A Role for Protein Kinase CβI in the Regulation of Ca\(^{2+}\) Entry in Jurkat T Cells*

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T cell activation leading to cytokine production and cellular proliferation involves a regulated increase and subsequent decrease in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). While much is understood about agonist-induced increases in [Ca\(^{2+}\)]\(_i\), less is known about down-regulation of this pathway. The understanding of this down-regulation is critical to the prevention of cell death that can be the consequence of a sustained elevation in [Ca\(^{2+}\)]\(_i\). Protein kinase C (PKC), activated by the diacylglycerol produced as a consequence of T cell receptor engagement, has long been presumed to be involved in this down-regulation, although the precise mechanism is not wholly clear. In this report we demonstrate that activation of PKC by phorbol esters slightly decreases the rate of Ca\(^{2+}\) efflux from the cytosol of Jurkat T cells following stimulation through the T cell receptor or stimulation in a receptor-independent manner by thapsigargin. On the other hand, phorbol ester treatment dramatically reduces the rate of Ca\(^{2+}\) influx following stimulation. Phorbol ester treatment is without an effect on Ca\(^{2+}\) influx in a different T cell line, HSB. Down-regulation of PKCβI expression by 18-h phorbol ester treatment is associated with a loss of the response to acute phorbol ester treatment in Jurkat cells, suggesting that PKCβI may be the isozyme responsible for the effects on Ca\(^{2+}\) influx. Electroporation of an anti-PKCβI antibody, but not antibodies against PKCα or PKCγ, led to an increase in the rate of Ca\(^{2+}\) influx following stimulation. Taken together, these data suggest that PKCβI may be a component of the down-regulation of increases in [Ca\(^{2+}\)]\(_i\), associated with Jurkat T cell activation.

Activation of T lymphocytes via stimulation through the T cell receptor for antigen (TCR) leading to proliferation, cytokine production, or effector function requires a regulated increase and subsequent decrease in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). The biochemical events associated with activation of T lymphocytes and leading to Ca\(^{2+}\) entry have been extensively studied (1). Engagement of the TCR leads to the activation of phospholipase C, which subsequently cleaves phosphatidylinositol bisphosphate into inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) binding to an intracellular receptor, induces the release of Ca\(^{2+}\) from an intracellular storage depot (2). In a receptor-independent manner, the Ca\(^{2+}\)/ATPase inhibitor thapsigargin (3) causes an uncompensated leak of Ca\(^{2+}\) from this internal storage pool. In T lymphocytes, as well as in most electrically nonexcitable cells, this depletion of the intracellular storage pool induces the opening of the plasma membrane Ca\(^{2+}\) entry pathway and permits influx of extracellular Ca\(^{2+}\) (1). Previously, we have provided evidence that the pathway between release of intracellular Ca\(^{2+}\) and influx of extracellular Ca\(^{2+}\) is mediated by Ca\(^{2+}\)-activated calmodulin (4), which permits Ca\(^{2+}\) entry carried by a current we have called \(I_\text{C}\) (5).

The DAG produced subsequent to TCR stimulation is the naturally occurring activator of a family of serine-threonine kinases known collectively as protein kinase C (PKC). It has been proposed that DAG, via activation of PKC, represents the down-regulatory arm of Ca\(^{2+}\) signaling initiated by IP\(_3\) generation (for example, see Ref. 6). Four points in Ca\(^{2+}\) signaling have been implicated as targets for PKC action. First is a PKC-mediated phosphorylation of the \(\gamma\) subunit of the TCR-CD3 complex and subsequent down-regulation of this complex at the surface of the cell (7, 8). Second is the PKC-dependent phosphorylation of phospholipase C, with a consequent reduction in phospholipase C activity and IP\(_3\) generation (9). Third is the phosphorylation of the plasma membrane Ca\(^{2+}\)/ATPase, leading to its activation and a facilitation of efflux of Ca\(^{2+}\) (10). Depending upon the methodology used and the cell type examined, activation of PKC can (11) or cannot (12) be shown to affect the rate of Ca\(^{2+}\) efflux following stimulation-induced increases in [Ca\(^{2+}\)]\(_i\). The fourth potential target for a PKC effect is on the rate of Ca\(^{2+}\) influx itself, as suggested for HPB-ALL cells (13). In this report, we show that in Jurkat T cells the rate of Ca\(^{2+}\) influx is reduced by approximately 50% following activation of PKC.

The PKC family consists of at least 11 isozymes that vary in their requirements for lipid and Ca\(^{2+}\) for activation and are differentially expressed in various cell types (reviewed in Refs. 14 and 15). The study of the functions of this family of isozymes in cellular regulation has been facilitated by the use of phorbol esters, such as phorbol myristate acetate (PMA), which activate PKC, although with little specificity for any individual isozyme (14). In the past, the lack of isoform-specific inhibitors (16) has made assignment of a PKC effect to a specific isoform difficult. However, the recent development of some isoform-specific inhibitors (17) and the use of technology such as RNA aptamers to block transcription of individual isozymes (18) is...
FIG. 1. Increases in [Ca2+]i in Jurkat cells are reduced in the presence of phorbol ester. Changes in [Ca2+]i were monitored spectrofluorometrically as described under "Materials and Methods." A, Jurkat cells were stimulated with 100 nM thapsigargin at 90 s in the absence (solid line) or presence of the indicated concentrations of PMA added 60 s prior to thapsigargin. B, Jurkat cells were stimulated with 100 nM thapsigargin at 30 s. PMA (0–100 nM) was added at 150 s. C, cells were stimulated with 1 μg/ml of anti-CD3 antibody (OKT3) in the absence (solid line) or presence of the indicated concentration of PMA added 30 s prior. D, cells were stimulated at 60 s with OKT3. PMA (0–100 nM) was added at 150 s. Traces are means of three determinations and are representative of five experiments.

MATERIALS AND METHODS

Cell Lines—Jurkat E6.1 and HS B cells were purchased from ATCC (Rockville, MD). Cells were maintained in RPMI 1640 (BioWhitaker, Walkersville, MD) containing 5% fetal bovine serum (Hyclone, Logan, UT), SerXtend (Irvine Scientific, Santa Ana, CA), and glucose (Bio- Whitaker, Walkersville, MD) at 37 °C in a CO2 incubator.

Reagents—Indo-1 and BAPTA were purchased from Molecular Probes (Eugene, OR). Anti-PKC isoform-specific antibodies and the peptides used for immunization were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Unless otherwise indicated, all other reagents were purchased from Sigma.

Intracellular [Ca2+]i Measurements—Cells at a concentration of 4 × 10^6/ml were incubated with 1 μM indo-I/AM for 1 h at 37 °C in culture medium. Cells were washed three times in buffer A (10 mM HEPES, pH 7.4, 3 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 140 mM NaCl, 0.1% glucose, 1% fetal bovine serum) and suspended at a final concentration of 1 × 10^6/ml in buffer A. The (Ca2+)i, was determined from the fluorescence ratio (398/480 nm) with excitation at 360 nm in an SLM 8100 spectrofluorometer (SLM/Aminco, Urbana, IL) in the T format as described previously (4, 22). Calibration was conducted as described previously (22, 23) using the equation developed for an earlier generation of Ca2+ indicator dyes (24, 25).

Western Blotting—Cells were lysed in SDS-polyacrylamide gel electrophoresis sample buffer and 50 μl, representing 5 × 10^6 cells, were applied to a 10% acrylamide gel. Proteins were then transferred to blotting membrane and probed for isoform expression using enhanced chemiluminescence (ECL) detection (Amersham Corp.) according to the manufacturer’s directions.

Electroporation of Antibodies—The protocol for electroporation was based on that published by Lukas et al. (26). Briefly, cells were washed three times in buffer B (10 mM PO4, pH 7.4, 150 mM NaCl) and suspended to a final concentration of 2 × 10^6/ml. Cells (50 μl) were mixed with 50 μl of antibody at 100 μg/ml in buffer B plus 0.1% NaN3 and 0.2% gelatin (as supplied by Santa Cruz Biotechnology) and placed in the electroporation chamber. For those experiments using antibody plus peptide, 50 μl of buffer B or 50 μl of peptides (200 μg/ml in buffer B plus 0.1% NaN3 and 200 μg/ml bovine serum albumin as supplied by Santa Cruz Biotechnology) was mixed with the anti-PKC antibodies and incubated on ice for 30 min prior to the addition of 50 μl of cells to the electroporation chamber. Electroporation (CellPorator, Life Technologies, Inc., Bethesda, MD) was carried out with the following settings: high resistance, 270 V, and 800 microfarads. These settings were chosen based on testing of multiple settings and balancing percentage of cell survival with antibody incorporation (determined by Western blotting and immunofluorescence; data not shown). Cells were then removed from the electroporation chamber, suspended to 2 × 10^6/ml in culture medium, and placed in a CO2 incubator for a period of recovery (6–18 h) prior to use. Controls include cells mixed with antibody but not electroporated or cells electroporated in the absence of antibody.

Immunofluorescence—Cells subjected to the electroporation procedure and prepared for use in experiments to measure changes in [Ca2+]i, were allowed to settle on poly-t-lysine-coated microscope slides. The cells were lightly permeabilized by incubation with 9% buffered formalin and washed three times in buffer B. Anti-PKC antibody was visualized by incubating the slide with fluorescein isothiocyanate-labeled goat anti-rabbit antibody. For the images presented in Fig. 8, all fluorescence fields were photographed using T-Max 400 film (Eastman Kodak Co., Rochester, NY) using a 16-s exposure time. Phase contrast images were taken of the same fields immediately thereafter using a 5-s exposure time.

Statistics—All statistical analyses were performed using GraphPad Prism or Microsoft Excel.

RESULTS

Effects of Phorbol Ester on Agonist-induced Changes in [Ca2+]i—In most electrically nonexcitable cells, the Ca2+ entry pathway can be opened by depletion of the intracellular Ca2+ storage depot (1). In T cells, the Ca2+ entry pathway can be opened in a receptor-dependent or -independent manner, using, respectively, an activating monoclonal antibody to the T cell receptor (OKT3) or thapsigargin (4). The effects of activation of PKC by the phorbol ester PMA on these two methods of opening the Ca2+ entry pathway are shown in Fig. 1. The addition of PMA prior to stimulation of Jurkat cells with thapsigargin results in a concentration-dependent decrease in the maximum [Ca2+]i, reached (Fig. 1A). Fig. 1B demonstrates that the inhibitory effect of PMA also occurs once the Ca2+ entry pathway has been opened. The addition of PMA at a time when the storage pool has refilled but the Ca2+ entry pathway is still...
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open (4) results in a concentration-dependent reduction of [Ca$^{2+}$]. Similar results were observed when the Ca$^{2+}$ entry pathway was opened in a receptor-dependent manner using the anti-CD3-specific antibody OKT3 (Fig. 1, C and D). Previously, it has been reported that such inhibitory effects of PMA are not seen in all T lymphocytes (19). Therefore, a different T cell line, HSB, was examined as well. This human T cell line has lost expression of the T cell receptor for antigen; thus, the Ca$^{2+}$ entry pathway can only be opened by thapsigargin treatment. As shown in Fig. 2, there was no effect of up to 3 μM PMA on the changes in [Ca$^{2+}$] induced by thapsigargin in this cell line.

It has been proposed that the decreased magnitude of agonist-stimulated changes in [Ca$^{2+}$], after PMA pretreatment is due to a PKC-dependent phosphorylation and inactivation of phospholipase C (9). Such a phosphorylation would result in decreased production of IP$_3$ with a corresponding reduction in the amount of Ca$^{2+}$ released from the intracellular storage depot. To measure the amount of Ca$^{2+}$ released from the intracellular storage pool without interference from Ca$^{2+}$ influx, extracellular Ca$^{2+}$ was chelated with EGTA prior to stimulation. Consistent with inhibition of phospholipase C, prior PMA treatment results in a reduction in the amount of Ca$^{2+}$ released from the intracellular storage pool following receptor stimulation in Jurkat cells (Fig. 3A). However, the same inhibition of Ca$^{2+}$ release is seen with thapsigargin treatment (Fig. 3C). Since thapsigargin increases [Ca$^{2+}$], without the involvement of the T cell receptor, it seems unlikely that this latter effect is due to inhibition of phospholipase C. Whatever the site of PKC action is, no significant effect on [Ca$^{2+}$] was observed when PMA was added after stimulation of Ca$^{2+}$ release in the absence of Ca$^{2+}$ influx (Fig. 3, B and D).

**Phorbol Ester Treatment Specifically Affects Ca$^{2+}$ Influx in Jurkat Cells**—It has been proposed that activation of PKC results in activation of the plasma membrane Ca$^{2+}$ pump, such that there is a more rapid efflux of intracellular Ca$^{2+}$ following PMA treatment (10). Additionally, it is possible that activation of PKC inhibits Ca$^{2+}$ influx. To examine which of these mechanisms might be active in Jurkat cells, it was necessary to measure influx and efflux separately. To measure Ca$^{2+}$ pump activity, cells were stimulated to open the Ca$^{2+}$ entry pathway, and then extracellular Ca$^{2+}$ was rapidly chelated by the addition of 3 mM BAPTA to the extracellular medium (Fig. 4A). At this point, the rate at which Ca$^{2+}$ returns to base line is a reflection of the activity of the Ca$^{2+}$ pump, since there is no Ca$^{2+}$ entry. Because the rate of pump activity is dependent primarily upon the [Ca$^{2+}$] (27), a calculation of the exponential rate of decay was used to provide an estimate of pump activity; i.e. calculation of the $t_1/2$ for the return of Ca$^{2+}$ to basal levels can be used to compare efflux under various experimental conditions (Fig. 4B).

Unlike a calcium channel blocker such as Ni$^{2+}$ (4), chelation of extracellular Ca$^{2+}$ with BAPTA does not close the Ca$^{2+}$ entry pathway. Therefore, readdition of extracellular Ca$^{2+}$ to the cell suspension (Fig. 4A) allows for the measurement of entry specifically during the first 10–15 s after readdition of Ca$^{2+}$ (27).
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100 nM PMA added at zero time.

Figure 4. Measurement of Ca\(^{2+}\) influx and Ca\(^{2+}\) efflux in Jurkat cells. Jurkat cells were incubated with indo-1 and monitored for changes in [Ca\(^{2+}\)], as outlined under "Materials and Methods." A, thapsigargin (100 nM) was added at 30 s, and the change in [Ca\(^{2+}\)], was monitored until 250 s, at which time 3 mM BAPTA was added to the cuvette to chelate extracellular Ca\(^{2+}\). After [Ca\(^{2+}\)], had returned to base line, 3 mM CaCl\(_2\) was added to the cuvette (350 s). The solid line represents the experiment conducted in the absence of prior PMA treatment; the dashed line represents the experiment conducted with 100 nM PMA added at zero time. B, calculation of the rate of Ca\(^{2+}\) efflux. The rate of the return of Ca\(^{2+}\) to base line was measured using the [Ca\(^{2+}\)], values from 250 to 290 s. The solid line represents the [Ca\(^{2+}\)], measurements, and the dashed line represents the curve calculated to fit these data. C, calculation of the rate of Ca\(^{2+}\) influx. The rate of rise in [Ca\(^{2+}\)], measured from 350 to 375 s, was calculated using linear regression analysis of the data. The solid line represents the [Ca\(^{2+}\)], measurements; the dashed line represents the line calculated to fit these data. This figure is representative of the experiments used to generate the data of Fig. 5.

is governed by [Ca\(^{2+}\)], during this time when [Ca\(^{2+}\)], is low, pump activity will be greatly reduced. Therefore, a straight line function can be applied, and the rate of Ca\(^{2+}\) influx in nmol/s can be determined and compared under the various experimental conditions (Fig. 4C).

Influx and efflux rates in the presence and absence of PMA were calculated from several independent experiments (Fig. 5). PMA caused a slight, but reproducible, decrease in the rate of influx (measured at 154 s) of Ca\(^{2+}\) efflux, measured during the 30 s following the addition of BAPTA in Jurkat cells (Fig. 5B). In paired experiments, this difference ranged from a 13 to 23% change but was not, overall, statistically significant. However, PMA caused a pronounced, statistically significant decrease in the rate of Ca\(^{2+}\) influx measured in Jurkat cells during the 15 s following readdition of extracellular Ca\(^{2+}\). This reduction ranged from 37 to 56% when thapsigargin was used and up to 71% when cells were stimulated through the T cell receptor with OKT3 (Fig. 5A).

Because the release of Ca\(^{2+}\) from the internal stores is reduced in the presence of PMA (Fig. 3), it is possible that this reduction in the rate of influx is merely a consequence of reduced release from internal stores. However, when the rate of influx was measured in cells stimulated with a concentration of OKT3 that resulted in a 50% reduction in the amount of Ca\(^{2+}\) released from internal stores (0.1 \(\mu\)g/ml of OKT3, data not shown), the rate of influx was not different from the rate of influx in the presence of the maximally effective concentration of 1 \(\mu\)g/ml of OKT3 (83–102 nmol/s with 1 \(\mu\)g/ml OKT3 versus 98–114 nmol/s using 0.1 \(\mu\)g/ml OKT3, n = 3).

As a control for the calculations of influx and efflux, the same experiment was performed using HSB cells, which show no response to PMA on thapsigargin-induced changes in [Ca\(^{2+}\)]. No differences in Ca\(^{2+}\) influx or efflux were observed between cells that were treated with 100 nM PMA and those that were not (Fig. 5, right bars). For HSB cells, influx rates in the presence of PMA varied from 82 to 105% of control, and efflux rates varied from 93 to 110% of control.

In many cell types, treatment with PMA for 12–18 h ablates the effects of acute PMA addition; under most circumstances, this is due to extensive proteolytic degradation of PKC (28). To determine whether this same phenomenon occurred in Jurkat cells, cells were treated for 18 h with 250 nM PMA, and the effects of acute PMA addition on stimulated changes in [Ca\(^{2+}\)], were examined. Fig. 6 demonstrates that the rapid return to basal levels that occurs following acute PMA addition (Fig. 6A) was lost in cells that had been treated overnight with PMA (Fig. 6B). Similarly, when influx and efflux were measured as in Fig. 4, the effect of PMA on the rate of Ca\(^{2+}\) influx was completely lost in cells that had been treated for 18 h with PMA prior to stimulation (Fig. 6C).

Involvement of PKC\(_{\beta1}\) in the Effects of PMA on Ca\(^{2+}\) Levels—As outlined above, PKC is a family of serine-threonine kinases, and although the isozymes appear to be differentially sensitive to PMA within any single cell type, there is little consistency among various cell types (29, 30). To determine whether the lack of phorbol ester-induced inhibition of Ca\(^{2+}\) influx in HSB cells was due to the absence of a certain PKC isozyme, isozyme expression in Jurkat and HSB cells was compared. As shown in Table 1, although there were some differences in the level of expression of a specific isozyme (for exam-
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**Fig. 6.** The effect of PMA treatment on agonist-induced changes in [Ca\(^{2+}\)], in cells treated for 18 h with PMA. A, Jurkat cells, incubated for 18 h with vehicle (0.01% ethanol), were monitored for changes in [Ca\(^{2+}\)], following OKT3 treatment at 30 s. PMA (100 nM) was added at 130 s. B, the same experiment as in A, except that cells had been incubated with 250 nM PMA for 18 h, and 3 \(\mu\)M PMA was added at 130 s. Traces are means of three determinations and representative of four experiments. C, influx (right bars) and efflux (left bars) of Ca\(^{2+}\) were measured in the absence (open bars) and presence (hatched bars) of the prior addition of 100 nM PMA in Jurkat cells that had been treated for 18 h with 250 nM PMA prior to use.

Because the effect of acute PMA addition was ablated by 18 h of PMA exposure, the Western blot analyses were repeated in Jurkat and HSB cells that had been incubated for 18 h with 250 nM PMA. Expression of PKCa, -\(\beta III\), and -\(\zeta\) were unchanged following this treatment (data not shown). However, PKC\(\beta I\) expression was markedly reduced following PMA treatment in Jurkat cells but only minimally affected in HSB cells (Fig. 7). This lack of an effect of PMA in HSB cells is similar to that observed by others (31, 32).

Since isozyme-specific inhibitors (16) are not commercially available, the effect of electroporation of isozyme-specific antibodies on Ca\(^{2+}\) entry was examined to garner additional support for a role for PKC\(\beta I\) in inhibition of Ca\(^{2+}\) entry. Jurkat cells were electroporated in the presence of isozyme-specific antibodies as outlined under “Materials and Methods.” As shown in Fig. 8, cells mixed with antibody but not electroporated showed no incorporation of antibody (Fig. 8A). However, electroporation resulted in a reasonably uniform incorporation of antibody, as visualized by immunofluorescence (Fig. 8B). Prior incubation of antibody with the peptide used for immunization did not affect the incorporation of antibody (Fig. 8C).

**Table I**

**PKC isozyme expression by Jurkat and HSB cells**

Cells were lysed and proteins were separated by polyacrylamide gel electrophoresis as outlined under “Materials and Methods.” Proteins were transferred to blotting membrane, incubated with isozyme-specific anti-PKC antibodies, and visualized with ECL reagents. Relative signal intensity (greater to lesser) is designated by +++, +, and −. − indicates no antibody binding detected.

| Isozyme-specific antibody | Jurkat | HSB |
|--------------------------|--------|-----|
| \(\alpha\)               | ++     | +   |
| \(\beta I\)              | +      | +   |
| \(\eta\)                 | ++     | +++ |
| \(\theta\)               | +      | +   |
| \(\zeta\)                | +      | +   |
| \(\delta\)               | −      | −   |
| \(\epsilon\)             | −      | −   |
| \(\gamma\)               | −      | −   |

**Fig. 7.** Down-regulation of PKC\(\beta I\) expression with overnight PMA treatment. Cells were lysed with SDS-polyacrylamide gel electrophoresis sample buffer, and proteins were resolved on a 10% acrylamide gel; proteins were transferred and blotted for PKC\(\beta I\) expression as described under “Materials and Methods.” Left arrows, molecular weight markers; lane A, Jurkat cells incubated for 18 h with vehicle (0.01% ethanol); lane B, Jurkat cells incubated for 18 h with 250 nM PMA; lane C, HSB cells incubated overnight with vehicle (0.01% ethanol); lane D, HSB cells incubated for 18 h with 250 nM PMA; right arrow, PKC\(\beta I\) marker.
The effects of electroporation were more striking when the influx of Ca\(^{2+}\) was examined. In individual experiments, a 25–40% increase in the rate of Ca\(^{2+}\) influx was observed in cells electroporated in the presence of anti-PKC\(\beta_i\) compared with a less than 5% decrease in Ca\(^{2+}\) influx in cells electroporated in the presence of anti-PKC\(\alpha\) or anti-PKC\(\gamma\) (Fig. 10A).

Additionally, the effect of PMA treatment on Ca\(^{2+}\) influx was lost in cells electroporated in the presence of anti-PKC\(\beta_i\), while the PMA effect was still seen in cells electroporated with anti-PKC\(\alpha\) or anti-PKC\(\gamma\). As with the effects of anti-PKC\(\beta_i\) antibody shown in Fig. 9, the effects of anti-PKC\(\beta_i\) on Ca\(^{2+}\) influx could be blocked by a 2-fold mass excess of the peptide used for immunization but were not blocked by the peptide used for the production of anti-PKC\(\alpha\) antibody (Fig. 10A).

**DISCUSSION**

Control of intracellular free Ca\(^{2+}\) concentration is a critical component of cellular homeostasis for all cell types. Increases in [Ca\(^{2+}\)]\(_i\), subsequent to receptor stimulation are necessary for the regulated progression of most cells through the cell cycle (33), for appropriate expression of gene products such as cytokines in T lymphocytes (1), and for some T cell effector functions, such as perfisin-dependent cell killing by cytolytic T lymphocytes (34). Equally necessary is the regulated decrease with a return to prestimulation calcium levels subsequent to any increase. The consequence of a sustained increase in [Ca\(^{2+}\)]\(_i\), is generally cell death, often due to induction of apoptosis (21).

Changes in [Ca\(^{2+}\)]\(_i\), subsequent to engagement of the T cell receptor for antigen are initiated by release of Ca\(^{2+}\) from an internal storage depot mediated by the IP\(_3\) generated by receptor-induced activation of phospholipase C. This release of stored Ca\(^{2+}\) is followed by an influx of extracellular Ca\(^{2+}\). This process has been called by a number of names in electrically nonexcitable cells, such as capacitative calcium entry and the storage-operated calcium entry pathway (1). Regardless of the nomenclature, most current models for this component of cellular signaling indicate a feed forward mechanism.

In general, PKC activation, mediated by the other product of phospholipase C, DAG, has been associated with down-regulation of this pathway, although the data are not wholly consistent. The discrepancies may be due to differences in methodologies employed as much as to differences in cell lines examined. For example, in mixed populations of peripheral blood lymphocytes, phorbol ester treatment has been shown to have either an inhibitory (12) or augmenting (8) effect on receptor-mediated changes in [Ca\(^{2+}\)]\(_i\). These opposing results may be due to the relative state of differentiation of the cells as at least one study has implied (19). The data are more consistent when cell lines are examined. When changes in [Ca\(^{2+}\)]\(_i\) are examined in Jurkat cells using fluorescent dyes, stimulation through the TCR with prior activation of PKC is associated with a reduction in the magnitude of increases in [Ca\(^{2+}\)]\(_i\) (this report and Refs. 8, 11, and 35). These results are in contrast to stimulation of Jurkat cells with a megengic lectin, such as concanavalin A, where there appear to be no effects of PKC activation on the subsequent changes in [Ca\(^{2+}\)]\(_i\) (35).

In addition to controversy over the effects of PKC activation, multiple targets of PKC in the Ca\(^{2+}\) entry pathway have been proposed. While in both a murine T lymphoma line (9) and in neutrophils (7, 36) phosphorylation and down-regulation of receptors by PKC has been implicated, this observation cannot explain the effect of PMA on receptor-independent changes in
ments for activation of the various isozymes suggest that specific functions are associated with each. Isozyme-specific functions in T cells were recently elegantly demonstrated in a study examining a subclone of Jurkat cells transfected with constitutively active expression vectors for different isozymes (39). Regulation of some transcription factors was enhanced in cells that overexpressed PKCβ but not PKCζ (39).

Several observations in the present study support a role for PKCζ in inhibition of Ca^{2+} influx. First, although PKCζ isozyme expression did not significantly differ between Jurkat cells that respond to PMA and HSB cells that did not, PKCζ was down-regulated by long term PMA treatment only in Jurkat cells (Fig. 8). Second, this long term PMA treatment abolished the PMA-induced inhibition of Ca^{2+} influx (Fig. 7). Third, electrophoration of an antibody to PKCζ, but not an antibody to PKCα or PKCγ, reduced the rate of Ca^{2+} influx and abolished the PMA effect (Fig. 10).

The presence of PKCζ in HSB cells seems difficult to reconcile with the failure of these cells to exhibit PMA-induced down-regulation of [Ca^{2+}], if this isozyme is responsible for the effect. However, the failure of PMA to down-regulate this isozyme in HSB cells (Fig. 7) suggests that PMA may fail also to activate the isozyme in these cells. This is consistent with previous reports suggesting that HSB cells fail to respond to PMA (31, 32). Other possible explanations for the failure of these cell to down-regulate [Ca^{2+}], despite the presence of PKCζ include a defective enzyme and altered or missing downstream substrates.

Collectively, the results of these and other studies provide support for a closed loop pathway initiated by engagement of the TCR in human lymphocytes. Following TCR ligation and activation of phospholipase C, IP_3 induces the release of Ca^{2+} from the intracellular stores. This released Ca^{2+} activates calmodulin, which initiates the influx of extracellular Ca^{2+} (23), carried by a current we have named I(T) (5). This feed forward portion of the pathway is down-regulated by DAG-activated PKCζ, which inhibits Ca^{2+} influx. Whether or not this inhibition is by an action on I(T) is currently under investigation.

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Fig. 10. Influx and efflux of Ca^{2+} in Jurkat cells electroporated in the presence of anti-PKC antibodies. Jurkat cells were electroporated in the absence or presence of the indicated anti-PKC isozyme-specific antibody without or with immunization peptides as indicated. Influx (A) and efflux (B) of Ca^{2+} were measured following stimulation with 1 μg/ml of OKT3 as outlined in Fig. 4 and under “Results.” Data represent means of triplicate determinations in four experiments.

[Ca^{2+}], Similarly, a reduction in IP_3 generated due to a PKC-dependent phosphorylation of phospholipase C (9) fails to explain the observed reduction in release of Ca^{2+} from internal stores following thapsigargin treatment (Fig. 3).

The effects of PKC activation on Ca^{2+} influx versus efflux also have been examined. In peripheral blood lymphocytes, no effect on efflux following PMA treatment was seen when a straight function line was applied to the decrease in [Ca^{2+}], following chelation of extracellular Ca^{2+} (12). However, in Jurkat cells, activation of PKC was associated with enhanced efflux of Ca^{2+} when a nonlinear, biexponential calculation was used (11). Efflux of Ca^{2+} has been associated with a pump mechanism, most likely the plasma membrane Ca^{2+}/ATPase (27). The activity of the pump has been shown to be dependent upon the intracellular concentration of Ca^{2+} (27), which we believe makes this application of a nonlinear function more appropriate. The observation of a PMA effect on Ca^{2+} efflux is confirmed by the data in this report, although the magnitude of the change was slightly less in the current study than observed in the previous one (11), perhaps due to the different curve fitting method used in this study.

There is more consistency when the effects of PKC activation on Ca^{2+} influx are examined. In nonlymphoid cells, PKC activation was associated with inhibition of Ca^{2+} influx when the current induced by Ca^{2+} influx was measured (37). In another study, a current others (38), but not ourselves (5), have implicated as the mediator of storage pool depletion-induced Ca^{2+} entry was reduced in the presence of activated PKC (38). Consistent with these observations, we observed a 30–50% inhibition of the rate of Ca^{2+} influx in Jurkat cells following PKC activation with PMA (Fig. 5).

Some of the discrepancies reported among various cell types may be due to differences in the expression of individual isozymes of PKC. Differences in the Ca^{2+} and lipid require-
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