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FACTORS REGULATING PROSTAGLANDIN E_2 BIOSYNTHESIS IN RENAL CORTICAL TUBULAR CELLS

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Abstract—Prostaglandin synthesis by isolated rat renal cortical tubular cells was studied *in vitro* with a superfusion system. The cells were introduced in Teflon chambers and intermittently stimulated. The PGE_2 production was measured in the effluent. ANG II (10^{-10}–10^{-6}M) induced a dose-dependent increase in PGE_2 synthesis. Saralasin antagonized the response to ANG II. Hyperosmolar mannitol or NaCl and Ca^{2+}-ionophore A23187 also stimulated PGE_2 synthesis. The PGE_2 response to all stimuli was blocked in Ca^{2+}-free media containing EGTA. The Ca^{2+}-channel blocker nifedipine (10^{-10}–10^{-6}M) did not significantly inhibit the PGE_2 response to ANG II, hyperosmolar mannitol or NaCl, and A23187, whereas the phospholipase-inhibitors *p*-bromophenacyl bromide (10^{-4}M) and chloroquine (10^{-4}M) inhibited the response. Thus, PGE_2 synthesis in response to these stimuli in rat renal cortical tubular cell is a Ca^{2+}-dependent process, acting via phospholipases by a mechanism which does not appear to involve voltage-dependent Ca^{2+}-channels.

Several studies have shown that prostaglandins are synthesized in various sites in the kidney [1–3], and that renal prostaglandin synthesis is a Ca^{2+}-dependent process [4–5]. Ca^{2+} is required to activate phospholipase A_2, which hydrolyses arachidonic acid from membrane phospholipids [6,7]. In addition, a phospholipase C pathway has been described as a mechanism of prostaglandin formation [8–10]. Phospholipase C liberates diacylglycerol, which yields arachidonic acid under the action of monoacylglycerol- and diacylglycerol-lipase. We have previously reported that rat renal cortical tubular cells produce PGE_2 and $PGF_{2\alpha}$ in response to various stimuli, including ANG II, hyperosmolar mannitol and sodium chloride, and Ca^{2+}-ionophore A23187 [11]. The aim of this study was to investigate some of the mechanisms activating prostaglandin synthesis in these cells, using a Ca^{2+}-channel blocker and phospholipase-inhibitors as pharmacological tools in a superfusion system. Part of this work has been presented in abstract form [12].

MATERIALS AND METHODS

Materials. Collagenase (CLS II, 156 U/mg) was from Worthington Diagnostic Systems. A23187 (free acid), chloroquine, *p*-bromophenacyl bromide and bovine serum albumine (BSA) were from Sigma Chemical Company (St. Louis, MO). ANG II and [Sar^1-Ala^8]ANG II were from Bachem AG (Bubendorf, Switzerland). Nifedipine was a gift of Bayer (Leverkusen, F.R.G.). [^{3}H]PGE_2 (160 Ci/mmol) was purchased from Amersham International (Bucks, U.K.) and the PGE_2 antiserum was purchased from the Institut Pasteur (Paris). The Krebs–Ringer bicarbonate buffer had the following composition: 118 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM $MgSO_4 \cdot 7H_2O$, and 25 mM $NaHCO_3$. Glucose (11.1 mM) was added, and 2% BSA (media I) for collagenase dispersion of the cortical tissue, or 0.2% BSA (media II) for the superfusion of the cortical tubular cells.

Dispersion of renal cortical tubular cells. The kidneys from three to four female Wistar rats (180–220 g body wt) were excised immediately after decapitation and chilled in 0.9% NaCl. After removal of the capsules, the outer cortical tissue (2.5–3.5 g) was dissected away from the medulla and papilla and minced to a paste-like consistency. The cortical tissue was incubated for 30 min at 37° with media I containing 0.03% (w/v) collagenase, and disrupted by repeated aspiration through a plastic tube connected to a disposable 20 ml syringe. The cell suspension containing the isolated cells was filtered through a 70 μm nylon gauze to retain glomeruli and tissue fragments. The cells were then washed twice with media I, filtered again and resuspended in 4 ml of media I. More than 95% of the cells excluded trypan blue before and after superfusion. Electron microscopy was performed on the pellets of the cortical cell suspensions and showed well preserved epithelial cells, and no glomeruli. The majority of the cells were of proximal tubular origin, characterized by the typical brush-border membrane, the heterochromatin of the nucleus and the abundance of mitochondria.

Experimental design. 1.5 ml of the cell suspension containing $7–12 \times 10^6$ cells/ml was introduced in each of two specially designed Teflon chambers, which were superfused in parallel. The cells were retained by a 4 mm Bio-Gel layer [P2, 200–400 mesh polyacrylamide gel beads, Bio-Rad Laboratories, (Richmond, Ca)]. Red cells and a few small cells crossed the Bio-Gel layer and were not retained. The beads were supported by a 30 μm nylon gauze.

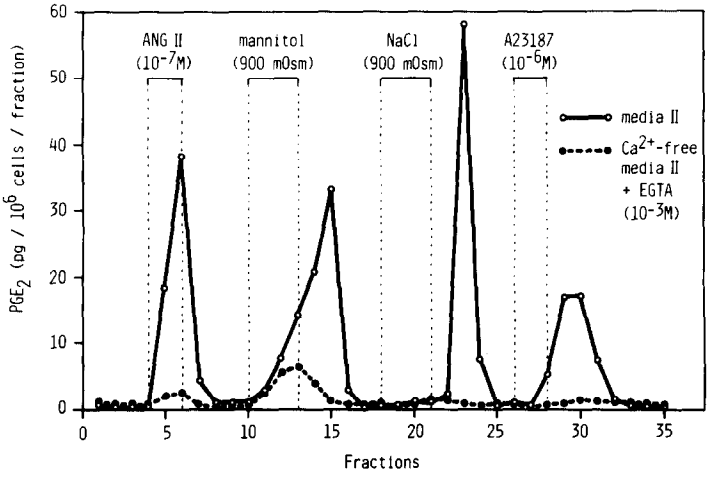

Fig. 1. Each of two Teflon chambers were loaded with 15.7×10^6 cells and superfused with media II. After 60 min of stabilization, one chamber was superfused with regular media II, and one chamber with Ca^{2+}-free media II containing 10^{-3}M EGTA. The cells were then intermittently stimulated with 10^{-7}M ANG II, 900 mOsm mannitol and NaCl, and 10^{-6}M A23187, in the presence of media II or Ca^{2+}-free media II containing EGTA. Five minute fractions were collected, and the PGE_2 content in each fraction was measured.

The Teflon chambers were fixed in a water bath at 37° and the cells were continuously superfused with media II at a flow rate of 250 μl/min. The media was gassed with 95% O_2:5% CO_2. After 60 min of stabilization, the cells were intermittently stimulated for 10 or 15 min at 20–30 min intervals, by adding sequentially angiotensin II, hyperosmolar mannitol and NaCl, and Ca^{2+}-ionophore A23187. One superfusion chamber was superfused with the stimuli and served as the control, and the second chamber was superfused with the stimuli in the presence of an inhibitor. The effluent was collected in 5 min fractions, stored at −20° and assayed within two days.

PGE_2 assay. PGE_2 was directly measured in a 50 μl aliquot of each fraction by radioimmunoassay without prior extraction, using $[^3H]PGE_2$ and specific rabbit antiserum, whose crossreactivity was less than 0.5% for $PGF_{2\alpha}$, PGA_2, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2. All fractions were assayed in duplicate. Charcoal was used for separation, and bound radioactivity was counted with a Packard liquid scintillation counter. The limit of detection for a 50 μl aliquot was 1 pg. Specific binding ranged from 36 to 44%, and nonspecific binding from 3 to 6%. Fifty per cent tracer displacement occurred around 13 pg. Recovery measurements of added standard to the superfusion medium ranging from 0 to 400 pg PGE_2 were performed. Regression analysis gave a slope of 0.9 ($r = 0.998$) and an extrapolated blank (intercept on the y-axis) of 0.83 pg. The mean coefficient of variation for triplicate determinations of undiluted, 1:2 and 1:4 diluted 50 μl fraction aliquots was 7.8 ± 1.3% (mean ± S.E., $N = 22$). Intra- and interassay variability were 6.6 and 10.5%, respectively.

Analysis of data. The measured PGE_2 content in each fraction was divided by the number of cells. The PGE_2 response upon stimulation was calculated as the increment above the corresponding basal release. Results are expressed as mean ± S.E. Statistical differences were evaluated at the 95% confidence level using Student's two-tailed *t*-test.

RESULTS

Effect of ANG II, changes in osmolality and A23187 *on PGE_2 synthesis*

Figure 1 shows that renal cortical tubular cells responded to ANG II, hyperosmolar mannitol and NaCl, and A23187 by an increase in PGE_2 production. The response to NaCl was delayed and occurred when osmolality declined to normal values (290 mOsm/kg H_2O). In Ca^{2+}-free superfusion media containing 10^{-3}M EGTA, the response to all four stimuli was abolished. ANG II (10^{-10}–10^{-6} M) induced a dose dependent increase in PGE_2 synthesis (Fig. 2). In the presence of the specific receptor antagonist [Sar^1-Ala^8]ANG II (10^{-5}M), the response to 10^{-7}M ANG II was reduced by 68.6% ($N = 5$, $P < 0.005$).

In the presence of the Na^+–K^+-ATPase inhibitor ouabain (10^{-3}M), the cells responded reversibly with an increase in the PGE_2 production (basal release: 0.9 ± 0.4, stimulated release after 10 min of application: 54.0 ± 4.6 pg/10^6 cells/fraction, $N = 3$, $P < 0.01$). Concentrations of ouabain of 10^{-4}M or lower did not stimulate the PGE_2 production. K^+-free medium, a condition during which Na^+-K^+-ATPase is inhibited, increased also reversibly the PGE_2 production (not shown).

Effect of nifedipine, p-bromophenacyl bromide and chloroquine on PGE_2 synthesis

In the presence of the specific Ca^{2+}-channel blocker nifedipine (10^{-10}–10^{-6}M), the PGE_2 response to ANG II, hyperosmolar mannitol and NaCl, and A23187 was not significantly inhibited.

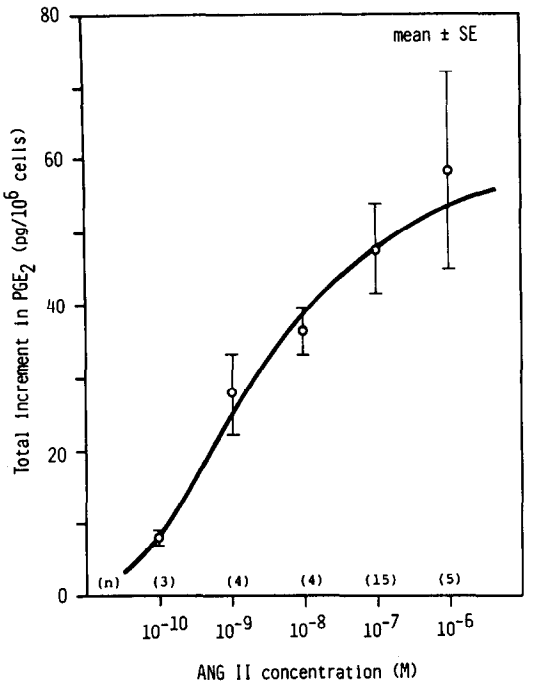

Fig. 2. Dose–response curve for ANG II. Cortical tubular cells were loaded into Teflon chambers and superfused during 10 min with different concentrations of ANG II. The increase in the PGE_2 production over the basal release in four fractions was calculated for each dose of ANG II. Each point of the curve represents the mean ± S.E. from three to 15 separate cell preparations.

Table 1 indicates the absence of inhibition on PGE_2 synthesis with 10^{-6}M nifedipine. On the other hand, both *p*-bromophenacyl bromide and chloroquine inhibited significantly renal cortical tubular PGE_2 synthesis (Table 1), at concentrations known to be effective on phospholipase. *p*-bromophenacyl bromide (10^{-4}M) inhibited the ANG II-, mannitol- and NaCl-stimulated PGE_2 synthesis by 78, 62 and 100% respectively, whereas chloroquine (10^{-4}M) blocked by 73, 43 and 86%, respectively. The PGE_2 response to 10^{-6}M A23187 was inhibited by 98% in the presence of chloroquine.

DISCUSSION

We have previously reported that superfused rat renal cortical tubular cells release PGE_2 and $PGF_{2\alpha}$, but not 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) in response to ANG II, changes in osmolality, and Ca^{2+}-ionophore A23187 [11]. The present study demonstrates that the superfusion of renal cortical tubular cells is a useful method to elucidate the intracellular mechanisms of prostaglandin biosynthesis by means of pharmacological agents.

ANG II stimulated PGE_2 synthesis dose-dependently between 10^{-10} and 10^{-6}M. [Sar^1-Ala^8]ANG II, a potent antagonist, inhibited the response to ANG II. This indicates that the observed increase in PGE_2 synthesis was consecutive to the interaction of ANG II with a specific membrane receptor. In Ca^{2+}-free medium containing EGTA, the PGE_2 response to ANG II was blocked, demonstrating that Ca^{2+} is required for prostaglandin synthesis in rat renal cortical tubular cells. Other investigators have also demonstrated that ANG II-stimulated prostaglandin synthesis in renal medullary slices [4] and glomerular mesangial cells in culture [13] is reduced in Ca^{2+}-free media. Nifedipine, a selective inhibitor of voltage-sensitive Ca^{2+}-channels [14], did not inhibit ANG II-stimulated PGE_2 synthesis, suggesting that prostaglandin synthesis in response to ANG II is not mediated by these channels or that these channels are absent in cortical tubular cells. Both *p*-bromophenacyl bromide and chloroquine inhibited the PGE_2 response to ANG II at concentrations known to be inhibitory on phospholipases [8,15–18]. The alkylating agent *p*-bromophenacyl bromide is a phospholipase A_2-inhibitor thought to interact directly with a specific histidine residue in the enzyme [15]. Chloroquine in turn, interferes with membrane

Table 1. Effect of *p*-bromophenacyl bromide, chloroquine and nifedipine on PGE_2 synthesis

Stimulus	Control	+ *p*-Bromophenacyl bromide (10^{-4}M)	+ Chloroquine (10^{-4}M)	+ Nifedipine (10^{-6}M)
Basal release (pg/10^6 cells/fraction)	2.5 ± 0.6	3.2 ± 0.3	2.0 ± 0.8	0.7 ± 0.2
Increment (Δpg/10^6 cells)				
ANG II, 10^{-7}M	47.8 ± 6.1	10.6 ± 0.8*	13.1 ± 4.9*	54.3 ± 14.7
Mannitol, 900 mOsm	52.7 ± 3.7	19.9 ± 3.9*	30.0 ± 11.7†	52.4 ± 6.4
NaCl, 900 mOsm	66.5 ± 5.1	< 3.2	9.0 ± 2.9*	77.5 ± 18.9
A23187, 5 × 10^{-6}M	26.5 ± 3.9	not done	not done	21.5 ± 5.2

Cortical tubular cells were superfused in Teflon chambers and intermittently stimulated for 10 min with ANG II or A23187, or for 15 min with 900 mOsm mannitol or NaCl, in the presence or absence of p-bromophenacyl bromide, chloroquine or nifedipine. Basal values represent the non-stimulated PGE_2 release (pg/10^6 cells/fraction) with regular media II. The increment in PGE_2 synthesis (pg/10^6 cells) over the corresponding basal release was calculated in four to six fractions. Results are mean ± S.E. from four to 14 experiments.

* $P < 0.001$.

† $P < 0.05$.

phospholipids by way of its amphiphilic nature [19,20], inhibiting thereby the metabolic degradation of phospholipids by phospholipases [16,17,19]. More recent studies in platelets indicate that *p*-bromophenacyl bromide inhibits also other enzymes, especially phospholipase C [18,21,22]. Chloroquine is also known to inhibit phospholipase C [16], like the close analogue mepacrine [22,23]. Our results suggest that ANG II-stimulated prostaglandin synthesis in renal cortical tubular cells may be mediated by phospholipase A_2 or C, although the lack of specificity of these inhibitors has to be considered.

Changes in osmolality induced with mannitol and NaCl stimulated cortical tubular PGE_2 synthesis. Like for ANG II, the response was also dependent on the presence of extracellular Ca^{2+}, was not inhibited by nifedipine, and was significantly blocked by the phospholipase-inhibitors *p*-bromophenacyl bromide and chloroquine. This suggests that a similar mechanism for prostaglandin formation is activated by these stimuli. By increasing the intracellular sodium concentration, hyperosmolar NaCl can cause a secondary rise in intracellular Ca^{2+} [23, 24], which may activate phospholipases. Increasing the intracellular sodium concentration by inhibiting the Na^+-K^+-ATPase with ouabain or K^+-free medium enhanced also the PGE_2 synthesis, suggesting again that an increase in intracellular Na^+ and/or Ca^{2+} can trigger prostaglandin synthesis in renal cortical tubular cells.

Ca^{2+}-ionophore A23187, which causes an increase in cytosolic Ca^{2+}, increased also PGE_2 formation. The response was dependent on extracellular Ca^{2+}, was not influenced by nifedipine, and was blocked with the phospholipase A_2-inhibitor chloroquine. This indicates that a rise in intracellular Ca^{2+} may activate phospholipases for prostaglandin synthesis in these cells.

In summary, prostaglandin synthesis in rat renal cortical tubular cells can be stimulated by ANG II in a dose-dependent manner. Ca^{2+}-influx not mediated by voltage-dependent Ca^{2+}-channels might activate phospholipase A_2 and/or phospholipase C, yielding intracellular free arachidonic acid, the substrate of cyclooxygenase.

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REFERENCES

1. M. G. Currie and P. Needleman, *A. Rev. Physiol.* **46**, 327 (1984).
2. M. J. Dunn and V. L. Hood, *Am. J. Physiol.* **233**, F169 (1977).
3. J. Sraer, W. Siess, L. Moulonguet-Doleris, J.-P. Oudinet, F. Dray and R. Ardaillou, *Biochim. biophys. Acta* **710**, 45 (1982).
4. J. E. Benabe, L. A. Spry and A. R. Morrison, *J. biol. Chem.* **257**, 7430 (1982).
5. P. A. Craven and F. R. DeRubertis. *Biochim. biophys. Acta* **804**, 450 (1984).
6. G. J. Blackwell and R. J. Flower, *Br. med. Bull.* **39**, 260 (1983).
7. H. R. Knapp, O. Oelz, L. J. Roberts, B. J. Sweetman, J. A. Oates and P. W. Reed, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4251 (1977).
8. Y. Fujimoto, N. Akamatsu, A. Hattori and T. Fujita, *Biochem. J.* **218**, 69 (1984).
9. J. Pfeilschifter, A. Kurtz and C. Bauer, *Biochem. J.* **223**, 855 (1984).
10. D. Schlondorff, J. Perez and J. A. Satriano, *Am. J. Physiol.* **248**, C119 (1985).
11. R. P. Wuthrich, R. Loup, L. Favre and M. B. Vallotton, *Am. J. Physiol.* (in press).
12. R. P. Wuthrich, C. Wicht, L. Favre and M. B. Vallotton, *Eur. J. clin. Invest.* **15**(2) part II, A31 (1985).
13. L. A. Scharschmidt and M. J. Dunn, *J. clin. Invest.* **71**, 1756 (1983).
14. A. M. Katz, W. D. Hager, F. C. Messineo and A. J. Pappano, *Am. J. Med.* **77**(2B), 2 (1984).
15. J. J. Volwerk, W. A. Pieterson and G. H. de Haas, *Biochemistry* **13**, 1446 (1974).
16. Y. Matsuzawa and K. Y. Hostetler, *J. biol. Chem.* **255**, 5190 (1980).
17. K. Y. Hostetler and D. D. Richman, *Biochem. Pharmac.* **31**, 3795 (1982).
18. L. Best, A. Sener, P. C. F. Mathias and W. J. Malaisse, *Biochem. Pharmac.* **33**, 2657 (1984).
19. J. K. Seydel and O. Wassermann, *Biochem. Pharmac.* **25**, 2357 (1976).
20. H. Lüllmann and M. Wehling, *Biochem. Pharmac.* **28**, 3409 (1979).
21. E. M. Kyger and R. C. Franson, *Biochim. biophys. Acta* **794**, 96 (1984).
22. S. L. Hofmann, S. M. Prescott and P. W. Majerus, *Archs. Biochem. Biophys.* **215**, 237 (1982).
23. D. A. Lowe, B. P. Richardson, P. Taylor and P. Donatsch, *Nature* **260**, 337 (1976).
24. M. P. Blaustein, *Am. J. Physiol.* **232**, C165 (1977).