Biochemical Pharmacology, Vol. 39, No. 6, pp. 1019–1028, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

THE AUTORADIOGRAPHIC LOCALIZATION OF ADENYLATE CYCLASE IN RAT KIDNEY USING [^{3}H]FORSKOLIN

LYNNE R. MCMARTIN and ROGER J. SUMMERS*
Department of Pharmacology, University of Melbourne, Parkville 3052, Australia

(*Received* 7 *August* 1989; *accepted* 10 *October* 1989)

Abstract—The localization of [^{3}H]forskolin binding to microscope slide mounted sections of rat kidney has been examined using autoradiography. Saturation studies showed [^{3}H]forskolin binding to two sites, a high affinity site (K_D = 8.7 nM, B_{max} = 0.14 pmol/mg protein) and a low affinity site (K_D = 6.7 μM, B_{max} = 11.0 pmol/mg protein). Autoradiographs showed high affinity binding (thought to identify stimulatory guanine nucleotide binding protein (Gs)-linked adenylate cyclase) to all renal structures known to possess hormone sensitive adenylate cyclase, including all tubular segments, glomeruli and blood vessels. High concentrations of binding were associated with a portion of the proximal tubule and with papillary collecting tubules and ducts.

In the kidney many different receptor types are linked to the stimulation of adenylate cyclase, including those which respond to parathyroid hormone (PTH†), calcitonin, glucagon, vasopressin, dopamine, histamine, serotonin, prostaglandins and noradrenaline. Only some of the physiological consequences of adenylate cyclase activation by these receptors are known, such as PTH stimulation of adenylate cyclase in the epithelial cells of the proximal tubule which inhibits the reabsorption of phosphate, bicarbonate, and associated fluid [1], and vasopressin stimulation of adenylate cyclase, via V_2 receptors, which increases the osmotic permeability of collecting tubules to water resulting in an antidiuretic effect [2]. There are still, however, many examples of hormone-activated adenylate cyclase in the kidney for which the associated physiological responses are not known, including the vasopressin stimulated adenylate cyclase activity in the thin ascending limb of the loop of Henle [2] and glucagon stimulation of adenylate cyclase in collecting tubules [1]. The picture is further complicated by the finding that in some tubule segments different receptors activate the same adenylate cyclase pool. In the cortical segment of the thick ascending limb of the loop of Henle PTH, glucagon, calcitonin and vasopressin stimulate the same adenylate cyclase and presumably produce the same physiological response in these cells [2].

Much of the knowledge of hormone-responsive adenylate cyclase in the kidney is the result of adenylate cyclase microassays of microdissected nephron segments. Another approach now available uses forskolin, a diterpine compound, which stimulates hormone-sensitive adenylate cyclase via an interaction with sites on the catalytic subunit or an associated protein [3] and has been employed to investigate the adenylate cyclase system in a variety of cells and tissues. The development of radiolabelled [^{3}H]forskolin has allowed the identification and autoradiographic localization of its binding sites in rat brain [4, 5]. This study uses autoradiography to localize [^{3}H]forskolin binding sites in a peripheral tissue, the rat kidney. The distribution of binding is discussed in relation to the known distribution of neurotransmitter- and hormone-receptors linked to adenylate cyclase in the kidney.

MATERIALS AND METHODS

Materials

[^{3}H]Forskolin (49.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA); forskolin, Napthol AS-MX phosphate solution, Fast Blue RR salt and Pyronin Y were purchased from the Sigma Chemical Co. (Poole, U.K.).

Preparation of tissue sections. Female Wistar rats (240–260 g) were anaesthetized with methohexitone sodium (100 mg/kg i.p.) containing heparin sodium (50 IU/mg). Kidneys were perfused *in situ* with a mixture of equal parts of 0.32 M sucrose and Krebs phosphate buffer (composition mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.27, Na_2PO_4 10.0, pH 7.4) via the abdominal aorta until clear of blood, followed by the same solution containing 0.1% formaldehyde. Kidneys were removed and frozen in isopentane cooled in liquid nitrogen. Sections (10 μm) were cut using a Reichert–Jung cryostat at −18° and thaw-mounted onto cold gelatin-coated microscope slides.

Labelling of slide-mounted kidney sections with [^{3}H]*forskolin.* Slide-mounted sections were incubated at room temperature for 10 min in 50 mM Tris buffer (pH 7.7) containing 5 mM $MgCl_2$, 100 mM NaCl [6] and [^{3}H]forskolin (10 nM). To minimize the quantity of radioligand used, small aliquots of incubation medium (130 μL) were added to the slide-mounted sections. For the determination of non-specific binding, unlabelled forskolin (10 μM) was

* To whom correspondence should be addressed.

† Abbreviations used: Gs, stimulatory guanine nucleotide binding protein; PTH, parathyroid hormone; PGE_2, prostaglandin E_2.

included. Following incubation, sections were rinsed in cold buffer and washed for two periods of 2 min in buffer at 4°. Following a brief rinse in distilled water (4°) sections for biochemical studies were wiped from the slide using GF/B filters and counted by conventional scintillation techniques and sections for autoradiography were dried with a stream of cool dry air.

Biochemical studies of [^{3}H]*forskolin binding.* Association of [^{3}H]forskolin (10 nM) was evaluated by incubation of slide-mounted sections for different periods of time at room temperature. Dissociation was determined by the incubation of sections with the ligand (10 nM) for 10 min followed by washing in Tris buffer at room temperature for up to 90 min. The polyexponential curve fitting program ESTRIP [7] was used to determine the rate constants for dissociation of [^{3}H]forskolin using the data obtained in the kinetic experiments. The saturation of binding sites was studied by the incubation of sections with concentrations of [^{3}H]forskolin ranging from 500 pM to 500 nM. Saturation data were analysed using two computer programs, EBDA [8] to provide preliminary estimates of binding constants, and LIGAND [9], a nonlinear iterative curve fitting program, to obtain final binding parameter estimates. Protein was determined using the Bio-Rad protein assay, based on the method of Bradford [10], and bovine serum albumin as the standard.

Autoradiography. [^{3}H]Forskolin-labelled sections were apposed to either Amersham ^{3}H-Hyperfilm or to nuclear emulsion- (Kodak NTB3) coated coverslips and exposed in the dark for 9–10 weeks. Hyperfilm was developed in Kodak D19 developer and coverslips in Kodak Dektol developer. Both emulsions were fixed using Kodak Rapid Fixer. Sections apposed to coverslips were then fixed in 3:2 acetone : McIlwains solution (composition mM: citric acid 46.4, Na_2HPO_4 107.2) and stained with 0.1% pyronin Y in McIlwains. Every fifth section cut was set aside for histological examination and stained for alkaline phosphatase activity and counterstained with pyronin Y.

RESULTS

Washing conditions

To establish appropriate washing conditions, slide-mounted sections were washed for different periods of time following 10 min incubation with 15 nM [^{3}H]forskolin. Washing for two periods of 2 min at 4° gave 80% specific binding. Longer washing increased the percentage of specific binding but significantly reduced the amount of binding. Two 2-min washes were consequently used in all experiments.

Characterization of [^{3}H]*forskolin binding to kidney sections*

Association of [^{3}H]forskolin to binding sites in slide mounted sections of rat kidney at room temperature was rapid and reached equilibrium after 7 min (Fig. 1). Dissociation was also rapid with more than 90% of specific binding lost after 10 min. It was also biphasic and two dissociation rate constants were calculated, an initial fast phase (K_{-1} = 0.45 ± 0.11/min) and a prolonged slow phase (K_{-2} = 0.04 ± 0.02/min, N = 3). The presence of two binding sites was confirmed in saturation experiments. A representative saturation curve and the corresponding Scatchard transformation of the binding data are shown in Fig. 2. Two [^{3}H]forskolin binding sites were observed, a high affinity site (K_D = 8.7 ± 1.7 nM) and a low affinity site (K_D = 6.7 ± 3.7 μM) with B_{max} values of 0.14 ± 0.02 pmol/mg protein and 11.0 ± 5.8 pmol/mg protein respectively (N = 4). The gradient of the Hill plot (nH) was 0.795 ± 0.04 (N = 4).

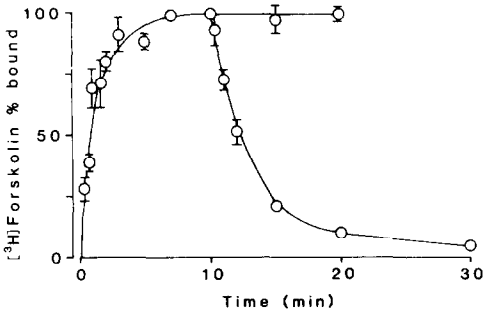

Fig. 1. Kinetics of specific [^{3}H]forskolin binding to slide-mounted sections of rat kidney. Association of 10 nM [^{3}H]forskolin, determined by the incubation of sections for different times at room temperature followed by two 2-min washes in buffer at 4°, was rapid and reached equilibrium after 7 min. In the dissociation experiments a 10-min incubation with 10 nM [^{3}H]forskolin was followed by incubation in buffer containing no radioligand at room temperature for different time periods followed by two 2-min washes at 4°. The resulting curve was biphasic and two dissociation rate constants were determined, K_{-1} = 0.45/min and K_{-2} = 0.04/min. Points are mean values ± SE, of three experiments in duplicate.

Autoradiographic studies

The autoradiographic localization of [^{3}H]forskolin binding was studied in 16–24 sections of kidney from each of six rats. The ^{3}H-Hyperfilm image of [^{3}H]forskolin binding to a slide mounted section of rat kidney and a photograph of a serial section stained for alkaline phosphatase activity to identify proximal tubules are shown in Fig. 3. Binding was present in all regions of the kidney but higher levels of binding were confined to strips extending from the cortico-medullary border to the outer cortex and throughout the papilla. No image was obtained from sections coincubated with 10 μM forskolin.

A more detailed study of the localization of [^{3}H]forskolin binding was made with nuclear emulsion coated coverslips exposed to labelled sections and serial sections stained for histology. Figure 4 shows binding to the cortex and dense binding to some proximal tubules. Higher magnification (Figs 5 and 6) showed binding localized to glomeruli, proximal and distal tubules and blood vessels, and higher binding to a portion of the proximal tubules. A high level of binding was also associated with the papilla (Fig. 7).

DISCUSSION

In this study [^{3}H]forskolin bound to two sites in

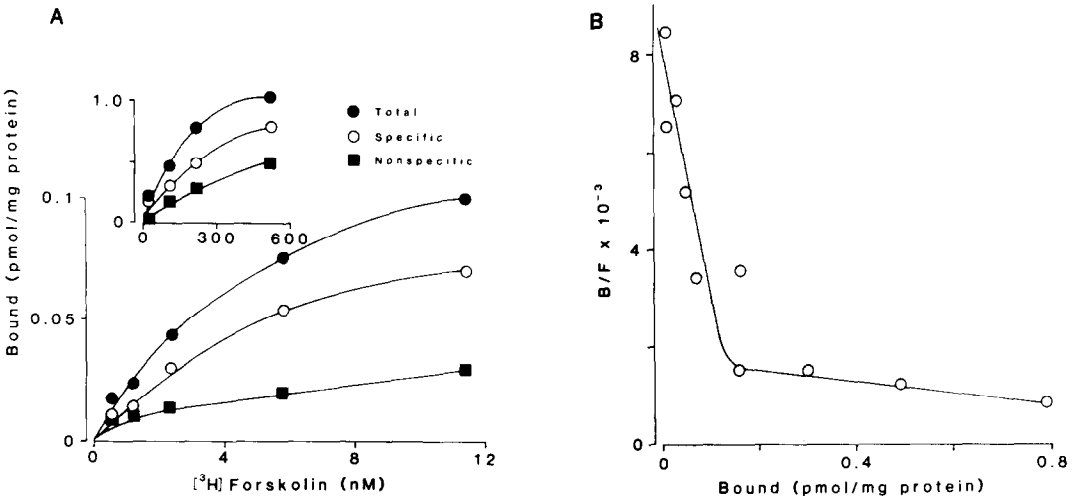

Fig. 2. Saturation of [^{3}H]forskolin binding to slide mounted sections of rat kidney in one of four experiments. (A) Saturation binding curves for total, specific and nonspecific binding of [^{3}H]forskolin at concentrations of 0.5–12 nM and 20–500 nM (inset) in the same experiment. (B) The corresponding Scatchard plot of specifically bound [^{3}H]forskolin, versus the ratio of bound/free (B/F), was nonlinear (nH = 0.79) indicating binding to two sites, a high affinity site (K_D = 8.7 nM, B_{max} = 0.14 pmol/mg protein) and a low affinity site (K_D = 6.7 μM, B_{max} = 11.0 pmol/mg protein).

slide-mounted sections of rat kidney, a high affinity site with a dissociation constant of 8.7 nM and a low affinity site with a K_D of 6.7 μM. Seamon *et al.* [4] using a centrifugation binding assay, also found that [^{3}H]forskolin bound to two sites in rat brain membranes (K_{D1} = 15 nM, K_{D2} = 1.1 μM). Although at concentrations of 10–100 nM forskolin can markedly enhance hormonal stimulation of adenylate cyclase, the EC_{50} for direct stimulation of adenylate cyclase is 5–10 μM [4]. It has been suggested that these two different affinities of forskolin for stimulation of adenylate cyclase correspond to the two different binding sites of forskolin, and furthermore that the high affinity site is associated with the presence of the stimulatory Gs protein subunit, while low affinity activation of adenylate cyclase by forskolin does not require the Gs protein [3]. Since hormone activation of adenylate cyclase requires the presence of the Gs subunit, it is believed that the high affinity [^{3}H]forskolin binding sites correlate with hormone–sensitive adenylate cyclase. In the present study [^{3}H]forskolin was used at a concentration of 10 nM, the calculated K_D of the high affinity binding, resulting in the labelling of only high affinity sites.

Autoradiographic images showed [^{3}H]forskolin binding in all regions of the kidney. Dense binding was confined to proximal tubules and to papillary collecting tubules and ducts, while more diffuse binding was associated with renal blood vessels, glomeruli and other cortical and medullary tubules.

Adenylate cyclase studies and particularly those using microdissected nephron segments, have revealed a large number of agents which can stimulate adenylate cyclase in different segments and cells of the mammalian kidney. In the proximal tubule, particularly the convoluted portion, PTH potently stimulates adenylate cyclase [1]. There have also been reports of small populations of β-adrenoceptors [11] linked to stimulation of adenylate cyclase in the proximal tubules. In slide-mounted sections of rat kidney, [^{3}H]forskolin binding was associated with all proximal tubules (identified by a positive reaction for alkaline phosphatase activity), however, dense binding was confined to only a portion of proximal tubules. At the light microscopic level it is very difficult to differentiate between the convoluted segment and the straight segment of the proximal tubule, however it has been reported that there is about 40% more alkaline phosphatase activity in the convoluted segment of the proximal tubule than the straight portion [12]. Since the dense [^{3}H]forskolin binding did appear to be associated with those proximal tubules which showed heavy staining for alkaline phosphatase (Figs 4 and 5), it is likely that most of the binding was to the convoluted segment.

Specific binding of [^{3}H]forskolin was also localized to other cortical tubules and these have all been reported to contain hormone-sensitive adenylate cyclase. The cortical thick ascending limb of the loop of Henle contains adenylate cyclase responsive to PTH, calcitonin, vasopressin, glucagon and isoprenaline [1] and there is evidence that PTH, calcitonin, vasopressin and glucagon stimulate the same cyclase pool in this region [2]. The adenylate cyclase of the distal convoluted tubule and cortical collecting tubule is also stimulated by these agents to varying degrees [1], and it has been reported that PGE_2 also stimulates adenylate cyclase in the latter region [13, 14].

[^{3}H]Forskolin also bound to sites in the glomeruli, where cyclase has been shown to respond to β-adrenoceptor agonists [15, 16], histamine via H_2 receptors [17, 18], serotonin [19], PTH [17, 20] and PGE_2 [14]. Whether these agents activate the same adenylate cyclase pool is still unknown. Renal blood vessels are also influenced by hormones acting via

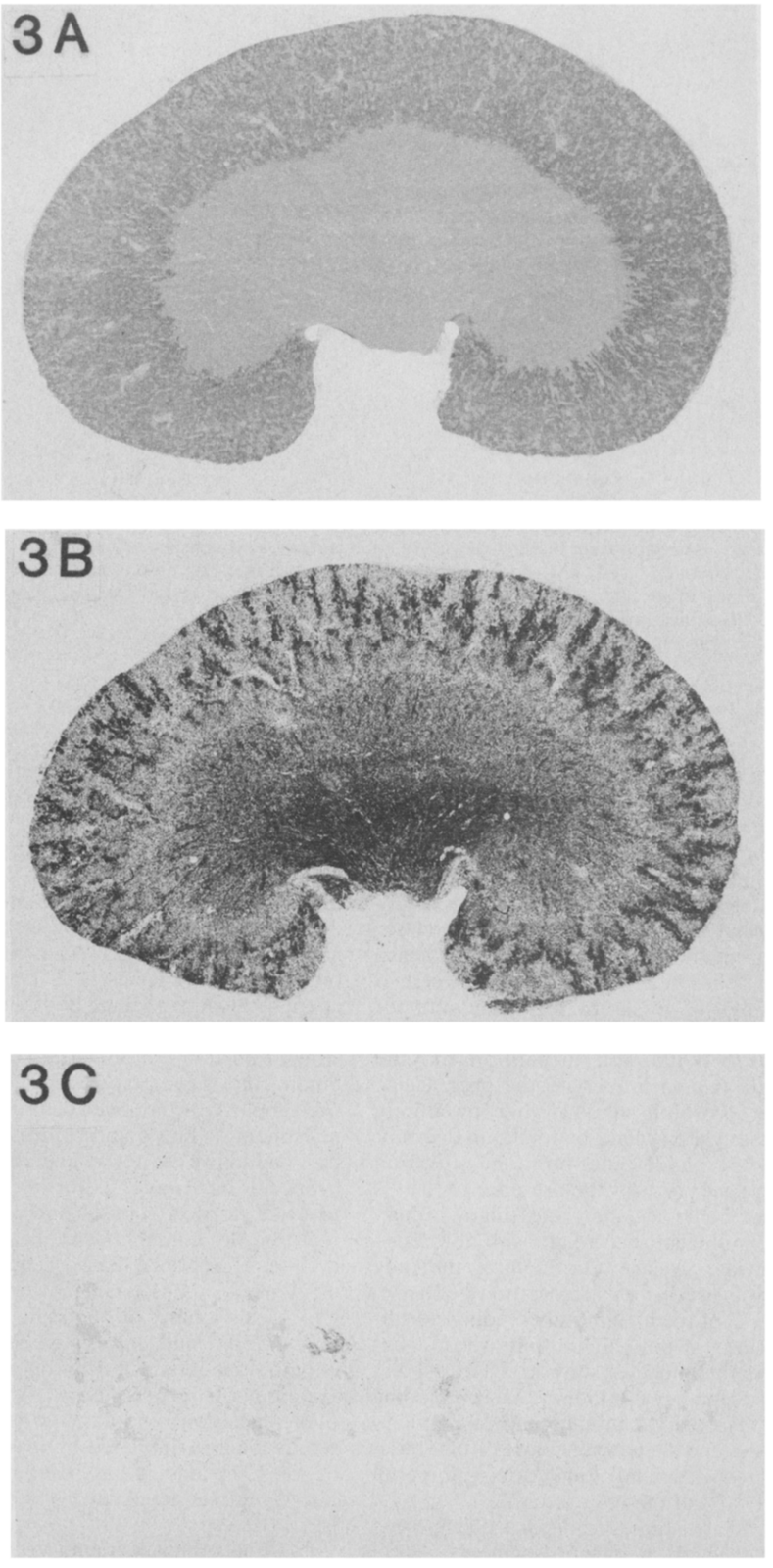

Fig. 3. Binding of 10 nM [^{3}H]forskolin to sections of rat kidney. (A) Photograph of a section of rat kidney stained for alkaline phosphatase activity to identify proximal tubules (dark staining). (B) The image from ^{3}H-Hyperfilm apposed to a serial section incubated with 10 nM [^{3}H]forskolin. Although binding was associated with all regions of the kidney, dense binding was confined to narrow strips extending from the cortico-medullary junction to the outer cortex, and throughout the papilla. No image was obtained from sections coincubated with 10 μM forskolin to define nonspecific binding (C).

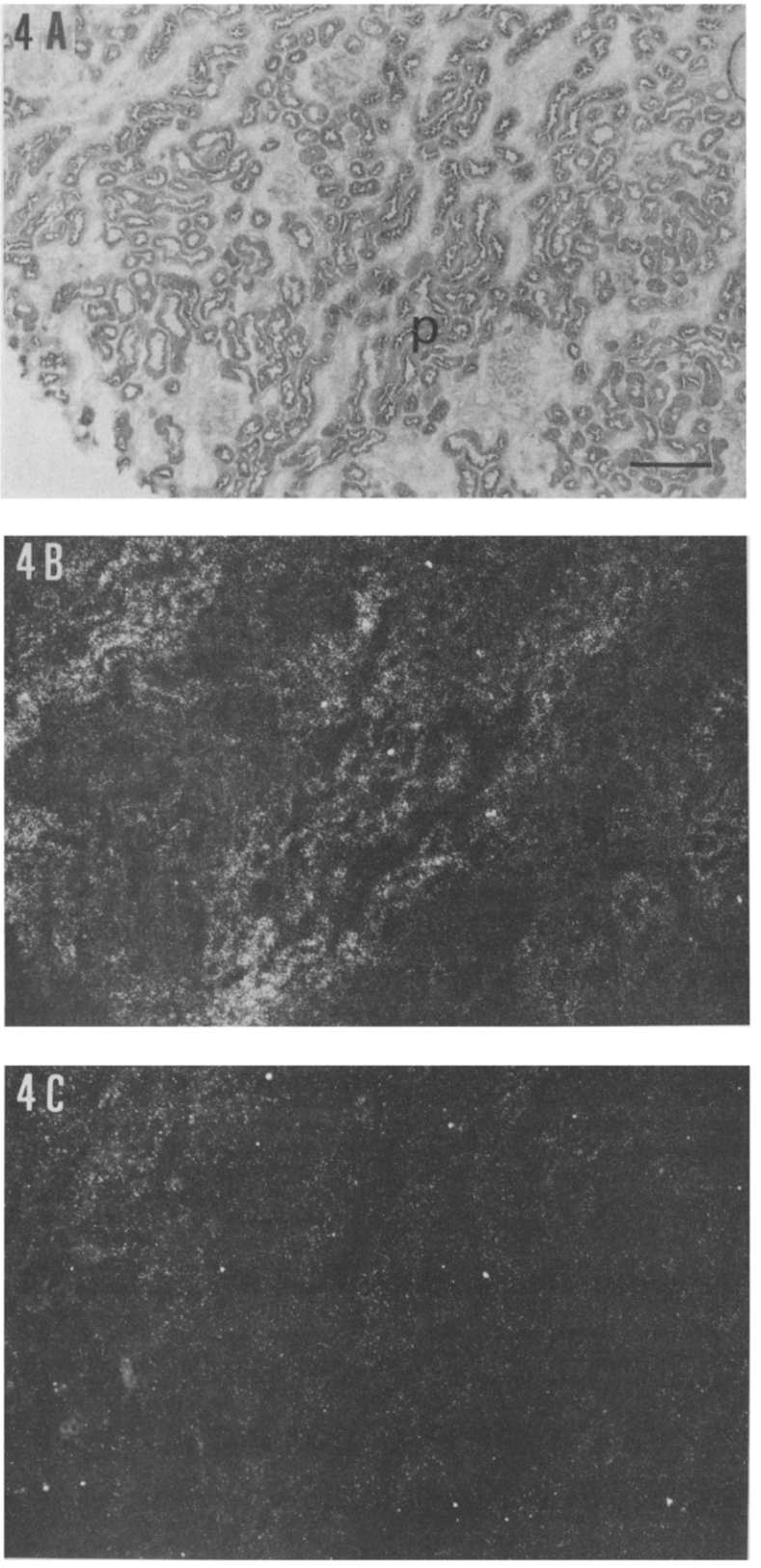

Fig. 4. Low power micrograph of [^{3}H]forskolin binding to the renal cortex. (A) Lightfield micrograph of a section of rat kidney stained for alkaline phosphatase and counterstained with pyronin Y. (B) Darkfield micrograph of silver grains. (C) Darkfield view of corresponding section coincubated with 10 μM forskolin. A high density of silver grains was associated with proximal tubules which stained heavily for alkaline phosphatase (p). Bar represents 200 μm.

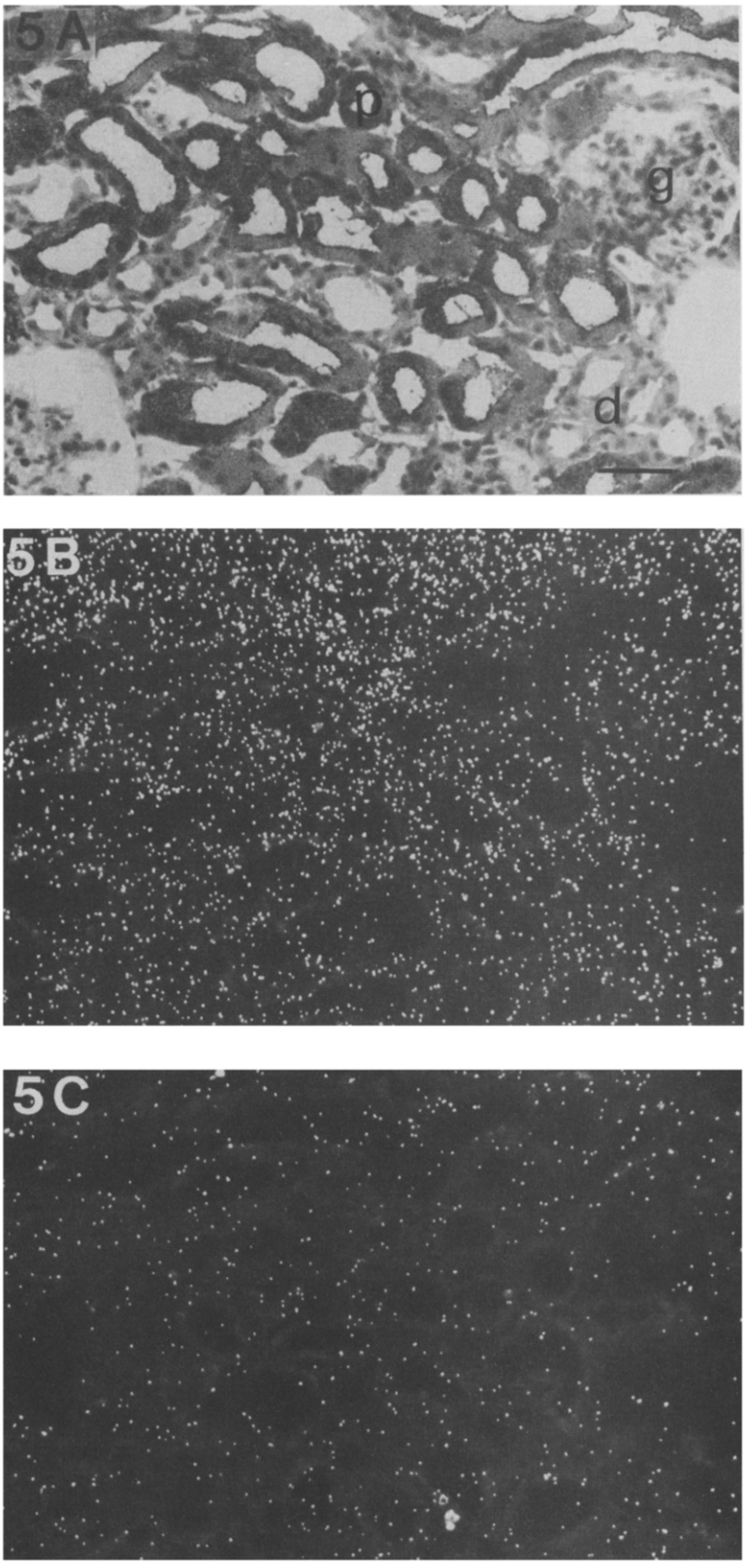

Fig. 5. High power micrograph of [^{3}H]forskolin binding to a region of the outer cortex of rat kidney. (A) Lightfield micrograph of a section stained for alkaline phosphatase activity. (B) Darkfield micrograph of the silver grains. (C) Darkfield view of grains over a section coincubated with 10 μM forskolin. Binding was associated with proximal tubules (p), distal tubules (d) and glomeruli (g). Bar represents 50 μM.

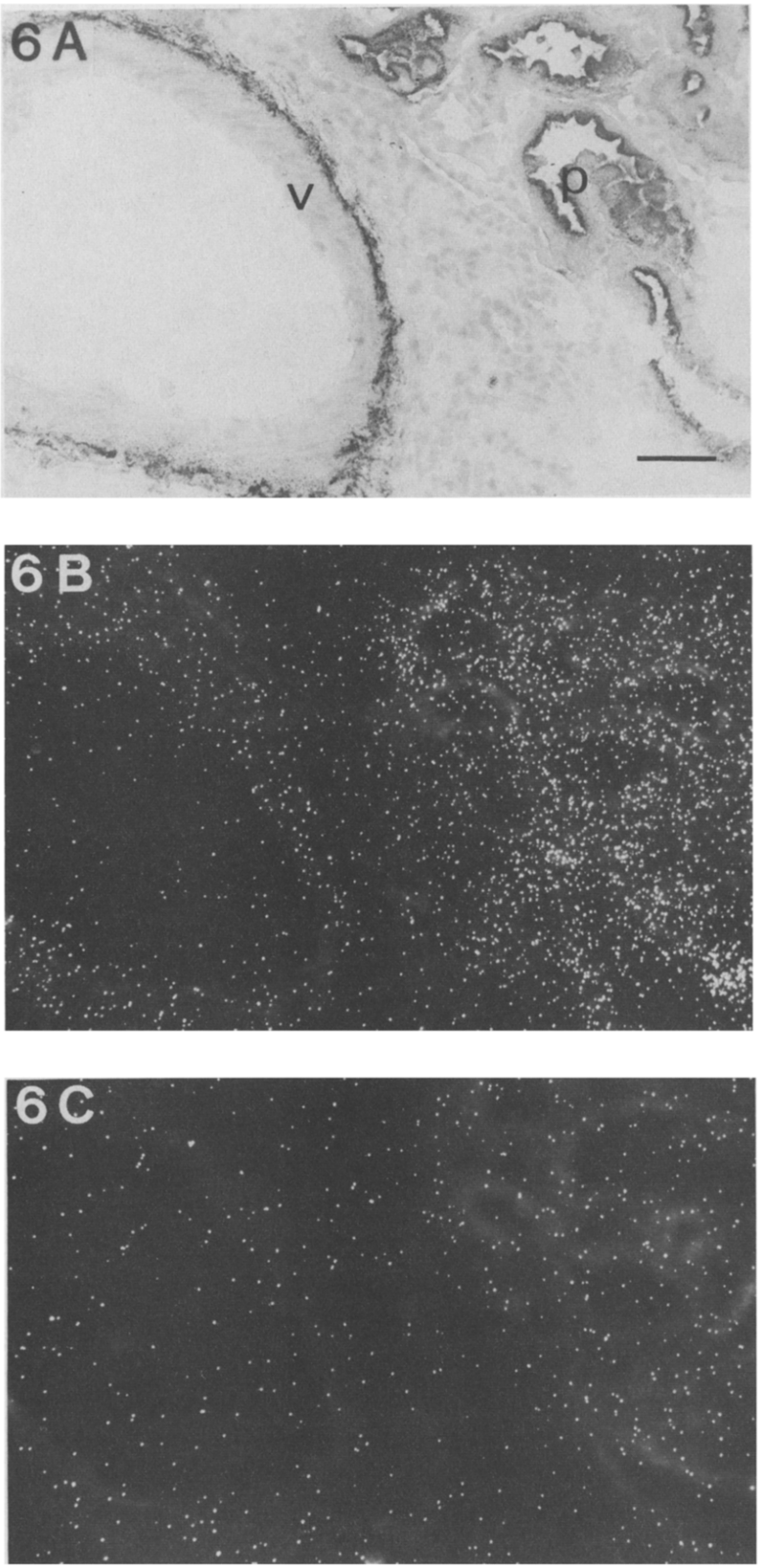

Fig. 6. Localization of [³H]forskolin binding to a cortical renal blood vessel. (A) Lightfield view of section stained for alkaline phosphatase activity. (B) Darkfield view of silver grains. (C) Darkfield view of a section coincubated with 10 μM forskolin. Silver grains were localized to renal blood vessels (v), although less densely than those associated with proximal tubules (p). Bar represents 50 μM.

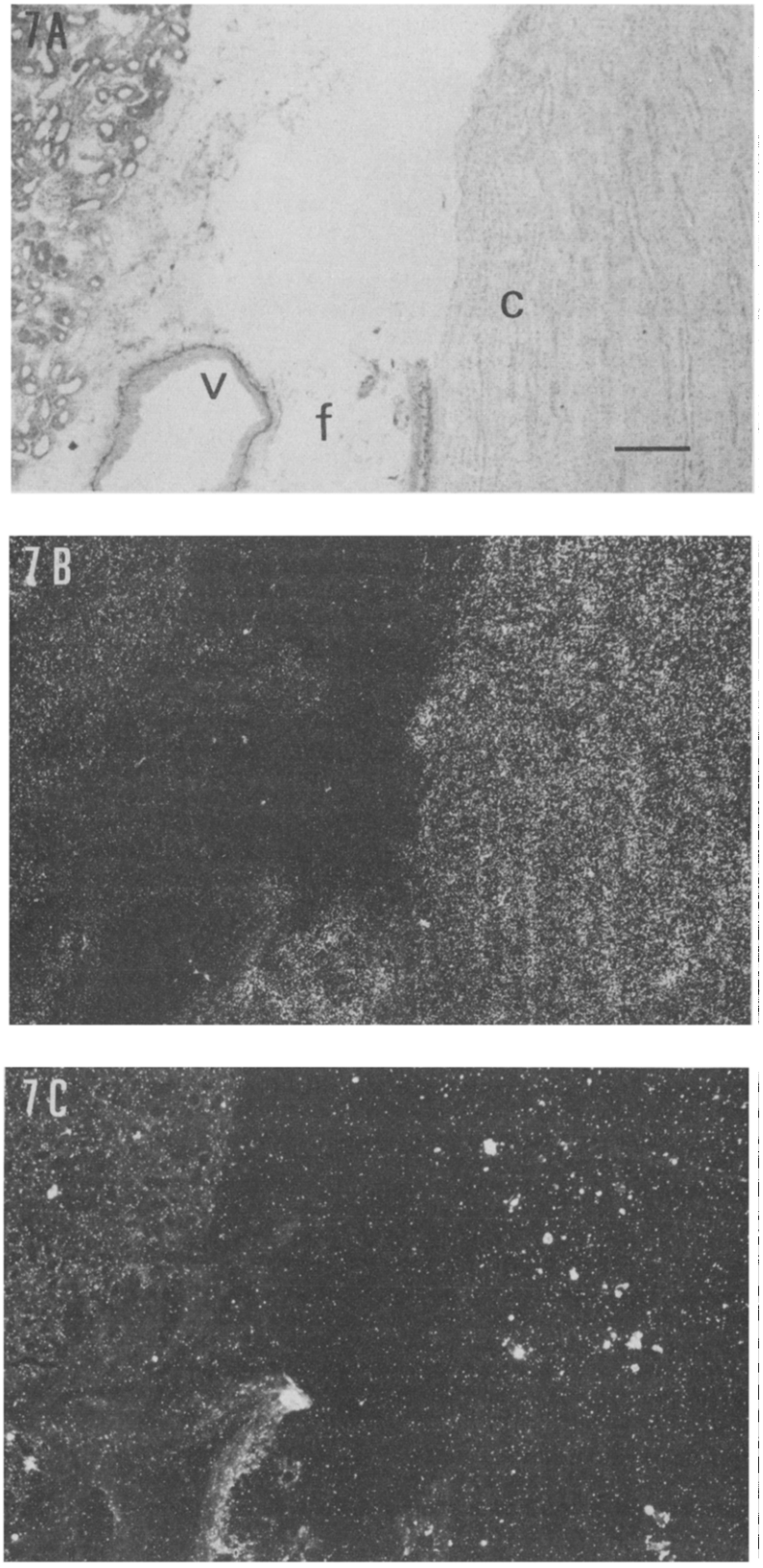

Fig. 7. Localization of [^{3}H]forskolin binding to the papilla of rat kidney. (A) Lightfield micrograph of a section stained with alkaline phosphatase and pyronin Y. (B) Darkfield view showing the density of silver grains associated with the papillary collecting tubules and ducts (c), an adjacent blood vessel (v), remnants of surrounding fat (f) and renal cortex. (C) Darkfield micrograph of a section coincubated with 10 μM forskolin. Bar represents 200 μm.

adenylate cyclase especially dopamine which causes vasodilatation via D_1 receptors [21, 22], purines via A_2 purinoceptors [23] and PTH [24]. Specific [^{3}H]forskolin binding was associated with all blood vessels observed in kidney sections.

Medullary nephron segments could not be differentiated but there was diffuse [^{3}H]forskolin binding throughout the medulla, suggesting the presence of hormone-sensitive adenylate cyclase in all segments. The thin descending limb of the loop of Henle is the only segment which appears to have little hormone-sensitive adenylate cyclase [1], although PGE_2 has been reported to produce a small cyclase response in this segment [13, 14]. Both the thin ascending limb and the medullary thick ascending limb have vasopressin-stimulated adenylate cyclase, and the cyclase of the medullary thick ascending limb is also responsive to glucagon and calcitonin [1]. The medullary collecting tubules have been shown to have cyclase sensitive to vasopressin, via V_2 receptors, and glucagon [1]. The dense binding of [^{3}H]forskolin to the inner medulla and papilla, apparent in Figs 3 and 7, provides evidence for the presence of hormone-sensitive adenylate cyclase in these areas. Studies using rat renal papillary collecting tubule cells in culture have shown the presence of cyclase responsive to glucagon [25], vasopressin and PGE_2 [26].

It has been reported that the density of [^{3}H]forskolin binding sites correlates with the ability of forskolin to stimulate adenylate cyclase in a tissue [3, 27]. It could be expected, therefore, that forskolin might greatly potentiate the PTH activation of adenylate cyclase in the proximal tubules where a high number of [^{3}H]forskolin binding sites were present. It is still unclear, however, whether there is any correlation between the density of [^{3}H]forskolin binding and the total amount of hormone sensitive adenylate cyclase present in a tissue.

In conclusion, [^{3}H]forskolin (10 nM) binds to a high affinity site in slide-mounted sections of rat kidney, thought to be associated with hormone-sensitive adenylate cyclase. Specific [^{3}H]forskolin binding was heterogeneously distributed and localized to all renal structures known to possess hormone-sensitive cyclase, including all cortical and medullary tubular segments, glomeruli and blood vessels.

Acknowledgements—This work was supported by a grant from the National Health and Medical Research Council of Australia. L.M. is an N.H. & M.R.C. Postgraduate Research Scholar.

REFERENCES

1. Morel F and Doucet A, Hormonal control of kidney functions at the cell level. *Physiol Rev* **66**: 377–468, 1986.
2. Morel F, Imbert-Teboul M and Chabardes D, Receptors to vasopressin and other hormones in the mammalia kidney. *Kidney Int* **31**: 512–520, 1987.
3. Seamon KB and Daly JW, Forskolin: its biological and chemical properties. In: *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Eds. Greengard P and Robison GA), Vol. 20, pp. 1–150. Raven Press, New York, 1986.
4. Seamon KB, Vaillancourt R, Edwards M and Daly JW, Binding of [^{3}H]-forskolin to rat brain membranes. *Proc Natl Acad Sci USA* **81**: 5081–5085, 1984.
5. Gehlert DR, Dawson TM, Yamamura HI and Wamsley JK, Localization of [^{3}H]-forskolin binding sites in the rat brain by quantitative autoradiography. *Eur J Pharmacol* **106**: 223–225, 1984.
6. Worley PF, Baraban JM, De Souza EB and Snyder SH, Mapping second messenger systems in the brain: Differential localizations of adenylate cyclase and protein kinase C. *Proc Natl Acad Sci USA* **83**: 4053–4057, 1986.
7. Brown RD and Manno JE, Estrip: A basic computer program for obtaining initial polyexponential parameter estimates. *J Pharm Sci* **67**: 1687–1691, 1978.
8. McPherson GA, A practical computer-based approach to the analysis of radioligand binding experiments. *Comp Prog Biomed* **17**: 107–114, 1983.
9. Munson PJ and Rodbard D, LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **197**: 220–239, 1980.
10. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
11. Jacobs WR and Chan YL, Evidence for the presence of functional beta-adrenoceptor along the proximal tubule of the rat kidney. *Biochem Biophys Res Commun* **141**: 334–339, 1986.
12. Guder WG and Ross BD, Enzyme distribution along the nephron. *Kidney Int* **26**: 101–111, 1984.
13. Torikai S and Kurokawa K, Distribution of prostaglandin E_2-sensitive adenylate cyclase along the rat nephron. *Prostaglandins* **21**: 427–438, 1981.
14. Umemura S, Smyth DD and Pettinger WA, Regulation of renal cellular cAMP levels by prostaglandins and α_2-adrenoceptors: microdissection studies. *Kidney Int* **29**: 703–707, 1986.
15. Judes C, Helwig JJ, Bollack C and Mandel P, Isoproterenol-sensitive adenylate cyclase in the glomeruli isolated from young and adult rat renal cortex. *Gen Pharmacol* **16**: 205–210, 1985.
16. Freissmuth M, Kraupp O, Hausleithner V, Tuisl E and Schutz W, Alterations of the glomerular β-adrenoceptor-linked adenylate cyclase system in perinephritis hypertension. *J Cardiovasc Pharmacol* **8**: 60–66, 1986.
17. Torres VE, Northrup TE, Edwards RM, Shah SV and Dousa TP, Modulation of cyclic nucleotides in isolated rat glomeruli. *J Clin Invest* **62**: 1334–1343, 1978.
18. Wargo AA, Slotkoff LM, Jose PA, Pelayo JC and Eisner GM, Cyclic nucleotide response to stimulation in isolated glomeruli from dog kidney. *Nephron* **32**: 165–169, 1982.
19. Shah SV, Northrup TE, Hui YSF and Dousa TP, Action of serotonin (5-hydroxytryptamine) on cyclic nucleotides in glomeruli of rat renal cortex. *Kidney Int* **15**: 463–472, 1979.
20. Sraer J, Ardaillou R, Loreau N and Sraer JD, Evidence for parathyroid hormone sensitive adenylate cyclase in rat glomeruli. *Mol Cell Endocrinol* **1**: 285–294, 1974.
21. Schmidt M and Imbs JL, Pharmacological characterization of renal vascular dopamine receptors. *J Cardiovasc Pharmacol* **2**: 595–605, 1980.
22. Steinhausen M, Weis S, Fleming J, Dussel R and Parekh N, Responses of *in vivo* renal microvessels to dopamine. *Kidney Int* **30**: 361–370, 1986.
23. Kenakin TP and Pike NB, An *in vitro* analysis of purine-mediated renal vasoconstriction in rat isolated kidney. *Br J Pharmacol* **90**: 373–381, 1987.
24. Helwig JJ, Schleiffer R, Judes C and Gairard A, Distribution of parathyroid hormone-sensitive adenylate cyclase in isolated rabbit renal cortex microvessels and glomeruli. *Life Sci* **35**: 2649–2657, 1984.

25. Ishikawa S, Saito T and Kuzuya T, Effects of osmolality on the interaction between vasopressin and prostaglandins in rat renal papillary collecting tubule cells in culture. *Biomed Res* **6**: 269–278, 1985.
26. Ishikawa S, Saito T and Kuzuya T, Calmodulin regulation of cellular cyclic AMP production in response to arginine vasopressin, prostaglandin E_2 and forskolin in rat renal papillary collecting tubule cells in culture. *J Endocrinol* **107**: 15–22, 1985.
27. Jackman GP and Bobik A, Forskolin-mediated activation of cardiac, liver and lung adenylate cyclase in the rat. *Biochem Pharmacol* **35**: 2247–2251, 1986.