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Retinoid induced changes in cAMP-dependent protein kinase activity detected by a new minigel assay

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A new method for the resolution of protein kinases in nondenaturing minigels was used to analyze the type of PKA induced by retinyl acetate in transformed mouse 10T1/2 cells. Protein kinases in 0.5 μl aliquots of cell extracts were resolved on nondenaturing gradient Phastgels and assayed for PKA activity in situ using a specific peptide substrate and [^{32}P]ATP. Retinyl acetate caused a decrease in type I and an increase in type II PKA isoforms after about 36 h in culture. The system provides a quick and sensitive means for analyzing the activity of PKA isoforms from small numbers of cells.

Retinoid; Protein kinase, cAMP-dependent; Phastgel

1. INTRODUCTION

Recent observations from a number of laboratories suggest that retinoids act, at least in part, by modulating protein phosphorylation/dephosphorylation reactions in cells, possibly through their actions on the activities of protein kinases, specifically cAMP-dependent protein kinase (PKA) [1-5]. Recent experiments from this laboratory have shown that retinyl acetate can increase the PKA activity of transformed mouse 10T1/2 cells (MCA cells) about 3-fold after about 36-48 h in culture [6]. PKA, however, is comprised of two major isoforms, type I and type II, which, in turn, have further subforms which may differ in the structure either of their regulatory [7] or catalytic [8] subunits. It has also been noted that the type I and type II isoform classes have disparate functions in cells. In general, type II isoforms predominate in slower growing, differentiated cells while type I isoforms are more typically associated with proliferating cells [9]. In our previous studies, we did not fractionate the PKA isoforms. In the present work, we introduce a new method for the electrophoretic fractionation of PKA isoforms in nondenaturing minigels. This method is capable of resolving several PKA fractions, and it clearly reveals that the addition of retinyl acetate to growing cultures of 10T1/2 cells causes a drop in the relative activity of type I and an increase in the activity of type II.

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2. MATERIALS AND METHODS

2.1. *Growth of cells*

MCA cells were grown as monolayers in 75 cm^2 flasks using BME (Basal Medium, Eagle) supplemented with 5% heat-inactivated fetal bovine serum (Gibco) as previously described [6]. The standard protocol consisted of subculturing confluent monolayers at a 1:9 dilution. After 24 h, test cultures were given medium containing 0.5 μg/ml retinyl acetate (RAC), while control cultures received unsupplemented BME. Cells were harvested and protein kinase activities were isolated after an additional 18, 36 and 48 h. The content of DNA and the rate of DNA synthesis were measured as described previously [6].

2.2. *Isolation of crude protein kinase activities*

Cells were detached with 1 mg/ml EDTA in 0.1 M potassium phosphate, pH 7.5, and homogenized in about 1 ml of 20 mM Tris, pH 7.2, containing 1 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF and 0.01% Triton X-100. Nuclei were removed by centrifugation. The crude nuclear fraction was then extracted with 20 mM Tris, pH 7.2, containing 1 mM DTT and 0.4 M NaCl. The crude cytoplasmic and nuclear extracts were combined (about 2 ml) and dialyzed against 10 mM Tris, pH 7.2, containing 5 mM $MgCl_2$, 2 mM EGTA, 5 mM β-glycerophosphate, and 1 mM DTT. The dialyzed extracts were centrifuged briefly to remove any insoluble material. The dialyzed extracts could be assayed in this state, or frozen and thawed about 3 times. We have also lyophilized the extract (after dialysis against water) and taken the dry powder up in a small volume of water to concentrate the activities. Again, this is stable to only about 2 additional freeze/thaw cycles.

2.3. *Nondenaturing minigel electrophoresis*

The Pharmacia 'Phast' system was used with 8-25% precast acrylamide gradient gels and native buffer wicks. Up to 8 samples of 0.5 μl each were run per gel, and the gels were electrophoresed for 270 Vh (about 1 h) at 15°C. After electrophoresis, the gels were soaked in ca 5 ml of substrate (the 'A' peptide, described below, dissolved in water at a concentration of 3 mg/ml) in a small, heat-sealable (Scot-

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chpak) plastic bag for 30 min in the cold. A corner of the bag was then cut and the substrate was removed by pipet (it can be reused.) A protein kinase assay buffer (5 ml) composed of 10 mM Tris, pH 7.2, 5 mM $MgCl_2$, 1 mM DTT and containing 5×10^{-6} M cAMP was then added to the bag. The reaction was initiated by adding 50 μl of 5 mM ATP containing 25 μCi of [^{32}P]ATP to the bag, yielding a final ATP concentration of 50 μM. The bag was then resealed and incubated at 37°C for 30 min. The bag was then reopened, the radioactive reaction mixture was discarded, the gel fixed in 5% TCA containing 0.01 M silicotungstic acid for 1 h and then washed extensively (overnight) in several changes of 5% TCA to remove unreacted [^{32}P]ATP. The bands were visualized by autoradiography using Kodak X-ray film. Exposure times varied from 1 to 7 days.

2.4. *Peptide substrate for PKA*

As a substrate for phosphorylation by PKA in minigels, the 'A' peptide is ca 5 times more reactive than histone IIAS (Sigma) on a weight basis. The A peptide was synthesized using a Biosearch 9500 automated solid phase peptide synthesizer. Approximately 750 mg of peptide was synthesized using 1 g of 4-methylbenzhydralamine resin and t-BOC amino acids. The A peptide corresponds to the sequence surrounding the PKA-sensitive phosphorylation site of calf thymus H1 histone:

Ala-Arg-Arg-Lys-Ala-Ser[P]-Gly-Pro-Pro-Val-Ser-Glu-Leu-Ile-Thr-Lys

2.5. *Differentiation of PKA from other protein kinases*

Preincubating the kinase extract with 10^{-5} M cAMP for 30 min prior to electrophoresis in the gel causes the dissociation of the regulatory and catalytic subunits of PKA, and, under the conditions of the electrophoresis, the catalytic subunit does not enter the gel [10,11].

3. RESULTS AND DISCUSSION

As shown in fig.1A, the type I and II PKA isoforms obtained from rabbit muscle and bovine heart (Sigma) can be readily separated using 8–25% gradient gels. Preincubating the PKA with cAMP prior to electrophoresis (panel A, lanes 2–5) causes the dissociation of the catalytic and regulatory subunits, and, under the conditions of the electrophoresis, the catalytic subunit does not enter the gel. These commercial PKA preparations can readily be seen to be mixtures of the two isoform classes. The bovine heart preparation is predominantly type I. In fig.1B, we show a serial dilution of PKA from bovine heart (Sigma). We estimate that we can readily detect 25 ng of enzyme in a 0.5 μl load using the assay conditions described. In the following studies we have therefore used 25 ng of bovine heart PKA (Sigma) as a mobility reference marker in our gels.

Table 1

Effect of RAC on the growth of MCA cells

Time (h)	DNA/75 cm^2 flask (μg) (average of 3 expts)		Specific activity of DNA (cpm/μg) (average of 3 expts)	
	−RAC	+RAC	−RAC	+RAC
18	41.5	45.3	3491	2295
36	74	61.5	2074	1422
48	104	57.4	1381	820

MCA cells were cultured at a 9-fold dilution, and allowed to establish in BME for 24 h. At this time (0 h) the medium in the experimental flasks was changed to BME containing 0.5 μg/ml RAC. The DNA content, and specific activity, was determined at 18, 36 and 48 h after time 0 [6]

Based on our previous observations, it takes at least 24 h for the retinoid-dependent increase in PKA activity to become detectable in MCA cells [6]. As shown in table 1, the addition of RAC lowered the growth rate and saturation density of the cells over the 48 h period. The total PKA activity, measured in solution as described previously [6] was about 75% higher in the RAC-treated cultures (data not shown, but compare to about a 3-fold increase in our previous work [6].) As shown in fig.2A, there is no difference in the PKA isoforms after 18 h. In fig.2 we have included two lanes wherein the protein kinase extracts have been preincubated with cAMP for 30 min prior to electrophoresis. Panel B demonstrated that, by 36 h, PKA type I is decreased in the retinoid-treated cultures and by 48 h the decrease in type I is accompanied by a relative increase in type II PKA in the retinoid-treated cultures. It is apparent that

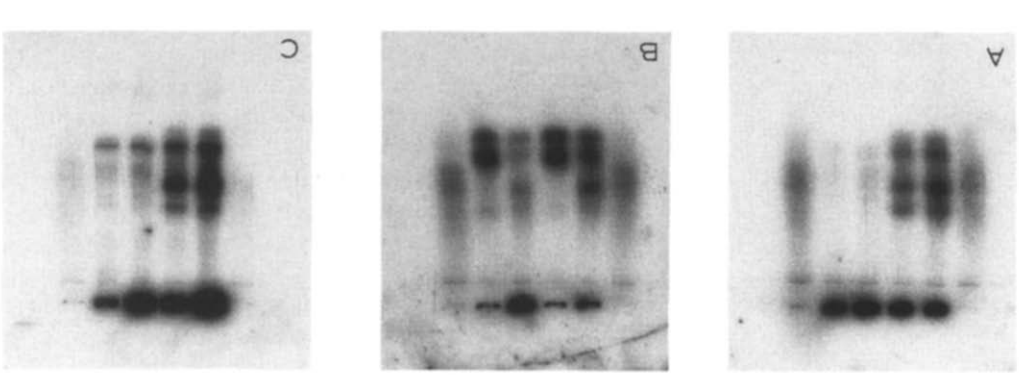

Fig.1. Nondenaturing gel electrophoresis of PKA. 8–25% gradient Phastgels were run as described in section 2. The PKA isoforms were obtained from Sigma, and the A peptide was used as the substrate. (A) Resolution of type I and type II PKA isoforms. (Lane 1) Bovine heart (100 ng); (lane 2) bovine heart (100 ng) preincubated with 10^{-6} M cAMP; (lane 3) bovine heart (200 ng) preincubated with 10^{-6} M cAMP; (lane 4) rabbit muscle type I preincubated with 10^{-6} M cAMP; (lane 6) rabbit muscle type II preincubated with 10^{-6} M cAMP; (lane 7) rabbit muscle type II (100 ng); (lane 8) rabbit muscle type I (100 ng). (B) Serial dilution of bovine heart PKA. (Lane 1) 100 ng; (lane 2) 50 ng; (lane 3) 25 ng; (lane 4) 12.5 ng; (lane 5) 6 ng; (lane 6) 3 ng; (lane 7) 1.5 ng; (lane 8) 0.75 ng.

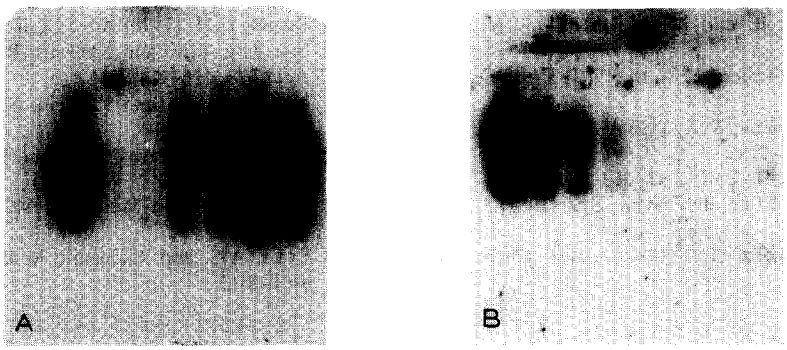

Fig.2. Effect of retinyl acetate on PKA isoforms of MCA cells. 8–25% gradient Phastgels were run as described in section 2. (A) Lanes 1 and 6) bovine heart PKA marker, 25 ng; (lane 2) MCA cells grown 18 h in the absence of RAC; (lane 3) MCA cells grown 18 h in the presence of RAC; (lane 4) same as lane 2, preincubated with cAMP; (lane 5) same as lane 3, preincubated with cAMP. (B) (Lanes 1 and 6) bovine heart PKA marker, 25 ng; (lane 2) MCA cells grown 36 h in the absence of RAC; (lane 3) MCA cells grown 36 h in the presence of RAC; (lane 4) MCA cells grown 48 h in the absence of RAC; (lane 5) MCA cells grown 48 h in the presence of RAC. The gels in (A) and (B) were soaked in A peptide substrate. (C) This gel is the same as the gel in panel (A), but it was soaked in histone IIAS (Sigma) as the substrate.

the modest (75%) increase in total PKA activity we observed in this experiment is a complex function of the decrease in type I PKA activity compensated by an increase in type II activity.

The finding that retinoids decrease the activity of type I PKA while increasing the activity of type II is consistent with other observations wherein DEAE chromatography was used to fractionate the PKA isoforms [5]. The high degree of resolution, speed, sensitivity and commercial availability of the minigel system offers a convenient new way to study PKA isoforms.

There are two factors to consider when applying the minigel system to studies of other protein kinases. First, different substrates will react with different protein kinases, yielding different patterns in the gel. Second, not all substrates will work in the gel system. We tried visualizing PKA with a number of substrates, including casein, histone H1, histone IIAS (Sigma) and Kemptide, another peptide substrate for PKA [12]. Casein and Kemptide gave little or no signal while histones H1 and IIAS gave good signals. Fig.2C shows an autoradiogram of a gel identical to the gel in panel A except that it was soaked in histone IIAS (10 mg/ml) instead of the A peptide. Note that, in addition to some modest quantitive changes in the intensity of labelling, there are some faint faster-moving bands, and a prominent band is now visible in the area occupied by type II PKA. This illustrates the fact that mixed histones and the A peptide recognize overlapping, but different sets of kinase families. Since histone IIAS is available commercially, and acts as a good substrate, we can recommend that it be used as a general first substrate for visualizing protein kinases in these gels. Casein is a poor substrate probably because it does not penetrate the gel well, while Kemptide, a heptapeptide, probably can not be fixed in the gel, even with silicotungstic acid. Given a general substrate, such as histone IIAS, one must, however, be cautious in interpreting a single band as arising from a single protein kinase species. For example, the region of the gel where PKA migrates has also been reported to contain PKC [13], but we have not included either calcium or phospholipids in the assay, and it is therefore not possible to confirm this report at present. Since it has been reported that retinoids can influence the activity of PKC in cells [4], further studies will be needed to clarify this issue.

Paradoxically, the sensitivity and degree of resolution of this minigel system can be a complicating factor in the interpretation of the patterns obtained. The nondenaturing gels used by McClung and Kletzein [10,11] showed PKA and little else. Improvements in this system by Grove and Mastro [13] and Halleck et al. [15] began to reveal the complexity of protein kinases actually found in cell extracts. Using the present system and a general substrate such as core histones, we often obtain a degree of resolution that is striking in its detail of bands. In order to fully realize the potential of this system, we are presently defining the conditions for the specific activation and assay of different protein kinase families. We expect that the application of this system to a variety of problems involving the fractionation and estimation of different protein kinases will quickly expand its utility.

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