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Response of Alkalinization or Acidification by Phytohemagglutinin is Dependent on the Activity of Protein Kinase C in Human Peripheral T Cells

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Abstract The increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) and protein kinase C (PKC) activity are two major early mitogenic signals to initiate proliferation of human T cells. However, a rapid change in intracellular pH (pH_i), acidification or alkalinization during the activation, is also associated after these two signals. The aim of this study was to define whether the change in pH_i is affected by calcium and protein kinase C (PKC), in phytohemagglutinin (PHA)-stimulated T cells. T cells were isolated from human peripheral blood. The $[Ca^{2+}]_i$ and the pH_i were measured using, respectively, the fluorescent dyes, Fura-2, and BCECF. In addition, down-regulation of PKC activity by PMA (1 μM, 18 h) was confirmed in these cells using a protein kinase assay. The results indicated that, (1) alkalinization was induced by PHA or PMA in T cells; the results of alkalinization was PKC-dependent and Ca^{2+}-independent, (2) in PKC down-regulated T cells, PHA induced acidification; this effect was enhanced by pre-treating the cells with the Na^+/H^+ exchange inhibitor, 5-(N,N-dimethyl)-amiloride, (DMA, 10 μM, 20 min), (3) the acidification was dependent on the Ca^{2+} influx and blocked by removal of extracellular calcium or the addition of the inorganic channel blocker, Ni^{2+}, and (4) Thapsigargin (TG), a Ca^{2+}-ATPase inhibitor, confirmed that acidification by the Ca^{2+} influx occurred in T cells in which PKC was not down-regulated. These findings indicate two mechanisms, alkalinization by PKC and acidification by Ca^{2+} influx, exist in regulating pH_i in T cells. This is the first report that PHA stimulates the acidification by Ca^{2+} influx but not alkalinization in T cells after down-regulation of PKC. In conclusion, the activity of PKC in T cells determines the response in alkalinization or acidification by PHA. J. Cell. Biochem. 81:604–612, 2001.

Key words: pH_i; Ca^{2+}; protein kinase C; T cells

Signal transduction following T cell activation and proliferation is characterized by the activation of various biochemical processes of which the two most important are sustained increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) by Ca^{2+} influx and protein kinase C (PKC) activity [Berry and Nishizuka, 1990; Weiss and Littman, 1994]. Stimulation of T cell proliferation by lectins can be mimicked by the combination of PKC activation, induced by phorbol esters, and Ca^{2+} influx, induced by ionophores [Clevers et al., 1985]. Blocking the increase in $[Ca^{2+}]_i$ by removal of extracellular calcium, using chelators or calcium channel blockers, results in inhibition of IL-2 secretion and cell proliferation [Mills et al., 1985; Gelfand et al., 1986].

A rapid increase in intracellular pH (pH_i), alkalinization, is also associated with those two second messengers during the activation of T cells. However, in mouse T cells, two sequential steps in intracellular alkalinization are induced by mitogens. The first of which coincides with early biochemical signalling events, whereas, the second coincides with the entry of the T cells into the S phase of the mitotic cell cycle and results in enhanced thymidine incorporation into DNA [Gerson et al., 1982]. T cells from bone marrow transplantation recipients have a defect in PMA-induced alkalinization and also show a low proliferative response to T cell mitogens [Izquierdo et al., 1989].

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However, in addition to alkalinization, acidification is also reported to associate with the appearance of second messengers during triggering of the proliferation of T cells [Cheung et al., 1988; Fischer et al., 1988; Gelfand et al., 1988]. The amiloride-sensitive Na^+/H^+ exchange 1 (NHE 1) has been identified in human T cells by immunoblotting and is one of the main mechanisms responsible for pH_i regulation in these cells [Siczkowski et al., 1994]. However, studies on pH_i changes and NHE function in T cells have yielded some conflicting results. For example, pH_i changes mediated by the NHE in stimulated T cells have been reported to depend, on the one hand, on an increase in $[Ca^{2+}]_i$ as a result of Ca^{2+} influx [Rosoff and Cantley, 1985] and, on the other, on PKC activation and to be unrelated to $[Ca^{2+}]_i$ changes [Civitelli et al., 1989]. In addition, discrepancies are also apparent in studies of the molecular functions on NHE. Point mutations in, and deletion of, the calmodulin-binding site of NHE-1 have demonstrated that NHE is activated by Ca^{2+} [Bertrand et al., 1994], and calmodulin is reported to inhibit NHE-1 function in defined host cells [Takaichi et al., 1993]. In our previous study, alkalinization is found to occur after an increase in $[Ca^{2+}]_i$ or PKC activity by stimulation T cells with PHA or LPS [Chien et al., 2000]. Thus, these discrepancies in changes of pH_i during triggering the proliferation of T cells or NHE function may be due to lack of information about how pH_i is controlled by the second messengers, calcium and PKC, such as whether PKC can affect calcium-induced pH_i changes. The aim of the present study was therefore to examine the roles of the second messengers, calcium and PKC, in pH_i control in human peripheral T cells. PHA-induced pH_i and $[Ca^{2+}]_i$ responses were carefully studied in T cells with intact PKC or with it down-regulated by PMA. $[Ca^{2+}]_i$ or pH_i was respectively measured using the fluorescent dyes, fura-2, and BCECF.

MATERIALS AND METHODS

Chemicals

Fura-2/AM and BCECF/AM, nigericin and valinomycin were purchased from Molecular Probes (Eugene, OR). Phytohemagglutinin (PHA), RPMI 1640 medium (RPMI), Hank's balanced salt solution (HBSS) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY) and phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), dimethylsulphoxide (DMSO), ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and Ficoll/Hypaque from Sigma Chemical Co. (St. Louis, MO). P-81 phosphocellulose paper was supplied by Whatman (Maidstone, UK) and myelin basic protein (MBP4-14) by Research Biochemicals International (Natick, MA). PHA was dissolved in distilled water. The culture media were supplemented with 10 % FCS (v/v).

Separation of T Cells

Heparinized peripheral blood samples were obtained from healthy male volunteers (ages 20–25 years old) and blood mononuclear cells (MNCs) isolated using the Ficoll-Hypaque gradient-density method. The MNC suspension (15 ml) was added to a 100×15-mm plastic Petri dish and the cells incubated for 50 min in a humidified incubator at 37°C, 5% CO_2, then adherent cells were harvested using a rubber policeman and washed; this entire process was repeated three times. The nonadherent T cells were prepared by E-rosetting and lysed of the rosetted erythrocytes using cold distilled water. To verify the effectiveness of the separation procedure, the isolated T cells were incubated for 30 min at 4°C with phycoerythrin-labeled monoclonal anti-CD3 antibodies (Ortho Pharmaceuticals, Raritan, NJ) and the antibody-coated T cells separated on a fluorescence-activated cell sorter (Coulter EPICS C, Hialeah, FL); the results showed that the T cell suspension contained almost 100% CD_3-positive cells [Lin and Lo, 1991]. Phytohemagglutinin (PHA, 10 μg/ml), a mitogenic lectin, and the co-mitogen, phorbol 12-myristate 13-acetate (PMA, 0.1 nM), were used as stimulants.

Measurement of the $[Ca^{2+}]_i$

T cells (2×10^7 cells/ml) were loaded for 30 min at 25°C with fura-2/AM (5 μM) in RPMI 1640 containing 10% FCS (v/v), washed free of extracellular fura-2/AM by 3 washes with RPMI 1640 and resuspended (4×10^8 cells/ml) in RPMI 1640 containing 10% FCS. To determine the $[Ca^{2+}]_i$, 2×10^6 cells were washed twice, resuspended in 2.5 ml of loading buffer (152 mM NaCl, 1.2 mM $MgCl_2$, 1.6 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 10 mM Hepes, pH 7.4) and placed in a plastic cuvette at 37°C in a dual-wavelength spectrofluorimeter (Spex Industries, model CM1T11I, Edison, NJ). The

fluorescence emission at 505 nm using excitation wavelengths of 340 nm and 380 nm was measured and the $[Ca^{2+}]_i$ determined from the fura-2 fluorescence-ratio signal using Spex DM3000 software according to the calculation formula derived by Grynkiewicz et al. [1985].

Measurement of the pH_i

T cell suspensions (2×10^7 cells/ml) were incubated at 37°C for 30 min with BCECF/AM (3 μM) in HBSS containing 5 mM glucose and 0.2% BSA, then the cells were washed three times with HBSS and resuspended in RPMI 1640 containing 10% FCS. For pH_i measurements, 1×10^6 cells were washed twice with HBSS, resuspended in 2.5 ml of the same solution, transferred to a plastic cuvette at 37°C and allowed to stabilize for 15 min before stimulation. BCECF fluorescence emission at 525 nm at the excitation wavelengths of 435 nm and 500 nm was measured using a spectrofluorimeter and the emission ratio calculated. To prepare the calibration curve, a mixture of 1×10^6 cells and 3 μM nigericin was added to K^+ HBSS at pH values of 2–10, then valinomycin (3 μM) was added and allowed to react for 5 min before the fluorescence signals were measured. The pH of the K^+ HBSS was measured to the nearest 0.001 unit using a pH meter (Radiometer Copenhagen, model PHM 93). The calibration values were fitted to a standard sigmoid curve that was then used to calculate the unknown pH_i values.

PKC Assay

Cells were washed twice with 200 μl of buffer A (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mM calcium chloride, 1 mg/ml of glucose, 20 mM Hepes, pH 7.2, 30°C). To permeabilize the cells and initiate the protein kinase assay, the medium was replaced with 40 μl of buffer A containing 50 μg/ml of digitonin, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM [γ-^{32}P] ATP and 300 μM myelin basic protein fragment (MBP4-14) and the kinase reaction allowed to proceed for 10 min at 30°C before being terminated with 10 μl of 25% (w/v) trichloroacetic acid. Aliquots (45 μl) of the reaction mixtures were spotted on to 2×2 cm phosphocellulose strips (Whatman P-81), which were then washed four times by immersion in ice-cold 75 mM phosphoric acid and dried at 80°C. The radioactivity in the phosphorylated protein was measured using a liquid scintillation counter (Wallac 1409, Pharmacia, Finland) and quantified as described by Roskoski [1983]. The protein content of T cells was determined using the Lowry assay [Lowry et al., 1951].

Statistical Analysis

The $[Ca^{2+}]_i$, pH_i and PKC data were analyzed by Student's paired or unpaired t-test with the significance level set at $P < 0.05$. All values are quoted as the mean ± standard error of the mean (SEM).

RESULTS

Effects of External Calcium on the PHA-Induced $[Ca^{2+}]_i$ and pH_i Responses in T Cells

To be used in experiments, the isolated T cells had to show the responses as described in Chien et al. [2000]. As shown in Figure 1a, a similar PHA-mediated increase in $[Ca^{2+}]_i$ was seen when T cells were suspended in loading buffer containing either 1.6 or 3.2 mM calcium (traces B and C, respectively). However, only a small increase was seen using Ca^{2+}-free buffer containing 0.2 mM EGTA (trace A). In contrast, the PHA-mediated increase in the pH_i (alkalinization) was Ca^{2+}-independent and not influenced by Ca^{2+} concentration in the buffer (Fig. 1b).

Down-Regulation of T Cell PKC Activity by Different Doses of PMA

PKC activity, expressed as [γ-^{32}P] phosphorylation of the myelin basic protein fragment, was measured in T cells after incubation for 18 h with different doses of PMA. Compared with the activity in vehicle (DMSO)-treated cells, the basal PKC activity decreased significantly ($n = 3$, $P < 0.01$) at PMA concentrations greater than 100 nM (Fig. 2).

PMA- or PHA-Induced pH_i Changes in T Cells After PKC Down-Regulation

Before stimulation, T cells were treated for 18 h either with the vehicle (DMSO) or with 1 μM PMA to down-regulate PKC activity. As shown in Figure 3, in vehicle-treated cells, PMA- or PHA-induced alkalinization was first seen 5 min after stimulation and, by the end of the 30 min stimulation period, the pH_i had increased significantly from a resting value of 7.15 ± 0.28 to 7.53 ± 0.29 (n=6, $P < 0.01$) in PMA-treated cells (Fig. 3a) and from a resting value of 7.16 ± 0.24 to 7.61 ± 0.26 ($n = 6$, $P < 0.01$) in

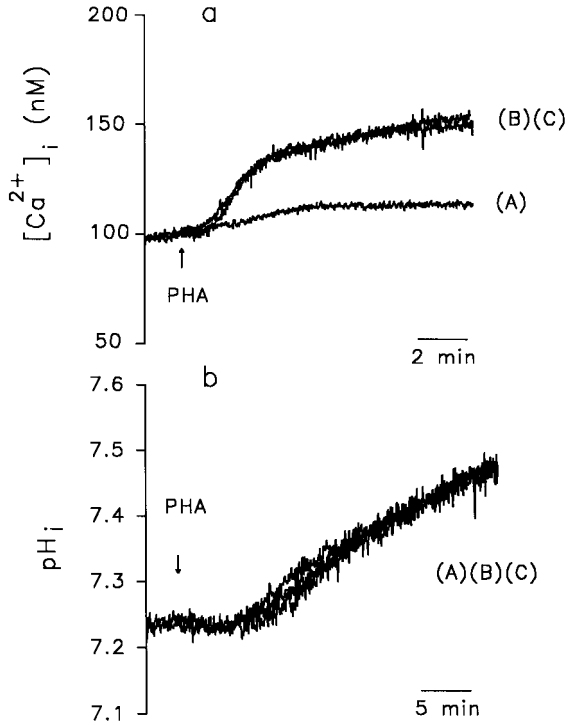

Fig. 1. Effect of exogenous Ca^{2+} on PHA-induced $[Ca^{2+}]_i$ and pH_i changes in human peripheral T cells. Fura-2/AM- and BCECF/AM-loaded cells used in the $[Ca^{2+}]_i$ and pH_i studies were suspended in medium containing 0 **(A)**, 1.6 **(B)** or 3.2 (C) mM Ca^{2+}. (A) $[Ca^{2+}]_i$ and (B) pH_i responses were monitored after stimulation with PHA (10 μg/ml), indicated by the arrows. The traces shown are representative of six experiments.

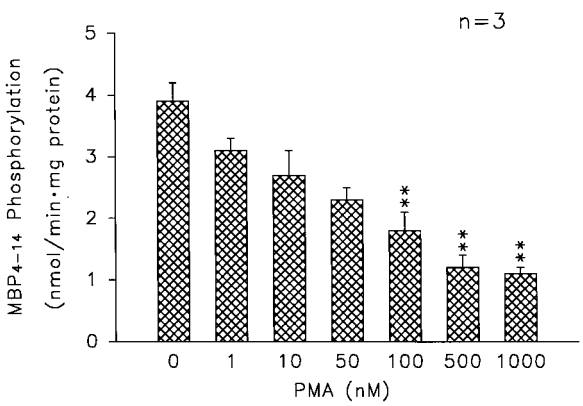

Fig. 2. PKC activity in T cells after down-regulation by PMA.T cells were treated with different concentration of PMA for 18 h before measurement of PKC activity by phosphorylation of myelin basic protein fragment (MBP_{4-14}) assayed using cell-free extracts from each group of stimulated cells. PKC activity decreased significantly ($^{**}P < 0.01$) after administration of a concentration of PMA greater than 100 nM. Each value represents the mean ± SEM, n = 3.

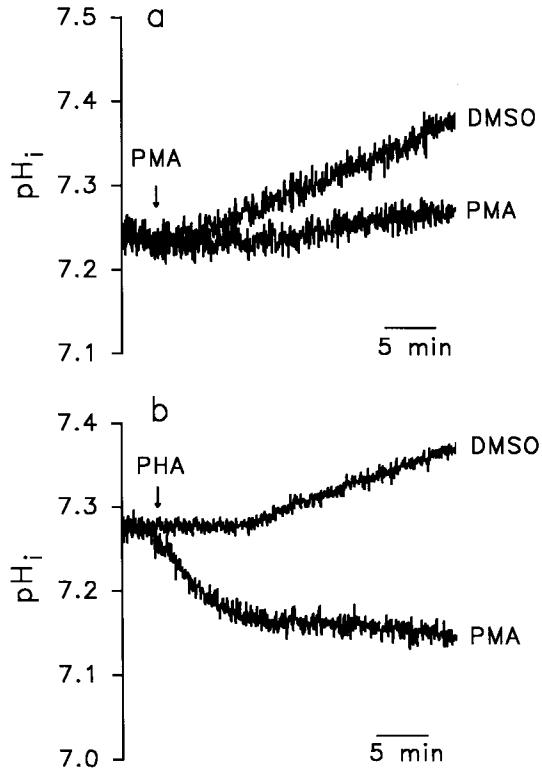

Fig. 3. pH_i responses to PMA or PHA in PKC down-regulated T cells. BCECF/AM-loaded T cells were preincubated with PMA (1 μM) or vehicle (DMSO) for 18 h before stimulation with **(a)** PMA (0.1 nM) or **(b)** PHA (10 μg/ml), indicated by the arrows. The traces are representative of six experiments.

PHA-treated T cells (Fig. 3b). When compared with vehicle-treated cells, no significant alkalinization was seen when PKC down-regulated T cells were stimulated with either PMA or PHA. In the case of PMA stimulation, the pH_i showed a non-significant increase from 7.18 ± 0.38 to 7.28 ± 0.39 (n=6, NS) (Fig. 3a) by the end of the 30 min stimulation period. This was, in contrast to PHA-stimulated cells, where acidification was seen, with the pH_i decreasing significantly from 7.22 ± 0.40 to 6.89 ± 0.40 ($n = 6$, $P < 0.01$) (Fig. 3b).

Effect of NHE Inhibitor, 5-(N,N-dimethyl)-Amiloride (DMA), on PHA-Stimulated Acidification in PKC Down-Regulated T Cells

It was of interest to know whether the NHE was activated to counter the acidification which was induced by PHA after the down-regulation of PKC in T cells. The NHE inhibitor, 5-(N,N-dimethyl)-amiloride (DMA), has a high affinity for the NHE and a low cell permeability and only exhibits non-specific effects at concentrations 10- to 100-fold higher than that used in the

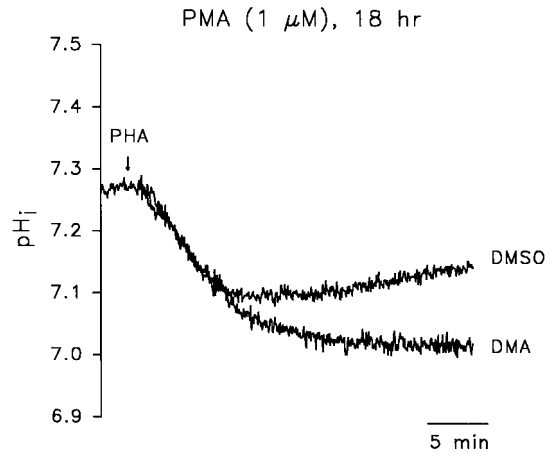

Fig. 4. Effect of 5-(N,N-dimethyl)-amiloride (DMA) on the PHA-induced acidification of PKC down-regulated T cells. BCECF/AM-loaded T cells were preincubated with PMA (1 μM) for 18 h, then suspended in medium containing DMA (10 μM) or vehicle (DMSO) for 20 min before stimulation with PHA (10 μg/ml), indicated by the arrow. The traces are representative of three experiments.

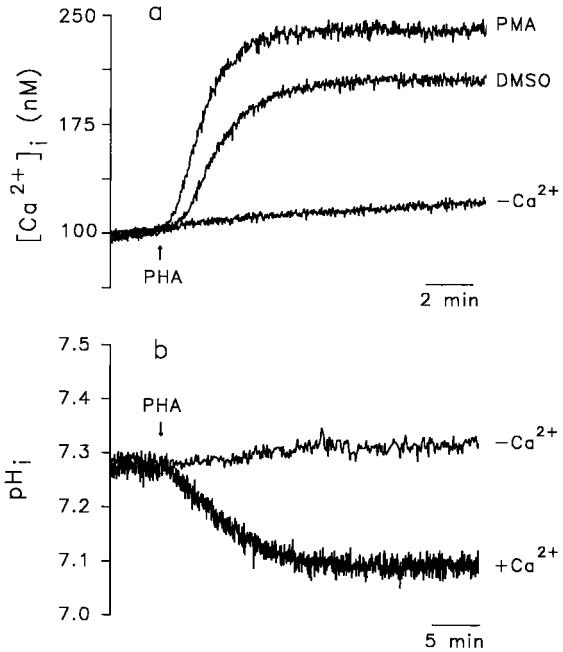

Fig. 5. Effect of PKC down-regulation on PHA-induced $[Ca^{2+}]_i$ changes and effect of Ca^{2+} on PHA-induced acidification in PKC down-regulated T cells. **(a)** Cells were preincubated with PMA (1 μM) or vehicle (DMSO) for 18 h, then loaded with fura-2/AM. The loaded cells were suspended in measuring medium with the addition of 1 μM PMA or vehicle (DMSO), or in Ca^{2+}-free measuring medium containing 1 μM PMA before stimulation with PHA (10 μg/ml), indicated by the arrow. The traces are representative of more than seven experiments. **(b)** T cells were preincubated with PMA (1 μM) for 18 h, then suspended in buffer with or without Ca^{2+} before stimulation with PHA (10 μg/ml), indicated by the arrow. The traces are representative of more than three experiments.

present study (10 μM) [Kleyman and Cragoe, 1988]. When PKC down-regulated cells were preincubated for 20 min with 10 μM DMA before PHA stimulation, the PHA-induced acidification was enhanced (Fig. 4).

PHA-Induced $[Ca^{2+}]_i$ Responses in T Cells After PKC Down-Regulation

The effect of PKC on PHA-induced $[Ca^{2+}]_i$ changes in T cells was studied in PKC down-regulated (1 μM PMA, 18 h) or vehicle (DMSO)-treated cells (Fig. 5a). In PMA-pre-treated cells, the PHA-stimulated $[Ca^{2+}]_i$ increase was 151 ± 9 nM above the resting level, significantly greater than the value of 83.5 ± 8.1 nM seen in vehicle-pre-treated cells ($n=7$, $P < 0.01$). In contrast, in Ca^{2+}-free medium, no PHA-induced $[Ca^{2+}]_i$ increase was seen. Experiments were then performed to investigate whether the acidification seen in PKC down-regulated T cells resulted from the enhancing PHA-induced $[Ca^{2+}]_i$ increase. As shown in Figure 5b, when PKC down-regulated T cells were stimulated with PHA in medium with or without Ca^{2+}, the PHA-induced acidification was blocked in the absence of Ca^{2+}.

Effects of the Inorganic Channel Blocker, Ni^{2+}, on the PHA-Induced $[Ca^{2+}]_i$ Increase and Acidification in PKC Down-Regulated T Cells

In order to further confirm that the acidification in PKC down-regulated T cells was induced by Ca^{2+} influx, the inorganic channel blocker, Ni^{2+}, was used to block the $[Ca^{2+}]_i$ increase. When Ni^{2+} was added 20 min after PHA stimulation, it reversed both the PHA-induced $[Ca^{2+}]_i$ increase (Fig. 6a) and the acidification effect (Fig. 6b).

Effect of Thapsigargin (TG) on $[Ca^{2+}]_i$ and pH_i Changes in T Cells in Which PKC is not Down-Regulated

In order to investigate if the mechanism of acidification by Ca^{2+} influx was also present in PMA-untreated T cells, thapsigargin (TG), a Ca^{2+}-ATPase inhibitor, was used. The dose-response relationship between the concentration of TG used and the increase in the $[Ca^{2+}]_i$ in T cells is shown in Figure 7a. As the response seen with 0.1 nM TG was closest to that produced by 10 μg /ml of PHA, this concentration was chosen to study the effect of $[Ca^{2+}]_i$ changes on the pH_i. As shown in Figure 7b, in the

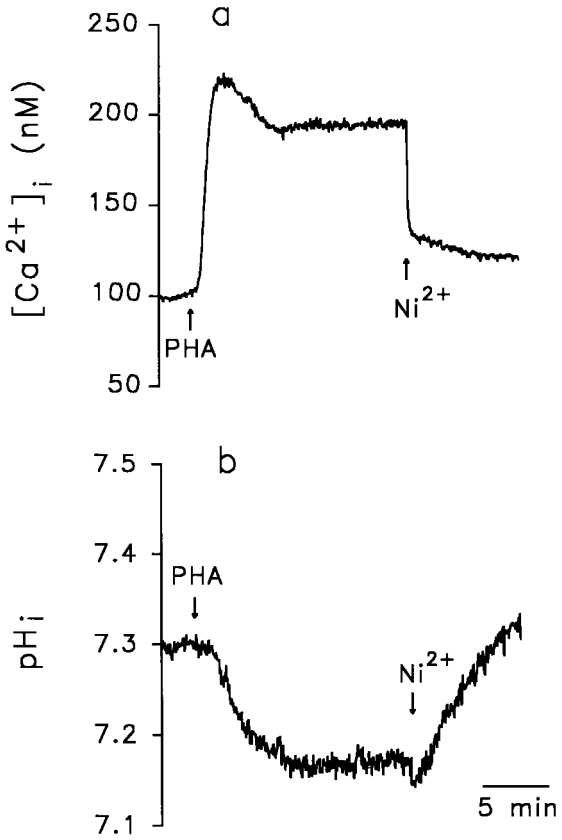

Fig. 6. Effects of the Ca^{2+} channel blocker, Ni^{2+}, on the PHA-induced $[Ca^{2+}]_i$ increase and acidification PKC down-regulated T cells.T cells were preincubated with PMA (1 μM) for 18 h, then loaded with fura-2/AM or BCECF/AM before stimulation with PHA (10 μg/ml), then changes in **(a)** $[Ca^{2+}]_i$ and **(b)** pH_i were measured. The inorganic Ca^{2+} channel blocker, Ni^{2+} (5 mM) was added 20 min after PHA stimulation. The arrows indicate the addition of the ligands. The traces are representative of at least three experiments.

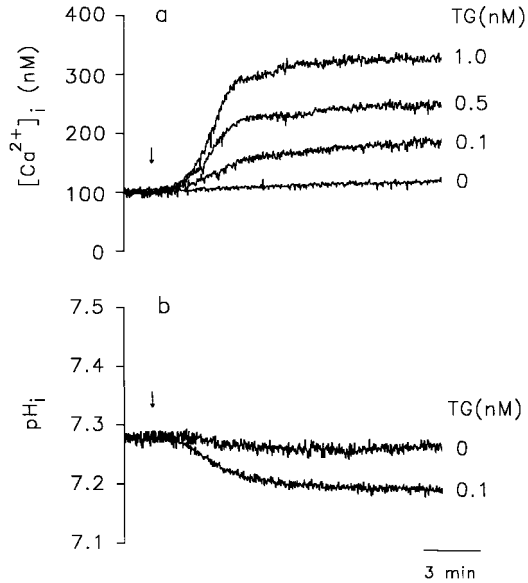

Fig. 7. Effects of thapsigargin (TG) on $[Ca^{2+}]_i$ and pH_i changes in T cells.T cells were loaded with fura-2/AM or BCECF/AM before stimulation with TG. The traces show **(a)** the dose-response for TG-induced $[Ca^{2+}]_i$ changes and **(b)** the effect of 0.1 nM TG on the pH_i. The arrows indicate the addition of TG. The traces are representative of three experiments.

presence of this concentration of TG, acidification was again induced.

DISCUSSION

The PHA-induced $[Ca^{2+}]_i$ increase was blocked in the absence of external Ca^{2+} (Fig. 1a, trace A). In T cells, the PHA-induced sustained increase in $[Ca^{2+}]_i$ is reported to depend on external Ca^{2+} influx [Allwood et al., 1971]. But, the PHA-induced alkalinization was not affected and independent of external Ca^{2+} influx (Fig. 1b, trace A). A 2-fold difference in Ca^{2+} concentration had no effect on either the resting levels of $[Ca^{2+}]_i$ and pH_i or the magnitude of the PHA-induced increases in $[Ca^{2+}]_i$ and pH_i (Fig. 1a,b, traces B and C). This indicates that T cells either possess a relatively good homeostatic system or the Ca^{2+} influx pathway has already been saturated at 1.6 mM $[Ca^{2+}]_o$.

The PMA (1 μM) was used in subsequent experiments to down-regulate PKC activity. At this concentration, the PKC activity was significantly suppressed (Fig. 2). The down-regulation of PKC activity blocked the alkalinization by PMA or PHA (Fig. 3) and, therefore, the alkalinization seen in stimulated T cells is PKC-dependent. As shown in Figure 4, in PKC down-regulated T cells, the PHA-induced acidification was increased in the presence of the NHE inhibitor, DMA, demonstrating that the NHE had already been stimulated by acidification to prevent a further decrease in pH_i. This is because the NHE could be partially stimulated by protons. Recently, alkalinization has been shown to occur by stimulation of the distinct cytoplasmic Ca^{2+}- and PKC-dependent domains of NHE [Ikeda et al., 1998]. In addition, in human erythrocytes, PKC is required for activation of NHE by Ca^{2+} [Lijnen et al., 1998]. If the activation of NHE in T cells had similar PKC requirement, it could explain why NHE was unable to counteract the PHA-induced acidification after PKC down-regulation. Furthermore, the results of DMA also implied that other mechanisms might be involved in PKC

down-regulated T cells to cause the PHA-induced intracellular acidification.

The sustained $[Ca^{2+}]_i$ increase induced by PHA was significantly enhanced in T cells after the down-regulation of PKC (Fig. 5). This could be attributed to the Ca^{2+} influx being inhibited by modulation via receptor/PLC-linked PKC activation [Cheung et al., 1988; Balasubramanyam and Gardner, 1995]. Hence, the enhanced $[Ca^{2+}]_i$ increase is probably due to the release of the PKC-mediated suppression of Ca^{2+} influx in T cells after PKC down-regulation. Moreover, down-regulation of the PKC_β isozyme by phorbol ester is reported to increase Ca^{2+} influx in Jurkat T cells [Haverstick et al., 1997]. It is not clear whether the PHA-induced acidification is due to enhancement of Ca^{2+} influx in T cells after PKC down-regulation. According to the new findings shown in Figures 5b and 6, acidification was blocked by removal of Ca^{2+} or immediately reversed with the addition of the inorganic channel blocker, Ni^{2+} in PKC down-regulated T cells. These indicate that the main effect of PHA stimulation on the pH_i is acidification as a result of enhanced Ca^{2+} influx in PKC down-regulated T cells.

More than one PKC-independent pathway may contribute to intracellular acidification as a result of enhanced Ca^{2+} influx. Firstly, activation of the Ca^{2+}/H^+ ATPase drives H^+ in and Ca^{2+} out, and, in the presence of external Na^+, H^+ is returned to the medium by NHE [Berthe et al., 1991]. Secondly, in human T cells, an increase in the concentration of cytosolic free Ca^{2+} leads to the generation of metabolic acid, such as by glycolysis, with consequent cytoplasmic acidification which might overwhelm the normal pH homeostatic mechanisms of Na^+/H^+ exchange system [Gelfand et al., 1988]. Thirdly, as demonstrated in both cardiac tissues and invertebrate neurons, H^+ can be displaced from intracellular buffers by high $[Ca^{2+}]_i$ levels [Vaughan-Jones et al., 1983]. More studies are required to define what mechanism is involved in PHA-induced acidification mediated by Ca^{2+} influx in T cells after PKC down-regulation.

Concomitant PHA-induced increases in pH_i, Ca^{2+} influx and PKC activity were reported in T cells [Chien et al., 2000]. The results of this study imply that the second messengers, PKC and Ca^{2+}, may activate a dual antagonistic mechanism, i.e. PKC-mediated alkalinization and Ca^{2+} influx-mediated acidification, to control pH_i changes in stimulated T cells. However, PHA induced alkalinization occurred in non-PKC-down-regulated T cells (Fig. 1), irrespective of whether Ca^{2+} influx occurred. Some evidence in T cells indicates that the response of acidification by Ca^{2+} influx can occur separately with a lack of PKC activation. For example, the diacylglycerol analogue, 1,2-sn-dioctanoyl glycerol can stimulate Ca^{2+} influx and acidification in T cells via a PKC-independent pathway [Ebanks et al., 1989]. In addition, the divalent cation ionophore, ionomycin can also stimulate acidification in T cells [Gelfand et al., 1988]. To investigate whether the mechanism of Ca^{2+} influx-mediated acidification also occurs in non-PKC-down-regulated T cells, we used thapsigargin (TG), an inhibitor of Ca^{2+}-ATPase, that can deplete intracellular Ca^{2+} stores and induce a sustained Ca^{2+} influx without perturbing the levels of phosphatidyl inositol and PKC activity in T cells [Gouy et al., 1990; Sei and Reich, 1995]. The concurrent stimulation by TG of a $[Ca^{2+}]_i$ increase and acidification in Figure 7, indicates that the mechanism of acidification by Ca^{2+} influx probably operates not only in PKC-down-regulated T cells, but also in intact T cells after PHA stimulation. Thus, the result of alkalinization by PHA is presumably due to the PKC-induced alkalinization dominating the Ca^{2+} influx-induced acidification.

Homeostasis of the intracellular calcium concentration and pH, as well as PKC activity, is important in terms of the cell's ability to respond to the initiation of differentiation and proliferation and the maintenance of normal metabolic pathways. A sustained cytoplasmic alkalinization is seen in cells stimulated by a variety of growth factors or mitogens [Hesketh et al., 1985; Civitelli et al., 1989; Li et al., 1991] and acidification is recognized as a general character of apoptosis that can activate the cation-insensitive acidic endonuclease [Sharma and Srikant, 1998] and thus genome destruction in cells [Gottlieb et al., 1996]. Further studies are planned to examine whether the pH_i responses can be utilized as an indicator of intracellular PKC activity and allow the determination of whether cells are proceeding down a pathway to growth or apoptosis.

This is the first report that the activity of PKC in T cells determines alkalinization or acidification by PHA. These findings demonstrate that if PKC activity in cells is not taken into account, it may lead to discrepancies in results when studying of pH_i changes or NHE functions, for

example, when the alkalinization or acidification is stimulated by proliferation of T cells [Cheung et al., 1988; Fischer et al., 1988; Gelfand et al., 1988]. Similarity, it is important to note that pH_i changes mediated by the NHE are PKC or Ca^{2+} influx dependent in stimulated T cells [Rosoff and Cantley, 1985; Civitelli et al., 1989] and thus can affect analysis of the molecular functions on NHE [Takaichi et al., 1993; Bertrand et al., 1994]. In conclusion, the physiological status of cellular levels of PKC probably determines the response of pH_i in T cells after stimulation.

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