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Rosiglitazone transiently disturbs calcium homeostasis in monocytic cells

J. Caddy [a], N. Singh [a], L. Atkin [a], M. Ahluwalia [a], A. Roberts [b], D. Lang [b], A.W. Thomas [a], R. Webb [a,*]

[a] Centre for Biomedical Sciences, Cardiff School of Health Sciences, University of Wales Institute Cardiff, Cardiff CF5 2YB, UK
[b] School of Medicine, Cardiff University, Heath Park Campus, Cardiff CF14 4XN, UK

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Abstract

The PPARγ agonist Rosiglitazone exerts anti-hyperglycaemic effects by regulating the long-term expression of genes involved in metabolism, differentiation and inflammation. In the present study, Rosiglitazone treatment rapidly inhibited (5–30 min) the ER Ca^{2+} ATPase SERCA2b in monocytic cells ($IC_{50} = 1.88\ \mu M$; $p < 0.05$), thereby disrupting short-term Ca^{2+} homeostasis (resting $[Ca^{2+}]_{cyto} = 121.2 \pm 2.9\%$ basal within 1 h; $p < 0.05$). However, extended Rosiglitazone treatment (72 h) induced dose-dependent SERCA2b up-regulation, and restored calcium homeostasis, in monocytic cells (SERCA2b mRNA: $138.7 \pm 5.7\%$ basal (1 μM)/ $215.0 \pm 30.9\%$ basal (10 μM); resting $[Ca^{2+}]_{cyto} = 97.3 \pm 8.3\%$ basal (10 μM)). As unfavourable cardiovascular outcomes, possibly related to disrupted cellular Ca^{2+} homeostasis, have been linked to Rosiglitazone, this effect may be of clinical interest. In contrast, in PPRE-luciferase reporter-gene assays, Rosiglitazone induced non-dose-dependent PPARγ-dependent effects (1 μM: $152.5 \pm 4.9\%$ basal; 10 μM: $136.1 \pm 5.1\%$ basal ($p < 0.05$ for 1 μM *vs.* 10 μM)). Thus, we conclude that Rosiglitazone can exert PPARγ-independent non-genomic effects, such as the SERCA2b inhibition seen here, but that long-term Rosiglitazone treatment did not perturb resting $[Ca]_{cyto}$ in this study.

Keywords: Rosiglitazone; Monocyte; PPARγ; Intracellular Ca^{2+}; SERCA2b; Anti-hyperglycaemic effects

It has been widely reported that increased cytoplasmic free calcium concentrations ($[Ca^{2+}]_{cyto}$) are involved in the pathophysiology of diabetes and its complications in a variety of cell types, including monocytes [1]. Disrupted Ca^{2+} homeostasis may—depending on cell type—contribute to hypertension, cardiac dysfunction and other diabetic complications [1–3]. For example, monocyte dysfunction has been linked to increased cytoskeletal rigidity and reduced cell deformability, leading to microchannel occlusions and microvascular diabetic complications [4].

Of direct interest in this regard is SERCA2b, the predominant 'Ca^{2+} pump' enzyme of non-muscle and smooth muscle cells, which is responsible for sequestration of Ca^{2+} into the endoplasmic reticulum after Ca^{2+} signalling events, and compensatory to non-specific leakage into the cytoplasm [5]. Many studies have reported decreased SERCA2b expression/activity in diabetes [3,6,7]. Indeed, it has been reported that decreased SERCA2b expression/activity correlates with both increased resting $[Ca^{2+}]_{cyto}$ and progression of diabetes [7].

Thiazolidinedione (TZD) drugs exert anti-hyperglycaemic effects by acting as ligands for the nuclear receptor PPARγ, which regulates the expression of a plethora of genes involved in metabolism, cell differentiation and inflammation [8]. However, it has been questioned whether all effects of TZDs depend on their metabolic action, or whether some effects may occur via distinct mechanism(s) [9]. We have previously shown [4] that short-term treatment with Rosiglitazone, at doses not toxic to cells (1 h; $IC_{50} = 3.72\ \mu M$), significantly reduces monocyte f-MLP-induced actin polymerisation, and that this change involves modulations in Ca^{2+}signalling. We suggested that this constituted a 'non-genomic PPARγ-independent' effect

* Corresponding author. Fax: +44 2920 416982.
E-mail address: rwebb@uwic.ac.uk (R. Webb).

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involving modulation of pre-existing intracellular signalling pathways by Rosiglitazone [4]. Thus, to bring about non-genomic effects, Rosiglitazone may interact with as-yet-undefined cellular component(s) to influence signalling events downstream of surface receptors to modulate cell signalling processes in a PPARγ-independent manner. Consequently, the net effects of TZD therapy may be due to a combination of PPARγ-dependent and -independent events [4].

Given that Ca^{2+} signalling is a fundamental component of cellular function, particularly within the cardiovascular (CV) system, and in the light of recent literature associating Rosiglitazone with negative CV outcomes [10,11], the investigation of links between the TZDs and modulations in $[Ca]_{cyto}$ assumes great importance. However, to our knowledge few studies have explored this topic. One study reported that long-term treatment with high doses of Rosiglitazone (10 μM; 72 h) induces ~1.5-fold increases in SERCA2 promoter activity and mRNA levels in cardiomyocytes, and consequent improvements in Ca^{2+} handling in Rosiglitazone-treated cells [12]. However, no evidence of a PPARγ response element (PPRE) in the SERCA2 promoter was found, and so the mechanism by which Rosiglitazone upregulated SERCA2 is not yet known.

Here, we report that incubation with Rosiglitazone rapidly (within 5 min) induces minor but statistically significant increases in resting $[Ca^{2+}]_{cyto}$ via a non-genomic mechanism of action in monocytic cells. We also identify the mechanism underpinning this effect as dose-dependent inhibition of SERCA2b. We further show that, after extended incubation with Rosiglitazone (>24 h), cells compensate for consequent increases in $[Ca^{2+}]_i$ by upregulating SERCA2b, and thereby provide a mechanism for restoring Ca^{2+} homeostasis, suggesting—at least at the level of the individual monocytic cell—that long-term Rosiglitazone therapy may not bring about adverse calcium-related effects in the monocytes of patients with diabetes.

Materials and methods

Materials. All reagents were purchased from Sigma–Aldrich (Poole, UK) unless stated otherwise. Cell permeabilisation reagents, Fluo 3/AM and Rosiglitazone were obtained from Harlan SERA-LAB Ltd. (Loughborough, UK), Molecular Probes, Inc. (Eugene, OR), and GlaxoSmithKline (Uxbridge, UK), respectively. Cultured MM6 monocytic cells were obtained from the German Collection of Micro-Organisms and Cell Culture (Braunschweig, Germany). PPARγ-responsive luciferase reporter (PPRE-luc) constructs and rabbit sarcoplasmic reticulum (SR) preparations were kindly provided by Professor W. Wahli (Lausanne University, Switzerland) and Professor A.G. Lee (Southampton University, UK).

Maintenance of cells in culture. Monocytic MM6 cells were utilised as an *in vitro* model for peripheral monocytes. Cells were cultured (37 °C; 5% CO_2) in RPMI medium plus 10% foetal calf serum and 1% penicillin (50 IU/ml)/streptomycin (100 μg/ml), and were sub-cultured when a cell density of $0.85–1.00 \times 10^6$ was attained; cells of passage number < 25 were used in all cases.

Subcellular fractionation and Western blot analysis. To confirm the expression and subcellular localisation of SERCA2b in MM6 cells, whole-cell homogenates, total protein extracts, cytoplasmic, mitochondrial and microsomal subcellular fractions (prepared using the method of Maruyama & MacLennan [13]) were subjected to Western blotting analysis. To immunologically characterise PPARγ expression, total cell protein lysates were subjected to Western blotting analysis.

In all cases, Western blot analyses were performed using 32.5 μg protein/sample, and anti-SERCA2b, anti-PPARγ or anti-phospho-MAPK primary (1:500 dilution in TBST (16 h)), followed by HRP-labelled anti-goat IgG/anti-rabbit IgG secondary antibody (1:2000 dilution in TBST (2 h); all from Cell Signalling Technology, Beverley, MA). Immunogenic proteins were detected by ECL-enhanced chemiluminescence, and band intensities determined and quantitated using AC-1 BioImaging and VisionWorksLS Software systems (UltraVioletProducts Ltd, Cambridge, UK).

Measurement of intracellular Ca^{2+} concentrations. $[Ca^{2+}]_{cyto}$ was measured by the method of Elsner et al. [14], in which fluo-3 fluorescence as detected via flow cytometry was used as an indicator of resting $[Ca^{2+}]_{cyto}$. Monocytic cells were pre-incubated ± 1–10 μM Rosiglitazone (or 0.1% v/v dimethylsulfoxide (DMSO) vehicle) for up to 72 h at 37 °C, and then incubated with 3 μM fluo-3/AM for 40 min at 37 °C, harvested by centrifugation, and the cell pellet resuspended in 0.5 ml PBS before analysis using a Cytomics FC500MPL flow cytometer ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 530$ nm; Beckman Coulter, Buckinghamshire, UK). Data were expressed as percentages of fluorescence due to resting $[Ca^{2+}]_{cyto}$ in untreated cells.

Ca^{2+}-ATPase assays. Ca^{2+}-dependent ATP hydrolysis was measured using a coupled enzyme assay, and free Ca^{2+}concentrations calculated, as described previously [15,16]. Briefly, samples (2–50 μg protein) were incubated at 25 °C in 2.5 ml buffer (100 mM KCl, 40 mM Hepes; pH7.2) containing ATP (2 mM), phospho(enol)pyruvate (2.5 mM), NADH (0.25 mM), pyruvate kinase (7.5 U), and lactate dehydrogenase (8.0 U). The re-synthesis of ATP consumed by Ca^{2+}ATPase activity was coupled by the pyruvate kinase and lactate dehydrogenase to NADH oxidation, which was recorded at A_{340} (Lambda25 spectrophotometer/UV Winlab software, Perkin Elmer, UK), and ATPase activity (expressed as IU (μmol of ATP hydrolyzed/mg of total protein/min)) was calculated using an extinction coefficient for NADH of 6200 l/mol/cm. Ca^{2+}-ATPase activity was defined as the activation seen on addition of calcium. ATPase activity was measured after a 30-min preincubation (25 °C) with Rosiglitazone (0–20 μM), thapsigargin (100 nM), cyclopiazonic acid (10 μM), or DMSO (0.1% v/v).

SERCA2b mRNA quantitation. MM6 cells were lysed and RNA extracted with Trizol® Reagent according to manufacturer's instructions (Invitrogen, Paisley, UK). RNA samples were converted to cDNA using an Applied Biosystems® High-Capacity cDNA Archive Kit (Invitrogen, Paisley, UK) and stored at −20 °C. SERCA2b mRNA expression was investigated using an Applied Biosystems 7500 Real-time PCR system and assessed semi-quantitatively (relative to Glyceraldehyde Phosphate Dehydrogenase (GAPDH)) via Taqman® Gene Expression Assays (Applied Biosystems, Warrington, UK) for SERCA2b (Gene Expression Assay Hs01566028_g1 [14]: Forward Primer: 5′-GAGATCACAGCTA TGACTGGTGATG-3′; Reverse Primer: 5′-CCCGATTTCCGACTT CTTCA-3′; Probe: 5′-/56-FAM/TGTGAACGACGCGCCCGC/36-TAMRA/-3′) and GAPDH (Gene Expression Assay Hs99999905_m1). Thermocycling was as follows: initial denaturation (2 min; 50 °C/10 min; 95 °C), followed by 50 cycles of denaturation (15 s; 95 °C)/annealing-extension (60 s; 60 °C).

Reporter gene assays. Cultured monocytic cells were transfected with PPRE-Luc plasmid using electroporation. 0.3 ml aliquots from a cell suspension (46×10^6 cells/mL in RPMI 1640) and plasmid (30 μg) were incubated in electroporation cuvettes for 5 min at room temperature before electroporation (975 μF; 250 V; Bio-Rad® Gene Pulser II) using an exponential decay pulse system. Transfected cells were transferred into an ice bath for 20 min, and then resuspended in 10 ml RPMI-1640. 100 μL cell suspension aliquots were then incubated with different stimuli (37 °C; 5% CO_2; 0–72 h). Cell lysis and assay of luciferase activity were performed using a Steady-Glo® Luciferase assay system (Promega, Southampton, UK) following the manufacturer's instructions. Luminescence was quantified using a Dynex MLX Microtitre Plate Luminometer (Dynex technologies, VA, USA).

Statistical analysis. Data are expressed as mean ± standard error of the mean. Multiple and non-multiple comparisons were performed using one-way analysis of variance (ANOVA), and paired or unpaired *t*-tests, as appropriate. Significance levels were set at $P < 0.05$.

Results

Cell viability

Cell viability, as determined by MTB assay and trypan blue exclusion, was not significantly reduced by incubation of cells with maximal doses of Rosiglitazone (20 μM) for >24 h [data not shown; $p < 0.05$].

SERCA2b and PPARγ protein expression in cultured monocytic cells

In subcellular fractionation studies, SERCA2b was detected by Western Blot analysis as a ~105 kDa band that was found predominantly in microsomal membranes from MM6 cells ($n = 3$; Fig. 1A), and which co-fractionated with the ER marker protein Calnexin [data not shown]. In separate Western Blotting experiments ($n = 4$; Fig. 1B), PPARγ was detected in total protein extracts as a doublet of ~55 kDa (non-phosphorylated PPARγ) and ~60 kDa (phosphorylated PPARγ) bands.

SERCA2b Ca^{2+} ATPase activity

In SR preparations from rabbit skeletal muscle, and in microsomes isolated from MM6 cells, maximal Ca^{2+}-ATPase activities were identified (at ~pCa 4.82) of 3.030 ± 0.300 IU ($n = 4$) and 0.061 ± 0.013 IU ($n = 18$) respectively. As expected, maximal activities were higher in SR preparations but Ca^{2+} dependencies and susceptibilities to the SERCA inhibitors thapsigargin and cyclopiazonic acid for the two preparations were almost identical (data not shown), and in approximate agreement with previously published values for SERCA enzymes [17]. Incubation with 0.1% DMSO alone did not cause inhibition ($103.6 \pm 9.6\%$ maximal activity, $p > 0.05$, $n = 3$). Thus, we conclude that SERCA2b is expressed as an active ER-resident Ca^{2+} pump enzyme in human monocytic cells.

Preincubation (30 min; 25 °C) with Rosiglitazone brought about complete dose-dependent inhibition of SERCA2 b activity in MM6 microsomes (Fig. 2; $IC_{50} = 1.88$ μM; ANOVA $p < 0.05$; $n \geqslant 6$; the apparent negative activity at very high Rosiglitazone concentrations is likely due to variability in Ca^{2+}-independent ATPase activity). Interestingly, these values are similar to that ($IC_{50} = 3.72$ μM) which we have previously reported for Rosiglitazone's Ca^{2+}-dependent actin cytoskeleton remodelling action [4]. Given this similarity, and as the timescale seen for the associated increase in $[Ca^{2+}]_{cyto}$ in both the present study (Fig. 3) and previously [4] is suggestive of leakage of Ca^{2+} ions into the cytoplasm rather than triggering of a specific Ca^{2+} signalling cascade, we propose that inhibition of SERCA2b is responsible for the Rosiglitazone-mediated cytoskeletal remodelling previously seen in monocytic cells [4].

Measurement of $[Ca^{2+}]_{cyto}$

As with our previous study [4], pre-incubation with 10 μM Rosiglitazone for short periods (<1 h) brought about a small but statistically significant increase in resting $[Ca^{2+}]_{cyto}$ in monocytic cells ($121.2 \pm 2.9\%$ basal; $n = 8$; $p < 0.05$; Fig. 3). This increase was statistically significant after 24 h ($140.5 \pm 9.5\%$ basal; $n = 3$; $p < 0.05$), but after 72 h, resting $[Ca^{2+}]_{cyto}$ had reverted to basal levels ($92.3 \pm 7.8\%$ basal; $n = 6$; $p > 0.05$). In contrast, 1 μM Rosiglitazone had no statistically significant effect with any duration of pre-incubation ($n = 15$).

Gene expression in monocytic cells after Rosiglitazone treatment

In reporter gene assays using monocytic cells transfected with a PPRE-luciferase construct, 24 h incubation with 1 or 10 μM Rosiglitazone induced increases in luminescence ($p < 0.05$ vs. basal in both cases; $n = 4$; Fig. 4A), but 1 μM Rosiglitazone produced a significantly greater increase than 10 μM Rosiglitazone (1 μM: $152.5 \pm 4.9\%$ basal; 10M: $136.1 \pm 5.1\%$ basal ($p < 0.05$)).

Incubation with Rosiglitazone for shorter durations (⩽6 h) did not induce a significant change in reporter gene expression (1 μM: $113.9 \pm 12.4\%$ basal (6 h); 10 μM: $100.8 \pm 3.6\%$ basal ($p > 0.05$; $n = 4$; Fig. 4A)), indicating that PPARγ ligands take several hours to exert their genomic effects. Treatment (24 h; 37 °C) with 100 ng/ml phorbol 12-myristate 13-acetate (PMA), an

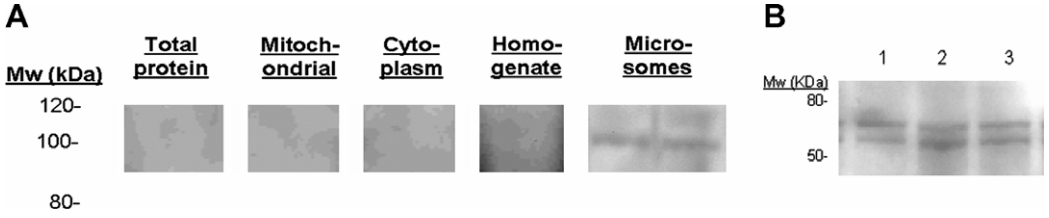

Fig. 1. Western blot detection of SERCA2b and PPARγ in MM6 cells. (A) MM6 subcellular fractions were probed with anti-SERCA2b antibodies (1:1000) and HRP-linked donkey anti-goat 2° antibodies (1:1000); (B) MM6 total protein extracts were probed with anti-PPARγ antibodies (1:1000) and HRP-linked mouse anti-rabbit 2° antibodies (1:1000). Lane 1: 10 μM Rosiglitazone (24 h); Lane 2: 1 μM Rosiglitazone (24 h); Lane 3: Control.

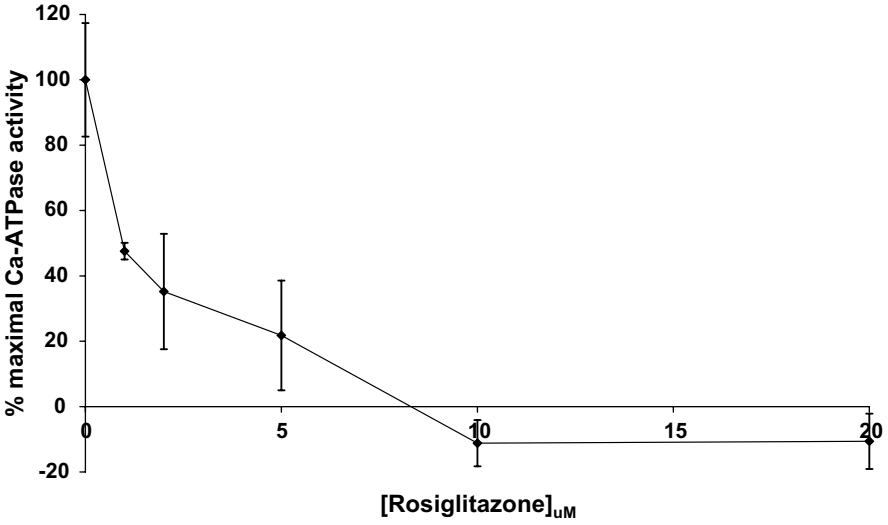

Fig. 2. Ca^{2+}ATPase assay data. Percentages of maximum Ca^{2+}ATPase activity (normalised to maximal activity) are displayed for MM6 microsomes after pre-incubation (30 min) with the indicated concentrations of Rosiglitazone.

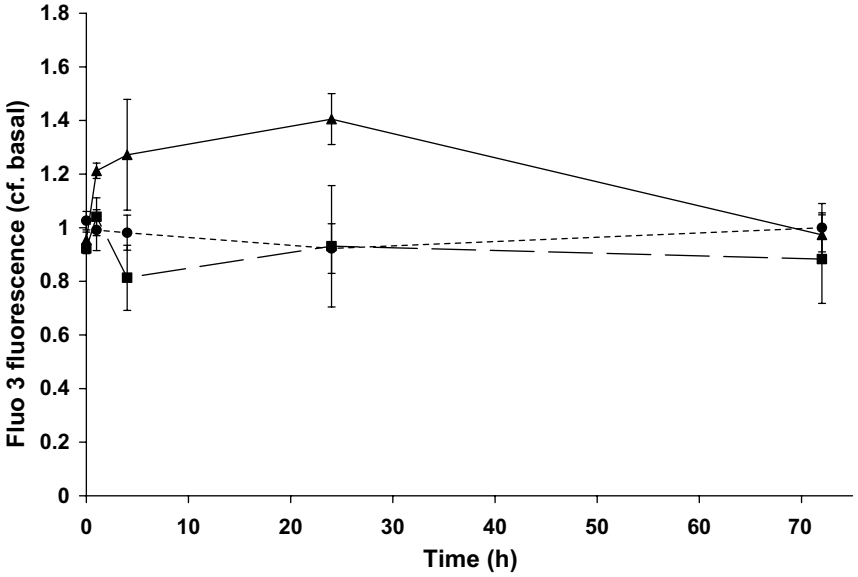

Fig. 3. The effect of Rosiglitazone on intracellular Ca^{2+} concentrations. Ca^{2+}-dependent fluo-3 fluorescence in monocytic cells is displayed relative to basal after incubation for the indicated times with 0.1% v/v DMSO (dotted line; circles), 1 μM Rosiglitazone (dashed line; squares), or 10 μM Rosiglitazone (solid line; triangles).

agent that induces differentiation of monocytic cells into macrophages [18], brought about an increase in luminescence ($159.8 \pm 4.5\%$ control; $p < 0.05$; $n = 4$; Fig. 4B) that was further increased by incubation with 1 μM Rosiglitazone ($182.8 \pm 5.4\%$; $p < 0.05$ vs. PMA alone; $n = 4$), and to a lesser extent by incubation with 10 μM Rosiglitazone ($163.1 \pm 7.6\%$; $n = 4$)). As MM6 cells express PPARγ in relatively small quantities, but differentiation is accompanied by increased expression of PPARγ [18], this provides further supporting evidence that reporter gene assay luminescence correlates to PPARγ-dependent genomic actions of Rosiglitazone.

As seen in Fig. 4C, RT-PCR analysis showed that levels of SERCA2b mRNA expression were increased in MM6 cells after $\geqslant 24$ h incubation with either 1 or 10 μM Rosiglitazone (ANOVA; $n \geqslant 3$; $p \leqslant 0.05$ in both cases), but not by treatment for shorter periods, or with vehicle alone (e.g. 72 h 0.1% DMSO: $100.3 \pm 3.2\%$ basal expression; $n \geqslant 3$; $p > 0.05$). In contrast to its effect on PPRE-luc, Rosiglitazone's effect was dose-dependent: at 72 h, 10 μM Rosiglitazone induced a larger upregulation than 1 μM ($215.0 \pm 30.9\%$ vs. $138.7 \pm 5.7\%$ basal; $n \geqslant 3$; $p < 0.05$). Furthermore, PMA-induced increases in expression in PPARγ [18] did not result in increased SERCA2b mRNA

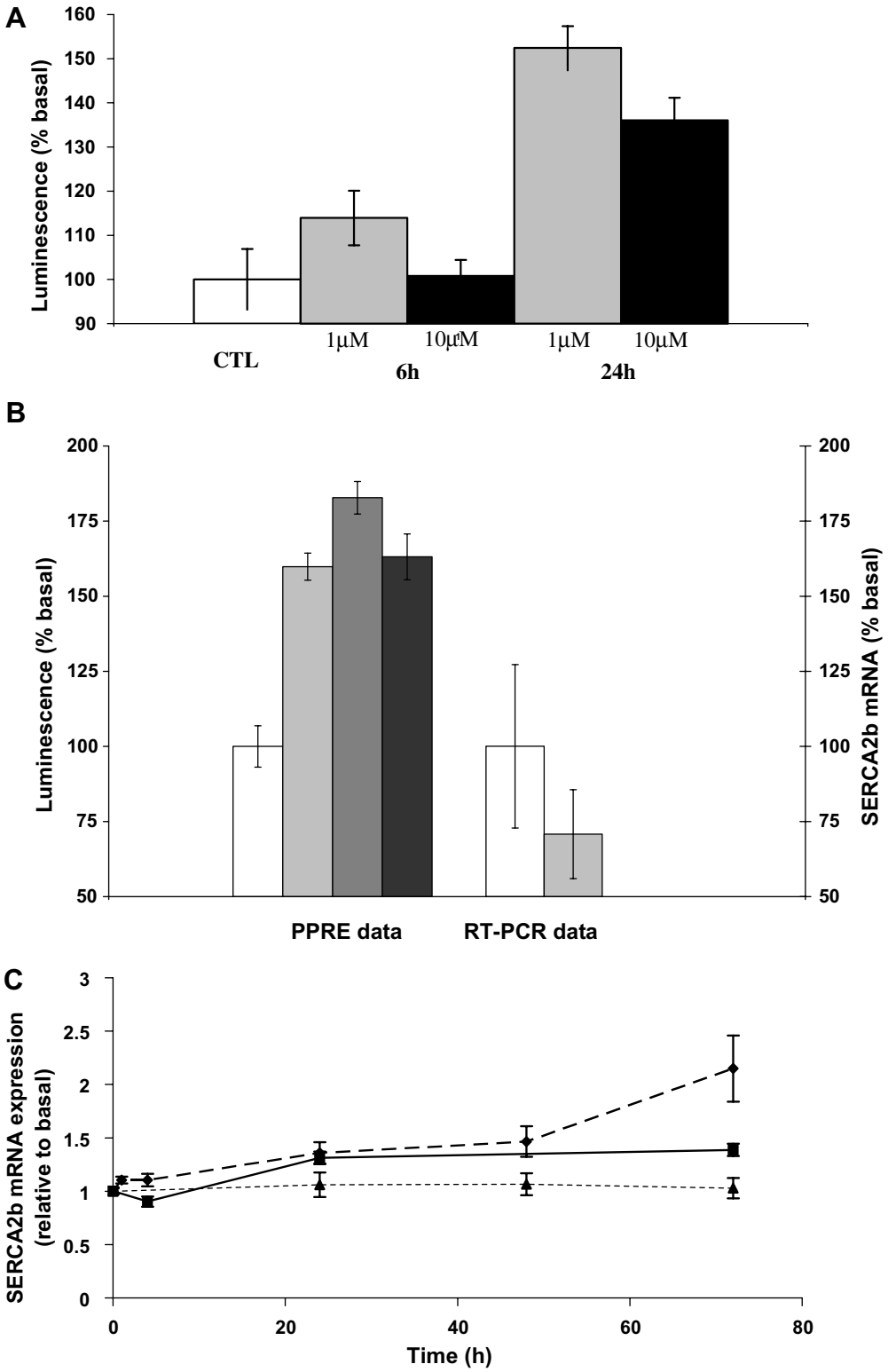

Fig. 4. The effect of Rosiglitazone on PPRE-Luc and SERCA2b expression. MM6 cells (±transfection with PPRE-Luc plasmid) were treated for the indicated times with the indicated concentrations of Rosiglitazone, or with PMA (100 ng/ml) or DMSO (0.1% v/v). (A) PPRE-Luc expression, as reported by luminescence, in control cells (white bar), and cells incubated for the indicated times with 1 μM (grey bars) or 10 μM (black bars) Rosiglitazone; (B) PPRE-Luc or SERCA2b mRNA expression, as determined by luminescence and RT-PCR respectively, in control cells (white bars), cells incubated with PMA (24 h; pale grey bars), and cells incubated with PMA and 1 μM (24 h; dark grey bars) or 10 μM (24 h; black bars) Rosiglitazone (PPRE-Luc only); (C) SERCA2b mRNA expression, as determined by RT-PCR, in DMSO-incubated cells (dotted line; triangles), and cells incubated with 1 μM (solid line; squares) or 10 μM (dashed line; diamonds) Rosiglitazone.

($70.9 \pm 14.7\%$ control; 24 h; $p > 0.05$ vs. basal; $n = 3$; Fig. 4B), suggesting that Rosiglitazone's effect on SERCA2b expression is PPARγ-independent (i.e. it is not due to Rosiglitazone-induced activation of PPARγ).

Discussion

This study provides an explanation for the apparent paradox that, while acute doses of Rosiglitazone can disrupt Ca^{2+} homeostasis within monocytes *in vitro* [4], ongoing TZD therapy has not been reported to adversely affect Ca^{2+} signalling within patients' cells. Rosiglitazone's inhibition of SERCA2b's Ca^{2+}ATPase activity (Fig. 2) causes $[Ca^{2+}]_{cyto}$ to increase within ~5 min (Fig. 3), but we propose that this can be linked to a negative feedback process in which cells respond within ⩾24 h by upregulating SERCA2 b expression (Fig. 4B) and restoring normal Ca^{2+} homeostasis in the long-term (Fig. 3).

Several factors suggest that the effects seen in the present study have not been brought about via a genomic mechanism involving interactions between PPARγ, its ligand and PPRE-bearing target gene(s). Firstly, the rapidity of the response (perturbations in $[Ca^{2+}]_{cyto}$ being seen within 5 min); secondly, the fact that inhibition of SERCA2 occurs not only in intact cells, but also in microsomal fractions that lack nuclear proteins such as PPARγ; thirdly, the lack of a PPRE in the SERCA2 gene promoter; and finally, the discrepancies between Rosiglitazone's effects on luciferase reporter gene expression, and its effects on SERCA2b activity and expression.

As shown in Fig. 4A and B, PPARγ-mediated effects of Rosiglitazone in inducing changes in PPRE-bearing target gene expression can be correlated to PPARγ expression, and are optimal at relatively low concentrations (1 μM), with higher doses (>10 μM) exceeding a threshold level beyond which antagonistic non-genomic effects such as MAPK phosphorylation (and therefore inactivation) of PPARγ are initiated [19,20]. Indeed, we observed that incubation of MM6 cells for 24 h with 10 μM (but not 1 μM) Rosiglitazone increased phosphorylation of both MAPK ($228.0 \pm 32.0\%$ basal (10 μM); $p < 0.05$; $n = 6$; [data not shown]) and PPARγ (phospho-PPARγ $= 53.1 \pm 1.4\%$ total PPARγ (10 μM Rosiglitazone) vs. $29.1 \pm 13.0\%$ total PPARγ (1 μM Rosiglitazone); $n = 4$; $p < 0.05$; Fig. 1B).

This contrasts with the dose-responsive nature of Rosiglitazone's induction of SERCA2b (Fig. 4C), and also with the similar SERCA2b mRNA levels seen in either non-differentiated (low PPARγ) or differentiated (i.e. PMA-treated [high PPARγ]) monocytic cells (Fig. 4B). Thus, we suggest that SERCA2b inhibition and SERCA2b mRNA induction may therefore be PPARγ-independent consequences of a non-genomic interaction between Rosiglitazone and either SERCA2b itself, or else some as-yet-undefined SERCA2b-associated intermediary molecule present in microsomes.

It has been demonstrated that rendering cell membranes leaky to Ca^{2+} using Ca^{2+} ionophore (A_{23187}) results in cytoplasmic Ca^{2+} elevation; this (and possibly the accompanying depletion of ER Ca^{2+} stores) induces increased SERCA2 mRNA and protein levels within 6–10 h via a signalling mechanism involving increased SERCA2 transcription [21]. Our data are in line with these findings, while our sequence analysis [data not shown] supported reports that no PPREs (AGGTCA-n-AGGTCA direct repeat sequences [8]) were present in the SERCA2 gene promoter [12]. However, we and others identified several sequences that resemble other response elements, including two UPREs, a thyroid RE, E-box (glucose RE), two fatty acid REs and nine Sp1-binding sites [data not shown]. The relative contributions of these elements in regulating SERCA2b gene expression remain to be defined [21], as does the precise nature of the mechanism by which SERCA2b is upregulated in response to Rosiglitazone.

In conclusion, it may be relevant to consider the pharmacological concentration range within which Rosiglitazone is found in the body. The peak plasma concentration of Rosiglitazone is estimated to be ~1 μM (e.g. $C_{max} = 285 \pm 50$ ng/ml, or 0.80 ± 0.02 μM [22]). In our experiments, this concentration does partially inhibit SERCA2b: at [Rosiglitazone] = 0.8 μM, SERCA2b is ~65% maximal (Fig. 2). However, we could not detect any effect of 1 μM Rosiglitazone on resting $[Ca^{2+}]_{cyto}$; this may be because cellular Ca^{2+} homeostatic mechanisms are able to buffer the relatively minor leakage of Ca^{2+} that results from partial SERCA2b inhibition. Nevertheless, the fact that 1 μM Rosiglitazone induces upregulation of SERCA2b (e.g. $138.7 \pm 5.7\%$ basal at 72 h; Fig. 4C) suggests that 1 μM Rosiglitazone does have an effect in this regard (and also that depletion of $[Ca^{2+}]_{ER}$ rather than increased $[Ca^{2+}]_{cyto}$ may be responsible for triggering SERCA2b upregulation). In any case, the present study demonstrates restoration of normal Ca^{2+} handling *in vitro*, and so we suggest that monocytes of a patient on long-term Rosiglitazone therapy may not undergo chronic perturbations in resting $[Ca^{2+}]_{cyto}$.

The recent publication of meta- and teleo-analyses of clinical Rosiglitazone studies [10,11] has raised safety concerns regarding the increased risk of myocardial infarction, and of death from cardiovascular causes in Rosiglitazone-treated subjects. However, an interim analysis of the RECORD study has refuted this suggestion [23]. Aberrant Ca^{2+} signalling in cardiomyocytes is linked to the pathophysiology of cardiovascular disease [24], but the data presented here suggest that Rosiglitazone does not, in monocytic cells at least, exert detrimental effects on intracellular calcium haemostasis. Indeed, the net effect of Rosiglitazone therapy seems to be increased SERCA2b expression, and as decreased SERCA2b expression/activity levels correlate with both increased resting $[Ca^{2+}]_{cyto}$ and the progression of diabetic complications [6,7], such Rosiglitazone-induced SERCA2b upregulation may constitute a beneficial effect of Rosiglitazone therapy on cellular Ca^{2+} handling.

As the current study consists of *in vitro* investigations into the mechanisms by which cellular effects are brought about, conclusions drawn from the present study should be interpreted cautiously with regard to the clinical setting. The mechanisms by which Ca^{2+} is handled vary widely in different cell types [25], and it remains to be seen whether the mechanisms by which the monocytic cells studied here restore normal Ca^{2+} homeostasis during long-term treatment with Rosiglitazone are also in operation in other cell types that may be more directly relevant to the patient outcomes analysed in recent studies [10,11,23].

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