THE EFFECT OF PARATHYROID HORMONE ON RESPIRATION IN ISOLATED RAT LIVER CELLS

A Thesis

Presented to

the Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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June 1967

ACKNOWLEDGMENTS

The author wishes to express sincere gratitude and appreciation to Dr. C. M. Dowse for his time, interest and valuable criticisms.

Similar acknowledgment is extended to Dr. I. Kleinberg and members of his faculty for their continued interest in this work.

I am deeply grateful to my wife Patricia for her patience and understanding during the course of this study.

The author was supported by a Research Fellowship from the Associate Committee on Dental Research, National Research Council of Canada.

ABSTRACT

The Effect of Parathyroid Hormone on Respiration in Isolated Rat Liver Cells

The role of parathyroid hormone (PTH) in calcium and phosphate regulation in the body is well established but there is no generally accepted hypothesis for the action of the hormone at the cellular level. Since PTH appears to involve oxidative phosphorylation in mitochondrial systems in vitro, the purpose of the present work was to establish and examine the conditions under which an in vitro effect of PTH on cellular respiration rate could occur and to study the specificity of this hormonal effect.

Liver cells were obtained by differential centrifugation after mild homogenization of perfused rat liver. Hormone was isolated by the phenol extraction gel-filtration technique of Aurbach and Potts (Endocrinology 75, 290, 1964). The cell preparation was incubated in Warburg flasks containing the optimal concentrations of 300mM mannitol, 20mM sodium phosphate pH 7.2, 15mM sodium succinate pH 7.2 and 1mM $MgCl_2$. Oxygen uptake was determined by standard manometric techniques.

The control rate in the above basic medium was 60 $\mu l O_2/hr/10^6$ cells. Oligomycin (3.85μg/ml) or 0.5mM ATP, separately, had no effect on the respiration rate but together reduced it to about 30 $\mu l O_2/hr/10^6$ cells. PTH (8.5μg/ml) had no effect on the control respiration rate but completely

overcame the reduction in respiration which occurred after oligomycin and ATP addition. Oxidized or heated (100°C) hormone did not function in this system whereas oxidized hormone that had been reduced again did. UTP, CTP, GTP and ITP had the same effect in the system as oligomycin and ATP combined. ATP itself could be completely replaced by ADP or AMP and partially by adenosine but oligomycin was still required. In the absence of oligomycin, interconversion of adenosine nucleotide was shown to occur governed by adenylate kinase, ATPase and oxidative phosphorylation activities. The reduction in respiration by ITP was overcome if ATP, ADP or AMP was also added to the medium. Other basic proteins (protamine sulfate and polylysine) and uncouplers (gramicidin and dinitrophenol) were as effective as PTH in relieving inhibition by oligomycin and ATP. If magnesium or phosphate was omitted from the incubation medium the respiration rate was reduced to about 30 $\mu l O_2/hr/10^6$ cells. In phosphate deficient but not in magnesium deficient mediums, PTH returned the oxygen uptake to control values only if nucleotide phosphate was present. In this case the hormone could not be replaced by dinitrophenol, polylysine, oxidized PTH or heated (100°C) PTH. However, oxidized hormone that had been reduced again functioned.

Although there is no definitive evidence, it is probable that PTH penetrates the cells and acts directly upon the mitochondrion. Effects of PTH on mitochondrial respiration

has been interpreted in several ways in the past. These have included stimulation of ATPase activity, uncoupling of oxidative phosphorylation and direction of energy into ion translocation. In the present system it is believed that the nucleotide phosphates improve the functional integrity of the mitochondrion and that the hormone is not stimulating an ATPase but rather functioning as an uncoupler of oxidative phosphorylation. However, its action is not identical with that of dinitrophenol nor is the basic nature of the hormone the only factor involved. These complex effects on isolated cells are compared to studies on mitochondria and discussed in detail.

ABBREVIATIONS

The following abbreviations are used:

PTH, parathyroid hormone; ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; IDP, inosine diphosphate; IMP, inosine monophosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; NAD, nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; FAD, flavin-adenine dinucleotide; DNP, dinitrophenol; $^{32}P_i$, radioactive inorganic phosphate; GSH, glutathione; CoA, coenzyme A; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetate; Tris buffer, tris (hydroxymethyl) amino methane; ^{o}C, degrees centigrade; mμ, millimicron; O.D., optical density; s.d., standard deviation; cm, centimetre; mm, millimetre; ml, millilitre; μl, microlitre; gr, gram; mg, milligram; μg, microgram; μμg, micromicrogram; N, normal; M, molar; mM, millimolar.

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INTRODUCTION

The physiological action of parathyroid hormone (PTH) in serum calcium and phosphate regulation has been known for many years. It has a direct effect on the mobilization of the skeleton, it stimulates calcium and phosphate uptake in the intestine and increases calcium reabsorption and phosphate excretion in the kidney. Although the liver is not an obvious target organ for PTH like the skeleton, intestine and kidney, there is considerable evidence that PTH does have a widespread action in the body. Various effects of PTH on mammary gland, lens, salivary gland, erythrocyte and muscle have been reported. More recently, effects of PTH at the subcellular level on liver and, to a lesser extent, on kidney mitochondria have been studied. However, there is no consensus of opinion as to how these physiological changes might be brought about at the cellular level.

The reasons for using rat liver cells in the present study were:

1) a suspension of isolated cells could be uniformly sampled and it appeared from the literature that the cells could be easily prepared in good yield from rat liver.
2) hormonal effects would be upon intact cells.
3) comparisons could be made with the recent work of others on liver mitochondria.

Briefly, the specific aims of the present study were:

1) to devise the optimal procedure for preparing isolated rat liver cells and a suitable medium in which to study cellular oxygen utilization.
2) to establish and examine the conditions under which an *in vitro* effect of PTH on cellular respiration rate could occur.
3) to study the specificity of this hormonal effect.

HISTORY OF THE LITERATURE

1) Parathyroid Hormone

General. Ever since the classic work of MacCallum and Voegtlin in 1909, it has been known that the parathyroid glands play an important role in controlling the concentrations of calcium and phosphate in the blood. However, a controversy existed concerning the mechanism of action of PTH. On the one hand, there were those investigators who favoured the view that the primary action of the hormone was upon renal excretion of phosphate and that changes in the concentration of calcium in the plasma and the subsequent mobilization of calcium from the bone were consequences of this renal effect. On the other hand, a number of investigators considered that the primary effect of PTH was upon resorption of the bone, the renal and other effects being secondary. The finding that parathyroidectomy resulted in a fall in the serum calcium of nephrectomized rats (Talmage et al, 1953) and the demonstration of a direct action of PTH on bone (Barnicot, 1948) and on kidney (Rasmussen and Deluca, 1963) made it obvious that neither of these diametrically opposed views was wholly correct.

Several studies have shown that calcium but not phosphate controls the secretion of PTH. Stoerk and Carnes (1945) demonstrated that parathyroid gland enlargement correlated almost perfectly with the serum calcium

concentration of the animals but showed no definite relation to the serum inorganic phosphate concentration. Roth et al (1964) have reported several effects of calcium on the ultrastructure of rat parathyroid glands. High calcium concentrations reduced the cell volume, size of the Golgi apparatus and the aggregation of ribonucleoprotein particles. These changes were correlated with a suppression of the liberation of ^{14}C-leucine labelled proteins. Low calcium concentrations had the opposite effects. More recently, the availability of sensitive radioimmunoassay has made possible direct determination of the level of circulating endogenous hormone. Melick et al (1965) have shown the biological half life to be relatively short, of the order of twenty-two minutes in the rat. Sherwood et al (1966) found the normal concentration range in bovine plasma to be between 400 and 1,800μμg/ml. They clearly established an inverse relationship existed between the concentration of PTH and of calcium but not phosphate. The plasma PTH concentration fell to less than 100μμg/ml when the plasma calcium was increased to 14mg % (4mg % above normal) by intravenous infusion of calcium chloride, and rose as high as 6,000μμg/ml by infusion of a solution of EDTA. The development of techniques for perfusion of isolated parathyroid glands in the sheep and goat permitted Care et al (1966) to measure the PTH concentration in the venous effluent of the glands. The rate of secretion of PTH was inversely proportional to, and responded rapidly

to changes in, the calcium concentration in the perfusate.

PTH is not the only hormone involved in the regulation of the blood serum calcium level. Thyrocalcitonin, which is secreted from the thyroid gland when the serum calcium level is high, has an effect opposed to that of PTH in reducing the calcium level (Munson, 1966). Thyrocalcitonin was suspected when surgical removal of the parathyroid glands resulted in a higher serum calcium level than occurred following destruction of the glands by electrocautery. It is probable that during electrocautery of the parathyroid glands the adjacent thyroid glands are directly stimulated to release thyrocalcitonin.

Potts et al (1965) have proposed a model structure for PTH consisting of a single chain polypeptide with a molecular weight of approximately 9,000. The amino acid composition is $lysine_{10}$, $histidine_4$, $arginine_5$, $aspartic\ acid_8$, $serine_6$, $glutamic\ acid_{10}$, $proline_3$, $glycine_4$, $valine_7$, $alanine_7$, $methionine_2$, $isoleucine_3$, $leucine_8$, $tyrosine_1$, $phenylalanine_2$ and $tryptophan_1$. Their partial analysis of amino acid sequence showed lysine and arginine to be clustered in certain areas giving these regions a highly positive charge. A minimum structure requisite for biological activity appeared to be approximately 25% of the molecule, a sequence of twenty amino acid residues at the carboxyl terminal of the polypeptide chain.

Oxidation of PTH by hydrogen peroxide or performic

acid resulted in the loss of both the calcium mobilizing and phosphaturic activities of the hormone in vivo (Tashjian, Ontjes and Munson, 1964). If the oxidized PTH was reduced at elevated temperatures with mercaptoethanol or cysteine hydrochloride, biological activity was again restored. They concluded that this activity was dependent on methionine because this was the sole amino acid residue found to undergo reversible oxidation-reduction. It is now known that oxidation of the tyrosine or tryptophan residues of PTH by N-bromosuccinimide in 8M urea also causes marked loss of biological activity (Potts et al, 1966). There was no loss of antigenic activity of the hormone following oxidation of methionine (Tashjian, Ontjes and Munson, 1964) or tryptophan (Aurbach and Potts, 1967) indicating that the sites of biological activity and immunological activity are not identical and that no major alteration in the conformity of the molecule occurred.

Effects of PTH on Bone. The first studies which clearly demonstrated a direct effect of PTH on bone were carried out by Barnicot (1948). He transplanted parathyroid tissue into direct contact with one surface of the bone of the skull. Bone resorption occurred on this surface and bone deposition on the opposite surface. The finding that PTH stimulates bone resorption has been verified by others (Chang, 1951 and Gaillard, 1959) but no generally accepted mechanism has yet been proposed. The resorption of bone is

a complex process in which the breakdown of ground substance and collagen fibers as well as the hydroxyapatite crystals occurs (Rasmussen, 1961).

Changes in carbohydrate metabolism may be involved. Neuman and Neuman (1957) suggested that the stimulation of citric and lactic acid production by PTH may be responsible for the mobilization of bone. The solubility of hydroxyapatite in the extracellular fluid would increase due to the increased citrate ion concentration and also due to the decrease in pH caused by an increase in lactate concentration. This suggestion by Neuman and Neuman was supported by other workers. Firschein et al (1958) found that following the injection of parathyroid extract into dogs, the serum citrate rose before the serum calcium. Martin et al (1964) reported that parathyroid extract increased the utilization of glucose and the production of lactate and citrate by calvaria in tissue culture. They proposed that the increase in citrate was due to a reduced isocitric dehydrogenase activity. On the other hand, Cohn et al (1965) found that tricarboxylic acid intermediates did not accumulate during <u>in vitro</u> incubation of bone slices prepared from rabbits injected with parathyroid extract despite a large inhibition of tricarboxylic acid cycle oxidation. Dowse et al (1963), adding PTH to rat calvaria incubated <u>in vitro</u>, also found no significant changes in citrate accumulation. The only significant finding was an increased production of lactate

which only occurred under aerobic conditions. Obviously, more data are required before the role of citrate and lactate in this complex process are understood.

Effects of PTH on bone probably also involve protein synthesis. Nichols et al (1965) showed that incorporation of ^{14}C-labelled amino acids into collagen of isolated mouse bone cells was depressed following injection of parathyroid extract. Rasmussen, Arnaud and Hawker (1964) and Tashjian, Ontjes and Goodfriend (1964) demonstrated that actinomycin D, a compound that blocks DNA-directed RNA synthesis, inhibited the mobilization effect of PTH on bone. Kunin and Krane (1965) point out that enzymes such as collagenases may be required for the resorption of bone matrix and their production enhanced by PTH.

Over three decades ago, it was established that deficiency of vitamin D led to hypocalcemia (Thomson et al, 1932). It was believed that the D vitamins exerted their efforts by stimulating the parathyroid glands. Au et al (1965) demonstrated that vitamin D did not appear to be involved in any way in the control of PTH secretion by the serum calcium level. Deluca et al (1962) and Harrison et al (1964) established that vitamin D on its own could raise the serum calcium level but that PTH required the presence of vitamin D. Zull et al (1966), working on the relationship between vitamin D action and the actinomycin-sensitive process, suggested that vitamin D was required to alter the nuclear

membrane permeability to calcium which in turn increased synthesis of a calcium-carrier protein so that more calcium could be mobilized from bone. PTH would accelerate the synthesis of the calcium-carrier.

Effects of PTH on Other Organs. The action of PTH appears to be widespread throughout the body because effects on a variety of tissues other than bone have been reported. PTH control of the renal excretion of phosphate is a well established fact (Greenwald, 1911, Thomson et al, 1932 and Greep, 1955). Nicholson (1959) and Rasmussen and Deluca (1963) demonstrated that the hormone increased the rate of phosphate secretion by the distal tubule. Talmage (1956) and Kleeman et al (1958) found that there was an increased excretion of calcium in the urine of rats following parathyroidectomy and a decrease in excretion of calcium in the urine of rats when parathyroid extract was added to a hypoparathyroid animal. In more recent work, Wildrow et al (1962) demonstrated that PTH caused an increased calcium reabsorption in the renal tubules.

The effects of parathyroid extract on the incorporation of subsequently injected radioactive phosphate ($^{32}P_i$) into various phosphate fractions of the rat kidney were investigated by Egawa and Neuman (1964). The extract increased the incorporation of $^{32}P_i$ into total phosphate, acid soluble phosphate, inorganic phosphate, acid soluble

organic phosphate and nucleic acid phosphate of rat kidney.

Earlier investigators believed that PTH had little or no effect upon the intestinal absorption of calcium and phosphate (Rasmussen, 1961). Talmage and Elliott (1958), working with rats, found that parathyroidectomy two to four hours before the measurements were carried out led to a significant decrease (approximately 50%) in the rate of absorption of radiocalcium from an isolated loop of small intestine *in vivo*. Rasmussen (1959) and Cramer (1963) obtained similar results. Borle et al (1963) found that the addition of parathyroid extract increased the influx of phosphate in duodenal loops of rats *in vitro*.

A possible effect of PTH upon mammary glands was reported by Munson et al (1954). They obtained data indicating that the calcium content of milk decreased during the hyperparathyroidism induced by low calcium diets given to lactating rats. Parathyroidectomy abolished this response.

Clark (1939) demonstrated that the calcium content in lens decreased if it was incubated *in vitro* with parathyroid extract. In 1962, Firschein reported that parathyroidectomy reduced glucose uptake and lactate production by lenses during subsequent *in vitro* incubation and that addition of PTH restored the rate of glycolysis to the control level.

In its action on all of the above organs, it may be seen that the effects of PTH could result in an increase in the calcium and a reduction in the phosphate levels of serum.

Effects of PTH on Mitochondria. In more recent studies, effects of PTH have been studied at a subcellular level. Liver mitochondria have been most widely used.

Both vitamin D and PTH, added *in vitro*, but not related sterols or other peptide hormones, stimulated the release of calcium by isolated rat kidney mitochondria (Deluca et al, 1962). The PTH effect could be demonstrated only in the presence of vitamin D, whereas the action of the vitamin did not require the presence of hormone.

PTH stimulated the *in vitro* accumulation of inorganic magnesium phosphate by isolated rat liver mitochondria in the absence of vitamin D (Sallis et al, 1963). A respiratory stimulation accompanied the translocation of ions. In this system, phosphate could be replaced by sulfate or arsenate (Rasmussen, Sallis et al, 1964) and magnesium by manganese (Fang and Rasmussen, 1964). PTH could also enhance an *in vitro* potassium ion uptake into mitochondria that was respiratory supported (Rasmussen, Fischer et al, 1964). It was postulated by a number of investigators (some of these are Sallis et al, 1964, Fang and Rasmussen, 1964, Kimmich and Rasmussen, 1966) that PTH stimulated ion transport by utilizing the energy of oxidative phosphorylation prior to the entrance of inorganic phosphate into the phosphorylative chain. Uncoupling of oxidative phosphorylation by PTH without any net accumulation of phosphate or magnesium ions has been reported (Fang and Rasmussen, 1964 and Sallis et al,