Sch-28080 depletes intracellular ATP selectively in mIMCD-3 cells

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Inhibitions of Sch-28080 inhibit K⁺ uptake, selective depletes ATPase activity in mIMCD-3 cells. Am J Physiol Cell Physiol 279: C1319–C1326, 2000.—Two H⁺-K⁺-ATPase isoforms are present in kidney: the gastric, highly sensitive to Sch-28080, and the colonic, partially sensitive to ouabain. Upregulation of Sch-28080-sensitive H⁺-K⁺-ATPase, or "gastric" H⁺-K⁺-ATPase, has been demonstrated in hypokalemic rat inner medullary collecting duct cells (IMCDs). Nevertheless, only colonic H⁺-K⁺-ATPase mRNA and protein abundance increase in this condition. This study was designed to determine whether Sch-28080 inhibits transporters other than the gastric H⁺-K⁺-ATPase. In the presence of bumetanide, Sch-28080 (200 μM) and ouabain (2 mM) inhibited 86Rb⁺ uptake (>90%). That 86Rb⁺ uptake was almost completely abolished by Sch-28080 indicates an effect of this agent on the Na⁺-K⁺-ATPase. ATPase assays in membranes, or lysed cells, demonstrated sensitivity to ouabain but not Sch-28080. Thus the inhibitory effect of Sch-28080 was dependent on cell integrity. 86Rb⁺-uptake studies without bumetanide demonstrated that ouabain inhibited activity by only 50%. Addition of Sch-28080 (200 μM) blocked all residual activity. Intracellular ATP declined after Sch-28080 (200 μM) but recovered after removal of this agent. In conclusion, high concentrations of Sch-28080 inhibit K⁺-ATPase activity in mouse IMCD-3 (mIMCD-3) cells as a result of ATP depletion.

Ouabain, inner medullary collecting duct; adenosine 5'-triphosphatase

Inhibition by ouabain of ATPase activity is a widely accepted marker of Na⁺ pump activity in vitro. Conversely, inhibition by Sch-28080 has been used to designate H⁺-K⁺-ATPase activity (21, 30). Specific binding sites for ouabain have been identified on the α₁-Na⁺-K⁺-ATPase (3, 23) but not on the gastric H⁺-K⁺-ATPase (HKα₁). In contrast, specific Sch-28080 binding sites have been identified on HKα₁ that are conspicuously absent in the α₁-Na⁺-K⁺-ATPase (3). On the basis of such observations, ouabain and Sch-28080 have been widely used to delineate which K⁺-ATPase is responsible for either K⁺ absorption or H⁺ secretion by the distal nephron. Accordingly, by convention, functions that are blocked by Sch-28080 have been assumed to be mediated by HKα₁ (15, 19, 28). Nevertheless, this assumption has been challenged in several experimental models. Chronic dietary K⁺ depletion increased the fraction of bicarbonate absorption (JtCO₂) sensitive to Sch-28080 in rat isolated perfused collecting duct segments (19, 28). Whereas this increase in JtCO₂ could be assumed to be the result of upregulation of HKα₁, both Northern and immunoblot analyses did not reveal changes in HKα₃ mRNA or protein abundance in rat renal medulla during chronic hypokalemia (9, 17). Rather, with hypokalemia, several groups have detected a selective increase in abundance of colonic H⁺-K⁺-ATPase (HKα₂) mRNA and protein that was site specific for the medullary collecting tubule (9, 17, 25).

High concentrations of Sch-28080 (~100 μM) have been used to delineate the role of HKα₁ in renal transport during respiratory acidosis and respiratory alkalosis (13). In that study, the ATPase activity of α₁-Na⁺-K⁺-ATPase was very similar to the level of activity of HKα₁ defined as "Sch-28080-sensitive ATPase activity." High concentrations of Sch-28080 (>100 μM) have also been used to identify three unique types (type I, type II, and type III) of K⁺-ATPase activity (5, 32). Nevertheless, designation of HKα₁ or HKα₂ as the functional equivalent of any of these ATPase activities has not been possible (8). Moreover, Sabolic et al. (24) reported that high concentrations of Sch-28080 and omeprazole (100 μM) inhibit the H⁺-ATPase nonselectively. This transporter is not involved in K⁺ homeostasis but is regulated in response to metabolic acidosis (2, 4).

The purpose of this study was to evaluate the specificity of Sch-28080 by determining whether Sch-28080 inhibits K⁺ transporters other than HKα₁. Our data demonstrate that both Sch-28080 at high concentrations (200 μM) and ouabain (2 mM) block Na⁺ pump activity in an established renal inner medullary cell line, mouse inner medullary collecting duct cells (mIMCD-3), in culture. Moreover, we demonstrate, for

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the first time, that in contrast to the direct inhibitory effect of ouabain on the α-subunit of the Na⁺ pump, the inhibitory effect of high concentrations of Sch-28080 was the result of depletion of intracellular ATP.

MATERIALS AND METHODS

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), cat. no. D-8900; newborn calf serum, cat. no. N-4637; gentamicin, cat. no. G-1272; Ham’s F-12, cat. no. N-3520; and soybean trypsin inhibitor, cat. no. T-9003, were purchased from Sigma (St. Louis, MO). Twenty-four-well dishes were purchased from Corning (Corning, NY; cat. no. 25820–24) or Nunc (Nalge Nunc, Naperville, IL; cat. no. 150628). Trypsin-EDTA was purchased from Life Technologies (Gaithersburg, MD; cat. no. 25300–062). Sch-28080 (a gift from Dr. J. Kaminiski at Schering-Plough Research Institute) was dissolved in 10% DMSO. DDT1MF-2 and BEAS-2B cells were grown in the presence of DMEM supplemented with newborn calf serum (10%) and gentamicin (50 μg/ml) and were adjusted to pH 7.4 by addition of NaHCO₃ (7.5%), as described previously by our laboratory (15, 20). BEAS-2B cells were grown in the presence of Ham’s F-12 containing gentamicin and serum at the concentration described above. Cells were grown to confluence at 37°C in a humidified environment in 24-well dishes. Before the assay, the cells were washed four times (1.5 ml/cell) with buffer A (145 mM NaCl, 1 mM KCl, 1.2 mM MgSO₄, 2 mM Na₂HPO₄, 1 mM CaCl₂, 200 μM bumetanide, and 32 mM HEPES, pH 7.4) at 4°C and then calibrated for ATP levels in the cells were determined using ATP assay.

RESULTS

Sch-28080 and ouabain block ⁸⁶Rb⁺ uptake in mIMCD-3 cells in culture. The results of a representative ⁸⁶Rb⁺-uptake experiment are displayed in Fig. 1. Figure 1A demonstrates that ouabain inhibited ⁸⁶Rb⁺ uptake in a dose-dependent manner (IC₅₀ ~30 μM). These results are consistent with the well-known inhibitory effect of ouabain on the renal Na⁺ pump.

![Fig. 1. ⁸⁶Rb⁺ uptake by mouse inner medullary collecting duct (mIMCD-3) cells. A: inhibitory effect of ouabain (plus bumetanide). B: inhibitory effect of Sch-28080 (plus bumetanide). Both ouabain and Sch-28080 inhibited ⁸⁶Rb⁺ uptake (>90%). These experiments were performed in the presence of 1 mM KCl, 145 mM NaCl, and 200 μM bumetanide.](http://example.com/image.png)
Because our experiments were performed in the presence of bumetanide (200 μM), our findings, in agreement with previously published data (14, 16, 29), substantiate that the Na\(^+\)-K\(^+\)-ATPase and the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter are the major pathways for K\(^+\) entry to the cell. Figure 1B demonstrates that Sch-28080 at concentrations >10 μM also inhibited \(^{86}\text{Rb}^+\) uptake in a similar dose-dependent manner (IC\(_{50}\) ≈ 60 μM). Because Sch-28080 (200 μM) inhibited \(^{86}\text{Rb}^+\) uptake (>90%), it seems reasonable to conclude that Sch-28080 acted by blocking the Na\(^+\)-K\(^+\)-ATPase.

To test whether the effects of either ouabain or Sch-28080 (to inhibit K\(^+\)-ATPase in mIMCD-3 cells) were reversible, we used the \(^{86}\text{Rb}^+\)-uptake assay during the application of, and after removal of, either ouabain or Sch-28080. In Fig. 2 (left), mIMCD-3 cells were incubated with ouabain (2 mM) and \(^{86}\text{Rb}^+\) uptake was blocked dramatically (as shown in Fig. 1). Removal of ouabain for 15 min at 37°C reestablished \(^{86}\text{Rb}^+\) uptake. Figure 2 (right) demonstrates similarly that the inhibitory effect of Sch-28080 (200 μM) was also reversible.

We prepared plasma membranes, as described previously by our laboratory (11, 22), and performed the experiment described in Fig. 3 to determine whether the Na\(^+\) pump of mIMCD-3 cells displayed a predictable pattern of response to either ouabain or Sch-28080. ATPase activity was measured in the presence of ATP I) under basal conditions (no K\(^+\) or Na\(^+\) added), 2) in the presence of 5 mM K\(^+\), 3) in the presence of 50 mM Na\(^+\), or 4) in the presence of 5 mM K\(^+\) and 50 mM Na\(^+\). The studies were performed in the presence or absence of either 1 mM ouabain or 200 μM Sch-28080. A representative experiment is displayed in Fig. 3. Basal activity was not modified by addition of 5 mM K\(^+\) or 50 mM Na\(^+\) to the assay. Basal ATPase activity and activity in the presence of K\(^+\) or Na\(^+\) alone was not sensitive to either ouabain or Sch-28080. However, addition of both K\(^+\) and Na\(^+\) to the assay induced an increase in ATP hydrolysis (Na\(^+\)-K\(^+\)-ATPase) that was sensitive to 2 mM ouabain but insensitive to 200 μM Sch-28080. This finding demonstrates that the mIMCD-3 Na\(^+\)-K\(^+\)-ATPase in broken cell preparations is sensitive to high concentrations of ouabain but totally insensitive to Sch-28080. However, in the \(^{86}\text{Rb}^+\)-uptake experiments in intact cells described above, a clear inhibitory effect by 200 μM Sch-28080 on the Na\(^+\) pump (inhibition by >90%) was observed.

The ATPase assay was performed in the presence of 50 mM NaCl with or without addition of KCl (10 mM; Fig. 4) in mIMCD-3 cells that were lysed as described...
in **MATERIALS AND METHODS**. The results demonstrate that on homogenization, 2 mM ouabain blocked ATPase (Na\(^+\)-K\(^+\)-ATPase) activity in both groups, consistent with a direct effect of ouabain on the Na\(^+\) pump. In contrast, addition of 200 μM Sch-28080 did not inhibit ATPase activity (in any group). The results from Figs. 1, 3, and 4 confirm that the effect of Sch-28080 on the Na\(^+\) pump was nonspecific. Moreover, the inhibitory effect of Sch-28080 on the Na\(^+\) pump was dependent on cell integrity.

Next, we investigated whether Sch-28080 blocked only the Na\(^+\) pump or if it blocked additional mechanisms of K\(^+\) entry into cells. These studies were performed by deleting bumetanide from the \(^{86}\)Rb\(^+\)-uptake experiments. A representative experiment is displayed in Fig. 5. Ouabain (2 mM) inhibited \(^{86}\)Rb\(^+\) uptake by 50–60% when bumetanide was not present (solid bar). Addition of 200 μM Sch-28080 inhibited \(^{86}\)Rb\(^+\) uptake (hatched bar) by >90%. Addition of 2 mM ouabain plus 200 μM Sch-28080 did not alter the inhibitory effect of Sch-28080. These data, taken together, demonstrate that the inhibitory effect of Sch-28080 is not specific for the Na\(^+\) pump but, rather, extends by a common mechanism to additional K\(^+\) transporters in mIMCD-3 cells in culture.

ATP is required for active transport by cells and for the activity of the Na\(^+\) pump. As displayed in Fig. 6, we measured total intracellular ATP content in control and in mIMCD-3 cells treated with 2 mM ouabain or 200 μM Sch-28080. Ouabain alone (solid bar) did not alter intracellular ATP content. However, incubation of cells with Sch-28080 caused a dramatic reduction in total ATP content. Removal of the Sch-28080 from the bathing solution for 15 min at 37°C reestablished both intracellular ATP (Fig. 7) and \(^{86}\)Rb\(^+\) uptake (see Fig. 2). In keeping with this observation in mIMCD-3 cells, we have also observed inhibition of \(^{86}\)Rb\(^+\) uptake by Sch-28080 in mOMCD 1 cells and in HEK-293 cells (data not shown). Nevertheless, the inhibitory effect of Sch-28080 on \(^{86}\)Rb\(^+\) uptake described in these cell lines.
did not extend to all cell lines studied. For example, 200 μM Sch-28080 did not inhibit 86Rb⁺ uptake (in the absence or presence of bumetanide) in DDT1MF-2 cells (Fig. 8), a hamster smooth muscle cell line; BEAS-2B cells, a human bronchial cell line; or in oocytes from *Xenopus laevis* (data not shown). In addition, 200 μM Sch-28080 did not decrease the content of ATP in DDT1MF-2 cells in culture (Fig. 9).

Several laboratories, including our own (6, 15, 19, 20, 28), have employed low concentrations of Sch-28080 (10 μM) in studies in medullary collecting duct cells in culture, or in inner medullary collecting ducts perfused in vitro, to define the role of HKα1 in pHᵢ recovery and K⁺ absorption during either chronic hypokalemia or metabolic acidosis. In experiments in inner and outer medullary collecting duct cells in culture, the activity of HKα1 was defined as inhibition of pHᵢ recovery after a NH₄Cl load. In studies in isolated inner medullary collecting ducts perfused in vitro, HKα1 activity was defined as Sch-28080-inhibitable JtCO₂. To simulate the effect of prolonged exposure of cells in culture or in isolated tubules perfused in vitro, we conducted the experiment displayed in Fig. 10 (left). In this study, mIMCD-3 cells in culture were incubated for an extended period (45 min) with either low (10 μM) or high concentrations (200 μM) of Sch-28080. Preincubation with high concentrations of Sch-28080, as demonstrated previously in Figs. 1, 2, and 5, resulted in a marked reduction in 86Rb⁺ uptake (>90%). In contrast, preincubation with low concentrations of Sch-28080 for 45 min resulted in a reduction of 86Rb⁺ uptake of only 20%. The data displayed in Fig. 10 (right) reveal that high concentrations of Sch-28080 (200 μM) decreased intracellular ATP concentration ([ATP]ᵢ) by >90%. This finding is in agreement with the data displayed in Figs. 6 and 7. In contrast, preincubation with low concentrations of Sch-28080 (10 μM) decreased [ATP]ᵢ by only 20%. Although the reductions in 86Rb⁺ uptake and in ATP concentrations were significant, the observed decrease in these parameters with low concentrations of Sch-28080 was significantly less marked than that seen with prolonged exposure to higher concentrations.
Our findings also reveal that ouabain decreased 86Rb uptake by 50% in mIMCD-3 cells (Fig. 5). Nevertheless, on addition of bumetanide to the assay, this degree of inhibition increased to almost 100% (Figs. 1 and 2). In contrast, Sch-28080 reduced 86Rb uptake by >90% in the presence or absence of bumetanide. It has been demonstrated previously that low concentrations of Sch-28080 (<10 μM) inhibit HKα1 activity by binding directly to the α-subunit (18, 30). In addition, however, Sabolic et al. (24) have reported that Sch-28080 and omeprazole (100 μM) inhibit H+-ATPase activity in renal cortical and medullary endosomes in the presence of ATP (1.5 mM). Our data demonstrate that Sch-28080, in high concentrations (200 μM), decreases the intracellular concentration of ATP. The predicted sequelae of intracellular ATP depletion would be to limit activity of the Na+ pump, which is entirely dependent on [ATP]. Depletion of [ATP], may also contribute to a decrease in activity of the Na+-K+-2Cl− cotransporter by increasing the intracellular Na+ concentration and diminishing Na+ entry. Nevertheless, our data cannot exclude a direct effect of Sch-28080 on the Na+-K+-2Cl− cotransporter.

Total JCO2 is increased by chronic hypokalemia in collecting ducts perfused in vitro. This increase is inhibited by low concentrations (~10 μM) of Sch-28080 (19, 28). In addition, Campbell et al. (6) demonstrated that low concentrations of Sch-28080 impaired intracellular pH recovery in RCCT-28A cells after a NH4 load. These results have been interpreted as evidence for a direct effect of Sch-28080 on HKα1 activity. However, a parallel increase in HKα1 mRNA and protein during chronic hypokalemia has not been observed (9, 17). On the basis of results from the present study, an indirect effect of Sch-28080 on collecting duct JCO2 in chronic hypokalemia should be considered a possibility in these experiments. In this regard, our findings (Fig. 1) demonstrate that Sch-28080 at low concentrations (~10 μM) does not block 86Rb uptake in mIMCD-3. However, by extending the preincubation time from 15 to 45 min, low concentrations of Sch-28080 (10 μM) inhibited 86Rb uptake by 20% (Fig. 10). We do not know if our observation using the 86Rb uptake assay can be extrapolated to JCO2 or pHi recovery experiments, where exposure of cells or tubules to Sch-28080 extends to periods of at least 45 min. On the basis of results obtained with prolonged incubation at low concentrations of this agent (Fig. 10), it seems logical to speculate that a portion of the inhibition attributed to a “specific” effect of Sch-28080 on HKα1 in kidney might represent, in part, a nonspecific response, attributable to a decrease in intracellular ATP. Because we have not examined the effect of Sch-28080 on ATP content in cells of stomach or colon origin, we concede that these observations may be pertinent only to renal cell lines.

In summary, our data demonstrate that Sch-28080 inhibits α1-Na+-K+-ATPase activity in mIMCD-3 cells by depletion of intracellular ATP. This nonspecific effect by an agent widely assumed to be a specific inhibitor of the gastric H+-K+-ATPase (30) now requires reconsideration, which takes into account the concentration of Sch-28080, as well as the setting and cell
type in which these observations have been made. With this view in mind, we can now offer a possible explanation for the disparity among results obtained from in vitro perfusion studies in the rat outer medullary collecting duct or inner medullary collecting duct and in established mouse cell lines from the same region of the nephron vs. results obtained in heterologous expression systems (10, 12, 19, 28).

In isolated tubules and in cells in culture, the increase in \( J_{\text{CO}_2} \) or the \( p_H \) recovery rate induced by chronic hypokalemia has been reported uniformly to be Sch-28080 sensitive (19, 28). Nevertheless, only HK\( \alpha_2 \), not HK\( \alpha_1 \), mRNA or protein was upregulated in the renal medulla by this condition. In addition, when expressed heterologously, HK\( \alpha_2 \) has been shown uniformly to be insensitive to Sch-28080.

Accordingly, if the “effect” of Sch-28080 observed in intact tubules or in these renal cell lines was the result of a nonspecific effect of Sch-28080 on the Na\(^+\)-K\(^+\)-ATPase or HK\( \alpha_2 \), such findings could then be reconciled. Moreover, it would be unnecessary to invoke the emergence of a Sch-28080-sensitive variant of HK\( \alpha_2 \) (1).

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