Protein Kinase C and Intracellular Free Ca^{++}: Relationship to Human Immunodeficiency Virus (HIV)-Induced Cellular Hyporesponsiveness (43818)

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Abstract. **Protein Kinase C (PKC) and Ca^{++} are both involved in the chain of events leading to T-cell activation. An impairment of the immune response is characteristic of T cells obtained from patients with HIV infection. In this report, the involvement of PKC and Ca^{++} in HIV-mediated cellular hyporesponsiveness was examined. Infection of peripheral blood mononuclear cells (PBMC)s from HIV-seronegative normal donors with HIV strain HTLV IIIB, or two fresh patient isolates produced a 1.4-, 10.7-, and 11.4-fold enhancement in PKC activity at 1 hr postinfection (PI) and a 1.8-, 2.3-, and 3.8-fold enhancement at 12 hr PI, respectively. A marked decrease of PKC content, as determined by Western Blot analysis, was observed in HIV-infected cells by Day 4 and 7 PI compared with mock-infected control cells. Furthermore, PKC synthesis was also inhibited in cells from immunosuppressed AIDS patients. PKC activity of PBMCs from HIV-infected patients did not change in response to 1 μM of phorbal myristate acetate (PMA). In contrast, the same dose enhanced the activity by 50%–100% in PBMCs from normal HIV-seronegative donors. A 40%, 50%, and 125% increase in intracellular free Ca^{++} in response to HIV infection was observed 12 hr PI in MT4, JURKAT, and PBMCs, respectively. However, the increase in intracellular free Ca^{++} in HIV-infected PBMCs obtained from normal donors in response to PHA was 56% and 17% compared with an increase of 100% and 120% in mock infected cells at 12 hr and 1 week PI, respectively. Comparing the Ca^{++} response to PHA in PBMCs from HIV-infected patients showed that patients with <250 absolute T4 cells/mm^3 had an impaired Ca^{++} response. These data suggest that there is a relationship between intracellular free Ca^{++} and PKC and HIV-induced T-cell hyporesponsiveness.** [P.S.E.B.M. 1994, Vol 207]

Infection of T cells by HIV-1 results in cellular activation and viral transcription, and ultimately to the production of infectious virus (1). Over a protracted period of time patients with HIV infection experience a progressive state of immunosuppression. A number of immune parameters have been described to be impaired in such patients and have been attributed to the lack of sufficient and/or functional T4 cells (2). T cells from HIV-infected patients do not respond normally to interleukin-2 (IL-2) (3) and have decreased IL-2 receptor expression (4), defective IL-2 production (5–7), impairment of γ-interferon production (8–9), and decreased T-cell proliferative responses, among the many abnormalities described to occur in cells from these patients. The mechanisms underlying the functional hyporesponsiveness of T cells have not been elucidated to date.

T-cell activation involves a partially understood process that includes a series of biochemical and molecular events that are responsible for relaying signals from receptor ligand complexes to the interior of the cell. Important signals in the chain of events are the

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intracellular free Ca^{++} and protein kinase C activation.

Several reports have documented the involvement of free Ca^{++} in receptor-mediated T-cell activation and T-cell-mediated cytotoxicity (10–14). The rise in Ca^{++} apparently results from receptor-mediated hydrolysis of phosphatidylinositol (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) by phospholipase C (10, 15–23). IP_3 mediates the release of Ca^{++} from the cellular organelles to the cytoplasm upon T-cell activation. In the presence of free Ca^{++}, DAG activates PKC, which in turn phosphorylates a specific group of cellular proteins that are involved in DNA synthesis (24). PKC can also be activated *in vivo* and *in vitro* with tumor promoting phorbol esters such as 12–0 tetradecanoyl phorbol 13 acetate (TPA) (25, 26). PKC's involvement in the regulation of the immune response is apparent from its role in T-cell proliferative response, as well as induction of IL-2 gene expression and subsequent events (27, 28).

A role of PKC in HIV-mediated events cannot be ruled out, since T-cell activation by PHA in the absence of infection has been shown to be preceded by enhanced PKC activity (27). In this report, the involvement of PKC and Ca^{++} in HIV-mediated cellular hyporesponsiveness was examined in CD4-positive cell lines and PBMCs from normal donors and HIV patients at different stages of immunosuppression.

Materials and Methods

Cells and Viruses. MT-4 and JURKAT cells (CD4-positive cell lines) as well as PBMCs from healthy donors and AIDS patients were used in this study. Cells were grown in RPMI 1640 medium and supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.075% $NaHCO_3$, and were maintained at 37°C. The media and FCS were endotoxin free.

HIV laboratory strain HTLV-IIIB and fresh patient isolates were used in these experiments, unless otherwise mentioned. HIV virus stocks were prepared from H9-HIV chronically infected cells and titrated as previously described (29).

The cells (5×10^6) were infected with HIV at an MOI of 0.02–0.05 $TCID_{50}$/cell for 90 min at predetermined times of infection. The cells were then cultivated at a density of 0.5×10^6/ml in RPMI 1640 medium in the presence or absence of the experimental treatment.

Mock infection was carried out using cell-free supernatants of noninfected H9 cells as controls for the HIV-infected cells.

PKC Extraction. Infected and uninfected cells were incubated, in presence or absence of the stimulants to be examined, in serum free RPMI 1640 medium for various times at 37°C. The cells were washed twice in identical medium, spun down, and resuspended in an ice cold extraction buffer (20 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 2 m*M* dithiothreitol [DTT], 0.25 *M* sucrose, 1 m*M* PMSF, and 0.01% Leupeptin) and sonicated for 1 min. The homogenates were then ultracentrifuged at 100,000*g* for 1 hr at 4°C. The supernatants (cytosolic fractions) were withdrawn and the pellet was reconstituted in an extraction buffer containing 0.1% Triton X100, incubated for 60 min at 4°C and centrifuged again for 1 hr at 100,000*g* to obtain the detergent soluble particulate fraction (30).

PKC Activity Determination. PKC was extracted from the cells on the day of harvest. The PKC activity was then measured by ^{32}P phosphorylation of a PKC-specific substrate using Amersham Kits with the same conditions recommended by the manufacturer (Amersham International, PIC, United Kingdom).

Western Blotting. Immunoblotting was carried out as previously described (31). Briefly, equal amounts of proteins (10 μg/lane) were loaded on 10% SDS-polyacrylamide gels. The nonstained gels were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) using Genie Electrophoretic Blotter (Idea Scientific Co., Minneapolis, MN) and Tris-glycine-methanol buffer pH 8.3 system at 24 volts for 90 min. The blots were then rinsed with Tris-buffered saline (TBS pH 7.5), then blocked with 5% milk/TBS for 2 hr. The blots were then washed three times with TBS each for 2–5 min, then washed three times with 0.2% NP-40 TBS and then three times with TBS. The nitrocellulose membrane was then incubated with the primary antibody Rabbit anti-PKC polyclonal Ab (Upstate Technology, Lake Placid, NY) (1 μg/ml in 1% milk/TBS) for 2 hr. The washing step was repeated as above and the secondary Ab alkaline phosphatase conjugate was then added (Bio-Rad, Richmond, CA) (1:3000 dilution) for 2 hr. The washing procedure was then repeated, as mentioned, two times each. The color reagent (1 ml each of nitro blue tetrazolium and 5-bromo-4-chloro-3-Indolyl phosphate, 15 mg and 7.5 mg/ml, respectively, in 70% dimethylformamide) was added to 50 ml carbonate buffer (pH 9.8), and, when the bands were clearly visualized, the reaction was stopped by washing the blots several times with distilled water.

Measurement of Reverse Transcriptase Activity (RT). Two milliliters of cell-free culture supernatants, were precipitated overnight at 4°C with 30% polyethylene glycol and 0.13 *M* NaCl. The virus pellet was then solubilized and the RT activity was then assayed in a buffer made of 42 m*M* Tris-HCl (pH 7.8), 8.5 m*M* DTT, 10 m*M* $MgCl_2$, 3.4 m*M* NaCl, 25 μCi/ml

of [^{3}H]TTP (10–20 Ci/mmol; New England Nuclear Corp., Boston, MA), and 0.5 units/ml of oligo dT poly rA template primer (Pharmacia/P-L Biochemicals, Piscataway, NJ). The acid precipitable radiolabeled material was collected by suction filtration on glass fiber filters (GF/C) and was then washed with 5% TCA containing 2% tetrasodium pyrophosphate. The filters were then dried and the radioactivity was measured by a liquid scintillation counter as previously described (32).

Determination of Cytoplasmic Free Ca^{++} Levels. Intracellular Ca^{++} was measured with a fluorescence Ca^{++} indicator, FURA-2. Briefly 1×10^7 cells were loaded with 10 μl of 4 μ*M* FURA-2 AM (Molecular Probes Inc., Eugene, OR) in RPMI 1640, enriched with 3% of FCS, for 15 min in the presence of 10 μl of pluronic acid (Molecular Probes Inc.) at 37°C. The cells were then diluted 10-fold and incubated for another 15 min. The cells were spun down gently and resuspended in fresh RPMI media and kept in the dark at 37°C. Immediately before use, cells were spun down, washed twice, and then resuspended in 2 ml of a buffer containing 140 m*M* NaCl, 5 m*M* KCl, 20 m*M* HEPES, 5.5 m*M* glucose, 1 m*M* $CaCl_2$, 1 m*M* NaH_2PO_4, and 1 m*M* $MgSO_4$. The samples were transferred to prewarmed quartz cuvettes, and the intensity of fluorescence was measured by Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instrument, Inc., Naperville, IL) at emission and excitation wavelengths of 500 nm for the former and 340 and 380 for the latter. Maximum fluorescence (F_{max}) was determined by lysing the cells with 0.1% Triton X100, and minimum fluorescence (F_{min}) by adding 1 m*M* EGTA. The levels of intracellular free Ca^{++} concentration were calculated according to the method of Grynkiewicz *et al.*, as previously described (33).

PBMC Proliferation Assay. PBMCs were obtained from heparinized blood of normal blood donors or patients infected with HIV by layering onto Ficoll-Hypaque gradients and separating them by density centrifugation. Mononuclear cells were washed three times, and 2×10^5 cells were incubated in quadruplicates for blastogenic transformation assays in 96-well microtiter plates in 0.2 ml of RPMI 1640 supplemented with L-glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml) in the presence or absence of optimum concentrations of antigens and mitogens. Control wells for PHA had RPMI media, while wells for CMV Ag control had antigen prepared from uninfected cell cultures treated the same way as CMV-infected cultures. Supernatants of cultures incubated at 37°C in a 5% CO_2 atmosphere and treated with PHA 1:500 dilution of (Difco, Detroit, MI) or 1:40 dilution (Davis strain) prepared as previously described (7). CMV Ag-treated cultures were harvested at 72 hr and 7 days, respectively. This dose of PHA or CMV Ag used gave an optimum response in PBMCs from healthy controls at the indicated time from preliminary kinetic experiments. At 24 hr before harvest tritiated thymidine was added to each well (0.5 μCi/ml, specific activity 6.7 Ci/mmol; ICN Chemical and Radioisotope Div., Irvine, CA). Cells were then collected onto glass filter paper discs and washed with a semiautomated microharvesting device (Skatron Inc., Sterling, VA). The discs were air dried, placed in scintillation cocktail (Scintiverse; Fisher Scientific Co., Pittsburgh, PA) and [^{3}H]thymidine uptake was then determined by a Beckman LS9000 liquid scintillation counter.

Results

Effect of HIV on PKC Expression: Relationship to Protein Kinase C Activity in PBMCs. In the first set of experiments, the relationship of HIV infection to PKC activity was examined in PBMCs from a normal donor. To determine the PKC response during HIV infection, cells were mock infected or infected with HIV strain IIIB, and at 12 hr, 4 and 7 days PI, they were treated with 1 μ*M* of PMA, an activator of PKC, for 30 min, then were extracted for determination of PKC by Western blot analysis. A marked decrease of cytosolic PKC content was observed by Day 4 and Day 7 (Fig. 1). The intensity of the bands at 12 hr PI was not much different from the control cells. However, when total steady-state PKC activity of infected cells was examined 1 and 12 hr PI using a PKC assay in which a PKC-specific substrate is phosphorylated, an enhancement of 1.4- and 1.8-fold above the control was observed (Table I). Comparable effects were detected when cells from four different cell donors were used. The enhancement was also observed when two fresh patient isolates were used; however, the degree of enhancement was even higher, with 10.7- and 11.4-, and 2.8- and 3.8-fold above the control at 1 and 12 hr PI, respectively. Similar observations were noticed when another four isolates were independently tested. The enhancement of PKC activity by HIV was observed as early as 1 hr PI (Table I), indicating that membrane perturbation may be involved in this phenomenon. Thus, HIV infection apparently is associated with an initial phase of PKC activation during the first 24 hr PI that is followed by a sustained phase of inhibition at later times PI.

PKC Activity in PBMCs from Patients with HIV Infection. Next we examined the PKC response to PMA of PBMCs from patients with HIV infection. PBMCs from HIV-seropositive and -seronegative individuals were incubated in the presence or absence of 1 μ*M* of PMA for 30 min. Ten million cells where then extracted, and total PKC activity was determined. Steady-state PKC activities in PBMCs from the patients and their controls were 19.1 ± 5.5 and 19 ± 5 pmol/min, respectively. PMA failed to activate PKC in

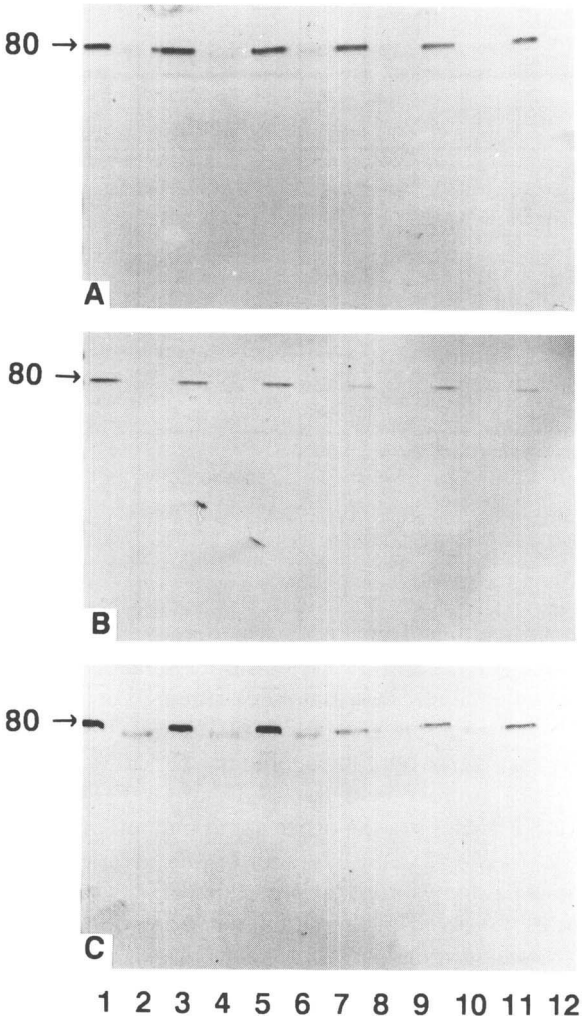

Figure 1. Western blot analysis of PKC levels in cytosolic and membrane fractions of normal and HIV-infected PBMCs using rabbit anti-PKC polyclonal antibody to PKC α isozyme. Lanes 1–6 show PKC in uninfected cells and Lanes 7–12 depict the protein in HIV-infected cells. Equal amounts (10 μg) of cytosolic and membrane protein samples were loaded in odd and even number lanes, respectively. Cells in Panel A were infected with HTLV IIIB for 12 hr. Those in Panel B were infected for 4 days, and those in Panel C for 7 days. RT activity was measured at Day 4 and 7 PI, and was 1.2 and 3.1 × 10^6, respectively. A representative of three independent experiments.

PBMCs in seven out of eight HIV-seropositive patients examined. The same dose enhanced PKC activity in PBMCs from normal HIV-seronegative donors by 50%–100% (Table II). Next the PKC content of PBMCs from HIV-infected patients was compared with healthy donor control cells (Fig. 2). Lanes 1–4 contain samples from two HIV patients with absolute T4 cell counts <250/mm^3. Lanes 5–8 were from PBMCs of two healthy HIV-seronegative donors. A marked decrease in patients' cytosolic PKC content was observed when compared with the control cells. The presence of 1 μ*M* PMA did not affect the levels of PKC in this patient population. No bands were observed when membrane fractions were analyzed in a parallel experiment. These findings indicate that PKC

Table I. Effect of HIV on PKC Activity in Human PBMCs

Treatment	PKC activity (pmol/min/5 × 10^6 cells)	Fold increase
Experiment 1[a]		
PBMCS	13.6 ± 1.5[d]	
PBMCS + HIV IIIB	19.1 ± 2.0	1.4
PBMCS + HIV_{852}[c]	145.6 ± 10.0	10.7
PBMCS + HIV_{789}[c]	154.6 ± 9.0	11.4
Experiment 2[b]		
PBMCs	4.5 ± 0.5[d]	
PBMCs + HIVIIIB	8.25 ± 1.0	1.8
PBMCs + HIV_{852}[c]	12.6 ± 2.3	2.8
PBMCs + HIV_{789}[c]	14.4 ± 3.0	3.2

[a] Determined at 1 hr PI. Equivalent infectious units of the different virus isolates were used in this experiment.
[b] Determined 12 hr PI.
[c] Wild-type isolates.
[d] PKC activity (Mean ± SD) of three samples.

Table II. Effect of PMA on PKC Activity in PBMCs from HIV-seropositive and -Seronegative Donors

Sample	T4/mm^3	HIV	PMA[a]	PKC activity (pmol/min/10^7 cells)
1	1015	+	−	20.0 ± 2.0[b]
		+	+	19.8 ± 1.5
2	518	+	−	8.9 ± 1.0
		+	+	13.8 ± 1.4
3	500	+	−	23.5 ± 2.3
		+	+	22.0 ± 2.1
4	560	+	−	19.0 ± 1.4
		+	+	19.0 ± 1.6
5	72	+	−	26.4 ± 3.0
		+	+	20.5 ± 2.5
6	16	+	−	21.5 ± 1.8
		+	+	13.6 ± 1.4
7	80	+	−	22.2 ± 4.0
		+	+	22.9 ± 3.0
8	85	+	−	12.0 ± 1.9
		+	+	10.0 ± 0.5
9	1200	−	−	14.0 ± 1.3
		−	+	24.8 ± 2.9
10	1100	−	−	26.9 ± 3.5
		−	+	53.3 ± 5.0
11	900	−	−	17.2 ± 1.4
		−	+	22.8 ± 2.1
12	1150	−	−	18.0 ± 1.6
		−	+	28.0 ± 2.3

[a] 1 μ*M* was added to viable cells for 30 min before PKC extraction.
[b] Mean ± SD of two samples.

production and activation are markedly affected in cells from AIDS patients as evidenced by the lack of response to PMA stimulation.

Effect of PMA and Ionomycin on the Proliferative Response of PBMCs from Patients with AIDS. Protein kinase C is involved in T-cell proliferation and the proliferative response of patients with AIDS to PHA has been shown to be impaired (6–8). To determine whether the impairment is due to lack of available intracellular free Ca^{++} required to activate PKC

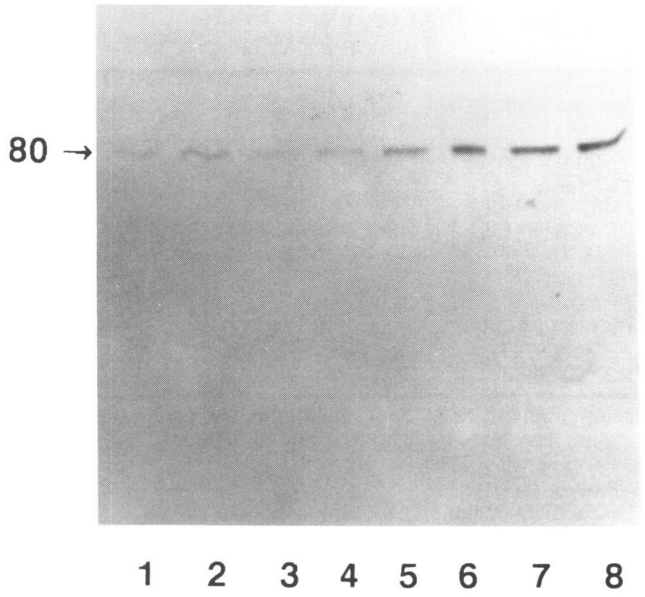

Figure 2. Western blot analysis of PKC in cytosolic fractions of PBMCs from two immunosuppressed AIDS patients. Lanes 1–4 show PKC content in patients, and Lanes 5–8 contain samples from control cells. Lanes 2, 4, 6, and 8 are from cells that were pretreated with 1 μ*M* of PMA. Additional PMA did not affect PKC levels.

in the presence of diacylglycerol (DAG), the effect of ionomycin (Ca^{++} ionophore) on PHA or CMV Ag-induced [^{3}H]thymidine uptake in patient's PBMCs were examined. Ionomycin did not enhance the proliferative response of the patient's cells (15 patients were examined with different degrees of immunosuppression), indicating that the lack of free Ca^{++} by itself could not account for the observed inhibition. Combining ionomycin with PMA (an analogue of DAG) at doses of 1–10 μ*M*, also did not enhance the PHA or the CMV Ag-induced proliferative response from the PBMCs of those patients (data not shown).

Effect of HIV on Intracellular Free Ca^{++} in PBMCs and CD4-Positive Cell Lines. Free Ca^{++} is an important trigger for T-cell activation, and is directly involved in the activation of PKC (34). Thus, we next examined the relationship of intracellular free Ca^{++} to HIV infection. Intracellular free Ca^{++} increased by about 40% and 50% in MT4 and JURKAT cells, respectively, in the first 12 hr PI (Table III). However, there was no difference in cellular free Ca^{++} between infected and uninfected cells at later times after infection (through Day 14) in both cell lines (data not shown). Similarly, infecting non-PHA-stimulated PBMCs with HIV increased intracellular free Ca^{++} by 125% from 100 n*M* in uninfected cells to 225 n*M* in HIV-infected cells 12 hr PI and to 300 n*M* (200%) by 1 week PI (Table III).

The Ca^{++} response of HIV-infected PBMCs to PHA is shown in Table IV. PHA was added for 5 min to the fura-2-loaded cells in the cuvettes, and then

Table III. Effect of HIV on Intracellular Free Ca^{++} in Human Cells

Sample	Time PI	Ca^{++}[a] (nm)	Percentage increase
Experiment 1			
JURKAT	12 hr	90 ± 5[b]	
JURKAT + HIV	12 hr	135 ± 7	50
MT4	12 hr	100 ± 5	
MT4 + HIV	12 hr	140 ± 9	40
Experiment 2			
PBMCs	12 hr	100 ± 10[b]	
PBMCs + HIV	12 hr	225 ± 15	125
PBMCs	1 week	110 ± 12	
PBMCs + HIV[c]	1 week	300 ± 28	200

[a] Measured by fura-2 AM.
[b] Mean of three samples ± SD, a representative of three independent experiments.
[c] Reverse transcriptase (RT) activity of culture supernatants were 1–2 × 10^5 CPM/ml.

peak intracellular free Ca^{++} was determined. At 1 hr PI, there was no difference in intracellular free Ca^{++} levels between infected and mock-infected cells. However, mock-infected cells at 12 hr and 1 week PI showed an increase of intracellular free Ca^{++} of 100% and 120% in response to 10 μg/ml of PHA. HIV-infected PBMCs, on the other hand, had an increase of 56% and 17% at the same intervals, respectively. These data suggest that HIV-induced Ca^{++} responses might be involved in the cellular hyporesponsiveness to external stimuli.

Intracellular Free Ca^{++} Response in PBMCs from Patients with HIV Infection. In the next group of experiments, we examined PBMCs from HIV-infected individuals with different degrees of immunosuppression (as determined by their absolute T4-cell count) for their Ca^{++} responses to PHA or antibody to OKT3. Patients were stratified into three groups: >500, 300–500, and <300 absolute T4 cells/mm^3. Eight, six, and five patients from the respective groups have been examined, as well as PBMCs from five normal individuals. The data indicate that patients with less than 250 absolute T4 cells had an impaired Ca^{++} response. On the other hand, patients with T4 cells >500/mm^3 had a response similar to that of PBMCs from normal individuals. The PBMCs of the 300–500 group, however, had a variable response with some showing a normal and some an impaired response. Figure 3 shows Ca^{++} traces from normal and HIV-infected patients. The data suggest that Ca^{++} could be involved in the pathogenesis of HIV-induced hyporesponsiveness in T lymphocytes.

Discussion

In this report, the relationship of intracellular free Ca^{++} and PKC activity in HIV infection was examined. The data indicated that the response of these

Table IV. Effect of HIV on PBMCs Intracellular Free Ca^{++} Response to PHA

Sample	Time PI	Ca^{++} (n*M*)		
		No PHA	PHA[a]	% change
Experiment 1				
PBMCs	1 hr	72 ± 4[b]	110 ± 10[b]	53
PBMCs + HIV	1 hr	74 ± 6	112 ± 12	51
Experiment 2				
PBMCs	12 hr	105 ± 10[c]	200 ± 12[c]	100
PBMCs + HIV	12 hr	220 ± 15	350 ± 10	56
PBMCs	1 week	110 ± 15	230 ± 10	120
PBMCs + HIV	1 week	300 ± 22	350 ± 15	17

[a] 10 μg/ml.
[b] Mean of peak Ca^{++} concentration of three samples ± SD. Represents two independent experiments.
[c] Mean of peak Ca^{++} concentration of three samples ± SD, a representative of three independent experiments.
[d] RT activity of culture supernatants were 8–1.5 × 10^6 CPM/ml.

parameters to external stimuli was impaired in PBMCs from HIV-infected patients. This would suggest that Ca^{++} and PKC are involved in HIV-induced T-cell hyporesponsiveness. This is particularly important since both participate early in the cascade of events leading to T-cell activation (10–12, 27, 35–37).

A biphasic pattern for PKC activity was observed, with an initial phase of activation followed by a phase of sustained inhibition (Table I). The initial phase of activity might be important for viral integration in the cellular genome. This step in the replicative cycle of HIV is dependent on cell activation. The inhibition phase was correlated with a concomitant decrease in PKC content in these cells at Day 4 and 7 PI (Fig. 1). This inhibition, however, was associated with viral replication and is possibly induced by the virus load and/or products of viral gene expression. The proliferative response to PHA, a measurement of T-cell activation, of PBMCs, from patients with HIV infection has been shown to be impaired (6–8). This response appeared dependent on the level of impairment induced by HIV infection. A link between the proliferative response and PKC is possible since a lack in the activity of the latter was associated with decreased expression of IL-2 and transferrin receptors with subsequent impairment of cell proliferation (38). However, attempts to restore the proliferative response, a marker for T-cell activation, of PBMCs from AIDS patients by ionomycin and PMA were not successful (data not shown). Moreover, the impaired PKC response to external stimuli was independent of the degree of immunosuppression. PKC did not respond to PMA, regardless of the absolute T4 cell count of the patients (Table II). However, there was no difference, in steady-state PKC activity, between normal and PBMCs from HIV-infected patients. This implies that the physiologic incompetence of the patient's cells (infected and uninfected) may be due to an imbalance of certain regulatory factors that feed back and inhibit the activation of PKC despite the availability of its inducers. Supporting this suggestion is the ability of the partially purified PKC obtained from the membranes or cytosol of HIV-infected cells or patients' PBMCs to phosphorylate its peptide substrates.

Steady-state intracellular free Ca^{++} was increased in cell lines only early after infection, however, in PBMCs from normal donors, it continued to increase through at least Day 7 PI. The progression of infection *in vitro* was accompanied by an impaired Ca^{++} response to PHA (Table IV). Although experiments performed on PBMCs from normal donors and cell lines indicated that there was a temporal coincidence between Ca^{++} and PKC activity in response to HIV infection, their patterns in PBMCs from HIV-infected patients did not always coincide. While the T4-cell counts did not affect the levels of PKC activity in PBMCs from HIV-infected patients, the Ca^{++} responses were intact among patients with higher T4-cell counts and were markedly diminished in cells from patients with less than 250 T4 cells/mm^3 (Fig. 3). This correlates with the *in vitro* data and further substantiates the suggestion that the lack of Ca^{++} could not be the sole factor responsible for PKC refractoriness. This might suggest the involvement of other factors that interfere with the activation of PKC despite the presence of Ca^{++} and PMA.

Increased intracellular Ca^{++} and activation of PKC are involved in the up-regulation of HIV expression in chronically infected cells stimulated by cytokines and other inducing agents (39). Additionally, gp 120 pretreated, tetanus toxoid-stimulated T-cell clones suppressed antigen-driven early activation signals, including PKC translocation, inositol phosphate turnover, and intracellular free Ca^{++} responses (40). It seems possible that the lack of Ca^{++} response in those with severe immunosuppression could be due to a lack of IP_3 secondary to inhibition of phospholipase C. The latter has been shown to be inhibited by cAMP

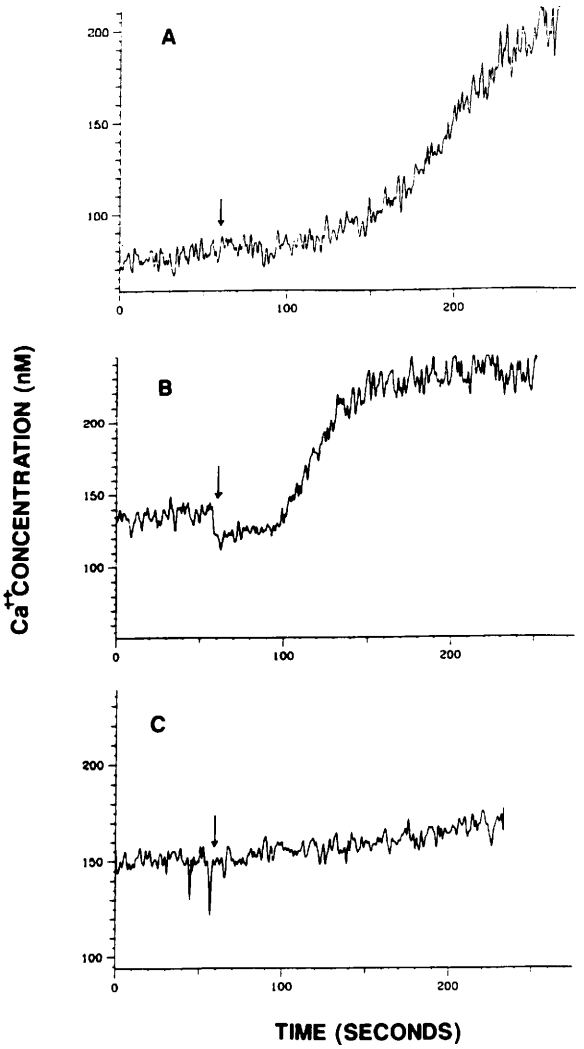

Figure 3. Intracellular free Ca^{++} response of PBMCs to PHA from (a) a normal individual, (b) HIV patient with >500 absolute T4 cells, and HIV patient with <250 T4 cells (c). The position of the arrows indicate the time of PHA addition. PHA was added to FURA-2-loaded cells in prewarmed cuvettes as described in Materials and Methods. These traces represent the pHA response of one individual from each of PBMCs from the different groups.

in other cell systems. HIV infection of CD4-positive cells has been reported to be associated with a sustained elevation of intracellular levels of cAMP (29, 41). Thus, it is not inconceivable that the HIV-induced increase in cAMP might feed back and inhibit the hydrolysis of PIP_2 in response to external stimuli like foreign antigens.

Taken together, the results of this study suggest that HIV-1 acts at multiple sites to alter PKC activity and intracellular free Ca^{++} levels, abrogating several distinct immune functions that are critical for an intact immune response and are defective in HIV-infected patients.

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