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Immunohistochemical localization of 3′:5′-cyclic AMP and 3′:5′-cyclic GMP in rat renal cortex: Effect of parathyroid hormone

(glomeruli/tubules/calcium)

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ABSTRACT **Adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) were localized in cells of rat kidney cortex by an immunocytochemical technique before and after perfusion with parathyroid hormone (PTH). In control tissues the cAMP antiserum detected approximately the same intensity of fluorescence in cytoplasmic epithelial cell elements of cortical tubules and glomeruli (cells of Bowman's capsule and podocytes). PTH increased fluorescence in these glomerular cells and increased cAMP fluorescence in cytoplasmic granules in proximal tubular cells. These granules, whose structure has not been identified, were located predominantly on the luminal side of the tubular cells. In control rats, the renal cortical fluorescence detected with the cGMP antiserum was more pronounced in glomeruli (predominantly in the mesangial areas) and lesser amounts of fluorescence were observed in tubules. After PTH treatment, cGMP fluorescence increased in glomeruli and in renal tubular cells. A bright linear pattern of fluorescence was found in the area of the tubular luminal membrane. Perfusion with PTH caused relatively small increases in total tissue cAMP and no consistent increases in total tissue cGMP. Our observations suggest that both cAMP and cGMP are involved in the glomerular and tubular responses to PTH and point out the added dimension that this immunocytochemical technique brings to studies of cyclic nucleotide dynamics in heterogeneous tissues.**

The renal effect of parathyroid hormone (PTH), an agent that regulates a number of functions including the reabsorption of filtered calcium and phosphate, is believed to be mediated by an increase in the generation of adenosine 3′:5′-cyclic monophosphate (cAMP) in renal cortical cells (1–3). Evidence for the role of cAMP as a "second messenger" includes the observations that: (*i*) PTH stimulates adenylate cyclase in renal cortical tissue (3) in both glomeruli (4, 5) and tubules (6); (*ii*) cAMP levels increase in renal cortex after PTH administration (2, 3); (*iii*) and the effects of PTH on renal cortex can be mimicked by administration of exogeneous cAMP, its analogs, or cyclic 3′:5′-nucleotide phosphodiesterase inhibitors (2, 7).

Guanosine 3′:5′-cyclic monophosphate (cGMP) is present in renal cortex (8) but its role in hormonal regulation has not been established. PTH neither increases total tissue cGMP in renal cortical slices *in vitro* (8) nor stimulates cGMP formation in either homogenates from renal cortex (9) or preparations of glomeruli or tubules (10). On the other hand, in several studies, an increase in urinary cGMP was observed after PTH administration or after elevation of endogenous PTH (11–14). It was not established in these studies whether increases in urinary cGMP excretion were due solely to a direct effect of PTH on intrarenal cGMP formation or were secondary to glomerular filtration of cGMP and active uptake of the nucleotide into tubule cells from the peritubular circulation (15, 16). Because there is a close correlation between cGMP levels and transcellular fluxes of calcium in various tissues (17), a process regulated in the kidney by PTH (2), it seemed pertinent to examine in further detail a possible role for cGMP in the action of PTH in the kidney. With an immunohistochemical method previously used for localization of 3′:5′-cyclic nucleotides in other tissues (18, 19), we studied the distribution of immunoreactive cAMP

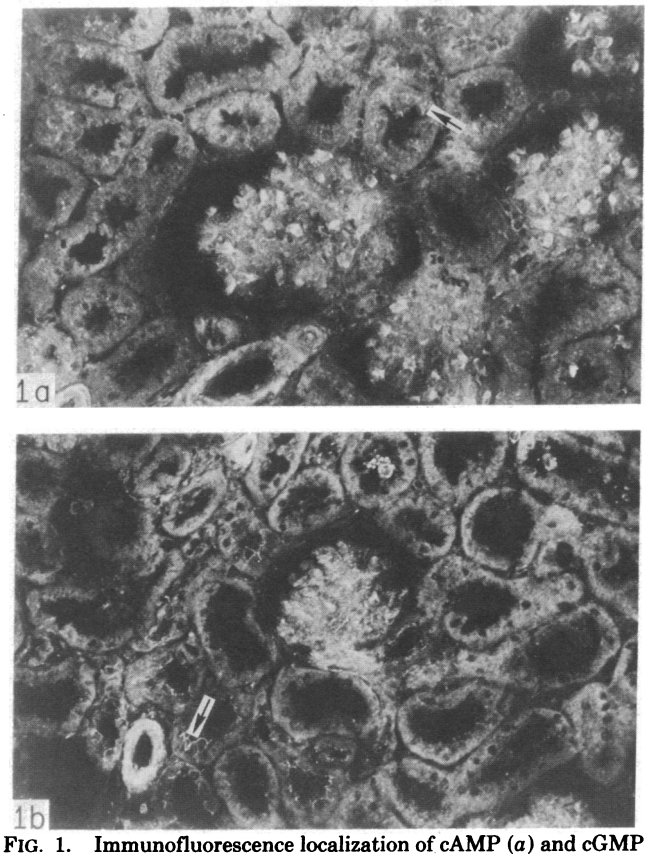

FIG. 1. Immunofluorescence localization of cAMP (*a*) and cGMP (*b*) in PTH-perfused renal cortex. In many proximal tubules, specific granules fluoresce with the cAMP antibody (arrow in *a*). In glomeruli, bright cytoplasmic fluorescence with the cAMP antibody is seen in podocytes and some cells in Bowman's space. Arrow in *b* marks linear fluorescence in the area of the luminal membrane of a proximal tubule after PTH administration. Note also the bright fluorescence in the glomerulus in areas of mesangial cells. (×235.)

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Abbreviations: PTH, parathyroid hormone; cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate.

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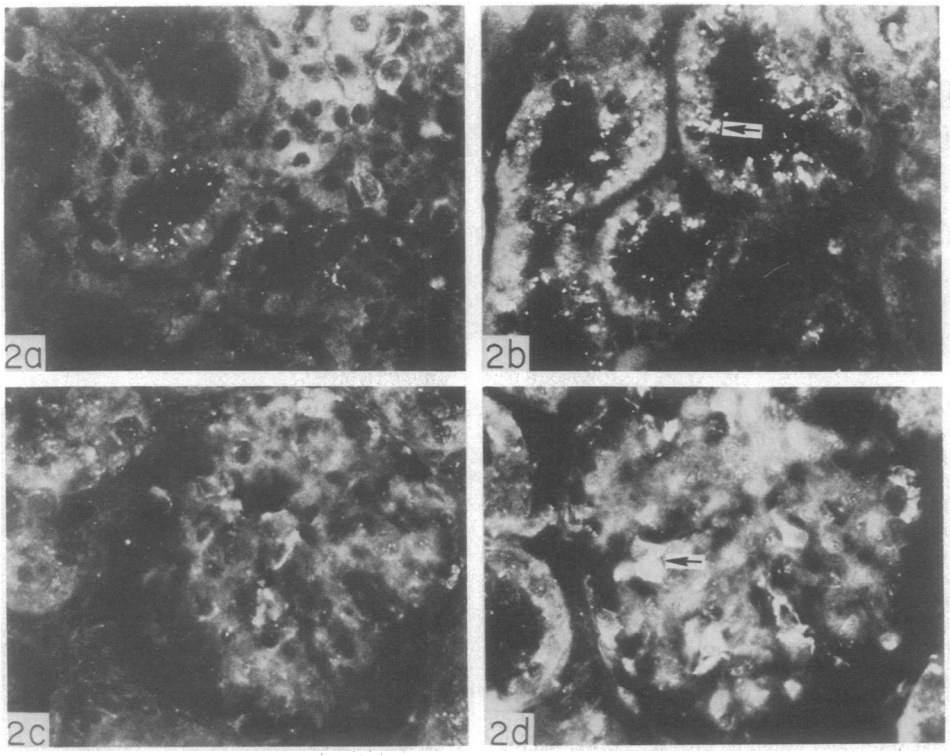

FIG. 2. Effects of PTH on immunoreactive cAMP in tubules (*a* and *b*) and glomerulus (*c* and *d*). (*a*) Tubules, control kidney. Note diffuse cytoplasmic staining in some tubules and scanty fluorescence in granules. (*b*) Tubules, PTH-perfused kidney. Note diffuse staining in cytoplasm and increase in the number and brightness of granules predominantly but not exclusively at periluminal areas of tubular cells (arrow). (*c*) Glomerulus, control kidney. (*d*) Glomerulus, PTH-perfused kidney. Note the increase in cytoplasmic fluorescence of podocytes (arrow) and cells in Bowman's space. (×500.)

and cGMP in rat renal cortex after PTH administration. In addition, total tissue levels of these cyclic nucleotides were determined.

METHODS AND MATERIALS

Preparation of Tissue Samples. Sprague–Dawley rats (about 200 g) were surgically thyroparathyroidectomized 4–6 hr before the experiment. Animals were then anesthetized (pentobarbital, 80 mg/kg intraperitoneally), and the abdominal and chest cavities were quickly opened. A polyethylene cannula was inserted into the thoracic aorta with the tip placed above the branching of the renal arteries; the right atrium was opened to allow outflow of perfusate passed through the kidney. Immediately after the insertion of the cannula, the splanchnic area including the kidney was perfused with Krebs–Ringer bicarbonate buffer containing 1 mM $CaCl_2$ and saturated with 95% O_2/5% CO_2, warmed to 37°. Perfusion was at a high rate (about 39 ml/min) for about 3–5 min until the surfaces of both kidneys were blanched completely, indicating removal of blood. Perfusion then was continued at 3–5 ml/min for about 10 min. At the end of this period the pedicle of one kidney was clamped with a hemostat and the kidney removed as a control specimen. Perfusion was continued for another 3–5 min; then the same solution but containing PTH at 7 units/ml was infused at the same rate. After infusion of 10 ml of solution containing PTH, the second (experimental kidney was removed). Twenty different experimental animals were infused.

Histochemical and Analytical Procedures. Kidneys (control or experimental), immediately upon removal, were stripped of their capsule, and the cortical portions were dissected with a razor blade. One portion of the cortical tissue was embedded in gum tragacanth and quickly frozen on a brass chuck in liquid nitrogen (it took less than 1 min from removal of the kidneys to completion of freezing). In some experiments, another piece of renal cortex tissue was homogenized in 6% (wt/vol) trichloroacetic acid for cAMP and cGMP determinations (8).

Blocks of cortical tissues embedded in gum tragacanth were stored in liquid nitrogen overnight; then tissue sections (4–6 μm) were cut in a cryostat. Tissue sections (about five of each specimen) were dried in open air and the intracellular location of both cAMP and cGMP was determined by an immunofluorescence procedure as described (18, 19). Antibodies were diluted in 0.01 M phosphate/0.15 M NaCl, pH 7.35. The specificity of the staining patterns was established by showing that prior incubation of the antiserum with the respective cyclic nucleotide at a concentration of 5×10^{-6} M could competively inhibit the cAMP or cGMP staining pattern (18). In addition, application of IgG from an unimmunized rabbit showed minimal fluorescence. For orientation in evaluation of cyclic nucleotide fluorescence, parallel sections from the same block of tissue were prepared by conventional hematoxylin/eosin and periodic acid-Schiff staining. For differences due to the effect of PTH, the intensity of cyclic nucleotide fluorescence in PTH-perfused kidney was always evaluated in relation to the fluorescence intensity of slices from the control kidney of the same animal. In some experiments, the cryostat sections from control or PTH-stimulated kidneys were overlayed with a solution of 5×10^{-4} M $CaCl_2$ in phosphate-buffered saline for 20 min at 30° and washed with phosphate-buffered saline prior to the application of the cyclic nucleotide antiserum. The staining procedure was then completed in the usual manner. To minimize bias in interpreting the tissue sections, a number of pairs were deliberately mislabeled. In every instance this was detected.

The precipitated proteins from the trichloroacetic acid homogenate of renal cortex were removed by centrifugation and the trichloroacetic acid was extracted with water-saturated

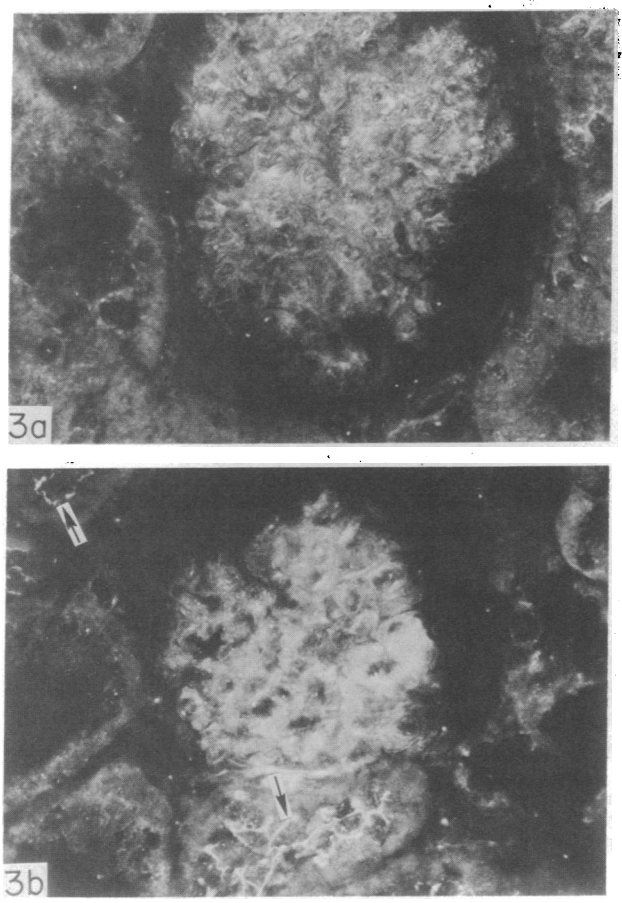

FIG. 3. cGMP fluorescence in control kidney (*a*) and after infusion of PTH (*b*). After PTH infusion, there is a marked increase in fluorescence in the area of mesangial cells and an increase in the appearance of linear cAMP fluorescence in the area of the tubular lumen (arrows). (×650.)

ether. cAMP and cGMP were separated on Dowex 1 × 8 columns by elution with formic acid (20) and were measured by radioimmunoassay (8). Cyclic nucleotide content was expressed per mg of tissue protein; the latter was determined by the method of Lowry *et al.* (21).

Materials. PTH was synthetic NH_2-terminal fragment (1–34) of bovine PTH (Beckman Co., Inc., Palo Alto, CA). Antibodies to cAMP and cGMP were prepared as described (18, 19); fluorescein-tagged goat antibodies against rabbit IgG were purchased from Miles Labs, Inc., (Kankakee, IL). All biochemicals were of the highest purity grade from standard suppliers.

RESULTS

Immunoreactive cAMP and cGMP were found in both glomerular and tubular cells of rat renal cortex, but the general distribution of the cyclic nucleotides was distinctly different (Figs. 1–3). Fluorescence with the cAMP antiserum was about the same intensity in cytoplasmic areas of cells in glomeruli and tubules; in contrast, the cGMP antibody was localized predominantly in glomeruli, but certain areas in tubular cells also fluoresced. After PTH treatment, increased areas of fluorescence in both glomeruli and proximal tubular cells were detected for both cyclic nucleotides (Fig. 1).

Localization of cAMP. In tubular cells, diffuse fluorescence was found in cytoplasm and bright fluorescence was present in unidentified cytoplasmic granules, aggregated mostly on the luminal side of the cells (Figs. 1*a* and 2*a*). Plasma membranes and nuclei were not stained. Tubular cells containing cAMP fluorescence were identified by appearance and location as cells of proximal convoluted tubules, and a similar pattern was present in tubular cells of more distal segments of the nephron. In glomeruli, cAMP appeared to be localized mainly in cells of Bowman's capsule and in cytoplasm of cells of capillary tufts; when compared to light microscopic photographs, these cells appear to be cells of visceral epithelium (podocytes) (Figs. 1*a* and 2 *c* and *d*). Cytoplasmic fluorescence increased in these cells after perfusion with PTH (Fig. 2 *c* and *d*). Fluorescence in tubular cytoplasm was accentuated after administration of PTH (Fig. 2 *a* and *b*) but the most prominent change was the increase in number and brightness of the luminally oriented granules (Fig. 2*b*). In occasional tubules, the fluorescent granules were found in the luminal space. This localization might represent a sectioning artifact or, alternatively, these granules might have been extruded from the tubular cells into the luminal space.

Localization of cGMP. In contrast with cAMP, cGMP was not detected in Bowman's capsule of glomeruli. Fluorescence appeared to be located mostly in areas between capillary loops, which contain mesangial cells (Fig. 3 *a* and *b*). Diffuse cytoplasmic cGMP fluorescence in tubular cells was of lower intensity than that of cAMP (Fig. 1*b*). In addition to this mild diffuse cytoplasmic fluorescence, cGMP fluorescence appeared as bright thin lines on cell surfaces facing the tubular lumen in both control and PTH-perfused kidney (Figs. 1*b*, 3, and 4). cGMP fluorescence in glomeruli was markedly intensified in the PTH-perfused kidneys (Fig. 3). Diffuse cGMP fluorescence in tubular cytoplasm did not change consistently, but the frequency and brightness of linear fluorescence on luminal tubular membranes markedly increased in response to PTH (Fig. 3).

Effects of Calcium. When calcium was omitted from the perfusion solution, cGMP fluorescence in glomeruli and on tubular membranes was lower in control and PTH-perfused kidneys. Addition of 1-methyl-3-isobutyl xanthine, a cyclic nucleotide phosphodiesterase inhibitor, to the perfusate intensified both cAMP and cGMP fluorescence under all conditions. To determine whether the increase in linear fluorescence at surfaces of the tubular lumen in PTH-perfused kidneys was due to cGMP formed in glomeruli and subsequently released into tubular fluid and absorbed at luminal cell surfaces from luminal fluid or whether it resulted from *in situ* generation of cGMP, the following experiment was performed. Frozen sections from the kidneys (either control or perfused with PTH) were overlayed with phosphate-buffered saline containing 0.5 mM $CaCl_2$ and incubated at room temperature for 20 min prior to staining with antiserum. In sections from control kidneys there was no difference in linear luminal fluorescence between slices incubated with or without calcium (Fig. 4 *a* and *b*). On the other hand, in PTH-perfused kidneys, preincubation with the solution containing calcium consistently intensified cGMP fluorescence at luminal surfaces of tubules (Fig. 4*d*), suggesting that the tissue sections that were previously perfused with PTH *in vivo* and then exposed to calcium *in vitro* were capable of synthesizing additional cGMP and binding the nucleotide to tissue receptors. Because the change in fluorescence was not seen in control renal cortical tissue, we believe that this enhanced linear fluorescence represents additionally synthesized cGMP and is not merely an effect of calcium on cGMP binding to receptors.

Total Tissue Levels of Cyclic Nucleotides. Total content of cAMP in renal tissue cortex was about 20 times higher than that of cGMP. PTH caused a consistent but relatively small elevation of total tissue cAMP (+41.2 ± 8%, $P < 0.05$). This increase was intensified in the presence of the diphosphoesterase inhibitor (+97.6 ± 17%, $P < 0.05$). Although the tissue levels of cGMP tended to be higher in PTH-perfused kidneys, the

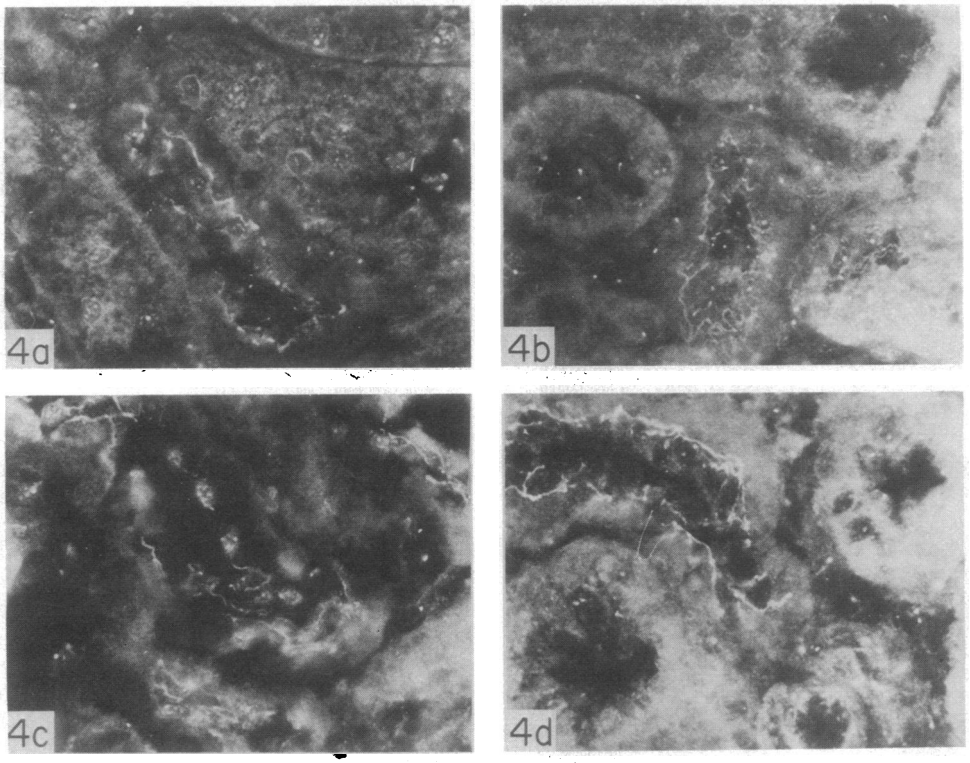

FIG. 4. Increase in cGMP fluorescence on the luminal surface of tubules in tissue sections incubated with phosphate-buffered saline containing 0.5 mM $CaCl_2$ prior to the application of the cGMP antiserum. (*a*) Without $CaCl_2$, control kidney. (*b*) Plus $CaCl_2$, control kidney. (*c*) Without $CaCl_2$, PTH-perfused kidney. (*d*) Plus $CaCl_2$, PTH-perfused kidney. (×560.)

differences were not statistically significant (data not shown).

DISCUSSION

In kidney tissue, cAMP and cGMP are not only actively generated and catabolized but also taken up from the peritubular capillary circulation (15, 16), filtered from plasma into urine through glomeruli (11, 13, 16), and reabsorbed from tubular fluid (1, 22). Thus, the total tissue level or rate of urinary excretion of cyclic nucleotides is a consequence of many factors. Results of the present study provide several new insights into the dynamics of cyclic nucleotide metabolism in the kidney in general and the cellular action of PTH in renal cortex in particular. The experimental design of the present study eliminates access of cyclic nucleotide from other organs to kidney, either by glomerular filtration or by peritubular uptake, and also avoids the effect of extrarenal humoral factors on cyclic nucleotide metabolism.

In tubular cells, much of the immunofluorescent cAMP was found in cytoplasm in granules, the majority of which were on the luminal side of the cells. The identity of these granules remains to be determined. Both the proximal and distal convoluted tubules contain a very active adenylate cyclase (23) which is most likely localized on peritubular (antiluminal) and lateral plasma membranes (24, 25). The immunocytochemical method used in this study most likely detects cAMP bound to proteins (18), conceivably on specific cAMP-binding proteins. The present finding of periluminally localized cAMP is consistent with reports showing the presence of high cAMP binding capacity of isolated renal brush-border membranes (26), presence of cAMP-dependent protein kinase (25), or cyclic nucleotide phosphodiesterase (27, 28) at the same subcellular structure. It is conceivable that periluminal cAMP, which increases in response to PTH, represents a fraction of the nucleotide involved in specific transport processes regulated by PTH and mediated by cAMP (e.g., transport of calcium or phosphate). Alternatively, the accumulation of cAMP in a periluminal location may reflect a site of active extrusion of cAMP from cells into the tubular lumen.

The presence of cAMP in glomeruli and its increase in response to PTH are in good agreement with the presence of a PTH-sensitive adenylate cyclase isolated from glomeruli (4, 5). Because cells of the parietal and visceral epithelium of Bowman's capsule are of the same embyrological origin as proximal tubules, it is not surprising that they contain similar PTH-sensitive cellular elements. Results of recent micropuncture studies on rats show that PTH causes a decrease in single-nephron glomerular filtration rate and a decrease in the ultrafiltration coefficient (29).

The large amount of immunoreactive cGMP in glomeruli in comparison with tubules supports data in rabbit kidney cortex, in which the specific activity of guanylate cyclase in homogenates from glomeruli was about 50 times higher than in the same preparation from tubules (10). Although the exact immunocytochemical localization of cGMP as either glomeruli or tubules should be interpreted with caution, its distribution is clearly different from that of cAMP. In glomeruli, most of the cGMP appears to be in areas of glomerular mesangium. Because mesangial cells contain structures reminiscent of contractile fibrils (30) and because smooth muscle contains significant amounts of cGMP (31), it is tempting to speculate that cGMP could be involved in regulation of the contractile function of the mesangium. Alternatively, or additionally, because another presumed function of mesangial cells is phagocytic uptake of particles (32), cGMP could be also involved in regulation of this mesangial function, in a manner similar to that in leukocytes (33). The possible roles of cAMP and cGMP in glomeruli are not fully elucidated. Either one or

both of these cyclic nucleotides may be involved in the mediation of the glomerular effect of PTH mentioned above (29). However, as stressed above, the increase in cAMP and cGMP in glomeruli in response to PTH cannot be accounted for by filtered nucleotides or by extrarenal factors.

Although the overall cGMP fluorescence in tubules is relatively low, the very bright cGMP fluorescence on luminal cell surfaces, accentuated by PTH, is of considerable interest. cGMP fluorescence increased further in the area of the luminal membrane in sections of renal cortical tissue perfused with PTH *in vivo* and exposed to calcium *in vitro* (Fig. 4). This finding suggests that this enhanced fluorescence is a result of *in situ* cGMP synthesis. Guanylate cyclase from rat kidney cortex is known to be stimulated by calcium (34), and experience with other tissues suggests that enzyme activities are not completely destroyed by freezing of tissue sections (18). Consequently, the calcium-induced increase in the cGMP luminal fluorescence suggests that cGMP is not merely adsorbed from tubular fluid but is probably formed and bound at this site.

These immunocytochemical observations suggest that not only cAMP but also cGMP is involved in the cellular actions of PTH in renal cortex. At least two hypotheses for a cGMP role may be entertained. PTH, probably via cAMP, increases calcium reabsorption in renal cells. The exact mechanism of PTH action is not known, but it should be noted that cAMP influences calcium fluxes across other membranes as well (35). Because cGMP formation can be stimulated by calcium (17, 31, 34), conceivably the increase in calcium access (either from an intracellular depot or from tubular fluid) to guanylate cyclase, evoked by cAMP, may result in the increase in cGMP formation concomitant with or consequent to the PTH-induced increase in cAMP. This model finds indirect support in the observations that nephrogenous urinary cGMP increases at the same time (13) or follows the increase in cAMP in response to exogenous or endogenous PTH (11–14). Supporting this idea is the observation that, in slices from PTH-perfused kidneys, in which cAMP was elevated, cGMP fluorescence may be stimulated *in situ* by exposure to calcium (Fig. 4).

Thus, the present studies suggest a role for cGMP in the area of renal cortical tubular membranes most likely associated with transport of calcium and possibly other cations. The function of the nucleotide could be as a negative feedback regulator to limit the transport of calcium or other cations into the renal tubular cell (31, 36). Consistent with this proposal is the finding that cGMP may accelerate cAMP breakdown in kidney (37); or, cGMP may regulate, with cAMP, either calcium or phosphate transport [a monodirectional control system (17, 36)].

Finally, as pointed out previously (19), this immunocytochemical technique detected changes in the cytochemical distribution of cGMP even though no significant change in total tissue cGMP content was found. Similar observations have been made in the process of liver regeneration—i.e., total cGMP content did not change but distinct redistributions in immunocytochemical cGMP occurred (38).

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