Inhibition of the Calcium Release-activated Calcium (CRAC) Current in Jurkat T Cells by the HIV-1 Envelope Protein gp160*

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The HIV-1 envelope glycoprotein gp120/160 has pleiotropic effects on T cell function. We investigated whether Ca\(^{2+}\) signaling, a crucial step for T cell activation, was altered by prolonged exposure of Jurkat T cells to gp160. Microfluorometric measurements showed that Jurkat cells incubated with gp160 had smaller (~40%) increases in [Ca\(^{2+}\)]\(_i\), in response to phytohemagglutinin and had a reduced Ca\(^{2+}\) influx (~25%). gp160 had similar effects on Jurkat cells challenged with thapsigargin. We used the patch clamp technique to record the Ca\(^{2+}\) current, which is responsible for Ca\(^{2+}\) influx and has properties of the calcium release-activated Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)). gp160 reduced I\(_{\text{CRAC}}\) by ~40%. The inhibitory effects of gp160 were antagonized by staurosporine (0.1 μM), an inhibitor of protein-tyrosine kinases and protein kinase Cs (PKCs), and by Gö 6976 (5 μM), an inhibitor acting especially on PKC\(\alpha\) and PKC\(\beta\). 12-O-Tetradecanoyl phorbol 13-acetate (16 nm), a PKC activator, reproduced the effects of gp160 in untreated cells. A Western blotting analysis of PKC isoforms α, β, δ, and ζ showed that only the cellular distribution of PKC\(\alpha\) and -β were significantly modified by gp160. In addition, gp160 was able to modify the subcellular distribution of PKC\(\alpha\) and PKC\(\beta\) caused by phytohemagglutinin. Therefore, the reduction in I\(_{\text{CRAC}}\) caused by prolonged incubation with gp160 is probably mediated by PKC\(\alpha\) or -β.

The functions of CD4\(^+\) T cells are impaired early in HIV-1 infection (1–2) at a time when very few (less than 1%) cells appear to be productively infected (2–3). This might be due to the multiple effects of virus proteins such as HIV-1 Tat (4), virus protein R (5–6) and gp120, which are released by infected cells. The major HIV-1 surface glycoprotein, gp120, is present at a high concentration in peripheral lymphoid organs (7) and, thus, may interact with uninfected cells. HIV-1 gp120 and its precursor gp160 have several effects on T lymphocytes. These include membrane depolarization (8), altered protein-tyrosine kinase p56\(^{lck}\) activity (9–12), and down-modulation of cell surface CD4 molecules (13). The gp120 protein also causes changes in PKC activity and in the intracellular free Ca\(^{2+}\) concentration (14–16) of T lymphocytes and opens Ca\(^{2+}\)-inward currents in astrocytes (17). The induction of gp120 but not that of gp120 has also been reported to increase the intracellular free Ca\(^{2+}\) concentration in CD4\(^+\) cells (18). The alterations in Ca\(^{2+}\) signaling due to gp120/160 (14–16) are particularly noteworthy because of the role of Ca\(^{2+}\) in T cell activation and proliferation (19–20). Last, gp120 alters the apoptotic/proliferative processes in T cells (12, 21–22).

Activation of T cell antigen receptors by antigens or mitogens such as the lectin phytohemagglutinin (PHA) leads to a biphasic rise in [Ca\(^{2+}\)]\(_i\), resulting from an initial inositol 1,4,5-trisphosphate-dependent release of Ca\(^{2+}\) from intracellular stores followed by an influx of Ca\(^{2+}\) across the plasma membrane (for review, see Ref. 23). Recent studies suggest that the Ca\(^{2+}\) influx across the plasma membrane is carried by a store-operated Ca\(^{2+}\) current (24) whose biophysical properties are very similar to those of the calcium release-activated calcium current (I\(_{\text{CRAC}}\)) of mast cells (Ref. 25, for review, see Ref. 20). Thus the sustained rise in [Ca\(^{2+}\)]\(_i\), which leads to T cell proliferation (15, 26–27), requires an appropriate I\(_{\text{CRAC}}\). Impairment of the Ca\(^{2+}\) influx has been reported to be responsible for severe immunodeficiency (28). We have therefore investigated the prolonged effects of HIV gp120/160 protein on (i) the intracellular Ca\(^{2+}\) response to PHA and the reticulum Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, (ii) the calcium release-activated calcium current I\(_{\text{CRAC}}\), and (iii) the subcellular distribution of several PKC isozymes in Jurkat T cells.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Jurkat E6.1 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 mM l-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin (all from Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO\(_2\) as previously described (8). HIV-1 NL/MAI gp160 glycoprotein was provided by Dr. Kiely (Transgene, Strasbourg, France) and Dr. El Habib (Aventis, Marcy l’Etoile, France). Cells were grown in medium containing 25 μg/ml gp160 for 5 days. This concentration depolarizes Jurkat cell membranes and decreases the voltage-gated K\(^+\) current (8). In PKC subcellular distribution studies, PHA 10 μg/ml was added to the cell culture media for 30 min.

All reagents and chemicals were from Sigma except Gö 6976 (Calbiochem). Herbimycin A, staurosporine, 12-O-tetradecanoyl phorbol 13-acetate (TPA), okadaic acid, and Gö 6976 were added 30 min before recording CRAC currents.

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‡ The abbreviations used are: HIV-1, type 1 human immunodeficiency virus; I\(_{\text{CRAC}}\), calcium release-activated calcium current; PKC, protein kinase C; TG, thapsigargin; Ca\(^{2+}\) \(_{i}\), cytosolic free Ca\(^{2+}\) concentration; PHA, phytohemagglutinin; fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-amino1,4-benzofuran-5-oxyl-2-(2′-amino-5′-methylphenoxo)-ethane-N,N′,N″,N‴-tetraacetic acid; fura 2-AM, fura 2 acetoxymethyl ester; TPA, 12-O-tetradecanoyl phorbol 13-acetate.
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All anti-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-goat secondary antibodies coupled to peroxidase were from Sigma.

Intracellular free Ca\(^{2+}\) Concentration ([Ca\(^{2+}\)\(_i\)]) Measurements—Cells were first allowed to adhere to a 0.17-mm thick, poly-D-ornithine-coated glass coverslip inserted into a laboratory-made perfusion chamber (~15-μl volume). Cells were rinsed twice with PBS, then incubated in phosphate-buffered saline supplemented with 1% bovine serum albumin (Invitrogen) and 4 μM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. Then cells were superfused with saline containing 160 mM NaCl, 4.5 mM KCl, 2 mM Ca\(^{2+}\)Cl\(_2\), 1 mM MgCl\(_2\), 5 mM Hepes, pH 7.25 KOH, plus 20 μM fura-2 (Molecular Probes). Nine free Ca\(^{2+}\) concentrations (1–705 nM) were generated by adding appropriate amounts of EGTA according to stability constants for all reactions between Ca\(^{2+}\), Mg\(^{2+}\), H\(^+\), and EGTA (29–30).

Electrophysiology—Cells for patch clamp recordings (31) were adhered to a poly-o-ornithine-coated plastic culture dish for 10 min. Each dish was then rinsed twice with a Ca\(^{2+}\)-free saline containing 160 mM NaCl, 4.5 mM KCl, 2 mM Mg\(^{2+}\)Cl\(_2\), 5 mM Hepes and adjusted to pH 7.4 with NaOH. Pipettes were pulled from borosilicate glass capillaries (GC150, Clark Electromedical Instruments, Pangbourne Reading, England) coated with Sylgard\(^\text{TM}\) (Dow Corning, Midland, MI) and fire-polished. The pipette tip resistance was 5–10 M\(\Omega\) megahms. Membrane currents were recorded with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA). Voltage clamp protocols were implemented, and data were acquired with pClamp 6.0 software (Axon Instruments). Capacitative currents were cancelled.

\(I_{\text{CRAC}}\) was measured in the whole-cell configuration using the following pipette solution: 140 mM cesium aspartate, 2 mM Mg\(^{2+}\)Cl\(_2\), 10 mM EGTA, 10 mM Hepes, pH 7.25/2.5 CO\(_2\) (calculated free Ca\(^{2+}\) concentration ~ 1 nM). At the disruption of the membrane patch, the holding potential (\(V_h\)) was set at 0 mV to minimize basal Ca\(^{2+}\) influx. \(I_{\text{CRAC}}\) was induced by passively depleting intracellular Ca\(^{2+}\) stores (24–26, 32–33), by incubating cells in Ca\(^{2+}\)-free solution for 10 min, and by dialyzing its interior with a Ca\(^{2+}\)-free solution. A 200-msec voltage-protocol (–100 to +50 mV) was used to test for voltage dependence. \(I_{\text{CRAC}}\) was also evoked by applying a 100-mV step during 200 msec. \(I_{\text{CRAC}}\) time course was monitored over a 10-min time range by measuring \(I_{\text{CRAC}}\) 10 ms after pulse imposition every 2 s (Figs. 3B and 4A). The time courses were fitted by a Weibull equation with five parameters using SigmaPlot 5.0 software (SPSS ASC, Erkrath, Germany).

Membrane potential (\(V_m\)) values were estimated by a non-invasive method (34). The pipette solution contained 140 mM KCl, 5 mM NaCl, 10 mM EGTA, 5 mM Hepes, pH 7.25 KOH. A –200 to +100 mV ramp of 500 ms was applied in the cell-attached configuration. Under the condition used, the equilibrium potential for K\(^+\) across the membrane patch was close to 0 mV, and the potential value corresponding to zero current was taken as an estimate of \(-V_m\). This technique allowed us to monitor \(V_m\) in intact cells.

Protein Extraction—After corresponding treatment, Jurkat cells at 5 × 10\(^5\) cells/ml were centrifuged at 900 × g during 10 min. The pellet was resuspended in 0.5 ml of buffer A (20 mM Tris–HCl, pH 7.5, 0.25 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 tablet of Complete\(^\text{TM}\)/10 ml (Mini protease inhibitor from Roche Molecular Biochemicals). The suspension was sonicated for 5 min and centrifuged at 39,000 rpm, 4 °C, for 1 h. The supernatant was used as the cytosol fraction. The pellet was resuspended in buffer A plus 1% Triton X-100 and mixed on ice. The suspension was centrifuged at 39,000 rpm, 4 °C, for 1 h. This supernatant was used as the Triton-soluble membrane fraction. The second pellet was resuspended in buffer B (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 tablet of Complete\(^\text{TM}\)/10 ml, 0.1% sodium deoxycholate, 0.1% SDS plus 1 tablet of Complete\(^\text{TM}\)/10 ml), mixed for 10 min at 4 °C, and then centrifuged at 39,000 rpm, 4 °C, for 1 h. The supernatant was used as Triton-insoluble fraction. Protein concentrations were determined using the Folin reaction and bovine serum albumin as a standard.

Immunoblotting—Equal amounts of cytosolic, membrane, and Triton-insoluble fractions (30 μg) were solubilized in an electrophoresis buffer (66 mM Tris–HCl pH 6.8, 10% glycerol, 2.5% SDS, 0.1% bromphenol, and 2% β-mercaptoethanol). The proteins were subjected to 8%polyacrylamide gel electrophoresis. Gels were calibrated using precasted molecular mass standards (Bio-Rad). After electrophoresis, proteins were transferred to 0.45-μm polyvinylidene difluoride membranes (Immobilon-P, Sigma). Then the membranes were blocked by incubation in phosphate-buffered saline containing 1% milk and 1% bovine serum albumin for 1 h at room temperature. The membranes were washed and incubated in blockade buffer plus FRC isoyzme antibody at 1:500 overnight at 4 °C. The blots were then washed three times in phosphate-buffered saline containing 1% milk and 1% bovine serum albumin and incubated with an anti-rabbit or anti-goat secondary antibody linked to peroxidase. The blots were washed three times in phosphate-buffered saline. Western blots were revealed with ECL plus (Amersham Biosciences, Inc.). Autoradiograms were analyzed and quantified by Scion Image software (Scion Corporation, Frederick, MA).

Statistical Analysis—Values are the means ± S.E. Student’s unpaired t test was used. A p value < 0.05 was considered significant. n values represent the number of cells investigated, and N is the number of independent microfluorimetric experiments performed.

RESULTS

PHA-induced [Ca\(^{2+}\)], Increase in Untreated and gp160-treated Jurkat Cells—The resting [Ca\(^{2+}\)]\(_i\), values in untreated (68 ± 21 nM, n = 241, N = 5) and gp160-treated (45 ± 24 nM, n = 150, N = 5) Jurkat cells in the presence of 2 mM external Ca\(^{2+}\) were not statistically different. The [Ca\(^{2+}\)]\(_i\), of both cell groups increased ~1 min after adding 10 μg/ml PHA, but the magnitudes of the responses were clearly different (Fig. 1, A and B). In untreated cells (Fig. 1A), [Ca\(^{2+}\)]\(_i\), reached a plateau at 458 ± 79 nm within 13 ± 1 min (n = 125, N = 2), whereas [Ca\(^{2+}\)]\(_i\), in gp160-treated cells (Fig. 1B) reached a plateau at a significantly (p < 0.01) lower level of 276 ± 55 nm after 6 ± 0.5
min (n = 75, n = 2). These plateau levels were dramatically dependent on the presence of external CaCl₂ (Fig. 1, A and B), which is consistent with a PHA-induced entry of Ca²⁺ (26); thus, [Ca²⁺], decreased immediately when external Ca²⁺ was removed. The calcium influx rate was measured for the first 10–20 s after adding extracellular Ca²⁺ back to the bath (Fig. 1, A and B). During this period, [Ca²⁺] was still low, and the Ca²⁺ pump activity was greatly reduced (35). gp160 reduced the Ca²⁺ influx by 25%; it was 1 ± 1 nA/s in gp160-treated cells (n = 75) and 14.5 ± 1 nA/s in untreated cells (n = 125, p < 0.05).

Thapsigargin-induced [Ca²⁺], Increase in Untreated and gp160-treated Jurkat Cells—The reduction of Ca²⁺ influx caused by gp160 may be due to an effect on the CRAC channels that are responsible for Ca²⁺ entry or to an alteration in the PHA transduction signal. We bypassed a possible alteration of the T cell antigen receptor complex by gp160 by testing the effects of thapsigargin (TG), which inhibits Ca²⁺ re-uptake by the endoplasmic reticulum and opens CRAC channels (32).

The changes in Ca²⁺ in response to TG were first measured in the absence of external CaCl₂. Under this condition, basal [Ca²⁺], values in untreated cells (17 ± 8 nM, n = 134, N = 3) and in gp160-treated cells (15 ± 7 nM, n = 84, N = 3) were not different, and adding 1 μM TG caused similar transient [Ca²⁺], increases in the two cell groups, indicating that gp160 did not affect internal Ca²⁺ stores (Fig. 1, C and D). Thus, [Ca²⁺], reached a peak of 99 ± 20 nM (n = 134, N = 3) in untreated cells (Fig. 1C) and 106 ± 20 nM (n = 84, N = 3) in gp160-treated cells (Fig. 1D). The delays (1.6 ± 0.4 min for control and 1.5 ± 0.4 min for gp160-treated cells) were similar to those in the presence of external Ca²⁺ (Fig. 1, A and B). This agrees with the fact that the initial rise in [Ca²⁺], after mitogen stimulation in the presence of external Ca²⁺ is due to a release of Ca²⁺ from the endoplasmic reticulum (32). As also shown in Fig. 1, C and D, [Ca²⁺], spontaneously returned to its basal concentration after 4.1 ± 0.5 min (n = 134, N = 3) in untreated cells and after 4.5 ± 0.7 min (n = 84, N = 3) in gp160-treated cells in the absence of external Ca²⁺. This suggests that gp160 did not alter the activity of the plasma membrane Ca²⁺-ATPases, as extrusion of Ca²⁺ from the cytosol is essentially due to plasma membrane Ca²⁺-ATPases in the presence of TG.

Adding 2 mM CaCl₂ to the bath in the continued presence of 1 μM TG induced strikingly different responses in untreated and gp160-treated cells (Fig. 1, C and D, [Ca²⁺]), peaked at 583 ± 66 nM and had a value of 399 ± 15 nM (n = 134, N = 3, Fig. 1C) 7 min after Ca²⁺ re-addition in untreated cells, but the peak was only 405 ± 31 nM, and the value at 7 min was 161 ± 14 nM (n = 84, N = 3, Fig. 1D, p < 0.01) in gp160-treated Jurkat cells. The calcium influx rate measured after adding extracellular Ca²⁺ back to the bath in the presence of TG was reduced by 31% in gp160-treated cells (9 ± 1.5 nA/s (n = 84) versus 13 ± 2 nA/s (n = 134), p < 0.05). This reduction was in the same range as those obtained in PHA experiment. This indicates that gp160 reduces the Ca²⁺ influx by acting at some point downstream of the binding of PHA to cell surface receptors.

CRAC Current Properties in Jurkat Cells—IₙCRAC was induced by passively depleting intracellular stores (see "Experimental Procedures"). Hyperpolarization of Jurkat cell membranes evoked a weak inward current in the absence of external Ca²⁺ (Fig. 2A). Adding 10 mM external CaCl₂ resulted in the appearance of an inward, rapidly inactivating current (Fig. 3A) that was further increased by 20 mM external CaCl₂. The opening of the inward current was independent of voltage (Fig. 2B). This current was inhibited by the Ca²⁺ current blockers, 5 mM Ni²⁺ ions and 1 mM Cd²⁺ ions (Fig. 2C) (36). These inhibitors act on the same current because their effects

Fig. 2. Properties of IₙCRAC in control Jurkat cells. A, whole-cell currents elicited by a 100-mV hyperpolarizing pulse were recorded in the presence of the extracellular CaCl₂ concentrations given on the right hand side of each trace (in mM). The pulse protocol is shown at the top of the traces. B, whole cell currents elicited by the ramp protocol (see "Experimental Procedures") in the absence (−Ca²⁺) and in the presence (+Ca²⁺) of 10 mM external CaCl₂. C, the inward Ca²⁺ current obtained in the presence of 2 mM external CaCl₂ was blocked by 5 mM NiCl₂ (left) and by 1 mM CdCl₂ (right).

Fig. 3. Effect of gp160 on IₙCRAC kinetics in Jurkat cells. A, the peak current evoked by a −100-mV pulse was plotted against time to monitor the change in the current over 5 min. The inward current increased rapidly upon adding 10 mM CaCl₂ (black bar) and then decreased slowly. Inactivation was more rapid in gp160-treated than in untreated Jurkat T cells. These recordings are representative of five experiments. B, histograms for CRAC peak current (open bar) and half-inactivation time (black bar) in gp160-treated (n = 5) and untreated (n = 5) Jurkat T cells. *p < 0.05, significant differences between gp160-treated and untreated cell IₙCRAC intensity. §, p < 0.05, significant difference between gp160-treated and untreated cell IₙCRAC half-inactivation time.

are not additive (not shown). These kinetic and pharmacological characteristics identified this current as IₙCRAC (25). The time course of IₙCRAC showed slow inactivation. IₙCRAC, measured by a hyperpolarization pulse, reached a peak of −39.6 ± 5.1 pA in 28 ± 1 s (n = 5) after adding 10 mM CaCl₂ and then slowly decreased to close to the initial basal level in ~6 min (352 ± 64 s, n = 5, half-inactivation time of 106 ± 12 s, see Fig. 3A). The peak IₙCRAC obtained in the presence of 20 mM CaCl₂ was ~36% higher than the IₙCRAC obtained in the presence of 10 mM CaCl₂ (~53.7 ± 6.4 pA), and it was reached in half the time (12 ± 2 s, n = 5, not shown). The IₙCRAC slow inactivation was accelerated, as the current returned to its basal amplitude in less than 2 min (82 ± 22 s, n = 5, not shown).
Fig. 4. Implication of PKC in the gp160-induced decrease in $I_{\text{CRAC}}$. The peak current evoked by a $-100$-mV pulse was plotted against time to monitor the change in the current over 5 min and determine the maximal peak current and the time course of inactivation. A, peak current recording from gp160-treated Jurkat cells in control condition (solid line) and after exposure for 30 min to TPA (16 nM, dotted line) or staurosporine (0.1 μM, dashed line). B, histograms for CRAC peak current (open bar) and half-inactivation time (black bar) in gp160-treated Jurkat T cells showing the effects of staurosporine (stauro., 0.1 μM), TPA (16 nM), and okadaic acid (OA) (1 μM) (n = 5–6); *, p < 0.05, significant difference between corresponding treatment and control $I_{\text{CRAC}}$ intensity. §, p < 0.05, significant difference between corresponding treatment and control $I_{\text{CRAC}}$, half-inactivation time. C, peak current recording from untreated Jurkat cells under control conditions (solid line) or after exposure for 30 min to TPA (16 nM, dotted line) or staurosporine (0.1 μM, dashed line). D, histograms for CRAC peak current (open bar) and half-inactivation time (black bar) in untreated Jurkat T cells showing the effects of staurosporine (stauro., 0.1 μM), TPA (16 nM), and okadaic acid (OA) (1 μM) (n = 5–6). E, CRAC current recorded from gp160-treated Jurkat cells under control conditions (solid line) or after exposure for 30 min to Gö 6976 (5 μM, dashed line). F, histograms for CRAC peak current (open bar) and half-inactivation time (black bar) in untreated and gp160-treated Jurkat cells showing the effects of Gö 6976 (n = 4–5).

Reduction in the CRAC Current of Jurkat Cells after gp160 Treatment—The $I_{\text{CRAC}}$ properties of Jurkat cells incubated with gp160 for 5 days were drastically altered (Fig. 3A). The time to reach the peak was shorter (14 ± 5 s, n = 5) than in untreated cells (28 ± 1 s, n = 5, p < 0.01). The peak $I_{\text{CRAC}}$ in the presence of 10 mM external Ca$^{2+}$ was reduced by ~40% (24.6 ± 3.9 pA, n = 5 versus 39.6 ± 5.1 pA, p < 0.02), and the half-inactivation time was reduced by ~38% (106 ± 12 s, n = 5 versus 12 s, Fig. 3B).

We investigated the mechanisms by which gp160 decreased $I_{\text{CRAC}}$ by adding a variety of phosphorylation modulators to the bath solution for 30 min before the patch clamp measurements. Neither genistein (100 μM), a general protein-tyrosine kinase inhibitor, nor herbimycin A (10 μM), a specific inhibitor of src-family protein-tyrosine kinase (for example, p56$^{\text{Lck}}$), had any effect on $I_{\text{CRAC}}$ in gp160-treated Jurkat cells (genistein (-22.2 ± 2 pA (n = 5)) and herbimycin A (-20.1 ± 3.5 pA (n = 5)) versus -24.6 ± 3.9 pA (n = 5)) or in untreated cells (genistein (-35.8 ± 1 pA (n = 5)) and herbimycin A (-39.4 ± 2.8 pA (n = 5)) versus 39.6 ± 5.1 pA (n = 5)), indicating that the effects of gp160 are not directly mediated by p56$^{\text{Lck}}$ (not shown).

Staurosporine (0.1 μM), a PKC and protein-tyrosine kinase inhibitor, increased the peak $I_{\text{CRAC}}$ in gp160-treated Jurkat cells (Fig. 4, A and B, n = 5, p < 0.02); the time needed to reach the current peak was longer (32 ± 3 s) than in controls (14 ± 3 s, n = 5, p < 0.01), and the return of $I_{\text{CRAC}}$ to its basal level was slower (Fig. 4, A and B, p < 0.01). Thus, staurosporine antagonized all the effects of gp160 on $I_{\text{CRAC}}$. The profound effects of staurosporine and the ineffectiveness of genistein and herbimycin A strongly suggest that a PKC-mediated phosphorylation is involved in the decrease of $I_{\text{CRAC}}$ by gp160. Adding TPA (16 nM), a PKC activator, to gp160-treated cells had no effect on the time course of $I_{\text{CRAC}}$ (Fig. 4, A and B, peak current of -23.9 ± 2.1 pA, n = 5, p = 0.32).

Measurements of $I_{\text{CRAC}}$ in untreated Jurkat cells also implicate a PKC-mediated phosphorylation in the regulation of this current. Staurosporine (0.1 μM) had no effect on $I_{\text{CRAC}}$ intensity in untreated cells (p = 0.23, Fig. 4, C and D). In contrast, 16 nM TPA significantly decreased $I_{\text{CRAC}}$ in untreated cells (p < 0.02, Fig. 4, C and D) and accelerated the slow inactivation. Okadaic acid (1 μM), which inhibits phosphatases PP1 and PP2A, thus favoring phosphorylation activity, had effects similar to those of TPA and decreased $I_{\text{CRAC}}$ (p < 0.01, Fig. 5D) in untreated cells. Conversely, this inhibitor had no effect on $I_{\text{CRAC}}$ (-19.9 ± 5 pA, n = 5, p = 0.19, Fig. 4B) in gp160-treated cells.

The above results indicate that $I_{\text{CRAC}}$ is reduced in gp160-treated cells via a staurosporine-sensitive PKC pathway. We investigated the effects of Gö 6976, which is considered as inhibiting preferentially PKCα and PKCβ1 (37–38). This agent is interesting because PKCα is the predominant isoform in Jurkat cells, and PKCβ1 has been implicated in $I_{\text{CRAC}}$ down-modulation (39). On gp160-treated cells, Gö 6976 (5 μM) increased the time to reach the peak current after the addition of 10 mM CaCl$_2$ (Fig. 4E) and slowed current inactivation (Fig. 4F). In contrast, Gö 6976 had no effect in untreated cells (Fig. 4F).
Subcellular Distribution of Protein Kinase C Isozymes—Because HIV-1 envelope protein disturbs the activity of PKC (14–16), an immunoblotting analysis was performed to investigate the translocation of several PKC isozymes from the cytosol to the membrane and the Triton-insoluble fraction in response to gp160 treatment, PHA stimulation, and PHA stimulation after gp160 treatment. The PKCs were chosen according to their subgroups, which are classical (α, β), novel (δ, and atypical (ζ)). Two independent experiments have been done for the four PKCs under all conditions. The PKCs and β appeared at an apparent molecular mass of 80 kDa. PKCδ antibody detected two bands with apparent molecular masses of 95 and 110 kDa and the antibody against PKCζ, two bands of 74 and 80 kDa. The results show that gp160 induced large alterations of the cellular distribution of PKCα and PKCβI (Fig. 5, A and B), whereas PKCδ and PKCζ were less affected (Fig. 5A).

In untreated cells, PKCα was detected predominantly in the cytosol (~92% of protein amount), with a weak amount in the membrane (~4%) and the Triton-insoluble fraction (~4%, Fig. 5, A and B). In gp160-treated cells, the total amount of PKCα was increased by ~25%. The newly synthesized protein appeared in the membrane fraction (~7-fold increase) and, to a lesser extent, in the Triton-insoluble fraction (~3-fold increase), since the amount of cytosolic protein was unchanged. PHA treatment (30 min) had no effect on PKCα total amount. However, PHA induced a translocation of PKCα from cytosol to the membrane (~6-fold increase) and Triton-insoluble fractions (~2.5-fold increase). Interestingly, gp160 antagonized the effect of PHA by reducing the amount of PKCα in the membrane (Fig. 5, A and B).

PKCβI was only detected in cytosolic and Triton-insoluble fractions. In untreated cells, ~75% of PKCβI was detected in the Triton-insoluble fraction, and ~25% was detected in cytosol. gp160 induced a 3-fold increase in total PKCβI amount; thus, the cytosolic and Triton-insoluble fraction amounts increased by ~400% and ~150%, respectively (Fig. 5, A and C). In contrast, PHA, which did not modify the total amount of protein, induced a 14-fold increase of protein amount in the cytosol and the almost complete disappearance of the Triton-insoluble PKCβI. gp160 antagonized the effect of PHA by preventing the disappearance of PKCβI from the Triton-insoluble fraction (Fig. 5, A and C).

PKCδ was only detected in cytosolic and Triton-insoluble fractions. In untreated cells, 90% of PKCδ was cytosolic (~% for the 110-kDa band, ~% for the 95-kDa band). gp160 treatment had no significant effect on total PKCδ amount but induced a slight increase in the Triton-insoluble fraction, keeping the two isoforms in the same proportions. PHA had no effect per se but could partly counteract the effect of gp160; PHA reduced the Triton-insoluble fraction and slightly increased the cytosolic fraction.

The PKCζ was only detected in the cytosol under the form of
the hyperpolarization of gp160-treated cells was transient (Fig. 6). Adding 10 μM PHA rapidly caused membrane hyperpolarization (Fig. 6) that reached a plateau at −87 ± 4 mV (ΔVm = −25 ± 4 mV, n = 5) in 4 ± 1 min (n = 5). Hyperpolarization was maintained in the presence of PHA throughout the recording time (~11-min check, Fig. 6). The resting Vm of gp160-treated cells (Vm = −50 ± 3 mV, n = 29; p < 0.05) was significantly less negative as compared with untreated cells. Although PHA hyperpolarized gp160-treated cells by −30 ± 5.5 mV (n = 5) within in 3.2 ± 0.8 min (n = 5), the hyperpolarization of gp160-treated cells was transient (Fig. 6). Vm rapidly returned to its resting value within 7.3 ± 1.3 min (n = 5) in the continued presence of PHA.

**Inhibition of ICRAC by gp160**—PHA triggers dramatically different Ca2+ responses in gp160-treated and control cells. Although untreated cells showed a 400 nM increase in [Ca2+]i, sufficient for IL-2 synthesis and cell activation (41), [Ca2+]i increased by only half that amount in gp160-treated cells. A short incubation of untreated Jurkat cells with the phorbol-ester PKC activator, had a reduced increase in [Ca2+]i, showing that Jurkat cells incubated with PMA, another phorbol-ester PKC activator, had a reduced increase in [Ca2+]i, after activation by anti-CD3 antibody or TG.

There are at least 11 PKC isoforms with different requirements for lipid and Ca2+, and the PKC profile varies from 1 cell type to another (46). Our results mainly implicate the classical PKCα and βII into the modulation of ICRAC by gp160. First, the electrophysiological experiments showed that Gø 6976 impaired the gp160-induced decrease of ICRAC. Gø 6976 is a more specific classical PKC inhibitor (37–38). Thus, gp160 seems to decrease CRAC channel activity by increasing the PKC activity. Indeed, a short incubation of untreated Jurkat cells with the phorbol-ester TPA, a broad spectrum activator of classical and novel PKC isozymes, mimicked the effects of gp160 and reduced the CRAC current. This agrees with a study by Haverstick et al. (39) showing that Jurkat cells incubated with PMA, another phorbol-ester PKC activator, had a reduced increase in [Ca2+]i, after activation by anti-CD3 antibody or TG.

**DISCUSSION**

Inhibition of ICRAC by gp160—PHA triggers dramatically different Ca2+ responses in gp160-treated and control cells. Although untreated cells showed a 400 nM increase in [Ca2+]i, sufficient for IL-2 synthesis and cell activation (41), [Ca2+]i increased by only half that amount in gp160-treated cells. A qualitatively comparable attenuation was reported in human CD4+ T cells challenged with antigen in the presence of gp120 (15). It is well recognized that gp120/160 interacts with cell surface receptors and disturbs the T cell antigen receptor/CD3/CD4/p56lck transduction machinery (9, 15). But this interaction cannot explain the present effect of gp160, since a similar result was obtained when TG was used in the presence of extracellular Ca2+. Indeed, as a specific inhibitor of Ca2+ re-uptake into the endoplasmic reticulum, causes a large Ca2+ response in lymphocytes that is independent of translocation signals.

The attenuated increase in [Ca2+]i, in the presence of gp160 appears to be due to a decreased Ca2+ entry rather than to an increased Ca2+ pumping out of the cytosol. TG caused the same increase in [Ca2+]i, in untreated and gp160-treated cells in the absence of bath Ca2+. In addition, Ca2+ extrusion from the cytosol in the presence of TG is essentially due to the Ca2+ ATPases of the plasma membrane, since Ca2+ uptake into the endoplasmic reticulum is inhibited. Under extracellular Ca2+-free conditions, [Ca2+]i, returned to its basal value with the same time course in gp160-treated and untreated cells, suggesting that gp160 does not modify the activity of the plasma membrane Ca2+-ATPases. In contrast, the Ca2+ influx, as measured when extracellular Ca2+ was added back to the bath, was 25% lower in gp160-treated cells than in untreated cells. Direct measurement confirmed that incubating Jurkat cells with gp160 reduces ICRAC, the current responsible for Ca2+ entry. Thus, gp160 was able to accelerate the CRAC current inactivation, resulting in ICRAC intensity decrease and limited entry of Ca2+ ions.

**Inhibition of ICRAC Is a PKC-mediated Process**—Several signal pathways inhibit ICRAC, depending on the cell type; they are the FcγRII receptor in B lymphocytes (42), sphingosine and several camadins in Jurkat T cells (43), and PKC in mast cells (44) and Jurkat T cells (39). A PKC-dependent process also seems to be involved in the reduction of ICRAC by gp160. Although herbimycin A and genistein (protein-tyrosine kinase inhibitors) had no effect on the gp160-induced reduction of CRAC, staurosporine, an inhibitor of the classical and novel PKCs and to a lesser extent of protein-tyrosine kinases (45), restored the ICRAC of gp160-treated cells to control values. A similar result was obtained with Gø 6976, a more specific classical PKC inhibitor (37–38). Thus, gp160 seems to decrease CRAC channel activity by increasing the PKC activity. Indeed, a short incubation of untreated Jurkat cells with the phorbol-ester TPA, a broad spectrum activator of classical and novel PKC isozymes, mimicked the effects of gp160 and reduced the CRAC current. This agrees with a study by Haverstick et al. (39) showing that Jurkat cells incubated with PMA, another phorbol-ester PKC activator, had a reduced increase in [Ca2+]i, after activation by anti-CD3 antibody or TG.

**gp160 Alters the Subcellular Distribution of PKC Induced by PHA**—In our experiments, the stimulation of ICRAC by PHA, induced by the depletion of internal calcium stores and, thus, was independent of PHA. Therefore the inhibitory effect of the PKC on ICRAC is not related to an alteration of the PHA transduction pathway. However, immunoblotting experiments suggest that gp160, besides inhibiting ICRAC, also modulates the PHA transduction pathway. PHA is known to induce the activation of
Ca²⁺-dependent PKCs (49). In our hands, the addition of PHA for 30 min mainly modified the distribution of PKCa, the main isozyme in Jurkat cells, and PKCβ1. We observed that gp160 pretreatment profoundly altered the effects of PHA on these two isozymes. After exposure to gp160, PHA was no longer able to induce PKCa translocation to the membrane, thus preventing the activation of this PKC. Using an antisense strategy, Lopez-Lago et al. (50) showed that inhibition of PKCα impairs the expression of IL-2 receptor, tumor necrosis factor-α production, and the induction of IL-2 gene in stimulated Jurkat cells. gp160 also prevented the major effect of PHA on PKCβ, which is the disappearance of the protein from the Triton-insoluble fraction. The meaning of this observation is not clear since Koterek et al. (51) have previously shown that PKCβ is not necessary to IL-2 secretion in Jurkat cells.

**Conclusion**—The decreases in CRAC (this study) and Kv1.3 currents (8) by HIV-1 gp160 may be considered within the context of T cell activation and proliferation. Mitogens cause an increase in Ca²⁺ concentration in both gp160-treated and untreated cells that is sufficient to hyperpolarize the membrane via the opening of Ca²⁺-dependent K⁺ channels (52). However, the reduced \( I_{\text{CRAC}} \) and its accelerated inactivation result in a lower \([\text{Ca}^{2+}]_i\) elevation (at about 250 nm) that cannot support the activity of Ca²⁺-dependent K⁺ channels (52).² The driving force for Ca²⁺ is reduced as a result of the lower activity of both Kv1.3- and Ca²⁺-dependent K⁺ channels. In addition, we have shown that HIV-1 gp160 alters the PKC activation pattern caused by PHA. Altogether, this may impair the activation of Ca²⁺-binding proteins and the synthesis of IL-2 and eventually diminish T cell activation and proliferation.

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² O. Delis, unpublished results.
Inhibition of the Calcium Release-activated Calcium (CRAC) Current in Jurkat T Cells by the HIV-1 Envelope Protein gp160

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