The role of calcium in the stimulation of prostaglandin synthesis by vasopressin in rabbit renal-medullary interstitial cells in tissue culture

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The role of Ca^{2+} in the stimulation of prostaglandin (PG) biosynthesis by vasopressin was investigated in rabbit renal-medullary interstitial cells in tissue culture. A decrease in extracellular Ca^{2+} to less than $25\text{ }\mu\text{M}$ did not affect basal PGE_2 production, but inhibited PGE_2 synthesis stimulated by vasopressin, angiotensin and the Ca^{2+} ionophore A23187 by 55, 65 and 95% respectively. The study of vasopressin-stimulated PGE_2 synthesis in the absence of extracellular Ca^{2+} demonstrated that: (a) hormone-sensitive phospholipase activity was inhibited as measured by [^3H]arachidonic acid release; (b) the maximal rate of vasopressin-stimulated activity was decreased without a change in the vasopressin concentration that evoked half-maximal stimulation of PGE_2 synthesis; and (c) the Ca^{2+} -channel blocker verapamil and the Ca^{2+} -calmodulin antagonist trifluoperazine mimicked the inhibitory effects of removing extracellular Ca^{2+} . These agents had no effect in the absence of Ca^{2+} . In contrast with their effects on vasopressin action, neither the removal of extracellular Ca^{2+} nor the addition of verapamil altered the ability of hyperosmotic mannitol to increase PGE_2 synthesis. These data are consistent with the hypothesis that a component of vasopressin-stimulated PGE_2 biosynthesis involves the influx of extracellular Ca^{2+} , followed by the activation of Ca^{2+} -calmodulin-stimulated phospholipase(s).

Prostaglandins are synthesized at multiple sites within the kidney (Morrison & Needleman, 1979), and their biosynthesis is regulated by many agents, including peptide hormones (Zusman & Keiser, 1977a,b), uni- and bi-valent cations, and osmolality (Zusman & Keiser, 1980; Craven & DeRubertis, 1981). The isolation of rabbit renal-medullary interstitial cells in tissue culture has provided a model to study the interrelationship of these stimulatory agents. Vasopressin and angiotensin increase PGE_2 biosynthesis in these cells. These hormones exert their effect by an increase in arachidonic acid release via stimulation of a cellular phospholipase (Zusman & Keiser, 1977b). The biochemical steps involved in this process remain unknown, however.

Vasopressin does not stimulate cyclic AMP production in rabbit renal-medullary interstitial cells (D. A. Ausiello & R. M. Zusman, unpublished work), and [1-desamino, 8-D-arginine]vasopressin,

a pure antidiuretic analogue of vasopressin, does not stimulate PG synthesis in comparable cells from rat kidney (Beck *et al.*, 1980). These data raise the possibility that vasopressin is coupled to its 'pressor' receptor in renal-medullary interstitial cells, as in smooth muscle (Altura, 1970) and hepatocytes (Cantau *et al.*, 1980), and that Ca^{2+} rather than cyclic AMP is the messenger responsible for the cellular effects of the hormone. Ca^{2+} also has been implicated as a second messenger for angiotensin stimulation of glycogenolysis in the liver (Chan & Exton, 1977) and aldosterone production in the adrenal glands (Foster *et al.*, 1981).

Vasopressin has been shown to stimulate PG synthesis in slices of rat renal inner medulla in the presence, but not in the absence, of Ca^{2+} (Craven & DeRubertis, 1981). The kidney slice preparation, however, prevents the study of the cellular regulation of PG synthesis in homogeneous cell populations. The present study was thus conducted to determine whether extracellular Ca^{2+} is important in the stimulation of rabbit renal-medullary-

Abbreviation used: PG, prostaglandin.

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cell PG synthesis by vasopressin, and whether the effects of the hormone could be modulated by Ca^{2+} antagonists. A preliminary account of part of this work has been published (Ausiello & Zusman, 1982).

Materials and methods

Rabbit renal-medullary interstitial cells were isolated and grown in tissue culture as previously described (Zusman & Keiser, 1977a). Confluent cultures were maintained in 75 cm² plastic flasks containing RPMI-1640 tissue-culture medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) containing 25 mM-Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid] buffer and 20% (v/v) foetal bovine serum. Confluent cell layers were dispersed with 0.25% trypsin in Dulbecco's phosphate-buffered saline containing 0.1% EDTA and suspended in fresh medium for subculture. Experimental studies were performed with cells subcultured in Costar-Cluster 24-cup plates (Costar, Data Packaging Corp., Cambridge, MA, U.S.A.). Experiments were performed after cells reached confluence, typically 24–48 h after subculture.

All studies were performed in NaHCO_3 -buffered media at pH 7.4 at a final osmolarity of 300 mosM as previously described (Zusman & Keiser, 1977a,b), unless otherwise indicated. Ca^{2+} -containing medium was prepared by addition of 1.3 mM- CaCl_2 to the basic incubation medium. Cells were washed three times with Ca^{2+} -containing or Ca^{2+} -free media before each experiment. The Ca^{2+} content of the medium of cells studied in Ca^{2+} -free media was less than 25 μM as measured by atomic-absorption spectrophotometry.

Experimental agents were added at the following concentrations except when otherwise noted: [8-arginine]vasopressin, 0.5 μM ; angiotensin, 5 nM; Ca^{2+} ionophore A23187, 10 μM (gift from Eli Lilly Co., Indianapolis, IN, U.S.A.); verapamil, 25 μM (gift from Knoll Pharmaceutical, Whippany, NJ, U.S.A.); trifluoperazine, 25 μM (gift from Smith, Kline and French, Philadelphia, PA, U.S.A.).

The PG content of the incubation medium was measured by direct radioimmunoassay of PGE_2 (Pong & Levine, 1977). For the measurement of arachidonic acid release, ^3H -labelled renal-medullary interstitial cells were prepared by addition of [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (New England Nuclear, Boston, MA, U.S.A.; 72 Ci/mmol) to the tissue-culture medium 24 h before each experiment, as previously described (Zusman & Keiser, 1980). Before exposure to the experimental conditions, the cells were washed three times with incubation buffer with or without Ca^{2+} . After

incubation under the experimental conditions, the radioactivity released into the incubation medium was determined by scintillation counting in Aquasol-2 (New England Nuclear). The radioactivity released into the medium represents the unmetabolized arachidonic acid as well as the arachidonic acid converted into PG during the incubation period. Previous studies have shown that under these conditions approx. 90% of the radiolabelled material released into the incubation medium is unmetabolized arachidonic acid (Zusman & Keiser, 1977b).

Data are expressed as prostaglandin and/or arachidonic acid produced per h per incubation well; the protein content of each well is approx. 500 μg and varies by less than 5%. Both basal and peptide-stimulated production of prostaglandin and arachidonic acid is linear in this cell type for at least 1 h (Zusman & Keiser, 1977a,b). Statistical analysis of the data was made by the use of Student's *t* test.

Results

Ca^{2+} -dependence of PGE_2 synthesis

As illustrated in Fig. 1, basal PGE_2 synthesis in the presence of Ca^{2+} was 0.22 ± 0.03 ng/h. When Ca^{2+} was present in the extracellular bathing media, A23187, angiotensin and vasopressin increased PGE_2 synthesis to approx. 5–15-fold above basal values. The removal of extracellular Ca^{2+} had no effect on basal PGE_2 synthesis. In Ca^{2+} -free media, PGE_2 synthesis in response to A23187, angiotensin and vasopressin decreased by 95, 65 and 55% respectively. These values are each significantly lower ($P < 0.05$) than the rate of PGE_2 synthesis in the presence of Ca^{2+} .

The removal or re-addition of extracellular Ca^{2+} results in a rapid decrease or increase, respectively, in the rate of vasopressin-stimulated PG synthesis (Fig. 2). In Ca^{2+} -free media, the synthetic rate of PGE_2 is approx. 40% of that observed in the presence of Ca^{2+} . Within 10 min of the addition of 1.3 mM- Ca^{2+} , the rate of PGE_2 synthesis reaches control values. Thus the effect of Ca^{2+} -free media is not the result of irreversible cell damage.

Fig. 3 illustrates the dose-response for the effect of vasopressin on PGE_2 synthesis in the presence and absence of Ca^{2+} . The maximum synthesis rate is approx. 4-fold greater in media containing Ca^{2+} than in Ca^{2+} -free media, (2.1 and 0.54 ng/h respectively). There is no significant effect of Ca^{2+} on the vasopressin concentration that produces half-maximal stimulation of PGE_2 synthesis (0.45 μM). This prominent effect of Ca^{2+} on V_{max} without an alteration of the K_D makes it unlikely that changes in hormone-binding affinity are responsible for the observed changes in PG synthesis.

Fig. 1. Effects of the Ca²⁺ ionophore A23187, angiotensin (AII) and vasopressin (VP) on PGE₂ synthesis by rabbit renal-medullary interstitial cells in tissue culture in the presence (a) or absence (b) of Ca²⁺

Removing Ca²⁺ from the tissue culture medium decreased PGE₂ synthesis (**P* < 0.05) in response to A23187, AII and VP. Each incubation was for 1 h and results (means ± S.E.M.) are expressed as total ng of PGE₂ produced from a minimum of six individual determinations.

Effect of Ca²⁺ antagonists

Verapamil, a Ca²⁺ antagonist, decreased the vasopressin response in the presence of extracellular Ca²⁺, but had no effect in the absence of Ca²⁺ (Table 1). Verapamil had no effect on basal PGE₂ synthesis in the presence or absence of Ca²⁺ (results not shown). Verapamil also decreased PGE₂ synthesis in response to angiotensin in the presence of Ca²⁺, but not in the absence of Ca²⁺.

The hormonal effect on PG synthesis in rabbit renal-medullary interstitial cells results from phospholipase activation and an increase in the release of arachidonic acid (Zusman & Keiser, 1977b; Zusman & Brown, 1980). Therefore experiments were performed to determine the effect of Ca²⁺ on hormone-stimulated arachidonic acid release (Table 1). The results are qualitatively similar to those observed for PG synthesis. These data are expressed as fmol of radiolabelled materials released/h; the total quantity of arachidonate released cannot be determined, since the specific radioactivity of the endogenous arachidonic acid pool(s) is unknown. Basal arachidonic acid release

was similar in the presence and absence of Ca²⁺ (results not shown). Vasopressin increased arachidonic acid release by greater than 10-fold in the presence of Ca²⁺, but only 5-fold in its absence. Verapamil decreased arachidonic acid release in response to vasopressin in the presence of Ca²⁺, but had no effect on vasopressin-stimulated arachidonic acid release in Ca²⁺-free media. Previous data from our laboratory have confirmed polypeptide-hormone activation of phospholipase(s) in these cells (Zusman & Brown, 1980); the present data are consistent with a role of Ca²⁺ in this activation process.

Phenothiazines have been demonstrated to be potent inhibitors of Ca²⁺-calmodulin-dependent enzymes, including phospholipases (Wong & Cheung, 1979). We therefore decided to determine whether the Ca²⁺-sensitivity of vasopressin action was inhibitable by the phenothiazine trifluoperazine. As illustrated in Table 1, trifluoperazine inhibited vasopressin-stimulated PGE₂ synthesis by 35% in the presence of Ca²⁺. In the absence of Ca²⁺, trifluoperazine had no effect.

The above data are consistent with the presence

Fig. 2. Reversibility of the effect of Ca^{2+} on vasopressin-stimulated PGE_2 synthesis

After 20 min of initial incubation in either Ca^{2+} -containing (○, ●) or Ca^{2+} -free (△, ▲) medium, cells were rapidly washed (arrow) and then incubated in Ca^{2+} -free (△, ●) or Ca^{2+} -containing (○, ▲) medium for an additional 20 min. PGE_2 synthesis is expressed as the amount produced per total incubation time. Data are means of duplicate experiments.

Fig. 3. Dose-response for vasopressin-stimulated PGE_2 synthesis in the presence and absence of Ca^{2+}

$V_{\text{max-Ca}^{2+}}$ and $V_{\text{max-0}}$ indicate maximal rate of PGE_2 synthesis in the presence or absence of Ca^{2+} respectively. $K_{\text{D-Ca}^{2+}/0}$ indicates the concentration of vasopressin which produces half-maximal PGE_2 synthesis in the presence or absence of Ca^{2+} .

Table 1. Effect of Ca^{2+} antagonists on hormone-stimulated PGE synthesis and arachidonic acid release in the presence and absence of extracellular Ca^{2+}

Data are expressed as amount of PGE synthesized or arachidonic acid released during a 1 h incubation. Values are means $\pm$ S.E.M. ($n = 6-24$): * $P < 0.05$ compared with $+\text{Ca}^{2+}$; ** $P < 0.05$ compared with vasopressin or angiotensin alone.

	PGE synthesis (ng/h)		Arachidonic acid release (fmol/h)	
	$+\text{Ca}^{2+}$	$-\text{Ca}^{2+}$	$+\text{Ca}^{2+}$	$-\text{Ca}^{2+}$
Control	0.16 ± 0.02	0.14 ± 0.02	1.8 ± 0.2	2.0 ± 0.2
Vasopressin ($0.5 \mu\text{M}$)	1.81 ± 0.08	$0.65 \pm 0.03^*$	21.8 ± 0.6	$9.8 \pm 0.4^*$
+ verapamil ($25 \mu\text{M}$)	$0.48 \pm 0.05^{**}$	0.44 ± 0.07	$9.5 \pm 0.2^{**}$	9.5 ± 0.3
+ trifluoperazine ($25 \mu\text{M}$)	$1.25 \pm 0.11^{**}$	$0.58 \pm 0.04^*$	—	—
Angiotensin (5 nM)	2.19 ± 0.17	$1.17 \pm 0.07^*$	—	—
+ verapamil ($25 \mu\text{M}$)	$1.14 \pm 0.04^{**}$	1.08 ± 0.09	—	—

of both Ca^{2+} -dependent and Ca^{2+} -independent hormone-sensitive mechanisms for arachidonic acid release and PGE_2 synthesis. To provide further evidence for this, the effect of increasing concentrations of the Ca^{2+} -antagonists on vasopressin-sensitive PG synthesis was studied. As illustrated in Fig. 4, the effects of trifluoperazine and verapamil are quantitatively similar. Half-maximal inhibition is observed at less than $10 \mu\text{M}$ for both substances. At concentrations as high as $100 \mu\text{M}$, however, these agents only gave 40% inhibition.

Effect of Ca^{2+} and osmolarity on PGE_2 synthesis

Mannitol stimulates PG synthesis in rabbit renal-medullary cells, but is unlike vasopressin or angiotensin in that it has no effect on phospholipase activity (Zusman & Brown, 1980). For this reason the effect of Ca^{2+} on mannitol stimulation of PG synthesis was examined. As seen in Table 2, increasing osmolarity of the medium from 300 to 1200 mosM increased PG synthesis by approx. 80%. This was true at a Na^+ concentration in the medium of 50 or 150 mM. Neither the removal of extracellular Ca^{2+} nor the addition of verapamil

Table 2. Stimulation of PGE₂ synthesis by hyperosmotic solutions in the presence and absence of extracellular Ca²⁺ and verapamil (25 μ M)

PGE₂ biosynthesis is expressed as pg produced/h of incubation in the presence of each of the solutions described below. Each value represents the mean $\pm$ s.e.m. for a minimum of four experiments. **P* < 0.001, A versus C and B versus D; †D versus E, no significant difference.

Solution	Final osmolarity (mosM)	Verapamil	Na ⁺ salts (mosM)	Mannitol (mosM)	PGE ₂ synthesis (pg/h)	
					Without Ca ²⁺	With Ca ²⁺
A	300	—	300	—	363 $\pm$ 23	336 $\pm$ 31
B	300	—	100	200	251 $\pm$ 18	266 $\pm$ 24
C	1200	—	300	900	535 $\pm$ 10*	546 $\pm$ 8*
D	1200	—	100	1100	486 $\pm$ 12*	460 $\pm$ 24*
E	1200	+	100	1100	436 $\pm$ 31†	420 $\pm$ 25†

Fig. 4. Dose-response for the inhibition of vasopressin-stimulated PGE₂ synthesis by verapamil (●) and trifluoperazine (○)

Data are expressed as ng of PGE₂ produced during a 1 h incubation and represent means of duplicate experiments.

altered the ability of hyperosmotic media to increase PG synthesis

Discussion

The study of renal PG synthesis and its regulation by hormones has been complicated by the multiplicity of sites for PG synthesis and the heterogeneity of the cellular response to hormones (Morrison & Needleman, 1979). This is particularly true for vasopressin, which can exert its effects in the kidney through at least two apparently disparate receptors, one coupled to adenylate cyclase, as in cortical and medullary collecting ducts (Ausiello & Orloff, 1982), and the other one characterized as a 'pressor' receptor, as in glomerular mesangial cells (Ausiello *et al.*, 1980) and in

cultured renal-medullary interstitial cells (Beck *et al.*, 1980). All these cell types have been shown to be sources of renal PG synthesis (Morrison & Needleman, 1979), but only glomerular mesangial cells (Scharschmidt & Dunn, 1983) and the renal-medullary interstitial cells (Zusman & Keiser, 1977a,b) have been shown to increase immuno-reactive-PG synthesis acutely *in vitro* in response to vasopressin. Stimulation of PG synthesis in rat renal-medullary slices has been demonstrated in the presence of vasopressin (Craven & DeRubertis, 1981; Zipser *et al.*, 1981), but the identity of the vasopressin-responsive cell(s) in these kidney slices remains to be defined. The availability of a homogeneous population of renal-medullary cells with angiotensin and vasopressin (pressor) receptors allowed us to determine whether the hormonal stimulation of PG synthesis in these cells shared a common dependence on Ca²⁺. The data presented support this conclusion.

The removal of extracellular Ca²⁺, such that concentrations in media are less than 25 μ M, inhibited the ability of vasopressin and angiotensin to stimulate PG synthesis. Similar dependence on extracellular Ca²⁺ has been demonstrated for angiotensin- or vasopressin-stimulated glucose formation in renal cortex (Guder, 1979) and liver (Chan & Exton, 1977), contraction of glomerular mesangial cells (Ausiello *et al.*, 1980) and inhibition of renal renin secretion (Van Dongen & Peart, 1974; Van Dongen, 1975). Inhibition of vasopressin-stimulated PG synthesis by removal of extracellular Ca²⁺ has been reported to occur in rat renal-medullary slices; however, this effect required the presence of 2 mM-EGTA (Craven & DeRubertis, 1981). The need for Ca²⁺ chelation might reflect the inability to remove extracellular Ca²⁺ with washing alone in the slice preparation.

The inhibition of hormone-stimulated PG synthesis by verapamil occurred only when extracellular Ca²⁺ was present. This is consistent with the concept that the Ca²⁺ antagonist exerts its effect by inhibiting Ca²⁺ influx through Ca²⁺

channels (Triggle, 1981). Two categories of these channels exist, potential-operated and receptor-operated (Triggle, 1981; Bolton, 1979; Putney, 1978), and both can be inhibited by verapamil (Triggle, 1981). The potential-operated channels are activated by membrane depolarization induced by raising extracellular K^+ (Triggle, 1981). We would therefore expect that increasing K^+ in the bathing media of renal-medullary interstitial cells would mimic hormone action and stimulate PG synthesis if potential-operated channels were present. The addition of K^+ to these cells, however, inhibits PG synthesis (Zusman & Keiser, 1980) and raises the possibility that vasopressin and angiotensin activate receptor-operated Ca^{2+} channels that can be inhibited by verapamil. Indirect support for an inhibitory site of action for verapamil at the level of plasma-membrane Ca^{2+} channels has been obtained from studies with hyperosmotic bathing media. Mannitol stimulated PG synthesis in rabbit renal-medullary cells, but, unlike the experiments with vasopressin, neither the removal of extracellular Ca^{2+} nor the addition of verapamil altered the ability of hyperosmotic mannitol to increase PG synthesis. These data therefore support the presence of an alternative pathway for stimulation of PGE_2 synthesis which is independent of extracellular Ca^{2+} .

It should be noted that the mechanism of action of verapamil has been most thoroughly studied in excitable cells where Ca^{2+} -channel blockade can be effected at concentrations of 0.1–1 μM (McAllister, 1982; Lee & Tsien, 1983). In contrast, Ca^{2+} -transport inhibition in pancreatic islet cells (Mallaisse *et al.*, 1978), fat-cells (Martin *et al.*, 1975), toad bladder (Humes *et al.*, 1980) and adrenal medulla (Aquirre *et al.*, 1977) requires 10–300 μM -verapamil. It is not known whether all such effects of the drug in non-excitabile tissue are due to Ca^{2+} -channel blockade. In this regard verapamil has a potentially important additional role as a Ca^{2+} antagonist. The drug can interact directly with calmodulin and inhibit the stimulation of phosphodiesterase by calmodulin (Epstein *et al.*, 1982). The concentration at which this inhibition is half-maximal, however, is 150 μM , a concentration at least an order of magnitude larger than is necessary to inhibit vasopressin-sensitive PG synthesis (<10 μM ; Fig. 4). The determination of the mechanism of action of verapamil in renal-medullary interstitial cells will require the direct measurement of the effect of the drug on Ca^{2+} transport and phospholipase activity.

The ability of trifluoperazine to inhibit Ca^{2+} -dependent processes has been attributed to this agent's ability to block the action of the Ca^{2+} -calmodulin complex (Levin & Weiss, 1979). If trifluoperazine is serving a similar role in renal-

medullary interstitial cells, then vasopressin probably activates a Ca^{2+} -calmodulin-sensitive phospholipase. Wong & Cheung (1979) described a Ca^{2+} -calmodulin-sensitive phospholipase A_2 in human platelets. Studies in rat renal-medullary slices have suggested the presence of a Ca^{2+} -calmodulin-dependent phospholipase, which may account for the Ca^{2+} -sensitivity of vasopressin-stimulated PG synthesis (Craven & DeRubertis, 1981). It should be noted that Craven & DeRubertis (1981) found that approx. 10 μM -trifluoperazine gave half-maximal inhibition of both vasopressin stimulation of PG synthesis and of calmodulin stimulation of phospholipase, and 25 μM -trifluoperazine was required for complete inhibition. A similar dose-response for trifluoperazine was observed in the present study.

In conclusion, extracellular Ca^{2+} is necessary to elicit maximum stimulation of PG synthesis by vasopressin in renal-medullary interstitial cells. This is in contrast with osmotic stimulation of PG synthesis, which is independent of a Ca^{2+} concentration in the medium as low as 25 μM . The results of studies with verapamil and trifluoperazine support the hypothesis that vasopressin stimulates the uptake of extracellular Ca^{2+} , which in turn activates calmodulin-sensitive phospholipase.

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