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Regulation of 1α,25-dihydroxyvitamin D_3 synthesis in macrophages from arthritic joints by phorbol ester, dibutyryl-cAMP and calcium ionophore (A23187)

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Phorbol 12-myristate 13-acetate (100 nM), a potent protein kinase C and macrophage activator, has a biphasic affect on 25(OH)D_3-1α-hydroxylase activity in synovial fluid macrophages from arthritis patients. After 5 h, 1α,25(OH)D_3 synthesis fell from 5.2 ± 0.1 to 1.6 ± 0.2 pmol/h per 10^6 cells, however, after 24 h and 48 h, synthesis increased to 17.4 ± 0.3 and 22.3 ± 1.4 pmol/h per 10^6 cells, respectively. Although an independent short-term mechanism is suggested, protein kinase C may promote macrophage activation, thus increasing long-term 25(OH)D_3-1α-hydroxylase expression. Intracellular calcium and cAMP are unlikely to activate the enzyme, since 0.1 μM of the calcium ionophore, A23187, and 1 mM dibutyryl-cAMP inhibited synthesis by 87% and 79%, respectively, after 24 h.

Macrophage; 1α,25-$(OH)_2D_3$; Inflammatory arthritis; Phorbol ester; Cyclic AMP; Calcium ionophore A23187

1. INTRODUCTION

The active metabolite of vitamin D_3, 1α,25$(OH)_2D_3$ (1α,25-dihydroxyvitamin D_3), is synthesized from 25(OH)D_3(25-hydroxyvitamin D_3) by activated macrophages (Mø), as well as by kidney and placental cells. 1α,25$(OH)_2D_3$ synthesized by all these cells not only fulfils different functions, but is regulated by different mechanisms [1,2]. For example, hypocalcaemia promotes parathyroid hormone-mediated 1α,25$(OH)_2D_3$ synthesis by a cAMP-dependent mechanism in kidney. Renal 1α,25$(OH)_2D_3$ increases intestinal calcium absorption, stimulates osteoblast function, inhibits its own synthesis and increases formation of 24,25$(OH)_2D_3$, which is normally the predominant renal metabolite [3]. In contrast, 1α,25$(OH)_2D_3$ is the primary non-placental extra-renal metabolite formed by Mø activated by pulmonary sarcoidosis [4–6], tuberculosis [7], lymphoma [8], peritonitis [9] and arthritis [10–13]. 24,25$(OH)_2D_3$ is not normally formed by activated Mø but can be formed by monocytes and immature Mø [14,15]. In patients, excess 1α,25$(OH)_2D_3$ synthesis may cause hypercalcaemia which can be controlled by glucocorticoids; this phenomenon is typical of extra-renal formation [16]. Synthesis of 1α,25$(OH)_2D_3$ is induced by Mø-activating factors including, interferon-gamma (IFN-γ) [6,17], tumour necrosis factor-alpha (TNF-α) [18] and bacterial lipopolysaccharides (LPS) [19]. In addition, leukotriene C_4, a distal metabolite in the arachidonic acid 5-lipoxygenase pathway may also stimulate [20], whilst, prostaglandins E_1 and E_2 formed by the cyclo-oxygenase pathway, inhibit synthesis [12]. Further studies have suggested that the calcium ionophore, A23187, can increase 1α,25$(OH)_2D_3$, whilst phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator and cyclic adenosine monophosphate (cAMP) do not affect synthesis [6,20,21], however, PMA will promote synthesis in the monoblastic U937 cell line [22]. Since the intracellular regulation of 25-(OH)D_3-1α-hydroxylase activity in Mø is still poorly understood, particularly with respect to arthritis, we have investigated the effects of PMA, dibutyryl cAMP and the calcium ionophore, A23187, on 1α,25$(OH)_2D_3$ synthesis by synovial fluid Mø cultured from arthritic joints.

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Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; cAMP, cyclic adenosine monophosphate; Mø, macrophage; IFN-γ, interferon-gamma; TNF-α, tumour necrosis factor-alpha; LPS, bacterial lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PKC, potent protein kinase C; Ca_i, intracellular calcium.

2. MATERIALS AND METHODS

2.1. *Culture of synovial fluid Mø*

Synovial fluid was obtained from the knees of 3 patients with active rheumatiod arthritis and 2 with inflammatory pseudogout. Each patient was diagnosed on the basis of clinical criteria and synovial fluid total and differential white cell counts supplemented by appropriate immunological, radiological and crystallographic investigations. Samples were diluted (4–16% v/v) in RPMI-1640 medium containing 2% heat-inactivated foetal calf serum, 2 mmol/l glutamine, 100 μg/ml

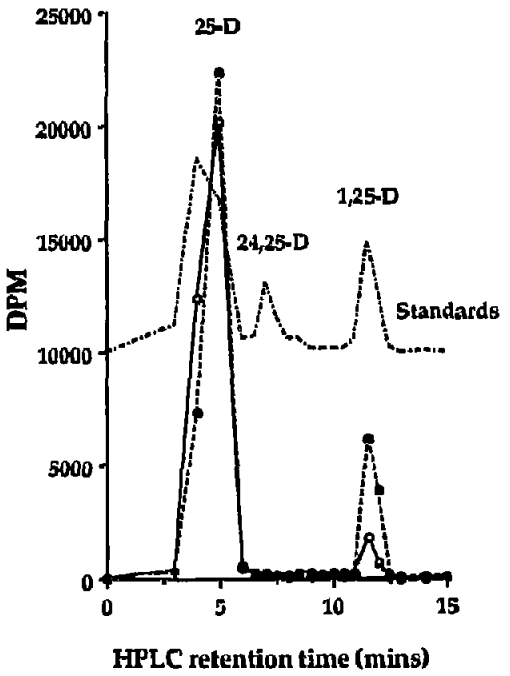

Fig. 1. HPLC analyses of [^{3}H]25(OH)D$_3$ metabolism by untreated synovial fluid Mø (○—○) compared to cells treated with 100 nM PMA for 24 h (●---●). 0.31×10^6 cells were incubated with [^{3}H]25(OH)D$_3$ for 5 h after a total of 13 days in culture following isolation from a patient with pseudogout. Each point represents the radioactivity in eluent fractions collected following chromatography on a normal phase HPLC system developed with *n*-hexane/isopropanol/methanol (110:3.3:4 by vol.) at a flow rate of 2 ml/min. The standards trace (-•-) shows the retention times of authentic [^{3}H]25(OH)D$_3$, [^{3}H]24,25-(OH)$_2$D$_3$ and [^{3}H]1α,25(OH)$_2$D$_3$.

streptomycin and 100 IU/ml penicillin (Imperial Laboratories); for each sample the dilution depended on the volume and the cell density. Aliquots (2 or 4 ml) were plated directly into one or more 24- or 12-well culture dishes (Imperial Laboratories). Following 48–72 h culture in an atmosphere of 95% air/5% $C0_2$ at 37°C the non-adherent cells, predominantly lymphocytes and apoptopic polymorphs, were decanted leaving adherent Mø; the 2% serum-RPMI-1640 medium was then changed every 3–4 days. The Mø were used after 6–24 days in primary culture during which time they had developed from immature monocytes into adherent Mø. Experiments examined the time-coures and dose-response to 1–100 nM PMA (Sigma) on the synthesis of 1α,25(OH)$_2$D$_3$ by cultured Mø. Further experiments examined the affects of dibutyryl-cAMP (0.01–1 mM; Sigma) and the calcium ionophore, A23187 (0.01–1 μM; Sigma) on 25-(OH)D$_3$-1α-hydroxylase activity.

2.2. *Assay of 25-(OH)D$_3$-1α-hydroxylase activity*

The assays of 25(OH)D$_3$ metabolism were carried out in situ in 2 ml of serum-free RPMI-1640 incubation medium containing 1.5 mg/ml of bovine serum albumin (Immuno Diagnostika), 50,000 dpm [^{3}H]25(OH)D$_3$ (6.5 TBq/mmol, Amersham International), 0.125 nmol 25(OH)D$_3$ (25 ng/ml, Roussel UCLAF) for 5 h in an atmosphere of 95% air/5% $C0_2$ at 37°C. Incubations were carried out in triplicate for each concentration of test substance and were terminated by mixing the incubation medium with 3 ml of chloroform and 2 ml of methanol. The extracts were centrifuged at 500 *g* for 15 min at 4°C and the lower chloroform layer containing vitamin D$_3$ metabolites removed, dried under a stream of nitrogen gas, resuspended in 1 ml of ethanol and stored at −20°C. Each chloroform extract was analyzed by normal phase high performance liquid chromatography (HPLC) using a mobile phase of *n*-hexane/propan-2-ol/methanol, 110:3.3:4 by vol. at 2 ml/min, a model 6000A HPLC solvent delivery pump (Waters Associates) and a Zorbax-Sil column (6.2 mm × 4.6 cm, Du Pont). Fractions were collected using a model 202 fraction collector (Gilson) and radioactivity estimated on a liquid scintillation counter (1217 Rackbeta LKB Wallac). Authentic tritium-labelled 25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$ (Amersham International) were also chromatographed to determine the HPLC retention time for each metabolite. For each experiment cells were counted in duplicate and the values used to normalise 25(OH)D$_3$-1α-hydroxylase activity for cell number. Synthesis of 1α,25(OH)$_2$D$_3$was expressed as pmol/h per 10^6 cells (means ± S.E.M.) of triplicate determinations. Statistical analysis was carried out using Student's *t*-test and significance expressed as *(P<0.05) or **(P<0.01) compared to controls.

3. RESULTS AND DISCUSSION

After 6–24 days in primary culture and a 5 h incubation with [^{3}H]25(OH)$_2$D$_3$ the conversion of substrate to [^{3}H]1α,25(OH)D$_3$by untreated synovial fluid Mø ranged from 1.7 to 39.4%, representing 1.8–72.4 pmol 1α,25(OH)$_2$D$_3$/h per 10^6 cells. Fig. 1 shows typical HPLC analyses of [^{3}H]25(OH)D$_3$ metabolism by Mø from a patient with pseudogout. Cells exposed to the PKC activator, PMA, (100 nM) for 24 h synthesized significantly more [^{3}H]1α,25(OH)$_2$D$_3$ (17.7%) from the substrate compared to untreated cells (5.3%); Fig. 1 also shows that the Mø did not synthesize [^{3}H]24,25(OH)$_2$D$_3$, unlike kidney cells [3]. Fig. 2a illustrates that stimulation of 1α,25(OH)$_2$D$_3$ synthesis by PMA in two samples dose-dependently increased from 1.8 ± 0.2 to 14.1 ± 0.8 and 5.7 ± 0.9 to 20.9 ± 1.8 pmol/h per 10^6 cells (P<0.01), respectively, after 24 h. However, after 5 h exposure PMA inhibited 1,25(OH)$_2$D$_3$ synthesis compared to 24 h and 48 h treatments (Fig. 2b). These results suggest that independent long- and short-

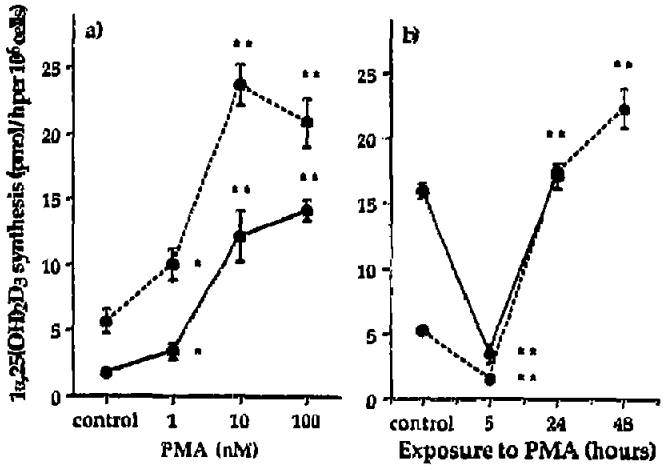

Fig. 2. Effects of PMA on 1α,25(OH)$_2$D$_3$ synthesis by synovial fluid Mø (a), PMA dose-response (24 h) using cells from two patients with rheumatoid arthritis after 10 (●---●) and 13 days (●—●) in culture, and (b) PMA time-course (100 nM) using cells from patients with pseudogout (●—●) and rheumatoid arthritis (●---●) after 6 and 24 days culture, respectively. Assays of 25(OH)D$_3$-1α-hydroxylase activity were carried out over 5 h using 0.28–0.35×10^6 Mø. Values are the mean ± S.E.M. of 3 determinations (*P<0.05, **P<0.01).

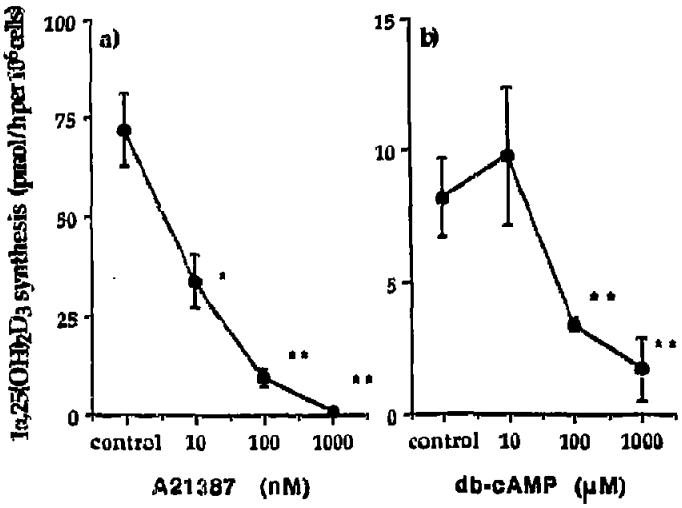

Fig. 3. Effects of (a) dibutyrl-cAMP and (b) calcium ionophore (A23187) after 24 h on $1\alpha,25(OH)_2D_3$ synthesis by synovial fluid Mø from patients with rheumatoid arthritis and pseudogout after 13 and 11 days culture, respectively. Assays of $25(OH)D_3$-1α-hydroxylase activity were carried out over 5 h using $0.08–0.1\times10^6$ Mø. Values are the mean ± S.E.M. of 3 determinations (*$P<0.05$, **$P<0.01$).

term mechanisms may be involved. The long-term stimulation of $25\text{-}(OH)D_3$-1α-hydroxylase by PMA may be mediated by PKC-mediated Mø maturation and activation [21,22]. These results also suggest that induction of $1\alpha,25(OH)D_3$synthesis by other Mø activators, including IFN-γ [6,17], TNF-α [18] and LPS [19], may in part be mediated by PKC.

Normally PKC activation results from ligand–receptor-mediated synthesis of diacylglycerol and inositol-1,4,5-triphoshate (IP_3) from phosphoinositol-4,5-bisphosphonate (PIP_2). Diacylglycerol stimulates PKC, which in turn phosphorylates proteins that mediate specific biological actions, including cell differentiation [21–23]. In contrast, IP_3 mediates calcium release from the endoplasmic reticulum which promotes various cellular events [23]. The calcium ionophore, A23187, may mimic the effects of IP_3 by allowing extracellular calcium to enter cells; in our experiments, A23187 inhibited $25\text{-}(OH)D_3$-1α-hydroxylase activity in synovial fluid Mø in a dose-dependent manner; likewise, dibutyryl cAMP inhibited $1\alpha,25(OH)D_3$synthesis (Fig. 3). These results suggest that IFN-γ, TNF-α or LPS-induced $1\alpha,25(OH)D_3$synthesis is unlikely to be mediated by raised intracellular Ca (Ca_i) or by cAMP. However, our results differ from those reported for sarcoid Mø where 10^{-6} M A23187 stimulated synthesis [20]. Our results suggest that the short-term response to PMA or perhaps prostaglandin E_1- and E_2-mediated inhibition of $25\text{-}(OH)D_3$-1α-hydroxylase [13] may be mediated by raised Ca_i or cAMP.

Severe bone, cartilage and synovial tissue destruction occurs in arthritis as a result of complex immune interactions [24–26], some of which may be modulated by $1\alpha,25(OH)D_3$ following binding to specific intracellular receptors that are expressed by lymphocytes [2,27–29], monocytes [2,28], synoviocytes (fibroblasts) [30] and bone cells [1]. Locally synthesized $1\alpha,25(OH)_2D_3$ formed by Mø in the arthritic joint may inhibit interleukin-2 production by T-lymphocytes reducing proliferation of T- and B-lymphocytes [2,31], promote the differentiation of monocytes into Mø [22,28,32] and the fusion of Mø to multinucleated Mø with bone resorbing activity [33]. It is therefore necessary to determine which factors regulate $1\alpha,25(OH)_2D_3$ synthesis in Mø at an extracellular and intracellular level in order to evaluate the role of this metabolite in inflammatory disease. In this study the data presented suggests that PKC may increase $1\alpha,25(OH)_2D_3$, whereas Ca_i and cAMP may inhibit synthesis.

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