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Mechanisms of $1,25(OH)_2D_3$-Induced Rapid Changes of Membrane Potential in Proximal Tubule: Role of Ca^{2+}-Dependent K^+ Channels

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Summary. Eleven different secosteroids or steroids (10^{-10} to 10^{-8} M) were acutely and reversibly introduced in solutions delivered to the lumen of single proximal tubules of the amphibian *Necturus* kidney while recording basolateral cell membrane potential V_m. Seven of these molecules ($1,25(OH)_2D_3$, $25(OH)D_3$, $24,25(OH)_2D_3$, 5,6-trans-$25(OH)D_3$, 19-diol-cholesterol, estradiol and testosterone) resulted in changes of V_m (ΔV_m) occurring in a few seconds, the largest ΔV_m being observed with $1,25(OH)_2D_3$, $+6.5 \pm 0.75$ mV ($n = 19$); these seven (seco)steroids, but not the four inactive sterols (vitamin D_3, cholesterol, $1\alpha D_3$ and aldosterone) possess a hydroxyl group on at least one carbon of the C_{17} to C_{25} lateral chain of the sterol ring. The ΔV_m effect was present in Na^+-free or Cl^--free media, but it was abolished in HCO_3-free media. Depolarization of cell membrane potential by addition of glucose, 11 mM, in luminal perfusion fluid abolished the $1,25(OH)_2D_3$-evoked ΔV_m effect, suggesting dependence of the latter on the absolute value of membrane potential. Barium, a blocking agent of K^+ conductances, suppressed the $1,25(OH)_2D_3$-evoked ΔV_m effect, even when the proper effects of barium of cell membrane potential were canceled by current clamp. Pretreatment with quinine, a putative blocker of Ca^{2+}-dependent K^+ channels also abolished the $1,25(OH)_2D_3$-evoked depolarization. Such observations are consistent with the presence of Ca^{2+}-dependent K^+ channels at the apical cell membrane of the proximal tubule, these channels being inactivated by $1,25(OH)_2D_3$ and probably by other (seco)steroids.

Key Words steroids · conductances · amphibian · proximal tubule · membrane potential · $1,25(OH)_2D_3$

Introduction

Steroid hormones traditionally act on target cells by binding with specific receptors in the nucleus, thereby inducing protein synthesis. This process requires in general more than 1 hr. More recently, it has been shown that some sterols such as estradiol [9] exhibit early effects detectable within a few minutes or less (for review, *see* Ref. 10), in addition to their usual mode of action. We have also reported that vitamin D metabolites, $1,25(OH)_2D_3$ and $25(OH)D_3$ (but not cholesterol), alter cell membrane potential of presumptive target cells, e.g. epiphyseal cartilage and renal proximal tubule cells but not myocytes, with a time course of the order of seconds [14, 15]. Since these early responses to steroids clearly cannot be ascribed to *de novo* protein synthesis, they most likely reflect direct effect(s) on the cell membrane, e.g. changes of its ionic conductances [14]. This effect is consistent with the view that vitamin D metabolites, by virtue of their cholesterol-like configuration, are rapidly incorporated into the membrane leaflet, thus altering its physical properties [27]. Since vitamin D metabolite-induced (rapid) changes of cell membrane potential may underlie some yet undefined physiologic action, the present work was undertaken to further characterize the ionic mechanisms associated with these changes. The experiments were performed in the proximal tubule of the amphibian *Necturus maculosus*, a preparation extensively investigated in this laboratory [1–4, 11–14] and shown to be sensitive to vitamin D metabolites [14]. A large number of steroids and secosteroids[1] was tested in the present study to determine which molecules are effective in changing cell membrane potential and to attempt structure/function correlations. In addition we examined in more detail the ionic dependence of the $1,25(OH)_2D_3$-evoked depolarization in proximal tubular cells and the effects of some inhibitors. The results indicate that $1,25(OH)_2D_3$ acts by decreasing a barium-sensitive, Ca^{2+}-dependent K^+-conductive pathway.

Materials and Methods

All experiments were performed in the proximal tubule of *Necturus* kidney *in vivo*. The methods for anesthesia, dissection of the animals and preparation of the kidney have been described elsewhere [1, 2, 11–14].

[1] Secosteroids are steroids in which the ring B is opened; they are mostly made up by vitamin D and its metabolites.

Table. Composition of perfusing solutions

	Control (mM)	Glucose (mM)	Na-free (mM)	HCO_3-free (mM)	Low-Cl (mM)	Low-Ca (mM)	Barium (mM)
Na	95.0	95.0		95.0	95.0	95.0	95.0
K	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mg	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ca	1.8	1.8	1.8	1.8	1.8	0.6	1.8
Cl	89.6	89.6	89.6	102.6	8.6	87.2	93.2
HCO_3	13.0	13.0	13.0		13.0	13.0	13.0
Hepes	5.0	5.0	5.0	5.0	5.0	5.0	5.0
TMA			82.0				
Gluconate					93.2		
Choline			13.0				
Glucose		11.0					
Barium							1.0
Ethanol	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%

pH was adjusted with NaOH to 7.4.

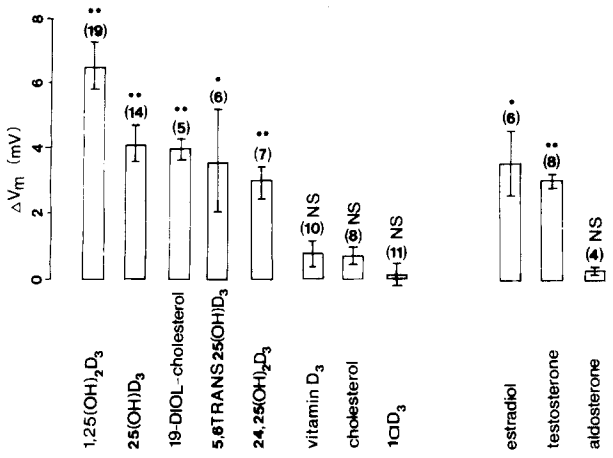

Fig. 1. Rapid effects of 11 test molecules on V_m (concentrations are indicated in Materials and Methods). Average changes of V_m ± SEM. The number of experiments is given in parentheses. ΔV_m statistically different from zero at the 0.05 and 0.01 level is indicated by * and **, respectively, NS = not significant

Luminal Microperfusions

A double-barreled micropipette (tip diameter 10 to 15 μm) was inserted in the lumen of a tubule. One channel was filled with a physiologic Ringer's solution (Table 1, control) and the other with a similar Ringer's solution supplemented with a secosteroid or a steroid (one of the molecules appearing in Fig. 1). All perfusates were collected downstream by means of a single micropipette. Rapid changes from control to test perfusing solution and vice versa were achieved by two independent gravimetric systems, each of them connected to one channel of the micropipette [12–14]. In a recent study, several pitfalls of the double-microperfusion technique have been emphasized in rat kidney [6]. The most important methodological aspect of that study was the necessity to ensure high perfusion rates and prevent retrograde contamination of the nonperfusing barrel. However, contrary to rat kidney [6], a large opening of the tubular wall in *Necturus* does not suffice to evacuate the perfusates downstream, because it is spontaneously resealed by the abundant surrounding connective tissue. Continuous collection was necessary to avoid tubular distension [12–14]. Using perfusing pipettes having a tip diameter of 10 to 15 μm and applying high perfusion rates (~ 1 μl/min), we empirically defined the minimal tip diameter of the collecting pipette ensuring nondistension of the tubule as 40 μm. Under these conditions, no retrograde contamination of the second barrel was noticed in preliminary tests in which a colored (methylene blue 1%) versus a plain Ringer's solution were alternatively delivered in the lumen of single tubules.

In a number of experiments it was necessary to assess the effects of a (seco)steroid on V_m when added in either a physiologic solution (A) or in a solution (B) of different composition. For such experiments, two double-barreled micropipettes were introduced into the lumen of single tubules (in addition to the collection pipette). They were filled with (i) solution A, (ii) solution B, (iii) solution A plus a (seco)steroid and (iv) solution B plus the same (seco)steroid. All solutions contained ethanol, 0.1%, the usual solvent of (seco)steroids. The composition of all perfusates is listed in Table 1.

Electrical Measurements

A conventional Ling-Gerard microelectrode filled with KCl, 1 M, was inserted into the cell of a tubule, previously impaled with one (two) double-barreled (perfusing) plus a single (collection) micropipette and luminally perfused with the control Ringer's solution; once a stable V_m was achieved the perfusion fluid was shifted to a test solution. The tip resistance of our microelectrodes was 25 to 40 MΩ. The reference 1 M KCl agar electrode and the measuring microelectrode were connected to ground and input of a WPI electrometer (Model 750, New Haven, Conn.). V_m and ΔV_m refer to cell membrane potential and to its rapid and reversible change during exposure to a (seco)steroid, respectively.

Steroids

Each of the solutions listed in Table 1 was used as such or supplemented with a (seco)steroid. Two groups of sterols may be distinguished. The first is made up of the molecules containing the lateral chain of vitamin D; in addition to the metabolites $1,25(OH)_2D_3$, $25(OH)D_3$, $24,25(OH)_2D_3$, vitamin D_3, 19-diol-cholesterol and cholesterol, the synthetic compounds $1\alpha D_3$ and 5,6-trans-$25(OH)D_3$ were also used. The second group comprises two sex steroids (estradiol and testosterone) and one mineralocorticoid (aldosterone).

Vitamin D_3 and its derivatives have the disadvantage of adhering to glassware and plastic pipes. To assess the importance of vitamin D metabolite losses, their respective concentrations were measured at the outlet of the micropipette, *in vitro*, by using Shepard's [24] or Preece's [23] techniques. We found that $1,25(OH)_2D_3$ concentration at the tip of the micropipette was in the 10^{-10} M range, irrespective of its nominal concentration in bulk solution (10^{-9} to 10^{-7} M). Using $24,25(OH)_2D_3$, $25(OH)D_3$ and vitamin D_3, the concentration at the delivery site was in the 10^{-9} M range. Concentrations of $1\alpha D_3$, 5-6-trans-$25(OH)D_3$, 19-diol-cholesterol and cholesterol have not been assayed at the delivery site. The 5,6-trans-$25(OH)D_3$ concentration in bulk solution was 10^{-9} M. Concentrations of $1\alpha D_3$, cholesterol and 19 diol-cholesterol in bulk solution, were 10^{-10} to 10^{-8} M, but these compounds were ineffective, irrespective of nominal concentration. Testosterone yielded a peak effect at 10^{-8} M. Estradiol was fully active at 10^{-10} M, but no increment was observed at 10^{-8} M. Nominal concentration of aldosterone was 10^{-7} M. In short, our attempts to establish dose-response curves were inconclusive.

The results are expressed as mean ± SE. The unpaired *t*-test was used for comparison of two sets of data or to evaluate the significance of a change of membrane potential.

Results

Effects of Various Test Molecules on V_m

Luminal microperfusion experiments were carried out to assess the effects of several secosteroids and steroids on V_m. Luminal microperfusions lasted 5 to 20 min, the test molecule being intermittently added to the perfusate (20 to 100 sec), then removed. The results are schematically depicted in Fig. 1. Seven molecules, including two sex steroids resulted in V_m depolarization (they will be referred to hereafter as active molecules), the most potent being $1,25(OH)_2D_3$. Four other molecules, $1\alpha D_3$, vitamin D_3, cholesterol and aldosterone did not alter V_m. In some experiments, the first or two first applications of an active compound were ineffective, suggesting that these tubules became progressively responsive. This transition was more carefully analyzed with two compounds, $1,25(OH)_2D_3$ and $25(OH)D_3$. The initial application of $1,25(OH)_2D_3$ failed to alter V_m in 10 tubules in which cell membrane potential was on the average -65.8 ± 2.0 mV; depolarization was obtained in 19 tubules (including the 10 initially non-responsive) displaying an average V_m of -73.8 ± 2.1 mV, the difference between these two values being statistically significant ($P < 0.02$). The corresponding V_m values when $25(OH)D_3$ was used instead of $1,25(OH)_2D_3$ were -56.9 ± 1.3 mV ($n = 7$) and -71.8 ± 1.9 mV ($n = 14$), including the seven initial ones) again statistically different from one another at the 0.001 level. The partial depolarization of some tubules at the beginning of perfusion suggests that perhaps their initial potassium permeability was lower than that of the responding cells. For the sake of homogeneity, the lower initial V_m's associated with lack of response to the test substances have not been included in the statistical analysis.

In other experiments in which repetitive application of the test-substance was pursued beyond 20 min, the ΔV_m response reversed from de- to hyperpolarization, suggesting sudden appearance of a different membrane process. This delayed effect was not investigated further.

Ionic Dependence of the $1,25(OH)_2D_3$-Evoked Acute V_m Change

To get some insight on the ionic mechanisms involved in the V_m depolarization, $1,25(OH)_2D_3$, 10^{-10} M, was added in solutions of altered ionic composition. Using Na-free solutions (choline or tetramethylammonium were the substitutes), $1,25(OH)_2D_3$ brought about $+6.5 \pm 2.3$ mV depolarization ($n = 8$), comparable to that obtained with the physiologic Ringer's solution. Similarly, gluconate for Cl^- substitution did not significantly affect the magnitude of the $1,25(OH)_2D_3$-induced depolarization: $+6.0 \pm 1.5$ mV ($n = 8$). We conclude that secosteroid-evoked V_m changes do not involve Na^+- or Cl^--dependent processes, carrying current across the membrane.

By contrast, removal of HCO_3^- from luminal perfusion fluids completely abolished the ΔV_m effect of $1,25(OH)_2D_3$: ΔV_m was -0.1 ± 0.1 mV, $n = 9$ (not significantly different from zero). This observation indicates that bicarbonate is directly (change of HCO_3^- conductance) or indirectly (i.e., by permissive action on unspecified membrane components) involved in the generation of secosteroid-induced change of V_m.

Potential Dependence of $1,25(OH)_2D_3$-Evoked Acute Changes of V_m

To evaluate the dependence of such V_m changes on the absolute value of cell membrane potential, $1,25(OH)_2D_3$ was shortly added to a physiologic

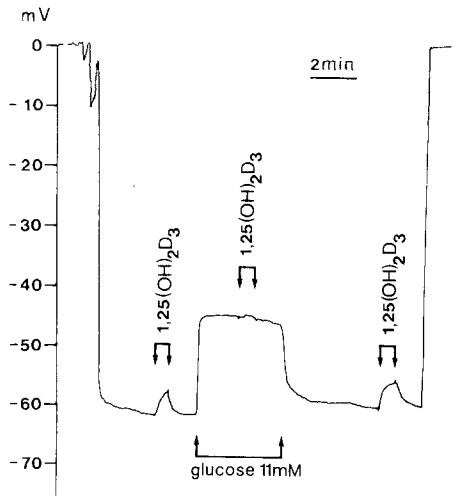

Fig. 2. Effects of $1,25(OH)_2D_3$, 10^{-10} M on V_m in the presence of a glucose-induced depolarization. Addition of the metabolite in a glucose-free perfusate resulted in ΔV_m depolarization, but this effect was reversibly abolished during exposure to a solution containing glucose, 11 mM

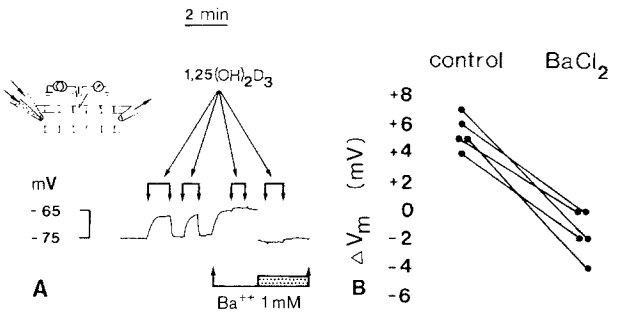

Fig. 3. *A.* Effects of barium on the $1,25(OH)_2D_3$ ΔV_m-evoked depolarization. Addition of Ba^{2+}, 1 mM, to the perfusate resulted in ~9 mV depolarization and abolishment of the specific effect of $1,25(OH)_2D_3$ on V_m (third release of the metabolite in luminal perfusate). Restoring V_m to its control level by current clamp (stippled area) during perfusion with a Ba^{2+}-supplemented solution failed to re-establish the metabolite-induced ΔV_m effect (fourth application of the metabolite). Inset: A double-barreled microelectrode was inserted in a proximal tubular cell. One of its barrels recorded V_m, the second barrel was used to inject a constant amount of current (~10^{-9} A) in order to bring V_m to its control value; current intensity was not varied thereafter. Although only one double-barreled luminal perfusion micropipette is represented in this drawing, two such pipettes were used to alternatively deliver four different solutions. *B.* Schematic representation of the effects of $1,25(OH)_2D_3$, 10^{-10} M, on V_m, under control conditions and during application of $BaCl_2$, 1 mM. Paired measurements in single tubules

Ringer's solution, then to a similar perfusate supplemented with glucose, 11 mM. High concentrations of glucose are known to depolarize V_m, by activating carrier-mediated rheogenic Na^+-glucose entry in the cell [13]. As shown in Fig. 2, addition of $1,25(OH)_2D_3$ to a physiologic Ringer's solution depolarized V_m in a reversible fashion. In five such experiments performed in single tubules, V_m was -67.2 ± 2.8 mV when the lumen was exposed to a physiologic Ringer's solution, -54.8 ± 2.6 mV, $n = 5$ after addition of glucose, 11 mM. Application of the metabolite during glucose exposure failed to affect V_m. Recovery to a glucose-free perfusate restored cell membrane potential and the depolarizing effect of $1,25(OH)_2D_3$.

Such observations are consistent with, but they do not unequivocally prove voltage dependence of the $1,25(OH)_2D_3$-evoked V_m depolarization.

Effects of Barium

Barium selectively blocks the partial conductance to potassium in several epithelia, including the apical [16, 19] and basolateral cell membrane [5, 22] of renal tubules. To assess whether the $1,25(OH)_2D_3$-evoked depolarization is mediated via a change (decrease) of K^+ conductance, the metabolite was briefly added to a physiologic Ringer's solution, then to a similar solution supplemented with $BaCl_2$, 1 mM. A representative experiment is shown in Fig. 3*A* and the composite data in Fig. 3*B*. It is seen that the $1,25(OH)_2D_3$-induced V_m depolarization under control conditions ($\Delta V_m = +4.5 \pm 0.5$ mV) is abolished (or even sometimes reversed) after addition of $BaCl_2$ to the perfusate ($\Delta V_m = -2.1 \pm 1.4$ mV; paired measurements in five single tubules). The suppression of the ΔV_m response in barium-treated conditions could be ascribed either to voltage dependence of the ΔV_m effect (ΔV_m was abolished when the membrane was depolarized by glucose, *see above*) and/or to barium blockage of a potassium-conductive pathway, presumably inactivated by $1,25(OH)_2D_3$. To distinguish between these two alternatives, the barium-induced depolarization was offset by current clamp (current was injected by a second intracellular microelectrode filled with KCl 0.1 M) to reset V_m at the recording site to its pre-barium level, and $1,25(OH)_2D_3$ was added again in the perfusate (Fig. 3*A*). In three such experiments, $1,25(OH)_2D_3$ failed to depolarize the membrane, whether V_m was depolarized by barium or restored to its control value by current injection. It is inferred that the $1,25(OH)_2D_3$-evoked V_m depolarization is mainly achieved by blockage of a barium-sensitive K^+-conductive pathway.

Involvement of Calcium

Calcium is a potential messenger of vitamin D metabolite action [26]. In addition, Ca^{2+}-dependent K^+ conductances have been identified in several tis-

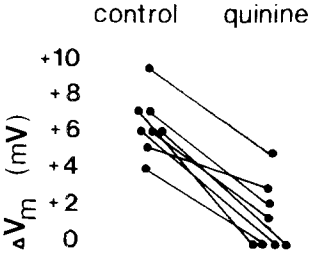

Fig. 4. Schematic representation of the changes of V_m (ΔV_m) induced by luminal application of $1,25(OH)_2D_3$, 10^{-10} M, under control conditions and after addition of quinine, 0.5 mM. Paired measurements in single tubules

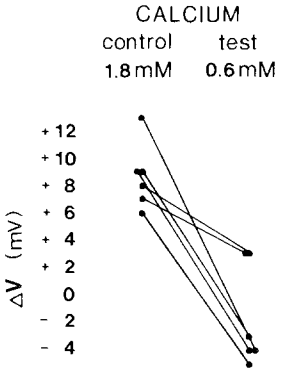

Fig. 5. Schematic representation of the effects of $1,25(OH)_2D_3$, 10^{-10} M, on V_m during alternate perfusion of the lumen with a solution containing physiologic (1.8 mM) or lowered (0.6 mM) Ca^{2+} concentration. Paired measurements in single tubules

sues, including epithelia [20]. To ascertain whether the barium-sensitive K^+ conductance of the apical proximal cell membrane, subject to reversible inhibition by $1,25(OH)_2D_3$, is calcium-dependent, the following experiment was designed: $1,25(OH)_2D_3$ was shortly added to a physiologic Ringer's solution, then to a similar solution supplemented with quinine (0.5 mM), a putative inhibitor of calcium-dependent K^+-conductive channels [7, 8]. In eight such experiments, quinine produced V_m depolarization ($+5.1 \pm 0.7$ mV), smaller than that obtained with glucose or barium; however, the $1,25(OH)_2D_3$-elicited ΔV_m effect was significantly decreased in the presence of quinine ($\Delta V_m = +1.5 \pm 0.7$ mV, $n = 8$, $P < 0.001$). The results are schematically depicted in Fig. 4. These observations strongly suggest that $1,25(OH)_2D_3$ depolarizes V_m decreasing a barium-sensitive, Ca^{2+}-dependent K^+ conductance.

To further characterize the role of calcium, the effects of $1,25(OH)_2D_3$ on cell membrane potential were assessed during alternate perfusion of the lumen with solutions of physiologic (1.8 mM) or lowered (0.6 mM) Ca^{2+} concentration. The depolarizing effect of the metabolite was $+8.7 \pm 1.1$ mV at physiologic Ca^{2+}, -1.7 ± 1.6 mV, ($n = 6$) at the low Ca^{2+} concentration (Fig. 5), the difference between these values being statistically significant ($P < 0.001$). Such observations support the notion that intraluminal (and, possibly through it, intracellular) calcium concentration contributes to the production and controls the magnitude of the V_m depolarization induced by $1,25(OH)_2D_3$.

Discussion

This study establishes that a class of biologically active molecules, i.e. vitamin D derivatives and sex steroids, elicit a rapid and reversible change of cell membrane potential when added in luminal perfusion fluid at physiologic concentrations. The ΔV_m response seems to be present only above a threshold V_m value (possibly different from one steroid to another), suggesting potential-dependence of the ΔV_m effect, a conclusion also supported by the glucose experiments. We demonstrate in addition that the $1,25(OH)_2D_3$-evoked V_m depolarization is due to decrease of a potassium-conductive pathway: not only this maneuver is associated with decrease of overall membrane conductance [14], but the depolarizing effect of the metabolite is abolished by pretreatment with barium, a blocker of K^+ conductances. Finally, the inhibitory action of quinine on the $1,25(OH)_2D_3$-evoked depolarization suggests that the K^+-conductive pathway, inactivated by the metabolite, is calcium dependent.

The postulated $1,25(OH)_2D_3$-induced inactivation of a potassium conductance resulted in a rather small depolarization: that V_m remained always close to the K^+ equilibrium potential is surprising in view of the fact that the apical cell membrane in the proximal tubule of *Necturus* is predominantly K^+-selective [3]. This apparent contradiction may be accounted for by either of the following mechanisms: (i) The "equivalent electromotive force" of the basolateral membrane contributes to a greater extent than that of the apical cell membrane, to the establishment of V_m [1]. (ii) If the ΔV_m changes are voltage-dependent, as suggested by the glucose experiments, increased depolarization would oppose the effects of the metabolite. (iii) In addition to $1,25(OH)_2D_3$-sensitive K^+ channels, $1,25(OH)_2D_3$-insensitive K^+ channels may be present at the apical cell membrane. Whatever the mechanism(s) limiting the ΔV_m depolarization to about 6.5 mV, the sensitivity of this change of membrane potential to extracellular Ca^{2+} concentration and quinine strongly suggest that the underlying mechanisms involve Ca^{2+}-dependent K^+ channels. It is recalled that potassium channels are unmasked at the apical membrane of frog skin by decreasing mucosal Ca^{2+}

concentration or intracellular pH [25]. Since divalent cations act by shielding negative surface charges [17], i.e., calcium removal should have facilitated K^+ permeation, the observation that the $1,25(OH)_2D_3$ depolarization (inactivation of K^+ channels) is blunted in low-Ca solutions suggests the Ca^{2+} removal promotes $1,25(OH)_2D_3$-insensitive K^+ pathways antagonizing the effect of the metabolite.

The failure of $1,25(OH)_2D_3$ to alter V_m when HCO_3^- is removed from a HEPES-buffered solution may be due to intracellular acidification. The commonly observed $1,25(OH)_2D_3$ depolarization can be hardly ascribed to an increase of HCO_3^- conductance, even though the bicarbonate equilibrium potential of about −12 mV [21] is consistent with this hypothesis; an increase of HCO_3^- conductance would increase cell membrane conductance, not decrease it, as observed [14]. In addition a change of a partial conductance to HCO_3^- would not have been abolished by barium. More likely, removal of luminal bicarbonate from a HEPES-buffered solution reduces the basolateral [4] or apical membrane permeability to potassium, presumably by decreasing intracellular pH (*see also* Ref. 25). It is known for example that acidosis is associated with a fall in basolateral K^+ conductance in the proximal tubule of *Necturus* [18].

Another interesting observation is that ΔV_m changes are selective, i.e. only seven out of 11 tested sterol molecules produced such changes. The common structural feature of the seven active (seco)steroids is that (i) the carbon C_{17} or one of the carbons attached to C_{17} is hydroxylated and (ii) an —OH bond (=O bond in the case of testosterone) is present on the A ring. The four inactive molecules possess one —OH (=O) radical on the A ring, but no hydroxyl function on the C_{17} to C_{25} carbon chain. These observations lend indirect support to the views of Willmer [27] who hypothesized that polar groups at spatially opposed ends of sterols are a prerequisite for their incorporation in bilayers and biological membranes. Apparently, hydroxylation of one carbon belonging to the C_{17} to C_{25} chain is a key factor for the appearance ΔV_m changes, reflecting changes of cell membrane conductance(s). In short, the "active" steroids probably alter the lipid environment surrounding K^+ membrane channels at the apical membrane of the proximal tubule in some relatively specific manner. This may be a generalized property of steroids on membranes, yet the effects of each class of steroids on K^+ and/or, possibly, on other channels may differ from one cell population to another, depending on the particular lipid composition and structural arrangement of each membrane.

It should also be noticed that the observed changes in V_m (through changes in K^+ conductance) cannot be considered specific in so far as the hormonal function is considered. No evidence is presented supporting steroid-dependent changes of transport rates, nor are we aware of rapid effects of (seco)steroids on renal function in amphibia. Information on a putative physiologic role of $1,25(OH)_2D_3$ could be obtained in the future by comparing prolonged effects of the metabolite on V_m and on transport rates of selected substrates, e.g., calcium. Further studies are needed to clarify this issue.

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