Immunohistochemical Localization of 3':5'-Cyclic AMP and 3':5'-Cyclic GMP in Rat Liver, Intestine, and Testis
(nuclear cyclic GMP/immunocytochemistry)

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ABSTRACT Cyclic GMP and cyclic AMP have been localized in rat liver, small intestine, and testis by a fluorescent immunocytochemical procedure. In liver, cyclic AMP is distributed along sinusoids predominantly, and increased fluorescence is seen in sinusoidal areas after glucagon administration. Cyclic GMP is located in nuclear elements and on the plasma membranes of hepatocytes. In jejunum, cyclic AMP is found predominantly at the basal and lateral sides of brush border cells and in the lamina propria, while cyclic GMP is located to the brush border membrane, smooth muscle, and nuclear elements. In testis, cyclic AMP is found in cytoplasm of cells at the perimeter of the seminiferous tubules and in interstitial cells, while cyclic GMP is visualized on the plasma membrane of the cells lining the tubules. Cyclic GMP is also seen on chromosomes of premeiotic spermatocytes and in sperm. These data provide histological evidence implicating diverse roles for the nucleotides in these tissues. The nuclear localization of cyclic GMP in all of these tissues suggests a role for the nucleotide in nucleus-directed events.

Accumulating evidence suggests that cyclic GMP is involved in a number of cellular events ranging from the action of acetylcholine at muscarinic receptors (1–3) to growth regulation in lymphocytes (4) and fibroblasts (5). In these studies increased tissue concentrations of cyclic GMP have been demonstrated after hormonal stimulation. Despite this evidence, the role of cyclic GMP in cell function has been difficult to determine, partly because exogenously administered cyclic GMP in most tissues acts as a weaker cyclic AMP (6).

To gain insight into the relative roles of cyclic AMP and cyclic GMP in cell function, we have recently applied the technique of cyclic nucleotide immunocytochemistry to studies in canine thyroid tissue, and contrasted the localization of cyclic GMP with that of cyclic AMP. We utilized canine thyroid tissue for these studies because thyrotrophin stimulating hormone increases cyclic AMP, without affecting cyclic GMP levels, and cholineric compounds increase cyclic GMP while cyclic AMP is unchanged. We found cyclic GMP to be located in the area of the follicular plasma membrane bordering the colloid, and showed an increase in cytoplasmic fluorescence after acetylcholine treatment. In contrast, cyclic AMP was found throughout the cytoplasm of the follicular cells, and increased cytoplasmic fluorescence was seen after administration of thyrotrophin stimulating hormone. The distinct differences in localization of cyclic AMP and cyclic GMP suggested that the specific roles and the intracellular localization of these two nucleotides may be related (7).

We have continued to apply this immunocytochemical procedure for the localization of cyclic AMP and cyclic GMP in a variety of rat tissues. In general, the two nucleotides have distinctly different staining patterns in individual cells. Cyclic AMP is found mostly in cytoplasm and sometimes on plasma membranes, but cyclic GMP is usually distinctly localized, most commonly in plasma membranes and nuclear elements. Since this methodology is applied to unfixed tissues, we are most likely detecting cyclic nucleotide bound to receptor sites. These studies then provide clues for the location of receptors for cyclic AMP and cyclic GMP, which in turn suggest separate roles for the nucleotides, particularly cyclic GMP in cell function.

MATERIALS AND METHODS

Preparation of Tissue Samples. Fed male Sprague–Dawley rats, weighing 180–200 g, were utilized in these studies. Intact animals were killed by blunt trauma in order to minimize stress and pieces of liver, jejunum, and testis were frozen immediately in an aluminum foil boat filled with Optimal Cutting Temperature Compound (Ames Co., Division Miles Laboratories, Inc., Elkhart, Ind.) by immersion in ice-cold acetone. Hypophysectomized rats, purchased from Hormone Assay Laboratories, Chicago, Ill., were sacrificed by cervical dislocation on the tenth day after hypophysectomy and samples of testicular tissue were frozen as described above.

Materials. Antibodies to cyclic nucleotides were prepared as described elsewhere (8). Rabbit gamma globulins and fluorescein-labeled goat antibodies to rabbit IgG (lot 16) were obtained from Miles Laboratories, Inc., Miles Research Division (Kankakee, Ill.). Crystalline glucagon (lot 62C-2450) was obtained from Sigma Chemical Co., St. Louis, Mo. and was dissolved in 1% albumin-0.9% saline for intraperitoneal administration. Control animals received an equal quantity of the vehicle solution intraperitoneally.

Histochromic Procedure. Histochromic localization (9) of both cyclic nucleotides was determined by an immunofluorescent procedure on frozen tissue sections 4–6 μm in thickness with the use of highly specific immunoglobulin IgG from rabbit antisera to either cyclic AMP or cyclic GMP as prepared by the method of Steiner et al. (8). The frozen sections were dried in air on slides and treated in sequence for 30 min with each of the following: antibody to nucleotide (1:10 dilution of antibody to cyclic AMP and 1:8 dilution of antibody to cyclic GMP or 1:10 control serum); phosphate-buffered saline (PBS); fluorescein-labeled goat antiserum to rabbit IgG in a 1:8 dilution; and PBS. The slides were

Abbreviations: cyclic GMP, guanosine 3':5'-cyclic monophosphate; cyclic AMP, adenosine 3':5'-cyclic monophosphate.

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Immunohistochemical Localization of cAMP and cGMP

FIG. 1. Immunofluorescent localization of cyclic AMP (a) and cyclic GMP (b) in rat liver (×120). (c) is a photograph illustrating nonspecific fluorescence. In (d), the cyclic GMP antibody has been incubated with cyclic GMP (5 μM) prior to staining.

mounted with 50% glycerin in PBS. To demonstrate the specificity of the procedure, we found that similarly prepared antisera from unimmunized rabbits failed to produce significant staining. Furthermore, when the antiserum was incubated overnight at 4°C with the corresponding cyclic nucleotide at 5 μM, the staining pattern was no longer present, indicating competitive inhibition of cyclic nucleotide binding. Incubation with 50–100 fold higher concentrations of the other cyclic nucleotide were required to eliminate the staining pattern (9). When the antibodies were incubated with ATP or 5'-AMP (10 μM) staining was not eliminated.

RESULTS

We have compared the distribution of cyclic GMP and cyclic AMP in three rat tissues.

Liver. In Fig. 1 is shown the distribution of cyclic AMP and cyclic GMP in liver from a control rat. Cyclic AMP is distributed predominantly along sinusoids (Fig. 1a). Both plasma membranes and cytoplasmic elements fluoresce brightly. Perinuclear staining is seen also. While the sinusoidal staining is most likely of hepatocytes, we cannot eliminate the possibility that the fluorescence includes elements of the reticuloendothelial system. Intranuclear elements do not fluoresce. In contrast, cyclic GMP is localized predominantly in nuclei and plasma membranes of the hepatocytes (Fig. 1b). The plasma membranes of each parenchymal cell fluoresce, outlining individual cells. There is no outlining of sinusoidal areas and fluorescence in cytoplasm is minimal. Nucleoli fluoresce brightly and in some nuclei a clumped chromatin pattern is seen. When the cyclic GMP antiserum is incubated with 5 μM cyclic GMP, both plasma membrane and nuclear fluorescence are eliminated (Fig. 1d). A control with serum from unimmunized rabbits is shown in Fig. 1c.

Glucagon has been shown to increase cyclic AMP levels in liver and increases the release of the nucleotide into the extra-

FIG. 2. Dark field fluorescence micrographs of rat liver, illustrating the relative intensity of fluorescent staining for cyclic AMP in liver from rats injected with glucagon (a) or from a control animal (b) (×86.5). The exposure times for the photographs were identical. At the bottom of (b) is fluorescent staining in the area of a central vein.
cellular fluid during liver perfusion (10). Rats were injected with glucagon, 1 mg, and the liver was frozen 10 min later. Cyclic AMP levels were measured by radioimmunoassay and increased 4-fold in the glucagon-treated rats. In the animals treated with glucagon (Fig. 2a), there was a marked increase in fluorescence compared to liver from a control rat (Fig. 2b). The increased fluorescence in the glucagon-treated animals is concentrated along sinusoids and membrane fluorescence appears to increase.

Small Intestine. The distributions of cyclic GMP and cyclic AMP in rat small intestine are distinctly different. Cyclic GMP is localized in the villus brush border membrane with minimal staining for cyclic GMP in other areas of the villus tip (Fig. 3a). This brush border localization of cyclic GMP is virtually eliminated when the cyclic GMP antibody is incubated with 5 μM cyclic GMP. Cyclic GMP is also found in nuclei, especially of cells in the crypts areas. In many cells, the nucleoli fluoresce (Fig. 3b). Smooth muscle also fluoresces brightly (Fig. 3b).

Cyclic AMP is localized in the lateral and basal sides of the cells of the villus tip (Fig. 3c and d). Minimal staining is found in the brush border area. This localization of cyclic AMP is consistent with the distribution of adenylate cyclase from mucosal epithelial cells of rabbit intestine (11). Cyclic AMP is also found in the laminal propria (Fig. 3c) and in smooth muscle (photograph not shown). Nuclear fluorescence with the cyclic AMP antibody is minimal.

Testis. In Fig. 4a the distribution of cyclic AMP is shown within seminiferous tubules and the interstitial area of a mature rat. Prominent staining is found in the cytoplasm and membranes of the cells on the perimeter of the tubule. These cells include both Sertoli and germ cells. Interstitial cells also fluoresce brightly. Cells in the center of the tubules show only minimal fluorescence (photograph not shown). Sperm fluoresce also.

In contrast, cyclic GMP is found predominantly on membranes, but not in the cytoplasm, of cells bordering the tubular membrane (Fig. 4b). In primary spermatocytes prior to meiosis chromosomes fluoresce brightly. The specificity of this chromosomal localization of cyclic GMP in this particular cell pool is demonstrated by the absence of chromosomal staining in the cells bordering the peritubular membrane and in cells in the center of the tubule that have completed meiosis. When the cyclic GMP antibody is incubated with 5 μM cyclic GMP, chromosomal staining disappears, but fluorescence remains when the antibody is incubated with 50-fold greater concentrations of cyclic AMP. Incubation of the antiserum with other nucleotides such as ATP and GTP at significantly

![Fig. 3. Immunofluorescent localization of cyclic AMP and cyclic GMP in jejunum of the rat (×120). (a) Cyclic GMP is localized on the brush border membrane and nucleus in this longitudinal section. (b) is an area from the junction of the mucosal and smooth muscle areas (tangential section). In (c) and (d) the sections have been stained with the cyclic AMP antiserum. (c) is a longitudinal section while (d) is a cross section from the crypts area. The arrows point to fluorescence at the lateral side of the brush border cells. Note the minimal fluorescence in (c) and (d) in the area of the brush border membranes.](image-url)
higher concentrations does not eliminate fluorescence. In addition, in animals hypophysectomized for 10 days, chromosomal localization of cyclic GMP is not seen, but cyclic GMP is seen in nuclear elements in a clumped chromatin pattern (Fig. 4c). Cyclic GMP is also found in sperm from mature animals (Fig. 4d).

**DISCUSSION**

The demonstration by this immunocytochemical method that cyclic AMP and cyclic GMP are uniquely distributed in a variety of rat tissues helps to elucidate the roles of the two nucleotides in cell function. The observation that cyclic GMP is found on the plasma membrane of a number of tissues, including canine thyroid (7), liver, small intestinal brush border, and testicular cells, suggests that the nucleotide is involved in membrane function. Since this immunocytochemical procedure is performed on unfixed tissue, the free cyclic nucleotides should be lost during the staining procedure and only those nucleotides bound to cellular receptors should be depicted. Our observation of plasma membrane staining for cyclic GMP thus suggests the presence of binding sites for cyclic GMP in plasma membranes in a variety of tissues.

It is important to emphasize that this immunocytochemical technique depends upon the recognition by the antibodies of specific antigenic determinants for the cyclic nucleotides in tissue. These determinants most likely include the 3':5' cyclic ring and specific substitutions on the purine nucleus. Fluorescent staining indicates available sites for antibody recognition, while the absence of staining is consistent with determinants that are unavailable to the immunological reagents. Thus, it is possible that either cyclic nucleotide might be present at specific cellular sites and not be recognized by antibody. Our experience with a number of cyclic GMP and cyclic AMP antisera from different rabbits is that the staining patterns are consistent for each cyclic nucleotide. Since each antiserum most likely contains a number of antibodies with different affinities for the cyclic nucleotide, this consistency of staining pattern decreases but does not eliminate the possibility that a cyclic nucleotide may be present at a receptor site, but is unrecognized by antibody. Studies that combine measurement of total nucleotide content in subcellular fractions with immunohistochemical localization should help to determine if cyclic nucleotide at certain sites is not detected by this immunohistochemical procedure.

The localization of cyclic GMP in nuclear elements suggests a role for the nucleotide in growth regulation in liver. The significance of the plasma membrane staining for cyclic GMP remains to be determined. Since insulin and cholinergic agents have been reported to raise cyclic GMP levels in liver slices

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**Fig. 4.** Immunofluorescent localization of cyclic AMP and cyclic GMP in rat testis. All views are in cross section. In (a) fluorescence is seen in three seminiferous tubules and an interstitial area stained with the cyclic AMP antiserum (X120). In (b) the sections have been stained for cyclic GMP (X120). Note the fluorescence on membranes of the cells lining the tubule, chromosomal fluorescence, and the absence of fluorescence in the center of the tubule. Insert shows the fluorescence of the premeiotic chromosomes (X180). (c) Testis from a rat hypophysectomized 10 days previously and stained for cyclic GMP (X120). Note nuclear fluorescence, but not on condensed chromosomes as in (b). (d) is a lower power view stained for cyclic GMP (X80). Note fluorescence in sperm.
in vitro (13), additional studies with these stimulants would be of interest. The absence of significant cytoplasmic staining for cyclic GMP might explain the low levels (10 nM) that have been observed for the nucleotide in this tissue.

The localization of cyclic GMP on the brush border membrane of the small intestinal villus suggests a role for the nucleotide in intestinal transport. It is of interest that guanylate cyclase activity in small intestine is mostly particulate (12). Further studies exploring the regulation of guanylate cyclase and cyclic GMP action in small intestinal transport mechanisms are indicated by the present immuno-cytochemical studies. The predominant localization of cyclic AMP in the basal and lateral sides of the cells of the villus and not at the brush border is consistent with the finding of adenylate cyclase at these sites in rabbit intestine (11).

The localization of cyclic GMP in nucleus in liver, small intestine, adrenal cortex (14), and testis suggests that this nucleotide serves a regulatory function in nucleus-directed events. The most striking staining is found in nucleoli of cells from these tissues, and along the chromosomes of primary spermatocytes in testis. The role that the nucleotide serves at these sites is not known, but this cytochemical localization provides clues for receptors for cyclic GMP in nuclear elements. Since ribosomal RNA synthesis in nuclei is a site for regulation of polymerase I (15), our studies suggest that the nucleotide might be involved in the control of this other nucleolar enzymes. We have found recently that the levels of cyclic GMP in rat adrenal increase within one hour after hypophysectomy and decrease to below control values within 15 min of adrenocorticotropic hormone administration, and in chronically hypophysectomized rats levels of cyclic GMP are nearly equal to those of cyclic AMP (14). Cyclic GMP is located predominately in nucleoli and other nuclear elements, while cyclic AMP is found in cytoplasm of the cells of the zona fasciculata (14). When adrenal homogenates are fractionated by differential centrifugation in sucrose, and the absolute amount of cyclic nucleotide is determined, we find that nuclear elements contain approximately 50% of total cellular cyclic GMP and less than 10% of cyclic AMP (14), confirming the immuno-cytochemical localization. These studies suggest that cyclic GMP may play a repressor role in adrenal growth regulation.

In testis the demonstration of cyclic GMP on condensed chromosomes prior to meiosis, but not on chromosomes in spermatocytes at other stages of development, suggests that the localization of the nucleotide varies during the cell cycle. In lymphocytes (4) and fibroblasts (5) cyclic GMP levels increase transiently after the addition of growth-promoting factors. While abundant evidence has accumulated which suggests that both cyclic AMP and cyclic GMP are involved in the regulation of the cell cycle, the mechanisms for such control have not been thoroughly determined. The application of cyclic nucleotide immunocytochemistry to cultured cells in studies of growth regulation by growth substances should be helpful in determining in greater detail the role of the cyclic nucleotides in the control of the cell cycle.

The localization of both cyclic nucleotides in individual cells in heterogeneous tissues and in specific sites within cells points out that the measurement of total cyclic nucleotide levels in tissues might not be a sensitive index of cyclic nucleotide levels within individual cells or in cellular compartments. It is conceivable that hormonal stimulation might cause a redistribution of either cyclic nucleotide in particular tissue without changing the total tissue level of the nucleotide. In preliminary experiments, we have found that the total concentration of cyclic GMP is unchanged in testis from 10 day hypophysectomized rats as compared to the mature animal, and yet by immunocytochemistry membrane and particularly nuclear (not on condensed chromosomes) localization of cyclic GMP is increased. Studies in other tissues should confirm whether this will be a general observation.

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