Activation of phospholipase C and prostaglandin synthesis by [arginine]vasopressin in cultures

Josef PFEILSCHIFTER, Armin KURTZ and Christian BAUER*
*Physiologisches Institut der Universität Regensburg, Postfach 397, D-8400 Regensburg, Federal Republic of
Germany*

[Arginine]vasopressin (AVP) stimulates maximal prostaglandin E_2 production in cultured rat renal mesangial cells within 2 min. As early as 10 s after addition of AVP (10^{-6} M) a significant loss of radioactivity from phosphatidylinositol 4,5-bisphosphate but not from phosphatidylinositol 4-phosphate and phosphatidylinositol was observed in cells prelabelled with $^{32}P_i$. Cells labelled with [^{14}C]arachidonic acid showed an increase of label in 1,2-diacylglycerol after 15 s and in phosphatidic acid after 30 s upon stimulation with AVP. Pretreatment of the cells with indomethacin (10^{-5} M) did not abolish the effect of AVP on the increased labelling of phosphatidic acid.

AVP is known to exert at least two major effects on the kidney. It enhances the reabsorption of water in the distal convolute and collecting tubule and has furthermore been shown to reduce the glomerular ultrafiltration coefficient, K_f (Ichikawa & Brenner, 1977; Schor *et al.*, 1981). This latter effect is thought to be caused by a contraction of mesangial cells (Mahieu *et al.*, 1980; Ausiello *et al.*, 1980; Scharschmidt & Dunn, 1983). AVP has also been shown to induce PGE_2 synthesis, the major cyclo-oxygenase product of cultured mesangial cells (Kreisberg *et al.*, 1982; Scharschmidt & Dunn, 1983). This increase in prostaglandin production is thought to modulate the contractile effect of AVP on mesangial cells (Kreisberg *et al.*, 1982).

In view of these connections experiments were designed to gather information on the mechanism by which AVP induces PG synthesis in mesangial cells. It is known that the rate-limiting step of PG synthesis is the availability of the cyclo-oxygenase substrate arachidonic acid (Isakson *et al.*, 1978). One of the mechanisms for arachidonic acid release is the activation of the so-called phosphatidylinositol cycle which leads to the liberation of

arachidonic acid from phospholipids (Bell *et al.*, 1979; Marshall *et al.*, 1980, 1981; Lapetina, 1982). Since it has been demonstrated that AVP activates this cycle in hepatocytes (Billah & Michell, 1979; Kirk & Michell, 1981; Michell *et al.*, 1981; Litosch *et al.*, 1983; Thomas *et al.*, 1983) we determined whether or not AVP induces PG synthesis in cultured mesangial cells by a stimulation of the phosphatidylinositol cycle.

Experimental

Materials

Lipid standards, AVP and indomethacin were obtained from Sigma. T.l.c. plates (pre-coated silica gel 60 with concentration zone, 0.25 mm thick) were from Merck, Darmstadt, Germany. [1-^{14}C]Arachidonic acid and carrier-free $^{32}P_i$ were purchased from Amersham International. All other chemicals for t.l.c. were from Merck.

Cell culture

Cultivation of rat mesangial cells was done as described (Kurtz *et al.*, 1982). For all experiments the first passage of mesangial cell cultures was used. The cells were grown in RPMI 1640 (Boehringer, Mannheim, Germany) supplemented with 10% foetal calf serum (Boehringer), penicillin (100 units/ml), streptomycin (100 μg/ml) (Boehringer) and bovine insulin at 0.66 unit/ml (Sigma). Tissue dishes (7 cm²; Greiner, Nürtingen, Germany) were incubated at 37°C in a humidified atmosphere in incubators under $CO_2/O_2/N_2$ (1:4:15).

Abbreviations used: AVP, [arginine]vasopressin; PG, prostaglandin; PI, PIP, PIP_2, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate; PA, phosphatidic acid; DG, diacylglycerol.
* Present address: Physiologisches Institut der Universität Zürich, Winterthurerstraße 190, CH-8057 Zürich, Switzerland.

Cell labelling

Medium was removed and the cells were incubated in MEM (Boehringer) supplemented with fatty acid-free bovine serum albumin (0.3mg/ml) (Sigma) and [^{14}C]arachidonic acid (0.25μCi/ml) for 24h. For the determination of polyphosphoinositides, cells were labelled with carrier-free $^{32}P_i$ (100μCi/ml) in phosphate-poor MEM (50μM-P_i) for 24h. After this prelabelling period the medium was replaced by fresh medium either with or without (control) AVP (10^{-6}M). Incubations were terminated by a rapid withdrawal of the incubation medium and the addition of 1ml of ice-cold methanol. The aspirated medium was frozen in liquid N_2 and stored at -60°C until use. Indomethacin (Sigma) was added where indicated at a final concentration of 10^{-5}M 15min before addition of agonists.

Extraction

The lipid extraction was done according to Bligh & Dyer (1959) with a final proportion of 2ml of methanol, 2ml of chloroform and 1.6ml of water (containing 0.74% KCl, 0.04% CaCl$_2$ and 0.034% MgCl$_2$). After removal of the first chloroform extract, the remaining methanol/water phase was acidified with HCl (final concentration 0.01M) and extracted twice with 2ml of chloroform. The extraction of polyphosphoinositides was done at acid pH in all three steps. The chloroform extracts were combined and dried in a rotation evaporator, dissolved in 200μl of chloroform/methanol (2:1, v/v) and an aliquot was taken for t.l.c.

Chromatography

For separation of phospholipids, chromatographs were developed in one dimension using chloroform/methanol/acetic acid/water (100:30:35:3, by vol.). This solvent system allowed the complete separation of sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid and cardiolipin (Thomas & Williamson, 1983). For separation of neutral lipids, chromatographs were developed in one dimension using n-heptane/diethyl ether/acetic acid (75:25:4, by vol.). This solvent system allowed the complete separation of free fatty acid, monoacylglycerol, 1,2-diacylglycerol, 1,3-diacylglycerol, triacylglycerol and cholesteryl ester (Korte & Casey, 1982). Polyphosphoinositides were separated on thin layer plates pretreated with 1% potassium oxalate containing 2mM-EDTA using chloroform/methanol/4M-NH$_4$OH (9:7:2, by vol.) (Billah & Lapetina, 1982). For phospholipid separation standards of PA, PI, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine

were added as carriers; for neutral lipid separation standards of triacylglycerol, cholesterol ester, 1,2-DG and 1,3-DG and for polyphosphoinositides separation standards of PIP$_2$ and PIP were added as carriers. The lipids were visualized by iodine staining and ^{32}P-labelled lipids additionally by autoradiography.

Analyses of radiochromatograms

The developed t.l.c. plates were analysed with a TLC-Linear-Analyzer LB 2820 from Berthold, München, Germany. The detection efficiency for ^{14}C was about 10%. ^{32}P-labelled lipids were scraped off and counted by liquid scintillation in water (Cerenkov counting).

PG and protein analysis

PGE$_2$ concentrations of the culture mediums were determined by radioimmunoassay for PGE$_2$ (New England Nuclear). Protein determination was done according to the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as standard.

Results

Effect of AVP on PGE$_2$ synthesis in rat mesangial cells

The time-dependence of basal and AVP-stimulated synthesis of PGE$_2$ by mesangial cells is shown in Fig. 1. Stimulation of PGE$_2$ synthesis by 10^{-6}M-AVP was found to occur rapidly; 64% and 93% of the total amount of PGE$_2$ present after 10min were already produced 30s and 120s respectively after AVP stimulation. The stimulation of PGE$_2$ synthesis was therefore essentially complete within 2min. PGE$_2$ synthesis was also
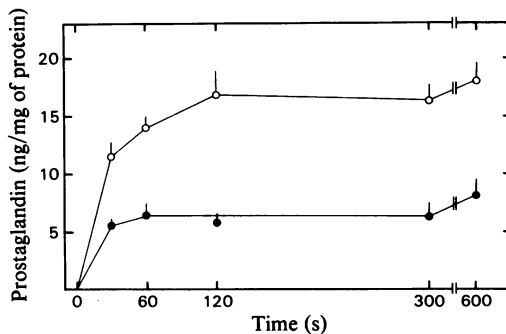
Fig. 1. *Time dependence of PGE$_2$ production by cultured mesangial cells incubated in MEM without or with AVP (10^{-6}M)*

Data are expressed as ng of PGE$_2$ produced per mg of cell protein with ($\bigcirc$) or without ($\bullet$) AVP. Values are mean $\pm$ S.E.M., $n = 5$.

found to be stimulated in the control experiments but to a much lesser degree than in those with AVP. The net rate of production during the first 2min after stimulation with AVP was 8.4ng of *PGE$_2$/min per mg of protein (control 2.9ng/min per mg) and then returned to a basal level of about 0.15ng/min per mg.

Effect of AVP on phospholipase C in rat mesangial cells

Addition of AVP (10^{-6}M) to cultured mesangial cells prelabelled with [^{14}C]arachidonic acid stimulated the rapid and transient formation of [^{14}C]1,2-DG and [^{14}C]PA (Figs. 2 and 3). Maximal formation of 1,2-DG occurred as early as 15s after addition of AVP (182% of control value) and returned to control values after 10min. Phosphatidic acid levels had a maximum (174% of control value) 30s after addition of AVP and then reached
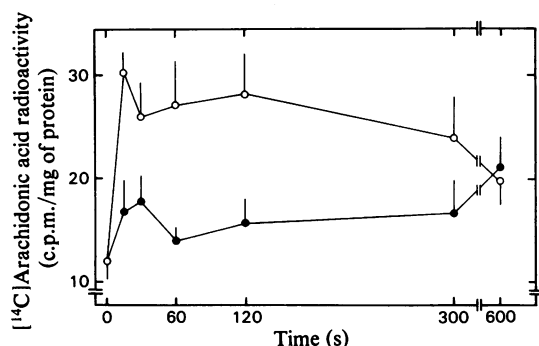
a plateau. After 5min PA levels slowly decreased and reached near-control values after 10min.

Although a small initial decrease in PI was observed during the first 15s (results not shown), this decrease was not statistically significant. As shown in Fig. 4, AVP treatment provoked a rapid decrease in the level of ^{32}P found in PIP$_2$. This effect was maximal within 10s (70% of control), the earliest time interval examined. At 1min after the addition of AVP the level of ^{32}P in PIP$_2$ increased and approached control level, followed by an increase above initial levels after 2min. PIP was also found to decrease initially. This decrease, however, was not statistically significant and PIP levels rapidly increased above the initial values. It should be noted in this connexion that no significant change of radioactivity was observed in phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine in AVP-stimulated cells.

AVP-induced stimulation of the PI cycle was not prevented by indomethacin

To examine whether the PI cycle was directly stimulated by AVP or only indirectly stimulated by cyclo-oxygenase products we determined the time course of PA in the presence of arachidonic acid (10^{-5}M), arachidonic acid (10^{-5}M) plus indomethacin (10^{-5}M) and AVP (10^{-6}M) plus indomethacin (10^{-5}M).

Fig. 5 shows that addition of arachidonic acid to cultured mesangial cells led to an increase of the ^{32}P level in PA. This increase in PA was abolished by the addition of indomethacin. Simultaneous addition of AVP and the cyclo-oxygenase inhibitor indomethacin, however, did not prevent the AVP-stimulated increase in PA.

Fig. 2. *Time course of* [^{14}C]arachidonic acid radioactivity in 1,2-DG in mesangial cells stimulated with AVP (10^{-6}M)
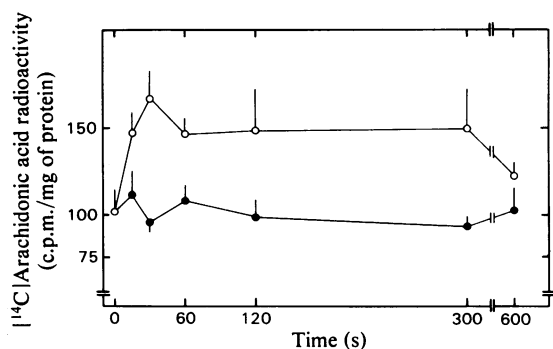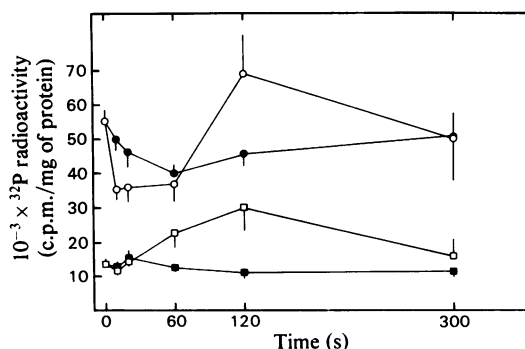●, Control (no agonist); ○, AVP (1 μM). Values are mean ± S.E.M., n = 5.

Fig. 3. *Time course of* [^{14}C]arachidonic acid radioactivity in PA in mesangial cells stimulated with AVP (10^{-6}M)
●, Control (no agonist); ○, AVP (1 μM). Values are mean ± S.E.M., n = 5.

Fig. 4. *Time course of* ^{32}P *radioactivity in polyphosphoinositides*
Open symbols represent stimulation with AVP (10^{-6}M); filled symbols represent control values. ○, ●, PIP$_2$; □, ■, PIP. Values are mean ± S.E.M., n = 5.
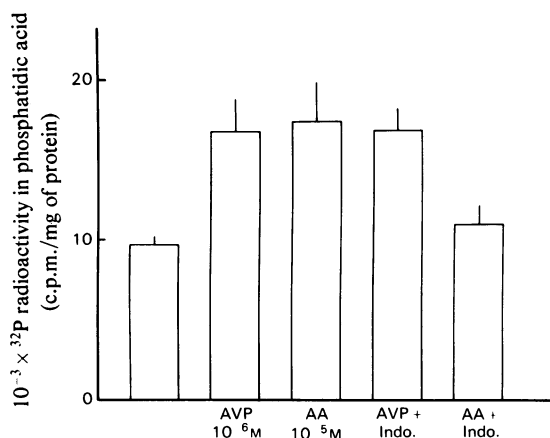
Fig. 5. *Effect of indomethacin on ^{32}P radioactivity in PA in presence of AVP and arachidonic acid*
^{32}P radioactivity in PA was determined 30s after addition of AVP or arachidonic acid (AA) alone or together with indomethacin (Indo.). Values are mean ± S.E.M., $n = 5$.

Discussion

The results presented in this paper are compatible with the hypothesis that AVP-stimulated PG synthesis is linked to an increased turnover of polyphosphoinositides in cultured rat mesangial cells. Since it is known that the rate limiting step in PG synthesis is the availability of free arachidonic acid as substrate for cyclo-oxygenase, AVP must cause the release of arachidonic acid from certain phospholipids. The bulk of arachidonic acid in mammalian cells is bound to the fatty acyl chains of glycerophospholipids, almost exclusively in the 2-acyl position (for review see Irvine, 1982).

There are two possible mechanisms by which arachidonic acid may be released from phospholipids: one is the action of a phospholipase A_2 (Lapetina, 1982) and the other is the combined action of a phospholipase C and DG lipase (Mauco et al., 1978; Bell et al., 1979). From the present data it is clear that AVP causes rapid hydrolysis of PIP_2 in cultured rat mesangial cells. This response is quite similar to that observed during AVP action in cultured rat hepatocytes (Thomas et al., 1983). It seems most likely that the decrease in ^{32}P label in PIP_2 is due to the activation of a specific phospholipase C. An alternative explanation for the decrease of ^{32}P labelling in PIP_2 would be the decreased synthesis of PIP_2 or the increased conversion of PIP_2 to PIP and PI via phosphomonoesterases. However, these are unlikely explanations as labelling of neither PIP nor PI (results not shown) increased during this early time period.

The conclusion that phospholipase C activity is increased is corroborated by the findings of several authors who have demonstrated a selective breakdown of PIP_2 in various tissues (for review see Berridge, 1984). This early decrease in PIP_2 was accompanied by the rapid and transient formation of $[^{14}C]1,2$-DG and $[^{14}C]PA$ (Figs. 2 and 3). Maximal formation of 1,2-DG preceded that of PA, indicating the sequential action of a specific phospholipase C and 1,2-DG kinase.

This result clearly shows that AVP stimulates the PI cycle in cultured renal mesangial cells. Since an activation of the PI-cycle provides possibilities for arachidonic acid release (Bell et al., 1979; Lapetina, 1982) and the availability of arachidonic acid is the rate-limiting step in PG synthesis, our results provide evidence that AVP enhances PG synthesis by an activation of the PI cycle. Conceivably, the PI cycle could also be activated by cyclo-oxygenase products, as demonstrated in platelets (Siess et al., 1983). However, this possibility can be excluded because indomethacin inhibited the stimulatory effect on the PI cycle of exogenous addition of arachidonic acid, but not the stimulation seen with AVP (Fig. 5). Our results furthermore suggest that any arachidonate liberated in response to AVP is either insufficient in quantity, or is in the wrong place to stimulate additionally phospholipase C.

The kinetics of the formation of 1,2-DG and PA on one hand, and of PGE_2 on the other (Fig. 1), are also in harmony with the hypothesis that an activation of the PI cycle liberates arachidonic acid from phospholipids. Three possibilities need to be considered in this regard: (a) increased availability of the substrate 1,2-DG to the DG lipase, (b) activation of the Ca^{2+}-stimulated phospholipase A_2 by PA, which acts as Ca^{2+}-ionophore (Putney et al., 1980; Salmon & Honeyman, 1980; Holmes & Yoss, 1983), and (c) the release of arachidonic acid by a PA-specific phospholipase A_2 (Billah et al., 1981). We are currently investigating which of these three mechanisms operates in AVP-stimulated renal mesangial cells.

References

Ausiello, D. A., Kreisberg, J. I. & Roy, C. (1980) *J. Clin. Invest.* **65**, 754–760
Bell, R. L., Kennerly, D. A., Stanford, N. & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3241
Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360

Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 12705–12708

Billah, M. M. & Michell, R. H. (1979) *Biochem. J.* **182**, 661–668

Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399–5403

Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

Holmes, R. P. & Yoss, N. L. (1983) *Nature (London)* **305**, 637–638

Ichikawa, I. & Brenner, B. M. (1977) *Am. J. Physiol.* **233**, F102–F117

Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16

Isakson, P. C., Raz, A., Hsuch, W. & Needleman, P. (1978) *Adv. Prostaglandin Thromboxane Res.* **3**, 113–120

Kirk, C. J. & Michell, R. H. (1981) *Biochem. J.* **194**, 155–165

Korte, K. & Casey, M. L. (1982) *J. Chromatogr.* **232**, 47–53

Kreisberg, J. I., Karnovsky, M. J. & Levine, L. (1982) *Kidney Int.* **22**, 355–359

Kurtz, A., Jelkmann, W. & Bauer, C. (1982) *FEBS Lett.* **137**, 129–132

Lapetina, E. G. (1982) *Trends Pharmacol. Sci.* **3**, 115–118

Litosch, I., Lin, S. H. & Fain, J. N. (1983) *J. Biol. Chem.* **258**, 13727–13732

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mahieu, P. R., Foidart, J. B., Dubois, C. H., Dechenne, C. A. & Deheneffe, J. (1980) *Invest. Cell Pathol.* **3**, 121–128

Marshall, P. J., Dixon, J. F. & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3292–3296

Marshall, P. J., Boatman, D. E. & Hokin, L. E. (1981) *J. Biol. Chem.* **256**, 844–847

Mauco, G., Chap, H., Simon, M. F. & Douste-Blazy, L. (1978) *Biochimie* **60**, 653–661

Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. Lond. Ser. B* **296**, 123–137

Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Nature (London)* **309**, 562–564

Putney, J. W., Jr., Weiss, S. J., Van de Walle, C. M. & Haddas, R. A. (1980) *Nature (London)* **284**, 345–347

Salmon, D. M. & Honeyman, T. W. (1980) *Nature (London)* **284**, 344–345

Scharschmidt, L. A. & Dunn, M. J. (1983) *J. Clin. Invest.* **71**, 1756–1764

Schor, N., Ichikawa, I. & Brenner, B. M. (1981) *Kidney Int.* **20**, 442–451

Siess, W., Siegel, F. L. & Lapetina, E. G. (1983) *J. Biol. Chem.* **258**, 11236–11242

Thomas, A. P. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 1411–1414

Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 5716–5725