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Effects of Bivalent Cations on Prostaglandin Biosynthesis and Phospholipase A_2 Activation in Rabbit Kidney Medulla Slices

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The bivalent cations Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+} and Ba^{2+} were compared for their stimulatory or inhibitory effect on prostaglandin formation in rabbit kidney medulla slices. Ca^{2+}, Mn^{2+} and Sr^{2+} ions stimulated prostaglandin generation up to 3–5-fold in a time- and dose-dependent manner ($Ca^{2+} > Mn^{2+} \simeq Sr^{2+}$). The stimulation by Mn^{2+} (but not by Sr^{2+}) was also observed in incubations of medulla slices in the presence of Ca^{2+}. Mg^{2+} and Co^{2+} ions were without significant effects on either basal or Ca^{2+}-stimulated prostaglandin synthesis. The stimulatory effects of Ca^{2+}, Mn^{2+} and Sr^{2+} on medullary generation of prostaglandin E_2 were found to correlate with their stimulatory effects on the release of arachidonic acid and linoleic acid from tissue lipids. The release of other fatty acids was unaffected, except for a small increase in oleic acid release. As both arachidonic acid and linoleic acid are predominantly found in the 2-position of the glycerol moiety of phospholipids, the stimulation by these cations of prostaglandin E_2 formation appears to be mediated via stimulation of phospholipase A_2 activity.

Mammalian tissues contain no significant amounts of preformed prostaglandins (Crowshaw, 1973) or their free precursor arachidonic acid (Morgan & Hanahan, 1963). Thus prostaglandin formation requires the hydrolysis of esterified arachidonic acid from tissue lipids (Bills & Smith, 1976; Hsueh *et al.*, 1977; Isakson *et al.*, 1977). Arachidonic acid is particularly abundant among the fatty acids in the 2-position of the glycerol moiety of phospholipids (Morgan & Hanahan, 1963) from which it could be hydrolysed by the action of phospholipase A_2. Both seminal-vesicle homogenate, which is highly active in prostaglandin synthesis, and seminal plasma, which is rich in prostaglandins, were found to contain phospholipase A_2 activity (Kunze *et al.*, 1974*a*,*b*). Furthermore addition of snake venom phospholipase A_2 preparation to incubated kidney medulla slices results in increased synthesis of prostaglandins (Kalisker & Dyer, 1972). The activity of phospholipase A_2 from snake venoms is stimulated by Ca^{2+} and inhibited by Ba^{2+} and other bivalent cations (Wells, 1972; Chang *et al.*, 1977). Renal prostaglandin biosynthesis is decreased in a Ca^{2+}-free medium (Kalisker & Dyer, 1972). The studies reported in the present paper were designed (1) to determine the selectivity of several bivalent cations for stimulation of kidney medulla prostaglandin generation, and (2) examine the relationship between the activity of the cations in stimulating prostaglandin biosynthesis and in inducing release of fatty acids from medulla slices.

Materials and Methods

Preparation and incubation of slices

Rabbits (male, New-Zealand-white-derived, local strain, 2.5–3.0kg) were killed by air injection into the heart, both kidneys were removed, and medulla slices (approx. 1mm thick) were quickly prepared. The slices were rinsed with a buffer and incubated in the same buffer (0.2g slices in 2.0ml of buffer) with shaking at 37°C for 10–120min. Incubations were performed in either Tris/HCl buffer (0.1M, pH8.0), Krebs–Henseleit buffer, pH7.4 (95mM-NaCl, 1.97mM-$CaCl_2$, 3.7mM-KCl, 0.97mM-$MgSO_4$, 25mM-$NaHCO_3$, 0.94mM-KH_2PO_4, and 1.8g of dextrose/litre) bubbled with O_2/CO_2 (19:1, v/v) or in modified Tyrode physiological solution (25mM-Tris/HCl, 137mM-NaCl, 2.68mM-KCl, 1.2mM-$MgSO_4$, 1.8mM-$CaCl_2$, 0.4mM-NaH_2PO_4, and 1.8g of dextrose/litre). Incubations were also carried out in which the slices were first preincubated for 60–90min with the medium changed every 30min before addition of cations.

Determination of prostaglandin products

After incubation the medium was assayed for prostaglandin E_2 content by bioassay on a rat stomach strip (Vane, 1957). In preliminary studies a portion of the medium was extracted with 2×3vol. of ethyl acetate (pH3.5), separated by t.l.c. and the prostaglandin E_2 zone was extracted from the thin-

layer plates and determined by bioassay. Two chromatography systems were employed: (*a*) chloroform/methanol/acetic acid/water (90:8:1:0.8, by vol.), which separates prostaglandins $F_{2\alpha}$, E_2, D_2 and thromboxane B_2 (Needleman *et al.*, 1976), but does not allow separation of 6-oxoprostaglandin $F_{1\alpha}$ from prostaglandin E_2, and (*b*) ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, by vol., organic phase), which allows separation of 6-oxoprostaglandin $F_{1\alpha}$ and prostaglandins $F_{2\alpha}$, E_2 and D_2. Bioassay of each prostaglandin zone from both chromatographic systems against a known prostaglandin standard solution indicated that the major prostaglandin produced in our incubation and recovered in the media is prostaglandin E_2. We also determined the prostaglandin-products profile obtained in incubation of slices from kidneys that were prelabelled with [1-^{14}C]arachidonic acid, as previously described (Isakson *et al.*, 1977). The major prostaglandin product (64–76%) generated by the medulla slices was prostaglandin E_2. The other prostaglandin products were prostaglandin $F_{2\alpha}$ (17–24%) and prostaglandin D_2 (6–12%). In the rat stomach bioassay, these prostaglandins were only one fifth to one tenth as active as prostaglandin E_2. Consequently comparison between direct bioassay of prostaglandin E_2 content in the medium and the determination of purified prostaglandin E_2 after chromatography (with correction for the 55–60% recovery during the overall extraction and chromatography procedure) indicated that over 95% of the bioassayable prostaglandin E_2 in the medium was due to the activity of this prostaglandin. Direct measurement of the rat stomach-contracting activity of the incubation medium thus provides an accurate measurement of the concentration of prostaglandin E_2 in the medium.

Determination of fatty acids

A portion of the incubation medium was acidified to pH 3.5 with 2M-citric acid, extracted with 2×3 vol. of ethyl acetate, dried over Na_2SO_4 and separated by t.l.c. [light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (35:15:1, by vol.)]. The fatty acids zone was extracted with chloroform/methanol (2:1, v/v), evaporated to dryness and treated with freshly prepared diazomethane in diethylether (5min at 25°C). The methyl esters were quantified by g.l.c. by using a model 417 Packard gas chromotograph equipped with a flame-ionization detector. Separation was carried out on a 7ft (2.13m) glass column packed with SP 2340 (Supelco, Bellefonte, PA, U.S.A.), with temperature programming from 175 to 250°C. A mixture of fatty acid standards containing palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, eicosa-8,11,14-trienoic acid, arachidonic acid, eicosa-5,8,11,14,17-pentaenoic acid, docosa-7,10,13,16-tetraenoic acid and docosa-4,7,10,13,16,19-hexaenoic acid was used for qualitative and quantitative calibration. $C_{20:5}$ fatty acid (25μg) was added to each medium sample before extraction, to correct for recovery losses during extraction and purification (overall recovery range 20–30%).

Materials

Prostaglandins E_2, D_2, $F_{2\alpha}$, and A_2 were kindly supplied by Dr. U. Axen and Dr. J. E. Pike of the Upjohn Co. (Kalamazoo, MI, U.S.A.). Fatty acid standards were obtained from Supelco. All other reagents were analytical grade.

Results and Discussion

Prostaglandin E_2 synthesis and release from rabbit medulla slices was found to be a time-dependent process. The release was linear during the first 40min with a rate of 80ng/min per g wet wt. of tissue and reached a plateau at 90–120min (Fig. 1). Addition of $CaCl_2$ (2mM final concentration) produced a 4–6-fold increase in the inital rate of prostaglandin E_2 release with a plateau of release obtained after 120min (Fig. 1). The initial higher rate of prostaglandin formation is probably due to the mechanical treatment of the tissue during preparation of the slices, as such treatment was shown to stimulate prostaglandin release from organs (Danon *et al.*, 1975). To

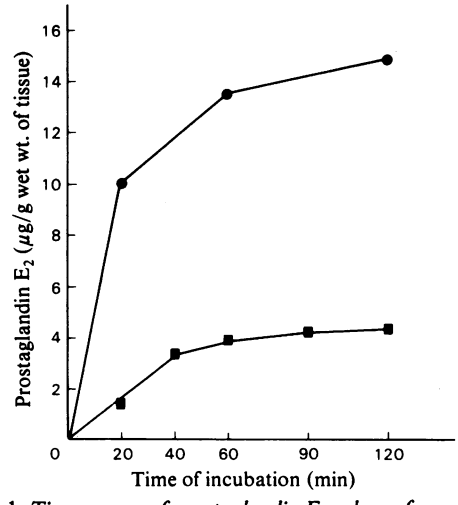

Fig. 1. *Time course of prostaglandin E_2 release from rabbit kidney medulla slices*

Incubations were done in Tris/HCl buffer in the absence (■) or the presence of 2mM-Ca^{2+} (●). For details, see the Materials and Methods section.

determine the effect of Ca^{2+} on prostaglandin release during the post-initial incubation period, kidney slices were first incubated in Tris buffer for 90min with the medium being changed every 30min. Prostaglandin E_2 release during the subsequent 90–120min period was approx. 25–33% of that observed during the initial 30min incubation. Nevertheless, addition of 2mM-Ca^{2+} during the 90–120min period produced the same stimulatory effect (range 2.5–6-fold) as that seen during the initial period.

We evaluated the possible effect of the incubation medium composition on the generation of medullary prostaglandins. Slices were incubated in Tris buffer (pH8.0), Krebs–Henseleit physiological buffer (pH7.4, Ca^{2+}-free), with continuous gassing with O_2/CO_2 (19:1, v/v) or in modified Tyrode physiological buffer (pH7.4, Ca^{2+}-free). Both basal and Ca^{2+}-stimulated generation of prostaglandin E_2 were essentially unaffected by the type of medium employed (Table 1).

The selectivity of the Ca^{2+}-stimulatory effect on medullary prostaglandin biosynthesis was evaluated in studies with other bivalent cations (Figs. 2 and 3). Ca^{2+}, Mn^{2+} and Sr^{2+} showed dose-dependent stimulation of prostaglandin E_2 production. In contrast, Ba^{2+}, Co^{2+} and Mg^{2+} ions were without effect at concentrations of 2mM or lower and showed only very small stimulation at higher concentrations. Neither Co^{2+} or Mo^{2+} had any effect on the stimulatory effect on Ca^{2+} (Table 2). Incubation in the presence of both Ca^{2+} (2mM) and Mn^{2+} (2mM) produced an additive stimulatory effect, whereas no additive effect was observed in similar incubations with Ca^{2+} (2mM) and Sr^{2+} (2mM).

Cellular biosynthesis of prostaglandins is dependent on deacylation of cellular lipids to provide the prostaglandin precursor arachidonic acid. As this fatty acid is found mainly in the 2-position of the glycerol moiety of phospholipids, phospholipase A_2 reaction is likely to be necessary for arachidonate release. We therefore determined the effects of the various bivalent cations on the release of fatty acids and prostaglandin E_2. Medullary slices were incubated with bovine serum albumin (2mg/ml) to facilitate 'trapping' the released fatty acids. The release of prostaglandin E_2 was quantified by bioassay; the fatty acids were methylated and analysed by g.l.c. The results (Table 3) indicated complete correlation between the effects of the bivalent cations to stimulate medullary prostaglandin pro-

Table 1. *Effect of incubation medium on the synthesis of prostaglandin E_2*

Rabbit medulla slices (0.2g) were incubated at 37°C for 60min in Tris/HCl, Krebs–Henseleit or modified Tyrode solution in the presence or absence of Ca^{2+} (2mM). Values given are means ± S.E.M. for four experiments. For other details see the Materials and Methods section.

Medium	Prostaglandin E_2 released (μg/g of medulla)
0.1M-Tris/HCl (pH8.0*)	6.1 ± 0.2
+Ca^{2+}	13.5 ± 0.3
Krebs–Henseleit (Ca^{2+}-free, pH7.4)	5.7 ± 0.3
+Ca^{2+}	12.3 ± 0.4
Tyrode (Ca^{2+}-free, pH7.4)	5.9 ± 0.4
+Ca^{2+}	13.6 ± 0.5

* This pH value was employed since it is optimal for the activity of medullary cyclo-oxygenase.

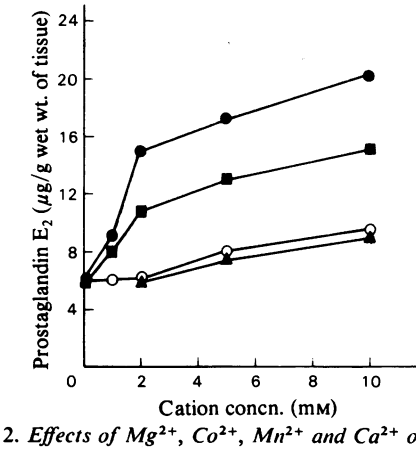

Fig. 2. *Effects of Mg^{2+}, Co^{2+}, Mn^{2+} and Ca^{2+} on prostaglandin biosynthesis*

Medulla slices (0.2g) were incubated with shaking at 37°C for 60min in the presence of different concentrations of Mg^{2+} (▲), Co^{2+} (○), Mn^{2+} (■) or Ca^{2+} (●).

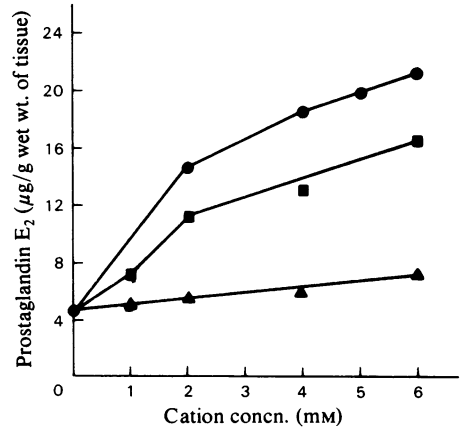

Fig. 3. *Effects of Ba^{2+}, Sr^{2+} and Ca^{2+} on prostaglandin biosynthesis*

Medulla slices were incubated in the presence of different concentrations of Ba^{2+} (▲), Sr^{2+} (■) or Ca^{2+} (●).

duction and to release linoleic acid and arachidonic acid. Ca^{2+}, Mn^{2+} and Sr^{2+} ions (but not Mg^{2+} ions) dose-dependently stimulated the release of prostaglandin E_2, arachidonic acid and linoleic acid; the release of other fatty acids, except for a small increase in oleic acid, was unaffected by these bivalent cations. As both arachidonic acid and linoleic acid are mainly found in the 2-position of the glycerol moiety of phospholipids, it appears likely that Ca^{2+}, Mn^{2+} and Sr^{2+} stimulate prostaglandin E_2 biosynthesis by stimulating a phospholipase A_2 reaction that provides the prostaglandin precursor arachidonic acid. It is noteworthy that kidney medulla phospholipase A_2 shows different cation specificity from that observed previously for phospholipase A_2 from venoms and other toxins, where inhibition by Ba^{2+} and Sr^{2+} was observed (Wells, 1972; Chang *et al.*, 1977).

Table 2. *Effect of Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+} and Co^{2+} ions on prostaglandin release from kidney medulla slices*

All cations were added at 2mM final concentration. Results are means ± S.E.M. for five experiments. For other details, see the legend to Fig. 2.

Incubation medium	Prostaglandin E_2 released (μg/g wet wt. of tissue)
Buffer	4.2±0.3
+Ca^{2+}	12.6±0.5*
+Mg^{2+}	4.4±0.3
+Mn^{2+}	10.5±0.6*
+Co^{2+}	4.4±0.4
+Sr^{2+}	9.0±0.6*
+Ca^{2+}+Mg^{2+}	13.5±0.5
+Ca^{2+}+Co^{2+}	13.4±0.6
+Ca^{2+}+Mn^{2+}	22.0±0.7†
+Ca^{2+}+Sr^{2+}	13.9±0.5

* Significantly different at $P<0.05$ from the buffer value.

† Significantly different at $P<0.05$ from the other three cation mixtures.

The cation-stimulated phospholipase A_2 activation in medulla slices is different from the recently described hormone-sensitive lipase (probably phospholipase) in kidney and heart, which is selective for the release of only arachidonic acid (Hsueh *et al.*, 1977; Schwartzman & Raz, 1979). The properties and selective products of the cation-dependent phospholipase A_2 activation also contrast with the non-selective lipolysis induced by extracellular serum albumin, which promotes the hydrolysis of all fatty acids (Table 3). The albumin-mediated lipolysis and the cation-stimulated lipolysis apparently work by independent mechanisms, since an additive effect of

Table 3. *Release of fatty acids and prostaglandin E_2 from medulla slices by bivalent cations and bovine serum albumin*

Medulla slices (200mg) were incubated at 37°C for 60min in 0.1M-Tris buffer, pH 8.0. Cations were added at 2 or 5mM final concentration and the bovine serum albumin (BSA) concentration was 2mg/ml when present. The medium was analysed for prostaglandin E_2 and fatty acids as described in the Materials and Methods section. Values given are means ± S.E.M. for six experiments.

Addition to incubation medium	Fatty acids released (μg/g wet wt. of tissue)						Prostaglandin E_2 released (μg/g wet wt. of tissue)
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Total $C_{20:4}$*	
—	58±2	24±1	28±1	12±1.4	3.1±0.2	8.7	6.6±0.3
BSA	77±3	49±2	56±2	29±1	9.3±0.4	14.4	6.1±0.4
2mM-$CaCl_2$	61±2	25±1	33±1	22±2	7.1±0.4	18.3	13.2±0.5
2mM-$CaCl_2$+BSA	75±3	47±2	63±2	38±2	16.6±0.6	26.7	12.0±0.6
5mM-$CaCl_2$	60±2	25±1	38±2	27±2	9.4±0.6	25.5	18.9±0.6
5mM-$CaCl_2$+BSA	77±2	50±3	69±3	44±3	21.6±1.4	34.3	15.5±0.7
2mM-$MnCl_2$	61±2	28±2	31±2	19±1	5.2±0.5	13.8	9.0±0.6
2mM-$MnCl_2$+BSA	81±3	46±3	60±2	33±2	13.3±0.8	20.0	7.9±0.4
5mM-$MnCl_2$	56±3	27±2	37±2	22±2	6.9±0.5	19.7	15.1±0.7
5mM-$MnCl_2$+BSA	74±2	48±3	65±3	34±2	13.9±0.9	26.4	14.7±0.8
5mM-$MgCl_2$	61±2	25±1	29±1	12±0.5	4.2±0.3	10.4	7.3±0.5
5mM-$MgCl_2$+BSA	79±2	46±1	59±2	32±2	10.9±0.7	17.1	7.3±0.4
2mM-$SrCl_2$	57±3	24±1	27±1	18±2	4.9±0.3	12.5	8.9±0.6
2mM-$SrCl_2$+BSA	71±3	51±3	53±3	34±2	16.0±0.8	23.5	8.8±0.5
5mM-$SrCl_2$	60±3	27±2	29±2	23±2	7.8±0.6	19.7	14.0±0.8
5mM-$SrCl_2$+BSA	76±3	48±2	56±3	37±3	18.0±0.9	27.6	11.3±0.7

* The total amount of arachidonate released from tissue lipids during the incubation is equal to the isolated amounts of free arachidonate and of prostaglandin E_2. The amount of prostaglandin E_2 released (μg) can be converted into arachidonate released (μg) by multiplication by the factor 304/352 (which is the ratio of the molecular weight of arachidonate to that of prostaglandin E_2). The total amount of arachidonate released is thus: $C_{20:4}$ (μg)+[prostaglandin E_2 (μg)×304/352].

both stimulants was observed in incubations with albumin and Ca^{2+}, Mn^{2+} or Sr^{2+}. Furthermore cation-stimulated lipolysis is accompanied by a significant increase in prostaglandin E_2 production, whereas albumin causes a slight decrease in the release of prostaglandin E_2. This difference indicates that arachidonate release by cation (but not by albumin) is coupled, in some measure, to the subsequent conversion of this acid into prostaglandins. In this respect, the cation-stimulated phospholipase A_2 activity resembles the hormone-sensitive lipase described previously (Hsueh *et al.*, 1977; Schwartzman & Raz, 1979).

From the results presented in Table 3, we plotted the quantitative relationship between the release of prostaglandin E_2 and of arachidonic acid or linoleic acid (Fig. 4). The total release of arachidonic acid (i.e. arachidonic acid and prostaglandin E_2) is also plotted and is quantitatively similar to the release of linoleic acid. The deviation of the curves from linearity was observed in all experiments. Thus for the three cations the ratio of μg of prostaglandin E_2 released to μg of arachidonate released was lower at 2mM cation concentration than at 5mM. It thus appears that at 5mM cation concentration, a larger percentage of released arachidonate was converted into prostaglandin E_2 than that at 2mM (Fig. 4). The possible effect of higher cation concentration on activation of medullary cyclo-oxygenase was ruled out, since this would have resulted in a selective increase in the ratio of μg of prostaglandin E_2 to μg of total arachidonate without affecting the prostaglandin E_2/linoleate ratio. The data can, however, be explained if, in addition to phospholipase A_2 activation, the bivalent cations at higher concentrations also accelerate re-acylation of the released fatty acids. Thus at higher cation concentration, the amount of fatty acids hydrolysed (i.e. rate of lipolysis) is proportionally higher, thereby leading to increased formation of prostaglandin E_2. However, the increase in re-esterification rate leads to a relative decrease in the amount of free fatty acids released into the medium. Such a combined mechanism, which involves activation of the lipolytic process coupled to prostaglandin generation, followed by activation of re-acylation, would serve to generate the required amount of prostaglandin E_2 and at the same time conserve cellular essential fatty acids.

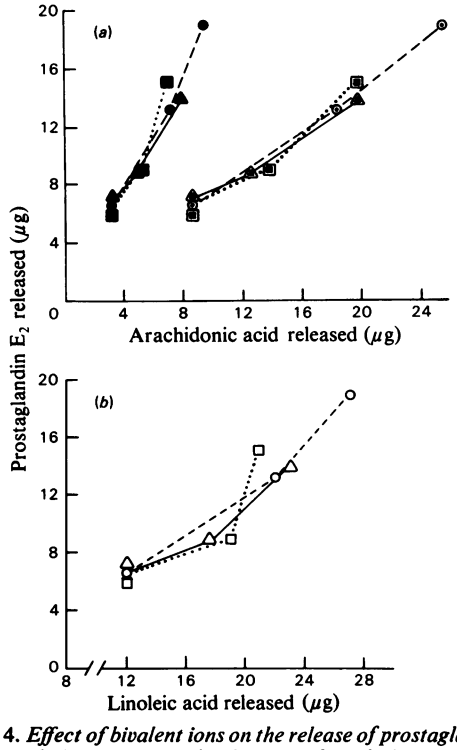

Fig. 4. *Effect of bivalent ions on the release of prostaglandin E_2, arachidonic acid and linoleic acid from kidney medulla slices*

Values in each three-point graph were obtained at 0, 2mM and 5mM cation concentrations. (*a*) shows the effect of Ca^{2+} (●), Mn^{2+} (■) and Sr^{2+} (▲) on the release of prostaglandin E_2 and arachidonic acid, and also the effect of Ca^{2+} (◉), Mn^{2+} (▣) and Sr^{2+} (◭) on the release of prostaglandin E_2 and 'total' (calculated) arachidonic acid (see the footnote to Table 3 for details). (*b*) shows the effect of Ca^{2+} (○), Mn^{2+} (□) and Sr^{2+} (△) on the release of prostaglandin E_2 and linoleic acid.

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