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VITAMIN A ACID-INDUCED ACTIVATION OF Ca^{2+}-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE FROM RABBIT RETINA

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Ca^{2+}-activated, phospholipid-dependent protein kinase from rabbit retina was partially purified. Vitamin A acid (retinoic acid) stimulated this protein kinase in the presence of Ca^{2+}, while other metabolites of vitamin A such as retinol or retinal were less effective. The order of the extent of phosphorylation of the various substrate proteins by this protein kinase was identical in the presence of vitamin A acid or phosphatidylserine. The major spots of the ^{32}P labeled peptide from histone H1 phosphorylated in the presence of vitamin A acid by this protein kinase did not differ from those obtained from histone H1 phosphorylated in the presence of phosphatidylserine. Retinol caused a further enhancement of the enzymatic activity, whereas the addition of retinal inhibited the activation by vitamin A acid. Thus, vitamin A and its metabolites may play an important role in the regulation of Ca^{2+}-activated, phospholipid-dependent protein kinase activity in the retina.

Ca^{2+} plays an important role as an intracellular mediator and is probably involved in the regulation of a variety of biological processes (1,2). Although the physiological functions of Ca^{2+} in various cellular processes remain obscure, recently obtained evidence suggests that one possible mechanism is the regulation of phosphorylation of endogeneous substrate proteins by Ca^{2+}-dependent protein kinase, with subsequent alterations in the protein functions and various biological cellular activities (3). Studies have shown that Ca^{2+}-dependent phosphorylation of endogeneous proteins in several tissues (4-6) is involved in the multifunctional intracellular Ca^{2+}-receptive protein, calmodulin, which plays important roles in Ca^{2+}-dependent activations of several

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The abbreviations used are : EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid; PS, phosphatidylserine; VAA, vitamin A acid.

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protein kinases by serving as either an integral subunit or an obligatory co-factor for the enzymes (7,8).

Another cyclic nucleotide-independent protein kinase, which was selectively activated by Ca^{2+} in the presence of phospholipids, such as phosphatidylserine and phosphatidylinositol, has been reported by Takai et al. (9). Low concentrations of diacylglycerols such as diolein remarkably increased the apparent affinity of the enzyme for Ca^{2+} as well as for phospholipids, thereby leading to a marked activation of the enzyme at less than micromolar concentrations of Ca^{2+} (10). This protein kinase activity is present in several mammalian or non-mammalian tissues (11). We now report that vitamin A acid markedly stimulate the Ca^{2+}-activated, phospholipid-dependent protein kinase from retina and the other retinoids, retinol or retinal, regulate this protein kinase activity in the presence of vitamin A acid, eitehr by enhancement or by diminution, respectively.

MATERIALS AND METHODS

Ca^{2+}-activated, phospholipid-dependent protein kinase was partially, purified from the rabbit retina soluble fraction, using methods previously described (12) but with slight modification. The molecular weight of this protein kinase determined by gel filtration on a Sephacryl S-300 column chromatography (2.2 x 94 cm) was 75,000, thus coinciding well with data reported by Takai et al. (9). The enzyme preparation thus obtained was practically free of endogeneous phosphate acceptors and interfering enzymes. The enzyme activity employed for the present study was about 1.5 nmol P/min/mg protein when lysine-rich histone was used as a substrate. Myelin basic protein was purified from rabbit brain by the method of Oshiro and Eylar (13) as described previously (14). [γ-^{32}P]ATP was obtained from the Radiochemical Center, Amersham. Phosphatidylserine was purchased from Serdary Research Laboratories, Inc. and retinol, retinal, retinoic acid, lysine-rich histone (Type III-S) and protamine sulfate from Sigma Chemical Co. Histone H1, H2B and H4 were purchased from Boehringer Mannheim Biochemicals. The protein kinase activity was determined by the method of Wrenn et al. (15). The standard reaction mixture contained, 25 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 50 μM [γ-^{32}P]ATP (5 - 10 x 10^5 cpm); 1 mM $CaCl_2$ or 0.05 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); 30 μg of type III-S; 10 μg of phosphatidylserine and 1 μg of the enzyme, in a final volume of 0.3 ml. The acid-precipitable radioactivity was determined as described previously (12). Protein concentrations were determined by the method of Lowry et al. (16) with bovine serum albumin as a standard protein. Two-dimensional mapping of tryptic peptides from radioactive histone H1 was carried out by the method of Yamaguchi et al. (17).

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic patterns of Ca^{2+}-activated, phospholipid-dependent protein kinase from rabbit retina, as developed on a DEAE-cellulose

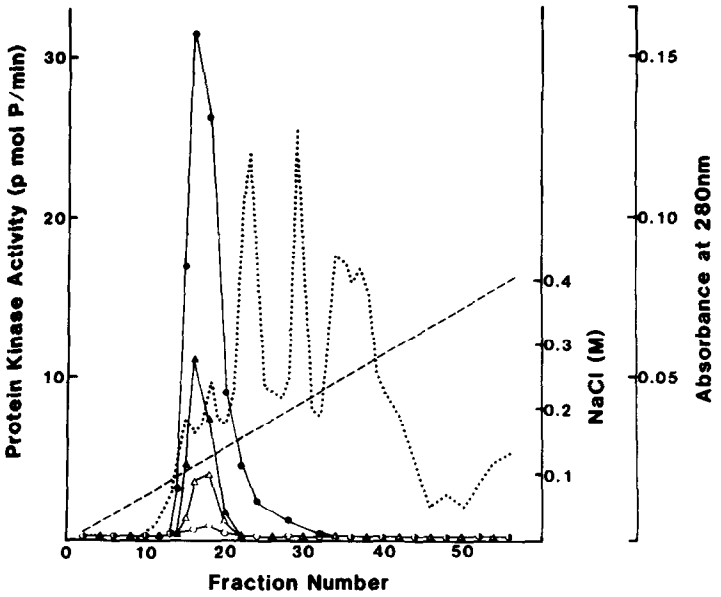

Fig. 1 DEAE-cellulose column chromatography of rabbit retina Ca^{2+}-activated, phospholipid-dependent protein kinase. The retinae were homogenized by sonication with 10 ml of 25 mM Tris-HCl at pH 7.5 containing 2 mM EGTA, 0.01% leupeptin, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl-fluoride, 10 mM $MgCl_2$ and 0.25 M sucrose. The 105,000 x g supernatant was applied to a DEAE-cellulose column (10 x 1.3 cm) previously equilibrated with 25 mM Tris-HCl at pH 7.5 containing 0.5 mM EGTA, 0.001% leupeptin and 50 mM 2-mercaptoethanol. After washing the column extensively with the same buffer, a linear gradient of NaCl (0-0.4 M) was then applied in a total gradient volume of 160 ml. The protein kinase activity was measured in the presence of 10 µg of phosphatidyl-serine or 9 µg of vitamin A acid, respectively, under the conditions described in "Materials and Methods". Phosphatidylserine with (●—●) or without (○—○) Ca^{2+}; vitamin A acid with (▲—▲) or without (△—△) Ca^{2+}; ------, absorbance at 280 nm; and — — —, NaCl concentrations.

column. When vitamin A (a fat-soluble vitamin) acid was used as an activator, the chromatographic pattern of this protein kinase from retina coincided with pattern obtained when phosphatidylserine was used as an activator. However, the peak activity in the presence of vitamin A acid was one-third that in the presence of phosphatidylserine. The protein kinase activity was eluted in a single peak at about 0.12 M NaCl and the peak fraction was used for the following experiments as a Ca^{2+}-activated, phospholipid-dependent protein kinase.

The apparent Km values for ATP of the partially purified retina Ca^{2+}-dependent protein kinase, using phosphatidylserine or vitamin A acid as an activator, were 5.9 and 11.8 µM, respectively (Fig. 2).

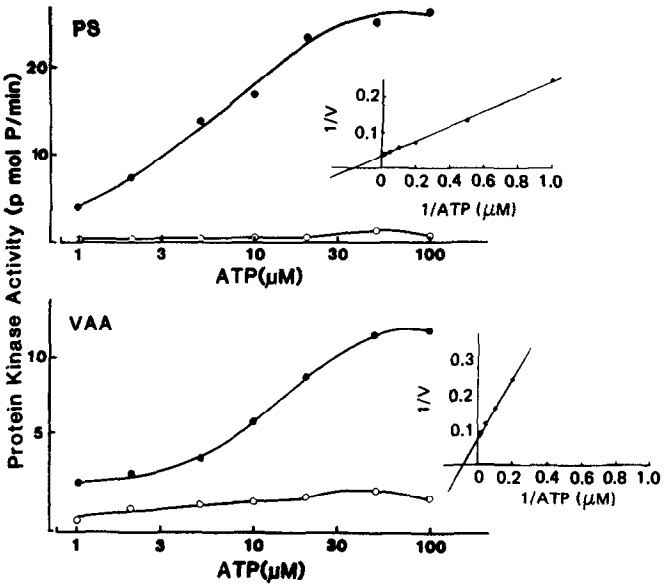

Fig. 2 Determination of apparent Km for ATP of rabbit retina Ca^{2+}-activated, phospholipid-dependent protein kinase. The enzyme activity was assayed in the presence of 10 μg of phosphatidylserine or 9 μg of vitamin A acid, respectively, as described under "Materials and Methods", except that the concentrations of [γ-^{32}P]ATP used ranged from 1 - 100 μM. Phosphatidylserine or vitamin A acid, respectively, with (●—●) or without (O—O) Ca^{2+}.

The substrate specificity of this Ca^{2+}-activated, vitamin A acid-dependent protein kinase from rabbit retina was investigated (Fig. 3). When phosphatidylserine was used as an activator, lysine-rich histone (Type III-S or histone H1) was the most Ca^{2+}-sensitive substrate for the Ca^{2+}-activated protein kinase. Under the present conditions histone H4 was much less effective. Protamine sulfate was phosphorylated in a Ca^{2+}-independent manner, whereas myelin basic protein served as an excellent substrate (18). Using vitamin A acid, the substrate specificity was much the same as observed with phosphatidylserine.

Autoradiograms of tryptic digests of ^{32}P labeled histone H1 phosphorylated by the Ca^{2+}-activated, phospholipid-dependent protein kinase with phosphatidylserine or vitamin A acid, respectively, as activators yielded identical major radioactive spots (Fig. 4).

The results indicate that the mechanism of activation with vitamin A acid was similar to that with phosphatidylserine, although some small differences exist with respect to ATP requirement, substrate specificity, the extent of

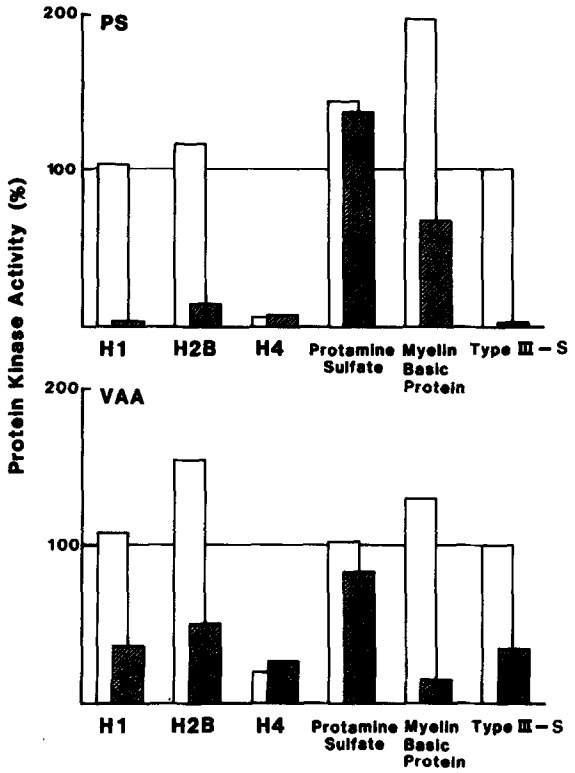

Fig. 3 Substrate specificity of rabbit retina Ca^{2+}-activated, phospholipid-dependent protein kinase stimulated by phosphatidylserine or vitamin A acid, respectively. The enzyme activity was assayed in the presence of 10 μg of phosphatidylserine or 9 μg of vitamin A acid, respectively, as described under "Materials and Methods", using various substrates, as indicated. The protein kinase activity, using type III-S as a substrate protein in the presence of phosphatidylserine or vitamin A acid, respectively, and Ca^{2+} was taken as a standard. Phosphatidylserine or vitamin A acid with (□) or without (▨) Ca^{2+}.

phosphorylation of histone H2B, and the Ca^{2+}-requirement for phosphorylation of myelin basic protein.

In contrast to vitamin A acid, neither retinol, nor retinal alone activated the protein kinase, up to 30 μg (data not shown). However, these inactive retinoids showed significant effects on this protein kinase activity in the presence of vitamin A acid (Fig. 5). Retinol further enhanced the activity. Conversely, the addition of increasing amounts of retinal diminished the activation by vitamin A acid. These findings indicate that the metabolites of vitamin A may play cooperative roles in the activation of this Ca^{2+}-dependent protein kinase from rabbit retina.

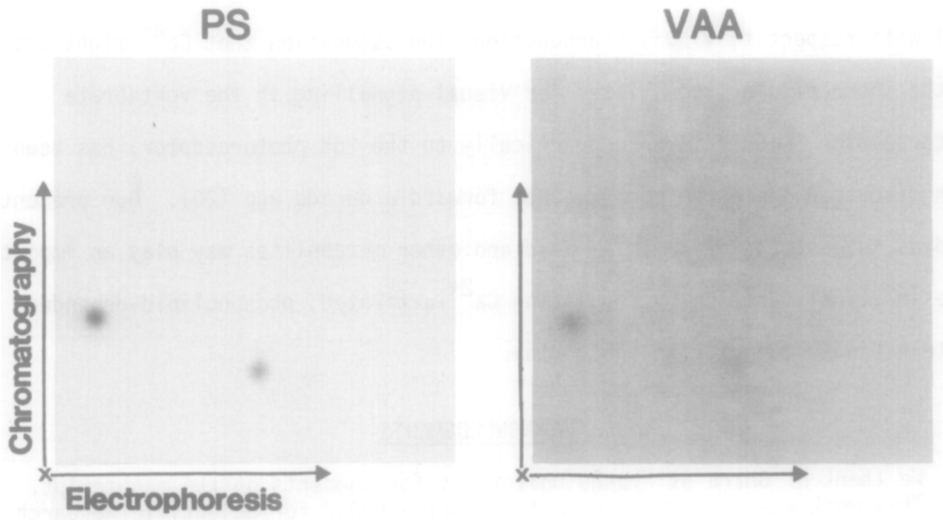

Fig. 4 Tryptic peptide mapping of radioactive histone H1 phosphorylated by rabbit retina Ca^{2+}-activated, phospholipid-dependent protein kinase stimulated by phosphatidylserine or vitamin A acid, respectively. Each radioactive histone H1 preparation was digested with trypsin and subjected to electrophoresis followed by ascending chromatography, under the conditions described (17).

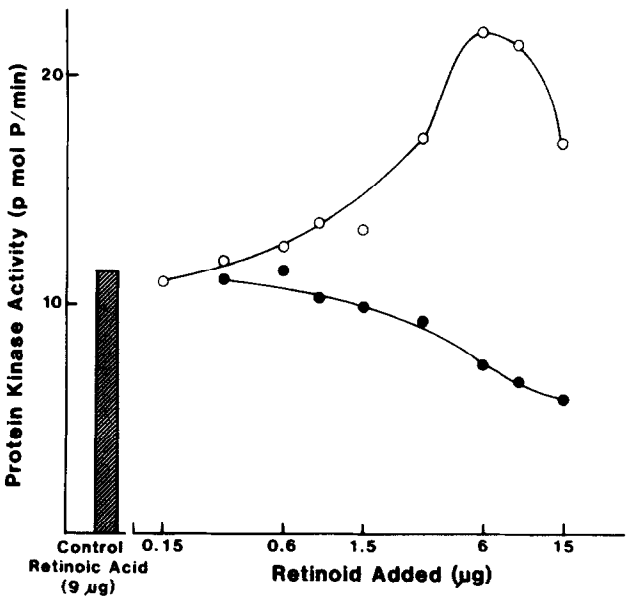

Fig. 5 Effects of retinol or retinal on rabbit retina Ca^{2+}-activated, phospholipid-dependent protein kinase stimulated by retinoic acid. The enzyme activity was assayed in the presence of fixed amounts of retinoic acid (9 µg) and Ca^{2+} (1 mM). In addition, various amounts of retinol or retinal, respectively, were supplemented. Column (▨) indicates the activity of the protein kinase stimulated by 9 µg of retinoic acid and 1 mM Ca^{2+} as a control. o—o, with retinol; ●—●, with retinal.

Although a role for cGMP in the retina is being given increasing attention (19) with respect to visual transduction, the suggestion that Ca^{2+} might act as the intracellular transmitter for visual signalling in the vertebrate photoreceptor (with reference practically to the rod photoreceptor) has been much discussed since the idea was put forward a decade ago (20). Our present studies suggest that vitamin A acid and other metabolites may play an important role in visual function by regulating Ca^{2+}-activated, phospholipid-dependent protein kinase activity in the retina.

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