Glucocorticoid modulation of Ca^{2+} homeostasis in human B lymphoblasts

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1. We determined the effect of cortisol (200 nM for 48 h) on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and parameters of Ca_i^{2+} signalling in 19 lymphoblastoid cell lines (LCLs).

2. Using the fluorescent dye fura-2, the basal $[Ca^{2+}]_i$ in Ca^{2+}-containing medium was $63{\cdot}5 \pm 2{\cdot}4$ nM in vehicle (ethanol)-treated LCLs and $55{\cdot}7 \pm 2{\cdot}6$ nM (mean ± S.E.M.) in cortisol-treated LCLs.

3. Ca_i^{2+} signalling following platelet-activating factor (PAF, 100 nM) addition was enhanced by cortisol treatment, with LCLs having small PAF responses showing the largest percentage increase after cortisol treatment. Mean peak $[Ca^{2+}]_i$ responses to PAF were enhanced 67·0% and 55·7% in Ca^{2+}-free and Ca^{2+}-containing medium, respectively.

4. The endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (100 nM) caused a transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium in which the peak change was increased in cortisol-treated cells ($98{\cdot}5 \pm 5{\cdot}8$ *vs.* $79{\cdot}8 \pm 4{\cdot}5$ nM). Peak changes in the freely exchangeable Ca^{2+} in response to 5 μM ionomycin were also enhanced in cortisol-treated cells ($923{\cdot}7 \pm 113{\cdot}9$ *vs.* $652{\cdot}2 \pm 64{\cdot}5$ nM) and correlated to the PAF-evoked $[Ca^{2+}]_i$ response.

5. Cortisol-treated LCLs exposed to thapsigargin to empty intracellular Ca^{2+} stores (10 min treatment in Ca^{2+}-free medium) and exposed to $CaCl_2$ or $MnCl_2$ had a greater rate of Ca^{2+} entry ($18{\cdot}6 \pm 1{\cdot}8$ *vs.* $13{\cdot}8 \pm 1{\cdot}5$ nM s^{-1}) and higher rate constant for Mn^{2+} entry ($0{\cdot}0345 \pm 0{\cdot}0029$ *vs.* $0{\cdot}0217 \pm 0{\cdot}0020$) than vehicle-treated cells. Peak $[Ca^{2+}]_i$ in cells exposed to $CaCl_2$ was also enhanced ($869{\cdot}4 \pm 114{\cdot}7$ *vs.* $562{\cdot}6 \pm 61{\cdot}7$ nM). Parameters of divalent cation influx were highly correlated to the peak $[Ca^{2+}]_i$ elicited by thapsigargin or ionomycin.

6. Inclusion of RU 486 (a glucocorticoid antagonist) with cortisol prevented the decrease in basal $[Ca^{2+}]_i$ and stimulatory actions of cortisol on all Ca_i^{2+} parameters. RU 486 alone had no apparent effects on basal $[Ca^{2+}]_i$ or Ca_i^{2+} signalling.

7. Based on data obtained over a wide range of responses (in the presence and/or absence of cortisol or RU 486), the results show that cortisol stimulation of glucocorticoid receptors decreases basal $[Ca^{2+}]_i$ and enhances PAF-evoked $[Ca^{2+}]_i$ signalling, most probably through its effects on intracellular Ca^{2+} stores. In turn, the extent of Ca^{2+} entry via store-operated plasma membrane Ca^{2+} channels is closely linked to the size of the Ca^{2+} stores.

Stimulation of B lymphocytes leads to diverse cellular functions including secretion, proliferation, differentiation or programmed cell death (apoptosis). Inflammatory agents binding or cross-linking surface membrane receptors increase protein kinase/phosphatase activities and activate phospholipase C, generating inositol 1,4,5-trisphosphate (IP_3). IP_3 mobilizes Ca^{2+} from intracellular Ca^{2+} stores and produces a transient rise in cytosolic Ca^{2+} (Ca_i^{2+}). The emptying of Ca^{2+} from IP_3-sensitive Ca^{2+} stores also results in enhanced Ca^{2+} influx across the plasma membrane, which is manifested as an enhancement of the Ca_i^{2+} transient, oscillatory increases in Ca_i^{2+}, or a secondary, sustained increase in $[Ca^{2+}]_i$. This Ca^{2+} entry pathway, termed store-operated Ca^{2+} entry (SOCE), is responsible for refilling intracellular Ca^{2+} stores (Putney, 1990) and is an important regulator of Ca^{2+}-dependent gene expression (Negulescu *et al.* 1994; Dolmetsch *et al.* 1998; for review, see Putney & Bird, 1993). In B lymphocytes, elevation of $[Ca^{2+}]_i$ serves as a differentiation signal towards antibody-secreting cells (Clevers *et al.* 1985; Huang *et al.* 1995) and enables

interleukin-2 (IL-2) synthesis (Taira *et al.* 1987) and cell proliferation (Ambrus *et al.* 1991). The inhibition of Ca^{2+} influx via the SOCE pathway results in marked decreases in $[Ca^{2+}]_i$ and IL-2 (Diegel *et al.* 1994), supporting the hypothesis that the B cell response is related to the degree of $[Ca^{2+}]_i$ elevation (Clark & Lane, 1991). Additional changes in Ca_i^{2+} regulation in anti-IgM-stimulated malignant B-1 cells suggest that dysfunctional Ca_i^{2+} signalling may increase the susceptibility for these cells to undergo apoptosis (Dang *et al.* 1995). Thus, the regulation of $[Ca^{2+}]_i$ represents a key factor in the control of B lymphocyte development and function.

Cortisol and synthetic glucocorticoids such as dexamethasone are important modulators of the immune system (Cupps & Fauci, 1982), and exert both inhibitory and stimulatory actions on lymphocyte development and cellular responses to inflammatory stimuli (Guyre *et al.* 1988; Wilckens & De Rijk, 1997). In murine precursor B cells, slight elevation of the circulating level of glucocorticoids decreased the number of cycling cells by inducing apoptosis (Garvey *et al.* 1993). In B cells undergoing lymphopoiesis, negative regulatory effects of glucocorticoids were countered by the presence of (T) lymphocyte-supportive stromal cells (Borghesi *et al.* 1997). Glucocorticoids produce variable responses in cell surface Ia (sIa) expression, the response being dependent on the duration of exposure and the state (i.e. resting or activated) of the B cell (McMillan *et al.* 1988). In a report examining cell activation of resting B cells, cortisol acted as an inhibitor of anti-Ig-antibody-mediated IP_3 synthesis, Ca_i^{2+} signalling and entry into the cell cycle (Dennis *et al.* 1987). These results suggest that, similar to T lymphocytes, cortisol effects on B cell function are dependent on the extent of glucocorticoid exposure and the context in which inflammatory mediators are presented. In light of the significant influence of Ca^{2+} homeostasis on B cell proliferation and differentiation, widely different effects of glucocorticoids on Ca_i^{2+} signalling and B cell physiology could be expected at different stages of B lymphocyte development.

To examine the glucocorticoid regulation of Ca^{2+} homeostasis in activated B lymphocytes, we studied cortisol-induced alterations of Ca^{2+} signalling in Epstein–Barr virus (EBV)-transformed B lymphoblasts. *In vitro* transformation of peripheral blood cells by EBV leads to partial differentiation and immortalization of cells of the B lymphocyte lineage, lending to their characterization as a model of activated B cells. EBV-generated lymphoblastoid cell lines (LCLs) are often used where stable, proliferating cultures of pure B cells are required. Their growth in tissue culture surmounts obstacles often associated with freshly isolated cell populations, including low cell number, deleterious effects of cell isolation chemicals or procedures, and exposure to leucocyte-derived cytokines or hormones. Several groups have suggested that LCLs exhibit signalling and membrane ion transport properties common to non-transformed cells (Schulam *et al.* 1990; Kuruvilla *et al.* 1993; Kojima *et al.* 1994; Siffert *et al.* 1995), supporting the idea that independent of viral transformation, LCLs retain membrane signalling properties representative of B lymphocytes. Thus, to study the role of cortisol on Ca^{2+} homeostasis in activated B cells, we examined parameters of receptor-mediated $[Ca^{2+}]_i$ signalling, intracellular Ca^{2+} stores and Ca^{2+} influx across the plasma membrane (SOCE) in LCLs.

METHODS

Chemicals

Unless otherwise specified, the chemicals used in this study were obtained from Sigma. Fura-2-acetoxymethylester (fura-2 AM) was from Molecular Probes; ionomycin and platelet-activating factor (PAF) were from Calbiochem; thapsigargin (TG) was from Alexis Corporation; mifepristone (RU 486) was from Biomol Research Laboratories, Inc.

Culture of B lymphoblasts

LCLs from the Centre d'Etude du Polymorphisme Humain (CEPH) collection (Coriell Institute for Medical Research, Camden, NJ, USA) were cultured as previously described (Brzustowicz *et al.* 1997) in RPMI 1640 culture medium containing 2 mM L-glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\,l^{-1}$ streptomycin and 15% heat-inactivated fetal bovine serum (Irvine Scientific). Cells were passaged 2–3 times per week, and cell density was maintained between $0{\cdot}5 \times 10^6$ and 1×10^6 cells ml^{-1}. Cells used for experiments were derived from fresh cultures thawed from frozen stocks at 3–4 month intervals. Cells were routinely monitored for mycoplasma contamination with the Mycotrim TC Triphasic Culture System (Irvine Scientific).

In preparation for experimental protocols, 10^7 cells ($0{\cdot}5 \times 10^6$ ml^{-1}) were resuspended for 48 h in fresh culture medium containing glucocorticoid (200 nM cortisol), glucocorticoid receptor (GR) antagonist (RU 486: Gagne *et al.* 1985) or vehicle (ethanol, final concentration of 0·04%). Cell numbers were determined with a Coulter Counter ZM.

Measurements of $[Ca^{2+}]_i$

Lymphoblasts were washed at 37 °C with Hepes-buffered solution (HBS) comprising (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (Hepes), 10 glucose (pH 7·4), and 0·1% fatty acid-free bovine serum albumin. Cells (2×10^7 ml^{-1}) were then incubated for 30 min at 37 °C with 2 μM fura-2 AM and 0·125 mM sulfinpyrazone (an anionic transport inhibitor used to retard fura-2 extrusion: Di Virgilio *et al.* 1988) as previously described (Dang *et al.* 1995; Brzustowicz *et al.* 1997). Aliquots of lymphoblasts (2×10^6 ($100\ \mu l$)$^{-1}$) were centrifuged to remove extracellular fura-2, washed once with HBS and resuspended in a cuvette containing 3 ml of either Ca^{2+}-free HBS (0·3 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) substituted for $CaCl_2$) or HBS. $[Ca^{2+}]_i$ measurements were performed in a CM3 spectrophotometer equipped with stirring and temperature control (Instruments SA, Inc.). Excitation wavelengths were set at 340 nm/380 nm and emission wavelength at 505 nm. $[Ca^{2+}]_i$ was calculated from the 340 nm/380 nm fluorescence ratio (R) according to the equation described by Grynkiewicz *et al.* (1985). Calibration was achieved by treating cells with 100 μM digitonin in the presence of 1 mM $CaCl_2$ (R_{max}), followed by 15 mM EGTA (pH 8·0; R_{min}). In some experiments, TG, a sarco(endo)plasmic

reticulum Ca^{2+}-ATPase (SERCA) inhibitor (Lytton *et al.* 1991), was used to inhibit Ca^{2+} reuptake by intracellular Ca^{2+} stores. Autofluorescence of unloaded cells in HBS was subtracted from the cellular fluorescence of the dye prior to all calculations for $[Ca^{2+}]_i$.

Mn^{2+} uptake assay

Mn^{2+} was used as a Ca^{2+} surrogate to study divalent cation entry (Hallam & Rink, 1985). Fura-2-loaded cells were centrifuged, resuspended in 500 μl microfuge tubes in Ca^{2+}-free HBS and treated with vehicle (DMSO; 0·5% final volume) or TG (500 nM) for 10 min. Cells were recentrifuged and suspended in cuvettes containing Ca^{2+}-free medium; after 10 s, 0·35 mM $MnCl_2$ was added. Fluorescence was monitored at excitation and emission wavelengths of 360 nm and 505 nm. Data were normalized using 360 nm values obtained immediately prior to addition of $MnCl_2$. Autofluorescence of Mn^{2+}-treated (1 mM), digitonin-permeabilized cells and buffer for each experiment was subtracted from the fluorescence record prior to normalization.

Data analysis and statistics

Mn^{2+} quenching of fura-2 (percentage fluorescence quenching at times 0, 10, 30 and 60 s compiled from individual experiments of vehicle- and TG-treated cells) was best-fitted to the mono-exponential function:

$$A = a_1 e^{-k_1 t},$$

where A is the cellular pool at time t, a_1 is the fura-2 fluorescence before treatment with Mn^{2+}, and k_1 is the rate constant. To obtain an estimate of the TG-sensitive rate of Mn^{2+} influx, Mn^{2+} quenching in DMSO-treated cells was subtracted from quenching in TG-treated cells and fitted to the equation:

$$A = a_1 e^{-k_1 t} + 55,$$

in which parameters were defined as above and a constraint on the maximal difference between DMSO- and TG-treated cells (45%, cf. Fig. 4*B*) was incorporated.

Repeated measures analysis of variance (ANOVA) was performed with an IBM-compatible PC using the general linear model procedure (PROC GLM, Statistical Analysis Systems (SAS) Institute, Cary, NC, USA). This analysis controlled between-cell line variability, which, as shown for PAF-evoked Ca_i^{2+} transients and other parameters of Ca^{2+} signalling, varies widely between LCLs from different individuals. Duncan's multiple range test was used to assess differences between treatment groups. Correlation analysis was performed using SAS PROC CORR. Non-linear regression analysis for computations of parameters of Mn^{2+} quenching of fura-2 quench experiments utilized SAS REG. Statistical analyses of the rate constants derived from curve-fitting the Mn^{2+} data were performed using weighted least squares according to the method described by Johnson & Milliken (1983). P values $< 0·05$ were taken as statistically significant. Data are reported as mean values ± S.E.M. for n LCLs.

RESULTS

Effects of cortisol on basal $[Ca^{2+}]_i$ and receptor-mediated $[Ca^{2+}]_i$ transient

To examine Ca_i^{2+} signalling in LCLs, we used platelet-activating factor (PAF), a potent cytokine that has diverse actions throughout the immune system (Snyder, 1990). In B cells, this lipid mediator alters several immuno-modulatory functions including nuclear-factor-κB-mediated immunoglobulin synthesis and secretion (Smith & Schearer, 1994; Rosskopf *et al.* 1995), cytokine expression (e.g. TNFα and IL-2; Smith *et al.* 1994) and Ca^{2+}-dependent expression of cell cycle genes and proliferation (Mazer *et al.* 1991, 1994; Rosskopf *et al.* 1995). The PAF receptor is expressed in both resting and activated cells (Nguer *et al.* 1992, 1996); its activation results in phosphatidylinositol bisphosphate hydrolysis, IP_3 generation and a transient Ca_i^{2+} response (Mazer *et al.* 1991). Previous work by the present authors and Siffert's group showed that the PAF-evoked $[Ca^{2+}]_i$ response in lymphoblasts displays marked variability between cell lines established from different individuals (Siffert *et al.* 1995; Brzustowicz *et al.* 1997). Based on widely differing $[Ca^{2+}]_i$ responses to PAF, we selected a panel of 19 LCLs from the CEPH collection to study the effects of cortisol on Ca_i^{2+} homeostasis. For these experiments, the effects of cortisol on the PAF-evoked $[Ca^{2+}]_i$ response, intracellular Ca^{2+} store size and SOCE were assessed. In addition, we determined whether modulation of Ca_i^{2+} occurred through the glucocorticoid–GR complex by performing the study in the presence of RU 486.

Preliminary experiments showed that 200 nM cortisol added 1 or 15 min prior to 100 nM PAF did not alter basal $[Ca^{2+}]_i$ or the PAF-evoked Ca_i^{2+} response. Furthermore, enhancement of the PAF responses was observed only after 12–24 h treatment with this concentration of cortisol (data not shown). We therefore performed all experiments after 48 h of exposure of cells to 200 nM glucocorticoid or inhibitor. Figure 1*A* shows examples of the PAF-evoked Ca_i^{2+} response in cortisol- or vehicle-treated cells suspended in HBS. Cells treated with glucocorticoid exhibited larger Ca_i^{2+} transients over the entire range of agonist concentration, with no apparent alteration of sensitivity or time course of response. As peak Ca_i^{2+} signals at 100 nM PAF are maximal and highly reproducible for a given LCL (Brzustowicz *et al.* 1997), this concentration of agonist was used to evaluate the influence of cortisol on Ca_i^{2+} signalling. In Ca^{2+}-containing medium, basal $[Ca^{2+}]_i$ in cortisol-treated cells was slightly decreased (55·7 ± 2·6 nM) compared with vehicle-treated cells (63·5 ± 2·4 nM, $P < 0·05$, $n = 19$). LCLs treated with RU 486, or the combination of RU 486 and cortisol, exhibited a basal $[Ca^{2+}]_i$ that was not significantly different from that of ethanol-treated cells.

Figure 1*B* and *C* shows the extent of stimulation following cortisol treatment on peak PAF-evoked $[Ca^{2+}]_i$ responses, in Ca^{2+}-free and Ca^{2+}-containing HBS. In the left panels, the range of responses in vehicle-treated cells is graphed as the dependent variable, and the percentage stimulation following cortisol treatment is shown on the ordinate. LCLs exhibiting small PAF responses demonstrated the greatest percentage stimulation following glucocorticoid treatment. This relation was maintained for individual LCLs in Ca^{2+}-free or Ca^{2+}-containing HBS, as analysis of the percentile stimulation of the cortisol-enhanced PAF responses in the two conditions showed a high degree of correlation ($r = 0·8890$, $P < 0·0001$, $n = 19$). Additional experiments showed the lack of cortisol responsiveness in LCLs with

inherently large responses was not a result of the high dose of agonist used; one cell line that showed no enhancement following cortisol treatment similarly failed to show increased $[Ca^{2+}]_i$ responses at lower (1–10 nM) PAF concentrations (data not shown). The right panels of Fig. 1*B* and *C* show that the mean PAF-evoked $[Ca^{2+}]_i$ response for 19 LCLs was significantly increased by cortisol treatment (by 67·0 and 55·7% in Ca^{2+}-free and Ca^{2+}-containing HBS, respectively). RU 486 had no discernible effect on the PAF response, and the stimulatory actions of cortisol were absent in cells treated with equimolar concentrations of RU 486 and glucocorticoid.

Effects of cortisol on intracellular Ca^{2+} stores

PAF responses in cortisol-treated cells were increased in the absence of extracellular Ca^{2+} influx (Fig. 1*B*). This result suggests that the size of the IP_3-releasable Ca^{2+} pool(s) was altered by glucocorticoid treatment. We examined whether cortisol increased intracellular Ca^{2+} stores by challenging LCLs with TG and ionomycin, two agents that mobilize Ca^{2+}

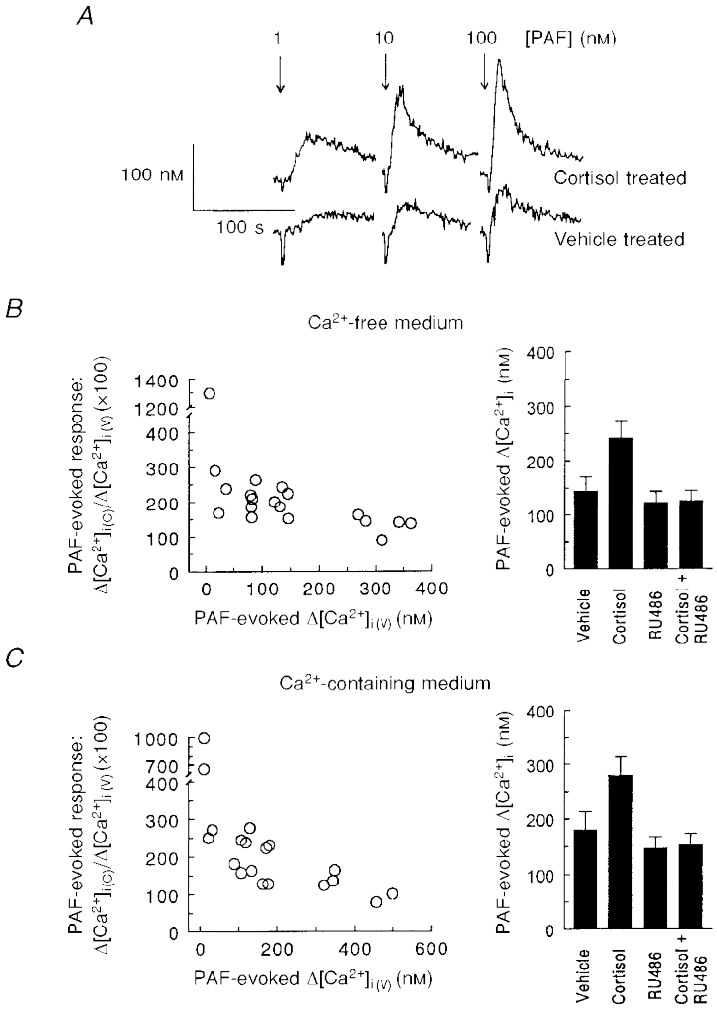

Figure 1. Cortisol treatment enhances PAF-evoked Ca_i^{2+} signalling

A, example of Ca_i^{2+} transients in an LCL exposed to 1, 10 or 100 nM PAF in HBS. 10×10^6 cells were treated with vehicle (ethanol) or cortisol (200 nM) for 48 h and processed for Ca_i^{2+} measurements as described in Methods. Basal $[Ca^{2+}]_i$ was determined as the mean $[Ca^{2+}]_i$ value immediately preceding addition of agonist, and the PAF-evoked $[Ca^{2+}]_i$ response was calculated as the difference between basal $[Ca^{2+}]_i$ and peak $[Ca^{2+}]_i$ after PAF addition. For this LCL, PAF addition (arrow) to cortisol-treated cells resulted in peak $[Ca^{2+}]_i$ increases of 123% (1 nM), 163% (10 nM) and 158% (100 nM) compared with vehicle-treated cells. *B* and *C*, PAF-evoked $[Ca^{2+}]_i$ responses in LCLs suspended in Ca^{2+}-free and Ca^{2+}-containing HBS. Left panels: percentage stimulation of PAF-evoked $[Ca^{2+}]_i$ response in cortisol-treated cells ($\Delta[Ca^{2+}]_{i(C)}$) to vehicle-treated cells ($\Delta[Ca^{2+}]_{i(V)}$) as a function of control response. Right panels: mean changes in PAF-evoked $[Ca^{2+}]_i$ in cells treated for 48 h with vehicle, cortisol (200 nM), RU 486 (200 nM), or cortisol (200 nM) and RU 486 (200 nM) ($n = 19$). ANOVA indicated overall P values $< 0{\cdot}0001$ for both Ca^{2+}-free and Ca^{2+}-containing HBS, with significant differences between cortisol and all other treatments.

from Ca^{2+} stores independently of IP_3. TG, a sarco(endo)-plasmic reticulum (SER) Ca^{2+}-ATPase (SERCA) inhibitor (Lytton *et al.* 1991), inhibits SERCA-mediated Ca^{2+} uptake into the SER. Its addition to lymphoblasts in Ca^{2+}-free HBS results in a transient increase in Ca_i^{2+}, owing to an endogenous leak of Ca^{2+} from these stores and subsequent extrusion of Ca^{2+} across the plasma membrane. Alternatively, addition of the Ca^{2+} ionophore ionomycin to cells results in its insertion into intracellular membranes and the electro-neutral exchange of Ca^{2+} for H^+. Addition of 100 nM ionomycin to cells in Ca^{2+}-free HBS results in a rapid increase in $[Ca^{2+}]_i$ that serves as an estimate of the freely exchangeable Ca^{2+} (FEC). Cortisol treatment (48 h, 200 nM) resulted in significant increases in the TG-mediated rise in $[Ca^{2+}]_i$ (Fig. 2*A*) and the FEC (Fig. 2*B*). The left panels illustrate the protocols used to obtain these parameters, and the right panels indicate the mean values for the four treatment groups. Treatment with the glucocorticoid antagonist had no effects of its own (on either parameter), and treatment of cells with RU 486 eliminated cortisol-induced increases in the TG-mediated rise in $[Ca^{2+}]_i$ and FEC. The TG-evoked rise in $[Ca^{2+}]_i$ appeared to reflect the FEC measurement, as comparison of these two parameters gave a correlation coefficient (r) of 0·5622 ($P < 0{\cdot}0001$, $n = 71$). These data suggest that cortisol enhances SERCA-regulated and freely exchangeable intracellular Ca^{2+} stores in LCLs.

Effects of cortisol on parameters of SOCE

The size, or capacity, of intracellular Ca^{2+} stores is tightly linked to the control of Ca^{2+} entry from the external medium. As cortisol enhanced intracellular Ca^{2+} stores, we examined whether a similar upregulation of store-operated Ca^{2+} entry (SOCE) was present in cortisol-treated cells. For these experiments, we measured both the rate of Ca^{2+} entry (change in $[Ca^{2+}]_i$ during the first 10 s) and peak change in $[Ca^{2+}]_i$ following Ca^{2+} reintroduction to cells with depleted Ca^{2+} stores (10 min of TG treatment in Ca^{2+}-free medium). Figure 3*A* shows an example of an experiment used to determine the rate and peak $[Ca^{2+}]_i$ after $CaCl_2$ was introduced into the medium (final extracellular $[Ca^{2+}] = 0{\cdot}15$ mM). Parameters of Ca^{2+} influx following Ca^{2+} readdition to TG-treated cells showed considerable variability between LCLs with regard to rate of Ca^{2+} entry (3·1–39·3 nM s^{-1}) and peak $[Ca^{2+}]_i$ levels (233·1–2233 nM). Within individual experiments, the correlation coefficient for the rate of Ca^{2+} entry and the peak $[Ca^{2+}]_i$ level was 0·7464 ($P < 0{\cdot}0001$, $n = 71$), indicating a high degree of consistency between the two measurements. Figure 3 also shows that cortisol treatment significantly increased both the rate of Ca^{2+} entry (Fig. 3*B*) and peak $[Ca^{2+}]_i$ (Fig. 3*C*). These results suggest glucocorticoids enhance the Ca^{2+} entry pathway activated by intracellular Ca^{2+} store depletion.

Estimates of rates of Ca^{2+} influx, or peak changes in $[Ca^{2+}]_i$ following Ca^{2+} readdition to cells, may be affected by Ca^{2+}-binding proteins and intracellular or plasma membrane Ca^{2+} transport mechanisms. We therefore assessed fura-2 quenching by Mn^{2+} as an indicator of store-operated divalent cation entry in TG-treated cells. Mn^{2+} has been used previously to characterize Ca^{2+} entry and SOCE in a variety of cells (Hallam & Rink, 1985; Missiaen *et al.* 1990).

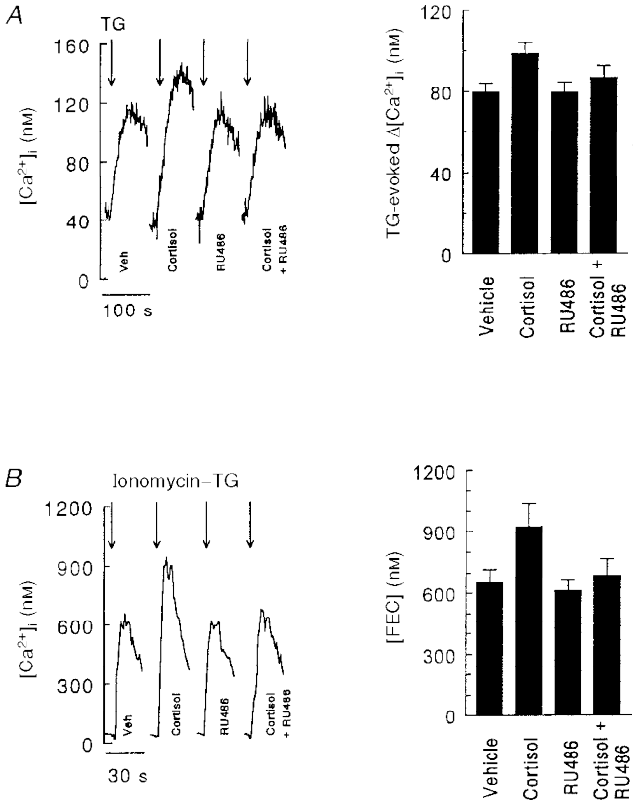

Figure 2. Cortisol treatment enhances intracellular Ca^{2+} stores in LCLs

The left panels show representative examples of the TG-evoked $[Ca^{2+}]_i$ response (*A*) and the ionomycin–TG-evoked rise in $[Ca^{2+}]_i$ (FEC) (*B*) in LCLs treated as described in the legend to Fig. 1. Arrows indicate addition of 100 nM TG (*A*) or 5 μM ionomycin–100 nM TG (*B*) to cells suspended in Ca^{2+}-free medium; TG was added to prevent reuptake of Ca^{2+} and diminution of the $[Ca^{2+}]_i$ transient. The peak $[Ca^{2+}]_i$ response minus the basal $[Ca^{2+}]_i$ was used to estimate TG-releasable Ca^{2+} pools defined by the SER and FEC. The right panels show the results from 19 (*A*) and 17 (*B*) LCLs. ANOVA indicated overall P values $< 0{\cdot}0001$ (TG-evoked $[Ca^{2+}]_i$) and $< 0{\cdot}0025$ (ionomycin–TG-evoked $[Ca^{2+}]_i$), with significant differences between cortisol and all other treatment conditions.

Table 1. Quenching of intracellular fura-2 by Mn^{2+}: k_1 (s^{-1})

Treatment	Vehicle	TG	TG sensitive
Control	0·446 ± 0·030	1·392 ± 0·077	2·169 ± 0·203
Cortisol	0·441 ± 0·047	1·731 ± 0·090*	3·450 ± 0·293**
RU 486	0·495 ± 0·044	1·483 ± 0·074	2·169 ± 0·178
Cortisol + RU486	0·489 ± 0·040	1·413 ± 0·079	2·005 ± 0·198

Cells were treated with vehicle, cortisol, RU 486 or cortisol and RU 486 (as described in text and legend to Fig. 4) and assessed for Mn^{2+} influx (cf. Fig. 4). Values are means ± asymptotic S.E.M. of k_1 ($\times 10^{-2}$), the rate constant. Percentage fluorescence quenching values at 0, 10, 30 and 60 s were compiled for vehicle- and thapsigargin (TG)-treated cells and were best fitted by the monoexponential function $A = a_1 e^{-k_1 t}$, described in Methods. TG-sensitive quenching of fura-2 describes the exponential fit of Mn^{2+} quenching of TG-treated cells minus the quenching in vehicle-treated cells. a_1 values ranged from 96·99 ± 1·44 to 99·18 ± 0·05 in vehicle- and TG-treated cells, and 44·37 ± 1·56 to 44·97 ± 4·53 for curve-fitted data describing TG-sensitive Mn^{2+} influx, and were not significantly different within treatments. $n = 18$ for each treatment. Significantly different at *$P < 0·05$, **$P < 0·01$.

It has substantially greater affinity than Ca^{2+} to fura-2, and is a poor substrate for SERCA and other Ca^{2+} transport systems (Gomes da Costa & Madeira, 1986; Missiaen *et al.* 1990). In addition, quenching is monitored at the isosbestic point of fura-2, enabling changes in the rate of Mn^{2+} entry to be monitored independently of changes in $[Ca^{2+}]_i$. Figure 4*A* illustrates a representative experiment showing fluorescence records of TG- and vehicle-elicited Mn^{2+} influx for the four treatment conditions. To quantify the rate of Mn^{2+} entry, data obtained with cells treated with TG, vehicle or the difference between these curves (i.e. TG-sensitive Mn^{2+} entry) were best-fitted to a monoexponential function (Fig. 4*B*, and see Methods and Table 1 for additional details). Rates of Mn^{2+} quenching of intracellular fura-2 showed considerable variability between LCLs; e.g. from 0·11 to 1·79 % s^{-1} for vehicle-treated cells and 0·14 to 2·46 % s^{-1} for cortisol-treated cells. Rate constants derived for each treatment indicated cortisol-treated cells had a significantly greater mean rate constant for TG-sensitive Mn^{2+} entry than control or RU 486-treated cells (Fig. 4*C* and Table 1). The parameters describing rates of TG-sensitive Ca^{2+} entry and Mn^{2+} entry (quenching at 30 s) were highly correlated with each other ($r = 0·5935$, $P < 0·0001$, $n = 69$). These experiments, coupled with the findings of Fig. 3, suggest that measurements of rates of Ca^{2+} entry and peak changes in $[Ca^{2+}]_i$ in TG-treated lymphoblasts are largely unaffected by Ca^{2+} transport or sequestration mechanisms. Importantly, the data indicate that LCLs treated with cortisol have enhanced SOCE.

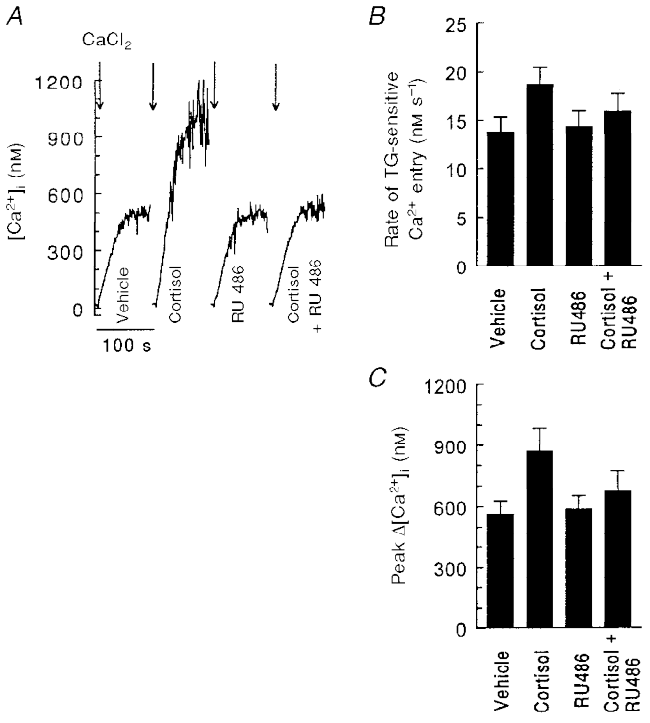

Figure 3. Cortisol enhancement of Ca^{2+} entry and peak $[Ca^{2+}]_i$ following Ca^{2+} addition to TG-treated cells

In representative experiments (*A*), cells challenged with 100 nM TG in Ca^{2+}-free medium for 10 min (not shown) had 0·45 mM $CaCl_2$ added to the cuvette (final $[Ca^{2+}]_o = 0·15$ mM). The first 10 s of the increase in $[Ca^{2+}]_i$ were used to estimate the rate of Ca^{2+} influx (nM s^{-1}, *B*), and peak changes in $[Ca^{2+}]_i$ were determined as the difference between $[Ca^{2+}]_i$ immediately prior to $CaCl_2$ addition and the maximal $[Ca^{2+}]_i$ (*C*). Control experiments with cells treated with DMSO (vehicle, 0·2 %) for 10 min had low (< 0·05 nM s^{-1}) rates of Ca^{2+} influx after $CaCl_2$ addition, hence no correction for non-TG-mediated increases in Ca_i^{2+} was performed. ANOVA indicated overall P values < 0·0001 for both analyses, with significant differences between cortisol and all other treatment conditions.

Table 2. Correlation analysis of PAF-evoked $[Ca^{2+}]_i$ response with intracellular Ca^{2+} store size and parameters of SOCE

	Parameter	*r*	*n*	*P*
PAF-evoked $[Ca^{2+}]_i$ in Ca^{2+}-free HBS	TG-evoked $[Ca^{2+}]_i$	0·2713	74	0·0194
	FEC	0·3544	72	0·0023
	Rate of Ca^{2+} entry	0·2701	74	0·0199
	Peak $[Ca^{2+}]_i$	0·2425	72	0·0401
	Mn^{2+} quenching	0·2703	70	0·0236

Analyses were performed for the PAF-evoked $[Ca^{2+}]_i$ response obtained in Ca^{2+}-free HBS *vs.* parameters of intracellular Ca^{2+} store size (TG-evoked $[Ca^{2+}]_i$ and FEC), rate of Ca^{2+} entry and and peak $[Ca^{2+}]_i$ in TG-treated cells, and the degree of TG-sensitive Mn^{2+} quenching at 30 s (obtained as described in legends to Figs 2–4).

These results indicate that glucocorticoid treatment enhances Ca^{2+} homeostasis in LCLs. Correlation of the extent of cortisol stimulation of PAF-evoked $[Ca^{2+}]_i$ signals (cf. Fig. 1*B* and *C*, left panels) with intracellular Ca^{2+} store or SOCE parameters could suggest potential determinants effecting the cortisol-induced stimulation. However, these analyses resulted in weak correlations showing high dependence on one or two data points. We therefore performed a correlation analysis of the entire data set of Ca^{2+} and Mn^{2+} parameters to examine the relation between PAF-evoked increases in $[Ca^{2+}]_i$ and Ca^{2+} store size and parameters of SOCE. Although this analysis does not specifically examine the cortisol-induced enhancement of the PAF response, it examines parameters of Ca^{2+}_i signalling throughout the range of cortisol-modulated Ca^{2+}_i responses. The correlation coefficients for these comparisons are indicated in Table 2. In Ca^{2+}-free medium, PAF-evoked $[Ca^{2+}]_i$ responses were positively correlated to Ca^{2+} stores

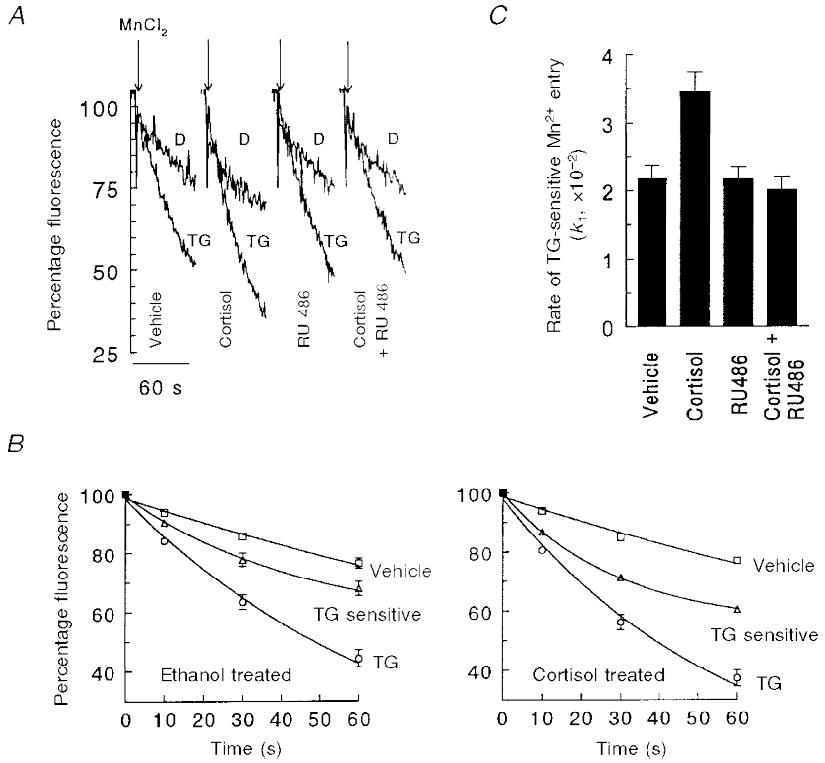

Figure 4. Cortisol treatment increases Mn^{2+} entry in TG-treated cells

A, representative experiments are depicted in which fura-2-loaded cells were centrifuged and pretreated with 500 nM TG (TG-labelled traces) or vehicle (D-labelled traces, for 0·5% DMSO) in Ca^{2+}-free medium for 10 min. Cells were then centrifuged and the pellet resuspended in 3 ml Ca^{2+}-free medium; traces show the normalized response of fluorescence decline immediately after addition of 0·35 mM $MnCl_2$ (arrows). *B*, data used to calculate the rate constant for TG-sensitive quenching of fura-2 in vehicle (left panel) and cortisol-treated (right panel) LCLs ($n = 18$) as described in Methods and legend to Table 1. Shown are means ± S.E.M. of LCLs evaluated for Mn^{2+} entry; S.E.M.S smaller than the symbol are not shown. Mn^{2+} entry in vehicle- (□, DMSO) and TG-treated cells (○); TG-sensitive Mn^{2+} influx is given by (△), and curve-fitted lines describing the exponential decay of fluorescence are given for each condition. *C*, results of weighted least-squares analysis of k_i (rate constant, see also Table 1) indicated a *P* value $< 0·01$ between vehicle- and cortisol-treated LCLs.

Table 3. Correlation analysis of measurements of intracellular Ca^{2+} store size and parameters of SOCE

	Parameter	r	n	P
TG-evoked $[Ca^{2+}]_i$	Rate of Ca^{2+} entry	0·4433	73	0·0001
	Peak $[Ca^{2+}]_i$	0·6905	71	0·0001
	Mn^{2+} quenching	0·2969	69	0·0132
FEC	Rate of Ca^{2+} entry	0·3551	71	0·0024
	Peak $[Ca^{2+}]_i$	0·4134	72	0·0003
	Mn^{2+} quenching	0·4804	67	0·0001

Analyses were performed for TG-evoked $[Ca^{2+}]_i$ and FEC and the rate of Ca^{2+} entry and peak $[Ca^{2+}]_i$ in TG-treated cells and the degree of TG-sensitive Mn^{2+} quenching at 30 s (obtained as described in legends to Figs 2–4).

and parameters describing SOCE. Similar correlations were obtained when the PAF response in Ca^{2+}-containing HBS was used. The strongest correlation occurred with FEC, and is shown in Fig. 5*A*. As intracellular Ca^{2+} stores can regulate Ca^{2+} entry, we also compared the relation between store size and parameters of SOCE (Table 3). In general, correlation coefficients for these comparisons were higher than those for PAF-mediated $[Ca^{2+}]_i$ responses and intracellular Ca^{2+} store size or SOCE. Two examples of the strong association are given by the correlations between the peak $[Ca^{2+}]_i$ in TG-treated cells and the peak $[Ca^{2+}]_i$ achieved following TG addition (Fig. 5*B*), and TG-sensitive Mn^{2+} entry and FEC (Fig. 5*C*). These results suggest that the magnitude of the PAF-evoked Ca_i^{2+} signal is dependent on the size of the intracellular Ca^{2+} stores, which in turn are related to Ca^{2+} entry through the SOCE pathway(s).

DISCUSSION

This report characterizes several novel aspects of glucocorticoid action on cellular Ca^{2+} homeostasis in human B lymphoblasts. Forty-eight hour treatment of cells with cortisol decreased basal $[Ca^{2+}]_i$ and increased the PAF-evoked $[Ca^{2+}]_i$ transient. Cortisol increased the size of intracellular Ca^{2+} stores and increased the rate of TG-sensitive Ca^{2+} and Mn^{2+} entry, indicating enhanced SOCE. These results identify links among the size of the Ca^{2+} stores, Ca^{2+} mobilization from these stores, and the pathways that refill the Ca^{2+} stores. As the stimulatory effects of cortisol on Ca_i^{2+} signalling occur in conjunction with increases in intracellular Ca^{2+} stores and SOCE, the results indicate a major

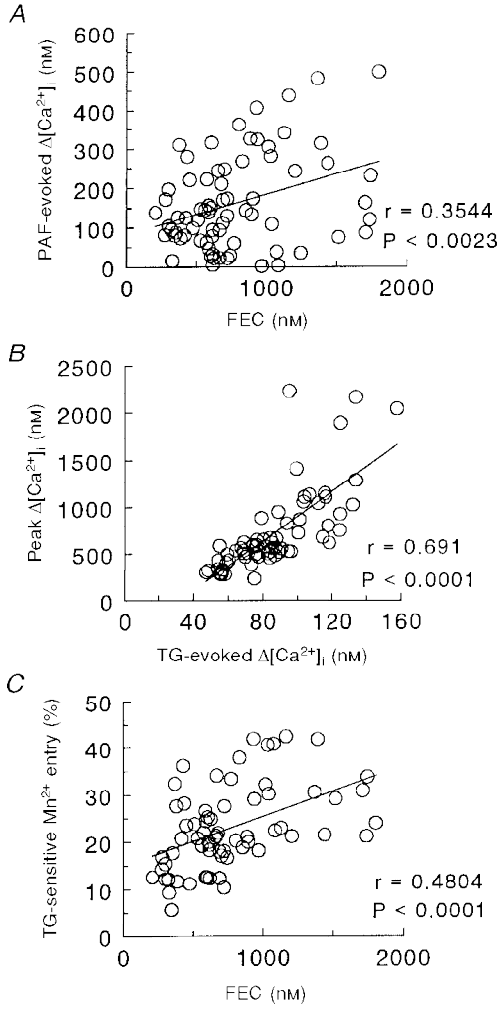

Figure 5. Correlations between PAF-evoked $[Ca^{2+}]_i$ response and intracellular Ca^{2+} store size (*A*) and intracellular Ca^{2+} store size and parameters of SOCE (*B* and *C*)

A, correlation of PAF-evoked $[Ca^{2+}]_i$ responses (in Ca^{2+}-free HBS) and FEC; parameters were obtained as described in legends to Figs 1 and 2. *B*, correlation of TG-evoked $[Ca^{2+}]_i$ and peak $[Ca^{2+}]_i$ levels achieved after 10 min TG treatment and readdition of $CaCl_2$. TG-evoked $[Ca^{2+}]_i$ was obtained as described in legend to Fig. 2, and peak $[Ca^{2+}]_i$ values were obtained as in legend to Fig. 3. *C*, intracellular Ca^{2+} store size (FEC) and TG-sensitive Mn^{2+} entry. FEC was determined as in the legend to Fig. 2, and Mn^{2+} entry was obtained from TG-sensitive decreases in fluorescence at 30 s after addition of $MnCl_2$ (cf. Fig. 4). Correlations were obtained with data from the four treatment conditions for 17–19 LCLs (for *A*, $n = 72$; for *B*, $n = 71$; for *C*, $n = 67$).

regulatory role of glucocorticoids on lymphoblast Ca^{2+} homeostasis.

Cortisol-induced changes in Ca^{2+} homeostasis could result via modulation of transcriptional events, or through a non-genomic mode of action initiated at the plasma membrane. In the former, more traditional, mechanism of steroid action, the triggering of genomic events is mediated by cortisol binding to a glucocorticoid receptor (GR) and subsequent modulation of nuclear transcription through the glucocorticoid–GR complex (Boumpas *et al.* 1993). Alternatively, a non-genomic mechanism of action is characterized as a rapid (< 2 min) onset, membrane-mediated response that occurs independently of DNA transcription or protein synthesis inhibition (for review, see Wehling, 1997). Non-genomic responses usually involve ion transport systems and are presumed to occur through a membrane-resident receptor. For example, cortisol both acutely and chronically affects cAMP concentrations and $^{45}Ca^{2+}$ accumulation in cichlid fish pituitary cells (Borski *et al.* 1991), and acutely hyperpolarizes guinea-pig neurons, an effect inhibited by GR antagonists (Hua & Chen, 1989). However, we failed to observe rapid effects of cortisol on Ca_i^{2+} signalling. Furthermore, the long (12 h) latency for enhancement of the Ca_i^{2+} signal is more consistent with a transcriptional/translational mechanism of action. As LCLs contain GRs (Tomita *et al.* 1985; Sinclair *et al.* 1994), we performed experiments with RU 486, a competitive antagonist of the cytosolic GR (Agarwal *et al.* 1987). The results of these experiments showed complete inhibition of cortisol-induced changes in all measured parameters of Ca^{2+} homeostasis and Mn^{2+} influx, suggesting activation of endogenous GR is critical for the modulatory effects of cortisol. Taken together, these results do not support a non-genomic action of cortisol and instead suggest the primary effect of cortisol occurs through the classically described, genomic mechanism of glucocorticoid action.

The present observations showing glucocorticoid-stimulated decreases in basal $[Ca^{2+}]_i$ and enhanced Ca^{2+} signalling in a model of activated lymphocytes are, with one exception, quite different from those reported in the literature for lymphocytes or lymphoid cells. That is, in response to increased cortisol, activated T cells and resting B cells exhibit reduced Ca_i^{2+} signalling (Dennis *et al.* 1987; Baus *et al.* 1996) whereas thymocytes demonstrate an increase in basal $[Ca^{2+}]_i$ (McConkey *et al.* 1989). S49 lymphoma cells exhibit increased basal $[Ca^{2+}]_i$ and decreased intracellular Ca^{2+} stores (TG-evoked $[Ca^{2+}]_i$ changes in Ca^{2+}-free medium: Lam *et al.* 1993; Bian *et al.* 1997) in response to dexamethasone (18–24 h) treatment. The discrepancies between glucocorticoid effects in these cells and immortalized lymphoblasts suggest that Ca_i^{2+} signalling pathways downstream of glucocorticoid–GR activation differ between resting and activated lymphocytes, subsets of lymphocytes, and lymphoma cells. These differences are likely to be key factors responsible for some of the well-described effects of glucocorticoids on lymphocyte function, i.e. inhibition of cell proliferation (Bowen & Fauci, 1984) and induction of apoptosis (McConkey *et al.* 1989; Lam *et al.* 1993; Bian *et al.* 1997). Interestingly, one recent report showed dexamethasone enhanced receptor-mediated $[Ca^{2+}]_i$ transients and other parameters of Ca^{2+} signalling in U937 and erythroleukaemia cells (Willmott *et al.* 1997). Although these investigators were unable to associate alterations in Ca^{2+} homeostasis with an increase in a glucocorticoid-regulated protein (lipocortin 1), their work lends support to the finding that glucocorticoids enhance Ca^{2+}-signalling pathways through modulation of intracellular Ca^{2+} stores. As this phenomenon is observed in monocytic, erythroid and lymphocytic cell lines, and occurs independently of viral transformation, glucocorticoid modulation of Ca^{2+} homeostasis may reflect differentiation-specific responses of haematopoietic cells to elevated levels of cortisol.

In cortisol-treated LCLs, the increase in the PAF-evoked $[Ca^{2+}]_i$ response was observed in Ca^{2+}-free medium and was strongly correlated to the FEC. The TG-evoked $[Ca^{2+}]_i$ response and FEC, two markers of the size of intracellular Ca^{2+} stores, were similarly enhanced by cortisol treatment. As PAF-evoked responses in Ca^{2+}-free HBS are approximately 70% of the response in Ca^{2+}-containing medium (Brzustowicz *et al.* 1997), it is apparent that not only the PAF-induced $[Ca^{2+}]_i$ signal is highly dependent on the size of the Ca^{2+} stores, but cortisol alters the size of these Ca^{2+} stores. Enhanced Ca^{2+} sequestration capabilities of these compartments could also explain the decrease in basal $[Ca^{2+}]_i$. The simplest explanation of the cortisol-induced enhancement of Ca_i^{2+} signalling would postulate an upregulation of SERCA Ca^{2+} pumps, SER Ca^{2+} binding proteins (e.g. calreticulin), or modifiers of these systems, assuming glucocorticoid–GR signalling events exist for enhancing the expression or activity of these proteins. However, assigning the involvement of a particular Ca^{2+} system as a target of glucocorticoid action, in the absence of specific experiments, is highly speculative. For instance, overexpression of a plasma membrane Ca^{2+} pump in endothelial cells downregulated SERCA Ca^{2+} pump and IP_3-channel expression, and enhanced store-dependent Ca^{2+} entry (Liu *et al.* 1996). This example illustrates the highly interdependent nature of Ca^{2+} signalling on multiple Ca^{2+} transport and buffering systems. We have ruled out the potential involvement of the Na^+–Ca^{2+} exchanger as a modifier of enhanced Ca^{2+} responses, as LCLs fail to exhibit measurable Na^+–Ca^{2+} exchange activity under conditions optimized to detect its activity (Balasubramanyam *et al.* 1996; Gardner & Balasubramanyam, 1996).

PAF-evoked $[Ca^{2+}]_i$ transients demonstrated cell line-specific responses with regard to the extent of stimulation by cortisol (Fig. 1*B* and *C*). The negative relation describing the percentage increase in the PAF response in cortisol- and vehicle-treated cells could be the result of several factors, including (but not limited to) a decreased ability of cortisol to effect GR activation, variable upregulation of GR in transformed LCLs, and/or a finite capacity of receptor-

mediated Ca^{2+} signalling to evoke Ca^{2+} mobilization. With regard to the first possibility, direct measurements of GR levels and their extent of activation in the presence of cortisol in individual LCLs may address this issue. In addition, several cultured cell types retain the metabolic pathways that convert active glucocorticoid to inactive metabolite (and vice versa: Nath *et al.* 1993; Reeves, 1995; Rajan *et al.* 1996; Brem *et al.* 1998). We have observed that LCLs treated with carbenoxolone (an inhibitor of 11β-hydroxysteroid dehydrogenase; Edwards *et al.* 1996) exhibit enhanced PAF responses, suggesting LCLs may express the cortisol 'inactivating' form of this isoenzyme (authors' unpublished observations). The second possibility, i.e. that certain LCLs are more responsive to cortisol because of increased GR levels, is supported by reports that EBV-transformed LCLs exhibit increased levels of GR protein compared with EBV-positive cells (e.g. Burkitt's lymphoma cells; Sinclair *et al.* 1994) or human mononuclear leucocytes (Tomita *et al.* 1985). This is in keeping with idea that LCLs are representative of cytokine-activated B cells, which also express enhanced glucocorticoid receptors (Falus *et al.* 1995). However, it is difficult to reconcile glucocorticoid responsiveness with the ability of an LCL to respond to an agonist such as PAF. Instead, it is more likely that LCLs exhibit a maximal capacity for receptor-evoked Ca_i^{2+} transients (with or without cortisol stimulation). This conclusion is supported by our study with a larger panel of CEPH LCLs (Brzustowicz *et al.* 1997), in which the maximal PAF-evoked $[Ca^{2+}]_i$ response in HBS was ~400 nM. The limits of receptor-mediated Ca^{2+} mobilization could be bound by the size of the intracellular Ca^{2+} stores, although the influence of IP_3 metabolic pathways would appear to be of major importance.

The PAF-evoked $[Ca^{2+}]_i$ transient and its dependence on the intracellular Ca^{2+} store–SOCE axis is supported by several converging lines of evidence: the significant correlation of the PAF response with intracellular Ca^{2+} stores, the correlation between intracellular Ca^{2+} store size and SOCE, and the positive, modulatory events of cortisol on up-regulation of the PAF response and the SOCE pathway and capacity of intracellular Ca^{2+} stores. Recently, Ca^{2+} entry via a store-operated channel (I_{CRAC}) was reported to be tightly linked to the filling status of intracellular Ca^{2+} stores (Hofer *et al.* 1998). Although our data do not address the time course of the activation of Ca^{2+} entry with Ca^{2+} depletion from intracellular stores, the results obtained with this limited number of LCLs suggest that over a range of intracellular Ca^{2+} store sizes, the magnitude of the Ca^{2+} entry process is coupled to the size of the Ca^{2+} store.

We observed that cortisol enhances Ca_i^{2+} signalling in a subset of minimally responsive cells. Physiological consequences of increased circulating glucocorticoid may thus serve to augment certain functions attributed to activated B cells, e.g. immunoglobulin secretion. This idea is derived from observations in B lymphocytes showing glucocorticoid-induced potentiation of IL-1 and IL-6 secretion (Emilie *et al.* 1988) and of IL-4-induced IgE formation (Nusslein *et al.* 1992). In this regard, immunoglobulin formation is highly dependent on endoplasmic reticulum (ER) synthesis, sorting and trafficking; these functions in turn are partially dependent on ER Ca^{2+} levels (Shachar *et al.* 1994). Furthermore, low-secreting variants of rat basal leukaemia cells have defective antigen-mediated $[Ca^{2+}]_i$ signals (Bingham *et al.* 1994), and LCLs with enhanced IgM and IgG secretion in response to PAF also have increased PAF-evoked $[Ca^{2+}]_i$ responses (Rosskopf *et al.* 1995). Based on this information and the findings in this report, we suggest intracellular Ca^{2+} stores and Ca_i^{2+}-signalling pathways could serve as a major physiological target for glucocorticoid action.

The major finding in this report is that glucocorticoids significantly affect parameters of Ca^{2+} homeostasis in B lymphoblasts. In addition to previously discussed Ca^{2+} transport or IP_3 metabolic pathways, several other candidate systems could be invoked to explain the mechanisms through which glucocorticoid produce its effects. Besides describing the modulatory effects of cortisol, the present study characterizes parameters of Ca^{2+} homeostasis in LCLs representative of the general population. The possibility exists that lymphoblasts may be used to examine the intrinsic regulation of numerous Ca^{2+} parameters, including G-protein-coupled receptors, SOCE and intracellular Ca^{2+} stores. These studies may be accomplished through the comparison of known or postulated modulators of Ca^{2+} metabolism in LCLs exhibiting markedly different responses. Alternatively, they may be performed through phenotypic expressions of Ca_i^{2+} signalling, which, when coupled with non-parametric methods of linkage analysis (Brzustowicz *et al.* 1997), can be used to identify genetic components that mediate or regulate a specific Ca^{2+} parameter.

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