Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 20-25

INHIBITION OF BASIC CALCIUM PHOSPHATE CRYSTAL-INDUCED MITOGENESIS BY PHOSPHOCITRATE

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Received June 29, 1990

Basic calcium phosphate crystals control the traverse of cells from the Go/G1 to S-phase of the cell cycle and initiate proliferation by rendering fibroblasts competent to respond to insulin-like growth factors in plasma. Simultaneous addition of phosphocitrate [a powerful inhibitor of hydroxyapatite crystallization] to cells exposed to basic calcium phosphate crystals caused a dose-dependent inhibition of crystal-induced DNA synthesis and c-fos transcription. This inhibition was specific for crystal-induced mitogenesis, since similar concentrations of phosphocitrate had no effects on either PDGF or 10% calf serum-induced thymidine incorporation and c-fos transcription.

Synovial hyperplasia is a feature of chronic synovitis associated with basic calcium phosphate [BCP] crystals (hydroxyapatite, octacalcium phosphate, and tricalcium phosphate [1]). Each of these crystals stimulates mitosis of cultured human skin fibroblasts or canine synovial fibroblasts in a dose-dependent fashion [2,3]. Both the onset and the peak of thymidine incorporation after crystal addition to quiescent cells lag by 2 to 3 hours behind the effect of serum. Conditioned media from these cultures were not mitogenic nor were control particles such as sodium urate,or latex beads [2].

BCP crystals can substitute for platelet-derived growth factor [PDGF] as a competence growth factor in vitro [4]. Moreover,BCP crystals and PDGF exert similar biologic effects on cultured cells,such as stimulation of PGE_2 production via the phospholipase A_2/cyclo-oxygenase pathway [5],activation of phospholipase C and inositol phospholipid hydrolysis [6],and induction of collagenase and neutral protease synthesis [7,8]. We also demonstrated that the proto-oncogenes [c-fos and c-myc] are induced with similar kinetics by both BCP crystals and PDGF. ß-interferon delays but does not block the transcription of these proto-oncogenes or DNA synthesis induced by BCP crystals and PDGF [9].

0006-291X/90 $1.50

Phosphocitrate [PC], a powerful inhibitor of hydroxyapatite crystallization [10,11] has been shown to prevent crystal-induced membranolysis of polymorphonuclear leukocytes [12]. While some responses to PC can be attributed to the crystal surface binding phenomena,PC is also known to influence some cellular events e.g. both low density lipoprotein uptake by cultured aortic smooth muscle cells [13],and aortic monocyte adhesion in hypercholesterol ischemic rats [14] can be restricted by PC. The apparent membrane protection afforded by PC led to the hypothesis that some underlying cellular events may well be influenced. In the present study,we examined the effects of PC on BCP crystal-induced transcription of c-fos and mitogenesis.

Materials and Methods

Cell Culture: Stock cultures of Balb/c-3T3 cells were grown in Dulbecco Modified Eagle's medium [DMEM] supplemented with 10% (v/v) calf serum, penicillin at 50 units/ml,and streptomycin at 50 ug/ml in humidified 10% CO_2/90% air at 37^0C. For all experimental procedures,cells were grown to confluence in culture dishes containing 24 wells each 16 mm in diameter [Multiwell,Gibco] in medium containing serum and then were rendered quiescent by removing the medium, washing once with 1 ml of DMEM containing 2% human platelet-poor plasma [PP],and subsequently incubating in this medium for 24 h before the experiments.

DNA Synthesis Assay: Cells in multi-well culture plates were incubated with [^{3}H]-thymidine (1 uCi/ml) for 24 h and processed for [^{3}H]-thymidine incorporation according the method published earlier [2]. In earlier work,we demonstrated that BCP crystal [100 ug/ml] produced the maximum induction of DNA synthesis in Balb/c-3T3 cells [9,15]. Unless otherwise specified,BCP crystals were used in this concentration throughout all experiments.

RNA Blot Analysis: RNA was prepared by scraping cells from 100 mm plates, washing in physiological buffered saline followed by disruption of cells in guanidinium isothiocyanate. The RNA was then precipitated in 4 M lithium chloride as described by Cathala et al [16]. RNA (5 ug/lane) was electrophoresed on formaldehyde gels and transferred to nitrocellulose filters as described previously [17]. The filters were hybridized with inserts purified from plasmid carrying sequences of the desired gene. The inserts were labeled to a specific activity greater than 10^8 cpm/ug using the random primer method [18]. The fos insert used as a probe in this work was a 1.0 Kb PstI v-fos fragment from the pfos-1 plasmid [19].

Preparation of BCP Crystals and PC : BCP crystals were prepared as described [4]. They were crushed and sieved to yield 10-20 um aggregates, sterilized by heating at 200^0C for 90 minutes,weighed,and suspended in DMEM. The suspensions were sonicated before use. PC was synthesized according to a procedure published earlier [20].

All analysis of statistical significance was performed by applying Student's T test. All experiments were run in quadruplicate, and repeated at least twice.

Highly purified PDGF was prepared as described [21]. [^{3}H]-thymidine and [α-^{32}P]-dCTP were from Amersham Corporation [Arlington Heights,IL]. Probes were randomly primed with kits from Boehringer Mannheim [Indianapolis,IN]. All cell culture supplies are products of Gibco Laboratory [Grand Island,N.Y.].

Results

To define the conditions under which PC may affect the proliferation of 3T3 cells,we first examined the effect of various concentrations of PC on the BCP crystal-induced [^{3}H]thymidine incorporation at 24 h. PC at a concentration 10^{-2}M was toxic to the cells. Up to 50% of cells detached from the culture dish. Otherwise,exposure of quiescent Balb/c-3T3 cells to medium containing concentrations [10^{-3}M to 10^{-5}M] of PC,and BCP crystals [100ug/ml] and 2% PP caused a dose-dependent inhibition of BCP crystal-induced DNA synthesis in cells harvested after 24 h of incubation. Similar concentrations of PC did not have any effect on the basal thymidine incorporation of the control cells incubated in 2% PP. This inhibition was specific for BCP crystal-induced mitogenesis,since similar concentrations of PC had no effects on either PDGF- or 10% calf serum-induced thymidine incorporation [Fig.1]. PC at a concentration of 10^{-3}M was used to inhibit the BCP crystal-induced mitogenic effect in all subsequent experiments.

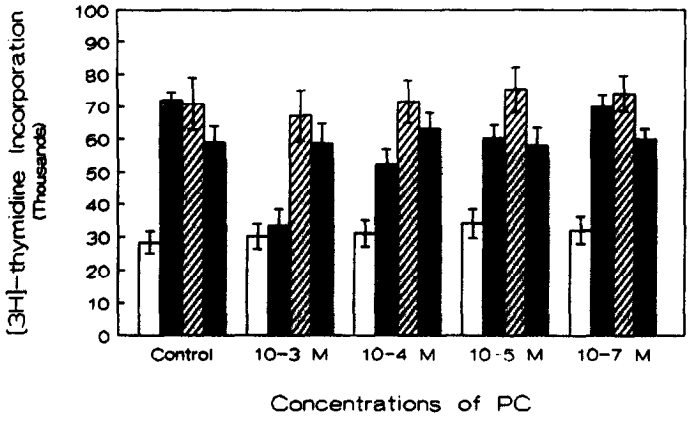

Fig.1. Relationship between PC concentrations and inhibition of BCP crystal-induced [^{3}H]-thymidine incorporation into DNA of Balb/c-3T3 cells. Cells were grown and maintained in 24-multiwell culture plate until confluence. They were then fed with DMEM and 2% PP containing one of the following : BCP crystals [100ug/ml], PDGF [50ng/ml], control [no crystal] and calf serum [10%] in the presence or absence of various concentrations of PC. ^{3}H-thymidine incorporation was estimated 24 hours after stimulation as described in the Material and Method. Values are expressed as the means ± SD, [n=4]. [^{3}H]-thymidine incorporation is expressed in cpm. ☐ 2%PP; ▨ BCP crystals; ▨ 10%CS; ■ PDGF.

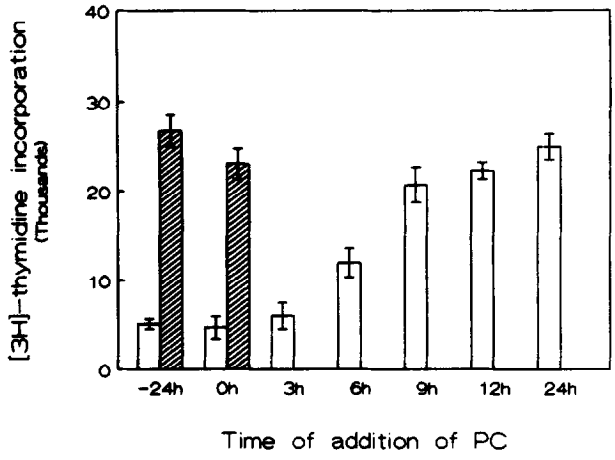

Fig.2. [^{3}H]-thymidine incorporation into DNA of Balb/c-3T3 cells as a function of delayed addition of PC [10^{-3} M] after stimulation with BCP crystals [100 ug/ml]. Values are expressed as the means ± SD. [n=4] ▨ PC was washed off after either 24 hr pre-incubation with cells alone or after 30 min. co-incubation with BCP crystals and cells at Time 0 hr.

The suppression of S-phase entry, as a function of the time of PC addition, was measured to determine the time in the cell cycle at which the PC-mediated block occurred. PC could be added as late as 3 h after the cells were stimulated with BCP crystals, thereafter the ability of PC to suppress entry into S-phase significantly [$P<0.01$] decreased [Fig.2].

The effects of PC on the induction of c-fos expression by BCP crystals and PDGF are summarized in Fig 3 a & b respectively. Stimulation of density-arrested Balb/c-3T3 cells with either BCP crystals or PDGF resulted in maximal accumulation of c-fos mRNA 30 min after stimulation. In the presence of PC, the level of BCP crystal-induced c-fos mRNA at 30 min was non-detectable. However, if cells were treated with PC for 1/2 hour, then the PC was washed off, BCP crystals were again able to stimulate c-fos transcription in 30 min. PC had no effect on the level of PDGF-induced c-fos, thus supporting the notion that PC specifically inhibits BCP crystal-induced cellular events, and its inhibitory effect can be reversed by washing.

Discussion

Stimulation of [^{3}H]-thymidine incorporation into mouse 3T3 cells by precipitates of calcium phosphate and pyrophosphate had been reported by a number of investigators [22,23,24]. Mitogenic properties can also be demonstrated for other calcium-containing crystals (see reviews [25]).

Our present study shows that PC inhibited the mitogenic effect of BCP crystals in a dose related fashion. This inhibitory effect appeared to be specific since the same concentrations of PC had no effect on the basal, PGDF- or serum-induced [^{3}H]-thymidine incorporation [Fig.1]. It is necessary to add

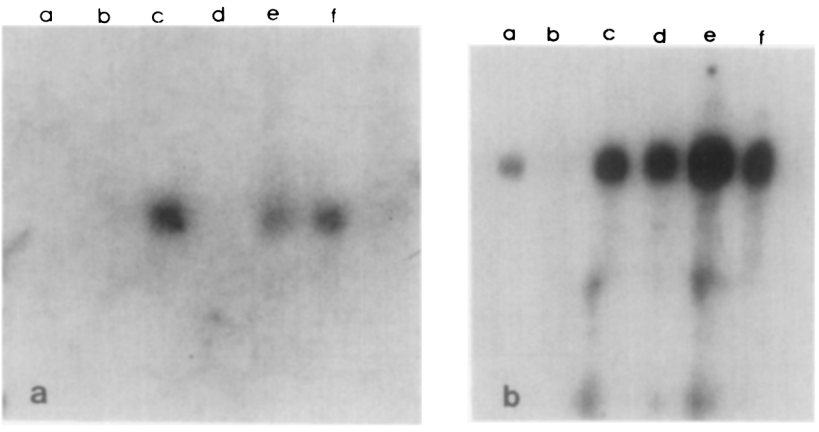

Fig.3a. Northern blot of c-fos mRNA of control and PC treated cells after stimulation with: (a) control; (b) control + PC [10^{-3}M]; (c) BCP crystals [100 ug/ml]: (d) BCP crystals [100ug/ml] + PC [10^{-3}M] (e) BCP crystals [100ug/ml] + PC [10^{-3}M]. PC was removed after 30 min. incubation and cells were reincubated for 30 min. (f) PDGF [50 ng/ml].

Fig.3b. Northern blot of c-fos mRNA of cells after stimulation with: (a) BCP crystals [100ug/ml]; (b) BCP crystals [100ug/ml] + PC [10^{-3}M]; (c) PDGF [50 ng/ml]; (d) PDGF [50 ng/ml) + PC [10^{-3}M]; (e) 10% calf serum; (f) 10% calf serum + PC [10^{-3}M].

PC within 3 h after stimulation with BCP crystals in order to have the maximum inhibitory effect, thereafter the ability of PC to suppress entry into S-phase significantly [$P<0.01$] decreased [Fig.2]. This suggests that PC interfered with one or more of the specific crystal-induced processes in early G_1 phase that are essential for entry into S-phase.

The induction of c-fos transcription is one of the earliest responses to serum or growth factors in cultured quiescent cells [26,27,28], and there is increasing evidence that the Fos protein, in combination with the protein products of the c-jun gene, can serve as a trans-acting activator of the expression of other genes [29,30]. We showed previously that stimulation of density-arrested Balb/c-3T3 cells with either BCP crystals or PDGF resulted in a transient increase in the level of c-fos with maximal c-fos mRNA levels 30 minutes after stimulation, followed by a rapid decline to the basal level in an hour [9,15].

Our present report confirms that stimulation of density-arrested Balb/c-3T3 cells with either BCP crystals or PDGF results in maximal accumulation of c-fos mRNA at 30 min. PC specifically blocks the BCP crystal-induced transcription of c-fos [Fig.3a & b]. PC had no effect on the level of PDGF- or 10% calf serum-induced c-fos, thus strengthening the notion that PC specifically inhibits an early BCP crystal-induced cellular event that may play an important role in the control of cell proliferation [26,27,28].

The inhibitory effect of PC is reversible. Even after preincubating cells with PC for 24 h or co-incubating cells with PC and BCP crystals for 30

min, the abilities of BCP crystal to induce c-fos transcription [Fig.3] and DNA synthesis were restored after PC was removed by washing [Data not shown].

To the best of our knowledge,we have demonstrated for the first time,that PC specifically blocks the BCP crystal-induction of DNA synthesis,and c-fos expression. Although the exact mechanism of its antagonistic actions is still unclear,our results suggest that PC blocks at least one of the specific BCP crystal-induced obligatory cellular events that are required for the progression phase of G_1 and S-phase entry.

Acknowledgments

This work was supported in part by USPHS grants AR38421 (HSC), and Arthritis Foundation Research Center Grant. We gratefully acknowledge the helpful comments of Drs. D.J. McCarty and L.M. Ryan.

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