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Lymphocyte Activation and Capping of Hormone Receptors

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In this study both a ligand-dependent treatment [concanavalin A (Con A)] and a ligand-independent treatment [high-voltage pulsed galvanic stimulation (HVPGS)] have been used to initiate lymphocyte activation via a transmembrane signaling process. Our results show that both treatments cause the exposure of two different hormone [insulin and interleukin-2 (IL-2)] receptors within the first 5 min of stimulation. When either insulin or IL-2 is present in the culture medium, the stimulated lymphocytes undergo the following responses: (1) increased free intracellular Ca^{2+} activity; (2) aggregation of insulin or IL-2 receptors into patch/cap structures; (3) tyrosine-kinase-specific phosphorylation of a 32-kd membrane protein; and finally (4) induction of DNA synthesis.

Further analysis indicates that hormone receptor capping is inhibited by (1) cytochalasin D, suggesting the involvement of microfilaments; (2) sodium azide, indicating a requirement for ATP production; and (3) W-5, W-7, and W-12 drugs, implying a need for Ca^{2+}/calmodulin activity. Treatment with these metabolic or cytoskeletal inhibitors also prevents both the tyrosine-kinase-specific protein phosphorylation and DNA synthesis which normally follow hormone receptor capping. Double immunofluorescence staining shows that actomyosin, Ca^{2+}/calmodulin, and myosin light-chain kinase are all closely associated with the insulin and IL-2 receptor cap structures.

These findings strongly suggest that an actomyosin-mediated contractile system (regulated by Ca^{2+}, calmodulin, and myosin light-chain kinase in an energy-dependent manner) is required not only for the collection of insulin and IL-2 receptors into patch and cap structures but also for the subsequent activation of tyrosine kinase and the initiation of DNA synthesis. We, therefore, propose that the exposure and subsequent patching/capping of at least one hormone receptor are required for the activation of mouse splenic T-lymphocytes.

Key words: hormone-receptor interaction, hormone receptor exposure, receptor patching/capping, contractile proteins

Lymphocytes are the primary cellular repositories for immune responsiveness. Specific binding between a surface receptor and ligand (e.g., antigen, antibody,

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hormone, or lectin) is absolutely required for lymphocyte activation and replication. Under the appropriate conditions, interactions between lymphocyte surface receptors and various ligands initiate a signal which travels from the plasma membrane to the cell nucleus and subsequently induces (1) proliferation and differentiation of the B-cells into antibody-secreting plasma cells [1–3]; (2) the development of an increased number of precursor cells which are responsible for immunological memory [2,4,5]; and (3) activation and proliferation of T-cells [6–8].

One possible mechanism for signal transduction at the cell surface involves ligand-induced surface receptor clustering and aggregation (so-called patching and capping, respectively). During the last 10 yr several laboratories, including our own, have determined that lymphocyte surface receptors are physically linked to actomyosin-associated cytoskeletal proteins [9–14] and that their redistribution is driven by a musclelike contraction of actomyosin filaments [15–18].

Surface receptor patching and capping appear to be important physiologically in a variety of different cell types. For example, histamine release by mast cells is known to require the clustering of IgE Fc receptors on the cell surface [19–23], and lymphocytes are stimulated to differentiate and proliferate by the binding of mitogens such as antiimmunoglobulin (anti-Ig) or lectins followed by aggregation of their receptors [24–28].

Electric stimulation of various types has been used clinically to promote healing of bone fractures and soft tissue injuries [29–35]. Although the mechanisms by which electric currents or fields promote healing are not known, activation of inflammatory cells such as lymphocytes is one possibility. Recently, we have determined that high-voltage pulsed galvanic stimulation (HVPGS), a common type of clinical electric stimulation, can cause the immediate aggregation of fibroblast membrane receptors into cap structures followed by increases in the rate of fibroblast protein and DNA synthesis [36,37].

To date, there has been no direct evidence to support the hypothesis that surface receptor capping is required for cell activation. In this study on mouse splenic T-lymphocytes, we have found that both Con A binding [a ligand-dependent process] and electric stimulation [a ligand-independent process] will induce the exposure of insulin and IL-2 surface receptors. If either one of these two hormones is available for binding, the lymphocytes will then carry out hormone receptor patching/capping followed by cellular activation (e.g., tyrosine-specific protein phosphorylation and DNA synthesis). However, if one blocks hormone receptor capping with metabolic or cytoskeletal inhibitors, there is a concomitant reduction in the amount of protein phosphorylation and DNA synthesis. We, therefore, propose that the exposure and subsequent patching/capping of at least one hormone receptor are required for the onset of T-lymphocyte activation.

MATERIALS AND METHODS

Cells

Mouse splenic T-lymphocytes were obtained from Balb C/J mice (Jackson laboratory, Bar Harbor, ME) and were prepared by passing the spleen cells over a nylon wool column as described previously [38].

Concanavalin A (Con A)-Stimulated Mitogenesis

Mouse splenic T-lymphocytes (5×10^5 cells) were dispensed into the wells of microtiter plates in a total volume of 0.2 ml of RPMI 1640 medium containing Con A

(Sigma Biochemical Co., St. Louis, MO) at final concentrations of 0.1–15 μg/ml. Following incubation at room temperature for up to 30 min, the Con A–treated cells were supplemented with either insulin (5×10^{-11}–5×10^{-8} M) or IL-2 (0.5–500 units) and incubated at 37° in 5% CO_2/95% air for various time intervals. Control samples were supplemented with RPMI 1640 medium only and incubated under the same conditions.

High-Voltage Pulsed Galvanic Stimulation (HVPGS)

The high-voltage pulsed galvanio stimulator used in this study was the EGS model 100-2 (Electro-Med Health Industries, Miami, FL). The stimulator delivers monophasic pulses of current (consisting of twin peaks with a total pulse duration of 0.1 msec) at intensities up to 500 V and frequencies between 1 and 120 pulses/sec (Fig. 1).

Mouse splenic T-lymphocytes (1×10^7 cells in 0.5 ml of RPMI 1640 medium) were placed in dialysis tubing which was located in the center of a rectangular plastic chamber filled with 20 ml of RPMI 1640 medium. Stainless-steel plate electrodes were located at each end of the chamber to deliver a uniform current through the medium [36,37]. Various voltages (ranging from 50 to 200 v) at a pulse rate of 100 pulses/sec were applied to the filled chamber for up to 30 min at room temperature. Control samples were incubated for the same amount of time in the chamber filled with either fresh or "conditioned" RPMI 1640 medium and with 0 V applied. "Conditioned" medium was prepared by treating the RPMI 1640 medium alone (i.e. no cells present) with HVPGS for 30 min at room temperature. No change in the pH of the RPMI 1640 medium (as determined by the pH color indicator) or in cell viability (as determined by trypan blue staining) was observed during any of the electric stimulation experiments.

The HVPGS-treated cells were then incubated in RPMI 1640 medium supplemented with either insulin (5×10^{-11}–5×10^{-8} M) (Eli Lilly & Co, Indianapolis, IN) or IL-2 (0.5–500 units) (Genzyme, Boston, MA) at 37° in 5% CO_2/95% air for various time intervals. Control samples were supplemented with RPMI 1640 medium only and incubated under the same conditions.

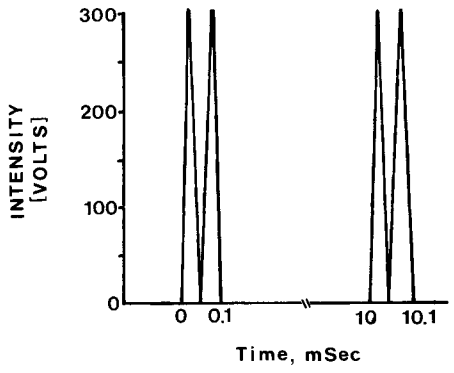

Fig. 1. High-voltage pulsed galvanic stimulation (HVPGS) waveform. (Settings: 300 V; 100 pulses/sec).

Insulin or IL-2 Induced Receptor Capping

Mouse splenic T-lymphocytes were treated with either Con A (6 μg/ml) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature in order to achieve the maximal exposure of their hormone receptors. Following binding of either insulin (5×10^{-11}–5×10^{-8} M) or IL-2 (0.5–500 units) to their respective receptors in a buffer containing 100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 1 mM EDTA, 10 mM glucose, and 1.5 mM sodium acetate (pH 7.8) [39] at 15° for 15–30 min, the cells were washed three times with the same buffer and fixed by incubation in 0.5% paraformaldehyde (in Dulbecco's phosphate-buffered saline, DPBS) for 20 min at 0°. The controls received RPMI 1640 medium only followed by fixation in 0.5% paraformaldehyde. All fixed cells were washed in DPBS plus 0.1 M glycine followed by incubation with fluorescein-conjugated human antiinsulin receptor antibodies (an affinity-purified fraction; a gift from Dr. Guenther Baden, University of Pennsylvania, Philadelphia, PA) or fluorescein-conjugated rabbit anti-IL-2 receptor antibodies (an affinity-purified fraction; obtained from Dr. Thomas Malek, University of Miami, Miami, FL). To verify the specificity of immunolabeling, some samples received fluorescein-conjugated preimmune serum or preabsorbed serum (antiinsulin or IL-2 receptor-free serum). The antiinsulin or IL-2 receptor-free serum was prepared by (a) incubating antiinsulin receptor antibody with insulin-receptor-containing IM-9 B-lymphoblasts to remove specific antiinsulin receptor antibody from the serum; or (b) incubating anti-IL-2 receptor antibody with IL-2 receptor containing CTLL T-lymphocytes to remove specific anti-IL-2 antibody from the serum. After IM-9 and CTLL cells were centrifuged down, the supernatant containing nonspecific serum (so-called preabsorbed serum or antiinsulin or anti-IL-2-free serum) was used as a control. Both preabsorbed serum or preimmune serum showed no obvious fluorescent staining; or fluorescein-conjugated guinea pig antiinsulin antibody (an affinity-purified fraction) (Miles-Yeda) or fluorescein-conjugated rabbit anti-IL-2 antibody (an affinity-purified fraction; Genzyme, Boston, MA). Controls received fluorescein-conjugated preabsorbed serum (antiinsulin antibody or anti-IL-2 antibody was incubated with an excess amount of insulin or IL-2, respectively, to remove specific antiinsulin or IL-2 antibody). This antiinsulin or anti-IL-2-free serum (preabsorbed serum) also showed no obvious fluorescent staining.

Double Immunofluorescence Staining

For simultaneous localization of intracellular proteins and surface molecules, fluorescein-labeled insulin or IL-2-capped cells were mounted on poly-l-lysine-coated slides. Cells were rendered permeable by treatment with 0.5% Triton X-100 (in DPBS-glycine for 15 min at 25°) followed by 95% ethanol for 5 min. Permeabilized cells were labeled with rhodamine-conjugated rabbit antiactin, rabbit antimyosin, sheep anticalmodulin (provided by Dr. Michael Welsh, University of Michigan, Ann Arbor, MI), or rabbit antimyosin light-chain kinase (a gift from Dr. Anthony Means, Baylor College, Houston, TX). The control samples received rhodamine-conjugated preimmune serum or preabsorbed serum (antiactin, myosin, calmodulin, or myosin light-chain kinase-free serum). They showed no detectable staining.

All fluorescent samples were examined by a Zeiss IM inverted microscope equipped with epiillumination. Fluorescein fluorescence was excited by using an Osram XBO 75-W bulb with a CZ 487710 filter, while rhodamine fluorescence was

excited by using a CZ 487714 filter. All samples were photographed using a ×63 oil-immersion lens and Kodak Tri-X film.

Hormone Receptor Binding Assays

Mouse splenic T-lymphocytes were treated with Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) at room temperature for various time intervals (t=5, 10, 20, and 30 min); or for 20 min at room temperature with various concentrations of Con A (0.5–15 μg/ml) or intensities of HVPGS (50–200 V). For measuring the exposed insulin or IL-2 receptors, these Con A– or HVPGS-treated T-lymphocytes (1×10^6 cells per assay point) were further incubated with ^{125}I-insulin (concentration ranging from 10^{-9} M to 10^{-14} M) or ^{125}I-anti-IL-2 receptor antibody (concentration ranging from 0.5 μg/ml to 5.0 μg/ml) in a buffer containing 100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 1 mM EDTA, and 10 mM glucose, 1.5 mM sodium acetate (pH 7.8) [39] at 15° for 15–30 min or 60 min followed by washing extensively at 4°C. In this binding assay, we measured the amount of both insulin and IL-2 receptors expressed after Con A or HVPGS treatment. The amount of hormone binding was the same regardless of whether the cells were incubated for 15–30 min or 60 min at 15°. As controls, (1) excess amounts of unlabeled insulin were added to compete with the binding of ^{125}I-insulin or (2) ^{125}I-anti-IL-2 receptor-free serum (preabsorbed serum) was used in order to confirm the specificity of insulin binding assay or anti-IL-2 receptor binding measurement; less than 5% of bound counts were nonspecific. The amount of ^{125}I-insulin and ^{125}I-anti-IL-2 receptor antibody binding was measured by an LKB Gamma counter.

To obtain the Scatchard plot, ^{125}I-insulin binding assays were carried out with ligand concentrations ranging from 1×10^{-9} M to 1×10^{-14} M; ^{125}I-insulin was diluted with unlabeled insulin in a fixed ratio of 1:9. Nonspecific binding was determined by performing identical assays in the presence of 1×10^{-6} M of unlabeled insulin. Specific binding was then obtained by substracting the nonspecific binding from the total binding. Data points from the Scatchard plot were extrapolated and fitted manually following the principles described previously [40,41].

Measurement of Intracellular Ca^{2+}

Mouse splenic T-lymphocytes (2×10^7 cells/ml) were first treated with either Con A (6 μg/ml) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature. These Con A– or HVPGS-treated splenic lymphocytes (2×10^7) were then loaded with 5 mM Fura-2 (Calbiochem, La Jolla, CA) by incubation for 30 min at 25° in 145 mM NaCl, 5 mM KCl, 0.1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, and 15 mM HEPES (pH 7.5). Cells were washed three times with this buffer and diluted to a final concentration of 1×10^7 cells/ml for both fluorimetric analysis and digital imaging analysis.

Fluorimetric analysis. For fluorimetric analysis, we have used a Hitachi MPF-2A fluorescence spectrophotometer equipped with a Perkin-Elmer 150 Xenon lamp and power supply; 2 ml of cells was placed in each cuvette and treated with either insulin (5×10^{-10} M) or IL-2 (1 unit) for various time intervals at 25°. Fluorescence was monitored at 500 nm with two different excitation wavelengths: 340 nm and 380 nm. Intracellular Ca^{2+} activity was determined by using the equation $[Ca^{2+}] = kd\ (R - Rmin/Rmax - R)\ (SF2/Sb2)$, with kd=135, Sf2/Sb2=constant calculated by Ca^{2+} calibration for each run, and R=ratio $F_{340\ nm}/F_{380\ nm}$ as described previously [40].

Digital imaging analysis. Fluorescence of individual Fura-2-loaded mouse splenic lymphocytes [treated with either insulin (5×10^{-10} M) or IL-2 (1 unit) for 2–3 min] was imaged by using a Zeiss IM inverted microscope equipped with a Zeiss Ultrafluor ×100 (NA=1.25) objective. Video images were obtained using a silicon-intensified target camera (Dage MTI, model 66) and an Image Technology video analyzer as described previously [41]. Images of Fura-2 500-nm fluorescence were excited by either 340- or 380-nm wavelength radiation using filters from Ditric Optics Inc. (Hudson, MA; emission filter, 500 nm, halfwidth, 20 nm; excitation filters, 340 nm, halfwidth, 10 nm; 380 nm, halfwidth, 10 nm). The ratio image ($R_{340/380}$) was obtained by division of the 340-nm image by the 380-nm image on a pixel-per-pixel basis. All images of Fura-2 fluorescence (e.g., 340-nm excitation, 500 nm emission; 380-nm excitation, 500-nm emission; and ratio 340/380) from a single splenic T-lymphocyte were photographed with a Kodak Tri-X film.

Membrane Isolation

The membrane preparation was obtained using the method described by Lang et al [39] with slight modification. Mouse splenic T-lymphocytes were first treated with Con A (6 μg/ml) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature. The Con A– or HVPGS-treated cells were incubated with either insulin (5×10^{-10} M) or IL-2 (1 unit) or RPMI 1640 medium alone at 37° in 5% CO_2/95% air for 6–12 hr. The lymphocytes were then lysed at 4°C with a Potter-Elvehjem homogenizer. The homogenization medium consisted of 405 mM sucrose in 50 mM HEPES-buffered Earle's salts (pH 7.5) with 1 mM PMSF, 4 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenate was centrifuged at $600 \times g_{av}$ for 10 min and the resulting supernatant was recentrifuged at $43,000 \times g_{av}$ for 30 min to yield a crude membrane pellet.

Protein Phosphorylation In Vitro

Mouse splenic T-lymphocytes were treated with either Con A (6 μg/ml) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with insulin (5×10^{-10} M), IL-2 (1 unit) or RPMI 1640 medium alone at 37°C for 6–12 hr. Crude membranes from unstimulated (i.e. resting) and Con A- and HVPGS-treated cells were isolated as described above. To carry out protein phosphorylation in vitro, the membranes were incubated with [γ ^{32}P]ATP (ICN) for 30 min at 24°C in a buffer containing 150 mM NaCl, 50 mM HEPES, 1% Triton X-100, 3 mM $MnCl_2$, 10 mM $MgCl_2$, 2×10^{-4} M Ca^{2+} [39]. Some samples were phosphorylated in the presence of various metabolic drugs or cytoskeletal inhibitors (cytochalasin D, 20 μg/ml; sodium azide, 50 mM; W5/W7/W12, 10 μM). Phosphorylated polypeptides were analyzed by SDS-PAGE and autoradiography as described below. Some of the gels were treated with 0.6 M KOH followed by autoradiography according to the procedures described previously [42,43]. The 32-kd phosphoprotein band was quantitated by densitometric tracing.

SDS-PAGE and Radioactivity Analysis

SDS-PAGE was conducted using an exponential polyacrylamide gradient (6.0–17%) and the discontinuous buffer system of Laemmli [46]. Samples were dissolved in buffer containing 2% SDS, 0.1 M 2-mercaptoethanol (w/v), 0.003% bromophenol blue, 20 mM Tri-HCl (pH 6.8), and 12.5% glycerol, and heated for 2 min at 100°C.

Electrophoresis was carried out overnight with a constant current of 3 mA at room temperature. The polypeptide banding pattern was determined by staining with Coomassie blue. Some gels were treated with 0.6 M KOH according to procedures described previously [42,43]. For autoradiographic analysis, all gels were vacuum-dried, exposed to Kodak X-ray (X-Omat XAR-5) film, and quantitated by densitometric tracing.

Phosphoamino Acid Analysis

The ^{32}P-labeled 32-kd protein band was recovered from the polyacrylamide gel slices by electroelution as described by Hunkapiller et al. [47] with the exception that a Hoeffer cylindrical gel apparatus was used as the elution tank. Acid hydrolysis of the eluted protein was performed at 110°C for 2 hr by the method of Cooper et al. [45], and the phosphoamino acids were separated by one-dimensional paper electrophoresis on 3-mm Whatman chromatographic paper (Whatman Inc., Clifton, NJ). Separation was achieved at pH 3.5 with 2,000 V for 2 hr. Phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine) were mixed with ^{32}P-labeled samples, coanalyzed, and identified by ninhydrin staining. Radioactively labeled phosphoamino acids were detected by exposure to Kodak X-ray (X-Omat XAR-5) film at −70°C.

Measurement of DNA Synthesis

Mouse splenic T-lymphocytes were first treated with Con A (0.1–15 μg/ml) or HVPGS (50–200 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with either insulin (5×10^{-11}–5×10^{-8} M) or IL-2 (0.5–500 units) at 37°C in 5% CO_2/95% air for 12–24 hr. Cells were then pulsed with 0.1 μCi ^{3}H-thymidine (1 mCi/ml, ICN Chemical and Radioisotope Division, Irvine, CA) for 4 hr at 37°C in 5% CO_2/95% air as described previously [36,37]. After killing the cells with ice-cold 10% trichloroacetic acid (TCA), The TCA-precipitated materials were collected on Millipore filters which were then dried and counted in a Beckman liquid scintillation counter.

RESULTS

Exposure of Hormone Receptors

Insulin and interleukin-2 (IL-2) receptors are not detected on the cell surface of unstimulated mouse splenic T-lymphocytes (Fig. 2A) [49–51]. In this study we have found that both Con A and HVPGS induce the exposure of insulin and IL-2 receptors during the first 5 min of the activation stimulus (Fig. 3). Both hormone receptors are maximally expressed after approximately 20 min of exposure to Con A or HVPGS at room temperature (Figs. 2B, 3). The relative amounts of exposed hormone receptors appear to be both Con A dose dependent (Fig. 4A) and HVPGS voltage dependent (Fig. 4B). Using ^{125}I-insulin-binding procedures and Scatchard plot analysis (Fig. 5), we have found that the newly expressed insulin receptors consist of two populations, high-affinity (kd= 2.1×10^{-2} M) and low-affinity (kd= 6.5×10^{-12} M) receptors (Fig. 5). There is a relatively small number of high-affinity binding sites (approximately 542 sites per cell), but a larger number of low-affinity sites (approximately 2,203 sites per cell). Similar high- and low-affinity IL-2 receptors were also ex-

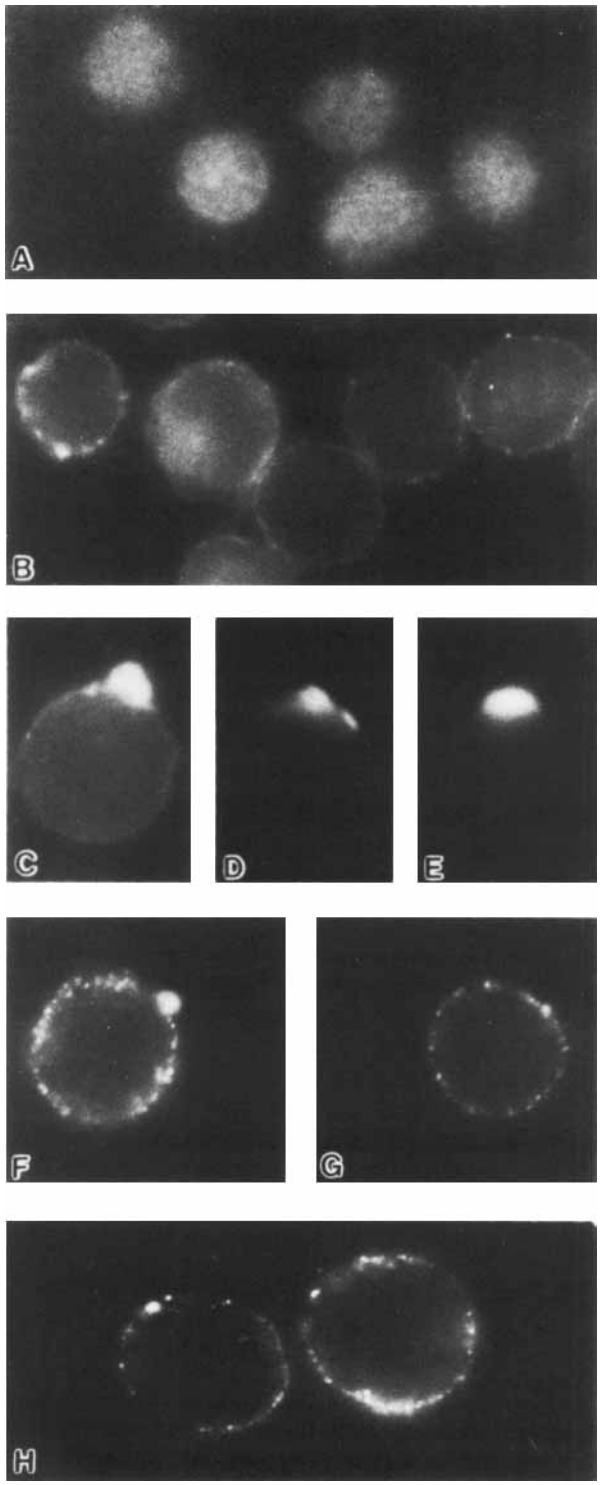

Figure 2

pressed during early onset of lymphocyte activation by both Con A and HVPGS (data not shown).

Hormone-Induced Receptor Patching and Capping

In the absence of any hormone, the insulin or IL-2 receptors on Con A– or HVPGS-treated T-lymphocytes are uniformly distributed on the cell surface (Fig. 2B). In the presence of either insulin or IL-2, splenic T-lymphocytes pretreated with either Con A or HVPGS form hormone receptor patch and cap structures (Fig. 2C–E) which reach a maximal level after 30 min of incubation at room temperature (Fig. 6).

Changes in Cytoskeletal Organization

We have used double immunofluorescence labeling to simultaneously localize intracellular cytoskeletal proteins and associated proteins together with insulin or IL-2 receptor molecules on the same cells. Our results indicate that actomyosin (Fig. 7B,D), calmodulin (Fig. 7F,H), and myosin light-chain kinase (Fig. 7J,L) are all accumulated directly beneath the insulin and IL-2 capped structures (Fig. 7A,C,E,G,I,K) in mouse splenic T-lymphocytes pretreated with either Con A or HVPGS.

In lymphocytes, cytochalasin D and sodium azide have been shown to inhibit multivalent-ligand-induced receptor capping by impairing microfilament function and ATP production, respectively [11]. W 5 along with W 7 and W 12 have been shown to inhibit anti-Ig-induced capping due to its anticalmodulin activity [52]. Recently, we have also reported that W 5 displays a more potent inhibitory effect than W 7 on insulin-induced receptor capping [18]. In order to further investigate the cellular components involved in hormone receptor patching and capping, we have tested the effect of adding these metabolic and cytoskeletal inhibitors during hormone receptor redistribution. Our results show that both insulin- and IL-2-induced capping can be inhibited by cytochalasin D (a microfilament-disrupting drug) (Fig. 2F, Table I), sodium azide (a metabolic energy poison) (Fig. 2G, Table I), and W 5, W 7, W 12 (calmodulin inhibitors) (Fig. 2H, Table I). In the presence of all these inhibitors, insulin and IL-2 receptors are found uniformly distributed in patches (small clusters) (Fig. 2F–H). Since most of the cells used in this drug-treated study remain viable (at

Fig. 2. Immunofluorescence staining of hormone receptors. **A:** Absence of insulin receptors on unstimulated (resting) mouse splenic T-lymphocytes. (Same result was observed for IL-2 receptors and unstimulated cells—data not shown). **B:** Exposure of insulin receptors on mouse splenic T-lymphocytes treated with Con A (6 μg) for 20 min at room temperature without the addition of any hormone. [Similar results were observed for HVPGS-treated cells (50 V at 100 pulses/sec) and for IL-2 receptors on Con A or HVPGS-treated cells—data not shown.] **C–E:** Hormone receptor cap structures on mouse splenic T-lymphocytes treated with Con A (6 μg) (C,D) or HVPGS (50 V at 100 pulses/second) (E) for 20 min at room temperature followed by incubation with insulin (5×10^{-10} M)or IL-2 (1 unit). C: Insulin receptor cap in cells treated with Con A followed by incubation with insulin. (Similar insulin receptor caps were observed in cells treated with HVPGS followed by incubation with insulin—data not shown.) D,E: IL-2 receptor cap in cells treated with Con A (D) or HVPGS (E) followed by incubation with IL-2. **F–H:** Effect of various inhibitors on cells treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec for 20 min at room temperature followed by incubation with either insulin or IL-2. F: Small clusters of insulin receptors formed during the treatment with cytochalasin D. (Similar small clusters of IL-2 receptors were observed during the identical cytochalasin D treatment—data not shown). G: Small clusters of IL-2 receptor formed during the treatment with sodium azide. (Similar small clusters of insulin receptors were observed during the identical sodium azide treatment—data not shown.) H: Small clusters of IL-2 receptor formed during the treatment with W 12. (Similar small clusters of insulin receptors were observed during the identical W 5 and W 7 treatment—data not shown.) (A, ×800; B, ×1,200; C–H, ×1,600)

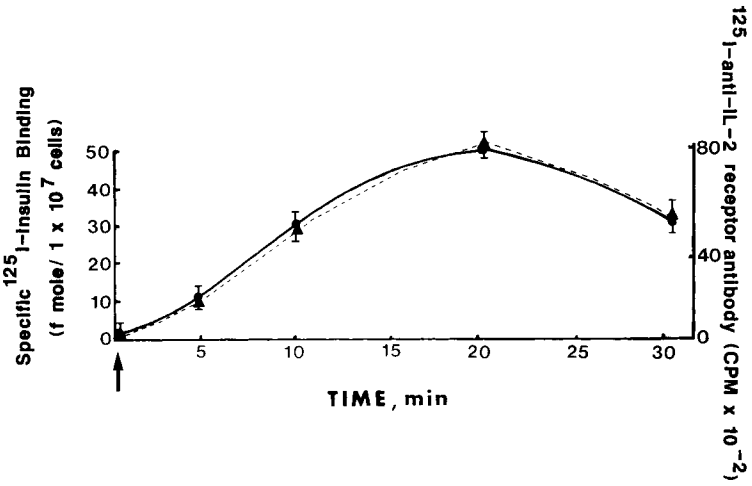

Fig. 3. Hormone receptor exposure on mouse splenic T-lymphocytes. Cells were treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) for various times at room temperature. ●—●, insulin binding sites; ▲-----▲, IL-2 receptors.

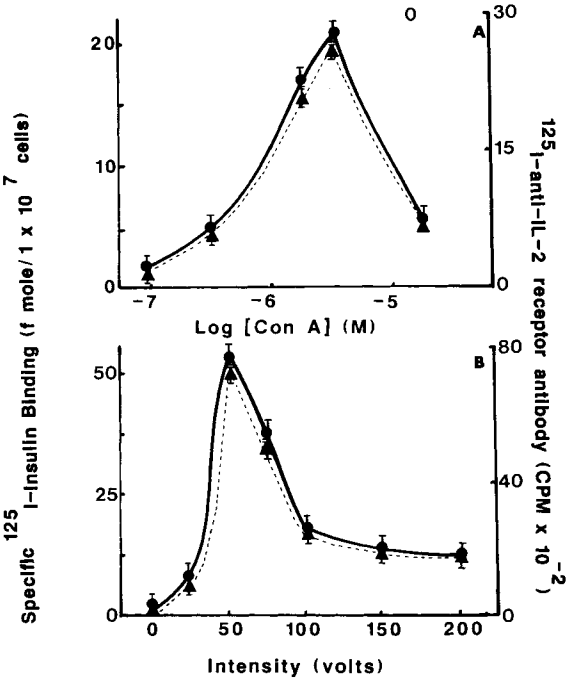

Fig. 4. Hormone receptor exposure on mouse splenic T-lymphocytes. Cells were treated with either Con A (0.1–15 μg) (**A**) or HVPGS (0–200 V at 100 pulses/sec) (**B**) for 20 min at room temperature. ●—●, insulin binding sites; ▲-----▲, IL-2 receptors.

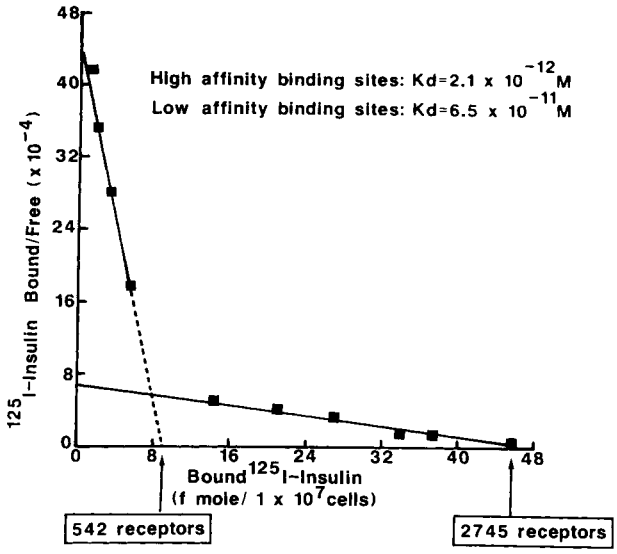

Fig. 5. Scatchard plot analysis of insulin binding to mouse splenic T-lymphocytes. The cells were first treated with HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature and incubated with ^{125}I-insulin in the presence of various concentrations of unlabeled insulin as described in Materials and Methods. Scatchard plot analysis of the data indicate that the kds of the high-affinity and low-affinity (2,203 sites per cell) insulin receptors are 2.1×10^{-12} M and 6.5×10^{-11} M, respectively. There are approximately 542 high-affinity insulin binding sites per cell and 2,203 low-affinity binding sites per cell. (Similar kds of high- and low-affinity insulin receptors were observed in Con A-treated cells; data not shown.)

least 95% viability as monitored by trypan blue exclusion) during various drug treatments, we are confident that these drug-related results are meaningful and important.

Changes in Intracellular Ca^{2+} Activity

Calcium (Ca^{2+}) ions have been implicated in the regulation of a variety of cellular activities [16,53–55]. We have used the fluorescent Ca^{2+} indicator, Fura-2, to measure the intracellular free Ca^{2+} concentration following insulin or IL-2 binding to mouse splenic lymphocytes pretreated with either Con A or electric stimulation. Our results indicate that the internal free Ca^{2+} concentration begins to increase at 2–3 min and reaches a maximum at about 15 min following the addition of either hormone (Fig. 8).

Using a digital imaging microscope (Fig. 9), the intracellular Ca^{2+} activity in individual cells was determined by comparing the intensity of emission (at 500 nm) when excitation is at 340 nm vs excitation at 380 nm (Fig. 9A,B,D,E). The image at 340 nm [the distribution pattern of total Fura-2 fluorescence indicating both intracellular Ca^{2+} concentration and dye (Fura-2) distribution] [43] shows an inhomogeneous fluorescence pattern throughout the cytoplasm (Fig. 9A,D). Division of the 340-nm image, on a pixel-by-pixel basis, by that obtained with 380-nm excitation (Fig. 9B,E) generates a 340/380 ratio image (Fig. 9C,F) which represents the free intracellular Ca^{2+} distribution. The ratio images indicate that there is a detectable accumulation of intracellular Ca^{2+} in either patched (Fig. 9C) or capped (Fig. 9F) forms underneath the lymphocyte membrane staining at about 2–3 min following addition of hormone.

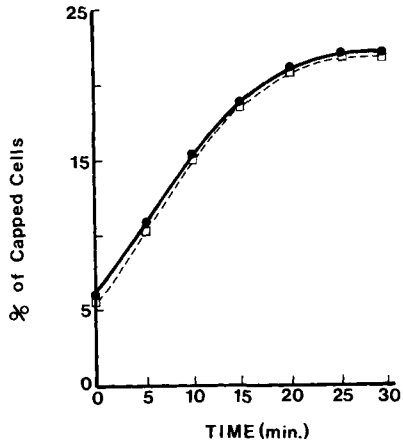

Fig. 6. Immunofluorescence analysis of insulin and IL-2 receptor capping in HVPGS-treated mouse splenic T-lymphocytes. ●—●, insulin receptor capping; □-----□, IL-2 receptor capping. (Similar insulin and IL-2 receptor capping were observed in cells treated with Con A followed by incubation with hormones—data not shown.)

This elevation of intracellular free Ca^{2+} (Fig. 8) appears to occur just prior to, or at the same time as, hormone receptor cap formation (Fig. 6).

Phosphorylation of 32-kd Membrane-Associated Protein

A well-known characteristic of phosphoamino acids is their differential sensitivity toward alkaline treatment. The phosphoamino bond in phosphotyrosine (P-tyr) is quite resistant to alkali whereas the bond in phosphoserine (P-ser) and phosphothreonine (P-threo) is very sensitive [44]. We have found in this study that essentially all of the membrane-associated phosphoproteins in unstimulated, resting lymphocytes are sensitive to alkaline treatment (Fig. 10A,B). However, during 15–30 min of incubation with insulin or IL-2, there is specific phosphorylation of a 32-kd membrane-associated protein which appears to be resistant to alkaline treatment (Fig. 10C–F). Further analysis by paper electrophoresis of the 32-kd protein amino acid hydrolysate indicates that phosphorylation of this protein occurs normally at serine and threonine residues in the unstimulated (resting) lymphocytes (Fig. 11A); but in the hormone-treated lymphocytes phosphorylation also occurs at tyrosine residues (Fig. 11B). Furthermore, tyrosine phosphorylation of the 32-kd membrane-associated protein can be effectively blocked by all five of the cytoskeletal protein inhibitors tested (Table I).

DNA Synthesis

In the absence of hormones, neither lectin-treated nor electric-stimulated splenic T-lymphocytes are activated to undergo DNA synthesis (Fig. 12A,B). However, in the presence of insulin or IL-2, both the Con A- and HVPGS-treated splenic T-lymphocytes are stimulated to synthesize DNA. The degree of hormone-induced DNA synthesis appears to be both Con A dose dependent (Fig. 12A) and HVPGS voltage dependent (Fig. 12B). As shown in Figure 13, the amount of DNA synthesized is also dependent on the concentration of hormone added to the Con A- or HVPGS-treated lymphocytes and it correlates very well with the percentage of cells displaying

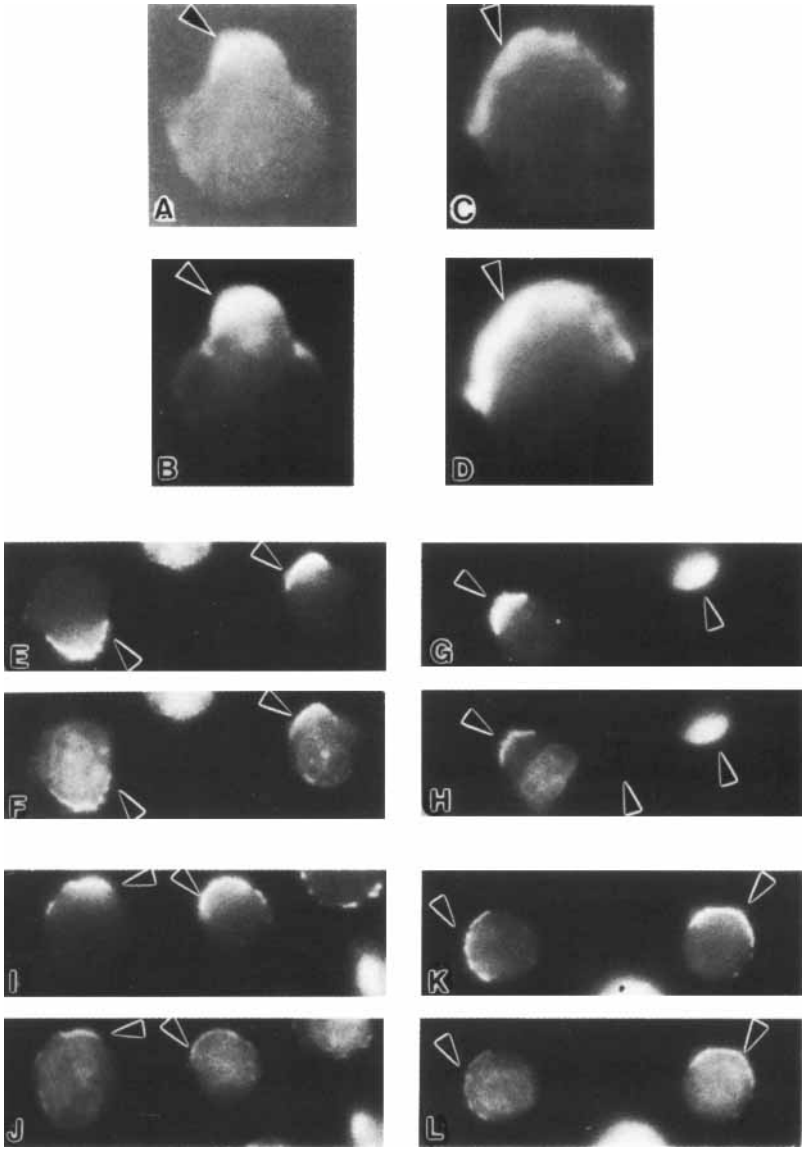

Fig. 7. Simultaneous localization of hormone receptors and intracellular cytoskeletal components by double immunofluorescence techniques. Mouse splenic T-lymphocytes were first treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with insulin (A,B,E,F,I,J) or IL-2 (C,D,G,H,K,L). **A,B:** Insulin receptor cap (A) and intracellular actin (B). **C,D:** IL-2 receptor cap (C) and intracellular actin (D). **E,F:** Insulin receptor cap (E) and intracellular calmodulin (F). **G,H:** IL-2 receptor cap (G) and intracellular calmodulin (H). **I,J:** Insulin receptor cap (I) and intracellular myosin light-chain kinase (J). **K,L:** IL-2 receptor cap (K) and intracellular myosin light-chain kinase (L). Arrowheads represent capped hormone receptors and aggregated cytoskeletal components. (A–D, ×1,600; E–L, ×700).

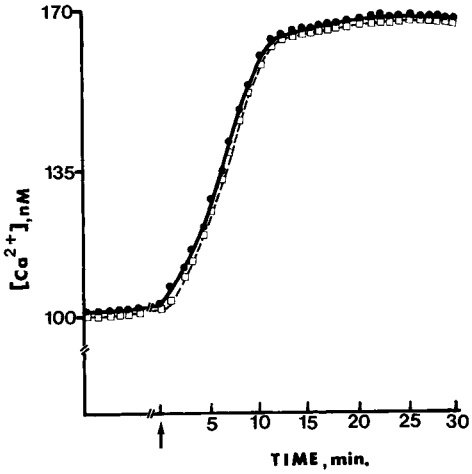

Fig. 8. Hormone-induced intracellular Ca^{2+} elevation by Fura-2 fluorescence measurement in HVPGS (50 V at 100 pulses/sec)-treated mouse splenic T-lymphocytes. ●—●, insulin-treated; □-----□, IL-2-treated. [Similar results were observed in cells treated with Con A (6 μg) followed by incubation with hormones—data not shown.]

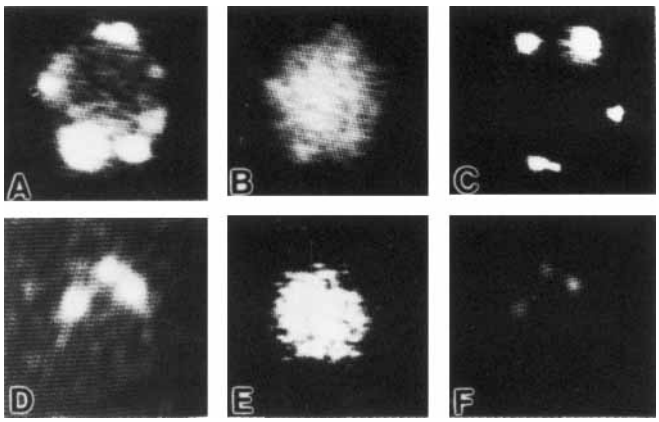

Fig. 9. Fura-2 fluorescence image of individual mouse splenic T-lymphocytes. **A,D:** 340-nm excitation, 500-nm emission. **B,E:** 380-nm excitation, 500-nm emission. **C,F:** Ratio 340/380 Image. Mouse T-lymphocytes were treated with HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with insulin (A–C) or IL-2 (D–F). [Similar Fura-2 fluorescence was observed in cells treated with Con A (6 μg) followed by incubation with insulin or IL-2—data not shown.] (×1,200).

hormone receptor caps. In addition, whenever insulin or IL-2 receptor capping is blocked by treatment with either metabolic or cytoskeletal protein inhibitors, there is always a concomitant reduction in both the amount of 32-kd tyrosine-phosphorylation and DNA synthesis in the T-lymphocytes (Table I).

DISCUSSION

The molecular mechanisms controlling eukaryotic cell proliferation are not well understood. Various ligands (e.g., peptide hormones, antibodies, and lectins) or

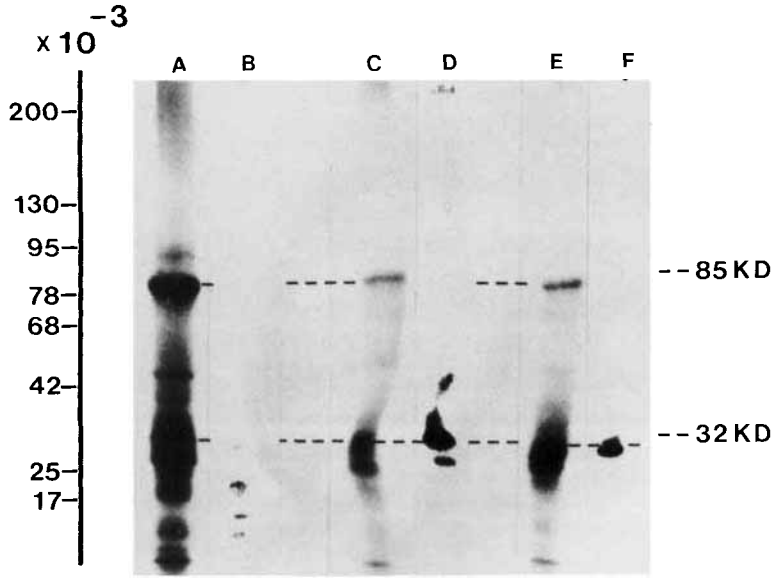

Fig. 10. Phosphorylation of 32-kd membrane-associated protein. Mouse splenic lymphocytes were treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with insulin (5×10^{-10} M) or IL-2 (1 unit) for 6–12 hr. SDS-PAGE was followed by alkaline treatment and autoradiography. **A,B:** No hormone treatment. **C,D:** Insulin treatment. **E,F:** IL-2 treatment. A,C,E: without alkaline treatment; B,D,F: with alkaline treatment.

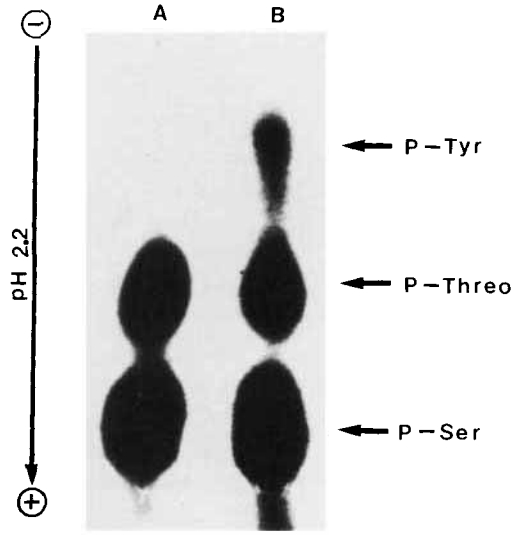

Fig. 11. Phosphoamino acid analysis of 32-kd membrane-associated protein. Cells were treated with either Con A or HVPGS followed by incubation with insulin or IL-2 as described in Figure 10. The ^{32}P-labeled 32-kd membrane-associated protein was isolated by SDS-PAGE and the phosphoamino acids were analysed according to the procedures described in Materials and Methods. **A:** No hormone treatment, **B:** With insulin treatment. (Similar result was observed in cells treated with IL-2.)

TABLE I. Effect of Various Inhibitors on Hormone Receptor Capping, 32-kd Protein Phosphorylation, and DNA Synthesis*

	Capping % of control		P-32 kd % of control		DNA synthesis % of control	
Inhibitors	Insulin	IL-2	Insulin	IL-2	Insulin	IL-2
Untreated control	100	100	100	100	100	100
Cytochalasin D	30	26	15	14	17	15
Sodium azide	25	25	20	23	7	8
W 5	24	23	17	16	7	7
W 7	26	25	13	14	8	9
W 12	27	29	15	12	16	12

*Mouse splenic lymphocytes were treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with insulin (5×10^{-11}–5×10^{-8} M) or IL-2 (0.5–500 units) in the presence of various drugs [e.g., cytochalasin D, 20 μg/ml; sodium azide, 50 mM; W 5/W 7/W 12 (10 μM)] for at least 20–30 min at room temperature. Both drug-treated as well as untreated control samples were examined for insulin and IL-2-induced receptor capping, 32-kd protein phosphorylation, and DNA synthesis as described in Materials and Methods.
All values presented are the average of 3–5 experiments and have a standard deviation of ± 5–10%.

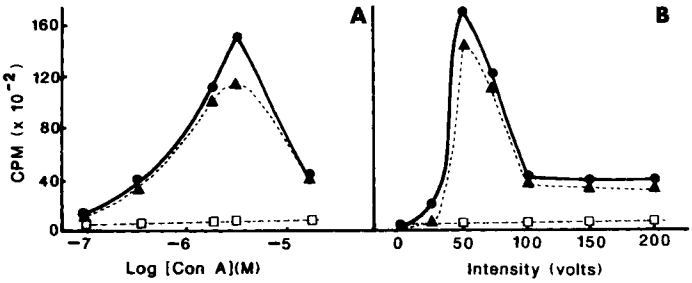

Fig. 12. Stimulation of DNA synthesis in mouse splenic T-lymphocytes. T-lymphocytes were first treated with either Con A (0.1–15 μg/ml) for 20 min (**A**) or HVPGS (0–200 V at 100 pulses/sec) for 20 min at room temperature (**B**) followed by incubation with no hormone (□-----□), insulin (●—●), or IL-2 (▲-----▲) for 12–24 hr.

treatments (e.g., electric stimulation) can be employed to induce mitogenesis in different types of cells [24–28]. In this study we have chosen to examine the mechanisms by which two different mitogenic treatments, Con A (ligand dependent) and HVPGS (ligand independent), activate resting mouse T-lymphocytes.

Among the various cellular events which occur during mitogenesis [i.e., the transition from the quiescent (G_0 phase) to G_1 phase and the proliferative state (S and M phases)], changes in and at the plasma membrane have been observed in a wide variety of cell types [56–59]. For example, multivalent ligands, such as lectins and antibodies, bind to their specific receptors on the cell surface and induce a dramatic redistribution of those receptors [9–16,60]. With lymphocytes, it has been shown that (1) "high" concentrations of Con A (above 50 μg/ml) appear to cause immobilization of the receptors and can be toxic [60,61]; and (2) "low" concentrations of Con A (approximately 0.1–15 μg/ml) can induce both surface receptor reorganization and cell proliferation [60]. Other membrane-associated activities, including increases in the transport of K^+, Ca^{2+}, and amino acids, have been reported to occur in thymocytes treated with a mitogen [62,63]. At the present time, the relationship between various membrane changes and other key intracellular events required for cell acti-

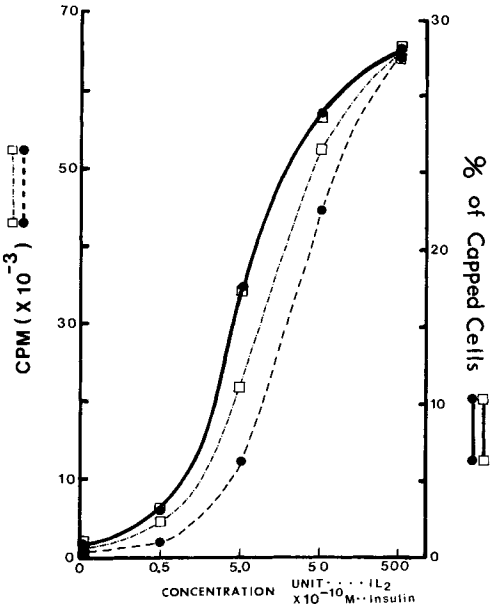

Fig. 13. Correlation of receptor capping and DNA synthesis. Mouse splenic T-lymphocytes were treated with either Con A (6 μg) or HVPGS (50 V at 100 pulses/sec) for 20 min at room temperature followed by incubation with various concentration of insulin (●—●, ●-----●) or IL-2 (□—□, □-·-·-□) for 30 min. Capping: (●—● insulin treated); (□—□ IL-2 treated). DNA synthesis: (●-----● insulin treated); (□-·-·-□ IL-2 treated).

vation and mitogenesis has not been clearly defined. It has been suggested that mitogens cause conformational changes in and/or reorganization of certain membrane proteins which then trigger a cascade of events leading to cell growth and division.

T-cell activation is generally initiated by the binding of external ligands such as mitogens, antigens, or antibodies to their specific surface receptors [24–28]. The role of peptide hormones, such as insulin and IL-2, in the regulation of T-lymphocyte activation has not been extensively investigated. It is known that both insulin and IL-2 receptors are not detectable on the surface of unstimulated (resting) T-lymphocytes [49–51]. However, detectable quantities of both hormone receptors have been reported as early as 4–8 hr after Con A treatment with maximum levels occurring at 24 hr [56–59]. Although both of these hormone receptors have been used as markers for lymphocyte activation [56–59], it is not clear whether they actually play a functional role in lymphocyte mitogenesis.

In this study various Con A concentrations (0.1–15 μg/ml) and intensities of HVPGS (0-200 V at 100 pulses/sec) were tested to determine the optimal amount required for both hormone receptor exposure (Figs. 2B, 3, 4) and subsequent DNA synthesis (Fig. 12). The major results obtained are as follows: (1) hormone receptor exposure can occur as early as 5 min poststimulation (Fig. 3); (2) the amount of hormone receptor exposed is closely correlated with the amount of Con A added (Fig. 4A) as well as with the intensity of HVPGS applied (Fig. 4B); (3) T-lymphocytes can cap the hormone-bound receptors and proceed to initiate DNA synthesis *only* when insulin or IL-2 is present in the medium (Fig. 12A,B). All together these results strongly suggest that lymphocyte activation requires first the exposure and

binding of hormone receptors, which is followed by aggregation of the bound receptors into cap structures. Transduction of the signal generated by the binding of insulin or IL-2 to their receptors appears to involve a number of biological steps. One of the first changes we have observed with the T-lymphocytes is a marked increase in the intracellular, free Ca^{2+} concentration (Figs. 8,9). Minutes later, hormone receptor patching/capping begins (Figs. 2C–E, 6), which is followed by tyrosine-specific protein phosphorylation (Fig. 10) and finally by DNA synthesis (Figs. 12, 13).

Cell surface receptors redistribute into small clusters (so-called patches) and/or large aggregates (so-called caps) upon binding of specific ligands (e.g., antibodies against surface antigens, lectins or insulin) [9–11,18]. Recent evidence indicates that certain membrane-associated proteins (e.g., ankyrin, band 4.1, and fodrin) [12–14] are involved in linking surface receptors to intracellular actin filaments. Aggregates (patches) of ligand-receptor complexes appear to be collected into a cap structure in an energy-dependent manner (involving Ca^{2+}/calmodulin/myosin light-chain kinase) analogous to the sliding filament mechanism which occurs during muscle contraction [9–16]. However, the physiological significance of receptor patching and capping has not been clearly established. One of the most important functions postulated for receptor patching and capping involves the initiation of intracellular activation signals. For example, receptor patching and capping may be responsible for signaling the initiation of (1) mitogenesis of antibody-producing cells [64,65], (2) degranulation of mast cells and basophils [20,23], (3) insulin-dependent glucose oxidation [66], (4) cellular activation by epidermal growth factor [67], and (5) toxin-mediated cellular responses [68].

In this study we have found that the capping mechanism with both hormone receptors appears to be identical to that induced by multivalent ligands (e.g, lectin or antibodies). Specifically, they all exhibit the following common characteristics: (1) inhibition of capping by cytochalasin D (a microfilament-disrupting agent), sodium azide (an energy poison), and W 5, W 7, W 12 (calmodulin inhibitors) (Fig. 2F–H, Table I); (2) a close association of calmodulin, myosin light-chain kinase and actomyosin with the cap structures (Fig. 7); (3) the regulation of cap formation by Ca^{2+}/calmodulin/myosin light-chain kinase; and (4) an energy-dependence on ATP (Figs. 2 C–H, 6–9, Table I).

A number of previous studies have indicated that protein phosphorylation by tyrosine-specific kinase(s) may be closely related to the overall regulation of cell proliferation [48,69–76]. Similarly, we have found that following insulin or IL-2 binding, a 32-kd membrane-associated protein becomes specifically phosphorylated at tyrosine residues in addition to the usual serine and threonine residues (Figs. 10C–F, 11B). It is conceivable that the 32-kd protein phosphorylation (presumably through the activation of a tyrosine-kinase) is directly involved in the regulation of T-cell mitogenesis. However, determining the precise function of the phosphorylation will require further investigation.

In summary, we have shown that lymphocyte hormone receptor capping plus the subsequent stimulation of tyrosine-kinase-specific protein phosphorylation and DNA synthesis require metabolic energy (ATP), Ca^{2+}/calmodulin, myosin light-chain kinase, and actomyosin components. Since (1) stimulation of hormone receptor capping correlates well with the subsequent activation of DNA synthesis (Fig. 13), and (2) inhibition of hormone receptor capping by several different drugs always blocks the following DNA synthesis, we propose that hormone receptor patching/capping is a requirement for the activation of lymphocytes.

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