

Inhibition of Parathyroid Hormone Secretion and Parathyroid Hormone-independent Diminution of Tubular Calcium Reabsorption by WR-2721, a Unique Hypocalcemic Agent

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Abstract

Hypocalcemia has been observed in patients receiving WR-2721 [*S*,2-(3-aminopropylamino)-, ethylphosphorothioic acid]. WR-2721 is a compound that, after being dephosphorylated, provides protection of normal tissues against radio- and chemotherapy. The hypocalcemic response was accompanied by a decrease in the plasma level of parathyroid hormone (PTH) and by hypomagnesemia. Our present studies in rats on the mechanism of the hypocalcemic effect of WR-2721 indicate that: (a) The phosphorylated and dephosphorylated form of WR-2721 induced an equal dose-dependent decrement in plasma calcium. (b) In intact rats a maximal hypocalcemic dose of WR-2721 reduced urinary cyclic AMP excretion from 70.5 ± 6.3 to 38.2 ± 3.1 pmol/ml glomerular filtration rate (GFR), a level comparable to that observed (35.9 ± 5.2 pmol/ml GFR) in thyroparathyroidectomized (TPTX) rats. (c) WR-2721 given to TPTX rats did not significantly interfere with the calcemic effect of bovine PTH 1-34 infused at 2.5 IU/h. Likewise, the drug did not impair the PTH actions on the renal Ca and inorganic phosphate (P_i) handling, and on the urine cyclic AMP excretion. (d) In TPTX rats made normocalcemic by low P_i diet, the hypocalcemic effect of WR-2721 was only about 25% of that observed in intact animals. However, it was associated with increased urine Ca per milliliter GFR, indicating a PTH-independent inhibitory effect on tubular Ca reabsorption. (e) In WR-2721-treated intact rats, prevention of hypomagnesemia by infusing magnesium chloride did not reduce hypocalcemia. In conclusion, the hypocalcemic effect of WR-2721 is not dependent upon the presence of a phosphate group in the molecule and is not causally related to hypomagnesemia. WR-2721 appears to be a unique hypocalcemic pharmacologic agent with strong inhibitory activity on PTH secretion and additional PTH-independent action on renal Ca reabsorption.

Introduction

The clinical expressions of primary hyperparathyroidism are various, and in some cases removal of the abnormal parathyroid gland is not the best therapeutic option (1). As a substitute for surgery, the medical management of primary hyperparathyroidism would be facilitated if a rapid and efficient inhibition of parathyroid hormone (PTH)¹ secretion could be achieved by the

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1. Abbreviations used in this paper: GFR, glomerular filtration rate; P_i , inorganic phosphate; PTH, parathyroid hormone; TPTX, thyroparathyroidectomized.

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use of a specific pharmacologic agent. So far, potential inhibitors of PTH secretion, such as receptor antagonists of either histamine or β -adrenergic catecholamines, have not proved to be effective in lowering consistently the plasma calcium level in patients with primary hyperparathyroidism (1-6).

Recently, a new hypocalcemic agent, WR-2721, which may become a useful drug in the treatment of hyperparathyroidism, was discovered (7). WR-2721 is an organic phosphorothioate (*S*,2-(3-aminopropylamino)-, ethylphosphorothioic acid) that provides selective protection of normal tissues against the toxicity of radiation and alkylating agents in animals, including human beings (8). In a group of normocalcemic cancer patients, the drug induced a fall in calcemia by ~ 0.4 mmol/liter within 3 h. This effect was associated with a decrease in the plasma level of PTH, suggesting that WR-2721 inhibits the production or release of PTH (7). This notion was sustained by *in vitro* experiments showing that the drug reduced the release of PTH from bovine parathyroid gland cell suspensions (7).

These data suggest that the major effect of WR-2721 on the plasma Ca level is due to an inhibition of PTH secretion. However, other mechanisms could contribute to the fall in calcemia. Indeed, WR-2721 contains a phosphate group that, either bound to or liberated from the molecule within the extra- and/or intracellular space (9-11), might play, if not a key, at least a partial role in the hypocalcemic response. Furthermore, evidence for a direct inhibition of PTH secretion does not exclude additional interference with the action of the hormone on its target organs. Likewise, the drug could still exert a PTH-independent hypocalcemic effect by affecting bone and/or kidney Ca fluxes. Finally, it has been reported (7) that the hypocalcemic effect of WR-2721 is associated with a fall in plasma magnesium. Thus, a change in Mg metabolism could also interfere with Ca homeostasis.

These different possible mechanisms of action have been considered in the present work by studying the effect of WR-2721 in intact and thyroparathyroidectomized rats. The reported results sustain the concept that WR-2721 exerts its hypocalcemic action mainly by inhibiting PTH secretion. However, an additional PTH-independent effect on the tubular reabsorption of Ca could contribute to the drug-induced hypocalcemia.

Methods

Animal preparation

Male Wistar rats weighing 180-200 g were used. During a 5-10-d period preceding the experiment, the animals were fed a semisynthetic diet (Kliba, Klingentalmühle AG, Switzerland) containing either 1.1% Ca and 0.8% phosphorus (normal P_i diet) or 1.1% Ca and 0.2% phosphorus (low P_i diet). 10 IU of vitamin D_3 dissolved in vegetable oil was added to the daily ration. Food was provided for the last time the evening preceding the experiment. Groups of animals were thyroparathyroidectomized (TPTX) at least 7 d before the study. Only animals displaying a calcemia equal to or below 1.88 mM (7.5 mg/100 ml) 48 h after the

operation were considered as TPTX and kept in the study. They were supplemented with thyroxine 2 $\mu\text{g}/100$ g of body weight subcutaneously every other day.

Standard experiment

All experiments were started between 8 and 9 a.m. Conscious animals were put into restrictive cages that allowed tail vein injections, and blood sampling from a hindlimb vein. After a first blood sampling, the drug was injected intravenously at the dose of 0.07 mmol/100 g of body weight unless otherwise indicated. This dose was dissolved in 0.4 ml Hepes containing solution with the pH adjusted to 7.4 by Tris buffer.

The study was carried out with either the phosphorylated [$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_2-\text{S}-\text{PO}_3\text{H}_2$] or the dephosphorylated [$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_2-\text{S}-\text{H}$] form of WR-2721. Both compounds were kindly provided by the National Cancer Institute (Washington, DC). The phosphorylated form of WR-2721 given intravenously or subcutaneously in various doses did not cause any toxic side effects at its injection sites. However, its dephosphorylated form led to a transient vasoconstrictory effect when injected intravenously. Nevertheless, this route of administration was preferred, because the drug, when given subcutaneously, provoked skin ulcers at the sites of injections. Blood samples were taken before and 2, 4, 6, and 8 h after the administration of the drug. Then, the animals were put back into their normal cages and kept fasted. A last blood sample was taken the next morning, 24 h after drug administration.

In one series of experiments, the role of hypomagnesemia was evaluated. Groups of rats received subcutaneously either WR-2721 or its solvent. Then, a continuous intravenous infusion of a 0.15 M NaCl containing 10 mM MgCl_2 was installed. Blood samples were taken just before and 2, 5, and 7 h after the drug administration.

Renal clearance studies

The general methodology of renal clearance measurement in conscious rats was described in an earlier article (12). In the present study the experiments were started between 8 and 9 a.m. and lasted until 5–6 p.m. A first dose of 0.4 μCi of [$\text{methoxy-}^3\text{H}$]inulin dissolved in isotonic saline was injected intravenously in a volume of 0.4 ml per rat. A 0.15 M NaCl solution containing 50 $\mu\text{Ci}/100$ ml of [$\text{methoxy-}^3\text{H}$]inulin was then infused at a rate of 4 ml/h into a tail vein throughout the experiment. Two types of renal clearance studies were conducted.

Influence of WR-2721 in intact rats. After an equilibration period of 60–90 min, a first clearance period was made by collecting urine during 30 min. Blood was sampled at the end of this period. Then the phosphorylated form of WR-2721 or its solvent was injected subcutaneously. Three additional blood samples and urine collections of 30 min were obtained 2, 4, and 6 h after the drug or solvent injection.

Effect of PTH infusion in TPTX rats treated or not treated with WR-2721. After a first 30-min clearance period, the dephosphorylated form of WR-2721 or its solvent was injected into a jugular vein catheter which was implanted and fixed to the skin of the neck 2 d before the experiment, as previously described (13). 90 min later and after a second clearance period, 2.5 IU/h of synthetic bovine PTH 1-34 was added to the 0.15 M NaCl. After another 90 min a third clearance period was made. The PTH infusion was then stopped and replaced by the initial solution. 45 min later a last clearance period was completed.

Analytical methods

Plasma and urine Ca and Mg were measured by atomic absorption spectrometry. P_i was determined by the malachite green colorimetric method. Sodium was measured by flame photometry, and urine cyclic AMP by protein binding assay (14). The [^3H]inulin radioactivity was measured in a scintillation spectrometer.

Statistical analysis

All results are expressed as mean \pm standard error of the mean. The significance of differences was evaluated by the two-sided unpaired or paired Student's *t* test whenever appropriate.

Results

Hypocalcemic activity of the phosphorylated as compared to the dephosphorylated form of WR-2721. In intact rats i.v. injection of the phosphorylated form of WR-2721 reduced calcemia within 2 h from 2.49 ± 0.04 to 1.88 ± 0.01 mmol/liter ($n = 5$, $P < 0.001$) (Fig. 1 a). The hypocalcemic effect was maintained for at least 6–8 h. A complete normalization of plasma Ca was observed 24 h after drug administration. In order to elucidate whether the phosphate group present in this form of the compound may contribute to the hypocalcemic effect, the dephosphorylated analogue was administered in the same experimental conditions. As depicted in Fig. 1 b, a single injection of the dephosphorylated form of WR-2721 (0.07 mmol/100 g of body weight) induced a comparable fall in calcemia. Furthermore, there was no significant difference between the hypocalcemic effect of the two forms when applied in various doses (Fig. 2). The decrement in calcemia was not related to an opposite change in phosphatemia (data not shown).

Effect of WR-2721 on urine cyclic AMP in intact and TPTX rats. WR-2721 reduced urine cyclic AMP excretion in intact but not in TPTX rats (Fig. 3). The reduction in intact rats was as pronounced as that observed after complete surgical removal of the parathyroid glands.

Effect of WR-2721 on PTH action. A possible peripheral interaction of WR-2721 with PTH was investigated in TPTX rats. The animals were perfused with bovine PTH 1-34 (2.5

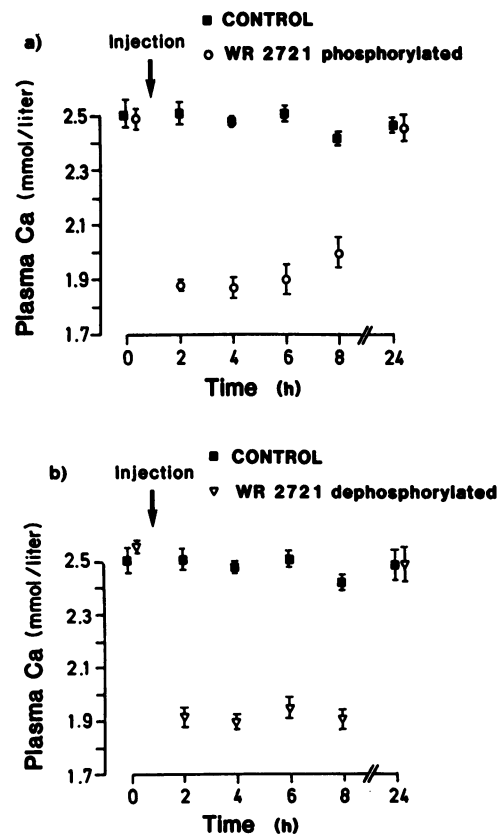


Figure 1. Influence of the phosphorylated (a) and dephosphorylated (b) form of WR-2721 on plasma Ca concentration in intact rats. The two forms of the drug were injected intravenously at the dose of 0.07 mmol/100 g of body weight. Each dot represents the mean \pm SEM of five to six rats.

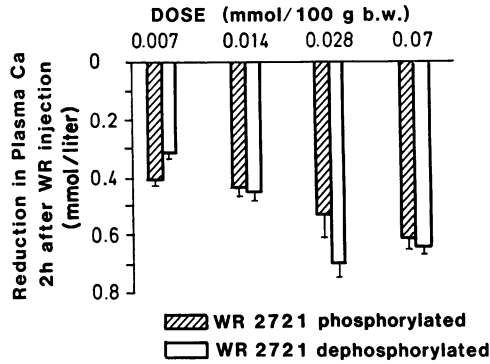


Figure 2. Comparative hypocalcemic activity of the phosphorylated (■) and dephosphorylated forms of WR-2721 (□). Plasma Ca concentration was determined 2 h after injecting the various doses. Each column represents the mean±SEM of three to four rats. Abbreviation: b.w., body weight.

IU/h) after being pretreated with the drug (0.07 mmol/100 g of body weight) or its solvent. The increase in urine cyclic AMP and P_i excretion in response to PTH was not attenuated in WR-2721-pretreated animals (Fig. 4). The PTH-induced rise in plasma Ca was not significantly different between the two groups, and the fall in urine Ca excretion was even more pronounced in the WR-2721-treated animals (Fig. 4).

Hypocalcemic effect of WR-2721 in TPTX rats. In order to investigate whether WR-2721 would exert a PTH-independent hypocalcemic effect, the phosphorylated and dephosphorylated forms of the drug were administered to TPTX rats fed a normal Ca (1.1%), normal P_i (0.8%) diet. The results of these experiments are presented in Table I and Fig. 5, where they are compared to those obtained in intact rats studied in the same experimental conditions. Note that a spontaneous fall in calcemia was observed in untreated TPTX rats in contrast to the maintenance of a steady value in intact counterparts. However, in TPTX rats treated with either form of WR-2721, the decrement in plasma Ca determined 6 h after the drug application was significantly greater than in the corresponding control group.

Nevertheless, in TPTX rats, the difference in the drop of calcemia observed at 6 h between the untreated and treated group corresponded only to ~25% of that recorded in the intact animals. It was associated with a mild rise in phosphatemia in TPTX animals injected with the phosphorylated, but not with the de-

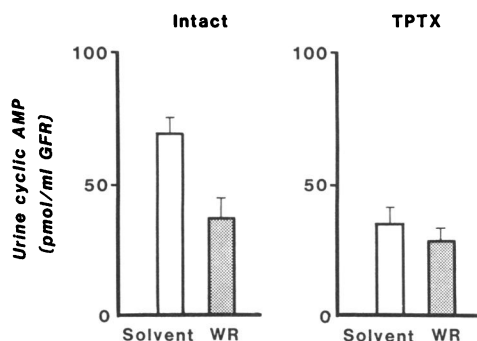


Figure 3. Urine cyclic AMP excretion in intact and chronically TPTX rats 2 h after intravenous injection of WR-2721 at the dose of 0.07 mmol/100 g of body weight. Each column represents the mean±SEM of five to six rats. $P < 0.01$.

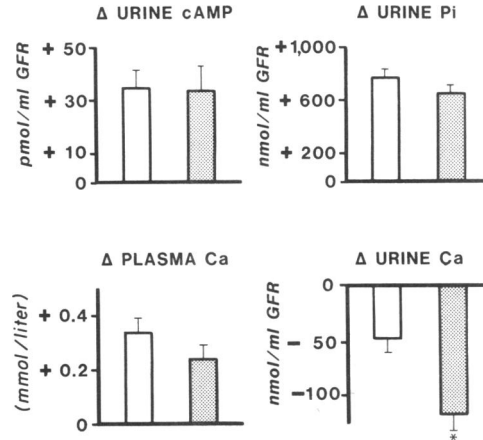


Figure 4. Acute response to bovine PTH infusion (2.5 IU/h per rat) in untreated (□) and WR-2721-treated (▨) TPTX rats. Each column represents the difference between the values recorded before and during the infusion of 2.5 IU/h bovine PTH. Values are the mean±SEM of five to six rats. The starting plasma Ca values were 1.80 ± 0.06 and 1.69 ± 0.02 mmol/liter in the untreated and WR-2721-treated rats, respectively.

phosphorylated form of WR-2721 (data not shown). Because the level of the initial calcemia could influence the magnitude of the hypocalcemic effect, WR-2721 was applied to TPTX rats made normocalcemic after feeding them with a normal Ca (1.1%) low P_i (0.2%) diet. In these initially normocalcemic TPTX rats, a spontaneous fall in plasma Ca was again recorded in the untreated group. WR-2721 did not elicit a greater hypocalcemic effect than that recorded in TPTX rats fed a normal P_i diet. In fact, in these conditions the difference in calcemia at 6 h did not reach statistical significance (Table I, Fig. 5).

Effect of WR-2721 on the renal handling of Ca. Renal clearance studies were conducted before and after the administration of WR-2721 in intact and TPTX rats. In both groups the clearance of inulin (glomerular filtration rate [GFR]) and the urinary excretion of sodium was not affected by the drug (data not shown). In contrast, a marked alteration in the renal handling of Ca was observed. This alteration can be particularly well vi-

Table I. Hypocalcemic Effect of the Phosphorylated and Dephosphorylated Forms of WR-2721 in Intact and TPTX Rats

Rats	Time after injection (h)		
	0	2	6
Intact (diet 0.8% P, 1.1% Ca)			
Control	2.52±0.03	2.48±0.03	2.51±0.03
Phosphorylated WR-2721	2.49±0.04	1.88±0.01*	1.90±0.06*
Dephosphorylated WR-2721	2.52±0.03	1.85±0.04*	1.95±0.04*
TPTX (diet 0.8% P, 1.1% Ca)			
Control	1.90±0.04	1.77±0.05	1.61±0.04
Phosphorylated WR-2721	1.84±0.08	1.65±0.07	1.42±0.05‡
Dephosphorylated WR-2721	1.89±0.04	1.69±0.04	1.45±0.04‡
TPTX (diet 0.2% P, 1.1% Ca)			
Control	2.54±0.05	2.32±0.08	1.98±0.06
Dephosphorylated WR-2721	2.63±0.06	2.32±0.08	1.92±0.10

Values are expressed in millimoles per liter and represent the mean±SEM of 10–12 rats. * $P < 0.001$. ‡ $P < 0.05$ as compared to control groups.

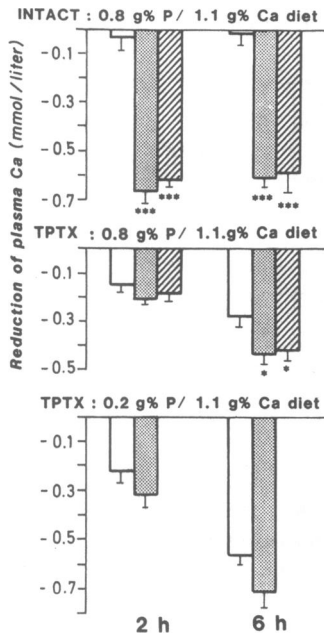


Figure 5. Hypocalcemic effect of WR-2721 in TPTX as compared to intact rats. Each column represents the changes in calcemia 2 and 6 h after application of either the phosphorylated (■) or the dephosphorylated (□) form of WR-2721, or its solvent (□). The dose applied was 0.07 mmol/100 g of body weight i.v. Each column represents the mean±SEM of five to six rats. **P* < 0.05; ****P* < 0.001.

sualized on the diagram relating urine to plasma Ca (Fig. 6). In intact rats the marked decrease in plasma Ca was accompanied by an increase in urine Ca, a change consistent with an inhibition of PTH secretion. In TPTX rats, however, WR-2721 still reduced the tubular Ca reabsorption capacity, indicating an additional PTH-independent effect on the renal Ca handling. Interestingly,

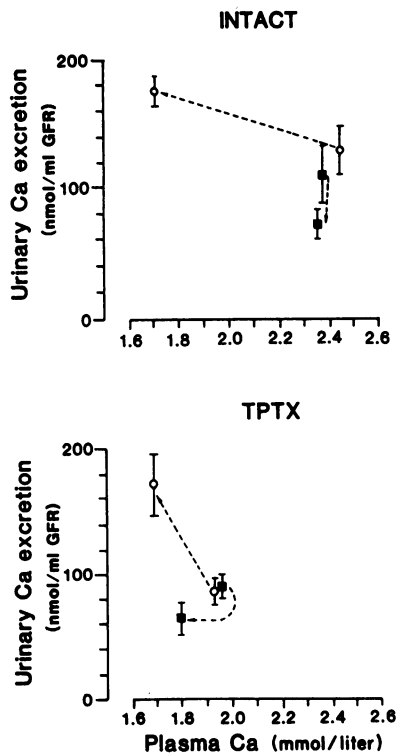


Figure 6. Influence of WR-2721 on the renal handling of Ca in intact and TPTX rats. Each diagram shows the relation between plasma concentration and urinary excretion of Ca before and 2 h after injecting 0.07 mmol/100 g of body weight of WR-2721 (○) or its solvent (■). The arrow points to the postinjection value. Each plot represents the mean±SEM of five to six rats.

plasma and urine Ca values after WR-2721 application were the same in intact and TPTX rats (intact: plasma Ca = 1.71±0.02 mmol/liter, urine Ca = 176±12 nmol/ml GFR; TPTX: plasma Ca = 1.69±0.02 mmol/liter, urine Ca = 172±25 nmol/ml GFR).

Relation between WR-2721 induced hypomagnesemia and hypocalcemia. As shown in Fig. 7, the hypocalcemic effect of WR-2721 was accompanied by a concomitant fall in the plasma Mg concentration. Prevention of hypomagnesemia by infusing magnesium chloride did not attenuate the hypocalcemic response to WR-2721.

Discussion

The availability of a pharmacological agent that has the property of inhibiting rapidly and selectively PTH secretion could contribute to the diagnosis and medical management of hyperparathyroidism. Such an inhibitor would also represent an invaluable tool in experimental and clinical investigations aimed at defining the role of PTH in various physiologic processes and disorders of Ca homeostasis. The organic phosphorothioate WR-2721 appears to be a serious candidate for filling this pharmacologic gap.

Our present experiments in rats demonstrate that the hypocalcemic effect of WR-2721 is dose-dependent, reaching a nadir after 2–6 h, with complete recovery 24 h after drug administration. The hypocalcemic response to WR-2721 can be obtained with both the phosphorylated and dephosphorylated forms which were equipotent when administered in various doses. Such a comparative study indicates first that an increase in inorganic phosphate resulting from any extra- or/and intracellular dephosphorylation of the molecule (9–11) cannot play a role in the hypocalcemic effect. Secondly, it suggests that, like for its radioprotective effect (9–11), the dephosphorylation of the molecule may be necessary for eliciting the hypocalcemic action.

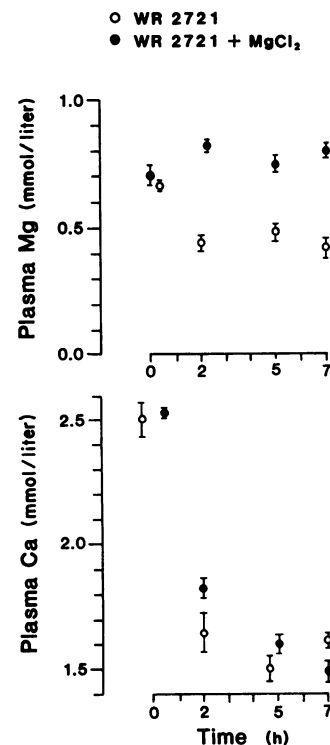


Figure 7. Relation between the hypocalcemic and hypomagnesemic effect of WR-2721. All animals were injected with WR-2721 at the dose of 0.07 mmol/100 g of body weight. They received intravenously a 0.150 M NaCl solution without and with 10 mM MgCl₂. Each plot represents the mean±SEM of five rats.

Our experiments indicate further that WR-2721 can lower calcemia and urine cyclic AMP excretion to the same extent as total parathyroidectomy. This observation, as well as the reported reduced PTH plasma levels in human beings (7) strongly suggest that the main action of WR-2721 consists in inhibiting PTH secretion. However, such an effect does not rule out other interactions with some of the various PTH-dependent and independent processes involved in extracellular Ca homeostasis.

Thus, the reduction in urine cyclic AMP excretion could reflect, at least in part, a PTH antagonistic action at the kidney level. However, our data do not sustain such an assumption. Indeed, pretreatment with WR-2721 does not attenuate the PTH-induced rise in urine cyclic AMP. Likewise, the acute tubular PTH effect of enhancing Ca and reducing P_i reabsorption is not affected by the drug. Therefore there is no reason to suspect that WR-2721 would exert an additional action at the PTH receptor or postreceptor level of the renal tubular cells. It could be argued, nonetheless, that the results observed in TPTX rats were obtained with a pharmacologic dose of PTH, and consequently, could not be extrapolated to the action of WR-2721 in intact animals. However, as indicated in the legend of Fig. 4, the administered dose of PTH increased calcemia of TPTX-untreated animals to a value still below the level recorded in intact rats (Fig. 1 a). In the same experiment, the urinary cyclic AMP excretion rose in response to PTH from 35.9 ± 5.2 to 70.8 ± 6.9 pmol/ml GFR, a value quite similar to that monitored (70.5 ± 6.3) in the intact untreated rats (Fig. 3). Finally, in a previous work (13) the same dose of PTH as that used in the present study was shown to normalize without overcorrecting the tubular Ca reabsorptive capacity of TPTX rats. Therefore, it appears unlikely that, in intact rats, WR-2721 could significantly interfere with the renal effects of PTH when secreted in normal or high amounts. Whether WR-2721 could display such an inhibitory activity when the PTH secretion rate is low cannot be excluded from the present studies.

As to a possible PTH antagonistic action at the skeletal level, our experiments in TPTX rats only show that the PTH-induced rise in plasma Ca was not significantly reduced by WR-2721 treatment. Obviously, this finding does not allow excluding some interference of the drug with the bone Ca-mobilizing action of PTH, in that it could be explained, at least in part, by the unimpaired stimulation of the tubular Ca reabsorption.

Our experiments demonstrate that both the phosphorylated and dephosphorylated forms of WR-2721 still exert a hypocalcemic influence in TPTX rats. This effect appears to be much smaller than that observed in intact rats. It is important to note that in the PTH-deprived state the plasma Ca level is unstable, decreasing as soon as the animals stop eating. Thus the fall in calcemia observed in WR-2721 treated TPTX rats appears to be quite impressive as compared to untreated intact counterparts. However, it becomes quite moderate when compared to control PTH-deprived animals (Fig. 5, Table I).

The hypocalcemic effect observed in TPTX rats could be considered as trivial if it were associated with a slight fall in calciuria. However, on the contrary, our experiments demonstrate that it occurs with a marked elevation in the urinary output of Ca. The plotting of the data on the diagram relating urine to plasma Ca allows assessment of the changes in the tubular Ca reabsorptive capacity (13). Examination of Fig. 6 strongly suggests that WR-2721 elicits a PTH-independent reduction in the tubular Ca reabsorptive capacity. Whether or not this renal effect accounts entirely for the hypocalcemic response observed in

TPTX animals cannot be ascertained from the present results. Particularly, one cannot rule out an additional PTH-independent action on bone resorption as suggested from the data presented in a recent preliminary communication (15).

Finally, our results confirm that WR-2721 induces a parallel decrement in the plasma levels of both divalent cations, Ca and Mg. Furthermore, they show that complete prevention of hypomagnesemia has no influence on the magnitude of the hypocalcemic response. Therefore, it appears very unlikely that decrease in extracellular Ca concentration is due to alteration in magnesemia. Preliminary data from our laboratory rather suggest that WR-2721 exerts a PTH-independent inhibitory activity on the tubular Mg reabsorption (16).

In conclusion, the present experimental study supports the suggestion that WR-2721 inhibits PTH secretion, and moreover, demonstrates that this drug exerts an inhibitory PTH-independent effect on the tubular reabsorption of Ca. An additional effect on bone resorption cannot be excluded. Therefore WR-2721 could be very useful in the management of clinical situations where the plasma level of PTH and Ca have to be rapidly reduced. We have recently reported on the case of a patient with parathyroid carcinoma (17). Administration of a single dose of WR-2721 led to a rapid reduction in the plasma level of PTH and Ca which was reversible within 24 h. This observation suggests that WR-2721 can also interfere with autonomous aberrant PTH secretion. Further studies should investigate whether the drug could be effective in the long-term treatment of hyperparathyroidism or hypercalcemia of other origins.

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