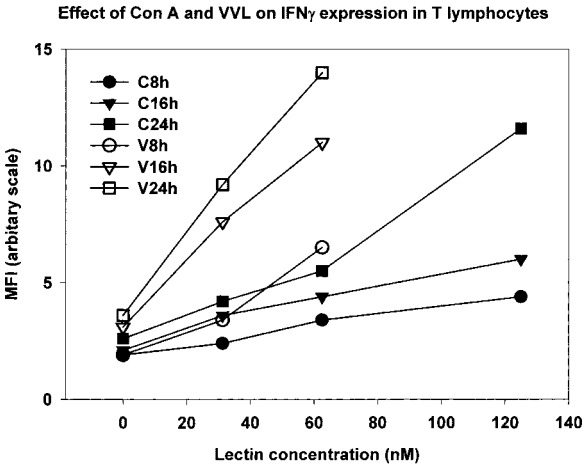

Fig. 6. Flow cytometric analysis of intracellular expression of interferon-γ (IFNγ) in T cells stimulated with VVL and Con A, respectively.

Two-Dimensional Gel Electrophoresis and Immunoblotting Analysis

Figure 7a shows NFAT and PCNA are undetectable in the untreated T lymphocytes, but they were expressed constitutively after treatment with Con A and VVL for 1 h. The two-dimensional gel electrophoresis–immunoblotting analysis showed 2.5- and 7-fold higher expression of NFAT and PCNA, respectively, in T cells after treatment with 8 nM VVL for 1 h than those with 120 nM Con A (Fig. 7b).

Binding of Transcription Factors to DNA

To further investigate the pathway by which VVL induces activation of T cells, we performed EMSA to study the binding of oligonucleotides for transcription factors to the promoter of IL-2 gene in nuclear extracts prepared from either untreated T cells or cells treated with VVL and Con A. As shown in Figure 8, both Con A and VVL promoted the formation of transcription factor–DNA binding complexes in T cells. A sustained elevation of NFAT, NFκB, and AP-1 DNA binding activities was observed in VVL-treated group as early as 30 min and lasted at least for 2 h. The binding for NFAT and AP-1 was only observed after treatment of Con A for 1 h but returned to the basal level at 2 h, while only binding for NFκB remained elevated for 2 h. Strong binding signal was observed in cells stimulated with a combination of 1 ng/ml ionomycin and 10 ng/ml PMA (I + P, the positive control), while the competition assay with 200-fold molar excess of unlabeled probes demonstrated a specific DNA-binding for individual transcription factor (Cp).

DISCUSSION

Lymphocytes play an important role in cell defense. These cells can be activated by binding to antigens or mitogens on their surface, triggering a series of signal cascade, activation of transcription factors and cytokine genes, and proliferation of lymphocytes. Con A and phytohemagglutinin A (PHA) are best examples of mitogenic lectins [Hutchinson et al., 1999], but not all lectins are mitogenic; as some of them, including *Solanum tuberosum* lectin (STL), ABL, and tomato lectin (LEL), are non-mitogenic or even anti-mitogenic lectins [Kilpatrick et al., 1986; Kilpatrick and McCurrach, 1987; Kilpatrick, 1999; Ho et al., 2004]. STL and LEL have a specificity for *N*-acetylglucosamine oligomers [McCurrach and Kilpatrick, 1988], WGA for *N*-acetylglucosamine and *N*-acetylneuraminic acid, and ABL for *N*-acetylgalactosamine, while mitogenic lectins, Con A and PHA, have affinities for α-D-mannose and α-D-glucose, and oligosaccharides, respectively. The specificity for particular saccharide structures on the lymphocyte surface is thus unlikely the exclusive factor for lectin mitogenesis. However, the binding of different saccharides on certain glycoproteins may initiate multiple signal transduction cascades, including activation of phosphorylation kinases, increase of ras activity [Zhong et al., 2002] and/or a rise of cytoplasmic calcium [Mascarell et al., 2000], nuclear translocation of the transcription factors, e.g., NFκB, AP-1, and NFAT, for the formation of a transcription complex which results in activation of cytokine genes and cell surface receptor genes [Masuda et al., 1998; Serfling et al., 2000; Ma et al., 2002], and subsequently lymphocyte proliferation [Kilpatrick, 1999]. Elevation of intracellular calcium is one of the crucial mediators of this process because the duration and amplitude of calcium signals determine the efficiency and specificity of gene expression and cellular events. A sustained calcium rise for 1–2 h is necessary for the transcriptional regulation of IL-2 gene by an active nuclear NFAT in proliferating lymphocytes [Lewis, 2001], while lower levels of calcium rise suppress T cell activities in aged mice [Miller, 1996] or mice bearing tumor [Mizoguchi et al., 1992]. NFAT is present in

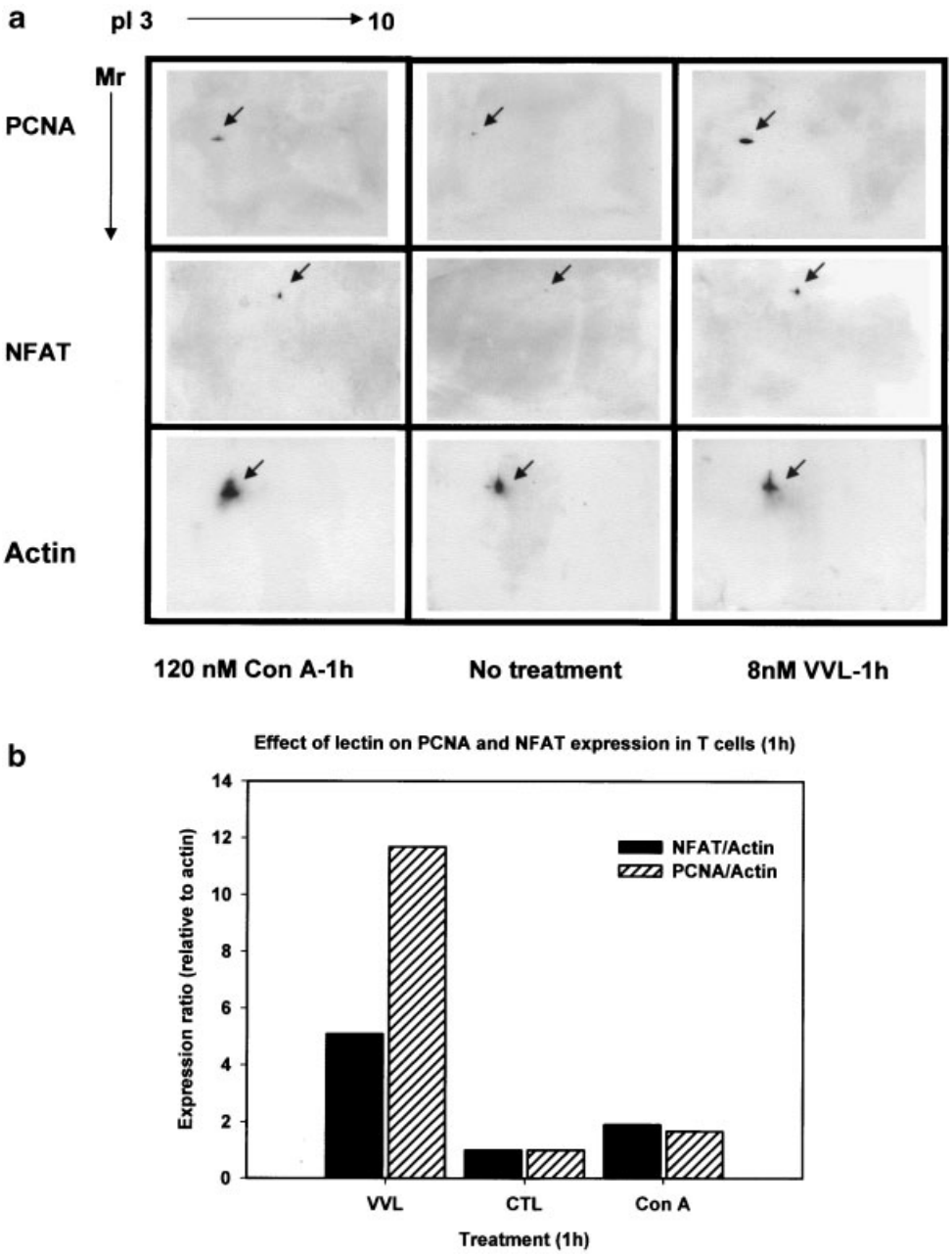

Fig. 7. A combination of two-dimensional gel electrophoresis and immunoblotting analysis of proliferating cell nuclear antigen (PCNA), nuclear factor of activated T cells (NFAT), and actin (all indicated by arrows) in total cell lysate of T cells treated with 120 nM Con A and 8 nM VVL for 1 h (**a**). Cells without treatment were used as control. The expression of PCNA and NFAT relative to actin is shown in **b**.

the cytoplasm in an inactive, highly phosphorylated state, but becomes activated when dephosphoylated by the calcium-dependent phosphatase calcineurin before translocates to the nucleus; a process that can be blocked by CsA [Patel and Gelfand, 1996; Sareneva et al., 1998; Baksh and Burakoff, 2000; Huang et al., 2000; Mascarell et al., 2000]. The present study confirmed these signaling cascades and further demonstrated that Con A induced a dose- and time-dependent increases of intracellular calcium within 5 min, the level at which both CD69 and CD25 were undetectable until 6 h when both began to increase in a parabolic pattern, reaching peak levels at about 100 nM at 16 and 24 h, respectively (Figs. 1–4). In contrast, there

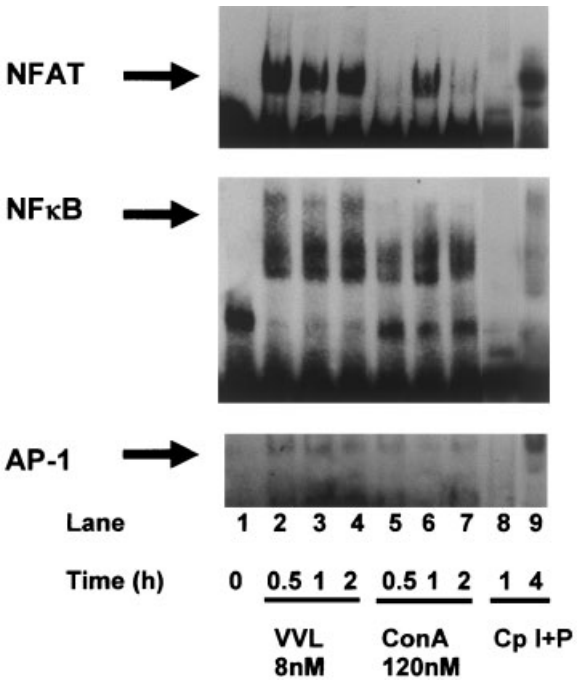

Fig. 8. Electrophoretic mobility shift assay (EMSA) demonstrates the binding of nuclear proteins from cells treated with 8 nM VVL (**lanes 2–4**) and 120 nM Con A (**lanes 5–7**) to biotin-labeled IL-2 specific NFAT, and NFκB and activation protein-1 (AP-1) oligonucleotides for 0.5, 1, and 2 h, respectively. Arrows denote transcription factor-specific DNA-binding complexes. Competition with 200-fold molar excess of unlabeled probes demonstrates the specificity of the bands (Cp, **lane 8**), and stimulation with a combination of 1 ng/ml ionomycin and 10 ng/ml PMA (I + P) for 4 h was used as a positive control (**lane 9**). No DNA-binding was observed in untreated cells (0 h) (**lane 1**).

was a dramatic increase of calcium influx within a minute (Fig. 2a), a 20% increase of CD69 that was already measured in T cells treated with 8 nM VVL for only 2 h (Fig. 3), and up to 80% at 6 and 16 h for CD69 and CD25, respectively, indicating that VVL possessed a more potent stimulatory activity towards T cells. In addition, the VVL-mediated calcium influx was blocked by nifedipine and verapamil (Fig. 2b), which are inhibitors for L-type calcium channel and prevent calcium influx, NFAT transcription, IL-2 production and cell proliferation in response to PHA and Con A [Birx et al., 1984; Kotturi et al., 2003]. L-type calcium channels present in the plasma membrane are crucial to TCR-mediated activation and proliferation. This activity can be inhibited by 10–200 μM nifedipine, a well known voltage-dependent inhibitor for L-type excitable cells [Kotturi et al., 2003]. Treatment of T cells with VVL in calcium-free culture medium abolished calcium influx, expression of CD25 and CD69 and cell proliferation (data not shown), further implicating that VVL mediates calcium-dependent mitogenesis in T cells.

IL-2 is the major cytokine controlling the expression of IL-2Rα and proliferation of T cells [Modiano et al., 1999; Rusterholz et al., 1999]. It can be stimulated by ligands of TCR, such as anti-CD3 [Sareneva et al., 1998] or lectin [Kilpatrick and McCurrach, 1987], resulting in proliferation of lymphocytes. Our previous studies have demonstrated that VVL is 10 times more potent than Con A in inducing IL-2 transcription in T cells [She et al., 1998; Ho et al., 2004]. IL-2 transcription can be mediated by either calcium-dependent or -independent pathways, indicating that more than one signal pathways control the expression of IL-2 gene [Lafont et al., 1998]. Several transcription factor binding sites, including NFAT, NFκB, and AP-1, are found in the promoter of IL-2 gene, and their cooperative interactions are required for the production of IL-2 in T cells [Rothenberg and Ward, 1996; Isakov and Altman, 2002]. Treatment with VVL resulted in constitutive elevation of DNA-binding activities to all three transcription factors for at least 2 h in comparison to the only 1 h time period using Con A (Fig. 8). A longer and stronger binding of NFAT, AP-1, and NFκB to regulatory regions of the IL-2 promoter enhances IL-2 transcription, which subsequently promotes PCNA expression and lymphocyte proliferation [Huang and Prystowsky, 1996]. PCNA is a cofactor for DNA polymerase δ, and is thus expressed constitutively in the nuclear matrix of proliferating cells [Gerner et al., 1998]. The 11.68- and 1.67-fold increases of PCNA in VVL- and Con A-treated groups, respectively, in comparison to untreated control indicate that T cells are more sensitive to VVL-mediated cell proliferation. Currently experiments are being conducted in our laboratories to elucidate the involvement of other transcription factors, including Ets and its family members [Li et al., 2000; Koskela and Lassila, 2002], in T cell activation by VVL. In comparison with Con A, VVL enhanced the influx of Ca^{++} and induced an earlier expression of the activation antigens, CD69 and CD25, as well as cytokines, IL-2 and IFNγ. All these events are of higher magnitude and are likely to contribute to a more potent VVL-mediated mitogenesis.

Since our initial study on the proliferative activity of VVL on mouse splenocytes [She et al., 1998] with mainly composed of both T- and B-lymphocytes, macrophages (i.e., antigen-presenting cells) and other cells (e.g., neutrophils), we have obtained preparations enriched in T- and B-lymphocytes by using negative selection columns, and compared their proliferative response with mouse splenocytes to VVL. We observe no proliferation in B lymphocytes with VVL stimulation, while that in enriched T cell preparation shows a slightly higher proliferation than splenocytes (data not shown). The T cells used in this study were not a pure population. Only 85% are T lymphocytes as claimed by the manufacturer, and about 80–85% CD3+ were demonstrated by our flow cytometric analysis, but all cells were non-B lymphocytes (i.e., CD19−) (data not shown), indicating that VVL is only mitogenic to T cells. The present results cannot exclude the involvement of CD28-B7 costimulation as required in the mitogenesis of Con A [Perrin et al., 2000]. Further studies are needed to elucidate the contribution of antigen-presentation and provide more detailed signal pathways mediated by VVL.

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