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Effects of dexamethasone and phorbol ester on P_2 receptor-coupled Ca^{2+} signalling and lipocortin 1 presentation in U937 cells

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1 Cell surface bound lipocortin 1 (LC1) is a putative mediator of the antiproliferative and anti-inflammatory effects of glucocorticoids. This study assessed the hypothesis that the glucocorticoid, dexamethasone-phosphate (dex-p), might exert the above effects via an LC1-mediated downregulation of receptor-coupled Ca^{2+} signalling, using P_2-receptor mediated intracellular Ca^{2+} accumulation in U937 cells as an appropriate model.

2 Addition of ATP (1–100 μM) to cells resulted in a transient increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Prior treatment of cells with dex-p (3–24 h) increased the magnitude of this Ca^{2+} transient at high, but not low concentrations of ATP, and increased thapsigargin (Tg)-induced Ca^{2+} influx, indicating that store-operated Ca^{2+} influx was potentiated in these cells. For cells treated with dex-p for 24 h, cell surface levels of LC1 were significantly reduced by 63%.

3 Differentiation of cells with 1 nM phorbol ester (PMA) for 24 h resulted in a 2.4 fold increase in the cell surface level of LC1 and inhibition of the ATP-induced Ca^{2+} response. However, the Tg-induced Ca^{2+} response was unaffected by long-term PMA treatment, and incubating cells with LC1 did not alter Tg-induced Ca^{2+} mobilization and influx, or the ATP-mediated Ca^{2+} response.

4 Data from this study suggest that: (1) dex-p does not inhibit P_2-receptor-coupled Ca^{2+} signalling in this cell line, (2) the observed modulation of the ATP-induced increase in $[Ca^{2+}]_i$ by dex-p and PMA, and store-operated Ca^{2+} influx by dex-p, is not linked to an increase in the cell surface level of LC1, and (3) differentiation of U937 cells with PMA downregulates the ATP-induced Ca^{2+} response, but does not affect the thapsigargin-sensitive Ca^{2+} pool or store-operated Ca^{2+} influx of these cells.

Keywords: Store-operated calcium influx; dexamethasone; lipocortin 1; U937 cells; ATP; P_2 receptors

Introduction

Many agonist–receptor interactions, including activation of certain members of the P_2 class of purinoreceptors (Barnard *et al*., 1994), result in an increase in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is often functionally coupled to cell response (Berridge, 1993). It has been proposed that purinoreceptor activation via release of adenine nucleotides following tissue damage may contribute to increased mitogenic and inflammatory signalling (Baricordi *et al*., 1996; Willmott *et al*., 1996; Dixon *et al*., 1997). As a functional coupling between receptor-mediated Ca^{2+} signalling and proliferation has been demonstrated in a number of cells (Partiseti *et al*., 1996; Baricordi *et al*., 1996; Willmott *et al*., 1996) it is conceivable that abrogation of the purinoreceptor-mediated increase in cytosolic Ca^{2+} may in turn lead to inhibition of mitogenic and inflammatory signalling pathways.

The glucocorticoid, dexamethasone, has been shown to induce the translocation of lipocortin 1 (LC1) to the cell surface (Solito *et al*., 1994). Surface bound lipocortin 1 (LC1) has been proposed to act as a mediator of the antiproliferative and anti-inflammatory actions of this steroid, *in vivo* and *in vitro*, via a mechanism that results in the inhibition of constitutive phospholipase A_2 (Flower & Rothwell, 1994). As in many cells, arachidonic acid release is thought to rely on the Ca^{2+}-dependent migration of constitutive phospholipase A_2 ($cPLA_2$) from the cytosol to the plasma membrane (Clark *et al*., 1995; Peters-Golden *et al*., 1996), it is possible that the antiproliferative and anti-inflammatory actions of dexamethasone and LC1 may be linked to a disturbance in receptor-mediated mechanisms that result in an elevation of $[Ca^{2+}]_i$. Indeed, several studies have demonstrated inhibition of Ca^{2+} signalling pathways by dexamethasone in a number of cell types, although differing mechanisms appear to account for these effects (Shipston & Antoni, 1992; Yamaguchi *et al*., 1993; Baus *et al*., 1996).

In undifferentiated and PMA (phorbol 12-myristate 13-acetate) differentiated U937 cells, dexamethasone has been shown to increase the membrane expression of LC1 (Solito *et al*., 1994; Perretti *et al*., 1996), presenting the possibility that antiproliferative effects may be mediated by LC1 in this cell line. Considering the above, this study set out to determine whether dexamethasone and LC1 downregulate receptor-mediated Ca^{2+} signalling. As P_2-receptor coupled Ca^{2+} signalling is operative, and has been implicated in mitogenic signalling in these cells (Willmott *et al*., 1996), this particular pathway was chosen as a worthy model to test the above.

Methods

Preparation of cells

Undifferentiated U937 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1 μg ml^{-1} penicillin, 1 μg ml^{-1} streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2, and were maintained in culture prior to use.

Measurement of free intracellular Ca^{2+}

For intracellular Ca^{2+} measurements, cells were loaded with 2 μM fura-2 AM in 2 ml Hank's medium (pH 7.2), in the

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presence of 0.1% pluronic for 60 min at room temperature. Cells were washed in 2×2 ml aliquots of Hank's medium to remove any residual dye, and were allowed to stand for 60 min at 22°C to ensure complete ester hydrolysis, prior to Ca^{2+} analysis.

Analysis of $[Ca^{2+}]_i$ was peformed using a Perkin-Elmer LS 50 fluorimeter. Cells were loaded with acetoxymethyl (AM) ester of fura-2 (see above) and equivalent aliquots of the resulting cell suspension (containing approximately 5 million cells) were added to a stirred fluorimetry cuvette in a final volume of 3 ml Hank's medium. Free cytosolic Ca^{2+} concentration was determined by calculating the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm, using an emission wavelength of 510 nm. Pairs of 340 and 380 nm fluorescence intensities were sampled every 0.8 s. Experiments were performed at 22°C. A low temperature was employed for Ca^{2+} measurements with fura-2 as this dye readily compartmentalises into cellular organelles at 37°C. Standard $CaCl_2$ solutions were used to calibrate the system and viscosity corrections were made (Poenie, 1990). The mean peak amplitude of Ca^{2+} responses were compared for statistical significance using Student's *t* test for unpaired observations.

Flow cytometric analysis of cell surface lipocortin 1

Flow cytometric analysis (FACS) of cell membrane lipocortin 1 was performed in a similar way as previously described (Perretti *et al*., 1996). Briefly, cells were incubated for 1 h at 4°C with a specific murine monoclonal antibody, anti-human LC1 (mAb 1B) at a final concentration of 20 μg ml^{-1}. Non-specific binding was blocked by 8 mg ml^{-1} human IgG. Control cells did not receive mAb 1B, or were treated with the isotype matched irrelevant mAb P3. Cells were then washed with PBS and incubated with 40 μl of a diluted (1 : 40) F(ab′) fragment of goat anti-mouse IgG conjugated to FITC for a further 45 min. FACS analysis was performed using a FACScan II analyzer (Becton Dickinson, Mountain View, California) with air-cooled 100 mV argon ion laser tuned to 448 nm and a consort 32 computer running Lysis II software. Units of mean fluorescence intensity (MFI) were measured in the FL1 channel and converted to the number of mAb molecules bound per cell with reference to microbeads labelled with standard numbers of FITC molecules. All results were compared for statistical significance using Student's *t* test for unpaired observations.

Drugs and solutions

Experiments were performed in Hank's medium (pH 7.2), containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.83 mM $MgSO_4$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 5 mM glucose. For experiments carried out in low Ca^{2+} medium, $CaCl_2$ was omitted from the above and 0.5 mM EGTA was added, yielding a free Ca^{2+} concentration of $\sim$10 nM. DMEM, FCS, penicillin and streptomycin were from Gibco. Fura-2 AM was from Molecular Probes Inc. Specific murine monoclonal antibody, anti-human LC1 (mAb 1B), was from Browning, Biogen Corp., Cambridge, Massachusetts. Microbeads labelled with standard numbers of FITC molecules were from Flow Cytometry Standard Corp., Research Triangle Park, North Carolina. Lipocortin 1 (32.5, 35 kDa) and all other drugs were from Sigma.

Results

Potentiation of the ATP-induced increase in $[Ca^{2+}]_i$ and thapsigargin-induced Ca^{2+} influx of U937 cells by dexamethasone-phosphate

Addition of 1–100 μM ATP to U937 cells resulted in a transient increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), via P_2 receptor activation (Figure 1a). The ATP-induced Ca^{2+} response was abolished by prior treatment of cells with the endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin (Tg), and was substantially reduced when cells were transferred to low Ca^{2+} medium (Figure 1b). This suggests that the response is comprised of a Ca^{2+} mobilization and a Ca^{2+} influx component, with the influx component being dependent on the mobilization component, and therefore presumably store-operated. Prior treatment of cells with dexamethasone phosphate (dex-p) for 3–24 h, resulted in a significant increase in the ATP-mediated Ca^{2+} transient (Figure 1c) evoked by high concentrations of ATP (100 μM). At 1 and 10 μM ATP, there was no difference in the Ca^{2+} responses of dex-p treated cells compared to untreated controls (Figure 1c), indicating that the above dex-p induced potentiation is probably dependent on a substantial depletion of intracellular Ca^{2+} stores. For cells transferred to nominally Ca^{2+} free medium and preincubated with Tg, in order to completely deplete intracellular Ca^{2+} stores, Ca^{2+} influx was significantly greater for cells treated with dex-p for 5 and 17 h, compared to untreated control cells, on reintroduction of Ca^{2+} to the bathing medium (Figure 2). These data suggest that store-operated Ca^{2+} influx is potentiated in dex-p treated cells. As a prolonged incubation (>3 h) with dex-p was required before these effects became apparent, the observed potentiation is probably a consequence of altered gene transcription. A similar effect was witnessed in human erythroleukaemia (HEL) cells, where incubation of these with 1 μM dex-p for periods greater than 3 h, resulted in an increased Ca^{2+} response to thrombin (data not shown), which is another Ca^{2+} release and influx mechanism. Furthermore, Tg-induced Ca^{2+} influx was also increased in these cells following dex-p treatment (data not shown), suggesting that potentiation of store-operated Ca^{2+} influx might be a general cellular effect of dexamethasone. Incubating cells with 1 μM dex-p for 1, 3, 17 or 24 h did not significantly alter the basal resting $[Ca^{2+}]_i$ of cells, and a 17 h incubation with this concentration of dex-p did not affect basal manganese quench of cytosolic fura-2 (data not shown), indicating no effect for dex-p on basal Ca^{2+} influx.

Differential modulation of the cell surface level of lipocortin 1 by PMA and dexamethasone-phosphate

Incubating U937 cells with 1 μM dex-p for 24 h failed to increase the cell surface level of LC1 (Figure 3). This parameter was significantly reduced by 63% compared to untreated controls after 24 h (Figure 3). Incubating cells with 1 nM PMA for 24 h resulted in a 2.4 fold increase in the surface level of LC1 above untreated control cells (Figure 3). Combined treatment of cells with 1 nM PMA and 1 μM dex-p for 24 h also increased the cell surface expression of LC1 (Figure 3) to a level 2.5 fold above untreated control cells. These data suggest that the effect of PMA and dex-p on the cell surface level of LC1 is not additive. Although the above treatments might have resulted in the release of lipocortin 1 or its fragments into the medium, it was considered impracticable to measure these parameters.

PMA-mediated abrogation of the ATP-induced increase in $[Ca^{2+}]_i$

Differentiation of U937 cells with 1 nM PMA resulted in the complete abrogation of the ATP-mediated Ca^{2+} response. After 24 h this response was significantly reduced in these cells (Figure 4a), and was completely abolished following 48 h (Figure 4a), suggesting that PMA-induced cell differentiation downregulates P_2-receptor mediated Ca^{2+} signalling. It has previously been demonstrated that PMA inhibits Ca^{2+} entry induced by Tg in rabbit and human neutrophils (Montero *et al*., 1993a; Shibata *et al*., 1994), in thyroid FRTL-5 cells (Tornquist, 1993), and in human leukaemia

HL-60 cells (Montero *et al*., 1993b). However, contrary to these reports, Tg-induced Ca^{2+} mobilisation and influx were apparently unaffected following the above treatments, as witnessed by the normal Ca^{2+} response profile to Tg (Figure 4c). For U937 cells, the Tg-induced increase in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} (Figure 4b). Removal of

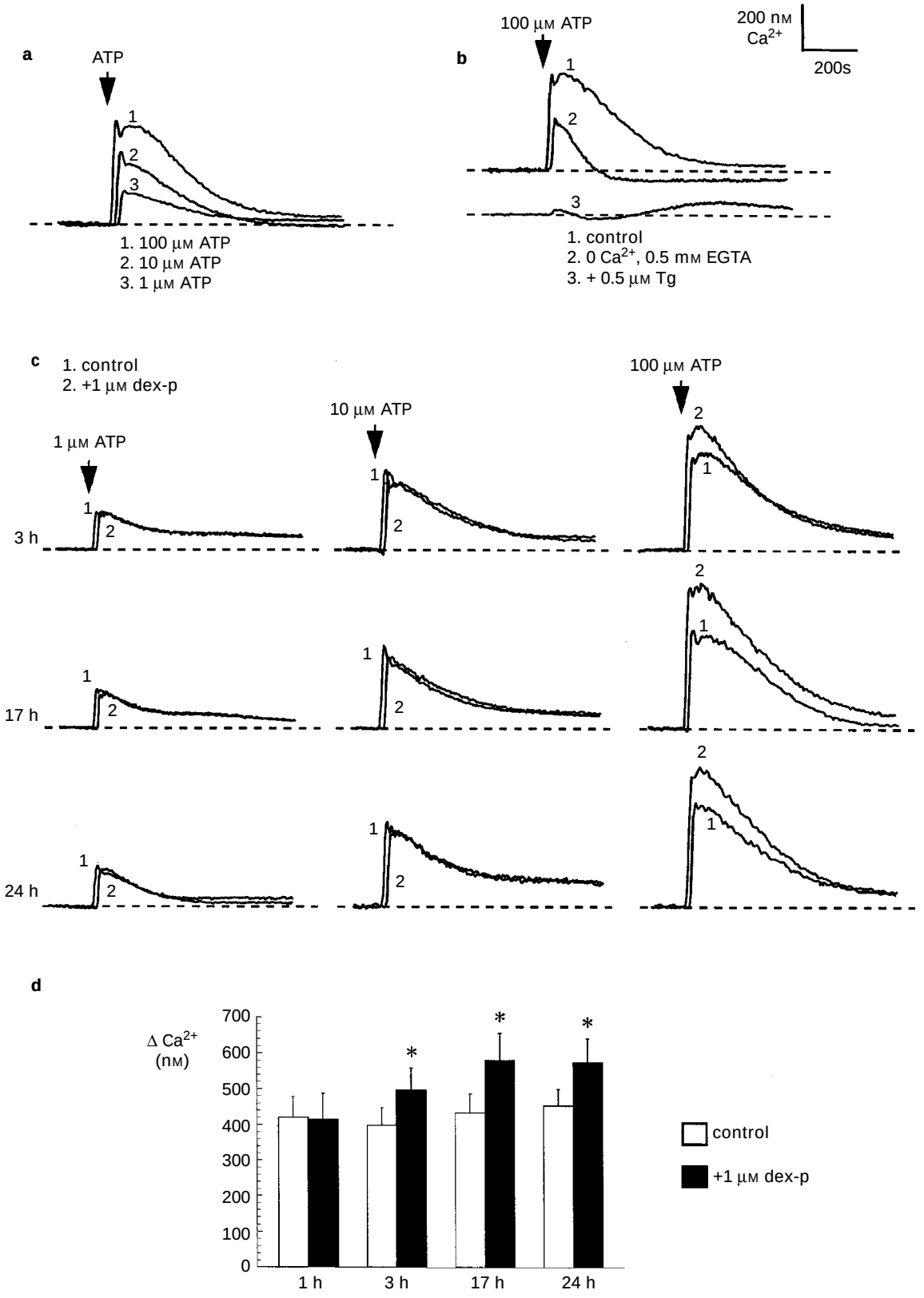

Figure 1 (a) ATP-induced increase in $[Ca^{2+}]_i$ of U937 cells. (b) Effect of transferring cells to Ca^{2+} depleted Hank's medium (0 Ca^{2+}, 0.5 mM EGTA) or pretreating for 15 min with 0.5 μM Tg on the ATP-induced Ca^{2+} response. (c) Treating cells with dexamethasone-phosphate (dex-p) for 3, 17 and 24 h (indicated) increased the ATP-induced Ca^{2+} transient of cells evoked at high (100 μM) but not low (1–10 μM) concentrations of ATP. In (b) and (c) dex-p treatment of cells did not alter response onset to ATP. (d) Mean maximum amplitude $\pm$ s.d. of Ca^{2+} transients elicited with 100 μM ATP, for untreated control cells and cells incubated with 1 μM dex-p for 1, 3, 17 and 24 h (indicated). Each bar corresponds to at least four separate experiments. $^*P<0.05$ vs control values.

extracellular Ca^{2+} from the bathing medium resulted in a decrease in the peak amplitude, and abrogation of the raised plateau phase of the Tg-induced Ca^{2+} response (Figure 4b). This suggests that Ca^{2+} mobilization and influx contribute to the overall magnitude of the response, while the sustained, elevated plateau phase is assumed to be due to store-operated Ca^{2+} influx.

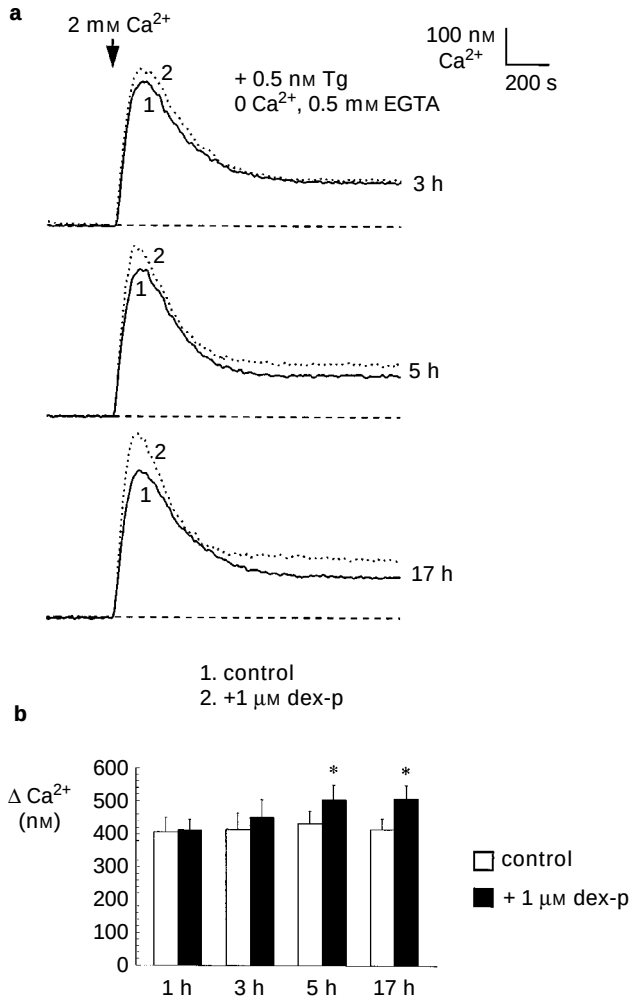

Figure 2 Potentiation of store-operated Ca^{2+} influx by dex-p. Prior to the experiment, cells were incubated in the absence, or the presence of 1 μM dex-p for 1, 3, 5, and 17 h. Cells were then transferred to low Ca^{2+} medium (0 Ca^{2+}, 0.5 mM EGTA) and were pretreated with 0.5 μM Tg for 15 min to deplete intracellular Ca^{2+} stores. Reintroducing Ca^{2+} to the bathing medium resulted in greater Ca^{2+} influx for cells incubated with dex-p at the indicated times, compared to untreated controls (a). (b) Mean maximum amplitude $\pm$s.d. of intracellular Ca^{2+} transients elicited on reintroduction of Ca^{2+} to the bathing medium for the Ca^{2+} store-depleted cells of (a), which had been preincubated in the absence or presence of 1 μM dex-p for the indicated times. Each bar corresponds to at least four separate experiments. *$P<0.05$ vs control values.

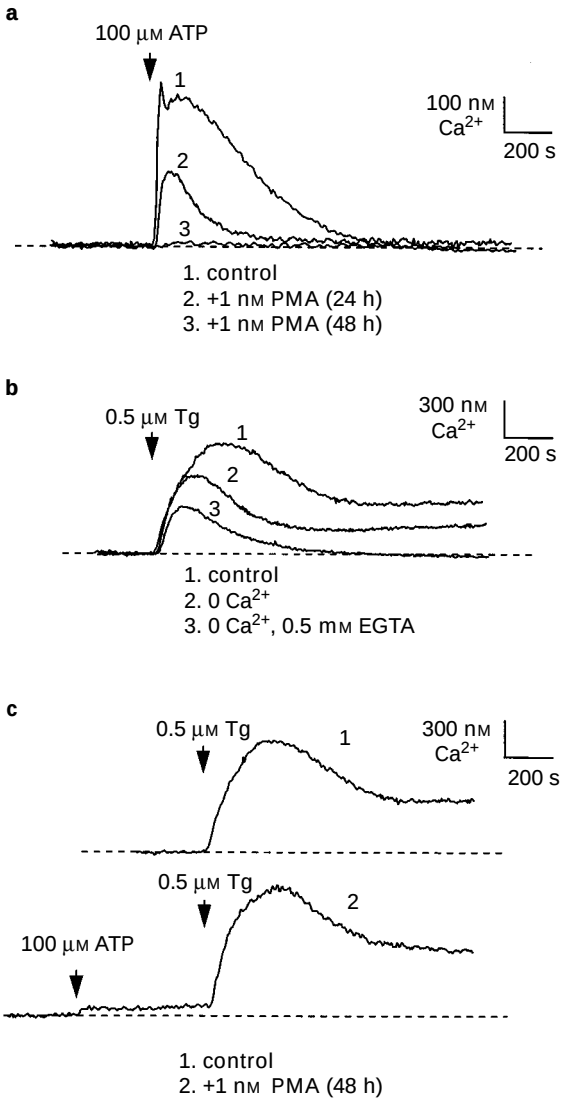

Figure 4 (a) Inhibition of the ATP-mediated Ca^{2+} response of U937 cells following incubation with 1 nM PMA for 24 and 48 h. (b) Lack of effect of incubating cells with 1 nM PMA for 48 h on the thapsigargin (Tg)-induced Ca^{2+} response of U937 cells, suggesting that Ca^{2+} store integrity and store-operated Ca^{2+} influx are unaffected by this treatment. Each trace of (a), (b) and (c) is the average of at least three separate experiments.

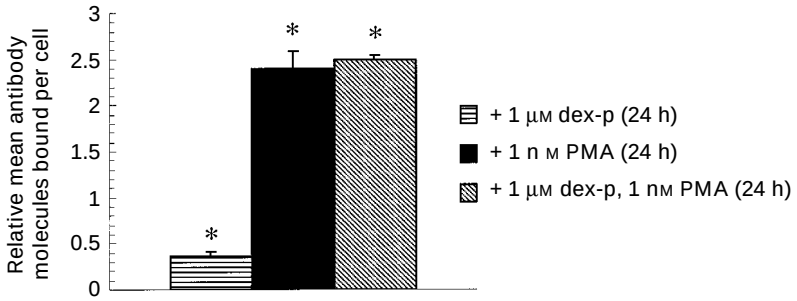

Figure 3 Cell surface expression of lipocortin 1. Cell surface lipocortin 1 was estimated by FACS, following incubation of cells with 1 μM dex-p, and 1 nM PMA separately or combined for 24 h. Results are expressed as the mean number of anti-LC1 mAb molecules bound per cell $\pm$s.d. relative to the level of untreated control cells which was $141.5 \pm 9.2 \times 10^3$. Each bar corresponds to duplicate experiments. *$P<0.01$ vs control values.

Lack of effect of incubating cells with lipocortin 1 on the ATP-induced increase in $[Ca^{2+}]_i$, or thapsigargin-induced Ca^{2+} influx

LC1 applied extracellularly binds to cell membranes via a Ca^{2+} dependent process (Becherucci *et al*., 1993). Surface bound LC1 has been proposed to act as a mediator of the antiproliferative and anti-inflammatory actions of dexamethasone, *in vivo* and *in vitro*, via a mechanism that results in the inhibition of $cPLA_2$ (Flower & Rothwell, 1994). It is unresolved whether this LC1-mediated inhibitory mechanism might be the result of aberrant cellular Ca^{2+} signalling or homeostasis. To test whether the modulatory effects of dex-p on P_2-receptor coupled Ca^{2+} signalling and Tg-induced Ca^{2+} influx were mimicked by an increased cell surface level of LC1, U937 cells were incubated with 5 μg ml^{-1} LC1 (32.5, 35 kDa) for up to 4 h. Over this time interval, and in contrast to dex-p treated cells, the ATP-mediated Ca^{2+} response (Figure 5a), and Tg-induced Ca^{2+} influx (Figure 5b and c) were unaltered. This result, combined with evidence that cell surface levels of LC1 are not increased following 24 h incubation with dex-p (see above), implies that an increased cell membrane expression of LC1 is unlikely to be responsible for the apparent potentiation of store-operated Ca^{2+} influx by dex-p in this cell line.

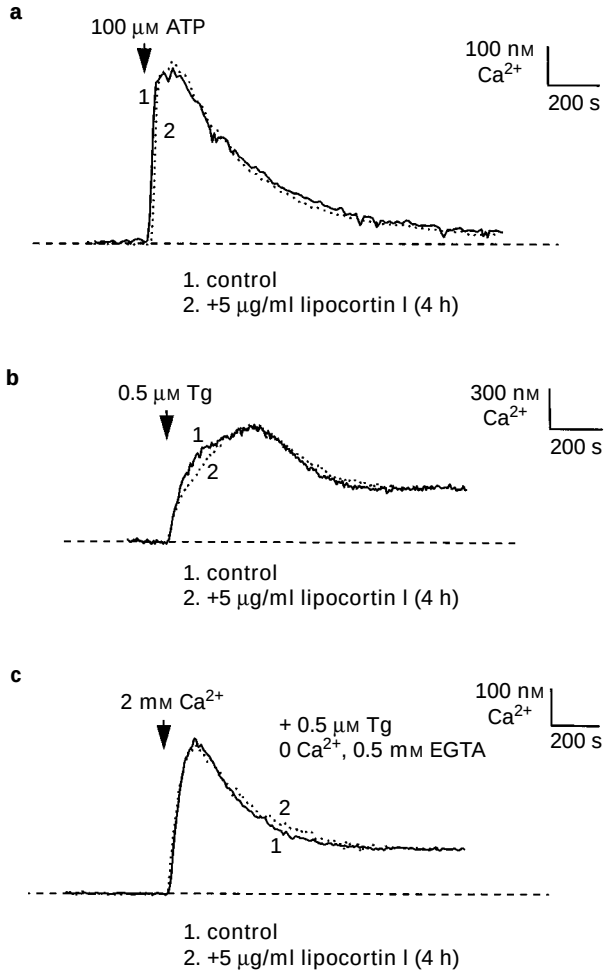

Figure 5 Lack of effect of incubating cells with 5 μg ml^{-1} LC1 for up to 4 h, on the ATP-mediated (a) and Tg-induced Ca^{2+} response (b) of U937 cells. For cells transferred to low Ca^{2+} medium (0 Ca^{2+}, 0.5 mM EGTA) and whose Ca^{2+}-stores were depleted by pretreating with 0.5 μM Tg for 15 min, Ca^{2+} influx was the same for cells incubated for 4 h with 5 μg ml^{-1} LC1, as for untreated controls, on reintroducing Ca^{2+} to the bathing medium (c). This suggests that an elevated membrane level of LC1 does not affect Ca^{2+} mobilization from Tg-sensitive stores, or store-operated Ca^{2+} influx. Each trace of (a), (b) and (c) is the average of four separate experiments.

Discussion

LC1 is a member of a family of phospholipid and Ca^{2+}-binding proteins which has been proposed to act as a mediator of the antiproliferative and anti-inflammatory actions of the glucocorticoid, dexamethasone, *in vivo* and *in vitro*. It has been suggested that LC1 exerts these effects via a mechanism that inhibits receptor-mediated activation of $cPLA_2$ (Flower & Rothwell, 1994), the enzyme responsible for the formation of arachidonic acid, a precursor of prostanoid biosynthesis. As the activation of this enzyme is Ca^{2+} dependent in many cells (Clark *et al*., 1995; Peters-Golden *et al*., 1996), it is conceivable that LC1 may inhibit $cPLA_2$ activation via the abrogation of receptor-mediated Ca^{2+} signalling, especially when also considering that dexamethasone has been reported to inhibit a number of Ca^{2+} signalling pathways in a variety of cell types (Shipston & Antoni, 1992; Yamaguchi *et al*., 1993; Baus *et al*., 1996). To determine whether dexamethasone and LC1 might inhibit a Ca^{2+}-signalling pathway that has been implicated in mitogenic and inflammatory signalling (Partiseti *et al*., 1994; Willmott *et al*., 1996; Baricordi *et al*., 1996; Dixon *et al*., 1997), the effect of these agents on P_2-receptor coupled Ca^{2+} signalling in the immature monocytic U937 cell line was assessed. Results from this study suggest that following prolonged treatment (>3 h) with dex-p, the Ca^{2+} response elicited with high concentrations of ATP (100 μM) is increased compared to untreated control cells. The endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin (Tg), has been shown to activate a dihydropyridine-sensitive, yet voltage-insensitive store-operated Ca^{2+} influx pathway in U937 cells (Willmott *et al*., 1996). As Tg-induced Ca^{2+} influx was also increased by dex-p, it is likely that this steroid is somehow affecting store-operated Ca^{2+} influx. It is noteworthy that Ca^{2+} response potentiation was only evident following prolonged incubation with dex-p (>3 h), suggesting that apparent effects might be dependent on increased gene transcription and expression of store-operated Ca^{2+} channels (SOCs) and/or components involved in SOC activation.

Although incubation of cells with dex-p or PMA for 24 h resulted in a modification in the cell surface level of LC1, the modulation of Ca^{2+} responses by these agents is likely to be LC1 independent because pretreatment of cells with LC1 for 4 h did not alter Tg-induced Ca^{2+} influx, or the ATP-mediated Ca^{2+} response. In addition, elevated levels of LC1 following 24 h PMA treatment did not affect the Tg-induced Ca^{2+} response of cells. These data suggest that increased cell surface levels of LC1 do not affect P_2-receptor coupled Ca^{2+} signalling, store-operated Ca^{2+} influx, or Ca^{2+} store integrity. It has been reported that several members of the annexin family, including type II and V, are able to form ion pores in the plasma membrane, which are permeable to divalent cations (Burger *et al*., 1996). It is unlikely that lipocortin 1 has the same property, however, as the basal $[Ca^{2+}]_i$ was not altered following addition of LC1 to cells (data not shown), suggesting that this protein does not act as a Ca^{2+} ionophore in U937 cells. It is noteworthy that we were unable to detect any increase in the cell surface level of LC1 following 24 h incubation of cells with dex-p. This might be explained by the apparent biphasic nature of the dexamethasone-induced increase in cytosolic and membrane-bound LC1 reported by Solito *et al*. (1994) in U937 cells, where elevated levels of LC1 were only seen at two time points over 48 h.

Although treating cells with PMA for 48 h resulted in the complete abrogation of the ATP-induced Ca^{2+} response, Tg-induced Ca^{2+} mobilisation and influx were apparently unaffected. This suggests that PMA treatment downregulates the P_2-receptor linked Ca^{2+} signalling pathway in these cells. Although the exact reason for this is unclear, it is possible that PMA-induced cell differentiation results in a functionally remodelled cell type which no longer expresses part or all of the above signalling pathway. Alternatively, protein kinase C (PKC) activation may result in receptor phosphorylation and desensitisation. As differentiating cells with 1% dimethyl

sulphoxide (DMSO) for 48 h failed to inhibit the ATP-induced Ca^{2+} response of cells, a scenario where PMA-mediated effects on Ca^{2+} signalling are the direct result of PKC activation, is perhaps the more likely. Contrary to previous reports (Montero *et al*., 1993a, b; Tornquist, 1993; Shibata *et al*., 1994), PMA treatment of cells (24 and 48 h) did not affect Tg-induced Ca^{2+} mobilisation and influx, suggesting that Ca^{2+} store integrity and store-operated Ca^{2+} influx remain intact following PKC activation and cell differentiation.

Taken as a whole, the results from this study suggest that the antiproliferative and anti-inflammatory effects of dexamethasone and LC1 may not be a consequence of the downregulation of receptor-coupled Ca^{2+} mobilisation pathways. Indeed, the apparent potentiation of store-operated Ca^{2+} influx by dex-p might be the result of increased expression of store-operated Ca^{2+} channels (SOCs) and/or components involved in SOC activation in U937 and HEL cells. Whether increased Ca^{2+} influx via this pathway is responsible for mediating some of dexamethasone's cellular effects is uncertain. It is nevertheless possible that enhanced store-operated Ca^{2+} influx might result in receptor desensitisation or the downregulation/inhibition of key components involved in mitogenic signalling. Alternatively, potentiation of this particular influx mechanism might play a role in dexamethasone-induced apoptosis. It is noteworthy that a similar increase in Ca^{2+} influx has been previously reported in dexamethasone-treated lymphocytes, which was suggested to mediate their apoptosis (Khan *et al*., 1996). The increased expression of the plasma membrane type 3 inositol-1,4,5-trisphosphate (IP_3) receptor was proposed to account for this. Hence, combined evidence suggests that increased Ca^{2+} influx might constitute a general cellular effect of dexamethasone, the physiological relevance of which remains to be fully understood.

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