Cellular Signalling 24 (2012) 1504–1514

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Protein kinase A-mediated phosphorylation of RhoA on serine 188 triggers the rapid induction of a neuroendocrine-like phenotype in prostate cancer epithelial cells

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ARTICLE INFO

Article history:
Received 21 November 2011
Received in revised form 12 March 2012
Accepted 22 March 2012
Available online 31 March 2012

Keywords:
Cyclic AMP
RhoA
Prostate cancer
Epithelial cells

ABSTRACT

Whilst androgen ablation therapy is used to treat locally advanced or metastatic forms of prostate cancer, side-effects can include the emergence of an androgen-independent neuroendocrine cell population which is associated with poor prognosis. Here we have examined how cyclic AMP elevation regulates early events in the neuroendocrine differentiation process. We demonstrate that selective activation of protein kinase A is necessary and sufficient for cyclic AMP (cAMP) elevation to rapidly promote a neuroendocrine phenotype in LNCaP cells independent of de novo protein synthesis. Furthermore, the effects of cAMP could be recapitulated by inhibition of RhoA signalling or pharmacological inhibition of Rho kinase. Conversely, expression of constitutively active Gln63Leu-mutated RhoA acted as a dominant-negative inhibitor of cAMP-mediated NE phenotype formation. Consistent with these observations, cAMP elevation triggered the PKA-dependent phosphorylation of RhoA on serine 188, and a non-phosphorylatable Ser188Ala RhoA mutant functioned as a dominant-negative inhibitor of cAMP-mediated neuroendocrine phenotype formation. These results suggest that PKA-mediated inhibition of RhoA via its phosphorylation on serine 188 and the subsequent inhibition of ROCK activity plays a key role in determining initial changes in cellular morphology during LNCaP cell differentiation to a neuroendocrine phenotype. It also raises the possibility that targeted suppression of this pathway to inhibit neuroendocrine cell expansion might be a useful adjuvant to conventional prostate cancer therapy.

1. Introduction

Prostate cancer (PCa[I]) is the most frequently diagnosed male-specific malignancy and causes the greatest number of male cancer-related deaths after lung cancer [1]. Prostate cell growth is reliant on the presence of androgens such as testosterone with traditional therapeutic strategies focussing on androgen ablation therapy [1]. However, such therapies have unwanted side effects including mood alterations, sexual dysfunction, skeletal complications and increased risk of diabetes and cardiovascular events [2,3], and selection for the emergence of androgen-independent cells such as neuroendocrine (NE) cells [1]. NE cells play a key role in governing differentiation and proliferation of the developing prostate gland and, whilst their role in the mature gland is less well defined, it is thought that they play important regulatory roles in secretion [4].

Clinically, PCa incidence and progression are assessed via monitoring serum prostate specific antigen (PSA) levels. However PSA is limited as a biomarker due to issues with false-positive results and lack of discrimination between aggressive and non-aggressive diseases [5]. Thus other markers are being assessed for use in the diagnosis and prognosis of PCa. Recently, the presence of NE cell products as a prognostic marker in PCa has come under investigation [6]. The presence of NE cells has been described in 47 to 100% of prostatic adenocarcinomas [7]. Expansion of the NE cell population has been described in late-stage PCa and has been suggested to be indicative of poor patient prognosis [6]. NE cells form foci of non-dividing cells surrounded by regions of proliferating tumour cells due to the release of mitogenic compounds such as bombesin [8] and chromogranin A [9]. Due to their resistance to many conventional chemotherapeutics, it is possible that inhibition of NE cell differentiation may be a useful avenue by which to complement existing therapies and thus prevent expansion of this cell type in late-stage PCa.

The LNCaP cell line was isolated from a lymph node metastasis of PCa [10] and is a well-established model of NE differentiation in PCa. LNCaP cells undergo differentiation to a NE phenotype under a number of conditions including androgen deprivation [11], chronic exposure to IL-6 [12], constitutive activation of gp130 [13] and elevation of intracellular cyclic AMP (cAMP) levels [7,12,14]. Initially,

Abbreviations: PCa, prostate cancer; cAMP, cyclic AMP; NE, neuroendocrine; PSA, prostate-specific antigen; PKA, cAMP-dependent protein kinase; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; 6-Bnz-cAMP, N^6-benzoyl-cAMP; Fsk, forskolin; C3T, cell-permeable *C. botulinum* C3 transferase; CREB, cAMP response element binding protein; ERK, extracellular signal-regulated kinase; RACK1, receptor of activated C kinase 1; EPAC, exchange protein directly activated by cAMP; ROCK, Rho-associated protein kinase.

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doi:10.1016/j.cellsig.2012.03.018

the differentiation of these cells to a NE phenotype is reversible [15] but chronic exposure to cAMP-elevating agents ultimately results in the terminal differentiation of LNCaP cells [7]. Inhibition of the initial stages of NE differentiation in prostate epithelial cells may therefore be a useful adjuvant to conventional chemotherapeutic strategies. Of particular interest is the role of cAMP-mediated signalling in this phenomenon and elucidation of the signalling mechanisms involved in initial NE differentiation.

Classically, cAMP was thought to exert its intracellular effects exclusively via activation of cAMP-dependent protein kinase (PKA). Binding of cAMP to the PKA holoenzyme promotes dissociation of the regulatory and catalytic subunits [16,17] and allows the catalytic subunits of PKA to phosphorylate downstream targets, such as the cAMP responsive element binding protein (CREB) [18–20]. Phosphorylation of transcription factors such as CREB is one mechanism by which elevation of intracellular cAMP concentrations can alter gene transcription. However it is now appreciated that in addition to PKA, cAMP can activate the exchange proteins directly activated by cAMP (EPACs) to promote intracellular effects [21–23]. It has been previously demonstrated that over-expression of constitutively active PKA catalytic subunits in LNCaP cells can promote NE differentiation in the absence of other stimuli [14], suggesting that PKA activation is sufficient to recapitulate this phenomenon following cAMP elevation. Furthermore, PKA-differentiated LNCaP cells can promote the anchorage-dependent and -independent growth of prostate cells [24], indicating that the kinase may play a crucial role in PCa progression. The ability of cAMP elevation to promote a neuronal cell phenotype is not restricted to LNCaP cells. In the PC12 pheochromocytoma cell line, elevation of cAMP can promote neurite outgrowth [25], Unlike LNCaP cells in which differentiation can be induced through constitutive PKA activity, PC12 cells require signalling through EPAC for neuronal differentiation [26]. Clearly different mechanisms are used in the two cell types to promote activation of similar downstream effectors. Of particular interest are the roles of the Rho GTPases which control cell morphology and motility via regulation of the actin cytoskeleton. Of particular interest is the role of RhoA, as this GTPase has been described as a master regulator of dendrite morphogenesis [27]. Neurite outgrowth from PC12 cells in response to basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) arises due to p190RhoGAP and ARAP3-mediated inhibition of RhoA [28,29]. Furthermore, activation of RhoA has been shown to inhibit NGF-induced neuronal extension and to promote neurite retraction in PC12 cells. In this study, NGF was shown to inhibit RhoA activity via PKA-mediated phosphorylation on Ser^{188}, a process which is required for NGF-induced neurite extension in PC12 cells [30].

Here, we demonstrate that selective activation of PKA entirely mimics the effect of cAMP elevation during initial changes in LNCaP morphology via a process independent of de novo protein synthesis. Furthermore, the effects of cAMP elevation on LNCaP cell morphology

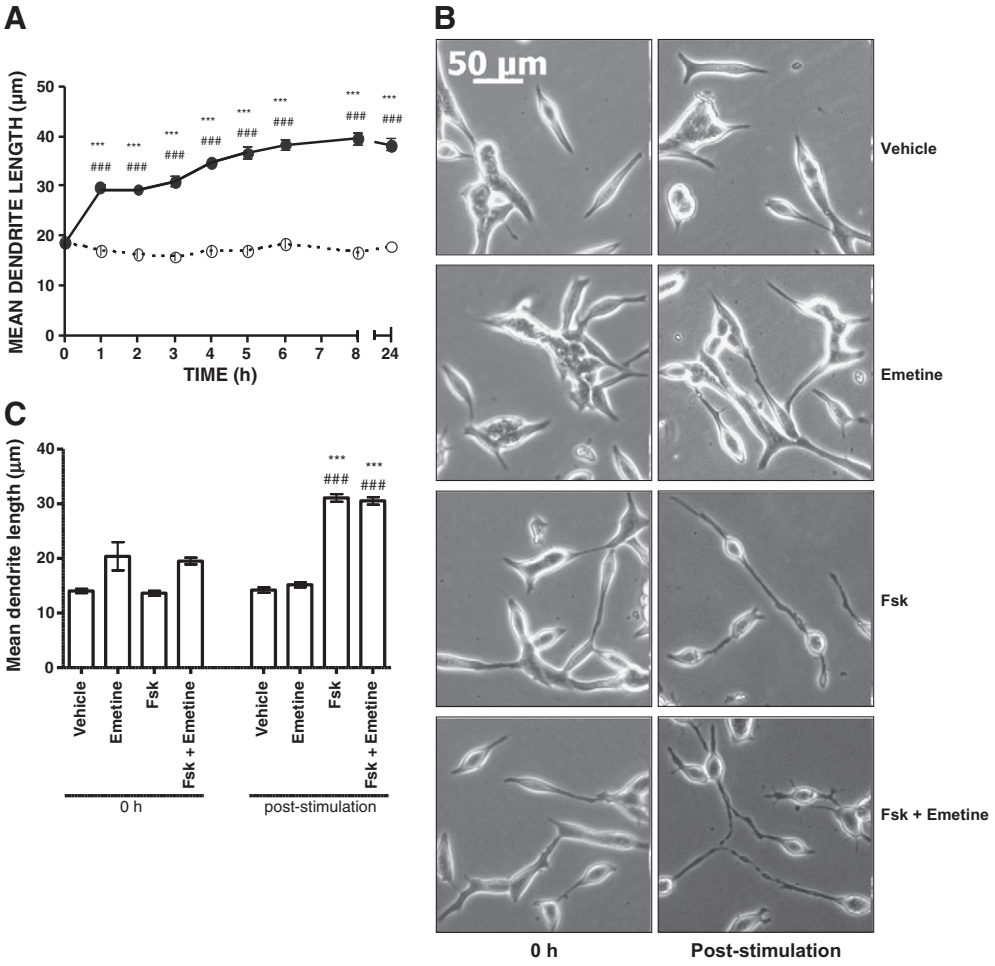

Fig. 1. The ability of Fsk to induce rapid changes in LNCaP cell morphology is independent of de novo protein synthesis. Panel A: LNCaP cells were pre-incubated for the indicated times in the presence of either vehicle (0.1% (v/v) EtOH) (open circles) or 100 μM Fsk (closed circles). Mean dendrite outgrowth was determined and results are presented as mean values ± SEM for $n = 3$ experiments. ***$p < 0.001$ vs. 0 h, ###$p < 0.001$ vs. vehicle at same time point. Panel B: LNCaP cells were pre-incubated for 2 h in the presence of either vehicle (0.1% (v/v) DMSO) or 100 μM protein synthesis inhibitor emetine prior to stimulation with vehicle (0.1% (v/v) EtOH) or 10 μM Fsk for a further 1 h. Phase contrast images were captured immediately prior to the experiment (0 h) and following the 1 h stimulation with Fsk (post-stimulation). Panel C: Mean dendrite outgrowth was assessed at 1 h post-stimulation as described previously. Results are presented as mean values ± SEM for $n = 3$ experiments. ***$p < 0.001$ vs. 0 h, ###$p < 0.001$ vs. vehicle at same time point.