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GENOME-INDEPENDENT EFFECTS OF 1,25-DIHYDROXY VITAMIN D-3 ON MEMBRANE POTENTIAL

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Cell membrane potential, V_m, was monitored in rabbit hypertrophic cartilage metatarsals, amphibian proximal tubule and muscle cells during application of 1,25-dihydroxy vitamin D-3, 25-hydroxy vitamin D-3 or cholesterol (10^{-10} M). 1,25-Dihydroxy vitamin D-3 elicited quick variations of V_m (in less than 1 min) in proximal tubular cells (whether injected in the lumen or in peritubular capillaries) and in cartilage. The precursor 25-hydroxy vitamin D-3 and cholesterol produced a small shift of V_m in proximal tubule only when applied from the luminal side, but this change was significantly smaller than that observed with 1,25-dihydroxy vitamin D-3. Muscle cells were unresponsive to both metabolites and cholesterol. It is concluded that rapid effects of 1,25-dihydroxy vitamin D-3 on V_m, in target cells, are specific, most likely due to permeability changes and not related to nuclear protein synthesis; they may contribute to early modulation of cell function.

It is widely recognized that steroid hormones act on target cells by a mechanism involving several steps: binding of the hormone to a cytosolic receptor, translocation of the hormone-receptor complex to the nucleus, and finally induction of protein synthesis. This is a slow process requiring at least one hour [1]. 1,25-Dihydroxy vitamin D-3 ($1,25(OH)_2D_3$), considered as the hormonal active form of vitamin D, stimulates the synthesis of a specific calcium binding protein in the intestine [2] via a similar genome mechanism [3,4]. It increases in this target tissue the phosphatidylcholine content of brush-border membrane vesicles [5], but it enhances also the net transport (absorption) of calcium, even before the occurrence of detectable increase in intracellular calcium-binding protein concentration [6]. This last observation raises the possibility of a rapid effect of this vitamin D metabolite on ion transport across cell membranes, in addition to its well established nuclear action [7].

The detection of instantaneous effects of a test substance on cell membrane properties requires the use of techniques liable to monitor rapid changes of some intrinsic membrane parameter(s). As one criterion for possible effects of $1,25(OH)_2D_3$ on cell membrane, we recorded the changes of the cell potential, V_m.

Three different cell preparations were chosen to perform the present study, i.e. kidney, cartilage and muscle. Kidney and cartilage are likely target tissues for $1,25(OH)_2D_3$ action. Muscle however is not considered as yet a classical target tissue for this metabolite [3]. Renal cells were studied in the proximal tubule of *Necturus* kidney, in vivo, by standard micropuncture techniques described elsewhere [8]. Metatarsal cartilage from 18-day-old New-Zealand rabbit and semitendinous muscle from *Rana pipiens* frog were studied in an in vitro perfusion chamber.

In the perfusion chamber, the stability of cartilage and muscle in vitro preparations, was achieved by embedding the tissue in a physiologic solution containing 2% agar. A surface window of about

20–30 mm^2 allowed the tissue to be continuously superfused with a physiologic, agar-free, Ringer solution. The perfusate could be shifted to a similar solution supplemented with the appropriate test substance. Perfusion fluids were removed by a vacuum pump. Washout of 90% of the surface

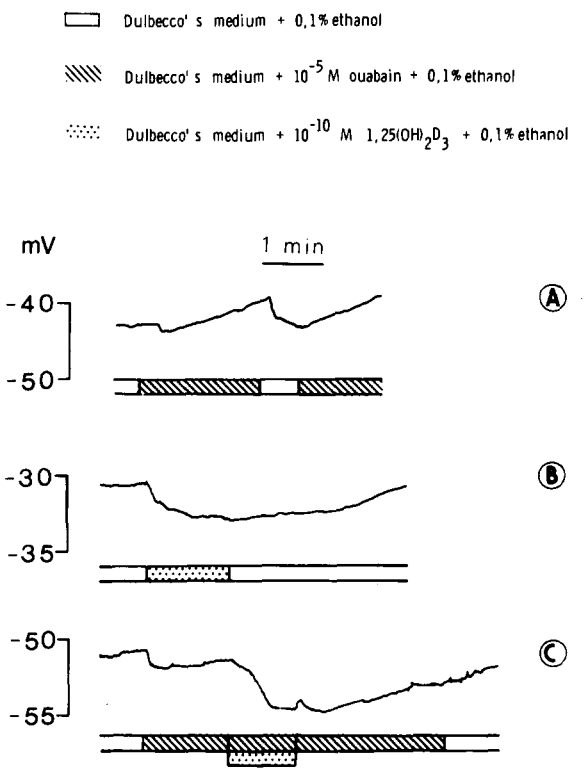

Fig. 1. Effects of ouabain and 1,25(OH)$_2$D$_3$ on cell membrane potential in rabbit chondrocytes. Membrane potential (mV) is plotted in ordinate as a function of time in abscissa (tracings redrawn). The composition of perfusion fluid is indicated below each tracing: the vehicle Dulbecco's medium containing 0.1% ethanol (hatched bars) throughout each experiment; addition of ouabain and/or 1,25(OH)$_2$D$_3$ to this medium is indicated by hatched and stippled bars, respectively. (A) Addition of ouabain to a Dulbecco's medium perfusate results in a delayed and transient hyperpolarization followed by a slow decline of V_m. (B) Addition of 1,25(OH)$_2$D$_3$ alone brings about an immediate hyperpolarization which is slowly reversible after the metabolite is removed from the perfusate. (C) Combined effects of ouabain and 1,25(OH)$_2$D$_3$. The first application of ouabain elicits a transient hyperpolarization followed by a slow decline of V_m. Addition of 1,25(OH)$_2$D$_3$ to the ouabain-containing perfusate results in hyperpolarization, as was the case with ouabain-free solutions. Subsequent removal of the metabolite unmasks the depolarizing effect of the drug which is not reversed after recovery to a Dulbecco's medium perfusate.

covering fluid was achieved within 30 s. The semitendinous muscle was placed in the perfusion chamber at room temperature and superfused with a Ringer's solution for amphibia supplemented with 0.1% ethanol [8]. Metatarsal fragments were inserted in the perfusion chamber at 37°C, their distal end being on top, directly in contact with the surface fluid (Dulbecco's medium also supplemented with 0.1% ethanol [9]). Preliminary experiments had shown that stable microelectrode impalements in the hypertrophic cartilage cells could be achieved only with sufficient hydration of the cartilage matrix. Such hydration was obtained by pre-incubation of the metatarsal fragments in Dulbecco's medium for 48 h at 37°C [9].

For all three tissues conventional microelectrodes were used to continuously record the intracellular potential of a superficial cell, before and after application of a test substance. Test substances were 1,25(OH)$_2$D$_3$, its precursor 25(OH)D$_3$, or cholesterol (all at 10^{-10} M).

Cell membrane potential of cartilage hypertrophic cells has not been determined in the past. The criterion for adequate impalements was the obtention of a stable potential within ± 1 mV for at least two minutes. The average V_m was -35.7 mV $\pm$ 0.8 S.E. ($n = 70$). The viability of the preparation was tested independently by adding 10^{-5}M ouabain to the perfusate. Ouabain addition elicited 7.3 $\pm$ 0.8 mV depolarization ($n = 10$), which started about 1 min after administration of the drug (Fig. 1A). The progressive development of the depolarization is probably related to the slow binding rate of ouabain to cell membranes [10]. Since ouabain affected V_m within 1 min after application, we assumed that changes of V_m produced by other substances before that period of time are also due to a direct effect on the membrane. The addition of 1,25(OH)$_2$D$_3$ in the surface fluid brought about 2.2 $\pm$ 0.3 mV hyperpolarization ($n = 34$) which was complete within less than 2 min (Fig. 1B). The effect was partly reversible and saturable, since the 3rd or 4th exposure of the tissue to the metabolite failed to affect V_m. Two min pre-treatment of the cartilage with 10^{-5}M ouabain did not alter the hyperpolarizing response of the tissue to subsequent addition of 1,25(OH)$_2$D$_3$ (Fig. 1C). It is inferred that the vitamin D metabolite effects on V_m are mediated

TABLE I

		V_m	$\Delta V_{1,25(OH)_2D_3}$ [a,b]	$\Delta V_{25(OH)D_3}$ [a]	$\Delta V_{Cholesterol}$ [c]
Cartilage		-35.7 ± 0.8 [d] $n = 70$	-2.2 ± 0.3 **,†† $n = 34$	$+0.4 \pm 0.2$ $n = 12$	$+0.2 \pm 0.3$ $n = 8$
Proximal tubule	Peritubular	-78.5 ± 1.7 $n = 14$	-3.2 ± 0.2 *,† $n = 5$	$+1.0 \pm 1.1$ $n = 5$	$+0.8 \pm 0.9$ $n = 4$
	Luminal	-70.1 ± 1.8 $n = 20$	$+6.8 \pm 1.2$*,† $n = 8$	$+2.9 \pm 0.3$ $n = 6$	$+1.9 \pm 0.7$ $n = 6$
Muscle fiber		-79.0 ± 1.6 $n = 18$	$+0.1 \pm 0.3$ $n = 9$	$+0.5 \pm 0.6$ $n = 5$	$+0.2 \pm 0.8$ $n = 4$

[a] $1,25(OH)_2D_3$ or $25(OH)D_3$ was added to the protein-free perfusate in ethanol (at a final concentration of 0.1%); the steroid-free perfusate (Dulbecco's medium or Ringer's for amphibia) also contained 0.1% ethanol. The $1,25(OH)_2D_3$ concentration, as assayed by the technique of Shepard et al. [13], was $2.5 \cdot 10^{-10}$M in the perfusion chamber. The $25(OH)D_3$ concentration determined by the method of Preece et al. [14] was $2 \cdot 10^{-10}$M.

[b] Hyperpolarization elicited by $1,25(OH)_2D_3$ is indicated by a negative sign, depolarization by a positive sign.

[c] Cholesterol was added to the protein-free perfusate (final concentration: 10^{-10}M, ethanol 0.1%).

[d] The results are expressed as mean $\pm$ S.E.

Statistical comparison between $\Delta V_{1,25(OH)_2D_3}$ and $\Delta V_{25(OH)D_3}$ for each preparation: ** = $P < 0.001$; * = $P < 0.01$; otherwise non-significant.

Comparison between $\Delta V_{1,25(OH)_2D_3}$ and $\Delta V_{Cholesterol}$: †† = $P < 0.001$; † = $P < 0.01$; otherwise non-significant.

via changes of cell membrane permeability(ies), not through interference with the Na^+,K^+ pump. Neither $25(OH)D_3$ nor cholesterol did produce changes of V_m (Table I).

In the proximal tubule of *Necturus* kidney, the effects of $1,25(OH)_2D_3$ were different depending on whether the metabolite was applied from the peritubular or the luminal side. Yet, in both cases, the response was completed in less than 1 min, suggesting again that it corresponded to a direct and rapid effect on the membrane. Peritubular addition of $1,25(OH)_2D_3$ resulted in 3.2 ± 0.2 mV hyperpolarization ($n = 4$), as in the case of hypertrophic cartilage. By contrast, the introduction of the metabolite in the lumen of the tubule brought about 6.8 ± 1.2 mV depolarization ($n = 8$). In this series of experiments the study was completed by the measurement of the transmembrane input resistance [11]. Briefly, two microelectrodes were inserted in the cell layer of single tubules; one of them was used to inject current (as rectangular pulses, 25 nA, 0.2 Hz), the second for recording V_m; their distance was kept constant. The addition of $1,25(OH)_2D_3$ in the luminal fluid resulted in 14% increase of the electrotonic potential (Fig. 2). Since junctional membrane resistance in epithelial cells, including renal tubules, is believed to be negligible [12], an increase of the electrotonic potential reflects a decrease of cell membrane conductance. The association of a decreased mem-

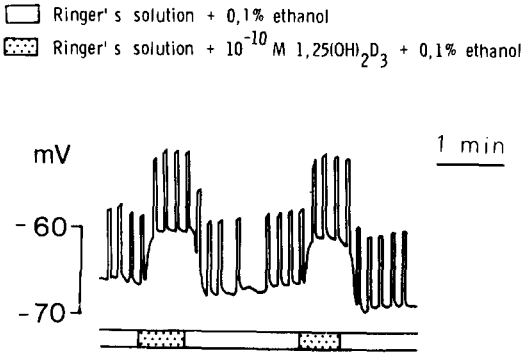

Fig. 2. Voltage recording from a microelectrode inserted in the cellular layer of a proximal tubule. Another microelectrode inserted in the same tubule was used to deliver rectangular current pulses. Interelectrode distance was 400 μm. The deflexions superimposed on V_m allow an estimate of transmembrane input resistance. White and stippled bars as in Fig. 1. Addition of $1,25(OH)_2D_3$ in peritubular fluid produced 6 mV depolarization and a detectable increase of input resistance. Both changes appeared rapidly and were quickly reversible.

brane conductance with V_m depolarization is consistent with a decrease of the partial conductance to potassium. Although, the difference in responses to $1,25(OH)_2D_3$ (peritubular vs. luminal application), is poorly understood, it reflects a specific action of the metabolite. Indeed, both the analog $25(OH)D_3$ and cholesterol were ineffective from the peritubular side; they produced a small depolarization when injected in the lumen, but this depolarization was significantly smaller than that produced by $1,25(OH)_2D_3$.

Muscle cells failed to respond to either cholesterol, $25(OH)D_3$ or $1,25(OH)_2D_3$ (Table I).

The main conclusion of this study is that one steroid hormone, $1,25(OH)_2D_3$ triggers detectable changes of cell membrane properties by a totally unconventional process. It acts very rapidly on the membrane by a direct mechanism which occurs prior to any de novo nuclear protein synthesis. Importantly, such effects are restricted to likely 'target cells' of $1,25(OH)_2D_3$ action (cartilage and kidney), muscle cells being totally unresponsive. Our data suggest in addition that $1,25(OH)_2D_3$ alters V_m via changes of cell membrane permeabilities. However, the dual effect of $1,25(OH)_2D_3$ on the luminal and the basolateral membrane of the proximal tubule presumably indicates that the metabolite may affect different permeabilities at different cell membranes. Clearly, a new field is open for investigation regarding the actions of vitamin D metabolites on cell physiology, and possibly, on similar effects of other steroid hormones.

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