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Insulin like growth factor-I, protein kinase-C, calcium and cyclic AMP: partners in the regulation of chondrocyte mitogenesis and metabolism

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The possible role of protein kinase-C (PKC), calcium and cyclic AMP (cAMP) in mediating the metabolic and mitogenic effects of insulin-like growth factor-I (IGF-I) on chondrocytes was investigated using a PKC activator (phorbol ester 12,13-dibutyrate, PDBU), a PKC inhibitor (compound H7), a calcium channel blocker, (verapamil) and a cAMP analogue (dibutyryl cAMP). IGF-I and PDBU stimulated sulphate and thymidine incorporation by chondrocytes. Both of these effects were inhibited by compound H7. Verapamil inhibited IGF-I- and PDBU-stimulated sulphate incorporation, but contrastingly stimulated basal and enhanced IGF-I and PDBU stimulation of thymidine incorporation. Dibutyryl cAMP increased basal and IGF-I-stimulated sulphate incorporation but inhibited both basal and IGF-I stimulation of thymidine incorporation. These results suggest a harmonic overlap between the activities of PKC and cAMP-dependent PKA enzyme systems, and calcium balance in the mitogenic and metabolic process of the chondrocyte.

Insulin-like growth factor-I; Protein kinase C; Ca^{2+}; cyclic AMP; Chondrocyte

1. INTRODUCTION

Insulin-like growth factor (IGF-I) is one of a family of growth hormone-dependent peptides which are mitogenic for a variety of tissues. They stimulate the uptake of sulphate into chondroitin sulphate of cartilage proteoglycans and thymidine into chondrocyte DNA [1].

Phorbol esters are mitogenic for a variety of tissues activating directly the enzyme protein kinase C (PKC) [2,3]. This is a phospholipid- and calcium-dependent enzyme normally activated by diacylglycerol produced from the breakdown of inositol phospholipids in cell membranes in response to external stimuli including receptor activation. Phorbol esters mimic the action of diacylglycerol thus activating PKC [4].

The nature of the intracellular factor(s) which may act as secondary messengers for IGF action on chondrocytes is uncertain. This preliminary study addresses the possible involvement of PKC, calcium and cyclic AMP (cAMP) in IGF-I stimulation of chondrocyte metabolism by observing the effects of a PKC activator (phorbol ester 12,13-dibutyrate, PDBU), a PKC inhibitor [1-(5-isoquinolinylsulphonyl)-2-methylpiperazine, H7), a calcium antagonist (verapamil) and a cAMP analogue (N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid) on basal and IGF-I-stimulated [^{35}S]sulphate and [^{3}H]thymidine uptake by monolayer cultures of porcine articular chondrocytes.

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2. MATERIALS AND METHODS

Porcine weanling knee joints were obtained from the Animal Virus Research Institute at Pirbright (Surrey, England). Collagenase (clostridial type II, Worthington) was obtained from Lorne Diagnostics (Bury St. Edmunds, England). Hyaluronidase, phorbol ester, compound H7, verapamil and dibutyryl cAMP were obtained from Sigma (Poole, England).

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Ham's F12 medium, penicillin/streptomycin and fungizone were obtained from Flow Labs (Irvine, Scotland). $Na_2^{35}SO_4$ and [^{3}H]thymidine were obtained from Amersham (Bucks, England).

IGF-I was prepared as reported in [5]. Due to paucity of pure IGF-I, partially purified IGF-I from an early stage of purification with an immunoactivity of 37 U/mg protein was used.

2.1. *Isolation of articular chondrocytes and cell culture*

The knee joints were opened under aseptic conditions shortly after slaughter; cartilage from the articular facets was removed and collected into a sterile solution of ice-cold phospho-saline buffer (PBS), supplemented with penicillin/streptomycin (100 U/100 μg per ml) and fungizone (2 μg/ml), and transported back to the laboratory.

The cartilage slices were chopped into fine pieces, washed extensively in PBS and digested overnight in an air incubator, on a magnetic stirrer, in Ham's F12 medium containing a 1:1 mixture of collagenase and hyaluronidase (1 mg/ml), with the addition of 5% fetal calf serum. The digestion volume per batch of cartilage was 50 ml. The final digest was filtered through sterile nylon gauze in order to remove any undigested material and the cells were centrifuged at 1500 rpm for 5 min. The cellular pellet was washed and centrifuged ($\times$ 3) in serum-free medium and resuspended in Ham's F12 medium supplemented with 10% fetal calf serum. Cells were seeded out at high density into 250-ml tissue culture flasks in order to maintain a chondrocytic phenotype, and grown to confluency.

For these experiments, sufficient cell numbers were usually obtained after three passages; cells were then seeded out at high density into 50-mm culture dishes, grown to confluency before being used for experiments (see fig.1). The mean cell number per culture dish at confluency was $(4.1 \pm 0.38) \times 10^5$ (SD), the coefficient of variation in cell number per dish being 9.2% ($n = 10$) by Coulter counter. Cell viability was determined by trypan blue dye exclusion and was consistently greater than 90%. Only cells isolated from the articular cartilage of one animal were used for each set of experiments, minimising intra-assay variation.

2.2. *Sulphate and thymidine incorporation*

Cell cultures were growth-arrested for 24 h in serum-free medium (Ham's). Duplicate cultures were then incubated for a further 24 h (sulphate incorporation) and 48 h (thymidine incorporation) in Ham's F12 medium in the presence of IGF-I and/or PDBU alone at various concentrations and with the addition of H7, verapamil and/or dibutyryl cAMP at various concentrations. [^{35}S]Sulphate and [^{3}H]thymidine were used at 2 μCi/ml.

At the end of the incubation the culture medium was aspirated and the cell monolayers were washed extensively with cold PBS until background levels of radioactivity were observed in the washes. The cell monolayers were then dissolved in 2 ml of 2 M KOH solution, 250 μl aliquots diluted 1:4 with distilled water and after addition of 4 ml toluene-based scintillant counted for radioactivity in a liquid scintillation β-counter.

Four aliquots from each of duplicate cultures represented each concentration of test substance. All results are expressed as a percentage of basal radionuclide incorporation by chondrocytes. Values of basal cpm (100%) $\pm$ SE of each group of 8 observations are stated for each figure. The standard error of the mean of each group of observations was always $\leq 8\%$.

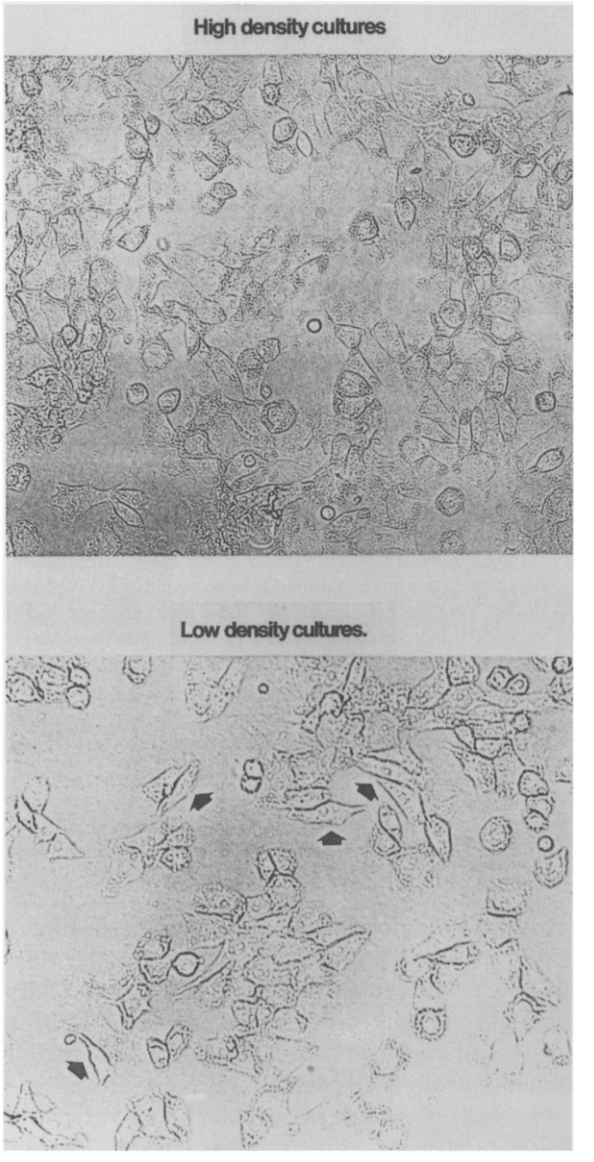

Fig.1. Articular chondrocyte monolayer cultures: comparison of high- and low-density seeding. Note the appearance of cells with a fibroblastic-like phenotype at low-density seeding, as indicated by the arrows.

.3 RESULTS

Chondrocyte uptake of both [^{35}S]sulphate and [^{3}H]thymidine was stimulated by IGF-I and PDBU (figs 2–5). Basal, IGF-I- and PDBU-stimulated [^{35}S]sulphate and [^{3}H]thymidine uptake by chondrocytes was inhibited by compound H7 (figs 2,3).

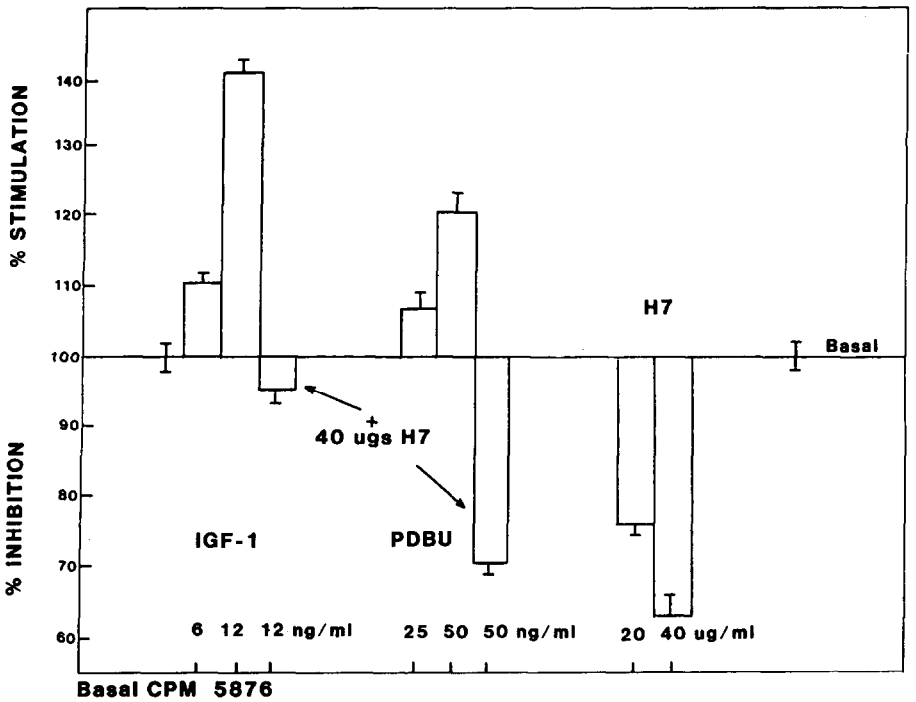

Fig.2. Effect of a protein kinase C inhibitor (H7) on basal, IGF-I- and phorbol ester (12,13-dibutyrate)-stimulated [^{35}S]sulphate uptake by chondrocytes.

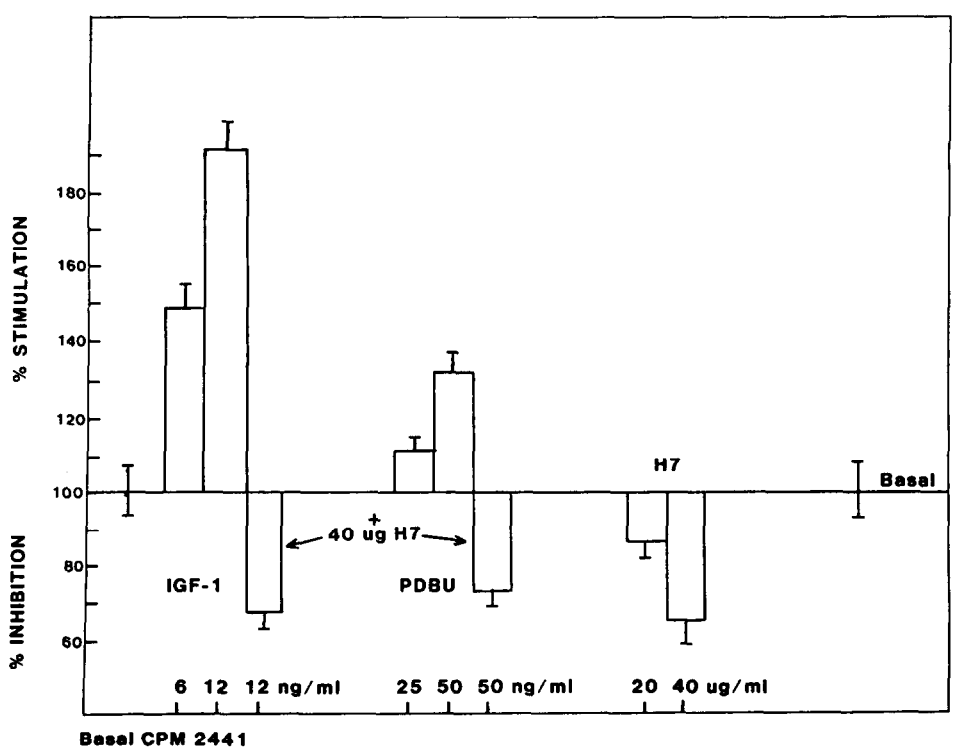

Fig.3. Effect of a protein kinase C inhibitor (H7) on basal, IGF-I- and phorbol ester (12,13-dibutyrate)-stimulated [^{3}H]thymidine uptake by chondrocytes.

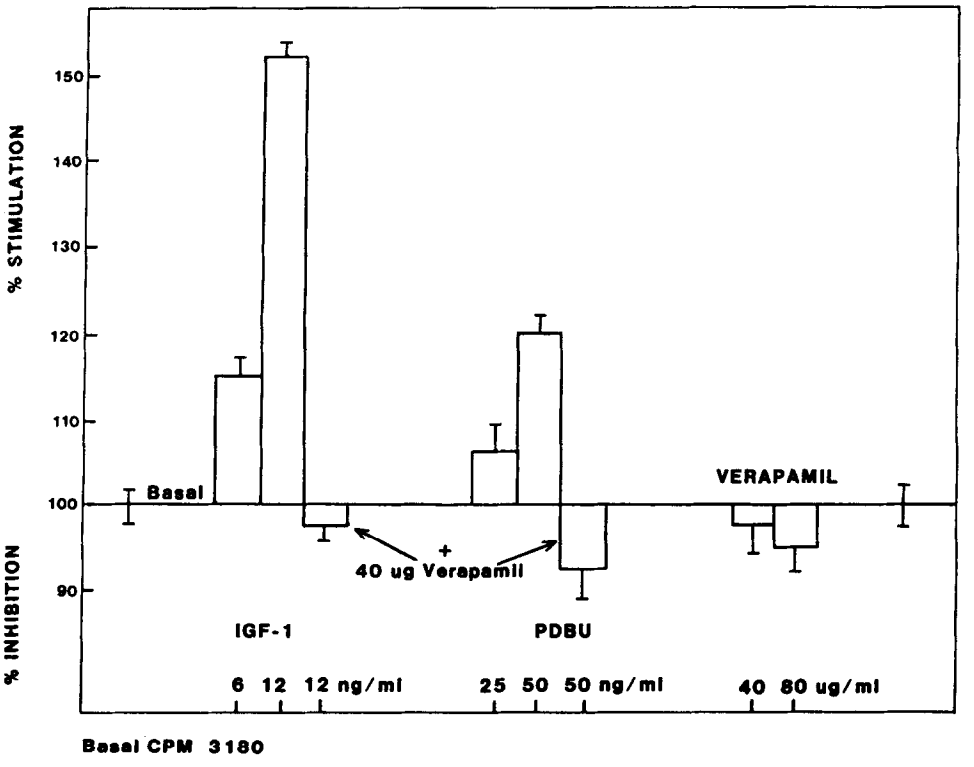

Fig.4. Effect of a calcium channel antagonist (verapamil) on basal, IGF-I- and phorbol ester (12,13-dibutyrate)-stimulated [^{35}S]sulphate uptake by chondrocytes.

Verapamil inhibited both IGF-I- and PDBU-stimulated [^{35}S]sulphate uptake, but had little effect upon basal sulphate incorporation by chondrocytes, suggesting that extracellular calcium influx may be involved in the metabolic effects of IGF-I and PDBU on chondrocytes (see fig.4). In contrast, verapamil caused an increase in basal and a marked enhancement of both IGF-I- and PDBU-stimulated [^{3}H]thymidine uptake by chondrocytes (see fig.5), suggesting that the mitogenic effects of IGF-I and PDBU may involve PKC activity independent of extracellular calcium influx (see fig.5). Dibutyryl cAMP increased basal and IGF-I-stimulated [^{35}S]sulphate uptake by chondrocytes, but constrastingly inhibited basal and IGF-I stimulation of [^{3}H]thymidine uptake by chondrocytes (see fig.6).

4. DISCUSSION

Our data demonstrate that IGF-I and PDBU stimulated [^{35}S]sulphate and [^{3}H]thymidine uptake by porcine articular chondrocytes in vitro. The uptake of these labels is inhibited by H7, a potent inhibitor of PKC [6]. Since phorbol esters stimulate PKC directly and the action of IGF-I is inhibited by H7, it was concluded that PKC mediates both the metabolic and mitogenic actions of IGF-I. However, since H7 inhibits the cAMP-dependent PKA enzyme system, this would not exclude a possible role for PKA in mediating the mitogenic and metabolic effects of IGF-I.

However, PKA activity is associated with an increase in levels of intracellular cAMP and in this system the addition of the cAMP analogue dibutyryl cAMP inhibited [^{3}H]thymidine incorporation.

The inhibition of PKA activity by H7 would be associated with low levels of cAMP and since dibutyryl cAMP itself inhibits thymidine incorporation by chondrocytes the inhibition of the mitogenic effects of both IGF-I and PDBU by H7 is unlikely to involve PKA activity, but rather involves the inhibition of PKC activity or some other

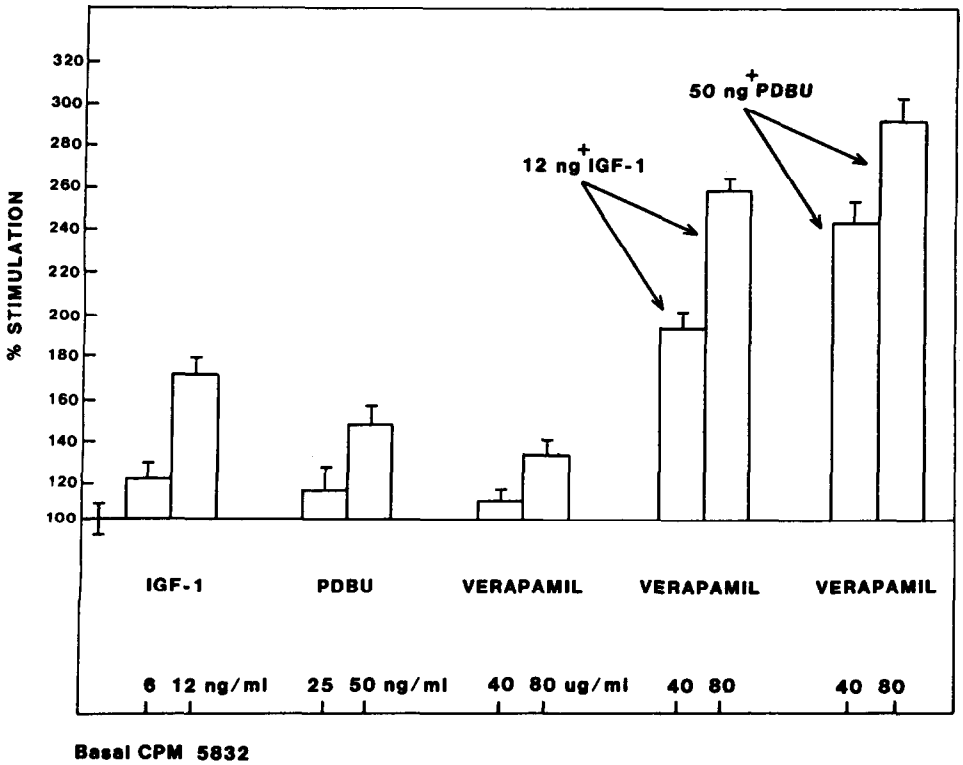

Fig.5. Effect of a calcium channel antagonist (verapamil) on basal, IGF-I- and phorbol ester (12,13-dibutyrate)-stimulated [^{3}H]-thymidine uptake by chondrocytes.

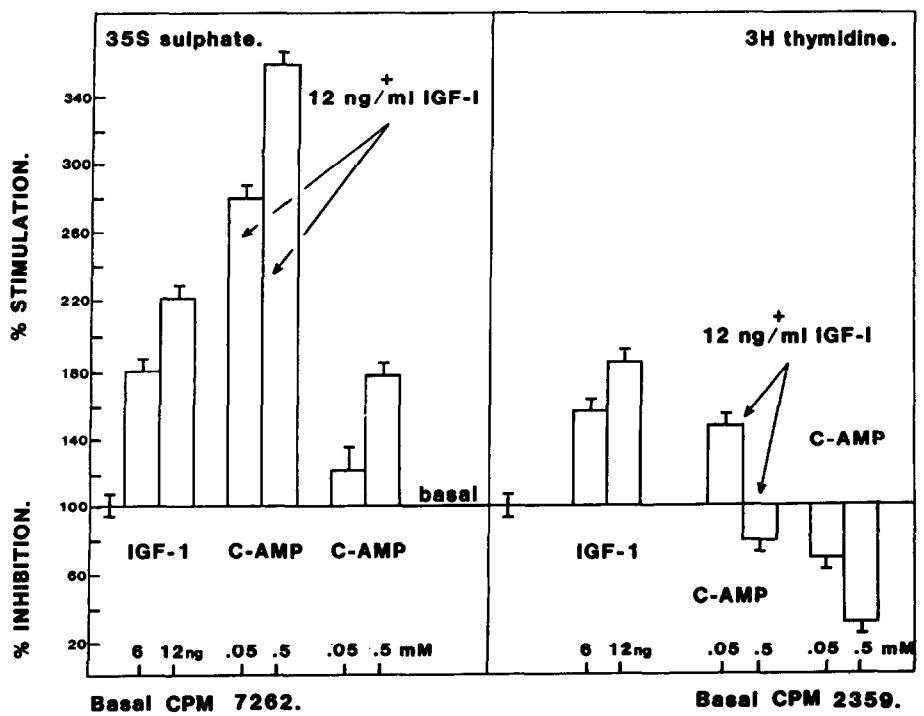

Fig.6. Effect of dibutyryl cAMP on basal and IGF-I-stimulated [^{35}S]sulphate and [^{3}H]thymidine uptake by chondrocytes.

as yet unknown enzyme system. Inhibition of H7 or IGF-I- and PDBU-stimulated [^{35}S]sulphate incorporation by chondrocytes is probably associated with inhibition of PKA activity, since dibutyryl cAMP increases basal and IGF-I stimulation of sulphate uptake by chondrocytes, suggesting that the metabolic effects of IGF-I may be mediated by the cAMP-dependent PKA system.

The effect of verapamil, a Ca^{2+} antagonist [7], on basal, IGF-I- and phorbol ester-stimulated [^{35}S]sulphate uptake by chondrocytes was also inhibitory; this is consistent with previous data on the role of intracellular calcium in mediating secretion and inducing stimulation in various cellular/tissue systems.

The most intriguing observation however, was that verapamil stimulated basal [^{3}H]thymidine uptake by chondrocytes and enhanced the stimulatory action of IGF-I and PDBU. Whereas verapamil has been shown to stimulate and calcium to inhibit secretory processes in some cell systems [8], verapamil has never been shown to have a divergent effect on the secretory and mitotic processes within the same cell. By implication, calcium plays different roles in regulating the secretory and mitotic processes in the chondrocyte. Thus, while calcium entry into the cell appears to be cardinal in mediating the metabolic effects of IGF-I and PDBU, calcium is inhibitory to mitogenesis. The mediation of mitogenesis due to IGF-I and PDBU may therefore be mediated by other postulated mechanisms, such as activation of the Na^+/H^+ pump; in our chondrocyte cell system, amiloride, an inhibitor of the Na^+/H^+ exchange pump inhibits IGF-I stimulation of [^{3}H]thymidine uptake by chondrocytes (unpublished).

The fact that a calcium channel blocker has differential effects on IGF-I-induced secretory and mitotic processes in the chondrocyte raises the possibility that IGF-I may mediate these effects through two distinct receptor and receptor-linked enzyme systems. However, the fact that verapamil has qualitatively similar effects on basal and phorbol ester-stimulated cells, implies that this differential effect of calcium blockade is mediated through intracellular enzyme-linked processes at a post-receptor level rather than through distinct receptors.

In conclusion, these preliminary results suggest that the PKC enzyme system may play a role in the mediation of the mitogenic effects of IGF-I and PDBU in the chondrocyte independently of extracellular calcium influx. The cAMP-dependent PKA enzyme system may play a counter-regulatory role in this process. Also, the metabolic effects of IGF-I may be mediated by PKA activity dependent upon extracellular calcium influx.

These results suggest a harmonic overlap between the activities of PKC and PKA enzyme systems in the mitogenic and metabolic processes of the chondrocyte. In addition, intracellular levels of cAMP and/or calcium balance may be important factors in determining the expression of either the metabolic or mitogenic message of IGF-I in the chondrocyte.

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