

Pineal Transduction

ADRENERGIC → CYCLIC AMP-DEPENDENT PHOSPHORYLATION OF CYTOPLASMIC 33-kDa PROTEIN (MEKA) WHICH BINDS $\beta\gamma$ -COMPLEX OF TRANSDUCIN*

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Juan A. Reig[‡], Li Yu, and David C. Klein[§]

From the Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Adrenergic regulation of phosphorylation of pineal proteins was studied. Norepinephrine treatment of intact pinealocytes incubated with ^{32}P i enhanced phosphorylation of a 33-kDa phosphoprotein (33PP). The effect of NE was rapid, sustained, and appeared to be mediated by a β -adrenergic → cyclic AMP mechanism. Studies using broken cell preparations revealed that 33PP was phosphorylated by cyclic AMP-dependent protein kinase (PKA). It was also possible to demonstrate PKA-dependent phosphorylation of the 33-kDa protein in cytosol from rat retina and in cow and sheep pineal glands. Two-dimensional polyacrylamide gel electrophoresis revealed that 33PP is acidic ($\text{pI} \approx 4.5$), appears to exist as two isoforms with slightly different charge, and has the same mobility as the retinal 33-kDa PKA substrate. Immunological analysis indicated 33PP in both tissues is a previously reported 33-kDa protein (MEKA); this protein is a PKA substrate which has been reported to form a cytoplasmic complex with the $\beta\gamma$ complex of transducin. Consistent with this, it was possible to identify the β -subunit in pineal cytoplasm and in the same ≈ 70 -kDa gel permeation fraction which contained the 33-kDa protein identified as MEKA. Thus, it appears possible that MEKA is present in pineal cytoplasm in a 70-kDa complex with $G\beta\gamma$, as is the case in retina. The finding of MEKA in the pineal makes it the latest addition to a family of retinal/pineal proteins which are thought to have evolved from a common ancestral photochemical transduction system.

The mammalian pineal gland functions as part of a photo-neuroendocrine transduction system which converts the length of the night period into the length of the period during which melatonin is synthesized and released (1, 2). The neural signals which stimulate the gland originate in an autonomous circadian clock in the suprachiasmatic nucleus of the hypothalamus. This clock is reset and adjusted on a daily basis by environmental lighting acting through the eye via a retinal-hypothalamic projection. Light entrains the circadian clock to the environmental lighting cycle and can block stimulation of the pineal by the clock. The suprachiasmatic nucleus communicates with the pineal gland by a neural pathway which passes through central neural structures and terminates

in sympathetic nerves which release norepinephrine (NE)¹ into the pineal extracellular space (1, 2). Recent studies also indicate that adenosine and vasoactive intestinal peptide may also be involved in neurotransmission in the pineal gland (3, 4).

NE acts through a biochemical "AND" gate² to stimulate cyclic AMP and cyclic GMP accumulation (5). For any increase in cyclic AMP or cyclic GMP to occur, β -adrenergic stimulation of the respective cyclase is required; this alone has a relatively small effect on cyclic nucleotides. Full 100-300-fold stimulation requires α_1 -adrenergic activation, which potentiates β -adrenergic stimulation of cyclases through mechanisms involving Ca^{2+} , phospholipid-dependent protein kinase C (PKC), and Ca^{2+} (6, 7).

Cyclic AMP plays a dominant role in the regulation of melatonin production by increasing 100-fold the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, E.C. 2.3.1.87), the first enzyme in the serotonin → *N*-acetylserotonin → melatonin pathway. This enzyme controls large changes in the production of melatonin (8). The cyclic AMP-dependent mechanisms involved in the stimulation of *N*-acetyltransferase activity are complex and appear to involve new gene expression, new protein synthesis, enzyme stabilization, and hyperpolarization of the membrane (1). The precise mechanisms involved have not been described, but it seems likely that cyclic AMP-dependent protein kinase (PKA) could play a role in some of these effects, as has been postulated (9).

The pineal gland is rich in PKA (9). However, the role of PKA in transmembrane signal transduction in the pineal has not been thoroughly described. For example, whereas it has been shown that NE stimulates the phosphorylation of a

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[‡] Present address: Biochemical Pharmacology Group, Department of Neurochemistry, University of Alicante, Alicante, Spain.

[§] To whom correspondence should be addressed: National Institutes of Health, 36/4A07, Bethesda, Maryland 20892.

¹ The abbreviations used are: NE, norepinephrine; DBcAMP, *N*⁶,2'-*O*-dibutyryl cyclic cAMP; DBcGMP, *N*²,2'-*O*-dibutyryl cyclic GMP; G protein, heterotrimeric GTP-binding regulatory protein; $G\alpha$, the α -subunit of GTP-binding regulatory proteins; $G\beta\gamma$, the $\beta\gamma$ complex of GTP-binding regulatory proteins; $G\beta$, the β -subunit of GTP-binding regulatory proteins; $G\gamma$, the γ -subunit of GTP-binding regulatory proteins; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PKA, cyclic AMP-dependent protein kinase; PKC, Ca^{2+} calcium, phospholipid-dependent protein kinase C; PMA, 4 β -phorbol 12-myristate 13-acetate; and, PVDF, polyvinylidene difluoride; DMEM, Dulbecco's Modified Eagle's medium; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; ISO, isoproterenol; DARPP-32, dopamine, cyclic AMP-regulated phosphoprotein.

² The term biochemical "AND" gate describes regulatory mechanisms which require that two independent receptor-regulated mechanisms must act simultaneously to produce a full response. This is analogous to the electronic "AND" gate which requires that two electronic signals must be received simultaneously at the two inputs of the gate for an output signal to be generated.

nuclear protein in the pineal gland (10), no evidence has been presented which indicates that this involves PKA. Similarly, it has been shown that addition of cyclic AMP to pineal homogenates causes phosphorylation of three proteins (11). However, intact cells have not been used to demonstrate that the same proteins are phosphorylated in intact cells in response to NE or *N*⁶,2'-*O*-dibutyryl cyclic AMP (DBcAMP) treatment.

In this report we present the results of a study on pineal protein phosphorylation in which intact pinealocytes and pineal supernatant fractions were used. A number of proteins were found to be rapidly and dramatically phosphorylated in response to adrenergic stimulation. Attention focused on a 33-kDa protein (33PP) because phosphorylation of it was rapid and prominent in both intact and broken cell preparations. The mechanism involved in phosphorylation and the identification of the protein are described here.³

EXPERIMENTAL PROCEDURES

Tissue Preparation

Preparation of Pinealocytes—Pinealocytes were obtained by enzymatic treatment of pineal glands obtained from 200-g female Sprague-Dawley rats (12). The cells were incubated (37 °C; 95% O₂, 5% CO₂; 4 × 10⁵ cells/ml) for 24 h in DMEM medium containing 10% fetal calf serum prior to subsequent experimental use.

Preparation of Pineal Cytosol—Cytosolic preparations used for phosphorylation studies were routinely prepared from cells or glands by sonication (5 s) in 20 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin. The supernatant was obtained by centrifugation (1 h, 4 °C, 100,000 × *g*).

Phosphorylation Procedures

Phosphorylation of Proteins in Intact Cells Using [³²P]Orthophosphate—A suspension of cells (4 × 10⁵ cells/ml) was centrifuged (10 min, 1,000 × *g*), and the cells were resuspended (1.6 × 10⁶ cells/ml) in a solution containing 5.3 mM KCl, 1.8 mM CaCl₂, 85 mM NaCl, 40 mM NaHCO₃, 5.5 mM D-glucose, 25 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 0.2% bovine serum albumin, and 1 mCi/ml ³²Pi (carrier free; ICN, Irvine, CA). Cells were transferred to 1.5-ml microtubes (4 × 10⁵ cells/tube) and incubated at 37 °C for 1 h. Drug treatment was initiated by adding a 100 × concentrated solution of an agent to a tube containing cells. The incubation was ended by placing the tube in a 4 °C bath. Cells were collected by centrifugation (2 min, 2,000 × *g*) and frozen on solid CO₂. Frozen cells were thawed by adding 20 μl of a solution containing 20 mM HEPES, pH 7.4, 20 μM digitonin, 1 mM EGTA, 0.5 mM EDTA, 1.5 mM ATP, 5 mM *p*-nitrophenylphosphate, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM leupeptin. The supernatant preparation was obtained by centrifugation (1 h, 4 °C, 100,000 × *g*) and either stored (-70 °C) or prepared for electrophoresis. Other procedures of cell disruption were evaluated including sonication and freeze thawing. Sonication and digitonin treatment were equally effective.

Phosphorylation of Proteins in the Cytosolic Extracts—Supernatant fractions (1–5 mg of protein/ml) obtained by sonication and centrifugation as described above were incubated with a solution (final volume = 40 μl) containing 20 mM MOPS, pH 7.4, 10 mM MgCl₂, and 10 μM [³²P]ATP (approximately 25 Ci/mmol); PKA (15 μg/ml) or cyclic AMP were added as indicated. Samples were incubated for 20 min at 37 °C; the reaction was stopped by addition of the electrophoresis sample buffer (see below).

Electrophoretic Techniques

One-dimensional Polyacrylamide Gel Electrophoresis (PAGE)—Samples were prepared for electrophoresis by adding 20 μl of sample buffer (0.125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.016

bromphenol blue, 0.002% pyronine, and 10% 2-mercaptoethanol) and heating for 5 min (100 °C).

SDS-slab gel electrophoresis was performed using 12.5 or 15% polyacrylamide/bis-acrylamide (30:1) according to the method of Laemmli (13). The running buffer was 25 mM Tris, pH 8.0, 190 mM glycine, and 0.1% SDS. Samples were run at 80 V for 2 h and at 180 V for 3 h. Gels were routinely electroblotted onto PVDF membranes (Immobilon P[®], Millipore Corp., Bedford, MA) as described below.

Two-dimensional PAGE—Samples were prepared by incubating (room temperature, 15 min) 20-μl volumes of extract with 20-μl volumes of lysis solution (9.8 M urea, 3% Nonidet P-40, and 100 mM dithiothreitol). A 1:5 mixture of pH 3–10 and pH 3–5 ampholytes (BioLytes, Bio-Rad) was added to a final concentration of 2% (v/v). Two-dimensional PAGE was done as described by O'Farrell (14) with some modifications. The 1:5 ratio of ampholytes was maintained in the isoelectric focusing gel. Isoelectric focusing gels were prepared in 2.0 mm (inner diameter) tubes using a sample of a gel mixture containing 6.9 g of urea, 2.5 ml of a 10% Nonidet P-40 solution, 1.7 ml of a 30% acrylamide solution, 0.6 ml of ampholytes, and 2.5 ml of water. A sample containing 30 μg of protein was layered on each gel. Voltage was applied as follows: 200 V for 30 min, 500 V for 17–20 h, and 1000 V for 1 h. The pH gradient was determined in a parallel gel by soaking 5-mm slices of the gel in distilled water for 1 h and measuring pH in the extract. Isoelectric focusing gels were stored at -70 °C prior to second dimension electrophoresis. Second dimension slab gels (12.5% acrylamide) were run at 8–10 °C at 80–180 V as described above. Gels were routinely electroblotted onto PVDF membranes (see below).

Electroblotting—Proteins from one- or two-dimensional-PAGE gels were transferred onto PVDF membranes according to the procedure of Towbin *et al.* (15). Gels were equilibrated in blotting buffer (25 mM Tris-glycine, pH 8.3, 20% methanol) for 10–15 min. A commercial blotting assembly (Bio-Rad Trans-blot cell) was used. Electrophoretic transfer was accomplished at 150 mA (constant current) for a period of about 15 h at 8–10 °C. Apparent molecular masses were estimated using prestained standards (Rainbow Standards, Amersham Corp.) containing myosin (200 kDa), phosphorylase b (92 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Blots were allowed to dry at room temperature.

Autoradiography—Autoradiography was performed with Kodak X-Omat AR2 film at -70 °C with an intensifying screen (Cronex Light-ning Plus Du Pont[®]).

Quantitation of Protein Phosphorylation—Autoradiographs were scanned by a densitometer (model 620, Bio-Rad) and ³²P incorporation into specific protein bands was estimated from the OD using the 1-D Analyst software package (Bio-Rad). In several figures data are presented as the height of the 33PP peak in OD units.

Sequence Analysis—Comparison of the amino acid sequence of the MEKA protein and the NBRF protein database was performed with the Wordsearch program contained in the Genetics Computer Group Sequence Analysis Software Package (16).

Assays

Cyclic Nucleotide Assays—Samples were assayed in duplicate for cyclic AMP by radioimmunoassay (5, 17).

Protein Assay—Protein was measured by a dye-binding method using bovine serum albumin as a standard (18).

Immunodetection—Electroblots were treated with 1% bovine serum albumin (1 h), incubated with antiserum for 18 h, washed, incubated for 18 h with second antiserum conjugated to peroxidase, washed, and color was developed using 4-chloro-1-naphthol (Bio-Rad, 19). Specific details regarding the duration of the incubations and the dilution of antiserum appear in the appropriate figure legend.

Materials

The following reagents were purchased: acrylamide, bisacrylamide, SDS, and other gel chemicals (Bio-Rad); Nonidet P-40, PMA, digitonin, MOPS, phenylmethylsulfonyl fluoride, leupeptin, DBcAMP, DBcGMP, synthetic protein kinase inhibitor, catalytic subunit of the cyclic AMP-dependent protein kinase (Sigma); [³²P]orthophosphate (ICN), [³²P]ATP (Amersham Corp.). Cyclic AMP antiserum was provided by Kevin Catt (National Institutes of Child Health and Human Development), DARPP-32 antiserum was provided by H. C. Hemmings and Paul Greengard (The Rockefeller University), anti Gβ-peptide serum (J-99) was provided by Allen Spiegel (National Institute of Diabetes and Digestive and Kidney Diseases), anti-

³ Portions of this investigation were presented at the Annual Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, January 29 to February 2, 1989, San Francisco (Reig, J. A., and Klein, D. C. (1988) *J. Cell Biol.* 107, 507A.).

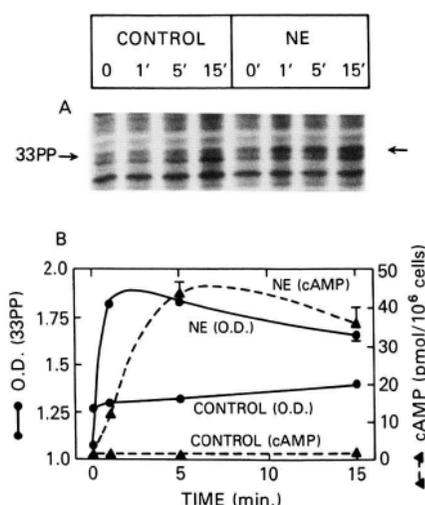


FIG. 1. Time course study on the effect of NE on phosphorylation of cytosolic pineal proteins and cyclic AMP. Pinealocytes were incubated for 24 h in DMEM medium, and then were collected, resuspended (800,000 cells/0.5 ml) in a low phosphate medium containing ^{32}P i (1 mCi/ml), and incubated. After 60 min NE (1 μM) was added. At the indicated time, cells were collected by centrifugation and resuspended in medium containing 20 μM digitonin. This preparation was then centrifuged and the supernatant was used for subsequent analysis (40 $\mu\text{g}/\text{lane}$) by 15% one-dimensional-PAGE and autoradiography. Phosphorylation was estimated by densitometry. A parallel experiment was run in which cells were resuspended in ^{32}P i-free low phosphate medium. *A*, autoradiograph of the blot exposed for 2 h. Similar results were obtained in two other experiments. *B*, densitometric analysis of 33PP phosphorylation (solid line) and radioimmunoassay analysis of cyclic AMP in the cytosolic fraction of the cells incubated without ^{32}P i (broken line). Data are given as the mean \pm S.E. of triplicate determinations. The techniques used are detailed under "Experimental Procedures."

lipocortin serum was provided by Blake Kapinsky (Biogen); and, anti-MEKA serum was provided through Toshimi Shinohara (National Eye Institute) by Tohru Abe (Akita University School of Medicine).

RESULTS

Studies with Intact Cells

To study protein phosphorylation in intact cells, a dispersed pineal cell preparation was used (12). Pinealocytes were incubated for 60 min in a low phosphate HEPES-buffered medium (0.2% bovine serum albumin) containing ^{32}P i.

Time Course Study of the Effect of NE on Phosphorylation of Cytosolic Pineal Proteins and on Cyclic AMP Content—NE was added at a dose (1 μM) which produces maximum stimulation of cyclic AMP and cyclic GMP accumulation in this system (5). Samples were collected 1–15 min later, and cells were permeabilized by treatment with digitonin. Cytosolic and membrane fractions were obtained by centrifugation.

NE-dependent changes in protein phosphorylation in the membrane fraction were not detectable within a 1–15 min test period.⁴ However, NE treatment caused prominent phosphorylation of a cytosolic 33-kDa protein (33PP, Fig. 1A). Densitometric analysis revealed that the largest response observed occurred 1 min after NE treatment had started (Fig. 1B) and had decreased only slightly at 15 min.

Analysis of the cytosolic fraction of cells incubated in ^{32}P i-free low phosphate culture medium (Fig. 1B) revealed that cyclic AMP had increased 10-fold 1 min after NE treatment; maximal stimulation (40-fold stimulation) was achieved at 5 min. These effects are smaller than the reported 100–200-fold

changes in cyclic AMP accumulation (5). This might reflect differences in experimental conditions; in the present study cells were incubated in a low phosphate salt solution (0.2% bovine serum albumin) rather than the complex medium containing 10% fetal calf serum, which is typically used in studies on cyclic nucleotide regulation.

Concentration-Response Relationship of NE and Cyclic AMP and of NE and 33PP Phosphorylation—A range of concentrations of NE was used to compare the effects of NE on cyclic AMP and 33PP phosphorylation (Fig. 2). A 10-min treatment period was used. NE produced parallel increases in 33PP phosphorylation and in cyclic AMP accumulation (Fig. 2); maximal stimulation was detectable at 1 μM ($\text{EC}_{50} = 0.11 \mu\text{M}$).

Studies with Adrenergic Agonists—To broadly classify the receptor involved in the effect of NE, effects of a single concentration (1 μM) of the β -adrenergic agonist isoproterenol (ISO) and the α -adrenergic agonist phenylephrine were compared. ISO was about as effective as NE (Fig. 3), but phenylephrine was without effect (data not shown). Although this indicates that the action of NE requires activation of a β -adrenergic receptor, it does not exclude the possibility that activation of α_1 -adrenergic receptors might also participate in the effect of NE on 33PP phosphorylation because NE acts on cyclic AMP through both β - and α -adrenergic receptors, as indicated in the Introduction (5).²

Studies with Second Messenger Substances—ISO (1 μM) elevates cyclic AMP and to a lesser degree cyclic GMP; in contrast PE (1 μM) has little or no effect on cyclic nucleotides. However, phenylephrine (1 μM) is known to increase $[\text{Ca}^{2+}]_i$, inositol phosphate production, and membrane-association of PKC, whereas ISO alone has little or no effect on these parameters (5–7). Accordingly, it would appear from the above that the likely candidate for the second messenger involved

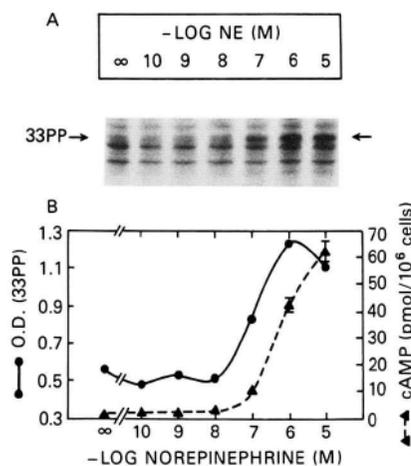


FIG. 2. Concentration-response relationship of NE and 33PP phosphorylation and cyclic AMP in intact cells. Pinealocytes were incubated for 24 h in DMEM medium, and then were collected and resuspended (800,000/0.5 ml) in a low phosphate medium containing ^{32}P i (1 mCi/ml). After 60 min NE was added at the indicated concentrations; 10 min later cells were collected by centrifugation and resuspended in medium containing digitonin. This preparation was then centrifuged and the supernatant was used for subsequent analysis (40 $\mu\text{g}/\text{lane}$) by 15% one-dimensional-PAGE; proteins were electroblotted. A parallel experiment was run in which cells were resuspended in ^{32}P i-free low phosphate medium. *A*, autoradiograph of the blot; this result was observed in three separate experiments. *B*, densitometric analysis of 33PP phosphorylation (solid line) and radioimmunoassay analysis of cyclic AMP in the cytosolic fraction of cells incubated without ^{32}P i (broken line). Data are given as the mean \pm S.E. of triplicate determinations; the absence of an error bar indicates it fell within the area of the symbol.

⁴ D. C. Klein, L. Yu, and J. A. Reig, unpublished results.

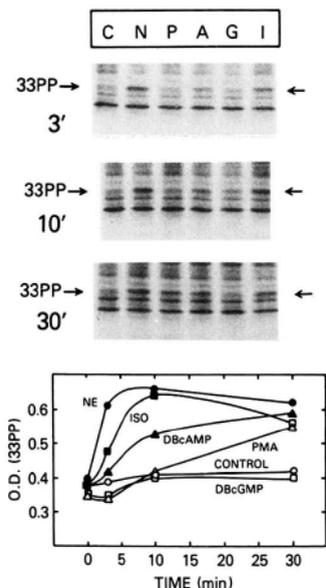


FIG. 3. Time-dependent effects of NE, ISO, PMA, DBcAMP, and DBcGMP. Pinealocytes were incubated for 24 h in DMEM medium, and then were collected and resuspended (800,000/0.5 ml) in a low phosphate medium containing ^{32}P i (1 mCi/ml). After 60 min norepinephrine (N, NE, 1 μM), isoproterenol (I, ISO, 1 μM), $N^6,2'$ -*O*-dibutyryl cyclic AMP (A, DBcAMP, 1 mM); $N^2,2'$ -*O*-dibutyryl cyclic GMP (G, DBcGMP, 1 mM); 4β -phorbol 12-myristate 13-acetate (P, PMA, 0.1 μM) or no drug (C, control) were added. After 3, 10, or 30 min, cells were collected by centrifugation and resuspended in medium containing digitonin. This preparation was then centrifuged and the supernatant was used for subsequent analysis (40 $\mu\text{g}/\text{lane}$) by 15% one-dimensional-PAGE. Top panel presents the autoradiograph of the gel, exposed for 2 h. The bottom panel presents the results of densitometric scan of the gels. These results were confirmed in two other experiments. The techniques used are detailed under "Experimental Procedures."

in the effects of NE on 33PP phosphorylation in intact cells is cyclic AMP or cyclic GMP.

To study the effects of cyclic nucleotides, cells were treated with DBcAMP or DBcGMP. It was also of interest to determine if exposure to a high concentration of K^+ (which elevates $[\text{Ca}^{2+}]_i$; 20) or treatment with 4β -phorbol 12-myristate 13-acetate (which activates PKC) could mimic the effect of NE on 33PP phosphorylation.

DBcAMP treatment increased 33PP phosphorylation, albeit not as rapidly as did NE (Fig. 3). In contrast, neither DBcGMP, PMA, nor K^+ had a similar marked effect. It was apparent, however, that PMA did produce a gradual increase in 33PP phosphorylation; this was distinctly less intense than that produced by NE, ISO, or DBcAMP (Fig. 3). These observations suggest that the effect of NE is mediated primarily by cyclic AMP and not to a significant degree by cyclic GMP, PKC, or Ca^{2+} acting alone.

The evidence that 33PP phosphorylation in intact cells was somewhat enhanced by an activator of PKC could be interpreted as evidence that the protein might also be PKC substrate. However, the evidence that PKC is rich in pineal cytosol (22, 23) makes it seem reasonable to expect that addition of PKC activators to cytosolic preparations would produce prominent phosphorylation of 33PP if it were a PKC substrate. This was not observed in these studies and in others which used both intact and broken cell preparations (data not shown). Accordingly, it seems that 33PP is not a PKC substrate.

An alternative explanation for the gradual and weak effect of PMA is that it indirectly stimulates 33PP phosphorylation,

via activation of cyclic AMP production. Although activators of PKC alone are not known to produce large changes in cyclic AMP in the pinealocyte (23), it is possible that very small changes occur and that these are sufficient to enhance PKA-dependent phosphorylation of 33PP.

Two-dimensional-PAGE Analysis—To determine the pI of 33PP and to test for the presence of isoforms, supernatant fractions of control and NE-treated ^{32}P -labeled cells were resolved using two-dimensional-PAGE. NE-induced phosphorylation of 33PP was clearly evident in autoradiographs (Fig. 4). Based on the ovoid shape of the 33PP spot, it seems that it might represent two distinct proteins or isoforms (pI \approx 4.5 and 4.6) of the same protein.

Studies with Cytosolic Preparations

To study cyclic AMP-dependent 33PP phosphorylation in cytosolic fractions, we used 100,000 $\times g$ supernatant preparations of sonicated cells and of pineal gland homogenates. These preparations were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Addition of cyclic AMP (20 μM) caused a time-dependent phosphorylation of 33PP (Fig. 5).

Concentration-Response Relationship of Cyclic AMP and 33PP Phosphorylation—Using a 100,000 $\times g$ preparation of sonicated pinealocytes (Fig. 6), it was found that addition of cyclic AMP produced a concentration-dependent ($\text{EC}_{50} \approx 30$ nM) increase in 33PP phosphorylation. In a parallel study cyclic GMP was found to be about 100-fold less potent than cyclic AMP in enhancing phosphorylation of 33PP ($\text{EC}_{50} \approx 3$

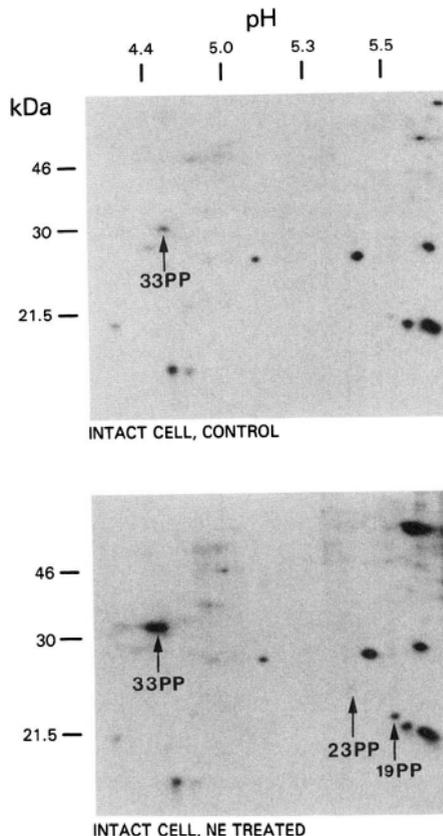


FIG. 4. Two-dimensional-PAGE analysis of cytosolic fractions from control and NE-treated cells incubated with ^{32}P i. Cells were incubated with ^{32}P i for 1 h and treated without or with 1 μM NE for 10 min. Samples were analyzed by two-dimensional gel electrophoresis and electroblotted. Autoradiographs were then prepared; for details see "Experimental Procedures." Autoradiographs are representatives of two separate experiments where each condition was tested in triplicate.

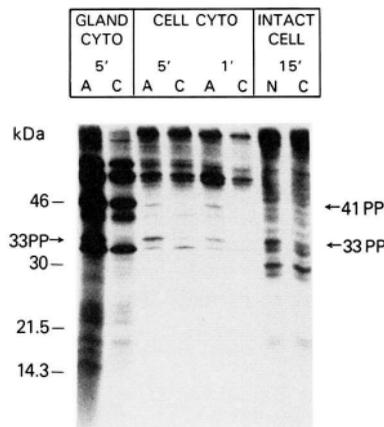


FIG. 5. Effect of cyclic AMP on phosphorylation of pineal proteins. In the blocks labeled *GLAND CYTO* and *CELL CYTO*, cytoplasmic preparations of intact glands or unlabeled pinealocytes were prepared by sonication and incubated with [γ - 32 P]ATP under control (C) conditions or with cyclic AMP (A, 20 μ M) for the indicated periods of time. In the section labeled *INTACT CELL*, pinealocytes were labeled as described in Fig. 1 and were treated under control (C) conditions or with NE (N) for 15 min. These samples were analyzed by 15% PAGE and autoradiography. The techniques used are described under "Experimental Procedures."

μ M). Ca^{2+} (1 mM) had no effect (data not shown).

Effect of PKA Inhibitor—It seemed probable that the cyclic AMP-dependent phosphorylation of 33PP involved PKA. This was tested using a specific inhibitor of PKA (PKI, 1 μ M), which clearly inhibited cyclic AMP-dependent phosphorylation of 33PP (Fig. 6). PKI also inhibited phosphorylation by the purified catalytic subunit of PKA (data not shown). As was the case for cyclic AMP, cyclic GMP-dependent 33PP phosphorylation was inhibited by PKI (data not shown). Thus, it would appear that these effects of cyclic GMP are due to activation of PKA.

Effect of Alkali Treatment on 33PP—Alkali treatment of a blot containing 32 P-labeled protein with 1 M KOH for 1 h at 55 $^{\circ}$ C removed radioactivity from 33PP indicating that it is phosphorylated at a serine and/or threonine residue (23).

Tissue Survey for 33PP—Soluble extracts of rat, cow, and sheep pineal glands were prepared and incubated with [γ - 32 P]ATP in the presence or absence of cyclic AMP. Phosphorylation of 33PP was observed in all cases (Fig. 7). Supernatant preparations of rat retina, adrenal, pituitary, testis, lung, heart, kidney, spleen, liver, and thyroid were also studied. Cyclic AMP-dependent phosphorylation of a 33-kDa protein was only evident in the retinal preparation (Fig. 7). Investigations with whole brain preparations were negative.⁴

Comparison of 33PP with Dopamine, Cyclic AMP-regulated Phosphoprotein (DARPP-32) and the Retinal 33-kDa Protein—Dopamine, cyclic AMP-regulated phosphoprotein (DARPP-32) (24, 25) is an acidic 32-kDa phosphoprotein, which is enriched in the caudatoputamen and substantia nigra regions of the central nervous system. Because of the similar sizes of DARPP-32 and 33PP, immunochemical studies were conducted to determine if these were the same protein. Supernatant extracts from pineal, retina, and putamen were phosphorylated using the catalytic subunit of PKA; proteins were resolved by PAGE and analyzed immunochemically.

PKA-dependent phosphorylation of 33-kDa proteins in pineal and retinal preparations was enhanced by the catalytic subunit of PKA; enhanced phosphorylation of a putamen 33-kDa protein was not evident (Fig. 8). DARPP-32 was detectable in the putamen but not in the pineal or retina. The failure to detect DARPP-32 in the retina is consistent with a

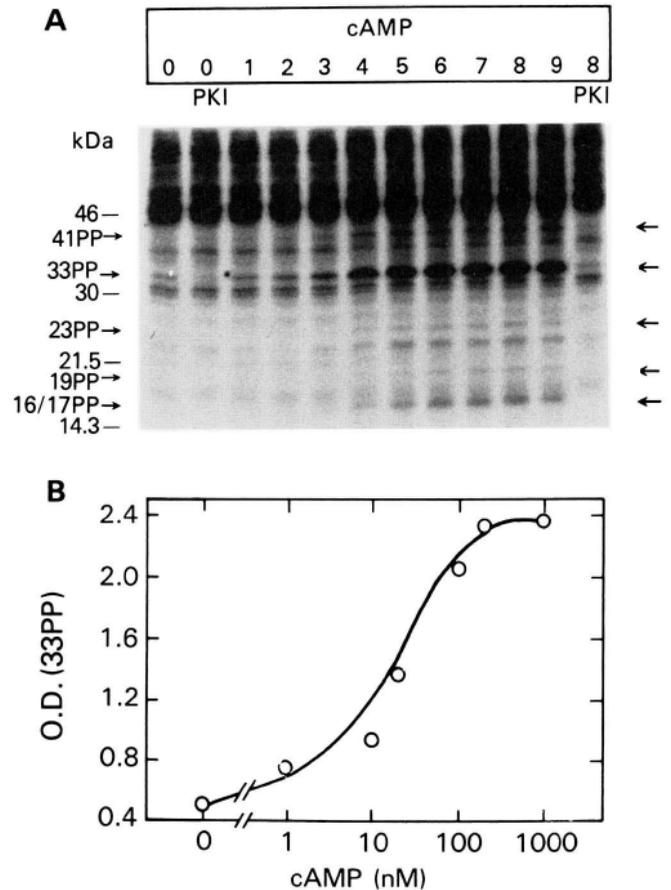


FIG. 6. Concentration-response relationship of cyclic AMP and 33PP phosphorylation in cytosolic preparations of pinealocytes. Cell extracts were prepared from pinealocytes and then treated in presence of [γ - 32 P]ATP and IBMX 0.5 mM without (O) or with the indicated concentrations of cyclic AMP: 1 nM (1); 10 nM (2); 20 nM (3); 100 nM (4); 200 nM (5); 1 μ M (6); 5 μ M (7); 10 μ M (8); 100 μ M (9). The inhibitor of cyclic AMP-dependent protein kinase (PKI, 1 μ M) was added in the absence or presence of cyclic AMP (10 μ M). The top panel presents a photograph of the autoradiograph, in which cyclic AMP-dependent phosphorylation of five proteins is apparent. The bottom panel presents the results of densitometric analysis of the intensity of 33PP phosphorylation as a function of cyclic AMP, which revealed the EC_{50} was \approx 30 nM. The EC_{50} for the cyclic AMP-dependent phosphorylation of other proteins was similar (41-kDa protein, \approx 80 nM; 23-kDa protein, \approx 120 nM; 19-kDa protein, \approx 250 nM; and, 16/17-kDa protein doublet, \approx 150 nM). Similar results were obtained in a second experiment. The techniques used are detailed under "Experimental Procedures."

previous report that DARPP-32 is about 1/20 as abundant in the retina as in the putamen (26). These findings suggest that 33PP and DARPP-32 are different proteins.

Two-dimensional PAGE Analysis of Retinal and Pineal Phosphoproteins—To determine if the pI values of retinal and pineal 33PP are the same, retinal and pineal proteins were labeled with [γ - 32 P]ATP using the catalytic subunit of the PKA (Fig. 9). Pineal and retinal 33PP appeared to have the same pI according to their relative position in the two-dimensional-PAGE patterns of retina and pineal and comigration in a mixture of retina and pineal proteins. Accordingly, these spots appear to represent the same protein.

Identification of 33PP as the 33-kDa Cytoplasmic Protein (MEKA) Which Binds the $\text{G}\beta\gamma$ Complex of Transducin

The evidence that pineal and retinal 33PP are the same protein led to a search for known 33PP retinal proteins. This revealed that 33PP might be a retinal PKA substrate first

described by Lolley and his co-workers (27). The deduced amino acid sequence of this protein was reported during the course of this investigation by Kuo *et al.* (28), who term the protein MEKA, based on the four N-terminal amino acids. This protein has the unusual characteristic of binding to the $\beta\gamma$ complex of transducin to form a soluble cytoplasmic heterotrimer (29, 30).

Immunological Studies—To further establish the identity of 33PP, we used a monoclonal antiserum against MEKA.⁵ Western blot studies indicated that this antiserum detects a immunoreactive material in pineal and retina cytosol (Fig. 10A), but not in other tissues (Fig. 10D); and, that immunoreactivity comigrated with 33PP (Fig. 10, B and C). In addition, immunoreactivity in pineal and retinal cytosolic preparations comigrated with 33PP resolved by two-dimensional-PAGE (Fig. 11). This analysis also revealed the presence of two distinct immunoreactive spots, with most of the immunoreactivity resolved in the slightly more acidic spot. This is consistent with the conclusion that there are two isoforms of MEKA.

Presence of $G\beta$ in Pineal Supernatant Fractions—A prediction from the above consideration is that MEKA in pineal cytoplasm will cause $G\beta\gamma$ to appear in the cytoplasm. This was tested by Western blot analysis with an anti- $G\beta$ -serum (J-99). This revealed an immunopositive band in the 100,000 $\times g$ supernatant fraction of the pineal gland and of retina, but not in that of the liver, adrenal gland, total brain, or cerebellum (Fig. 12).

Coisolation of $G\beta$ -subunits and 33PP—To test the hypothesis that the β -subunit exists as a complex with 33PP in rat cytoplasm, a 100,000 $\times g$ supernatant preparation was fractionated by gel permeation chromatography. 33PP and $G\beta$ were both found in a 70-kDa fraction (Fig. 13). To verify that the calibration established using standards was valid in this analytical run, Western blot analysis was performed in which antilipocortin serum was used to identify lipocortin (42 kDa). This identified an immunopositive band in the predicted fraction, which was eluted two fractions after that which contained MEKA and immunoreactive β -subunits. The recovery of 33PP and $G\beta$ in a ≈ 70 -kDa fraction is consistent with the conclusion that they exist in a ≈ 70 -kDa complex in pineal cytoplasm, and provides further indication that 33PP is MEKA (27–30).

Comment on Other PKA Substrates

During the course of this investigation it became apparent that there are several PKA substrates in pinealocytes; some are also present in the retina (Figs. 6 and 9).

⁵ The antigen used to generate the MEKA antibody was prepared from a 20,000 $\times g$ (60 min) supernatant of bovine retinae; the supernatant was saturated with ammonium sulfate, and the pellet was recovered and solubilized; the solution was dialyzed. Samples containing 1 mg of protein were injected intraperitoneally into Lewis rats; this was repeated twice at 2-week periods. Spleen cells were fused with mouse myeloma cells (P3-X63-AG8-U1). The supernatants of the hybridoma cell cultures were screened; one was found to produce an antibody which bound to a 33-kDa protein in the rat pineal and retina but not in other tissues. This antibody was used to select clones from rat pineal, rat retina, and human retinal cDNA libraries. These were sequenced; the rat clones were essentially identical to each other and were $>85\%$ homologous to the human sequence and to the published bovine MEKA sequence (27, 28). The antibody was prepared in the laboratory of T. Abe, Akita University School of Medicine; cloning and sequencing was done in the laboratory of T. Shinohara, National Eye Institute (T. Abe, H. Tamada, T. Takagi, S. Sakuragi, K. Yamaki, H. Nakabayashi and T. Shinohara, manuscript in preparation). Personal communication from Dr. T. Shinohara.

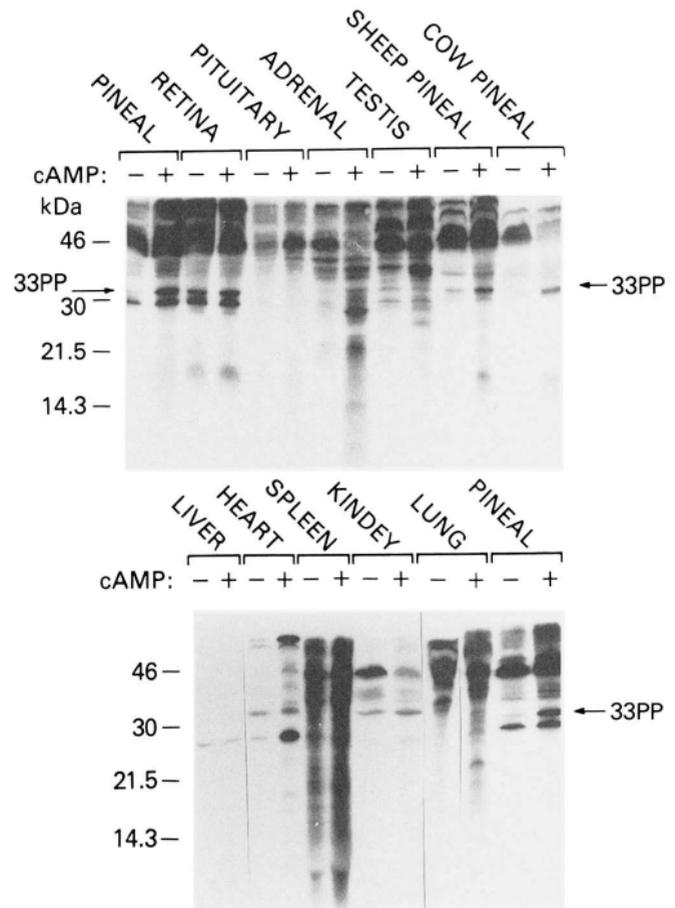


FIG. 7. Tissue survey for presence of cyclic AMP-dependent phosphorylation of a 33-kDa phosphoprotein. Extracts were prepared from a series of tissues by homogenization in 10 volumes of 20 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin. The supernatant was prepared (100,000 $\times g$, 60 min) and then used in phosphorylation studies. All tissues were obtained from rat unless otherwise indicated. Samples were treated $\pm 20 \mu\text{M}$ cyclic AMP in presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and analyzed by 15% PAGE (50 $\mu\text{g}/\text{lane}$) as described under "Experimental Procedures."

DISCUSSION

This investigation has revealed that the pineal contains MEKA, a protein kinase A substrate which was first identified in the retina. It is of special interest because it has been reported to bind the $\beta\gamma$ complex of transducin in a cytoplasmic complex (27–30). The presence of $G\beta\gamma$ complex in the cytoplasm is remarkable because it is generally thought of as being located exclusively in the plane of the membrane.

The presence of MEKA in only the pineal and retina place it among an interesting family of pineal/retinal proteins (for a broad review of pineal and retinal relationships see Ref. 32). These include the S-antigen and opsin kinase (33, 34), which are thought to be involved in signal transduction, and serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase (35, 36), which are involved in the synthesis of melatonin. Although the mammalian pineal does not contain all proteins involved in phototransduction (*i.e.* the α subunit of transducin and opsin), and therefore does not have the capacity to respond directly to light, this is not the case in lower vertebrates. Melatonin synthesis in the pineal organs of fish and frogs is controlled by light acting directly via rod-like structures which contain opsin and transducin (32, 37, 38). Light also acts directly on the bird pineal gland, which lacks the highly organized photoreceptor architecture of poikiloth-

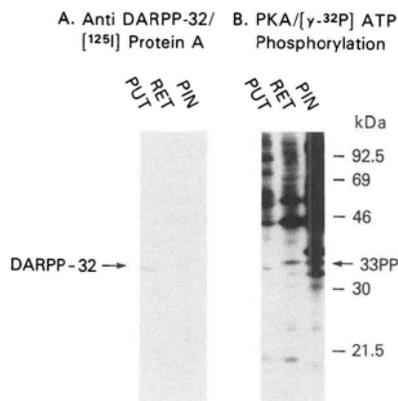


FIG. 8. Comparison of proteins of pineal, retina, and putamen. Unlabeled samples and samples labeled in presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the catalytic subunit of the PKA were analyzed by 12.5% PAGE. Samples were electroblotted on to PVDF membrane. The portion of the membrane containing unlabeled proteins was stained immunologically using an anti-DARPP-32 monoclonal antibody (1:10,000) and ^{125}I -labeled protein A according to published methods (30). The autoradiograph of this preparation is presented in A. An autoradiograph of the portion of the blot containing ^{32}P -labeled proteins is presented in B. The techniques used are detailed under "Experimental Procedures."

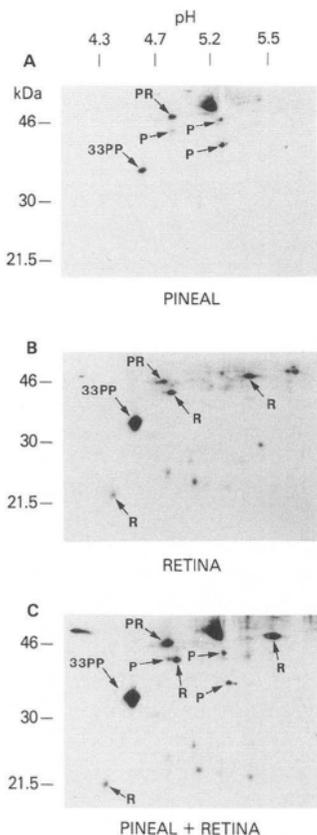


FIG. 9. Two-dimensional-PAGE analysis of rat pineal and retinal cytosol incubated with PKA and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Cytosolic fractions ($100,000 \times g$, 60 min) from rat pineal and retina were labeled for 10 min with $10 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase ($15 \mu\text{g}/\text{ml}$). Samples of cytosol ($50 \mu\text{g}$ of protein) from pineal (A) or retina (B) were analyzed. In addition, a mixture of $50 \mu\text{g}$ of pineal cytosol and $50 \mu\text{g}$ of retinal cytosol (C) was also analyzed as described under "Experimental Procedures." Phosphoproteins found only in the pineal are labeled P, those found only in the retinal preparation are labeled R, and those present in both preparations are labeled PR.

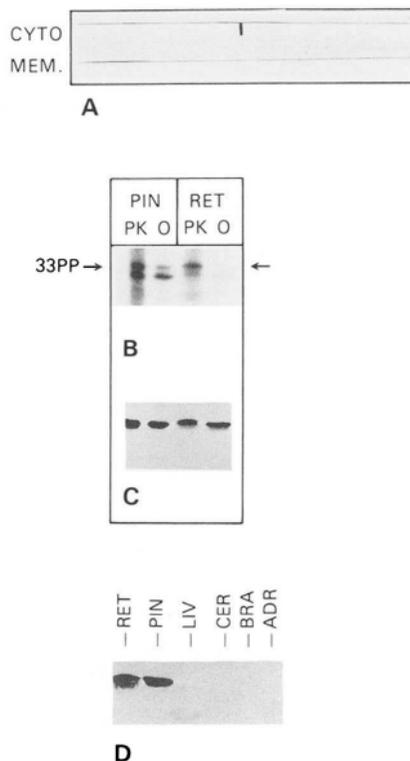


FIG. 10. Immunological analysis of 33PP. A, pineal cytosolic and membrane preparations were analyzed by 12.5% PAGE and electroblotted onto PVDF membrane. Unlabeled samples were stained using a monoclonal anti-bovine MEKA antibody (1:100 dilution, 18-h incubation; 18-h second antiserum incubation using goat anti-mouse serum).⁵ B, pineal and retinal samples were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (O) or presence of the catalytic subunit of the PKA (PK) and analyzed by 12.5% PAGE-electroblotting and autoradiography. C, the section of the blot in B containing the area of interest was removed and immunostained using the monoclonal anti-MEKA antibody as in A. D, cytosolic fractions from pineal (PIN), retina (RET), liver (LIV), cerebellum (CER), whole brain (BRA), and adrenal (ADR) were prepared after centrifugation ($100,000 \times g$, 60 min) and analyzed by 12.5% PAGE. Samples were electroblotted and immunostained as described in A.

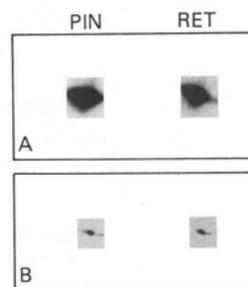


FIG. 11. Immunological identification of pineal and retinal 33PP in two-dimensional blots as MEKA. A, cytosolic fractions ($100,000 \times g$, 60 min) from rat pineal and retina were labeled for 10 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of the catalytic subunit of the PKA and analyzed by two-dimensional-PAGE electroblotting and autoradiography. B, sections of the blots in A containing 33PP were stained immunologically using an anti-MEKA antiserum as described in the legend to Fig. 10. These results were confirmed in a second study.

ermous vertebrates (39). The specialized structures and molecules shared by the vertebrate pineal and retina are the basis for the view that the pineal/retinal family of proteins evolved from a common ancestral photochemical transduction system.

As indicated above, MEKA is remarkable for its capacity to form a soluble cytoplasmic heterotrimer with $G\beta\gamma$. It seems

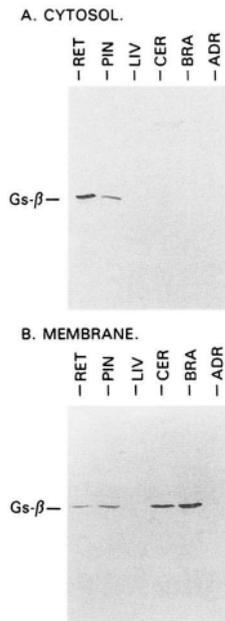


FIG. 12. Immunological detection of the $G\beta$ -subunit in cytosolic and membrane preparations. Cytosolic and membrane preparations were prepared from adrenal (*ADR*), whole brain (*BRA*), cerebellum (*CER*), liver (*LIV*), pineal (*PIN*), and retina (*RET*) after centrifugation at $100,000 \times g$, 60 min. Samples were resolved by 12.5% PAGE and electroblotted onto PVDF membrane. Membranes were stained using a polyclonal anti- $G\beta$ serum (J-99; 1:500, 18 h incubation; second antiserum = 18 h).

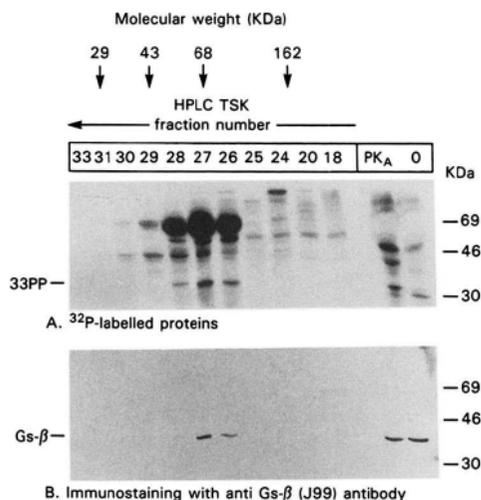


FIG. 13. Gel permeation chromatography of pineal cytosol. A 200- μ l sample of a cytosolic pineal preparation ($100,000 \times g$, 60 min) was applied to a TSK-3000 column and eluted at a flow rate of 1 ml/minute. The buffer was 0.1 M sodium phosphate buffer, pH 6.9, and 0.1 M sodium sulfate. Fractions (0.5 ml) were collected each 0.5 min. *A*, identification of 33PP. Fraction aliquots were labeled in the presence of [γ - 32 P]ATP and the catalytic subunit of the PKA and analyzed by 12.5% PAGE and autoradiography. Cytosolic preparations were labeled in absence (O) or in the presence of the catalytic subunit of PKA (PK_A). *B*, identification of the $G\beta$ -subunit. The presence of the $G\beta$ -subunit was analyzed in the corresponding blot by using a specific β -antiserum (J-99; 1:500 dilution, 18 h incubation; second antiserum incubation (goat anti-rabbit serum) period was 18 h. Lipocortin (42 kDa) was identified immunochemically in the second fraction after the one containing $G\beta$ -subunit and 33PP.

apparent that the soluble nature of MEKA reflects the strongly acidic nature of the protein. However, the basis of binding to the $G\beta\gamma$ complex is less obvious. The possibility

that binding of MEKA and all $G\alpha_s$ to $G\beta\gamma$ reflects a common amino acid sequence was investigated by sequence analysis; this failed to reveal a sequence shared by MEKA and all $G\alpha_s$.⁶

An issue which may help explain the formation of the MEKA- $G\beta\gamma$ complex is the precise identity of the γ -subunit. In the case of the retina it seems highly likely that it is the γ -subunit of transducin because it is abundant there. However, this has not been established directly. It is less likely that this is true of the mammalian pineal gland because it lacks several phototransduction-specific proteins, as indicated above, including the α -subunit of transducin. The eventual identification of the $G\gamma$ in the soluble heterotrimeric complex with MEKA will provide a better understanding of the role $G\gamma$ plays in both the formation of the complex and the tendency of the $G\beta\gamma$ complex to enter the cytoplasm and bind to MEKA.

The issue of function is perhaps most interesting. The characteristic of MEKA which brought it to our attention is that it is a PKA substrate. This suggests it functions in cyclic AMP transduction. Although cyclic AMP is generally thought of as being of little importance in signal transduction in the retina, it is the primary second messenger in the pineal gland and has several effects. There are several hypothetical modes through which this protein could participate in signal transduction in both tissues.

One is positive feedback. If phosphorylation of MEKA increased the affinity of MEKA for $G\beta\gamma$, this could draw it out of the membrane. The removal of $G\beta\gamma$ might tend to enhance signal transduction because this would increase the probability that α -subunit would not be bound as part of a G protein heterotrimer but would be free to act on G protein-regulated processes.

Another mode of action is feed forward. The complex could serve as a cytoplasmic analog of membrane-bound G proteins. In this hypothetical role phosphorylation of the binding protein is analogous to receptor occupancy. Phosphorylation might modulate association of the complex or of components of the complex with a target; the target could be an enzyme in the cytoplasm or membrane, or a membrane-bound ion channel. Modulation of the complex by phosphorylation could thereby control the function of a target. Cyclic AMP has a number of known effects on pineal metabolism, in addition to controlling melatonin production and *N*-acetyltransferase activity, as already mentioned. These include regulation of taurine release (40), GTP cyclohydrolase (biopterin synthesis) activity (41), and thyroxine 5'-deiodinase activity (42). Perhaps cyclic AMP regulates these or similar processes through PKA-dependent phosphorylation of MEKA.

A third hypothetical function is negative feedback. The cytoplasmic complex might serve as a reservoir for the $\beta\gamma$ complex. Phosphorylation could cause the heterotrimer to dissociate into free $G\beta\gamma$ and free MEKA. The free $G\beta\gamma$ might then enter the membrane and quench signal transduction globally by binding free $G\alpha_s$.

It will be of interest to pursue these hypothetical functions of MEKA and also to determine whether PKA substrates with binding characteristics similar to those of MEKA are present in the cytoplasm of other cell types.

⁶ The bovine MEKA protein showed limited homology with the α -subunits of various GTP-binding proteins in the NBRF data base (16). Homologous sequences consisting of four amino acids follow; positions of the first amino acid in the sequence of interest appear in parentheses (MEKA, α subunit); bovine retinal transducin $\alpha 1$ -subunit (rod), SLEE (8, 58); QFLE (122, 302); bovine retinal transducin $\alpha 2$ -subunit (cone), AAEY (159, 143); rat brain α -subunit of G_{α} , ELLS (196, 79); these sequences were shared with 135, 38, 51, and 119 other proteins, respectively.

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