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Parathyroid Hormone, 3′5′ AMP, Ca^{++}, and Renal Gluconeogenesis*

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Abstract. Isolated segments of renal tubules prepared from rat kidney cortex were capable of sustained rates of gluconeogenesis when lactate served as substrate. An increase in external Ca^{++}, or the addition of parathyroid hormone at a fixed Ca^{++} concentration led to an enhanced rate of gluconeogenesis. However, parathyroid did not increase gluconeogenesis in the absence of external Ca^{++} even though it caused a rise in 3′5′ AMP whether Ca^{++} was present or absent. Metabolite profiles showed that either an increase in external Ca^{++}, or the addition of hormone in the presence of calcium led to qualitatively very similar changes. However, the addition of hormone had little effect upon metabolite levels in the absence of extracellular calcium even though an increase in extracellular H^+ enhanced gluconeogenesis under similar conditions. These results are discussed in relationship to the close association of 3′5′ AMP and Ca^{++} in a variety of cellular systems.

Since the initial discussion, by Rasmussen and Tenenhouse, of the relationship between Ca^{++} and cyclic 3′5′ adenosine monophosphate (3′5′ AMP),[1] additional evidence has accumulated in support of the thesis that the activation of many cells by specific stimuli involves both an activation of adenyl cyclase and a requirement for external Ca^{++} which is believed to be related to an increase in calcium influx during activation of the particular cell.[2–11] It is not yet possible to define completely the relationship between Ca^{++} and 3′5′ AMP. However, the absence of extracellular calcium does not alter the change in 3′5′ AMP synthesis induced by specific stimuli in at least four different systems: (1) salivary gland;[1] (2) heart;[5] (3) thyroid gland;[9] and (4) pituitary hormone release.[8, 10, 11] Equally important is the fact that Ca^{++} is an inhibitor rather than an activator of the enzyme adenyl cyclase in plasma membrane fragments.[6, 12] These results clearly imply that calcium ions are required for the final physiologic expression of the initial specific effect of the stimulus on the cell, and might be interpreted as indicating a need for Ca^{++} in the expression of 3′5′ AMP action in these different cells, or alternatively that 3′5′ AMP alters the Ca^{++} permeability of the cell membrane. Data that suggest, but do not confirm, that glucagon, catacholamines, and 3′5′ AMP alter the permeability of the liver cell membranes to Ca^{++} have been reported by Friedmann and Park.[13]

In our earlier publications,[1, 4] we raised the possibility that the effect of parathyroid hormone upon calcium transport, as well as some of its metabolic effects

might be explained by an induced change in cellular calcium influx. In view of the data of Chase and Aurbach relating PTH action to a rise in 3′5′ AMP,[12, 14, 15] that of Borle showing an effect of PTH upon calcium uptake in tissue culture cells,[16, 17] and our own data showing that dibutyryl 3′5′ AMP infusion mimics many of the effects of PTH infusion in thyroparathyroidectomized rats,[18] we were led to the possibility that there was an intimate relationship between Ca^{++} and 3′5′ AMP in the action of this hormone as well. This possibility was first examined by studies in which the hormone, or Ca^{++} were given *in vivo*.[4] Data were obtained which were consistent with this possibility, but many critical questions could not be answered by this approach. It then became of interest to examine in detail the relationship between 3′5′ AMP and calcium in a well defined *in vitro* system responding to parathyroid hormone.

The system finally developed for these studies was the change in gluconeogenesis in isolated segments of renal tubules prepared from rat kidney cortex.

Methods. Rat renal tubules were prepared from parathyroidectomized or normal fed male Wistar rats weighing 150–200 gm. The tubules were prepared by a modification of the method developed by Howard and Pesch for preparing isolated liver cells[19] and depends upon the combined use of collagenase, Worthington CLS, and hyaluronidase, Sigma type I, in a calcium-free modified Hank's solution. Once prepared, the tubules were incubated in a concentration equivalent to 5–7 mg protein/ml of a modified Krebs-Ringer bicarbonate buffer containing variable amounts of calcium or EGTA (ethylene bisoxyethylenenitritotetraocetate), 2% bovine serum albumin, and 10 mM lactate as substrate. Incubations were carried out in siliconized 25-ml Ehrlynmeyer flasks at 37°, under 95% O_2–5% CO_2 with gentle shaking, 70 oscillations per minute. Each flask contained 1.2 ml of medium plus tubule.

At appropriate times, either trichloroacetic acid or perchloric acid extracts were prepared of the contents of respective flasks. The perchloric acid extracts were employed for metabolite assays by standard methods.[20] The trichloroacetic acid extracts were employed for 3′5′ AMP assays by a modification[4] of the method of Goldberg *et al.*[21] Glucose was measured on either extract by the method of Maitra and Estabrook.[22] Parathyroid hormone was prepared by the method of Hawker *et al.*[23]

Results and Discussion. When lactate was employed as substrate, the rate of glucose formation by isolated renal tubules was 140 μmoles glucose/gm dry weight per hour which is significantly greater than that observed by Krebs *et al.*[24] 117 μmoles/gm dry weight per hour, using slices of rat renal cortex. As shown by Krebs *et al.*[24] and Rutman *et al.*[25] calcium activates gluconeogenesis in renal cortical slices. A similar effect was seen when the tubules were employed (Fig. 1). It was reasoned that if parathyroid hormone (PTH) acted to increase intracellular calcium, it should also enhance renal gluconeogenesis. This proved to be the case. The characteristic response of isolated renal tubule to changes in external calcium and to hormone addition are shown in Fig. 1. The rate of gluconeogenesis from lactate was linear over a period of 30′ in the absence of added external Ca^{++} (0.1 mM EGTA). The addition of PTH at 15′ caused no increase in rate of glucose production under these circumstances. However, the addition of $CaCl_2$ to a final concentration of 0.25 mM led to a definite increase in glucose production, and the addition of both 0.25 mM calcium and PTH (5 μg/ml) led to an even greater increase in glucose production. If 0.25 mM $CaCl_2$ was presented throughout the period of incubation, the subsequent addition of PTH led to a stimulation of glucose production. Similar results have been ob-

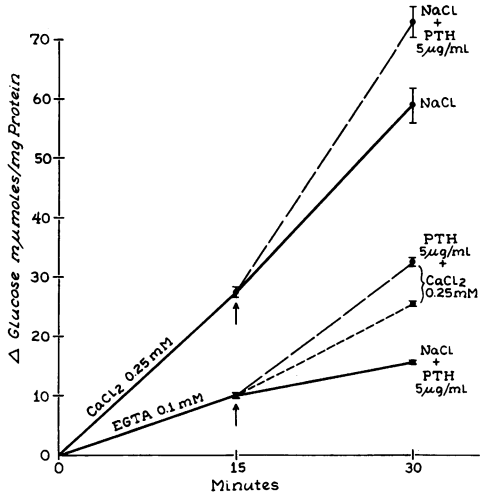

FIG. 1.—The formation of glucose from lactate as a function of time in isolated segments of rat renal tubules incubated in the absence of calcium (0.1 mM EGTA), lower line; and in the presence of 0.25 mM $CaCl_2$, upper line. At 15′ either PTH (parathyroid hormone) in 0.15 NaCl, or PTH and calcium were added to tubules incubated in the absence of calcium, and PTH in 0.15 *M* NaCl, or NaCl alone were added to tubules incubated in 0.25 mM $CaCl_2$. The incubation medium contained 2% bovine serum albumin; 0.5 mM palmitate; 10 mM sodium lactate; in Krebs Ringer bicarbonate modified in calcium content as noted above. Each flask contained 1.2 ml medium and tubules equivalent to 6 mg of protein. Incubations were carried out at 37° under 95% O_2–5% CO_2.

tained with a number of other substrates including pyruvate, glutamate, malate, oxalacetate, and ketoglutarate, but neither PTH nor an increase in extracellular calcium enhanced gluconeogenesis from glycerol.

The next question to be examined was the relationship between this requirement for Ca^{++} in the action of the hormone, and hormonally induced changes in 3′5′ AMP (Fig. 2). The addition of hormone to tubules in the presence of 2.5 mM $CaCl_2$ led to a significant rise in 3′5′ AMP within the tubules, and the rise was dose related. Also, the time course of the changes in 3′5′ AMP after the addition PTH are shown. Of particular importance is the fact that the hormone induced a rise in 3′5′ AMP whether Ca^{++} was present or absent in the medium. Thus under conditions of calcium lack, PTH caused a rise in 3′5′ AMP without producing a significant change in gluconeogenesis.

In the light of this result, it became of interest to examine the effect of dibutyryl 3′5′ AMP upon renal gluconeogenesis, and its relationship to extracellular calcium. As is shown in Figure 3, dibutyryl 3′5′ AMP also stimulated glucose production from lactate. Dibutyryl 3′5′ AMP was without effect in the absence of extracellular calcium. These data taken in conjunction with those

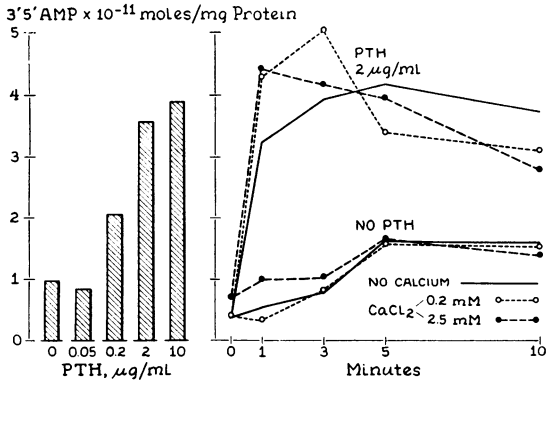

FIG. 2.—The effect of increasing doses of PTH upon the concentration of 3′5′ AMP in rat renal tubules (left) incubated in 2.5 mM Ca^{++} employing lactate as substrate. Also, the time course of the changes in 3′5′ AMP concentration (right) following the addition of PTH to tubules incubated in media containing either no calcium (0.1 mM EGTA), 0.25 mM, or 2.5 mM calcium.

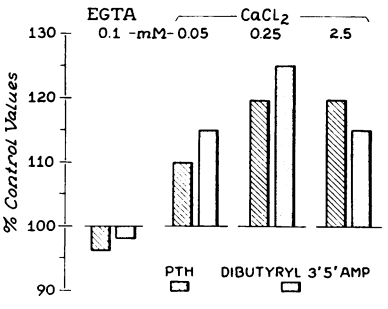

FIG. 3.—The effect of PTH (0.5 μg/ml) and dibutyryl 3′5′ AMP (0.5 mM) upon glucose formation from lactate during a 20 minute incubation in the absence (0.1 mM EGTA) and presence of increasing concentrations of extracellular calcium. The control value in each instance refers to the amount of glucose produced when neither PTH or dibutyryl 3′5′ AMP were present. The incubations were carried in the medium described in Figure 1 except for the variable calcium content. Control values for gluconeogenesis were 14.7, 28.6, 35.5 and 53.2 mμmoles glucose /formed mg protein at 0.1 mM EGTA, 0.05 mM, 0.25 mM, and 2.5 mM Ca^{++} respectively.

shown in Figure 2, indicate that calcium is required for expression of the effect of 3′5′ AMP and is not directly involved in the activation of renal adenyl cyclase by PTH.

We next examined the possibility that the rise in 3′5′ AMP after hormone addition might induce some metabolic changes even in the absence of calcium, i.e., that after hormone additions, both 3′5′ AMP and Ca^{++} act as second messengers and exert their effects upon metabolic processes presumably related to gluconeogenesis. Although this analysis is not yet complete, representative data employing lactate as substrate are shown in Table 1. In part 1, renal tubules were incubated in the presence of Ca^{++}. Metabolite concentrations after the addition of calcium or PTH are presented. An increase in extracellular Ca^{++} from 0.25 to 1.0 mM or addition of PTH in the presence of 0.25 mM Ca^{++} produced significant lowering of α-ketoglutarate with an enhanced formation of glucose. This is in clear contrast to the addition of PTH in the presence of 0.1 mM EGTA, where there was no significant change in the levels of any of the metabolites measured except for 3′5′ AMP (Part 2). However, this lack of hormonal effect upon metabolite levels in the absence of calcium cannot be attributed to a lack of gluconeogenic capacity under these circumstances because, as is shown on the same table, an increase in extracellular H^+ concentration will induce both a very significant increase in gluconeogenesis and marked changes in metabolite levels when tubules are incubated in the medium containing 0.1 mM EGTA. Decrease in α-ketoglutarate amount in this *in vitro* system caused either by additional calcium, PTH in the presence of calcium, or lowering pH is similar to that observed in rat kidney *in vivo* when calcium, PTH, or HCl are infused.[4] A lower α-ketoglutarate amount in the face of enhanced gluconeogenesis might be explained by an enhanced drain of α-ketoglutarate either by stimulation of the conversion of oxaloacetate to glucose or by direct stimulatory effect on citric acid cycle activity, as proposed in relation to acid-base balance.[26, 27] Also the possibility cannot be excluded that when gluconeogenesis is stimulated, increased oxaloacetate utilization to form phosphoenolpyruvate may reduce citrate formation and thereby the production of α-ketoglutarate.

Our interpretations of the present and previous data[6] were: (1) either an in-

TABLE 1. *Metabolites found in renal tubules and in medium after 20′ incubation with 10 mM lactate substrate in modified Krebs-Ringer bicarbonate. The pH of the medium shifted from 7.4 to 6.8 by the reduction of bicarbonate from 24 to 11 mM.*

mμmoles/mg protein	[I] 0.25 mM $CaCl_2$ pH 7.4	[I] 1 mM $CaCl_2$ pH 7.4	[I] 0.25 mM $CaCl_2$ pH 7.4 + PTH 5 μg/ml
α-Ketoglutarate	32.0 ± 2.2	20.8 ± 1.5	22.4 ± 1.5
Isocitrate	2.77 ± 0.14	2.35 ± 0.12	2.77 ± 0.18
Citrate	50.3 ± 1.5	51.3 ± 1.5	48.8 ± 2.0
Pyruvate	15.7 ± 0.7	20.4 ± 1.2	17.6 ± 1.0
Malate	10.6 ± 1.0	10.1 ± 0.8	8.30 ± 0.8
Phosphoenolpyruvate	0.47 ± 0.05	0.52 ± 0.04	0.49 ± 0.05
Glucose	34.5 ± 1.8	50.0 ± 3.0	42.4 ± 1.9
3′5′ cyclic AMP	0.0102 ± 0.0010	0.0098 ± 0.0011	0.0324 ± 0.0035
ATP	6.32 ± 0.25	6.95 ± 0.30	6.51 ± 0.20

mμmoles/mg protein	[II] 0.1 mM EGTA pH 7.4	[II] 0.1 mM EGTA pH 7.4 + PTH 5 μg/ml	[II] 0.1 mM EGTA pH 6.8
α-Ketoglutarate	61.7 ± 5.0	60.5 ± 4.8	18.5 ± 1.2
Isocitrate	2.96 ± 0.17	2.81 ± 0.15	2.07 ± 0.10
Citrate	51.3 ± 1.2	50.2 ± 1.5	46.2 ± 1.8
Pyruvate	5.8 ± 0.5	5.8 ± 0.8	29.1 ± 1.5
Malate	7.6 ± 0.7	7.4 ± 0.6	17.5 ± 1.5
Phosphoenolpyruvate	0.30 ± 0.04	0.32 ± 0.02	0.42 ± 0.04
Glucose	13.3 ± 1.0	13.0 ± 1.2	46.5 ± 3.1
3′5′ cyclic AMP	0.0104 ± 0.0012	0.0297 ± 0.0028	0.0102 ± 0.0015
ATP	5.18 ± 0.22	5.34 ± 0.18	6.73 ± 0.25

crease in external calcium concentration or the addition of PTH at a fixed calcium concentration led to an increase in intracellular Ca^{++}; (2) this increase in intracellular Ca^{++} was responsible for the increased rate of gluconeogenesis; (3) the hormone increased 3′5′ AMP whether external Ca^{++} was present or not, but this rise in 3′5′ AMP did not cause an increased rate of gluconeogenesis, nor activation of any key gluconeogenic enzymes in the absence of external Ca^{++} in spite of the fact that the activities of these enzymes could be greatly enhanced in the absence of calcium by other stimuli, specifically an increase in extracellular H^+. Although these striking effects have been observed *in vitro*, the role and controlling concentrations of Ca^{++} in renal gluconeogenesis *in vivo* have not been fully elucidated. Therefore, the physiological role in gluconeogenesis of agents supposed to act by altering Ca^{++} permeability cannot, as yet, be fully understood.

In terms of defining the relationship between 3′5′ AMP and calcium permeability, the present studies add to the list of circumstances in which a rise in 3′5′ AMP is seen when a cell is activated by a specific stimulus, but in which, in spite of the rise in 3′5′ AMP the expected physiological consequence is not observed. The present data also help to decide between independent effects of PTH upon adenyl cyclase and Ca^{++} permeability, or sequential effects. The fact that dibutyryl 3′5′ AMP enhanced gluconeogenesis from lactate, or other substrates, and this effect required the presence of external Ca^{++}, favors the view that cyclic AMP has direct effects upon calcium movements across the plasma membrane of

the cell. However, in either the presence or absence of Ca^{++}, the metabolite profiles are somewhat different after dibutyryl 3′5′ AMP from those seen after PTH addition. Also, the infusion of dibutyryl 3′5′ AMP *in vivo* causes some of the same changes in renal function as those seen after hormone infusion, but here again there are clear differences.[18] In order to conclude that the correct model is one in which the hormone first activates adenyl cyclase leading to an increase in 3′5′ AMP, which leads in turn to the changes in calcium distribution, one must assume that the addition of 5×10^{-4} *M* dibutyryl 3′5′ AMP to the external side of the cell membrane is equivalent to generating 10^{-7} or 10^{-8} *M* 3′5′ AMP on the internal surface of this membrane. This assumption remains to be validated.

The present results reemphasize the importance of the relationship between Ca^{++} and 3′5′ AMP, and make it even more likely that these two agents are integral parts of the response of many cell membranes to specific activating stimuli. This proposal implies that early in evolution, the adenyl cyclase-calcium permeability association was one of the few basic mechanisms developed for the coupling of events at the cell surface to those in the interior of the cell. The subsequent specialization of this mechanism has been of two kinds. On the one hand, the development of specific recognition sites on the cell surface so that particular differentiated cells respond only to a highly specific extracellular messenger, and specialization within the cell so that the same membrane events lead to different metabolic consequences depending on the structural and functional uniqueness of the particular cell. A particularly interesting aspect of this general model which is worthy of consideration is the relationship between adenyl cyclase and the specific phosphodiesterase involved in the hydrolysis of 3′5′ AMP to 5′ AMP.[28] In contrast to adenyl cyclase, the latter enzyme is not bound to the plasma membrane but appears in the cytosol. Thus, if this enzyme is highly active, very little unchanged 3′5′ AMP would be expected to diffuse far from its site of synthesis in the membrane. Hence by regulating the ratios of the activities of these two enzymes in particular cells the extent of 3′5′ AMP diffusion, and thus its role as a direct intracellular messenger can be regulated. On the other hand, a change in Ca^{++} entry across the plasma membrane and into the cell represent a second messenger which is not readily transformed as it diffuses into the cytosol. For this reason alone, calcium may be a more important second messenger than 3′5′ AMP in some cellular systems.

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