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

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In conclusion, high concentrations of Sch-28080 inhibit transporters other than the gastric H\(^+\)-K\(^+\)-ATPase. In the presence of bumetanide, Sch-28080 (200 \(\mu\)M) and ouabain (2 \(\mu\)M) inhibited \(^{86}\text{Rb}^+\) uptake (>90%). That \(^{86}\text{Rb}^+\) 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. \(^{86}\text{Rb}^+\)-uptake studies without bumetanide demonstrated that ouabain inhibited activity by only 50%. Addition of Sch-28080 (200 \(\mu\)M) blocked all residual activity. Intracellular ATP declined after Sch-28080 (200 \(\mu\)M) 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.

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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. Kaminski at Schering-Plough Research Institute) was dissolved in DMSO. DDT1MF-2 and BEAS-2B cells were gifts from Dr. R. B. Clark at the University of Texas Health Science Center at Houston. Plasma membranes and ATPases were gifts from Dr. R. B. Clark at the University of Texas Health Science Center at Houston. Plasma membranes and ATPases assays were performed as described previously (11, 22).

Cell culture and \(^{86}\text{Rb}^{+}\) uptake. mIMCD-3, mouse outer medullary collecting duct (mOMCD), human embryonic kidney (HEK-293), and DDT1MF-2 cells were grown in the presence of DMEM supplemented with newborn calf serum (10%) and gentamicin (50 \(\mu\)g/ml) and were adjusted to pH 7.4 by addition of NaHCO\(_3\) (7.5%), as described previously by our laboratory as well 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 concentrations described above. Cells were grown to confluency in a humidified environment at 37°C 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 MgSO\(_4\), 2 mM Na\(_2\)HPO\(_4\), 1 mM CaCl\(_2\), 200 \(\mu\)M bumetanide, and 32 mM HEPES, pH 7.4) at 4°C and then calibrating for 15 min with the same buffer. The buffer was removed and replaced by 250 \(\mu\)l of the corresponding solution containing \(^{86}\text{Rb}^{+}\) (3–8 \(\times\) 10\(^6\) counts/min). The reaction was allowed to proceed for 15 min at 37°C. The buffer was aspirated and washed five times with 1.5 ml of buffer B (100 mM MgCl\(_2\) and 10 mM HEPES, pH 7.4) at 4°C. Cells were dissolved by addition of 400 \(\mu\)l of buffer C (0.1 M NaOH and 2% SDS) at 65°C for 30 min. Resuspended cells (400 \(\mu\)l) were used to determine \(^{86}\text{Rb}^{+}\) uptake (16, 27). When experiments were performed using HEK-293 cells, Nunc dishes replaced Corning dishes to facilitate cell adherence.

**ATPase assays in cell lysates.** Cells were grown to confluency in 10-cm dishes, washed with saline, lifted by scraping, and centrifuged at 3,000 rpm for 5 min at 4°C in a top table centrifuge (Biofuge 17R). The cells were resuspended in buffer D (5 mM Tris-HCl, pH 8.0, 1 mM EDTA-Tris, 100 \(\mu\)M phenylmethylsulfonyl fluoride, 3 mM benzamidine, and 1 \(\mu\)g/ml soybean trypsin inhibitor) and were homogenized by passing the suspension five to six times through a 28-gauge needle. The ATPase assay was performed for 30 min at 37°C, as described previously by our laboratory, in an excess concentration of ATP (11).

**ATP assay.** ATP levels in the cells were determined using a bioluminescence assay as described by Wang et al. (31). Cells were grown to near confluency in 24-well dishes and incubated as described in the \(^{86}\text{Rb}^{+}\)-uptake experiments, except the \(^{86}\text{Rb}^{+}\) was omitted from the incubation medium. Somatic cell ATP-releasing agent (500 \(\mu\)l; Sigma, cat. no. FL-ASC) was added to each well and swirled. Different dilutions of the sample were performed with somatic cell ATP-releasing agent to ensure linearity of the assay. The amount of light emitted was measured immediately using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). A standard curve was constructed using known concentrations of ATP over the linear range of the assay (0–10 nM).

**RESULTS**

*Sch*-28080 and ouabain block \(^{86}\text{Rb}^{+}\) uptake in *mIMCD-3* cells in culture. The results of a representative \(^{86}\text{Rb}^{+}\)-uptake experiment are displayed in Fig. 1. Figure 1A demonstrates that ouabain inhibited \(^{86}\text{Rb}^{+}\) uptake in a dose-dependent manner (IC\(_{50}\) ~30 \(\mu\)M). These results are consistent with the well-known inhibitory effect of ouabain on the renal Na\(^{+}\) pump.
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 \(^{86}\)Rb\(^+\) uptake in a similar dose-dependent manner (IC\(_{50}\) ~ 60 μM). Because Sch-28080 (200 μM) inhibited \(^{86}\)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 \(^{86}\)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 \(^{86}\)Rb\(^+\) uptake was blocked dramatically (as shown in Fig. 1). Removal of ouabain for 15 min at 37°C reestablished \(^{86}\)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 \(^{86}\)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 \(^{86}\text{Rb}^+\) uptake experiments. A representative experiment is displayed in Fig. 5. Ouabain (2 mM) inhibited \(^{86}\text{Rb}^+\) uptake by 50–60% when bumetanide was not present (solid bar). Addition of 200 μM Sch-28080 inhibited \(^{86}\text{Rb}^+\) 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 \(^{86}\text{Rb}^+\) uptake (see Fig. 2). In keeping with this observation in mIMCD-3 cells, we have also observed inhibition of \(^{86}\text{Rb}^+\) uptake by Sch-28080 in mOMCD1 cells and in HEK-293 cells (data not shown). Nevertheless, the inhibitory effect of Sch-28080 on \(^{86}\text{Rb}^+\) uptake described in these cell lines...
did not extend to all cell lines studied. For example, 200 \( \mu M \) Sch-28080 did not inhibit \( ^{86}\text{Rb}^+ \) 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 \( \text{Xenopus laevis} \) (data not shown). In addition, 200 \( \mu M \) 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 \( \mu M \)) in studies in medullary collecting duct cells in culture, or in inner medullary collecting ducts perfused in vitro, to define the role of HK\(_{\alpha 1}\) 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 HK\(_{\alpha 1}\) was defined as inhibition of pHi recovery after a \( \text{Na}_2\text{HCO}_3\) load. In studies in isolated inner medullary collecting ducts perfused in vitro, HK\(_{\alpha 1}\) activity was defined as Sch-28080-inhibitable \( J_{\text{CO}_2} \). 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 \( \mu M \)) or high concentrations (200 \( \mu M \)) 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 \( ^{86}\text{Rb}^+ \) uptake (>90%). In contrast, preincubation with low concentrations of Sch-28080 for 45 min resulted in a reduction of \( ^{86}\text{Rb}^+ \) uptake of only 20%. The data displayed in Fig. 10 (right) reveal that high concentrations of Sch-28080 (200 \( \mu M \)) 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 \( \mu M \)) decreased [ATP]\(_i\) by only 20%. Although the reductions in \( ^{86}\text{Rb}^+ \) 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 Na\(^{+}\)-K\(^{+}\)-ATPase activity in mIMCD-3 cells in culture (Fig. 1). The mechanism of inhibition by ouabain and Sch-28080 differ, however. The inhibitory effect of ouabain was observed in intact cells (Fig. 1), membrane preparations (Fig. 3), and cell lysates (Fig. 4). These results are in agreement with the demonstration that the \(\alpha\_1\)-Na\(^{+}\)-K\(^{+}\)-ATPase contains a binding site for ouabain (7, 26). In contrast, Sch-28080 inhibited \(86\text{Rb}^+\) uptake only in intact mIMCD-3 cells (Fig. 1). Moreover, this inhibitory effect on K\(^{+}\)-ATPase activity disappeared after cellular homogenization when assays were performed in the presence of exogenous ATP (Figs. 3 and 4). This observation suggests an “indirect” effect by Sch-28080 on the Na\(^{+}\) pump through intracellular ATP depletion. In addition, this observation is compatible with the absence of a specific binding site for Sch-28080 on any of the known \(\alpha\)-Na\(^{+}\)-K\(^{+}\)-ATPase subunits (18, 30). Furthermore, our data demonstrate that the inhibitory effect of Sch-28080 on \(86\text{Rb}^+\) uptake is mediated by intracellular ATP depletion (Figs. 6 and 7). This interpretation is in agreement with the observation that Sch-28080 does not decrease Na\(^{+}\)-dependent K\(^{+}\)-ATPase (Na\(^{+}\) pump) activity in cell lysates or membrane preparations.

It is interesting to note, however, that Sch-28080 did not block \(86\text{Rb}^+\) uptake or affect ATP content in all cell lines. Indeed, an effect of Sch-28080 was not demonstrated in DDT-MF-2 or BEAS-2B cells or in oocytes from \textit{X. laevis}. A possible explanation for such a selective effect of Sch-28080 may be differences in cell or mitochondrial membrane permeability to the agent. Namely, if Sch-28080 does not enter the cell or mitochondria, it cannot decrease the intracellular ATP content and, therefore, an effect on \(86\text{Rb}^+\) uptake would not be observed.

Our findings also reveal that ouabain decreased \(86\text{Rb}^+\) 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 \(86\text{Rb}^+\) uptake by \(>90\%\) in the presence or absence of bumetanide. It has been demonstrated previously that low concentrations of Sch-28080 (<10 \(\mu\)M) inhibit HK\(_{\alpha_1}\) activity by binding directly to the \(\alpha\)-subunit (18, 30). In addition, however, Sabolic et al. (24) have reported that Sch-28080 and omeprazole (100 \(\mu\)M) 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 \(\mu\)M), 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 \(J_{\text{CO}_2}\) is increased by chronic hypokalemia in collecting ducts perfused in vitro. This increase is inhibited by low concentrations (~10 \(\mu\)M) of Sch-28080 (19, 28). In addition, Campbell et al. (6) demonstrated that low concentrations of Sch-28080 impaired intracellular pH recovery in RCC-28A cells after a NH\(_4\) load. These results have been interpreted as evidence for a direct effect of Sch-28080 on HK\(_{\alpha_1}\) activity. However, a parallel increase in HK\(_{\alpha_1}\) 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 \(J_{\text{CO}_2}\) 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 \(\mu\)M) does not block \(86\text{Rb}^+\) uptake in mIMCD-3. However, by extending the preincubation time from 15 to 45 min, low concentrations of Sch-28080 (10 \(\mu\)M) inhibit HK\(_{\alpha_1}\) activity by 20% (Fig. 10). We do not know if our observation using the \(86\text{Rb}^+\)-uptake assay can be extrapolated to \(J_{\text{CO}_2}\), or pH\(_2\) 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 HK\(_{\alpha_1}\) 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 \(\alpha_1\)-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 \( \text{J}_{\text{CO}_2} \) or the \( \text{pH}_i \) 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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