On the natriuretic effect of verapamil: inhibition of ENaC and transepithelial sodium transport

ALAN S. SEGAL, JOHN P. HAYSELETT, AND GARY V. DESIR

University of Vermont, Burlington, Vermont 05405; and Yale University School of Medicine and West Haven Veterans Affairs Medical Center, New Haven, Connecticut 06510

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Segal, Alan S., John P. Hayslett, and Gary V. Desir. On the natriuretic effect of verapamil: inhibition of ENaC and transepithelial sodium transport. Am J Physiol Renal Physiol 283: F765–F770, 2002. First published May 22, 2002; 10.1152/ajprenal.00253.2001.—The natriuretic effect of Ca2+ channel blockers has been attributed to hemodynamic changes and to poorly defined direct tubular effects. To test the possibility that verapamil may inhibit Na+ reabsorption at the distal tubule, its effect on transepithelial Na+ transport in aldosterone-stimulated A6 cells was determined. Cells were grown on permeable supports, and short-circuit current (Isc) measured in an Ussing chamber was used as a surrogate marker for transepithelial Na+ transport. Application of 300 μM verapamil to the apical side inhibited Isc by 77% and was nearly as potent as 100 μM amiloride, which inhibited Isc by 87%. Verapamil-induced inhibition of Isc was accompanied by a significant increase in transepithelial resistance, suggesting blockade of an apical conductance. Its action on transepithelial Na+ transport does not appear to occur through inhibition of L-type Ca2+ channels, since Isc was unaffected by removal of extracellular Ca2+. Verapamil also does not appear to inhibit Isc by modulating intracellular Ca2+ stores, since it fails to inhibit transepithelial Na+ transport when added to the basolateral side. The effect on Na+ transport is specific for verapamil, since nifedipine, Ba2+, 4-aminopyridine, and charybdotoxin do not significantly affect Isc. A direct effect of verapamil on the epithelial Na+ channel (ENaC) was tested using oocytes injected with the α-, β-, and γ-subunits. We conclude that verapamil inhibits transepithelial Na+ transport in A6 cells by blocking ENaC and that the natriuresis observed with administration of verapamil may be due in part to its action on ENaC.

VERAPAMIL; EPITHELIAL SODIUM CHANNEL; XENOPUS LAEVIS OOCYTE; SODIUM EXCRETION AND REGULATION; DIURETIC; NATRIURESIUS; A6 CELLS

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Calcium channel blockers are used extensively in the treatment of hypertension. They lower blood pressure by relaxing vascular smooth muscle and decreasing peripheral vascular resistance. Vasorelaxation is a result of blocking Ca2+ entry through voltage-gated Ca2+ channels. Ca2+ channel blockers also have significant effects on heart rate and renal function. They increase renal blood flow and glomerular filtration rate and decrease the activity of the renin-angiotensin-aldosterone system and the reabsorption of salt and water (4, 6, 10).

Although the natriuretic and diuretic effect of Ca2+ channel blockers is in part due to changes in renal hemodynamics, direct tubular actions have also been well documented. The dihydropyridine class of Ca2+ channel blockers has been studied most extensively. Intrarenal infusion of nifedipine significantly increased Na+ excretion without any detectable change in renal blood flow, creatinine clearance, and glomerular filtration rate (7). These effects are thought to result from a decrease in Na+ reabsorption in the proximal tubule. On the other hand, felodipine, another dihydropyridine, appears to increase Na+ excretion by blocking reabsorption at a distal tubular site (6). Verapamil, a phenylalkylamine, causes natriuresis when injected into the renal artery of the dog (1). Renal Na+ excretion is also significantly enhanced in hypertensive patients treated with verapamil (5, 10).

The molecular mechanisms underlying the tubular effects of Ca2+ channel blockers are unclear. Na+ reabsorption occurs all along the nephron, and inhibition at any site could account for the modest degree of natriuresis observed with these agents. The collecting duct is an important site for Na+ homeostasis. In this nephron segment, principal cells are responsible for Na+ reabsorption. The rate-limiting step is the apical entry of Na+ through the epithelial Na+ channel (ENaC) (2). This process is regulated by mineralocorticoids and plays a critical role in overall Na+ balance. The A6 cell line, derived from the Xenopus laevis kidney, possesses many of the properties of principal cells and has been used extensively as a model for the study of electrogenic transepithelial Na+ transport. When grown on permeable supports, these cells develop a high transepithelial resistance (TER), express the ENaC, and engage in transepithelial electrogenic Na+ transport that is regulated by insulin, aldosterone, and antidiuretic hormone. A6 cells and X. laevis oocytes expressing ENaC were used in the present study to examine the possibility that Ca2+ channel blockers exert their natriuretic effect by...
inhibiting Na⁺ transport in the cortical collecting duct (CCD).

MATERIALS AND METHODS

Cell culture. A6 cells were plated and maintained in culture as previously described (9). Briefly, cells were seeded at a density of 1 × 10⁶ cells/cm² on permeable supports in DMEM modified for amphibian culture and supplemented with 10% fetal bovine serum. They were grown in a humidified atmosphere of 2% CO₂ at 28°C. Aldosterone (1.5 μM) was added for 2 days after the initial seeding and then for 18 h before measurements.

Solution and drugs. Unless stated otherwise, the apical and basolateral solutions were identical and consisted of 97 mM NaCl, 1 mM CaCl₂, 1 mM KCl, 0.5 mM MgCl₂, and 5 mM HEPES (pH 7.4). For Ca²⁺-free solutions, CaCl₂ was omitted and 1 mM EGTA was added. Where indicated, Ba²⁺ was added as BaCl₂. Verapamil and nifedipine were dissolved in DMSO and 1 mM EGTA was added. Where indicated, Ba²⁺ was added as BaCl₂. Verapamil and nifedipine were dissolved in 100% DMSO and added from 100 mM stock.

Solution and drug concentrations were 0 or 1 mM. Verapamil (Vera) was added to the apical or basolateral compartment in increasing concentrations. The inhibition constant (Kᵢ) for verapamil was calculated from the best logistic fit of the dose response. Values are means ± SE.

Electrical measurements in A6 cells. Falcon inserts were mounted on plastic rings (effective surface area = 0.64 cm²) and placed in an Ussing chamber modified to allow continuous independent perfusion of apical and basolateral compartments. The compartments were connected by 1 M KCl-2% agar bridges. Current and transepithelial potential were measured using Ag-AgCl half-cells and a current-voltage clamp (model DVC-1000, WPI). Current flowing from the apical to the basolateral side was measured as positive by convention. The solution resistance was measured and compensated for before recordings began. Iₑₑₑ was measured by clamping the transepithelial voltage to 0 mV for 5 s. TER was calculated from the difference in current measured when the cells were voltage clamped to 0 or 60 mV, as follows: TER (Ω·cm²) = 60 mV(Iₘₐₓ(60 mV)) − Iₑₑₑ (0 mV) μA/cm².

Expression and measurement of ENaC current in X. laevis oocytes. Stage V-VI X. laevis oocytes were dissected from ovarian lobes and stored in modified Barth’s solution, as previously described. Oocytes were injected with 50 nl of solution containing either a mixture of in vitro-transcribed, 5’-capped α-, β-, and γ-ENaC RNA or water as a negative control.

Whole cell currents were recorded using a standard two-microelectrode voltage clamp (model OC-725, Warner Instruments) 1–8 days after injection. Oocytes were impaled with microelectrodes filled with 1 M KCl (resistance = 1–4 MΩ). The bath contained Na⁺ (or Li⁺)-ND-96: 96 mM NaCl (or LiCl), 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES (pH 7.4). Voltage-clamp protocols were controlled by PULSE (HEKA Lambrecht), and amplified currents were filtered at 1–2 kHz and then recorded and analyzed using PULSE-FIT (HEKA Lambrecht) and Igor-Pro (Wavemetrics).

RESULTS AND DISCUSSION

Effect of verapamil on transepithelial Na⁺ transport (Iₑₑₑ). Iₑₑₑ is used as a surrogate marker for transepithelial Na⁺ transport in A6 cells, because ~90% of the current is amiloride sensitive. We confirmed this for A6 cells grown on permeable supports and treated with aldosterone for 18 h before measurements. Under these conditions, 100 μM amiloride inhibited Iₑₑₑ by 87.3 ± 2% (n = 4). Therefore, the amiloride-sensitive Iₑₑₑ correlates well with Na⁺ transport from the apical to the basolateral side through the ENaC.

The effect of verapamil on Iₑₑₑ (and, therefore, on Na⁺ transport) was tested on A6 cells mounted in an Ussing chamber. As shown in Fig. 1, 0.2 mM verapamil added to the apical side significantly inhibited Iₑₑₑ compared with control conditions (16.0 ± 2.6 to 5.3 ± 1.1 μA/cm², n = 8, P < 0.05). Because verapamil is a known inhibitor of L-type Ca²⁺ channels, it might exert its effect on Iₑₑₑ by decreasing apical Ca²⁺ entry. To test the dependence of Iₑₑₑ on extracellular Ca²⁺, the effect of Ca²⁺ removal on Iₑₑₑ was measured. Figure 1 shows that removal of apical Ca²⁺ had no effect on Iₑₑₑ under control conditions and TER was unaffected. Furthermore, verapamil inhibited Iₑₑₑ to a similar degree in the absence and presence of Ca²⁺ (Fig. 1). Verapamil is lipophilic, so it penetrates the cell membrane and accumulates inside the cell. Therefore, it could inhibit Ca²⁺ release from intracellular stores, which could in turn modulate Iₑₑₑ. To further rule out the possibility that verapamil’s action on Iₑₑₑ might be Ca²⁺ dependent, its inhibitory effect on Iₑₑₑ was compared with that of nifedipine, a structurally unrelated, potent L-type Ca²⁺ channel blocker. Although nifedipine is a significantly more potent blocker of L-type Ca²⁺ channels than verapamil, it decreased Iₑₑₑ by only 7.3 ± 0.3% when applied to the apical side at the highest concentration (200 μM) achievable in aqueous media. Be-
cause, as shown in Fig. 1, 0.2 mM verapamil added to the basolateral side has no significant effect on $I_{sc}$, we conclude that the drug inhibits $I_{sc}$ and Na$^+$ transport only when applied to the apical side and through a mechanism unrelated to its action on plasma membrane L-type Ca$^{2+}$ channels and intracellular Ca$^{2+}$ stores.

**Effect of verapamil on TER.** It is possible that verapamil could inhibit $I_{sc}$ and yet cause no change in net Na$^+$ transport. Indeed, if addition of the drug leads to a nonspecific decrease in TER, through interaction with the paracellular junction or by damaging the plasma membrane, transepithelial voltage and $I_{sc}$ would be expected to decrease independently of a net decrease in Na$^+$ transport. If that were the case, the inhibition of $I_{sc}$ would be accompanied by a fall in membrane resistance. For instance, diltiazem is known to increase the permeabilities of anions and cations in photoreceptor rod outer segments and in intact red blood cells (3). To exclude this possibility, the effect of verapamil on membrane resistance was determined from the change in monolayer current resulting from a 60-mV voltage step. As shown in Fig. 2, control cells had a TER of 2,575 ± 350 Ω cm$^{-2}$ ($n = 11$). Increasing concentrations of verapamil from 100 to 400 μM led to progressive increases in TER (5,002 ± 406 Ω cm$^{-2}$, $n = 5$). These results indicate that the fall in $I_{sc}$ observed with the addition of verapamil does not occur because of nonspecific changes in the paracellular junction or damage to the cell membranes. Instead, verapamil appears to have a direct inhibitory effect on transepithelial Na$^+$ transport.

**Dose-dependent inhibition of Na$^+$ transport by verapamil.** The dose-response curve for verapamil with respect to inhibition of $I_{sc}$ in A6 cells was determined by adding increasing concentrations of the drug to the apical side. The amiloride-sensitive component of $I_{sc}$ was determined after each experiment by the addition of 100 μM amiloride. As shown in Fig. 3, the $K_i$ of $I_{sc}$ for verapamil is 104.5 μM.

Verapamil contains a nitrogen at position 9 that undergoes pH-dependent protonation ($pK_a = 8.5$). The nonprotonated form of verapamil is lipophilic and readily penetrates biological membranes. Figure 4 shows that the effect of verapamil is strongly influenced by the pH of the apical perfusate. As pH increases from 5 to 8, verapamil becomes deprotonated and its potency to block $I_{sc}$ increases. Indeed, verapamil predominantly blocks L-type Ca$^{2+}$ and K$^+$ channels from the cytoplasmic side (13). It is likely that verapamil also inhibits $I_{sc}$ from the cytoplasmic side, since, as observed for inhibition of Ca$^{2+}$ and K$^+$ channels, its onset of action on $I_{sc}$ is delayed (3–4 min) and slowly reaches a plateau within 10–15 min. Therefore, the true $K_i$ for verapamil-induced inhibition of $I_{sc}$ cannot be determined without measuring the intracellular concentration of the drug.

**Effect of nifedipine and K$^+$ channel blockers on Na$^+$ transport.** Verapamil is also known to inhibit voltage-gated K$^+$ channels, such as Kv1.3 and KCNA10 ($K_i = 50$ μM). Therefore, it is possible that it could mediate its effect on transepithelial Na$^+$ transport by blocking K$^+$ channels. This possibility was tested by examining the effect of K$^+$ channel blockers applied to the apical side of A6 cells. Ba$^{2+}$ (10 mM), which inhibits a variety of K$^+$ channels, caused only a small decrease in $I_{sc}$ (Fig. 5) and TER (from 2,520 ± 223 to 2,001 ± 216 Ω cm$^{-2}$, $n = 4$). This confirms the results of Thomas and Mintz (11), who showed that Ba$^{2+}$ depolarizes the apical membrane of A6 cells in culture and also reduces TER, probably by opening a paracellular conductive pathway. Inhibitors of voltage-gated and Ca$^{2+}$-activated K$^+$ channels (charybdotoxin and 4-aminopyridine, respec-
inability to block when added to the basal side argues otherwise. The latter observation does not completely rule out the possibility of blocks from the inside, since it is not known whether verapamil that enters from the basolateral side freely diffuse to the apical side.

**Physiological relevance.** On the basis of studies examining the urinary concentration of verapamil and its metabolites, ~5% of the dose of verapamil administered is excreted unchanged in the urine over 24 h (8). With a maximum daily dose of 500 mg, distal tubular fluid may contain 25 mg/l verapamil or ~50 μM. The data in the present study suggest that this concentration would be sufficient to exert a significant effect on Na⁺ transport through inhibition of ENaC. It should be noted, however, that inhibition of I_{sc} by verapamil was greater at alkaline than acidic pH. The pH at the distal tubule can be as low as 5.5 and would predict a significant decrease in the inhibitory potency of verapamil. On the other hand, verapamil tends to accumulate in cells, and the steady-state intracellular concentration at distal tubular cells might be even higher than expected from a luminal concentration of 50 μM.

**Conclusion.** The present study shows that verapamil, unlike nifedipine, inhibits Na⁺ reabsorption in A6 cells, a cell model for the principal cells of the CCD. The concentration of verapamil thought to be present in distal tubular fluid is more than sufficient to cause significant inhibition of Na⁺ reabsorption through ENaC. Therefore, the natriuresis observed with the administration of verapamil is likely mediated by a combination of hemodynamic factors and the direct tubular effect demonstrated in this study. The present study documents a previously unrecognized amiloride-like effect of verapamil on Na⁺ transport in A6 cells and a direct inhibitory effect of the drug on ENaC. We speculate that the antihypertensive action of verapamil results from a combination of a direct effect on

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**Fig. 4.** Alkaline pH potentiates inhibitory action of verapamil. Apical pH was varied from 5 to 8, and inhibition of I_{sc} by 100 μM verapamil was determined. Verapamil was 4.5 times more potent at pH 8 than at pH 5.

**Fig. 5.** Verapamil is a specific inhibitor of Na⁺ transport in A6 cells. Drugs were added to apical perfusate. Amiloride and verapamil specifically block I_{sc}, while nifedipine (a dihydropyridine) was ineffective. Classic K⁺ channel blockers such as Ba²⁺, 4-aminopyridine (4-AP), and charybdotoxin (CTX) were also without significant effect.
vascular smooth muscle and a small but significant decrease in total body Na\(^+\) stores through its action on Na\(^+\) reabsorption in the CCD. It may be possible to improve the efficacy and safety profile of verapamil and develop analogs with enhanced natriuretic properties.

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REFERENCES


