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Regional inflammation and systemic fever are hallmarks of host immune responses to pathogenic stimuli. Although the thermal element of fever is thought to enhance the activity of immune effector cells, it is unclear what the precise role of increased body temperatures is on the activation state and effector functions of lymphocytes. We report here that mild, fever-like whole body hyperthermia (WBH) treatment of mice results in a distinct increase in the numbers of tissue lymphocytes with polarized spectrin cytoskeletons and uropods, as visualized in situ. WBH also induces a coincident reorganization of protein kinase C (PKC) isozymes and increased PKC activity within T cells. These hyperthermia-induced cellular alterations are nearly identical with the previously described effects of Ag- and mitogen-induced activation on lymphocyte spectrin and PKC. Immunoprecipitation studies combined with dual staining and protein overlay assays confirmed the association of PKCβ and PKCδ with spectrin following its reorganization. The receptor for activated C kinase-1 was also found to associate with the spectrin-based cytoskeleton. Furthermore, all these molecules (spectrin, PKCβ, PKCδ, and receptor for activated C kinase-1) cotranslocate to the uropod. Enhanced intracellular spectrin phosphorylation upon WBH treatment of lymphocytes was also found and could be blocked by the PKC inhibitor bisindolylmaleimide I (GF109203X). These data suggest that the thermal element of fever, as mimicked by these studies, can modulate critical steps in the signal transduction pathways necessary for effective lymphocyte activation and function. Further work is needed to determine the cellular target(s) that transduces the signaling pathway(s) induced by hyperthermia. The Journal of Immunology, 1999, 162: 3378–3387.

Upon activation, lymphocytes undergo many morphological and biochemical changes. For example, activated T cells display polarization, with the formation of a well-defined single cytoplasmic projection termed the uropod (1, 2). These cells also exhibit a redistribution of adhesion molecules (i.e., ICAM-1, ICAM-3, and CD44) (3, 4) and cytoskeletal elements, including microtubules and ezrin-radixin-moesin family members (5–7), to the uropod. Our laboratory has previously shown that the uropod forms in close association with an aggregate of spectrin that develops in response to various activation signals (8, 9). While the uropod appears to be important in cell interactions and recruitment of immune effector cells to inflammatory sites, the precise mechanism and functional role of uropod formation are poorly understood.

The development of cell polarity seems to involve interactions between components of the cell membrane and cytoskeleton as well as reorganization of cytoskeletal elements. As a major component of the membrane cytoskeleton, spectrin has diverse functional motifs for protein-protein interactions, including an actin-binding domain (10), a Ca2+-binding domain (11, 12), an src homology domain 3 (SH3) (13), a pleckstrin homology (PH) domain (14, 15), and a membrane adhesion domain (16). Because a variety of proteins are known to interact with spectrin, it has been speculated that assembly of the spectrin-based skeleton participates within specialized membrane domains and in the construction of cell-cell contacts (17). Thus, spectrin is an important molecule to evaluate during lymphocyte activation (18).

We previously characterized a heterogeneous subcellular distribution of spectrin in lymphocytes and found that spectrin and other cytoskeletal proteins redistribute to distinct polar aggregates and caps upon various methods of lymphocyte activation (9, 18, 19). Spectrin aggregate formation has been shown to coincide with the positioning of the newly formed uropod following lymphocyte activation (9). Most interestingly, a key signaling molecule, protein kinase C (PKC) βII isozyme, also translocates to the spectrin aggregate upon cellular activation (20, 21). This strongly suggests a role for the dynamic reorganization of the spectrin-based skeleton in PKC-mediated signaling pathways that lead to lymphocyte activation and morphological polarization.

PKC is known to be involved in the regulation of a diverse range of cell functions, including cell proliferation and differentiation, and maintenance of cell morphology and adhesion (22). At least 12 subtypes of PKC have been identified in mammalian tissue and categorized into three subclasses based on differences in structure and cofactor requirements: conventional PKCs (α, βI, βII, and γ), atypical PKCs (δ, ε, η, θ, and ι), and novel PKCs (µ and ν).

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3 Abbreviations used in this paper: SH3, src homology 3; PH, pleckstrin homology; PKC, protein kinase C; RACK, receptor for activated C kinase; WBH, whole-body hyperthermia; TBS, 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl.

4 R. L. Campbell, S. Wright, E. A. Repasky, S. C. Watkins, and M. T. Lotze. Early spectrin aggregation during naive T cell activation distal to the site of antigen presenting cell (APC) interaction. Submitted for publication.
novel PKCs (δ, ε, η, θ, and μ) and atypical PKCs (ζ, τ, and λ) (23).
Although the importance of PKC activity during lymphocyte activation has been reported, little is known regarding the localization and role of each PKC isozyme in lymphocytes (24). A group of proteins termed receptors for activated C kinase (RACKs) appear to be important for increasing PKC phosphorylation of substrates, presumably by stabilizing the active form of PKC (25).
Furthermore, it was recently suggested that PKC binds to specific anchoring proteins located at various subcellular sites, thus providing another mechanism for isozyme-specific regulation (26, 27). Indeed, a number of signal-transducing proteins have recently been found to be associated with the membrane cytoskeleton interface (28–32), suggesting the importance of membrane cytoskeleton organization in the molecular regulation of PKC activity.
In conjunction with the lymphocyte activation caused by pathological stimuli or other immunomodulators, another cardinal feature of the host response to infection includes local increases in temperature at the site of inflammation and systemic fever. The complex immunological, neurological, and biochemical interactions that give rise to fever are becoming increasingly clear. However, the function of the resulting increased temperature in immune responses is not understood. This is primarily due to the difficulty in separating the effects that are due to the thermal element of fever alone from those caused by the myriad other physiological and neurological events that occur during the fever response. While there is no clear proof of a specific cellular effect of this evolutionarily conserved response to infection (i.e., increased body temperature), it is assumed that fever may have the ability to enhance immunological functions (33).

The use of externally applied heat has previously been used by our group in attempts to dissect the effects of the thermal element of fever. Application of this fever-like hyperthermia has been shown to induce multiple changes in lymphocytes that are indicative of altered activation levels. These include 1) significant alterations in the organization of the spectrin-based cytoskeleton (34), 2) activation and subcellular reorganization of PKC (34), 3) induction of heat shock proteins (notably heat shock proteins 70 and 110) (34), and 4) increased L-selectin-dependent adhesion of lymphocytes to vascular endothelium (35). Most of these cellular changes are not seen following the more severe and acute hyperthermia protocols that are usually employed in heat shock protein studies.

Here we attempt to characterize further the precise cellular targets of fever-like hyperthermia in immune responses and identify mechanisms by which fever may modify lymphocyte function. We first investigated the pattern of spectrin and PKC isozyme reorganization within T lymphocytes in vivo following a fever-like whole body hyperthermia (WBH) treatment. The potential for interactions among spectrin, PKC isozymes, and RACK1 were also determined. Lastly, the effects of WBH on PKC activity and spectrin phosphorylation were analyzed. Evidence is provided for the involvement of PKC in the dynamic spectrin organization of activated lymphocytes.

**Materials and Methods**

**Fever-range hyperthermia treatment**

Eight- to twelve-week-old female BALB/c mice (Springville Laboratories, Springville, NY) were placed in a humidified environmental chamber (VWR Scientific, Rochester, NY) that was preheated to 38°C. The incubator temperature was then increased 1°C every 30 min until mice reached a core temperature of 39.5 ± 0.5°C. Core temperatures were monitored during WBH using a thermocouple inserted into the rectum and connected to a digital readout (Sensortek, Clifton, NJ). Mice were maintained at a 39.5°C core temperature in the chamber for various periods up to 6 h. Control mice were kept at room temperature and were subjected to handling and temperature measurement manipulations similar to those of WBH-treated mice. After treatment, the mice were sacrificed, and lymphoid organs were removed and either imprinted or prepared in single cell suspensions. For in vitro hyperthermia, culture flasks containing cells were wrapped tightly with Parafilm (American National Can, Neenah, WI) and then totally submerged in a 39.5°C water bath (Lab-Line Instruments, Melrose Park, IL). Control cells were incubated at 37°C.

**Indirect immunofluorescence**

For in situ staining, freshly isolated spleen was cut and imprinted on coverslips, fixed in 2% formaldehyde, and permeabilized with PBS containing 0.2% Triton X-100. When localizing both spectrin and PKCs in the same cells, the imprints were first stained with goat anti-chicken α spectrin antisem (diluted 1/400 in PBS) (19, 36), followed by rhodamine-conjugated donkey anti-goat IgG (diluted 1/200; Miles/ICN). Lymph node imprints were then incubated with a 1/50 dilution of either rabbit anti-PKβ (Life Technologies, Grand Island, NY) or rabbit anti-PKβ (Santa Cruz Biotechnology, Santa Cruz, CA), followed by fluorescein-conjugated goat anti-rabbit IgG (diluted 1/200; Miles/ICN). Normal goat and rabbit IgG (Santa Cruz Biotechnology) were used as negative controls. Immunofluorescent results were analyzed with a confocal microscope MRC 600 (Bio-Rad, Hercules, CA).

Isolated T lymphocytes were prepared for immunofluorescence staining as previously described (19). Enriched populations of T lymphocytes were obtained by passing lymph node cell suspensions over nylon wool columns (37). Ninety percent purification was confirmed using FITC-conjugated anti-Thy.1.2 Abs (ICN Biomedicals, Costa Mesa, CA) (19).

**Subcellular fractionation**

Ice-cold PBS-washed cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mg/ml digitonin (Sigma, St. Louis, MO), 5 mM MgCl₂, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 25 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Alexis, San Diego, CA). Cytosolic and particulate fractions were separated by sedimentation at 100,000 × g for 50 min at 4°C (TL-100, Beckman, Fullerton, CA). The pellets were extracted in 1% Triton X-100 containing lys buffer and further spun at 15,600 × g for 20 min, and the supernatants (Triton-soluble membrane fraction) were collected. The Triton-insoluble pellets (cytoskeletal fraction) were resuspended in cytoskeletal buffer (125 mM Tris-HCl (pH 6.8), 1.25% SDS, 2 mM EDTA, 0.25 M sucrose, 10% glycerol, and 1% 2-ME) and sonicated three times for 5 s each time on ice. Protein content was determined using a BCA assay kit (Pierce, Rockford, IL).

**Western blot analysis**

Equivalent protein samples were subjected to 7.5–10% SDS-PAGE (38) and transferred onto an Immobilon-P membrane (Millipore, Marlborough, MA). Membranes were blocked with 5% nonfat milk in 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.05% Tween-20 for 1 h at room temperature followed by a 2-h incubation with either of the following primary Abs (diluted 1/100 in 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.05% Tween-20): rabbit anti-chicken erythrocyte α-spectrin serum (19, 36); mouse monoclonal or rabbit polyclonal anti-PKβ (Life Technologies/BRL); rabbit anti-PKCa, -βL,-βII,-βIII,-ζ, -θ, or -ε (Santa Cruz Biotechnology); and mouse anti-RACK1 or mouse anti-PKCa (Transduction Laboratories, Lexington, KY). After washing, membranes were incubated with horseradish peroxidase-conjugated dog anti-rabbit IgG or dog anti-mouse IgG (diluted 1/2000; Amersham, Arlington Heights, IL). Immunoreactivity was detected by the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

**PKC activity assay**

Total and subcellular fractions from T lymphocytes were prepared following WBH treatment and were partially purified with DEAE chromatography. PKC activity was measured using the PKC assay system from Life Technologies as previously described (39). For assays PKC activity in spectrin immunoprecipitates, the spectrin immunocomplexes were recovered by protein A-agarose beads and washed three times in kinase buffer (20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/ml leupeptin, and 25 μg/ml aprotinin). The kinase assay was performed in a final volume of 50 μl of assay solution containing 20 mM Tris (pH 7.5), 20 mM MgCl₂, 1 mM CaCl₂, 10 μM PMMA, 0.28 mg/ml phosphatidyserine, 20 μM ATP, and 50 μM Ac-myo-inositol basic protein peptide with 10 μCi of [γ-32P]ATP (ICN Biomedicals/assay). Incorporation of radioactivity was determined by liquid scintillation counting (Packard, Downers Grove, IL). PKC activity
was expressed as picomoles of phosphate incorporated per minute per 10^6 cells.

**Immunoprecipitation**

Lymphocytes were washed three times with cold PBS and lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Cell extracts were preabsorbed with 0.05 vol of preimmune serum together with 30 μl of protein A-agarose beads for 1 h at 4°C and centrifuged at 1500 rpm for 15 min. The lysate was incubated overnight at 4°C with rabbit anti-chicken spectrin Ab (diluted 1/100). Immune complexes were precipitated with 20 μl of protein A-agarose for 2 h at 4°C and washed twice with 1 ml of wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40), then washed with 50 mM Tris-HCl (pH 6.8) and 0.1% Nonidet P-40 and boiled in 30–40 μl of SDS sample buffer before loading on an SDS-PAGE gel.

**Overlay assay**

Binding of PKC to spectrin in vitro was analyzed using a modification of a previously described overlay assay (40). Immunoprecipitated spectrin was transferred to a membrane and blocked with 5% milk in TBS (10 mM Tris-HCl (pH 7.5) and 150 mM NaCl). Membranes were incubated with 20 μg/ml partially purified PKC fraction in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mg/ml BSA, 50 μg/ml phosphatidylserine, 0.1 mM CaCl₂, 1 mM calcium, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM β-ME, and 10 μM PMA (Sigma) for 1 h at room temperature. The membrane was washed three times in TBS containing cofactors. Samples were fixed with 5% formaldehyde in TBS for 20 min at room temperature, incubated in 2% glycine in TBS for 20 min, washed three times in TBS, and probed with the anti-PKC Abs as described above.

**In vivo phosphorylation**

Isolated T cells were incubated in phosphate-free RPMI medium supplemented with 5% dialyzed FCS for 30 min and then labeled for 2 h with 400 μCi/ml [³²P]orthophosphate. Cultures were treated with 10 μM of the PKC inhibitor bisindolylmaleimide I (GF109203X; Alexis Corp.) for 1 h followed by hyperthermia or PMA (100 ng/ml) treatment. Cells were lysed and precipitated with goat anti-chicken spectrin Abs (diluted 1/100). The resulting immunocomplexes were boiled in SDS sample buffer and electrophoresed on 7.5% SDS-PAGE gels. The gels were dried, and radioactive bands were detected by autoradiography using x-ray film (Kodak X-OMAT, Eastman Kodak, Rochester, NY) at −80°C.

**Results**

**Dynamic spectrin reorganization in WBH-treated T lymphocytes**

Following a 6-h WBH treatment, we compared the distribution patterns of spectrin in WBH-treated and control spleen tissue imprints, where the microenvironmental relationships are preserved compared with those in in vitro cell suspensions. As determined by in situ fluorescence staining, most cells exhibited a ring-like, membrane-associated spectrin distribution in untreated spleen tissue (Fig. 1A). When mice were treated with WBH, the percentages of splenocytes with a polar spectrin aggregate and with uropods at the site of the spectrin aggregate increased significantly (Fig. 1, B and C). Furthermore, we observed an increase in the numbers of splenocytes where spectrin had formed a distinct cap at one pole of the cell. These phenomena are consistent with the observed patterns of spectrin rearrangement and uropod formation during lymphocyte activation (8, 9, 19, 20). Fever-range WBH resulted in an ~4-fold increase in the percentage of lymphocytes with uropods (Fig. 1C). To directly examine the effects of WBH on the formation of spectrin aggregates in purified T lymphocytes, T cells were isolated from WBH-treated mice at various time points and stained with anti-spectrin Ab. After 6 h of WBH treatment, the percentage of T cells with spectrin aggregates or caps had increased from ~20 to 55% (Fig. 2A). The effects of WBH on the cellular distribution of spectrin were also determined by analysis of the solubility properties of spectrin (Fig. 2B). No changes in total expression levels of spectrin during WBH were observed. However, as shown previously following various activation signals (20), there was an obvious redistribution of spectrin from the Triton X-100-soluble (membrane) fraction to the Triton X-100-insoluble (cytoskeletal) fraction. Thus, WBH treatment appears to induce changes in lymphocyte morphology and spectrin localization that are identical with those seen following Ag- or mitogen-dependent activation or biochemical stimulation of PKC.

**PKC isozyme distribution and activity in WBH-treated T cells**

The ability of WBH to induce alterations in the cellular distribution and solubility of spectrin led us to next explore the intracellular localization patterns of PKC isozymes in T lymphocytes during WBH. T cells were isolated from the lymph nodes of WBH-treated mice at various times. Immunoblot analysis of total cell lysates and cell fractions (cytosol, membrane, and cytoskeletal fractions based on detergent solubility) with PKC isozyme-specific Abs revealed the effect of low hyperthermia stress on solubility fractions (membrane) of PKC isozymes in T lymphocytes during WBH. PKC isozymes were constitutively expressed in control and WBH-treated murine T lymphocytes. In nonstimulated cells (time zero), these PKCs were present in the cytosol (α, β1, βII, θ, ε, δ, ζ, and λ) associated with the cellular membrane (α, β1, βII, θ, ε, δ, ζ, and λ) or associated with the cytoskeleton (β1, βII, θ, ε). Although WBH was not capable of altering total PKC protein levels, all PKC isozymes displayed an obvious redistribution. PKCα, -βII, -θ, and -λ translocated from the cytosol to the membrane fraction, with the highest accumulation at the membrane after 2–4 h of WBH exposure.
PKCβI, -ε, -δ, and -ζ showed no significant reduction in the cytosol, indicating that only a small proportion of these PKC isozymes translocated to the particulate fraction. Interestingly, the cytoskeletal association of PKCβI, -βIII, -θ, and -ε increased significantly during WBH. In addition, double immunoreactive bands were seen in Western blots for PKCε, -ζ, and -α. The upper band is believed to be hyperphosphorylated PKC, and the lower band is believed to be hypophosphorylated PKC (41), indicating possible regulation of PKC activation through autophosphorylation or phosphorylation by other protein kinases.

The observed selective translocation of PKC isozymes suggests that PKCs are involved in hyperthermia-induced signaling transduction events. Thus, to determine PKC activity in WBH-treated cells, enzymatic assays were performed (Fig. 4). Interestingly, compared with the level in control cells (14.85 ± 1.22 pmol/min/10⁸ cells), total PKC activity was found to increase with time of WBH exposure and to reach a maximum level (23.60 ± 1.10 pmol/min/10⁸ cells) after 4 h of WBH treatment. Consistent with these results, WBH caused a decrease in PKC activity in the cytosol and simultaneous four- and 3-fold increases in membrane and cytoskeletal fractions, respectively. This increased PKC activity in the cytoskeletal fraction of T cells strongly suggests a role for activated PKC in cytoskeleton function.

**Determination of spectrin association with PKCβ and -θ**

Previous findings of colocalization of spectrin and PKCβII (20) led us to investigate whether other PKC isozymes could associate with spectrin. It was found that PKCβI, -βIII, and -θ were present in spectrin immunocomplexes from control, in vitro hyperthermia-treated, and PMA-treated T lymphocytes (Fig. 5), while the other PKC isozymes were not detected (data not shown). Protein overlay assays also revealed the direct association of PKCβ with immunoprecipitated spectrin from untreated, in vitro hyperthermia-treated, or PMA-treated T lymphocytes in a manner that could be blocked by the PKC inhibitor GF109203X (data not shown). These data provided evidence that the association between PKCβ and spectrin is dependent on PKC activation. The direct binding of PKCθ with spectrin was not detected by this protein overlay method (data not shown). However, this does not eliminate the possibility that PKCθ might associate with spectrin indirectly through other proteins, such as an unknown anchoring protein.

To further explore the localization patterns of spectrin, PKCβ, and PKCθ during WBH treatment, we performed in situ double staining of spleen tissue with anti-spectrin and anti-PKC Abs. PKCβ and spectrin have the same localization patterns in untreated splenocytes (Fig. 6A). When mice were treated with WBH for 6 h, these two molecules redistributed and colocalized within the aggregates (Fig. 6B). Dual staining for PKCθ and spectrin revealed the same pattern of redistribution to aggregates (data not shown). Since fever-range WBH treatment appears to induce lymphocyte spectrin aggregation in conjunction with uropod formation, it was of interest to determine whether PKCβ or PKCθ also colocalized with spectrin in the uropod of WBH-treated lymphocytes. Double-staining studies showed that both of these PKC isozymes translocated with spectrin to the uropod following WBH (Fig. 6, C and D), suggesting a role for the PKC/spectrin complex in lymphocyte uropod function.

**Identification of RACK1 in the spectrin-based skeleton**

Because RACKs appear to be important in PKC localization and function, we wanted to determine the role of the PKC binding protein RACK1 in the PKC isozyme localization phenomenon described above. Total cell extracts and cellular fractions of T lymphocytes from WBH-treated mice were analyzed by immunoblot using anti-RACK1 Ab (Fig. 7A). Interestingly, RACK1 was seen to be constitutively expressed in T lymphocytes and translocated from the cytosol to the membrane and cytoskeleton fractions following WBH. RACK1 was also present in the spectrin immunoprecipitates of T cells (Fig. 7B). Compared with control cells, the relative amount of RACK1 protein increased in the spectrin immunoprecipitates when cells were treated with either hyperthermia or PMA in vitro. Furthermore, following WBH treatment, RACK1 also localized to the lymphocyte uropod (Fig. 7C). Thus, these data...
support a role for RACK1 in the formation and maintenance of cytoskeleton-based morphological changes in stimulated T cells.

Effect of fever-range WBH on intracellular spectrin phosphorylation

Previous experiments have shown that pretreatment with PKC inhibitors reduced the percentage of cells with spectrin aggregates (20). Therefore, we wanted to determine whether spectrin-based skeleton reorganization is affected via phosphorylation by PKC. For this purpose isolated T lymphocytes were labeled with $^{32}$P and incubated with or without GF109203X followed by in vitro hyperthermia for 6 h or PMA stimulation for 30 min. Spectrin was immunoprecipitated and analyzed by SDS-PAGE (Fig. 8A). Compared with control cells, spectrin from in vitro hyperthermia and PMA-treated cells displayed increased phosphorylation. Pretreatment with PKC inhibitor reduced the phosphorylation of spectrin.

FIGURE 3. Western blot analysis of PKC isozyme distribution and translocation during fever-range WBH. T lymphocytes were isolated from mice treated with WBH for the indicated times. Cell lysates were separated into cytosolic and particulate fractions, and the particulate fractions were further separated into a membrane fraction (Triton X-100 soluble) and a cytoskeletal fraction (Triton X-100 insoluble). The expressions of conventional PKCs (A), novel PKCs (B), and atypical PKCs (C) were determined by immunoblotting using PKC isozyme-specific Abs. Double arrows (e.g., PKCa) indicate hyperphosphorylated and hypophosphorylated PKC isoforms. The results shown here are representative of three separate experiments.

FIGURE 4. Assay of PKC activity in T lymphocytes treated by fever-range WBH. Total cell lysates and cytosolic, membrane, and cytoskeletal fractions were prepared directly after 0, 2, 4, and 6 h of WBH treatment. PKC was partially purified from each fraction, and PKC activity was determined as picomoles of phosphate incorporated per minutes per $10^8$ cells. Data are shown as the average values of three experiments ± SE. *p < 0.05 compared with the control value (0 h) using unpaired Student’s t test.

FIGURE 5. Determination of interaction between spectrin and PKC isozymes. A-C, Identification of PKC isozymes in the spectrin immunocomplex. Spectrin was immunoprecipitated from T lymphocytes before (lane 1) or after 6 h of in vitro hyperthermia (lane 2) or 30 min of PMA (100 ng/ml) treatment (lane 3). Total cell lysate was used as a positive control (lane 4). Immunoblotting was performed using specific Abs for PKCβI (A), PKCβII (B), and PKCθ (C). The arrow indicates the PKC band; lower m.w. bands are the rabbit IgG used in the immunoprecipitation. These results are representative of three separate experiments.
in response to both in vitro hyperthermia and PMA. To determine whether the PKC associated with spectrin is an activated form, we directly assayed PKC activity in the spectrin immunocomplex (Fig. 8B). Compared with the level in control cells (0.54 ± 0.06 pmol/min/10^8 cells), PKC activity in spectrin immunoprecipitates were significantly elevated and reached the highest level after 6 h of WBH (2.26 ± 0.22 pmol/min/10^8 cells). These data are supportive of a hypothesis that PKC is involved in spectrin phosphorylation and subsequent spectrin reorganization.

### Discussion

Effective immune responses are heavily dependent on both intracellular and intercellular events mediated by specialized cell surface molecules and increased motility and homing. During the migration of lymphocytes toward an inflammatory site, one of the earliest changes to take place is cellular polarization and uropod formation (1, 2). While it is not clear whether there is a functional role for the uropod that is directly involved in cell motility, uropod formation has been observed during lymphocyte interaction with macrophages (42), mitogen or chemokine stimulation of lymphocytes (2, 43), and cell-mediated cytotoxicity (44). Furthermore, the uropod, with its increased concentration of adhesion and effector molecules (3, 4, 45, 46), appears to be an important site of cell interactions. Our observation of spectrin, PKCβI, PKCβII, PKCθ, and RACK1 redistribution to the uropod suggests that these elements play an important role in the mechanisms that are involved during this stage of T cell activation, and that WBH alone can enhance these cellular events. Moreover, the presence of spectrin-associated PKC in the uropod suggests a previously underappreciated role for kinase function in the initiation and/or maintenance of this site for cellular interactions.

PKC has been implicated in the activation of the transcription factor activating protein-1 and IL-2 synthesis regulation (24, 47). Recently, PKCθ was found to be translocated to the site of cell contact between T cells and APC upon T cell activation (48). In our studies, PKC isozymes (α, βI, βII, θ, δ, ε, ζ, and λ) in T cells display different distribution patterns, suggesting that an individual isozyme may mediate distinct signaling regulation. We found that fever-like WBH resulted in a 2-fold increase in total PKC activity and an increase in membrane- and cytoskeleton-associated PKC activity that is consistent with the translocation pattern of PKC isozymes (βI, βII, and θ) during WBH. This may indicate a possible role for these PKCs in membrane function and cytoskeletal reorganization. It may also be hypothesized that PKC located in the cytosol of resting cells will translocate to the membrane upon activation. Other investigators have shown that 42.5°C hyperthermia treatment resulted in an elevation of PKC activity (49, 50). In contrast, hyperthermia at 45°C caused a significant decrease in PKC activity and an increase in activity of phospholipid-independent protein kinases (51). However, the observations using hyperthermia in this nonphysiological range of 42–45°C, which dramatically inhibits protein synthesis and causes substantial cell death, probably should not be directly compared with our studies using a...
much milder fever-like hyperthermia treatment, where average temperatures are $<\!40^\circ C$ for a significantly longer duration. Consistent with our previous work (20), immunoprecipitation studies showed that PKC$\beta$II as well as PKC$\beta$I and PKC$\delta$I are detected in the spectrin immunocomplex, implying that these three PKC isozymes ($\beta$I, $\beta$II, and $\delta$I) may play an important role in spectrin reorganization. Furthermore, protein overlay assays showed the direct association between spectrin and PKC$\beta$, suggesting a possible PKC binding site in spectrin. Application of GF109203X in overlay assays abolished this binding process completely. GF109203X has been shown to inhibit PKC activity exclusively via the ATP binding site (52), indicating a requirement for PKC activation in this association. Overlay assays did not show the direct binding between spectrin and PKC$\delta$. However, dual staining showed that PKC$\theta$ colocalized with spectrin in T lymphocytes, suggesting that PKC$\theta$ might bind to spectrin through some other linker protein. The positioning of PKCs at specific intracellular locations is probably central to its ability to respond efficiently to second messengers and to have ready access to substrates (27). We speculate that PKC$\beta$I, -$\beta$II, and -$\delta$I associate with spectrin and mediate either the reorganization of the spectrin cytoskeleton or the phosphorylation of their specific nearby targets that are involved in T lymphocyte activation.

Although PKC inhibitors blocked the spectrin phosphorylation mediated by PKC, we cannot rule out the possibility that other protein kinases might also phosphorylate spectrin in response to hyperthermia. Based on the observation that the PKC inhibitor calphostin C blocks formation of the spectrin aggregate in response to PMA treatment (20) and WBH (data not shown), it seems likely that spectrin participates in membrane cytoskeleton organization following phosphorylation. We speculate that spectrin aggregate formation involves PKC activation and is regulated...
by PKC-mediated phosphorylation. Spectrin-based skeleton reorganization would then provide a framework for efficient PKC phosphorylation of its specific substrates.

Others have shown that PKC isozymes bind to specific cytoskeletal elements. For example, both PKCβII and PKCε bind to actin (53, 54), and PKCε also binds 14-3-3 proteins (55). PKCε colocalizes with vinculin and talin (56), while PKCδ associates with vimentin filaments upon cellular activation (57). PKCα and βII have been shown to bind a multienzyme scaffold protein, AKAP 79, which also binds protein kinase A and the Ca2+/calmodulin-dependent phosphatase (58). These findings have led to the suggestion that the specific localization of each isozyme by interaction with its binding proteins may be a critical regulatory step in the selection of the substrate to be phosphorylated.

Another group of anchoring proteins, RACKs, has been proposed to be critically required for the translocation and subsequent function of PKC (59). Our findings indicate that RACK1 is present at high levels in T lymphocytes and exhibits a similar translocation from cytosol to membrane and cytoskeleton during WBH as some PKC isozymes. RACK1 is also a component of the spectrin immunoprecipitates, and the amount of RACK1 in the spectrin complex increases following WBH and PMA treatment. Furthermore, RACK1 localizes at the uropod induced by WBH. All these data are consistent with the hypothesis that following WBH, RACK1 binds PKCβ and PKCθ, then associates with spectrin and assembles into an aggregate. Because the PH domain can bind to WD40 repeats within the β-subunit of G proteins (60, 61), it is of interest to note that RACK1 is a homologue of the G protein β-subunit, containing seven WD40 repeat elements (25). Thus, we speculate that spectrin might interact either directly with PKC or with RACK via its PH domain. However, the detailed molecular interaction of PKCs and their receptors with spectrin awaits further investigation.

The data presented here bring to light a hitherto largely ignored potential regulator of lymphocyte activation, i.e., core body temperature. We have shown here that external application of fever-like WBH alone, without any other exogenous immunological stimulant, results in PKC activation and reorganization of the spectrin-based cytoskeleton and its associated molecules. These data imply the existence of an as yet undefined cellular target(s) that is sensitive to minor changes in body temperature and is capable of interacting with signal transduction pathways involving PKC and the cytoskeleton. Spectrin-associated PKC isozymes (βI, βIIL, and θ) are likely to be involved in this spectrin reorganization through a phosphorylation event. The discovery of RACK1 in the spectrin immunocomplex provides a possible mechanism for PKC translocation and association with spectrin. Thus, it is suggested that PKC/RACK1 interact and assemble into a signaling complex, with the cytoskeleton providing a scaffold on which signal proteins and adhesion molecules function. Further investigations are necessary to gain an overall understanding of the functional significance of spectrin/PKC/RACK redistribution during fever-range WBH. However, the morphological and structural changes that are induced in lymphocytes by WBH are indicative of increased polarity, activation, and motility. We speculate that this increased level of lymphocyte activation would, in turn, increase the efficiency of Ag-dependent interactions, resulting in enhancement of immune responses.

Increased body temperature, which occurs naturally during an infection, has been previously suggested to enhance the immune response in a way that could benefit clinical practice (33). Even in cold-blooded animals (i.e., fish and lizards) a behaviorally induced response in a way that could benefit clinical practice (33). Even in cold-blooded animals (i.e., fish and lizards) a behaviorally induced increase in body temperature has been shown to be completely correlated to survival following various infections (62–64). This strongly supports the hypothesis that there is a fundamental immunological effect of increased body temperature. In fact, the use of externally applied hyperthermia in cancer therapies has been shown to potentiate the immune response to various cytokines (65–67), increasing ICAM-1 expression and adhesion of lymphocytes to endothelial cells (35, 68). Furthermore, the combination of IL-2 and local hyperthermia abrogated the growth of tumors in mice better than either modality alone (69) and increased trafficking of activated lymphocytes to the tumor area (70). In contrast to conventional high temperature, short duration WBH protocols, where core body temperatures are raised to ~41.8–42°C for anywhere from 30 min to 2 h, our laboratory has used a lower temperature, long duration WBH protocol that does not elicit the same cytotoxicity of normal tissue that can reduce therapeutic gain. Indeed, the fever-range WBH was found to induce little damage to
living cells while maintaining the ability to cause significant tumor growth delay (71). These data combined with the findings reported here on the effects of fever-like WBH on the dynamic properties of spectrin and PKC isozymes in T cells have important implications for the use of WBH in a clinical setting, particularly one involving immunotherapy.

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References