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Non-genomic Immunosuppressive Actions of Progesterone Inhibits PHA-Induced Alkalinization and Activation in T Cells

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Abstract Progesterone is an endogenous immunomodulator, and can suppress T-cell activation during pregnancy. When analyzed under a genome time scale, the classic steroid receptor pathway does not have any effect on ion fluxes. Therefore, the aim of this study was to investigate whether the non-genomic effects on ion fluxes by progesterone could immunosuppress phytohemagglutinin (PHA)-induced human peripheral T-cell activation. The new findings indicated that, first, only progesterone stimulated both $[Ca^{2+}]_i$ elevation and pH_i decrease; in contrast, estradiol or testosterone stimulated $[Ca^{2+}]_i$ elevation and hydrocortisone or dexamethasone stimulated pH_i decrease. Secondly, the $[Ca^{2+}]_i$ increase by progesterone was dependent on Ca^{2+} influx, and the acidification was blocked by Na^+/H^+ exchange (NHE) inhibitor, 3-methylsulphonyl-4-piperidinobenzoyl, guanidine hydrochloride (HOE-694) but not by 5-(*N*,*N*-dimethyl)-amiloride (DMA). Thirdly, progesterone blocked phorbol 12-myristate 13-acetate (PMA) or PHA-induced alkalinization, but PHA did not prevent progesterone-induced acidification. Fourthly, progesterone did not induce T-cell proliferation; however, co-stimulation progesterone with PHA was able to suppress PHA-induced IL-2 or IL-4 secretion and proliferation. When progesterone was applied 72 h after PHA stimulation, progesterone could suppress PHA-induced T-cell proliferation. Finally, immobilization of progesterone by conjugation to a large carrier molecule (BSA) also stimulated a rapid $[Ca^{2+}]_i$ elevation, pH_i decrease, and suppressed PHA-induced proliferation. These results suggested that the non-genomic effects of progesterone, especially acidification, are exerted via plasma membrane sites and suppress the genomic responses to PHA. Progesterone might act directly through membrane specific nonclassical steroid receptors to cause immunomodulation and suppression of T-cell activation during pregnancy. J. Cell. Biochem. 99: 292–304, 2006.

Key words: progesterone; non-genomic; immunosuppressive; Ca^{2+}; pH_i; IL-2; IL-4; proliferation; T cells

Maternal acceptance of the fetus during pregnancy results in T-cell tolerance rather than immunity. However, there is strong evidence to indicate that maternal T cells are not exposed to fetal alloantigens or if exposed, changes in the production of cortisol, progesterone, and estrogen play a major role in modulating the local immunosuppression at the maternal-fetal interface. These steroids can prevent the maternal immune system from activating effector T cells capable of attacking fetal cells and this results in T-cell tolerance during pregnancy. Clinically, systemic lupus erythematosus often flares during pregnancy, whereas rheumatoid arthritis commonly remits during pregnancy and then flares or initially develops in the postpartum period [Ostensen, 1999].

Progesterone is essential for the maintenance of pregnancy including ovulation, and for uterine and mammary gland development [Lydon et al., 1995]. The placenta is where

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progesterone is synthesized in a large amount, and its concentration reaches very high levels there (1–10 μM in human) [Stites and Siiteri, 1983]. Progesterone inhibits lymphocyte proliferation by mitogen [Van Voorhis et al., 1989]. The alterations involve a rapid increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) and a prolonged activation of PKC; these are known as the two major signals in T-cell differentiation and proliferation caused by various polyclonal mitogens [Berry and Nishizuka, 1990; Berry et al., 1990]. Blocking either PKC activity [Hengel et al., 1991] or the increase in $[Ca^{2+}]_i$ will inhibit IL-2 secretion and T-cell proliferation [Mills et al., 1985a,b; Gelfand et al., 1986]. However, it is still unclear if the two mitogenic signals, $[Ca^{2+}]_i$ increase, and PKC activation in T cells are affected by progesterone.

Recently, studies have found a non-genomic mechanism for progesterone-mediated immunosuppression by blockage of the potassium channels; this inhibits thapsigargin-induced calcium entry, and subsequent gene expression in T cells [Ehring et al., 1998]. However, the inhibition capacitative calcium entry by progesterone is reported to be independent of plasma membrane depolarization [Gamberucci et al., 2004] as human peripheral blood monocytes do not express the classical progesterone receptor. Thus, progesterone-induced immunosuppression has been found to be neither mediated through the classical progesterone receptor [Kontula et al., 1983; Schust et al., 1996] nor mediated by the glucocorticoid receptor [Szekeres-Bartho et al., 1990]. In our previous findings, alkalinization by a Na^+/H^+ exchanger (NHE) was found in phytohemagglutinin (PHA)-stimulated T cells to be dependent on PKC activation [Chien et al., 2001] and a concomitant increase in pH_i occurs due to Ca^{2+} influx into cells [Chien et al., 2000]. It should be noted that the primary ion transport target of the non-genomic signal transduction cascade is elicited by aldosterone for the NHE of the epithelium [Gekle et al., 1996]. However, until now, the cellular and molecular mechanisms of the non-genomic effects on T cells by progesterone have not been studied in detail and little is known about how progesterone exerts its non-genomic effects on PHA-stimulated ionic fluxes and immunosuppression in T cells. The aim of present study was therefore to investigate how progesterone exerted non-genomic effects on ion fluxes including changes in the intracellular Ca^{2+} ($[Ca^{2+}]_i$), pH (pH_i), and whether the non-genomic effects on ion fluxes by progesterone is able to immunosuppress PHA-induced cytokine secretion and proliferation in human peripheral T cells. Progesterone-induced pH_i and $[Ca^{2+}]_i$ responses were measured using the fluorescent dyes, BCECF and fura-2, respectively, in T cells. IL-2 and IL-4 were determined by ELISA. Proliferation was determined by [^{3}H]-thymidine incorporation into the T cells.

MATERIALS AND METHODS

Materials

Fura-2/AM and BCECF/AM, nigericin, and valinomycin were purchased from Molecular Probes (Eugene, OR). PHA, RPMI 1640 medium (RPMI), Hank's balanced salt solution (HBSS), and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). Progesterone, estradiol, testosterone, hydrocortisone, dexamethasome, phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), dimethylsulphoxide (DMSO), ethanol, EGTA, 5-(*N*,*N*-dimethyl)-amiloride (DMA), and Ficoll/Hypaque were purchased from Sigma Chemical Co. (St. Louis, MO). 4-pregnen-3, 20-dione 3-o-carboxymethyloxime:BSA (Progesterone 3-CMO:BSA) was obtained from Steraloids, Inc. (Newport, RI). 3-methylsulphonyl-4-piperidinobenzoyl guanidine hydrochloride (HOE-694) was kindly provided by Dr. S. H. Loh (National Defense Medical Center, Taiwan). PHA was dissolved in distilled water. The culture media were supplemented with 10% FCS (v/v). All sera were pretreated with dextran-charcoal to remove small molecules including steroids and thyroid hormone [Liu et al., 1993].

Preparation of T Cells

Heparinized peripheral blood samples were obtained from healthy male volunteers (ages 20–25 years old) and the 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. The adhering cells were harvested using a rubber policeman and washed; this entire process was repeated three times. The non-adhering T cells were prepared by E-rosetting and the rosetted

erythrocytes lysed 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 (EPICS C, Hialeah, FL). The results showed that the T-cell suspension contained almost 100% CD3-positive cells [Lin and Lo, 1991].

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 three 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). Using excitation wavelengths of 340 nm and 380 nm, the fluorescence emission at 505 nm was measured and the $[Ca^{2+}]_i$ determined from the fura-2 fluorescence ratio signal using Spex DM3000 software according to the 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. Upon excitation at wavelengths of 435 nm and 500 nm, the BCECF fluorescence emission at 525 nm was measured using a dual-wavelength spectrofluorimeter (Spex Industries, model CM1T11I) 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 units 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.

Enzyme-Linked Immunosorbent Assay

Cell culture supernatants were collected after treatment, and the levels of human IL-2 and IL-4 were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN), according to the vendor's instructions. The values were obtained using a DYNEX microplate reader (Microtiter Co., VA) and were fitted to a standard curve that was then used to calculate the unknown IL-2 or IL-4 values. The sensitivities for IL-2 and IL-4 were 7 pg/ml and 10 pg/ml, respectively.

Proliferation Studies

T cells (2×10^6 cells/ml) were plated in triplicates in a 96-well flat bottom plate (Corning, NY) and stimulated separately with PHA, progesterone, or combination of both. After 72 h, [^{3}H]-thymidine, (specific activity 1 μCi/mM, New England Nuclear, Boston, MA) was added to the wells, the cells were incubated for a further 18 h, and harvested with a Skatron Multiwell Harvester (Dynatech, Automash 2000, Billing Shourst, UK). Radioactivity incorporated into the DNA was measured using a the liquid scintillation counter (Wallac 1409, Perkin Elmer Inc., MA).

Statistical Analysis

The $[Ca^{2+}]_i$, pH_i, IL-2, IL-4, and proliferation data were analyzed by Student's *t*-test or one-way ANOVA analysis with a significance level set at $P < 0.05$. All values are quoted as the mean ± SEM.

RESULTS

Effects of Steroids on $[Ca^{2+}]_i$ and pH_i Changes

The stepwise dose-response of the $[Ca^{2+}]_i$ and pH_i changes was used to examine the specificity of steroids such as progesterone, estradiol, testosterone, hydrocortisone, and dexamethasone. As shown in Figure 1a, (2.5, 5, 12.5, 25, 50, 100 μM) of progesterone, estradiol, and testosterone exhibited a stepwise $[Ca^{2+}]_i$ elevation. Whereas, doses of hydrocortisone and dexamethasone did not affect $[Ca^{2+}]_i$. However,

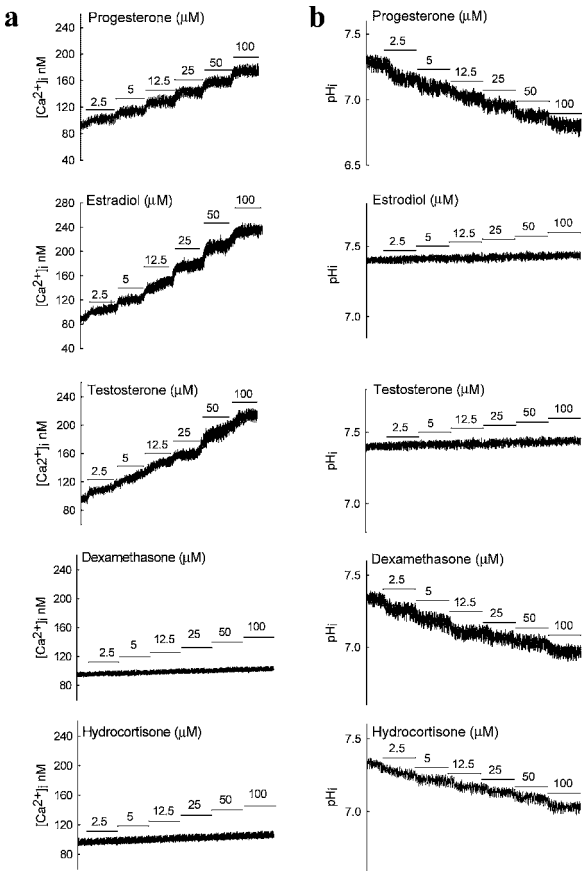

Fig. 1. Non-genomic effects of steroids on $[Ca^{2+}]_i$ and pH_i changes. Fura-2-loaded cells or BCECF-loaded cells were stimulated with (2.5, 5, 12.5, 25, 50, 100 μM) steroids such as progesterone, estradiol, testosterone, dexamethasone, and hydrocortisone, individually. **a**: Stepwise dose-response curves of various steroid hormone-induced $[Ca^{2+}]_i$ elevation in fura-2-loaded T cells. **b**: Stepwise dose-response curves of various steroid hormone-induced pH_i changes in BCECF-loaded T cells. The bar indicates the time of steroid addition recorded for 10 min. The tracings are from one representative of three experiments.

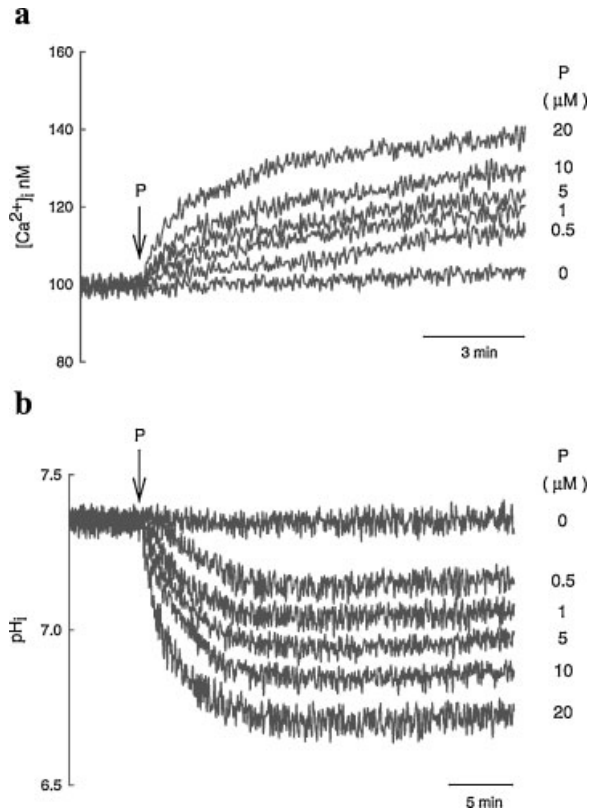

Fig. 2. Dose-response curves of progesterone-induced $[Ca^{2+}]_i$ and pH_i changes in T cells. **a**: Fura-2-loaded cells were stimulated with 0, 0.5, 1, 5, 10, or 20 μM of progesterone. **b**: BCECF-loaded cells were stimulated as in (a) above. The arrow indicates the time of addition. The tracings are from one representative of five experiments.

under the same situation, only progesterone, hydrocortisone, and dexamethasone gave a stepwise decrease pH_i. Estradiol and testosterone did not affect pH_i in T cells (Fig. 1).

Effects of Progesterone on $[Ca^{2+}]_i$ and pH_i Changes

The dose-response relationships between progesterone and $[Ca^{2+}]_i$ elevation and pH_i change in T cells were studied. Administration of progesterone (0.5, 1, 5, 10, 20 μM) resulted in an increase in $[Ca^{2+}]_i$, starting within 1 min, from a resting level of 94.3 ± 1.6 nM. The increase in $[Ca^{2+}]_i$ reached a plateau above resting of 12.6 ± 0.3 ($N = 5$, $P < 0.05$), 17.0 ± 1.1 ($N = 5$, $P < 0.01$), 26.1 ± 4.5 ($N = 5$, $P < 0.01$), 31.3 ± 3.1 ($N = 5$, $P < 0.001$), 40.8 ± 3.8 nM ($n = 5$, $P < 0.001$), respectively, at 3 min and these levels continued for 10 min or longer (Fig. 2a).

Administration of progesterone (0.5, 1, 5, 10, 20 μM) resulted in a decrease in pH_i starting within 1 min from a resting level of 7.37 ± 0.06 and the decrease reached a steady state of 0.07 ± 0.08 ($N = 5$, NS), 0.22 ± 0.04 ($N = 5$, $P < 0.05$), 0.26 ± 0.02 ($N = 5$, $P < 0.05$), 0.39 ± 0.02 ($N = 5$, $P < 0.01$), and 0.56 ± 0.04 ($N = 5$, $P < 0.001$), respectively, below resting at 5 min after stimulation and these values continued for 10 min or longer (Fig. 2b).

Effects of External Calcium on the Progesterone-Induced $[Ca^{2+}]_i$ Increase in T Cells

In order to confirm that the elevation in $[Ca^{2+}]_i$ was induced by a Ca^{2+} influx, the calcium chelator, EGTA (5 mM), was used to

modulate the $[Ca^{2+}]_i$ changes induced by progesterone. When EGTA was added 5 min after progesterone (10 μM) stimulation, it reversed the progesterone-induced $[Ca^{2+}]_i$ increase (Fig. 3a). However, only a small increase by progesterone was seen using Ca^{2+}-free buffer containing 0.2 mM EGTA (Fig. 3b, trace A). After the supplementation with 2.2 mM of $CaCl_2$ into buffer, the $[Ca^{2+}]_i$ response was rapidly elevated to reach almost the same levels as obtained with Ca^{2+} containing buffer (Fig. 3b, trace A, B).

Effects of External Calcium on the Progesterone-Induced pH_i Responses in T Cells

In order to confirm whether the decrease in pH_i (acidification) was induced by Ca^{2+} influx, the calcium chelator, EGTA (5 mM), was used to try and block the pH_i changes. When EGTA was added 5 min after progesterone (10 μM) stimulation, it did not affect the progesterone-induced pH_i response (Fig. 4).

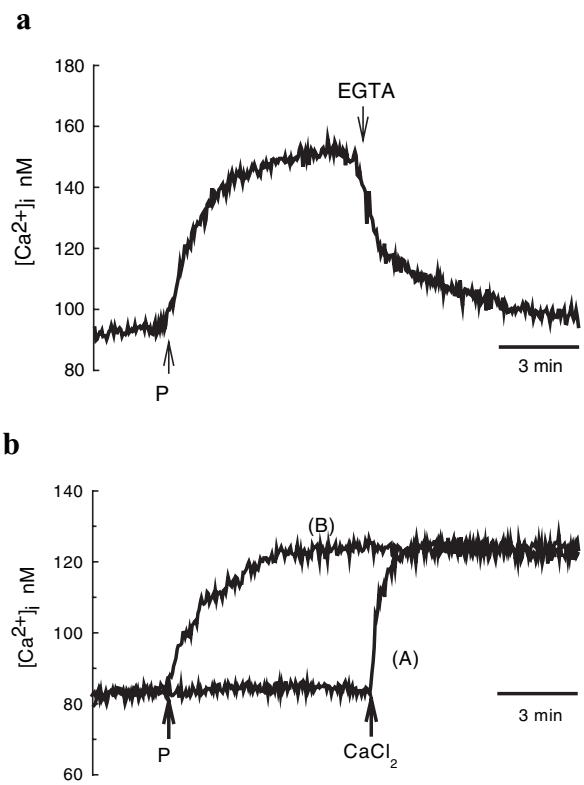

Fig. 3. Effect of exogenous Ca^{2+} on progesterone-induced $[Ca^{2+}]_i$ changes in human T cells. **a**: Fura-2-loaded T cells were suspended in normal loading buffer. EGTA (5 mM) was added 5 min after 10 μM progesterone stimulation. **b**: Cells were suspended in (A) Ca^{2+}-free loading buffer or (B) normal loading buffer. Arrows indicate the addition of 10 μM progesterone or 2.2 mM $CaCl_2$. The traces are representative of three experiments.

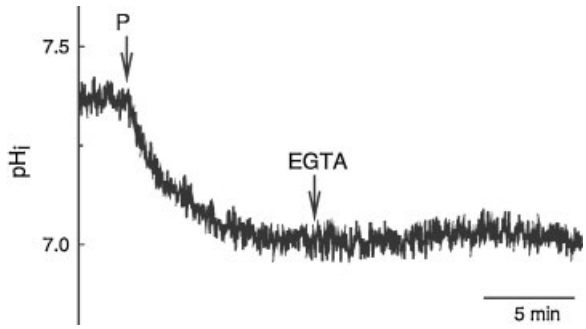

Fig. 4. Effect of exogenous Ca^{2+} on progesterone-induced acidification in human T cells. BCECF-loaded T cells were suspended in normal Na^+-Hank. EGTA (5 mM) was added 5 min after 10 μM progesterone stimulation. The traces are representative of three experiments.

Effects of NHE Inhibitors, 5-(*N*,*N*-Dimethyl)-Amiloride (DMA), and 3-Methylsulphonyl-4-Piperidinobenzoyl Guanidine Hydrochloride (HOE-694), on Progesterone-Stimulated Acidification in T Cells

It was of interest to investigate whether the acidification by progesterone could activate NHE to maintain pH_i homeostasis in cells. T cells were incubated with DMA (20 μM) for 20 min before the addition of progesterone. When compared to the control, the acidification by progesterone was not affected by DMA (Fig. 5a). In contrast, when T cells were preincubated with the specific NHE1 inhibitor, HOE-694 (15, 30, 60 μM), the acidification by progesterone showed a dose-dependent suppression (Fig. 5b).

Effects of Progesterone on PHA-Induced $[Ca^{2+}]_i$ Changes

In order to know whether the $[Ca^{2+}]_i$ changes by PHA and progesterone were additive, the $[Ca^{2+}]_i$ changes were observed by sequential administration to T cells of PHA and progesterone. For example, when progesterone (10 μM) was added 12 min after PHA (10 μg/ml), progesterone was observed to further stimulate the $[Ca^{2+}]_i$ increase in T cells (Fig. 6a). A similar result was obtained, when PHA (10 μg/ml) was added after progesterone (10 μM); PHA also enhanced the $[Ca^{2+}]_i$ increase by progesterone (Fig. 6b).

Effects of Progesterone on PHA- or PMA-Induced pH_i Changes

When progesterone (10 μM) was added 15 min after PHA (10 μg/ml), the increase of pH_i

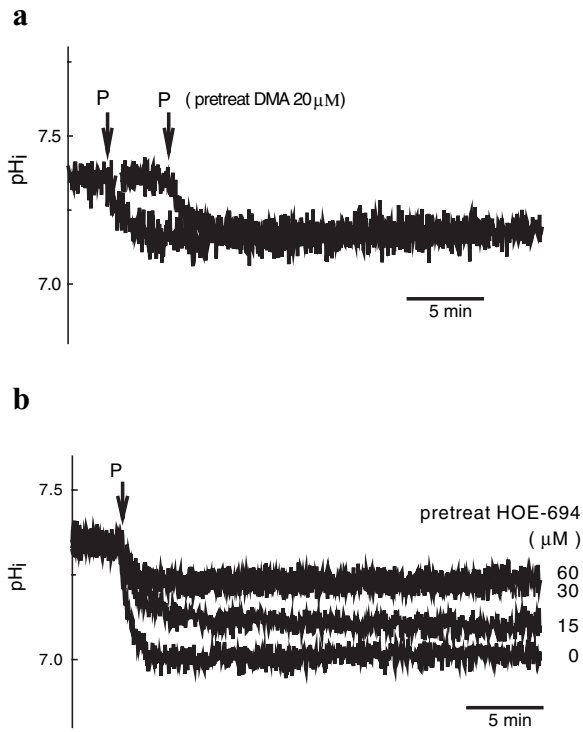

Fig. 5. The effect of Na^+/H^+ exchanger (NHE) inhibitors, 5-(*N*,*N*-dimethyl)-amiloride (DMA), and 3-methylsulphonyl-4-piperidinobenzoyl guanidine hydrochloride (HOE-694) on progesterone-induced acidification in T cells. **a**: Effect of DMA on acidification. BCECF-loaded cells were pretreated with or without 20 μM DMA for 20 min before the stimulation with 10 μM progesterone. **b**: Effect of HOE-694 on acidification. BCECF-loaded cells were pretreated separately with 0, 15, 30, and 60 μM HOE-694 for 30 min before the stimulation with 10 μM progesterone. The arrow indicates the addition of 10 μM progesterone. The traces are representative of three experiments.

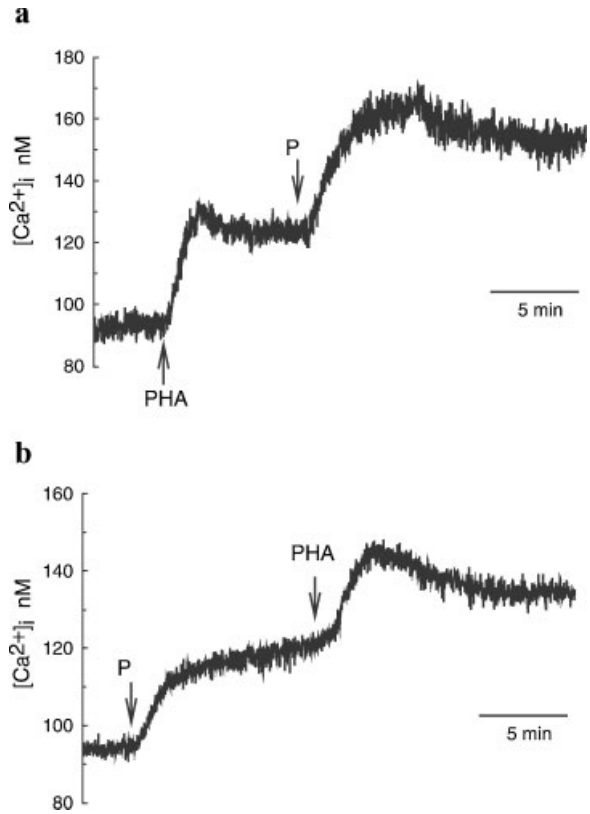

Fig. 6. Effects of progesterone on phytohemagglutinin (PHA)-induced $[Ca^{2+}]_i$ changes. **a**: Fura-2-loaded cells were stimulated with 10 μg/ml PHA for 10 min before the addition of 10 μM progesterone. **b**: Fura-2-loaded cells were stimulated with 10 μM progesterone for 12 min before addition of 10 μg/ml PHA. The arrows indicate the addition of progesterone or PHA. The traces are representative of three experiments.

(alkalinization) by PHA was blocked (Fig. 7a). In contrast, when PHA (10 μg/ml) was added 10 min after progesterone (10 μM), PHA could not reverse the acidification that had been induced by progesterone (Fig. 7b). Thus, it was necessary to identify if progesterone could block alkalinization by the PKC activator, PMA. As shown in Figure 7c, progesterone did block PMA-induced alkalinization.

Effects of Progesterone on PHA-Induced IL-2 and IL-4 Secretion

Here, the level of IL-2 and IL-4 secretion induced by progesterone was measured 24 h after stimulation. This was to further help to identify whether progesterone inhibited PHA-induced IL-2 and IL-4 secretion. T cells were stimulated with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 μM), PHA (5 μg/ml), or the combination of both, and IL-2 or IL-4 production was assessed 24 h after stimulation. By comparison with the controls, progesterone (0, 0.1, 0.5, 1, 5, 10, 20 μM) did not stimulate the production of IL-2 and IL-4 in T cells (Tables I and II). PHA did significantly stimulate IL-2 and IL-4 production in T cells. When progesterone and PHA were combined, it was found that progesterone (>0.5 μM) significantly inhibited PHA-stimulated IL-2 and IL-4 production in T cells (Figs. 8 and 9).

Effects of Progesterone on PHA-Induced [^{3}H]-Thymidine Incorporation into T Cells

The different concentrations of PHA (1, 2, 2.5, 5, and 10 μg/ml) significantly ($P < 0.001$) increased the degree of proliferation as measured by [^{3}H]-thymidine incorporation into T cells. In contrast, in comparison to the control, 10 μM of progesterone, testosterone, estradiol

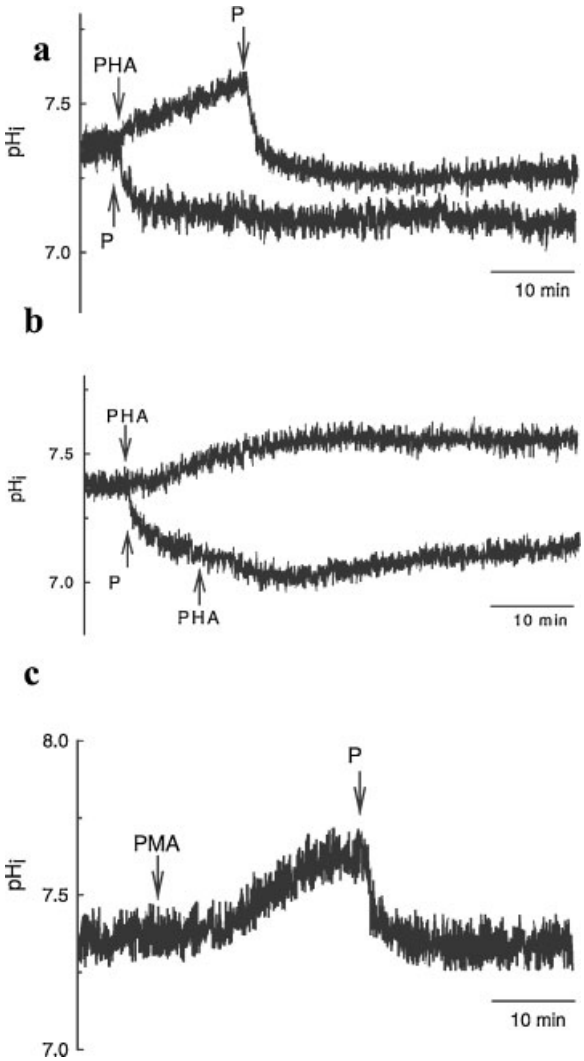

Fig. 7. Effects of progesterone on PHA- or Phorbol 12-myristate 13-acetate (PMA)-induced pH_i changes. **a**: BCECF-loaded cells were stimulated with or without 10 μg/ml PHA for 15 min before the addition of 10 μM progesterone. **b**: BCECF-loaded cells were stimulated with or without 10 μM progesterone for 10 min before the addition of 10 μg/ml PHA. **c**: BCECF-loaded cells were stimulated with 100 pM PMA for 25 min before the addition of 10 μM progesterone. The traces are representative of three experiments.

TABLE I. Effects of Progesterone on IL-2 Secretion in T Cells

Progesterone (μM)	0	0.1	0.5	1	5	10	20
IL-2	—	—	—	—	—	—	—

T cells were treated with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 μM) for 24 h and supernatants were collected to determine the levels of human IL-2, which were analyzed by ELISA. —, no response on comparison with the vehicle. The results from one experiment are representative of at least three experiments.

TABLE II. Effects of Progesterone on IL-4 Secretion in T Cells

Progesterone (μM)	0	0.1	0.5	1	5	10	20
IL-4	—	—	—	—	—	—	—

T cells were treated with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 μM) for 24 h and supernatants were collected to determine the levels of human IL-4, which were analyzed by ELISA. —, no response on comparison with the vehicle. The results from one experiment are representative of at least three experiments.

hydrocortisone, dexamethasone, and vehicle did not stimulate the uptake of [^{3}H]-thymidine and thus were not capable of inducing proliferation in T cells (Fig. 10). However, when compared with PHA (1 μg/ml), a combination of progesterone (0.1, 0.5, 1, 5, 10, 20 μM) with PHA (1 μg/ml) exhibited a significant dose-dependent suppression of the uptake of [^{3}H]-thymidine in T cells when the combination of progesterone was >0.5 μM (Fig. 11).

It was necessary to verify whether the suppression of T-cell proliferation by progesterone was attributable to the results of non-genomic steroid action. Therefore, instead of costimulation with progesterone (0.1, 0.5, 1, 5, 10, 20 μM) and PHA at same time, progesterone was added to T cells 72 h after PHA. Under these conditions, progesterone >0.1 μM was also found to exhibit a significant dose-dependent suppression of the PHA-induced [^{3}H]-thymidine uptake in T cells (Fig. 12).

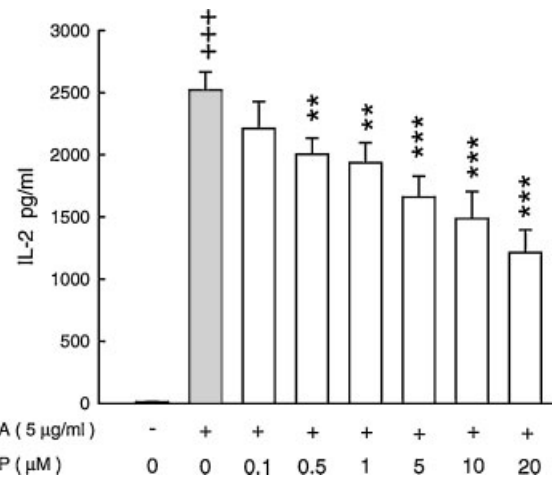

Fig. 8. Effects of progesterone on PHA-induced IL-2 secretion. Tcells were treated with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 μM) and with PHA (5 μg/ml) for 24 h and supernatants were collected to determine the levels of human IL-2, which were analyzed by ELISA. +++, $P < 0.001$ compared with the vehicle control. **, $P < 0.01$; ***, $P < 0.001$ compared with PHA. Each value represents mean ± SEM (n = 5).

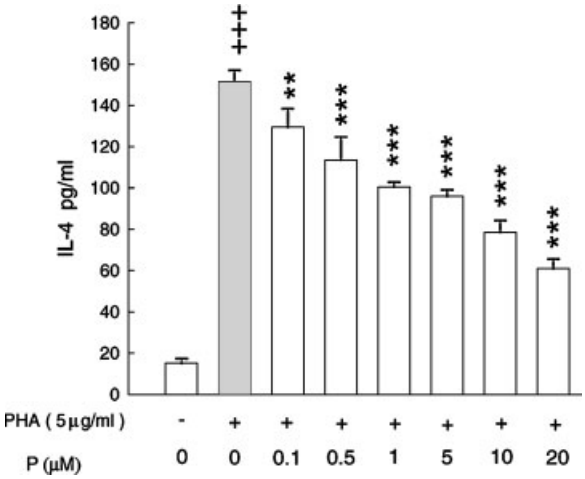

Fig. 9. Effects of progesterone on PHA-induced IL-4 secretion. T cells were treated with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 µM) with PHA (5 µg/ml) for 24 h and supernatants were collected to determine the levels of human IL-4, which were analyzed by ELISA. +++, $P < 0.001$ compared with the vehicle control. **, $P < 0.01$; ***, $P < 0.001$ compared with PHA. Each value represents mean ± SEM (n = 5).

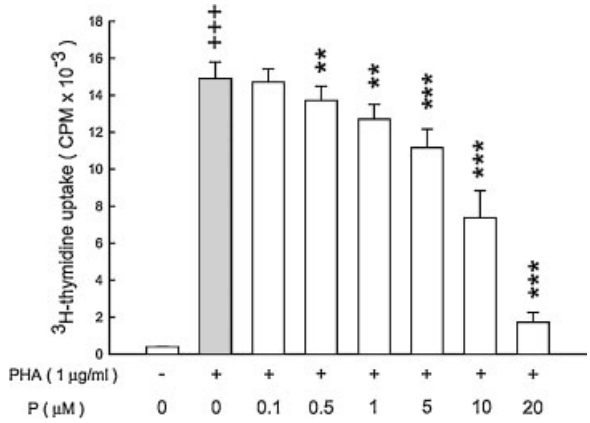

Fig. 11. Dose effect of progesterone on PHA-stimulated [^{3}H]-thymidine incorporation into T cells. The combination of PHA (1 µg/ml) with progesterone (0, 0.1, 0.5, 1, 5, 10, 20 µM) was used to study [^{3}H]-thymidine incorporation into T cells. The incorporation of [^{3}H]-thymidine is presented as CPM × $10^{-3}/10^5$ cells. +++, $P < 0.001$ compared with the vehicle control. **, $P < 0.01$; ***, $P < 0.001$ compared with PHA. Each value represents mean ± SEM (n = 5).

Effects of Progesterone-BSA Conjugate on $[Ca^{2+}]_i$, pH_i Changes and PHA-Induced [^{3}H]-Thymidine Incorporation Into T Cells

In order to demonstrate that the immunosuppression was due to non-genomic action by progesterone on plasma membrane sites, various doses of progesterone-BSA (PBSA) (10, 50, 100, 200, 400, 800, 1,600 nM) were used to stimulate T cells and the changes in $[Ca^{2+}]_i$ and pH_i or PHA-induced [^{3}H]-thymidine incorporation into T cells were observed. In human peripheral T cells, the membrane impermeable progesterone-BSA also resulted in a stepwise increase in $[Ca^{2+}]_i$, similar to that obtained with free progesterone as well as a decrease in pH_i (Fig. 13). In addition, progesterone-BSA above 50 nM resulted in a significant dose-dependent

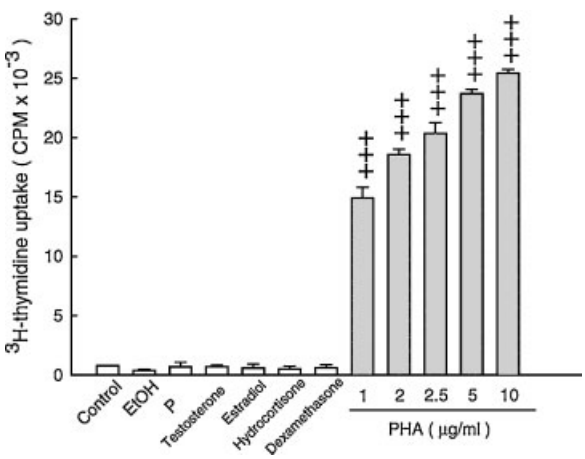

Fig. 10. Effects of progesterone, testosterone, estradiol, hydrocortisone, dexamethasone on [^{3}H]-thymidine incorporation into T cells. 10 µM of progesterone, testosterone, estradiol, hydrocortisone, dexamethasone, or PHA (1, 2, 2.5, 5, 10 µM) was used to study [^{3}H]-thymidine incorporation into T cells. The incorporation of [^{3}H]-thymidine is presented as CPM × $10^{-3}/10^5$ cells. +++, $P < 0.001$ compared with the vehicle control. Each value represents mean ± SEM (n = 5).

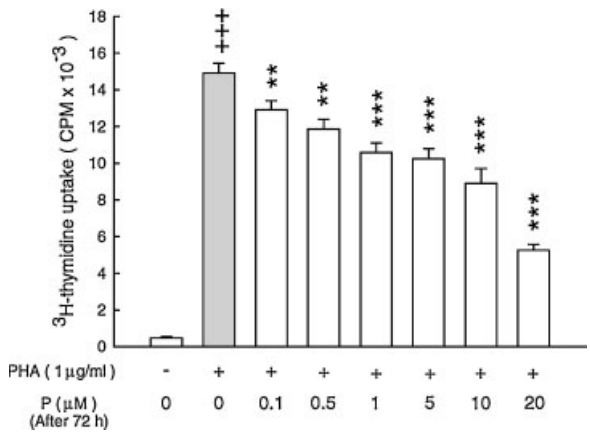

Fig. 12. Effect on [^{3}H]-thymidine incorporation into T cells by addition of progesterone 72 h after PHA. Progesterone (0, 0.1, 0.5, 1, 5, 10, 20 µM) was added with [^{3}H]-thymidine 72 h after PHA (1 µg/ml) in T cells. After addition, T cells were incubated for a further 18 h before the measurement of [^{3}H]-thymidine incorporation into T cells. The incorporation of [^{3}H]-thymidine is presented as CPM × $10^{-3}/10^5$ cells. +++, $P < 0.001$ compared with the vehicle control. **, $P < 0.01$; ***, $P < 0.001$ compared with PHA. Each value represents mean ± SEM (n = 3).

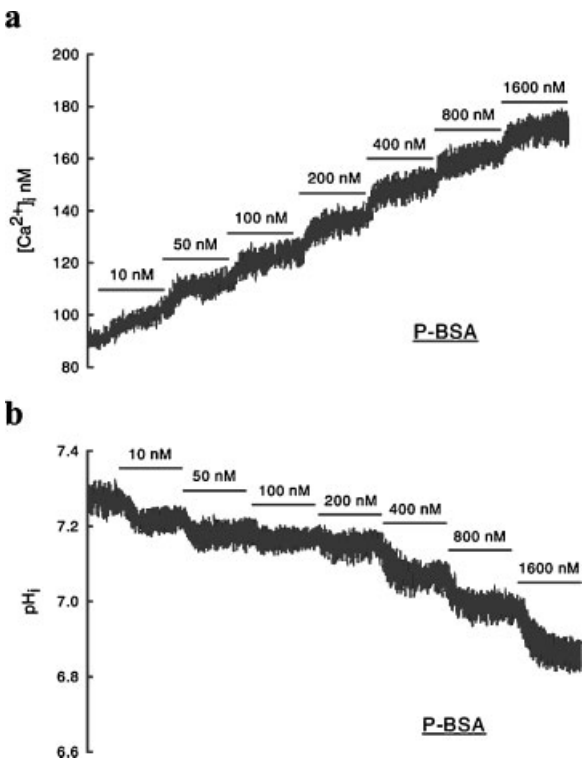

Fig. 13. The effect of progesterone-BSA (P-BSA) on $[Ca^{2+}]_i$ and pH_i changes. Fura-2-loaded cells or BCECF-loaded cells were stimulated with (10, 50, 100, 200, 400, 800, 1,600 nM) P-BSA. **a**: Stepwise dose-response curves of P-BSA-induced $[Ca^{2+}]_i$ elevation in fura-2-loaded T cells. **b**: Stepwise dose-response curves of P-BSA-induced pH_i changes in BCECF-loaded T cells. The bar indicates the time of P-BSA addition over a 10 min period. The tracings are from one representative of three experiments.

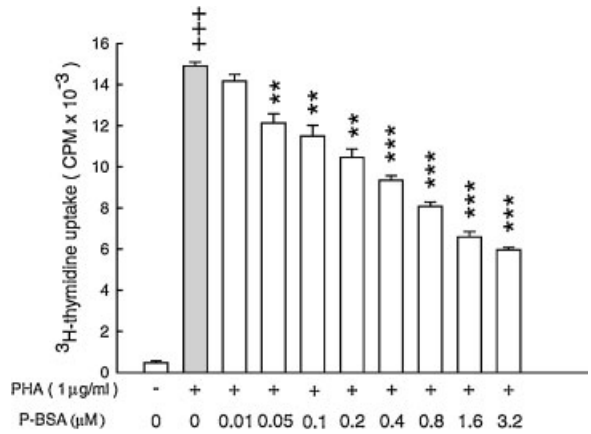

Fig. 14. Dose effect of P-BSA on PHA-stimulated [^{3}H]-thymidine incorporation into T cells. The combination of PHA (1 μg/ml) with P-BSA (0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μM) was used to study [^{3}H]-thymidine incorporation into T cells. The incorporation of [^{3}H]-thymidine is presented as CPM $\times 10^{-3}/10^5$ cells. +++, $P < 0.001$ compared with the vehicle control. **, $P < 0.01$; ***, $P < 0.001$ compared with PHA. Each value represents mean ± SEM (n = 3).

suppression of PHA-induced [^{3}H]-thymidine uptake in T cells (Fig. 14).

DISCUSSION

There is a difference between the stimulated responses induced by estradiol, testosterone, hydrocortisone, and dexamethasone and those stimulated by progesterone, because the latter resulted in an increase in intracellular Ca^{2+} and a decrease in pH (Figs. 1 and 2a,b). This is the first observation of a dose-response dependent acidification by progesterone in human peripheral T cells. Pretreatment of T cells with progesterone can inhibit potassium channels, thapsigargin capacitative calcium influx, and $[Ca^{2+}]_i$ oscillations that are stimulated by T-cell receptor ligation [Ehring et al., 1998]. It has been found that blocking of the potassium channel will cause depolarization of the membrane potential and inhibit receptor-operated calcium channels in Jurkat T cells [Sarkadi et al., 1990]. In this study, progesterone was found to have non-genomic effects on ionic fluxes in human peripheral T cells and the transient intracellular Ca^{2+} increase that was stimulated by progesterone has also been observed in human sperm [Blackmore et al., 1990; Falkenstein et al., 1999]. Recently, such rapid non-genomic signals by steroids are recognized as being transmitted by membrane receptors unrelated to the classic intracellular steroid receptors. A putative progesterone membrane binding protein has been identified in CHO cells. The antibody to this protein can suppress the rapid progesterone-initiated Ca^{2+} increase in sperm [Falkenstein et al., 1999]. A new family of membrane G protein-coupled progestin receptors has been discovered in spotted seatrout [Zhu et al., 2003b] and is also present in human, mouse, pig, and Xenopus [Zhu et al., 2003a].

On the removal of extracellular Ca^{2+} using EGTA, the increase in $[Ca^{2+}]_i$ caused by progesterone was abolished (Fig. 3). However, EGTA did not affect the acidification by progesterone (Fig. 4). Therefore, NHE inhibitors, DMA, and 3-methylsulphonyl-4-piperidinobenzoyl guanidine hydrochloride (HOE-694) were used to investigate whether NHE played a role in progesterone-stimulated acidification in T cells. We found the acidification induced by progesterone was inhibited by HOE-694 but not

by the amiloride inhibitor, DMA (Fig. 5). HOE-694 is reported to be a specific NHE inhibitor [Scholz et al., 1993]. The activation of the amiloride-sensitive NHE has been observed when T-cell surface receptors are activated by lectin or antibodies [Mills et al., 1985c, 1986]. If the acidification is induced by the inhibition of NHE, then the use of HOE-694 should further enhance the acidification by progesterone as occurs in Jurkat T cells under CD95 stimulation [Lang et al., 2000]. However, in this study, the acidification by progesterone was not further enhanced by HOE-694, but suppressed. Thus, more studies are required to identify why this discrepancy occurs and a kinetic study of NHE activity would seem necessary as part of this.

Pretreatment of T cells with progesterone was able to potentiate the PHA-induced $[Ca^{2+}]_i$ increase and vice versa (Fig. 6a,b). Since the $[Ca^{2+}]_i$ increase by PHA and progesterone was additive, progesterone and PHA might potentiate each other and share common calcium-mediated signals in the T cells. However, the results for pH_i with progesterone and PHA or PMA were different to the responses obtained for $[Ca^{2+}]_i$. In our previous study, PHA and PMA were shown to stimulate PKC-dependent alkalinization in T cells [Chien et al., 2001]. PHA did not block acidification by progesterone (Fig. 7b), but progesterone prevented alkalinization by PHA or PMA (Fig. 7a,c). Thus, whether the inhibition of PKC or of NHE by progesterone is the cause of the inhibition of alkalinization by PHA or PMA also needs to be further investigated. Progesterone can inhibit lymphocyte proliferation by a mitogen [Van Voorhis et al., 1989] and the pH_i response is the only diverse early signal induced by PHA and progesterone in T cells. This implies that the acidification induced by progesterone might play an important role in the inhibition of the thapsigargin capacitative calcium influx and T-cell proliferation by PHA. In this study, hydrocortisone and dexamethasome stimulated acidification in T cells (Fig. 1). It is still not clear whether the depolarization of the membrane potential is the downstream signal for transient $[Ca^{2+}]_i$ elevation by progesterone. However, it is well known that the loss of plasma membrane potential is an early important apoptotic feature in rat thymocytes after treatment with glucocorticoids or dexamethasone [Mann and Cidlowski, 2001; Lampert et al., 2003].

Since an increase of $[Ca^{2+}]_i$ is essential to induce T cells to secrete IL-2, it is clear that blocking the increase in $[Ca^{2+}]_i$ should be associated with an inhibition of IL-2 secretion and T-cell proliferation [Mills et al., 1985a,b; Gelfand et al., 1986]. Although progesterone could stimulate the $[Ca^{2+}]_i$ increase and also potentiated the $[Ca^{2+}]_i$ increase by PHA, it did block PHA-induced alkalinization. Thus, under this situation, it was necessary to investigate whether the $[Ca^{2+}]_i$ increase by progesterone was able to enhance the stimulation of IL-2 or IL-4 secretion in T cells by PHA. As shown in Tables I and II, progesterone alone did not stimulate secretion of IL-2 and IL-4, but it inhibited PHA-stimulated IL-2 and IL-4 production in T cells (Figs. 8 and 9). Recently, it has been shown that cytosolic Ca^{2+} increase by drug does not trigger the release of histamine in mast cells, but cytosolic alkalinization does [Pernas-Sueiras et al., 2005]. Thus, the acidification by progesterone might suppress the secretion of cytokines by PHA. In addition, progesterone did not stimulate T-cell proliferation, but it was able to suppress the responses caused by PHA involving T-cell proliferation (Figs. 10 and 11). The signal to transiently elevate $[Ca^{2+}]_i$ is produced by both progesterone and PHA in T cells. The non-genomic signal of acidification by progesterone seemed to exert an important role in suppressing PHA-induced IL-2 or IL-4 secretion and proliferation in T cells. In this study, hydrocortisone was found to stimulate the acidification but not $[Ca^{2+}]_i$ elevation in T cells (Fig. 1). However, hydrocortisone has been reported to block human mitogen-induced IL-4 secretion [Byron et al., 1992].

The production of diacylglycerol (DAG) following hydrolysis of phosphatidylinositol activates PKC, which in turn enhances the activity of NHE, and leads to an increase in pH_i in T cells [Gelfand et al., 1987]. Homeostasis of the intracellular pH is important in terms of the cell's ability to respond to the initiation of differentiation and proliferation and to allow the maintenance of normal metabolic functions. The down stream signal for intracellular alkalinization in T cells can be used as a physiological indicator of PKC activation [Chien et al., 2001]. Two shifts in the intracellular alkalinization have been observed in T cells after the addition of mitogens [Gerson et al., 1982]. 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 characteristic of apoptosis that can activate the cation-insensitive acidic endonuclease [Sharma and Srikant, 1998] and thus cause genome destruction in cells [Gottlieb et al., 1996].

When progesterone was administrated at the same time as PHA, or 72 h after PHA, a suppression effect on T-cell proliferation was obtained (Figs. 11 and 12). The suppression of PHA-induced T-cell proliferation by administration of progesterone 72 h later implied that the uptake of [^{3}H]-thymidine into T cells could be seriously affected by progesterone. Progesterone might exert its non-genomic action on the plasma membrane to reduce [^{3}H]-thymidine uptake as it suppresses T-cell proliferation. It would be interesting to know how this happens. A reduction in pH_i induced by lowering extracellular pH can attenuate the incorporation of [^{3}H]-thymidine into DNA. In contrast, an increase in pH_i is associated with acceleration in the rate of incorporation of [^{3}H]-thymidine into DNA in vascular smooth muscle cells [Bobik et al., 1991]. In addition, a second shift in the intracellular alkalinization was found in S phase during the cell cycle when T cells are activated by mitogen [Gerson et al., 1982]. Thus, the non-genomic effect of acidification by progesterone, against PMA or PHA-induced alkalinization in Figure 7, is suspected not only to inhibit the incorporation of [^{3}H]-thymidine into DNA (Fig. 12) but also to inhibit PHA-induced IL-2 or IL-4 secretion in T cells (Figs. 8, 9, 11).

The immobilization of progesterone by conjugation to a large carrier molecule BSA creating progesterone-BSA; progesterone-BSA was used at nM concentration to demonstrate the non-genomic effects on $[Ca^{2+}]_i$ elevation, pH_i decrease, and suppression of PHA-induced T-cell proliferation. The results show that the action of progesterone was at plasma membrane sites (Figs. 13 and 14). These results demonstrated that progesterone could perform its non-genomic effects at the membrane sites and affect the genomic responses caused by PHA. Further studies will be needed to investigate whether the non-genomic effects were mediated directly through the plasma membrane progesterone receptor. Non-genomic stimulation of $[Ca^{2+}]_i$ elevation and acidification by progesterone was first found in T cells. Progesterone can inhibit glucocorticoid-induced murine thymocyte apoptosis [McMurray et al., 2000]. As mentioned earlier, human peripheral blood monocytes do not possess a classical progesterone receptor [Kontula et al., 1983; Schust et al., 1996]. Thus, progesterone-induced immunosuppression is mediated through neither the classical progesterone receptor nor the glucocorticoid receptor. Our findings indicated the non-genomic effect on acidification might be an important early immunomodulation signal induced by progesterone in T cells. The action of progesterone was found to be specific and directly acted through nonclassical steroid receptors in T cells. Non-genomic effects of progesterone might play a pivotal role in the control mechanisms immunomodulating T cells during pregnancy.

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REFERENCES

Berry N, Nishizuka Y. 1990. Protein kinase C and T cell activation. Eur J Biochem 189:205–214.

Berry N, Ase K, Kishimoto A, Nishizuka Y. 1990. Activation of resting human T cells requires prolonged stimulation of protein kinase C. Proc Natl Acad Sci USA 87:2294–2298.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. 1990. Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. J Biol Chem 265:1376–1380.

Bobik A, Grooms A, Little PJ, Cragoe EJ , Jr., Grinpukel S. 1991. Ethylisopropylamiloride-sensitive pH control mechanisms modulate vascular smooth muscle cell growth. Am J Physiol 260:C581–C588.

Byron KA, Varigos G, Wootton A. 1992. Hydrocortisone inhibition of human interleukin-4. Immunology 77:624–626.

Chien EJ, Chien CH, Chen JJ, Wang SW, Hsieh DJ. 2000. Bacterial lipopolysaccharide activates protein kinase C, but not intracellular calcium elevation, in human peripheral T cells. J Cell Biochem 76:404–410.

Chien EJ, Hsieh DJ, Wang JE. 2001. Response of alkalinization or acidification by phytohemagglutinin is dependent on the activity of protein kinase C in human peripheral T Cells. J Cell Biochem 81:604–612.

Civitelli R, Teitelbaum SL, Hruska KA, Lacey DL. 1989. IL-1 activates the Na^+/H^+ antiport in a murine T cell. J Immunol 143:4000–4008.

Ehring GR, Kerschbaum HH, Eder C, Neben AL, Fanger CM, Khoury RM, Negulescu PA, Cahalan MD. 1998.

A nongenomic mechanism for progesterone-mediated immunosuppression: Inhibition of K^+ channels, Ca^{2+} signaling, and gene expression in T lymphocytes. J Exp Med 188:1593–1602.

Falkenstein E, Heck M, Gerdes D, Grube D, Christ M, Weigel M, Buddhikot M, Meizel S, Wehling M. 1999. Specific progesterone binding to a membrane protein and related nongenomic effects on Ca^{2+}-fluxes in sperm. Endocrinology 140:5999–6002.

Gamberucci A, Giunti R, Benedetti A. 2004. Progesterone inhibits capacitative Ca^{2+} entry in Jurkat T lymphocytes by a membrane delimited mechanism, independently of plasma membrane depolarization. Cell Calcium 36: 175–180.

Gekle M, Golenhofen N, Oberleithner H, Silbernagl S. 1996. Rapid activation of Na^+/H^+ exchange by aldosterone in renal epithelial cells requires Ca^{2+} and stimulation of a plasma membrane proton conductance. Proc Natl Acad Sci USA 93:10500–10504.

Gelfand EW, Cheung RK, Grinstein S, Mills GB. 1986. Characterization of the role for calcium influx in mitogen-induced triggering of human T cells. Identification of calcium-dependent and calcium-independent signals. Eur J Immunol 16:907–912.

Gelfand EW, Mills GB, Cheung RK, Lee JW, Grinstein S. 1987. Transmembrane ion fluxes during activation of human T lymphocytes: Role of Ca^{2+}, Na^+/H^+ exchange and phospholipid turnover. Immunol Rev 95:59–87.

Gerson DF, Kiefer H, Eufe W. 1982. Intracellular pH of mitogen-stimulated lymphocytes. Science 216:1009–1010.

Gottlieb RA, Nordberg J, Skowronski E, Babior BM. 1996. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc Natl Acad Sci USA 93:654–658.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hengel H, Allig B, Wagner H, Heeg K. 1991. Dissection of signals controlling T cell function and activation: H7, an inhibitor of protein kinase C, blocks induction of primary T cell proliferation by suppressing interleukin (IL)2 receptor expression without affecting IL2 production. Eur J Immunol 21:1575–1582.

Hesketh TR, Moore JP, Morris JD, Taylor MV, Rogers J, Smith GA, Metcalfe JC. 1985. A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. Nature 313:481–484.

Kontula K, Paavonen T, Luukkainen T, Andersson LC. 1983. Binding of progestins to the glucocorticoid receptor. Correlation to their glucocorticoid-like effects on in vitro functions of human mononuclear leukocytes. Biochem Pharmacol 32:1511–1518.

Lampert A, Muller MM, Berchtold S, Lang KS, Palmada M, Dobrovinskaya O, Lang F. 2003. Effect of dexamethasone on voltage-gated K^+ channels in Jurkat T-lymphocytes. Pflugers Arch 447:168–174.

Lang F, Madlung J, Bock J, Lukewille U, Kaltenbach S, Lang KS, Belka C, Wagner CA, Lang HJ, Gulbins E, Lepple-Wienhues A. 2000. Inhibition of Jurkat-T-lymphocyte Na^+/H^+-exchanger by CD95(Fas/Apo-1)-receptor stimulation. Pflugers Arch 440:902–907.

Li M, Morley P, Tsang BK. 1991. Epidermal growth factor elevates intracellular pH in chicken granulosa cells by activating protein kinase C. Endocrinology 129:2957–2964.

Lin CY, Lo SC. 1991. Treatment of hepatitis B virus-associated membranous nephropathy with adenine arabinoside and thymic extract. Kidney Int 39:301–306.

Liu TC, Pu HF, Wang PS. 1993. Unimpaired postreceptor regulation of luteinizing hormone secretion by gonadotropin-releasing hormone and estrogen in aged rat anterior pituitary cells. Endocrinology 132:1189–1194.

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW. 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 9:2266–2278.

Mann CL, Cidlowski JA. 2001. Glucocorticoids regulate plasma membrane potential during rat thymocyte apoptosis in vivo and in vitro. Endocrinology 142:421–429.

McMurray RW, Wilson JG, Bigler L, Xiang L, Lagoo A. 2000. Progesterone inhibits glucocorticoid-induced murine thymocyte apoptosis. Int J Immunopharmacol 22: 955–965.

Mills GB, Cheung RK, Grinstein S, Gelfand EW. 1985a. Increase in cytosolic free calcium concentration is an intracellular messenger for the production of interleukin 2 but not for expression of the interleukin 2 receptor. J Immunol 134:1640–1643.

Mills GB, Cheung RK, Grinstein S, Gelfand EW. 1985b. Interleukin 2-induced lymphocyte proliferation is independent of increases in cytosolic-free calcium concentrations. J Immunol 134:2431–2435.

Mills GB, Cragoe EJ, Jr., Gelfand EW, Grinstein S. 1985c. Interleukin 2 induces a rapid increase in intracellular pH through activation of a Na^+/H^+ antiport. Cytoplasmic alkalinization is not required for lymphocyte proliferation. J Biol Chem 260:12500–12507.

Mills GB, Cheung RK, Cragoe EJ Jr, Grinstein S, Gelfand EW. 1986. Activation of the Na^+/H^+ antiport is not required for lectin-induced proliferation of human T lymphocytes. J Immunol 136:1150–1154.

Ostensen M. 1999. Sex hormones and pregnancy in rheumatoid arthritis and systemic lupus erythematosus. Ann N Y Acad Sci 876:131–143.

Pernas-Sueiras O, Alfonso A, Vieytes MR, Botana LM. 2005. Mast cell exocytosis can be triggered by ammonium chloride with just a cytosolic alkalinization and no calcium increase. J Cell Physiol 204:775–784.

Sarkadi B, Tordai A, Gardos G. 1990. Membrane depolarization selectively inhibits receptor-operated calcium channels in human T (Jurkat) lymphoblasts. Biochim Biophys Acta 1027:130–140.

Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Scholkens BA. 1993. Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischaemia. Br J Pharmacol 109:562–568.

Schust DJ, Anderson DJ, Hill JA. 1996. Progesterone-induced immunosuppression is not mediated through the progesterone receptor. Hum Reprod 11:980–985.

Sharma K, Srikant CB. 1998. G protein coupled receptor signaled apoptosis is associated with activation of a cation insensitive acidic endonuclease and intracellular acidification. Biochem Biophys Res Commun 242: 134–140.

Stites DP, Siiteri PK. 1983. Steroids as immunosuppressants in pregnancy. Immunol Rev 75:117–138.

Szekeres-Bartho J, Philibert D, Chaouat G. 1990. Progesterone suppression of pregnancy lymphocytes is not

mediated by glucocorticoid effect. Am J Reprod Immunol 23:42–43.

Van Voorhis BJ, Anderson DJ, Hill JA. 1989. The effects of RU 486 on immune function and steroid-induced immunosuppression in vitro. J Clin Endocrinol Metab 69: 1195–1199.

Zhu Y, Bond J, Thomas P. 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci USA 100:2237–2242.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. 2003b. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci USA 100:2231–2236.