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Effects of Ionophore A23187 and External Calcium Concentrations on Renal Gluconeogenesis (40014)

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Since the initial suggestion of a close relationship between calcium and cyclic-3′,5′-adenosine monophosphate by Rasmussen and Tenenhouse (1), it has become evident that calcium acts as a second messenger in the control of many diverse cellular processes (1-3). The nearly universal nature of this close relationship between calcium and cyclic AMP has been the subject of several recent reviews (2, 3). It appears that in the many systems which are regulated by calcium, cyclic AMP plays an important role as a modulator of that signal (2, 3). In certain systems calcium and cyclic AMP seem to act synergistically, in that cyclic AMP augments the initial calcium effect, where in other systems cyclic AMP appears to oppose the calcium effect (3).

The activation and modulation of renal gluconeogenesis by parathyroid hormone is a system in which calcium and cyclic AMP are closely related (4). Borle has shown a significant increase in radiocalcium efflux from kidney cells in tissue culture following the addition of parathyroid hormone (5). After the demonstration of calcium requirement for maximal rates of gluconeogenesis in the perfused rat liver by Hems *et al.* (6), many investigators have examined the role of extracellular calcium and hydrogen ion concentrations in the control of renal gluconeogenesis (7-17). Parathyroid hormone stimulates renal gluconeogenesis in the presence of external Ca^{2+} but not in its absence, despite a similar increase in cyclic AMP levels under both conditions (10, 15). In addition, prostaglandin E_1 has been shown to increase gluconeogenesis (18), an effect which is inhibited by removal of external Ca^{2+}. Again, cyclic AMP generation in response to PGE_1 was identical in the absence and presence of external Ca^{2+}.

Ionophorous antibiotics modify the transport of specific ions across biological membranes. The ionophore A23187 is a carboxylic acid antibiotic derived from *Streptomyces chartreusensis* that is specific for the transport of divalent cations across biologic membranes (19). A23187 specifically binds cations at neutral pH with the following relative affinities $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ (18). The ionophore A23187 has recently been demonstrated to activate many calcium-dependent biological processes, including fluid secretion in the insect salivary gland (20), DNA synthesis in sea urchin eggs (21), secretion and aggregation in platelets (22), and insulin secretion in pancreatic β cells (23).

The present studies were undertaken to examine the roles of calcium and cyclic AMP in renal gluconeogenesis using the ionophore A23187 in an effort to alter intracellular calcium concentrations independent of any effect on cyclic AMP concentrations.

Materials and methods. Preparation of renal cortical tubules. Rat renal cortical tubules were prepared by a modification of the method of Nagata and Rasmussen (10). Adult Holtzman rats weighing 200-300 g, fed a regular diet, and not fasted prior to study were stunned by a blow to the head. The abdominal aorta was exposed through a midline incision and the kidneys were perfused via the aorta with 10 ml of a calcium-free Krebs-Ringer phosphate buffer, pH 7.4, containing 40 mg% of collagenase (Sigma Corp., St. Louis, Mo.). Following the perfusion, the kidneys were removed rapidly and placed in ice-

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cold, calcium-free Krebs-Ringer phosphate buffer, pH 7.4. Cortical slices were obtained using a Stadie-Riggs microtome. The slices were then incubated at 37° in 10 ml of the same calcium-free buffer containing 40 mg% of collagenase for 20-30 min in a Dubnoff metabolic shaker at a rate of 60-70 oscillations per minute. The slices were gassed using a mixture of O_2/CO_2 (95:5, v/v). Following this incubation the tubules were filtered through two layers of fine surgical gauze and washed with 50 ml of calcium-free Krebs-Ringer phosphate, pH 7.4. The suspension was then centrifuged at 90*g* for 90 sec at 4° in a Sorvall centrifuge (Model Rc2-B). The pellet was resuspended in 50 ml of the calcium-free Krebs-Ringer phosphate and the procedure was repeated ×3. The final preparation of tubules was resuspended in calcium-free Krebs-Ringer phosphate buffer.

Determination of glucose production. For the determination of glucose production a 500-μl aliquot of the suspension of renal cortical tubules containing 4-6 mg of protein was incubated in Krebs-Ringer phosphate buffer, pH 7.4, of the following final composition (millimoles per liter): Na^+, 130; K^+, 4; Mg^{2+}, 1.4; Cl^-, 136; PO_4^{2-}, 2.4. This solution also contained 2% BSA. Experiments were carried out with no calcium (Ca^{2+} was omitted and 0.1 mM EGTA was added), or with 0.1 or 1.0 m*M* Ca^{2+} concentration in the incubation medium. When Ca^{2+} was omitted, the Na^+ concentration of the Ringer's was 132 mmoles/liter. The final incubation volume was 1.0 ml and experiments were performed in 10-ml siliconized Erlenmeyer flasks containing either 10 m*M* α-ketoglutarate, 10 m*M* glutamine, 10 m*M* succinate, 10 m*M* lactate, 10 m*M* glycerol, or no substrate. The tubules were incubated at 37° in a Dubnoff metabolic shaker at 50 oscillations per minute and were constantly gassed using a mixture of O_2/CO_2 (95:5, v/v).

Experimental flasks contained ionophore A23187 at concentrations of 10^{-8} or 10^{-9} *M*. Control flasks contained DMSO, at the same concentration as that present in the experimental flasks, since the ionophore A23187 stock solution was made up in DMSO. Tubules were, therefore, incubated without substrate, with substrate and ionophore A23187, and with substrate and DMSO. After 60 min of incubation the reaction was stopped by boiling for 3 min. The suspension was then centrifuged at 3000*g* for 5 min and an aliquot was obtained for glucose determination. Glucose was assayed fluorimetrically using the hexokinase method (24). All measurements were done in duplicate. Protein was measured by the method of Lowry *et al.* (25).

Measurements of cyclic AMP. Suspensions of renal cortical tubules containing 4-6 mg of protein were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 m*M* glucose, 10^{-2} *M* theophylline, and 0.25% lyophilized bovine serum albumin. The calcium concentration was either 0, 0.1, or 1.0 m*M*. Experimental flasks contained ionophore A23187 at concentrations of 10^{-8} and 10^{-9} *M*, appropriate DMSO diluent, or parathyroid hormone, 1 unit/ml. Incubations were carried out for 20 min at 37° in a Dubnoff metabolic shaker using a gas mixture of CO_2/O_2 (5:95, v/v). The reaction was terminated by the addition of trichloroacetic acid. After ether extraction the suspension was centrifuged at 3000*g* for 5 min and the supernatant assayed for cyclic AMP. The pellet was placed into boiling 50 m*M* sodium acetate/acetic acid buffer, pH 4, for 10 min and homogenized by hand using a glass homogenizer. The homogenate was then centrifuged at 5000*g* for 20 min and the supernatant assayed for cyclic AMP. Cyclic AMP was determined by a modification of the method of Gilman (26). All measurements were done in duplicate. Similar experiments were carried out without theophylline in the medium and incubating for 10 min.

Reagents. [^{3}H]-Labeled cyclic-3′,5′-AMP was purchased from New England Nuclear (Boston, Mass.). Bovine parathyroid hormone synthetic 1-34, Lot No. 26013, was from Beckman. The ionophore, A23187, was a generous gift from Dr. Robert Hamill of the Eli Lilly Co., Indianapolis, Indiana. The ionophore was stored at 4°. Fresh solutions were prepared daily by adding 20 μl of DMSO/0.5 mg of ionophore and

bringing to a final volume of 1 ml with distilled water.

The results are expressed as means ± the standard error. Statistical analysis was performed using Student's t test for paired data.

Results. The effect of parathyroid hormone on glucose production by isolated rat renal cortical tubules is presented in Table I. In the presence of 0.1 and 0.25 mM external calcium, submaximal doses of parathyroid hormone stimulated significantly glucose production from α-ketoglutarate. Omission of calcium from the incubation medium (0.1 mM EGTA) abolished the stimulation of gluconeogenesis produced by parathyroid hormone. Glucose production was linear during the 60 min of incubation.

Table II presents the effect of ionophore A23187 on glucose production from α-ketoglutarate by isolated rat renal cortical tubules. The rates of glucose production from α-ketoglutarate at external Ca^{2+} concentrations of 1.0 mM (130.9 nmoles/mg of protein/60 min) are lower than those obtained by Nagata and Rasmussen (10) using an external Ca^{2+} concentration of 2.5 mM (179 ± 9 nmoles/mg of protein/hr). Ionophore A23187 (10^{-8} and 10^{-9} M) increased glucose production significantly ($P < 0.01$) in the presence of 10 mM α-ketoglutarate at external calcium concentrations of 0.1 mM. Increased ionophore concentrations, such as 10^{-6} M, inhibited glucose production at 0.1 mM external calcium concentrations. Omission of calcium from the incubation medium, which contained 2% BSA and 0.1 mM EGTA, decreased glucose production from α-ketoglutarate, and addition of ionophore, 10^{-8} and 10^{-9} M, did not increase glucose production significantly. At external calcium concentrations of 1.0 mM and higher, 10^{-8} and 10^{-9} M ionophore did not increase glucose production from α-ketoglutarate.

To explore further the role of external calcium present in bovine serum albumin (15), additional experiments were performed using dialyzed BSA. The BSA was dialyzed at 4° for 72 hr against a phosphate Ringer's solution containing no calcium. Table III shows the effects of ionophore on glucose production by isolated rat renal cortical tubules when dialyzed bovine serum albumin was used. In the absence of external calcium, 10^{-9} M ionophore did not significantly increase glucose production as compared to control flasks containing diluent (DMSO) at the same concen-

TABLE I. EFFECT OF PARATHYROID HORMONE ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/60 min)[a]		
	No external Ca^{2+}	0.1 mM External Ca^{2+}	0.25 mM External Ca^{2+}
No substrate	20.9 ± 2.5	24.6 ± 4.6	34.4 ± 1.4
10 mM α-Ketoglutarate	78.8 ± 3.7	93.2 ± 2.7	101.2 ± 3.2
Parathyroid hormone, 1 unit/ml	77.5 ± 4.2	121.2 ± 3.7	145.0 ± 5.8

[a] Values are the means ± SE of four experiments.

TABLE II. EFFECT OF IONOPHORE A23187 ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/60 min)		
	No external Ca^{2+}	0.1 mM External Ca^{2+}	1.0 mM External Ca^{2+}
No substrate	22.3 ± 2.1[a]	30.3 ± 1.8	46.5 ± 5.0
α-Kg + 10^{-8} DMSO diluent	76.3 ± 7.1	113.3 ± 10.9	130.9 ± 9.3
α-Kg + 10^{-8} M ionophore	81.5 ± 9.5	130.0 ± 12.6*	113.2 ± 4.4
α-Kg + 10^{-9} DMSO diluent	66.4 ± 6.6	115.4 ± 10.7	117.7 ± 4.1
α-Kg + 10^{-9} M ionophore	66.1 ± 7.3	136.4 ± 10.2*	115.6 ± 7.6

[a] Values are the means ± SE of four experiments in the cases of zero and 1.0 mM external Ca^{2+} and seven experiments with 0.1 mM external Ca^{2+}.

* Values significantly different from those obtained with α-Kg + DMSO 10^{-8} ($P < 0.01$).

tration. On the other hand, using dialyzed BSA and a 0.1 m*M* external calcium concentration, ionophore produced a significant ($P < 0.01$) increase in glucose production as compared to the values obtained using diluent alone.

Table IV depicts the effect of 10^{-8} *M* ionophore on glucose production from a variety of substrates at an external Ca^{2+} concentration of 0.1 m*M*. Under these conditions ionophore resulted in a significant increase in glucose production ($P < 0.05$) from α-ketoglutarate, glutamine, succinate, and lactate. Ionophore did not increase glucose production when glycerol was used as a substrate.

Since many compounds have been shown to stimulate gluconeogenesis by increasing cyclic AMP levels, we studied the effect of ionophore A23187 on cyclic AMP levels in isolated renal cortical tubules. Table V presents the results of such studies. Ionophore A23187 at concentrations of 10^{-8} and 10^{-9} *M* did not increase cyclic AMP production by renal tubules at external Ca^{2+} concentrations of 0.1 or 1 m*M*. Omission of Ca^{2+} from the external medium did not alter the results. On the other hand, 1 unit/ml of parathyroid hormone increased cyclic AMP production significantly both in the absence and presence of external Ca^{2+}.

Similar experiments (results not shown) were carried out without theophylline in the incubation medium at 0 (0.1 m*M* EGTA), 0.1, and 1 m*M* external Ca^{2+} concentrations. Ionophore did not increase cyclic AMP production under these conditions.

Additional experiments were performed to evaluate the effects of ionophore alone, cyclic AMP alone, and the two drugs in combination on glucose production by isolated rat renal cortical tubules. As shown in Table VI, both ionophore and cyclic AMP added alone increased glucose production significantly as compared to flasks containing only α-ketoglutarate. However, the combination of ionophore and cyclic AMP did not result in a further increase in glucose production as compared to the flasks containing either ionophore or cyclic AMP alone. These experiments would suggest that the effects of ionophore and cyclic AMP on glucose production are not additive.

Pentobarbital, a widely used inhibitor of calcium transport, completely abolished the increase in glucose production observed with ionophore A23187 at external calcium concentrations of 0.1 m*M*. Preincubation with 10^{-5} *M* pentobarbital for 20 min was required to observe this effect. The results of these experiments are shown in Table VII.

TABLE III. EFFECTS OF IONOPHORE A23187 ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.[a]

	Glucose production (nmoles/mg of protein/60 min)	
	No external Ca^{2+}	0.1 m*M* External Ca^{2+}
No substrate	10.9 ± 0.5	15.6 ± 3.3
α-Kg + 10^{-9} DMSO diluent	35.6 ± 4.8	60.4 ± 7.4
α-Kg + 10^{-9} *M* ionophore	30.3 ± 5.6	82.3 ± 8.1*

[a] These experiments were carried out using dialyzed bovine serum albumin (BSA). BSA was dialyzed at 4° for 72 hr against a phosphate Ringer's solution containing no calcium. The values are the means ± SE of six experiments.

* Values significantly different ($P < 0.01$) from α-Kg + 10^{-9} DMSO.

TABLE IV. EFFECT OF IONOPHORE A23187 ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.[a]

Substrate	No. of experiments	No substrate	Substrate and DMSO	Substrate and ionophore
α-Ketoglutarate	7	30.3 ± 1.8	113.3 ± 10.9	130.0 ± 12.6*
Glutamine	5	35.6 ± 4.2	93.9 ± 9.6	110.7 ± 10.1*
Succinate	4	39.3 ± 3.6	94.9 ± 17.8	116.4 ± 21.9*
Lactate	4	36.9 ± 3.2	87.1 ± 2.9	95.0 ± 2.3*
Glycerol	4	26.7 ± 2.4	61.2 ± 7.6	57.9 ± 7.2

[a] Values are means ± SE. External Ca^{2+} concentration, 0.1 m*M*. Substrates used at 10 m*M* concentration.

* Values significantly different ($P < 0.05$) from those obtained with substrate + DMSO.

TABLE V. EFFECT OF IONOPHORE A23187 AND PARATHYROID HORMONE ON CYCLIC AMP PRODUCTION BY RAT RENAL CORTICAL TUBULES.

	Cyclic AMP (pmoles/mg of protein/20 min)		
	1.0 m*M* External Ca^{2+}	0.1 m*M* External Ca^{2+}	No external Ca^{2+} (0.1 m*M* EGTA)
Control	12.4 ± 1.8[a]	9.9 ± 1.9	15.1 ± 0.6
10^{-8} DMSO diluent	11.4 ± 0.7	9.1 ± 2.7	16.7 ± 0.3
10^{-8} *M* Ionophore	10.7 ± 1.2	9.5 ± 3.3	16.1 ± 0.5
Parathyroid hormone, 1 unit/ml	47.5 ± 4.1	44.7 ± 10.8	68.1 ± 11.0
10^{-9} DMSO diluent	12.6 ± 0.9	8.9 ± 2.5	17.2 ± 1.0
10^{-9} *M* Ionophore	10.0 ± 0.3	8.9 ± 2.4	14.2 ± 2.0

[a] Values are the means ± SE of four experiments.

TABLE VI. EFFECTS OF IONOPHORE A23187 AND CYCLIC AMP ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.[a]

	Glucose production (nmoles/mg of protein/60 min)
No substrate	21.6 ± 1.3
α-Kg + 10^{-9} DMSO	109.4 ± 7.4
α-Kg + 10^{-9} *M* ionophore	134.4 ± 8.1*
α-Kg + 5×10^{-4} *M* cyclic AMP	147.7 ± 6.5*
α-Kg + 10^{-9} *M* ionophore + 5×10^{-4} *M* cyclic AMP	134.4 ± 4.7

[a] Values are the means ± SE of four experiments. External Ca^{2+} concentration was 0.1 m*M*.

* Values significantly different ($P < 0.01$) as compared to those obtained with α-Kg + DMSO.

TABLE VII. EFFECT OF 10^{-5} *M* PENTOBARBITAL ON IONOPHORE-STIMULATED GLUCOSE PRODUCTION BY RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/30 min)			
	α-Kg	α-Kg + pentobarbital	α-Kg + ionophore	α-Kg + ionophore + pentobarbital
Experiment 1	24.6	23.9	33.7	22.8
Experiment 2	34.4	32.3	40.4	31.6
Experiment 3	30.2	28.9	44.4	33.3
Experiment 4	23.7	30.0	56.4	27.2
Mean ± SEM	28.2 ±2.5	28.8 ±1.8	43.7 ±4.8	28.7 ±2.4

Discussion. The results of the present experiments demonstrate that the divalent cation ionophore A23187 increases glucose production from a variety of substrates in isolated renal cortical tubules. The effects of ionophore on gluconeogenesis were present at low levels of extracellular calcium (0.1 m*M*). In the absence of external calcium or at concentrations of extracellular calcium in excess of 1.0 m*M*, the ionophore did not stimulate renal gluconeogenesis. The critical role of calcium in the control of renal gluconeogenesis has been documented previously (10, 15, 18). In the absence of extracellular calcium, glucose production is not increased by a variety of stimuli capable of increasing gluconeogenesis when Ca^{2+} is present. On the other hand, cyclic AMP levels have been shown to increase in response to several agents even when calcium is omitted from the incubation medium (15, 18).

The lack of stimulation of gluconeogenesis by the ionophore at extracellular calcium concentrations of 1.0 m*M* might be due to the development of high cytosolic calcium concentrations. The fact that high concentrations of ionophore (10^{-6} *M*), rather than stimulating, inhibited gluconeogenesis also suggests that at these concentrations the ionophore may markedly increase cytosolic Ca^{2+} even when the external Ca^{2+} concentration was 0.1 m*M*.

The ionophore A23187 increased glucose production from α-ketoglutarate, glutamine, succinate, and lactate, but not from glycerol, suggesting that the ionophore may modulate (via changes in cytoplasmic calcium) the activity of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase. Previous studies by Roobol and Alleyne (27) have demonstrated this enzyme to be sensitive to changes in calcium concentration.

The increase in glucose production from α-ketoglutarate produced by the ionophore at external Ca^{2+} concentrations of 0.1 m*M* was completely abolished by pretreatment of the renal cortical tubules with 10^{-5} *M* pentobarbital. Pentobarbital has been shown to inhibit Ca^{2+} transport across

certain biological membranes (28). Thus, as calcium transport across the cell membrane into the cytosolic compartment was blocked, increases in glucose production did not occur using the ionophore, indicating that the ionophore most likely stimulated glucose production by altering cytosolic calcium concentrations.

Ionophore A23187 did not increase cyclic AMP levels in renal cortical tubules or slices at various calcium concentrations. The presence or absence of theophylline in the incubation medium did not alter this lack of effect. Glucose production was thus stimulated by the ionophore without an increase in cyclic AMP levels. Parathyroid hormone (4), prostaglandin E_1 (18), and catecholamines (29, 30) increase cyclic AMP levels and gluconeogenesis. In the case of both parathyroid hormone and prostaglandins, removal of external Ca^{2+} blocks the increased gluconeogenesis produced by these compounds without affecting cyclic AMP production. The ionophore in the insect salivary gland does not stimulate cyclic AMP levels (20), but in both the pancreatic islet cells (23) and in bone culture cells (31) it has been shown to cause an increase in cyclic AMP levels.

In the present studies, glucose production was stimulated by ionophore in the absence of an increase in cyclic AMP by a mechanism felt to be related to changes in cytosolic calcium concentrations. No additive effects on gluconeogenesis were observed when ionophore and exogenous cyclic AMP were added together (Table VI). At low levels of extracellular calcium, the ionophore stimulated glucose production presumably by increasing cytosolic calcium concentrations. This action was blocked by pretreatment with pentobarbital. At higher extracellular calcium concentrations, the ionophore did not stimulate gluconeogenesis, suggesting that there is an optimal concentration of cytosolic Ca^{2+} for gluconeogenesis and high Ca^{2+} concentration may be inhibitory of this process. Mechanisms designed to maintain cytosolic Ca^{2+} concentrations within narrow limits seem to be critical in the control of renal gluconeogenesis.

Summary. The divalent cation ionophore A23187 was found to increase glucose production from α-ketoglutarate, glutamine, succinate, and lactate, but not from glycerol in isolated rat renal cortical tubules at external Ca^{2+} concentrations of 0.1 m*M*. At an external Ca^{2+} concentration of 1.0 m*M* or in the absence of external Ca^{2+}, the ionophore did not increase glucose production from α-ketoglutarate. Ionophore A23187 did not increase cyclic AMP levels in renal cortical tubules. The increase in glucose production produced by ionophore A23187 was completely prevented by preincubation of the tubules with 10^{-5} *M* pentobarbital, a known inhibitor of calcium transport in biological systems. The experiments suggest that ionophore A23187 increases gluconeogenesis from a variety of substrates by altering the levels of cytosolic calcium without affecting the concentrations of cyclic AMP.

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