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DUAL EFFECTS OF A NEW HYPOCALCEMIC AGENT, WR-2721, ON CYTOPLASMIC Ca^{2+} AND PARATHYROID HORMONE RELEASE OF DISPERSED PARATHYROID CELLS FROM PATIENTS WITH HYPERPARATHYROIDISM

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Abstract—The effects of the new hypocalcemic agent, WR-2721, on calcium-regulated parathyroid hormone (PTH) release, cytoplasmic Ca^{2+} (Ca_i^{2+}) and membrane potential were measured in dispersed parathyroid cells from patients with hyperparathyrodisim (HPT). The drug had no effects in the absence of extracellular Ca^{2+} but acted synergistically with Ca^{2+} in the 0.5–1.5 mM range by depolarizing the cells, increasing Ca_i^{2+} and inhibiting PTH release. Although the depolarizing effect of 3.0 mM Ca^{2+} was unaffected by WR-2721 the drug antagonized the effect of Ca^{2+} by decreasing Ca_i^{2+} and stimulating PTH release. Whereas the inhibitory actions of WR-2721 on PTH release may result from the activation of the mechanism for Ca^{2+} gating in the parathyroid cell plasma membrane, the stimulatory effect probably reflects increased intracellular Ca^{2+} sequestration. The drug is considered potentially important for the treatment of HPT.

WR-2721 (*S*-2-(3-aminopropylaminoethyl)phosphorothioic acid) is an organic thiophosphate currently used in cancer therapy since it selectively protects normal tissue against the noxious effects of both ionizing radiation and alkylating agents [1]. Sustained hypocalcemia is a side effect of WR-2721 which has been attributed to an inhibition of parathyroid hormone (PTH) release [2]. A variety of regulators of PTH release, including Ca^{2+}, are believed to act by changing the cytoplasmic Ca^{2+} concentration (Ca_i^{2+}) but unlike most other secretory cells there is an inverse relation between Ca_i^{2+} and stimulation of the release process [3–10]. To explore the mechanism of action of WR-2721 and test its potential for treatment of hyperparathyroidism (HPT) we have measured the effects of Ca^{2+} and WR-2721 on PTH release, Ca_i^{2+} and membrane potential in dispersed parathyroid cells of patients with HPT.

MATERIALS AND METHODS

Distilled and deionized water was used. Collagenase was from Boehringer (Mannheim, F.R.G.), Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden) and Triton X-100 from Roth (Karlsruhe, F.R.G.). Dimethylsulphoxide, DNAase and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Quin2/AM (acetoxymethyl ester) and ionomycin was bought from Calbiochem-Behring (San Diego, CA) and human serum albumin from Hoechst-Behring (Marburg, F.R.G.). Bisoxonol originated from Molecular Probes (Junction City, OR), whereas WR-2721 was a kind gift from National Cancer Institute (Washington, DC). All other reagents were of analytical grade.

Cell preparation. Adenomatous and hyperplastic parathyroid glands were obtained at operations on patients with HPT. Bovine parathyroid glands were taken from adult cattle within a few minutes after slaughter. The glands were minced with scissors and cell suspensions were prepared by collagenase digestion followed by a short exposure to a calcium deficient medium containing 1 mM EGTA and filtration through nylon sieves [11]. After removal of dead cells and debris by centrifugation on a Percoll gradient, the cell viability was determined by Trypan blue exclusion. Preparations with a viability below 95% were not included. The cells were preincubated for 30–60 min at 37° in Hams' F10 medium [12] with 0.2% (w/v) bovine serum albumin and lacking bicarbonate and buffered at pH 7.4 with 20 mM Hepes.

Measurements of Ca_i^{2+}. In each experiment 5×10^6 cells were suspended in 10 ml of the F10 medium and 25 μM quin2/AM was added from a 50 mM solution in dimethylsulphoxide. After 30–40 min of gentle shaking at 37°, the cells were centrifuged (600 rpm, 5 min) and washed twice in a Hepes buffered medium (pH 7.4) containing 3 mM glucose, 1.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, 0.1% bovine serum albumin and physiologically balanced in other cations with Cl^- as the sole anion [13]. Finally, the cells were suspended in 1.3 ml of the medium containing 0.5 mM Ca^{2+}. The cell suspension was incubated with constant stirring at 37° in a 1 cm cuvette placed in a Perkin-Elmer LS 5 spectrofluorometer with excitation and emission wavelengths set at 339 and 492 nm, respectively. All additions to the cell suspensions were of concentrated stock solutions.

After each experiment the cells were lysed by addition of 10 μl Triton-X100 (10%; v/v) to obtain the maximal fluorescence at complete saturation of

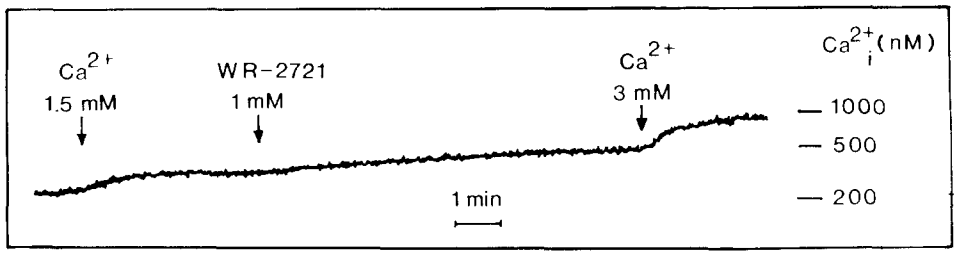

Fig. 1. Fluorescence trace (339/492 nm) from human parathyroid adenoma cells loaded with quin2. The cuvette initially contained 0.5 mM Ca^{2+}. Approximate values of Ca_i^{2+} are indicated as well as final concentrations of WR-2721 and Ca^{2+}.

quin2 (F_{max}). Estimates of Ca_i^{2+} were made essentially as described by Hesketh *et al.* [14] assuming a K_d for Ca^{2+}-quin2 of 115 nM. No correction was made for the presence of extracellular quin 2 resulting in a somewhat higher estimate of Ca_i^{2+} than previously reported [4, 8]. In separate control experiments the fraction of extracellular quin2 never exceeded 7% of F_{max} and leakage during the experiments was insignificant. In these experiments extracellular quin2 was determined by measurements of fluorescence of supernatants from experimental cell suspensions. The intracellular dye concentration was 1–2 mM assuming a cellular diameter of 10 μm. No effect of WR-2721 on the autofluorescence was found and PTH release from quin2 loaded cells were indistinguishable from controls.

Measurements of membrane potential. Membrane potential changes were assessed using the fluorescent probe bis-oxonol [15] added to a final concentration of 150 nM from a 1000-fold concentrated stock solution in dimethylsulphoxide. The fluorescence was recorded in a Perkin-Elmer LS 5 spectrofluorometer with excitation and emission wavelengths set at 540 and 580 nm respectively, in a 1 cm cuvette at 37° with constant stirring. Before the addition of 5–10 × 10^5 cells, the dye was allowed to equilibrate with the cuvette and the magnetic flea in a medium, similar to that used in the quin2 experiments but lacking

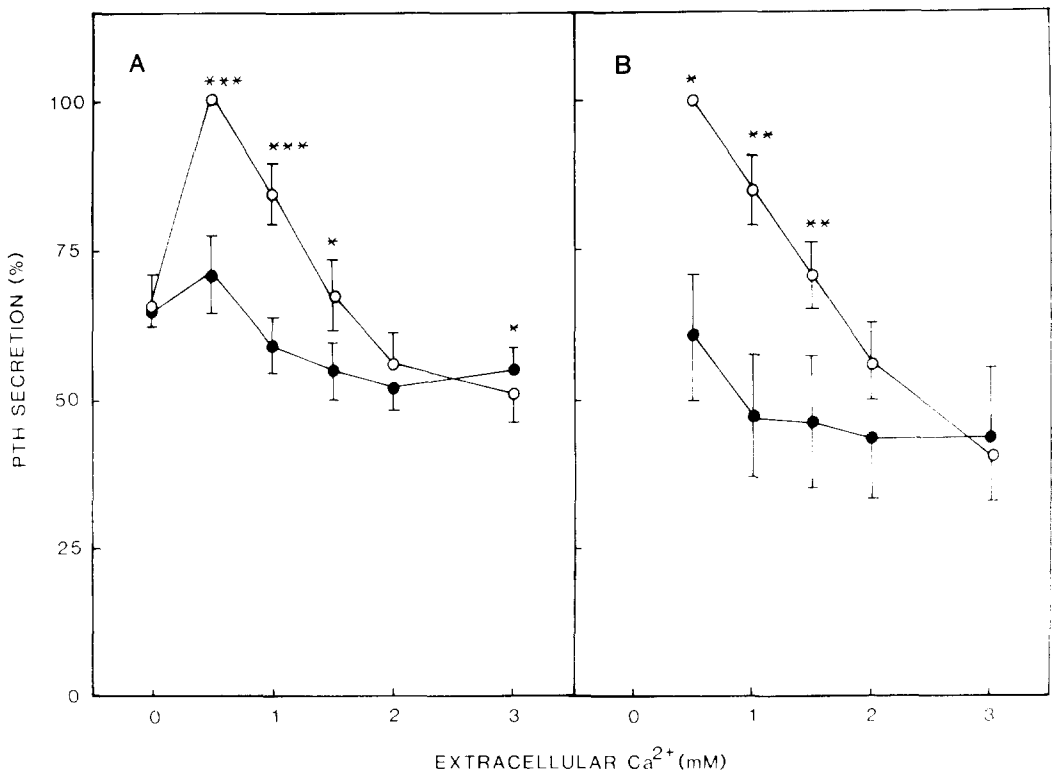

Fig. 2. Effects of Ca^{2+} on PTH release from pathological human parathyroid cells in the absence (○) and presence of 0.2 mM (●,A) or 1.0 mM (●,B) WR-2721. The results are expressed as percent of secretion at 0.5 mM external Ca^{2+} and presented as mean values ± SEM for 9 (A) and 5 (B) experiments. *$P < 0.05$; **$P < 0.025$; ***$P < 0.001$.

Table 1. Effect of Ca^{2+} and WR-2721 on cytoplasmic Ca^{2+} in pathological human parathyroid cells

Extracellular Ca^{2+} (mM)	Cytoplasmic Ca^{2+} (nM)		
	WR-2721 concentration (mM)		
	0	0.2	Difference
0	103 ± 9	100 ± 11	2 ± 2
0.5	194 ± 10	236 ± 14	−42 ± 7***
1.5	345 ± 29	432 ± 34	−87 ± 18**
3.0	588 ± 78	461 ± 48	127 ± 34*

The measurements of the cytoplasmic Ca^{2+} included 4–9 experiments.
The results are presented as mean values ±SEM.
* $P < 0.025$
** $P < 0.005$
*** $P < 0.001$.

albumin. Additions of reagents were made from concentrated stock solutions.

Measurements of PTH release. PTH release was determined by duplicate incubations of 5×10^5 cells for 2 hr at 37° in 0.5 ml of the Hepes buffered medium containing 0.1% human serum albumin and < 25 nM–3 mM Ca^{2+}. PTH was assayed radioimmunologically using a sheep antiserum (Giselle) raised against human PTH and with ^{125}I labelled 44–68 (Tyr) human PTH (Bachem Fine Chemicals, Torrence, CA) as a tracer. Bovine 1–84 PTH (Inolex) was used as standard. The assay mainly detects mid-C regional PTH [16].

Evaulation of results. The results are expressed as mean values ±SEM for the indicated number of experiments. Statistical significances were calculated from paired test and control data using Students' *t*-test.

RESULTS

A typical fluorescence trace from quin2 loaded human parathyroid cells is shown in Fig. 1. The addition of 0.2 mM WR-2721 to cells incubated at 1.5 mM Ca^{2+} clearly increased the quin2 fluorescence. The effects of Ca^{2+} and WR-2721 on Ca_i^{2+} and PTH release in pathological human parathyroid cells are summarized in Table 1 and Fig. 2.

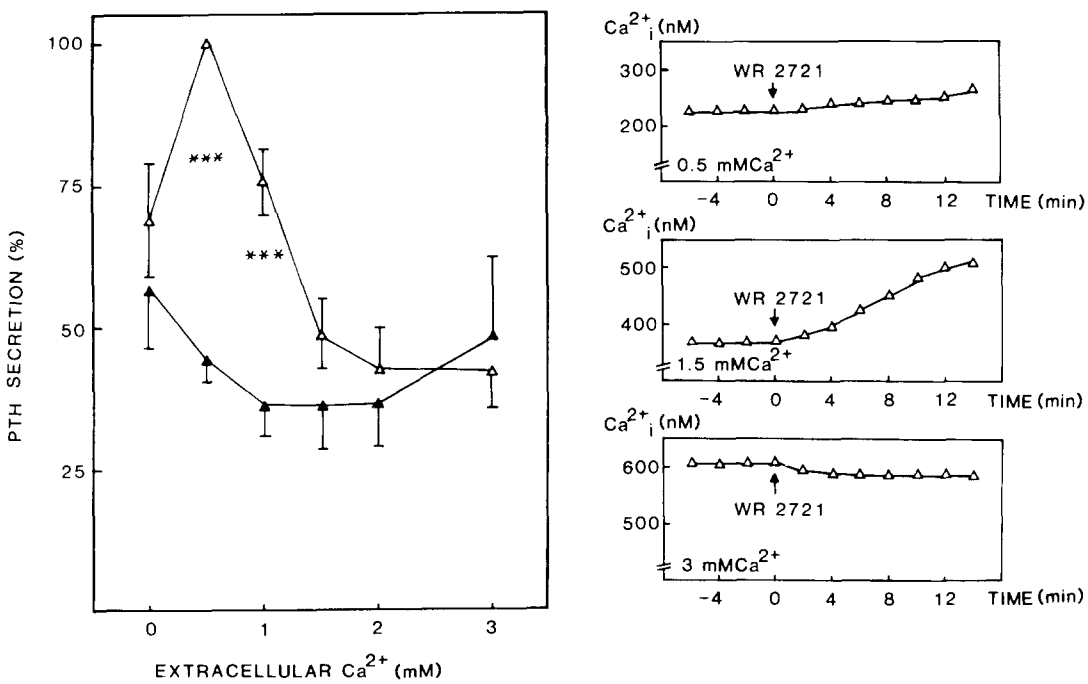

Fig. 3. Effects of Ca^{2+} on PTH release from dispersed normal bovine parathyroid cells in the absence (△) and presence (▲) of 0.2 mM WR-2721 (left panel) and on Ca_i^{2+} (right panels). PTH release is expressed as per cent of secretion at 0.5 mM extracellular Ca^{2+} and presented as mean values ± SEM for 4 experiments. Typical experiments are shown for the action of WR-2721 on Ca_i^{2+} at 3 different extracellular Ca^{2+} concentrations. Approximate values of Ca_i^{2+} as well as final concentrations of WR-2721 are indicated. ***$P < 0.001$.

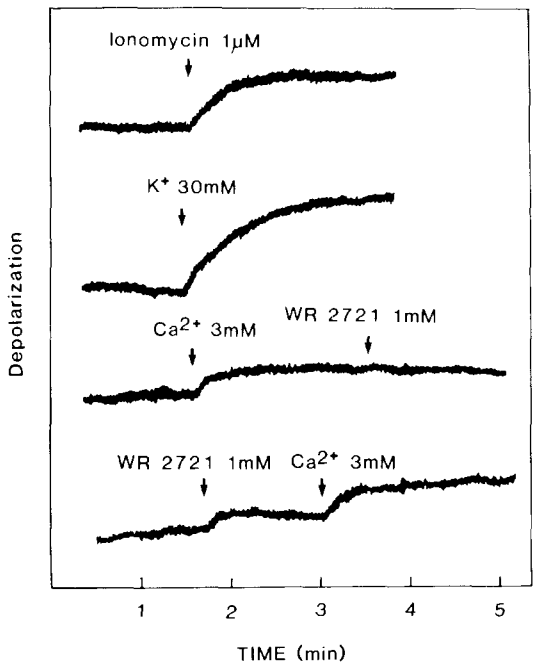

Fig. 4. Effects of ionomycin, K^+, Ca^{2+} and WR-2721 on membrane potential measured by the fluorescent dye bis-oxonol (540/580 nm). Upward deflection indicates depolarization. One of 3 similar experiments is shown.

Ca_i^{2+} increased with extracellular Ca^{2+} in the < 25 nM–3.0 mM range. PTH release increased between < 25 nM and 0.5 mM Ca^{2+} but was gradually inhibited by higher Ca^{2+} concentrations. WR-2721 had no effects in Ca^{2+} deficient media, increased Ca_i^{2+} and inhibited PTH release at 0.5–1.5 mM of the ion but had the opposite effects after raising the ambient Ca^{2+} concentration to 3.0 mM. Increasing the WR-2721 concentration from 0.2 to 1 mM had no marked additional effects on the secretory pattern (Fig. 2B). Largely the same effects of WR-2721 on PTH release and Ca_i^{2+} were obtained using normal bovine parathyroid cells (Fig. 3). When the membrane potential of the pathological human parathyroid cells was monitored with the fluorescent dye bis-oxonol the depolarizing effects of K^+ and ionomycin were readily demonstrated as well as that obtained when increasing the external Ca^{2+} concentration from 0.5 to 3.0 mM (Fig. 4). Also WR-2721 depolarized the cells at 0.5 mM external Ca^{2+} but had no effect at 3.0 mM Ca^{2+}.

DISCUSSION

Calcium inhibition of PTH release is associated with both depolarization of the parathyroid cell [17, 18] and a dose related increase in Ca_i^{2+} [3, 4]. The increase in Ca_i^{2+} probably results from the opening of Ca^{2+} channels which are insensitive to membrane potential [19]. It was therefore argued that depolarization is a consequence rather than cause of Ca^{2+} entry and the Ca^{2+} influx occurs through channels activated by Ca^{2+} itself. A malfunction of this unique messenger system has recently been reported for cells from patients with HPT. In these cells, a given extracellular Ca^{2+} concentration translates into an abnormally low Ca_i^{2+}, a phenomenon which may well explain the aberrant secretory behaviour of the disorder [4, 10]. A correction of Ca_i^{2+} consequently leads to normalization of the secretory pattern as demonstrated when exposing human pathological parathyroid cells to the Ca^{2+} ionophore A-23187 [9] or to D-600 [8], which blocks voltage dependent Ca^{2+} channels in other cells.

In the present study we provide evidence for different effects by WR-2721 on PTH release depending on the extracellular Ca^{2+} concentration with no action in the absence of Ca^{2+}. Considering the data on Ca_i^{2+} and the membrane potential WR-2721 may depolarize the cells by increasing the Ca^{2+} permeability of the plasma membrane at low and intermediate concentrations of Ca^{2+} with a subsequent inhibition of PTH release. At higher Ca^{2+} concentrations the drug lowers Ca_i^{2+} and consequently stimulates PTH secretion. However, there was not a complete quantitative correspondence between the effects of WR-2721 on Ca_i^{2+} and PTH release. This discrepancy may be attributed to the great difference in time of exposure to the drug. Indeed, when Ca_i^{2+} and PTH release were determined after 2 hr exposure to WR-2721, secretion was inhibited by 68% and Ca_i^{2+} increased by 66% when expressed in relation to their respective ranges of variation (not shown). These actions of WR-2721 on Ca_i^{2+} and PTH release are almost identical to those of the voltage-dependent Ca^{2+} channel blocker D-600 [8]. It was suggested that D-600 blocks the Ca^{2+} activated Ca^{2+} channels of the parathyroid cell at high concentrations of extracellular Ca^{2+} but instead has an activating effect at lower concentrations. The fact that WR-2721 seems to operate similarly might give clues to the molecular mechanism involved in this Ca^{2+} gating. WR-2721 is a sulphydryl agent which probably undergoes intracellular cleavage producing both a free phosphate ion and a free thiol [2]. Phosphate stimulates Ca^{2+} sequestration in different cells and should be expected to lower Ca_i^{2+} and stimulate PTH secretion. The thiol may have the opposite effect since reducing equivalents in the form of pyridine nucleotides and glutathione have been proposed to mediate the coupling between glucose stimulation and the ionic events leading to an increased Ca_i^{2+} in pancreatic β-cells [20–23]. Indeed, the observation by Morrissey and Klahr [24] that exposure of parathyroid cells to Ca^{2+} leads to an increased metabolic flux through the hexose monophosphate shunt is consistent with the concept that reducing equivalents are involved also in the physiological opening of the Ca^{2+} activated Ca^{2+} channels of the parathyroid cell. It is therefore tempting to speculate that WR-2721 increases Ca_i^{2+} and inhibits PTH secretion at low concentrations of extracellular Ca^{2+} by opening the Ca^{2+} activated Ca^{2+} channels. However, at higher concentrations of Ca^{2+} the channels are already opened and secretion inhibited. The production of intracellular phosphate after exposure to WR-2721 will be the dominating effect leading to organelle sequestration of Ca^{2+}, a decrease in Ca_i^{2+} and stimulation of PTH release. Since WR-2721 accumulates preferentially in non-malignant tissue [25], an effect on adenomatous and hyperplastic parathyroid glands

is not self-evident. The present demonstration that WR-2721 produces marked left shifts in the dose–response relationships for Ca^{2+} regulated PTH release and Ca_i^{2+} rejects all doubts on this point and has interesting clinical implications. WR-2721 is remarkably free from toxic side effects [26] and may therefore become a useful tool in the management of patients with hyperparathyroidism, especially in those with recurring disease and where surgical treatment is contraindicated.

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