Cyclic Nucleotide-Dependent Phosphorylation of Proteins in Rabbit Ciliary Processes

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Cyclic nucleotide-dependent protein phosphorylation in albino rabbit ciliary processes was studied in particulate and soluble fractions of the tissue by the technique of SDS-polyacrylamide gel electrophoresis and autoradiography. In the presence of gamma-32P-ATP, the soluble fraction showed increased phosphorylation of proteins of 200, 32 and 16 kDa molecular weight when 10 nM cAMP was added. Protein phosphorylation increased with time up to 5 min. No significant augmentation of phosphorylation was observed in the presence of 10 nM cGMP compared to control. In the particulate fraction, proteins with molecular weights of 200, 160, 105, 72, 58, 32 and 16 kDa showed increased phosphorylation in the presence of 10 nM cAMP. Phosphorylation caused by the addition of cAMP was maximal between 30 sec and 1 min for the particulate membrane fraction, but with longer incubation times the incorporation of phosphate residues decreased. The same molecular weight proteins of the membrane fraction that were phosphorylated in a cAMP-dependent manner were phosphorylated in the absence of exogenous cAMP by addition of either the catalytic subunit of cAMP-dependent protein kinase or activators of membrane-bound adenylate cyclase such as l-isoproterenol, vasoactive intestinal peptide, aluminum fluoride or forskolin. A cAMP-dependent dephosphorylation of a 56 kDa protein was observed in the membrane fraction. Cyclic GMP did not cause observable changes in the pattern of protein phosphorylation in the particulate fraction of rabbit ciliary processes. These results demonstrate that the particulate fraction of ciliary processes contains a complete cAMP signal transduction system comprising receptors coupled to adenylate cyclase, membrane-bound cAMP-dependent protein kinase (A-kinase), together with substrate proteins for the kinase enzyme(s). A-kinase activity and substrate proteins were also present in the soluble fraction of ciliary processes. Invest Ophthalmol Vis Sci 30:875–881, 1989

It is generally accepted that cAMP performs important roles in the ciliary process to modify aqueous humor production.1–5 Although adenylate cyclase may be a major second messenger system regulating water and/or electrolyte secretion, it is not clear whether an increase or a decrease in the intracellular level of cAMP in ciliary processes directly mediates the lowering of intraocular pressure. Cyclic nucleotides are considered to control cellular processes in eukaryotic cells by activating protein kinase enzymes, which in turn phosphorylate various substrate proteins regulating cellular functions.6 Relatively little information is available, however, on protein phosphorylation specifically dependent on the increased level of cyclic nucleotides in the ocular ciliary epithelium. Only recently, Coca-Prados reported cAMP-dependent phosphorylation of a 57 kDa protein, vimentin, in the ciliary epithelial cells.7 In previous papers we described the biochemical characterization of major classes of protein kinase enzymes in ciliary processes8 and signal-dependent protein phosphorylation in response to the Ca2+/calmodulin signal.9 In this report, we describe the stimulation of phosphorylation of endogenous proteins by exogenously added cyclic nucleotides in subcellular fractions of rabbit ciliary processes and also show that, in the particulate fraction of the tissue, essentially the same molecular weight proteins are phosphorylated by addition of cAMP, cAMP-dependent protein kinase (A-kinase), or activators of membrane-bound adenylate cyclase.

Materials and Methods

Materials

Gamma-32P-labelled ATP (specific activity, 25 Ci/mmol) was obtained from ICN Radiochemicals (Ir-
Preparation of Particulate and Soluble Fractions

The particulate and soluble fractions were separated essentially by the same method as described elsewhere. Briefly: New Zealand albino rabbits were anesthetized with intramuscular ketamine and were sacrificed by intravenous pentobarbital overdoses or air embolism. Enucleated eyes were kept in ice-cold saline and dissected within 30 min after death. Ciliary processes from two rabbits were carefully homogenized by hand in a glass-tellon homogenizer in 1 ml of Tris-HCl pH 7.4, 0.3 M sucrose, 5 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol and 10 μg/ml leupeptin (homogenizing buffer) with 30 strokes of the pestle. The homogenate was passed, through a double-layered nylon mesh (500 μm) and then centrifuged for 60 min at 100,000 g. The pellet was resuspended in the homogenizing buffer and used as the particulate fraction. The supernatant of the centrifugation was used as the soluble fraction. All procedures performed on rabbits conformed to the ARVO Resolution on the Use of Animals in Research.

Protein Phosphorylation

The reaction was performed in 1.5 ml tapered polypropylene microcentrifuge tubes. Twenty-five microliters of the reaction ingredients were preincubated at 30°C for 30 sec, and the reaction was started by adding 25 μl of the particulate (70-90 μg protein) or the soluble (100-130 μg protein) fraction. The reaction was stopped at times ranging from 10 sec to 5 min with 25 μl of stopping solution (125 mM Tris-HCl pH 6.8, 7.5% SDS, 15% 2-mercaptoethanol, 10 μg/ml leupeptin (homogenizing buffer) with 30 strokes of the pestle. The homogenate was passed, through a double-layered nylon mesh (500 μm) and then centrifuged for 60 min at 100,000 g. The pellet was resuspended in the homogenizing buffer and used as the particulate fraction. The supernatant of the centrifugation was used as the soluble fraction. All procedures performed on rabbits conformed to the ARVO Resolution on the Use of Animals in Research.

Electrophoresis and Autoradiography

Incubated samples treated with SDS and 2-mercaptoethanol as described above were subjected to electrophoresis on a 7.5 to 15% gradient SDS-polyacrylamide gel. Each gel lane was loaded with 30 μl of treated sample (28 to 36 μg protein for the particulate and 40 to 52 μg protein for the soluble fraction). After electrophoresis the gels were stained in Coomassie brilliant blue, destained by acetic acid/methanol and vacuum-dried. Molecular weights of separated proteins were calculated by comparing their relative mobility to the mobility of molecular weight marker proteins run in neighboring lanes. Proteins used as molecular weight markers were: myosin heavy chain (205 kDa), beta-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and lactalbumin (14 kDa). Phosphorylated proteins were detected by autoradiography on Kodak XO Mat AR film with a DuPont (Wilmington, DE) Cronex Lightning Plus intensifying screen. Film exposure was done for 3 to 48 hr at room temperature. Protein phosphorylation was quantitated by densitometry of autoradiograms using an LKB (Bromma, Sweden) UltroScan XL densitometer. Activation of phosphorylation was determined from the ratio of band density in the presence of cAMP to the density in the absence of cAMP (Tables 1, 2). Values of this ratio that differed from 1.00 by more than two SDs of the measurement were considered as significant.

Protein Determination

Protein concentration was determined with the dye-binding method using bovine serum albumin as a standard.

Results

Cyclic Nucleotide-Dependent Protein Phosphorylation in the Soluble Fraction

Addition of 10 μM cAMP to the soluble fraction augmented phosphorylation of proteins located at...
Table 1. Cyclic AMP-dependent phosphorylation of proteins in the soluble fraction of rabbit ciliary processes

<table>
<thead>
<tr>
<th>Protein band (kDa)</th>
<th>Activation Ratio (mean ± SEM, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cAMP/no activators)*</td>
</tr>
<tr>
<td>200</td>
<td>1.29 ± 0.05†</td>
</tr>
<tr>
<td>165</td>
<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>130</td>
<td>1.06 ± 0.09</td>
</tr>
<tr>
<td>105</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>62</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>50</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>42</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>32</td>
<td>1.25 ± 0.07†</td>
</tr>
<tr>
<td>21</td>
<td>1.02 ± 0.06</td>
</tr>
<tr>
<td>18</td>
<td>1.05 ± 0.02</td>
</tr>
<tr>
<td>16</td>
<td>1.57 ± 0.10†</td>
</tr>
</tbody>
</table>

* Denosimetric analysis of SDS-PAGE autoradiograms similar to Figure 1, lane F vs. lane E. Numerical values obtained from autoradiograms from three different experiments.
† Values greater than 1.00 ± 2 SD of the measurement indicate significantly increased phosphorylation.

200, 165, 62, 32 and 16 kDa apparent molecular weight by visual inspection (Fig. 1). Phosphate residues incorporated into the substrate proteins increased with the incubation time at least up to 5 min, the longest time point studied. Addition of 10 μM IBMX to inhibit phosphodiesterase activity did not change the pattern of protein phosphorylation (data not shown). Quantitation of increased phosphorylation by the addition of cAMP is shown in Table 1. After 1 min a 16 kDa protein was phosphorylated 57% more with 10 μM cAMP and 200 kDa and 32 kDa proteins were phosphorylated an average of 25% more compared to control. In contrast to cAMP, 10 μM cGMP did not increase the phosphorylation of proteins significantly. No changes were found with cGMP even at a very high concentration of Mg²⁺ (100 mM) (data not shown). Exogenously added catalytic subunit of bovine heart cAMP-dependent protein kinase (0.1 to 10 units/tube) did not increase phosphorylation of soluble proteins from rabbit ciliary processes (data not shown) in contrast to results on the particulate fraction (see below).

Table 2. Time course of cyclic AMP-dependent protein phosphorylation in the particulate fraction of rabbit ciliary processes

<table>
<thead>
<tr>
<th>Protein band (kDa)</th>
<th>30 sec incubation</th>
<th>1 min incubation</th>
<th>5 min incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cAMP/no activators)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1.32 ± 0.03†</td>
<td>1.26 ± 0.03†</td>
<td>1.17 ± 0.09</td>
</tr>
<tr>
<td>160</td>
<td>1.41 ± 0.08†</td>
<td>1.13 ± 0.10</td>
<td>1.33 ± 0.10</td>
</tr>
<tr>
<td>105</td>
<td>1.21 ± 0.02†</td>
<td>1.10 ± 0.03</td>
<td>1.19 ± 0.01</td>
</tr>
<tr>
<td>72</td>
<td>1.33 ± 0.04†</td>
<td>1.09 ± 0.03</td>
<td>1.17 ± 0.03</td>
</tr>
<tr>
<td>50</td>
<td>1.40 ± 0.09†</td>
<td>1.32 ± 0.04</td>
<td>1.18 ± 0.10</td>
</tr>
<tr>
<td>32</td>
<td>1.15 ± 0.06</td>
<td>0.85 ± 0.04†</td>
<td>0.67 ± 0.06†</td>
</tr>
<tr>
<td>21</td>
<td>1.19 ± 0.11</td>
<td>0.96 ± 0.06</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>18</td>
<td>1.43 ± 0.08†</td>
<td>1.16 ± 0.08</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>16</td>
<td>1.54 ± 0.10†</td>
<td>1.31 ± 0.09†</td>
<td>1.14 ± 0.03†</td>
</tr>
</tbody>
</table>

* Numerical values were obtained from densitometry of autoradiograms from three different experiments.
† Values greater than 1.00 ± 2 SD of the measurement indicate significant increased phosphorylation.
‡ Values less than 1.00 ± 2 SD of the measurement indicate a significant degree of dephosphorylation.

Cyclic Nucleotide-Dependent Protein Phosphorylation in the Particulate Fraction

In the membrane fraction, phosphorylation of proteins at 200, 160, 105, 58, 32 and 16 kDa in SDS gels was augmented by the addition of 10 μM cAMP in 1 min (Fig. 2). As found for the soluble fraction, addition of 10 μM IBMX did not increase the extent or change the overall pattern of protein phosphorylation seen with 10 μM added cAMP (data not shown). A 56 kDa protein showed an average of 33% less phosphorylation in the presence of cAMP at incubation times of 1 min or greater compared to the control (marked with arrow at left in Fig. 2). Densitometric analyses showed that substrate proteins were maximally phosphorylated at times between 30 sec and 1 min of incubation, and that with longer incubation less phosphate residues were incorporated into membrane-bound proteins (Fig. 2). Table 2 summarizes densitometric analyses of the time course of phos-
phorylation for each substrate protein. After 30 sec incubation, 200, 160, 105, 72, 58, 32 and 16 kDa proteins were all phosphorylated more with 10 nM cAMP present. However, after 5 min incubations, protein phosphorylation was less prominent than at 30 sec or 1 min. When whole homogenate was used instead of separated soluble or particulate fractions, more complicated autoradiograms were obtained that were essentially similar to superimposed autoradiograms of the soluble and particulate protein phosphorylations. Similar to the findings on the soluble fraction of the ciliary processes, when cGMP was compared to cAMP there was no significant enhancement of protein phosphorylation in the particulate fraction. Even when free Mg$^{2+}$ was raised from 6.6 mM to 96 mM, no increment in phosphate incorporation was observed with cGMP up to 5 min of incubation (data not shown).

Protein Phosphorylation by Exogenously Added cAMP-Dependent Protein Kinase

An exogenous kinase enzyme, the catalytic subunit of bovine heart cAMP-dependent protein kinase, was found to phosphorylate substrate proteins in the particulate fraction without adding cAMP (Fig. 3). SDS-polyacrylamide gel electrophoresis and autoradiographic analysis showed that essentially the same molecular weight proteins were phosphorylated by the exogenously added kinase as were found with cAMP activation of the endogenous kinase in the particulate fraction. Phosphorylation by the catalytic subunit of cAMP-dependent protein kinase was dose-dependent; the addition of ten enzyme units/tube gave stronger phosphorylation of the substrate proteins than did 10 μM cAMP (Fig. 3; compare lanes F and G).

Protein Phosphorylation by Activators of Adenylate Cyclase

Various activators of membrane-bound adenylate cyclase in the ciliary processes were incubated with the particulate fraction and gamma-labelled 32P-ATP. Isoproterenol at 10 nM (Fig. 4, lane B) or 1 μM VIP (Fig. 4, lane C) gave small increases in phosphorylation of membrane proteins best seen in the 16 and 32 kDa bands. More significant augmentation of protein phosphorylation was observed by the addition of a mixture of aluminum sulfate and sodium fluoride (0.1 mM and 2 mM) (Fig. 4, lane D) or 100 μM forskolin (Fig. 4, lane E), both of which can cause a maximal activation of adenylate cyclase via the G-protein or acting directly on the enzyme, respectively. Aluminum fluoride is apparently a more potent activator of protein phosphorylation than forskolin, and gave a similar degree of phosphorylation to that given by 10 μM cAMP itself (Fig. 4, compare lanes D, E and F, respectively). Densitometric analysis confirmed that endogenous protein substrates phosphor-
ulated by adding an activator of adenylate cyclase such as aluminum fluoride to the reaction are not essentially different from proteins which were phosphorylated in the presence of exogenously added cAMP (data not shown).

Discussion

The adenylate cyclase signal transduction system of the ciliary process membrane has been extensively investigated because a high activity of the cyclase is present in rabbit, bovine and human ciliary process tissue.13-16 Drugs that modify beta-adrenergic activity and/or the level of cAMP in the ciliary processes are known to affect the intraocular pressure, presumably by modifying the production of aqueous humor.17-19 The molecular mechanisms for cAMP-dependent modification of the intraocular pressure are unknown and few studies have been reported on steps beyond the generation of cAMP. Only recently Coca-Prados reported agonist-dependent phosphorylation of 78, 57, 48 and 36 kDa proteins in cultured cell lines derived from human and rabbit ciliary epithelial cells. The 57 kDa protein was identified as vimentin and immunocytochemistry showed that vimentin is a major cytoskeletal protein of the ciliary epithelial cell.7 While the use of intact cultured cells gives cellular specificity, there is a possibility that crossover effects involving more than one second messenger system, such as crossover between the Ca2+ and cAMP systems, may occur because the intracellular second messenger cannot be directly controlled by the investigator.20,21 To avoid this, we have used a simpler broken cell system where the second messenger concentration and the time of exposure can be controlled.

In this study, cAMP-dependent protein phosphorylation was found to be present in both the soluble and particulate fractions of rabbit ciliary processes (Figs. 1-2), but no cGMP-dependent protein phosphorylation was observed in either fraction or whole homogenate. This finding is in accord with our previous biochemical study of kinases in ciliary processes using exogenous substrates, where no significant cGMP-dependent protein kinase activity was detectable.8 Because ciliary processes do contain cGMP and a guanylate cyclase activated by atrial natriuretic peptide,22 our results suggest that cGMP is probably not a major second messenger for direct regulation of protein phosphorylation. Thus cGMP may have other functions in ciliary processes.

At the level of resolution of gradient gel electrophoresis in the current study, a total of 11 bands of phosphorylated protein substrates in the soluble and the membrane fraction were regulated in a cAMP signal-dependent manner. Three of these bands, at 200, 32 and 16 kDa, were found in both soluble and particulate fractions (Tables 1, 2). Whether these bands are the same proteins distributed in both soluble and membrane fractions cannot be determined at the level of resolution obtained by the one-dimension gradient electrophoresis method. When whole homogenate was used, more complicated autoradiograms similar to the superimposed pattern of the soluble and membrane fraction were observed. As no new bands appeared, no further information seemed to be available from the total homogenate system (data not shown). The 58 kDa substrate band seen in the membrane fraction (Table 2) may be the 57 kDa protein, vimentin, that Coca-Prados7 showed in his study. In our previous study on Ca2+-dependent protein phosphorylation in the particulate fraction of ciliary processes, we found Ca2+/calmodulin-dependent phosphorylation of vimentin.5 The magnitude of phosphorylation in this band is much greater with the Ca2+/calmodulin signal (3-fold increase), permitting identification by a Western blot, but it is not sufficient for identification in the case of the cAMP signal obtained in the current experiments.

The time course of protein phosphorylation was totally different in the soluble and in the particulate fractions (Figs. 1, 2). In the membrane fraction rather rapid dephosphorylation occurred after 1 min while in the soluble fraction phosphorylation steadily in-
creased with time up to 5 min (Figs. 1, 2). The significance of relatively rapid dephosphorylation is not known, but if signal-dependent phosphorylation is an important process in cellular function, then dephosphorylation might also take place fairly rapidly because it may function as a turn-off of the signal. In our earlier study of Ca<sup>2+</sup>-dependent phosphorylation, rapid dephosphorylation of proteins in the membrane fraction was found, and one specific protein at 43 kDa was dephosphorylated in a Ca<sup>2+</sup> signal-dependent manner. In this study we also noted one protein band at 56 kDa where more dephosphorylation was observed with cAMP than in the control (Fig. 2, Table 2). This indicates that there may be a cAMP-dependent protein phosphatase activity in the membrane fraction.

The catalytic subunit of cAMP-dependent protein kinase was not effective in phosphorylating ciliary process soluble proteins. The reason for this observation is not known, but could be due to the presence of an inhibitor of the exogenous kinase in the cytosol. By contrast, in the washed membrane fraction, proteins were effectively phosphorylated by exogenous catalytic subunit of cAMP-dependent protein kinase enzyme in the absence of cAMP. Activators of membrane-bound adenylate cyclase also caused the phosphorylation of essentially the same molecular weight proteins which were phosphorylated in response to cAMP itself (Figs. 3, 4).

These results provide strong evidence that protein bands that we observed to be phosphorylated in response to cAMP are the substrates of endogenous cAMP-dependent protein kinase enzymes. Furthermore, the activation of membrane-bound adenylate cyclase in the particulate preparations was sufficient to cause an increase in phosphorylation of the same membrane substrate proteins as seen with added cAMP. Under our standard assay conditions for adenylate cyclase, the level of maximal cyclase activation in ciliary process membranes is highest with forskolin, followed by aluminum fluoride, vasoactive intestinal peptide and isoproterenol. Using the same four substances and doses to activate adenylate cyclase, cAMP-dependent protein kinases, and substrate proteins, requires only the addition of a receptor agonist (e.g., VIP) in the presence of Mg ATP. Isolation and identification of these protein substrates will be required to provide further understanding of their cellular functions in ciliary processes and their role in responses to drugs that affect cellular cAMP and aqueous humor formation.

Key words: rabbit, ciliary processes, cyclic nucleotide-dependent protein phosphorylation, endogenous substrates, activators of adenylate cyclase

References