Electrophysiological characterization of volume-activated chloride currents in mouse cholangiocyte cell line

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1Department of Medicine, Division of Gastroenterology/Hepatology, Indiana University School of Medicine, and The Richard L. Roudebush Veterans Affairs Medical Center, and Departments of 2Pharmacology and Toxicology and 3Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

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Chen, Biyi, Grant Nicol, and Won Kyoo Cho. Electrophysiological characterization of volume-activated chloride currents in mouse cholangiocyte cell line. Am J Physiol Gastrointest Liver Physiol 287: G1158–G1167, 2004; doi:10.1152/ajpgi.00026.2004.—Recent electrophysiological and radioisotope efflux studies have demonstrated various Cl− channels in cholangiocytes including volume-activated Cl− channels (VACC). Because VACCs play prominent roles in many vital cellular functions and physiology in cholangiocytes, we have examined their electrophysiological characteristics in mouse cholangiocytes to provide an important framework for studying in the future. The present study is to characterize VACCs expressed in the mouse bile duct cell (MBDC) line, conditionally immortalized by SV40 virus. Conventional whole cell patch-clamp techniques were used to study the electrophysiological characteristics of VACC in MBDC. When the MBDCs were exposed to hypotonic solution, they exhibited an outwardly rectified current, which was significantly inhibited by replacing chloride in the bath solution with gluconate or glutamate and by administration of classic chloride channel inhibitors 5-nitro-2-(3-phenylpropylamino)-benzoate, glybenclamide, DIDS, and tamoxifen. These inhibitory effects were reversible with washing them out from the bath solution. Moreover, the ion selectivity of the volume-activated channel to different anions indicates that it is more permeable to SCN− > I− > Cl− > F− > acetate ≥ glutamate ≥ gluconate. These electrophysiological characteristics demonstrate that the volume-activated current observed is a VACC. In addition, the VACC in MBDC has electrophysiological characteristics similar to those of the VACC in human cholangiocarcinoma cell line. The present study is the first to characterize the VACC in mouse cholangiocyte and will provide an important framework for further studies to examine and understand the role of the VACC in biliary secretion and ion-transport physiology.

bile duct cell; cell volume; mouse cholangiocyte; volume-activated chloride channel; ion channel; regulatory volume decrease; patch clamping

Osmoregulation is a vital function of various cells to adapt and maintain cellular homeostasis in response to changes in the local environment and is involved in regulating cell volume, intracellular pH, ion transport, and even gene regulation (16). Under physiological conditions, bile duct cells or cholangiocytes are exposed to various osmotic stresses from the uptake of inorganic and organic solutes and bile secretion (11, 18). A recent study in Mz-ChA-1 human cholangiocarcinoma cell line (25) as well as our study in primary bile duct cell clusters (BDCC) from normal mouse livers (6) indicate that cholangiocytes can regulate their cell volumes back to baseline from swelling induced by exposure to hypotonic solution.

Chloride channels are involved in maintaining ionic homeostasis in a variety of cell types by regulating cell volume and intracellular pH as well as in more specialized functions, such as transepithelial transport and regulation of excitability in the nerve and muscle tissues (22). Functionally, a number of different types of chloride channels that show diverse electrophysiological and regulatory characteristics have been identified. These channels are often categorized by their modes of activation: elevation of the intracellular cAMP or Ca2+ concentration, cell swelling, hyperpolarization, and ligand binding (4, 22). Among these chloride channels, the volume-activated chloride channels (VACC) have been extensively studied due to their vital role in cell physiology and cell volume regulation. VACCs mediate the regulatory volume decrease (RVD) against cell swelling, which involves the extrusion of intracellular K+ and Cl− when the cells are exposed to hypotonic solution (13, 14, 22). The VACCs are shown to play a central role in volume regulation in conjunction with K+ channels (22, 23).

As in other cell types, bile duct epithelial cells have a number of different chloride channels, as demonstrated by various electrophysiological and radioisotope efflux studies in rat and human cholangiocytes (9, 10). An electrophysiological study in the human cholangiocarcinoma cell line (25) and our recent study in freshly isolated BDCCs (6) have shown that cholangiocytes have a RVD when exposed to hypotonic solution, which is mediated by K+ and Cl− conductances (6, 25). The chloride conductance during RVD in human cholangiocarcinoma cell line was mediated by an outwardly rectified chloride current, which had a time-dependent inactivation at depolarizing potentials and was inhibited by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (25). Although these electrophysiological channel characteristics are indicative of VACC, the molecular identity of this chloride channel was not well established. Moreover, no prior electrophysiological study was performed on mouse bile duct cells (MBDC). Thus, in the present study, we have performed more detailed electrophysiological characterization of these chloride conductance pathways using whole cell patch-clamp techniques in a conditionally immortalized primary MBDC line using SV40 virus (24). This work is the first in characterizing VACC in mouse cholangiocytes and should provide a significant framework for further studies of VACC in mouse cholangiocytes.

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MATERIALS AND METHODS

Materials

Bovine serum albumin, penicillin/streptomycin, EGTA, HEPES, DMSO, glybenclamide, tamoxifen, sodium gluconate, l-thyroxine, prostaglandin E1, and hydrocortisone were purchased from Sigma (St. Louis, MO). Matrigel dispase, epidermal growth factor, insulin, transferrin, and selenium were from Collaborative Biomedical (Bedford, MA), and mouse recombinant INF-γ was purchased from Boehringer Mannheim. MEM/F12 with glutamine, minimal essential media non-essential amino acid solution, fetal calf serum, and trypsin were from GIBCO (Grand Island, NY). DIDS was purchased from Molecular Probes (Eugene, Oregon), and NPPB and ionomycin were from Calbiochem (San Diego, CA). Monoclonal anti-cytokeratin 19 antibodies were from Amersham. The SV-40 transformed, conditionally immortalized MBDC line was a generous gift from Dr. Paradis (Montreal, Quebec, Canada).

Solutions

Isotonic and hypotonic solution compositions are as outlined below. The actual osmolalities of the solutions used were determined by a vapor pressure osmometer 5500 (Wescor, Logan, UT).

Bathing solution. 1) Isotonic bathing solution (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 2 MgCl2, 1 KH2PO4, 10 HEPES, and 5 glucose. Osmolarity: 304 mosM, pH adjusted to 7.4 with NaOH. 2) Hypotonic bathing solution (25, 31) (in mM): 100 NaCl, 4 KCl, 1 CaCl2, 2 MgCl2, 1 KH2PO4, 10 HEPES, and 5 glucose. Osmolarity: 225 mosM, pH adjusted to 7.4 with NaOH. 3) Isotonic gluconate bathing solution (in mM): 140 gluconate Na, 4 KCl, 2 MgCl2, 1 CaCl2, 1 KH2PO4, 10 HEPES, and 5 glucose. Osmolarity: 302 mosM, pH was adjusted to 7.4 with NaOH. 4) Hypotonic gluconate bathing solution (in mM): 100 gluconate Na, 4 KCl, 2 MgCl2, 1 CaCl2, 1 KH2PO4, 10 HEPES, and 5 glucose. Osmolarity: 221 mosM, pH was adjusted to 7.4 with NaOH.

Patch Clamp

For the patch-clamp experiments, the cells were plated on matrigel-coated rectangular glass coverslips (2–4 mm) and cultured at 37°C. After the cells were cultured 7–10 days, they were moved to a specimen chamber on an inverted Nikon microscope and were perfused with bath solution at room temperature (23–25°C). Whole cell currents were measured in the conventional configuration of the patch-clamp technique, using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Pipettes were prepared (resistance 4–5 MΩ) from borosilicate capillary tubes (1.0-mm OD, 0.5-mm ID, Sutter Instruments, Novato, CA) using a P-87 Flaming/Brown micropipette puller (Sutter Instruments, San Rafael, CA). The junction potentials were determined by immersing the pipette into the bath filled with pipette solution, zeroing the voltage reading (30). After a gigaseal was formed, the fast compensation system of the amplifier was used to compensate for the intrinsic input capacitance of the head-stage and the pipette. The whole-cell recording configuration was usually established by applying brief suction to disrupt the patch membrane. The membrane capacitance and series resistance were measured as the reference electrode. The current-voltage relationships between -120 and 130 mV were measured by voltage steps in 10-mV increments, 200 ms, 1 Hz, and holding potential of 0 mV in isotonic and hypotonic bathing solutions as indicated. The current was acquired and analyzed using pClamp 6.0 and pClamp 8.0 software (Axon Instruments), respectively. The chloride current amplitude was measured at the end of the 200-ms pulse. For determining the relative permeabilities of various anions, 91% of the chloride in the hypotonic solution was replaced with another anion, and the relative permeabilities were calculated according to the equation (29): \( P_{i}/P_{Cl} = \frac{[Cl]}{[Cl]} \times \frac{E_{rev} - E_{F/RT}}{E_{F/RT}} \times \frac{[Cl]}{[X]} \), where \( E_{rev} \) is the shift of the reversal potential, \([Cl]\) and \([Cl]\) are the extracellular Cl− concentrations in the normal and anion substituted hypotonic solutions, respectively, and \([X]\) is the concentration of the substituting anion X. R is the gas constant, T is absolute temperature, F is Faraday constant.

Characterization of Mouse BDCC

Immunocytochemistry using cytokeratin (CK)-19 or CFTR antibody (7, 19, 28) was performed in MBDCs. Immunofluorescent images were obtained using Olympus IX-71 inverted fluorescent microscope (Olympus America, Melville, NY) with a cooled charge-coupled device video camera (Hamamatsu Photonics Systems, Bridgewater, NJ) connected to a Power Mac computer with image-analysis software (Improvision, Boston, MA).

RESULTS

Characterizations of MBDCs

Viability of the BDCCs was >95%, as assessed by trypan blue exclusion, 24–72 h after culture. As with normal mouse isolated bile duct units and BDCCs characterized previously (6, 7), these MBDCs were characterized extensively (24). They were identified as bile duct epithelial cells by positive immunocytochemistry using a CK-19 antibody, whereas negative controls with secondary antibody alone were consistently negative for immunostaining. In addition, these MBDCs had a bright immunostaining with CFTR antibody, whereas the negative controls had no significant CFTR immunostaining.
Membrane Current Recordings in Isotonic and Hypotonic Solution

To study the effect of hypotonic challenge on the membrane currents in MBDCs, the currents were measured in isotonic solution then again after switching the bathing solution to hypotonic solution. Figure 1A shows a typical membrane current record and current-voltage relationship, and Fig. 1B shows time course of the membrane current. As shown in Fig. 1A, the voltage step elicited baseline currents that were small, outwardly rectified current with a reversal potential of $-0.5 \pm 14.5$ mV ($n = 4$, vs. calculated value of $-1$ mV). When the MBDCs were exposed to hypotonic solution, significantly ($n = 6$, $P < 0.01$) larger currents ($2,771.7 \pm 866.2$ pA in hypotonic solution vs. $234.2 \pm 87.9$ pA in isotonic solution) were observed within 3–5 min and reached a peak within 5–10 min (Fig. 1B) and had the same outwardly rectified configuration with time-dependent inactivation (Fig. 1A) as with the basal currents. The amplitude of the initial currents recorded at $+120$ mV was $-2.2$ times that of the currents recorded at $-120$ mV, and their reversal potential shifted slightly rightward ($9.8 \pm 4.9$ mV, $n = 4$, vs. calculated value of 6.8 mV) from baseline of 0 mV. When the cell was reexposed to isotonic solution, these volume-activated currents returned back to baseline level. The summary of these results is presented in Fig. 1C, which shows that hypotonic challenge significantly stimulated membrane currents in MBDCs and these volume-activated currents returned back to basal level when the cells were reexposed to isotonic solution.

Effect of Cesium on Volume-Activated Current

To determine the role of potassium in the observed membrane currents during the hypotonic challenge, the currents were measured after replacing potassium with cesium in the pipette solution. As shown in Fig. 2A, an exposure of MBDCs

Fig. 1. Volume-activated current in SV40 transformed mouse bile duct cells. Whole cell currents were measured using isotonic standard bathing solution or hypotonic solution and standard pipette solution. A: currents measured using voltage steps from $-120$ to $130$ mV in 10-mV increments, 200 ms, 0-mV holding potential. B: time course of current activated by hypotonic solution, 0-mV holding potential, 120-mV voltage step, for 200 ms. C: summary of currents activated by hypotonic solution ($n = 6$, means $\pm$ SD, **$P < 0.01$ vs. isotonic, ##$P < 0.01$ vs. hypotonic).
with the intracellular potassium replaced with cesium to hypo-
tonic solution also induced significantly \( P < 0.05 \) vs. isotonic control) large outwardly rectified currents. These large outwardly rectified currents also showed little time-dependent inactivation. The current-voltage \((I-V)\) relationship curve (Fig. 2A) shifted leftward when changing bathing solution from isotonic solution to hypotonic solution, while the reversal potential was shifted rightward \((5.4 \pm 2.9 \text{ mV}, n = 5, \) vs. calculated \(6.8 \text{ mV}\)). When the cells were re-exposed to the isotonic solution, the currents returned to basal level \((P < 0.001\) vs. hypotonic), and \(I-V\) relationship curve shifted back rightward. Figure 2B shows the time course of the volume-activated current. When the MBDC was exposed to hypotonic solution, the depolarizing steps elicited a large outward current, reaching the maximum at 5–10 min, then the currents returned to basal level within 5–10 min after reexposing the cells to isotonic solution. Figure 2C shows the mean values of the effect of hypotonic challenge on the cesium-insensitive currents, which are most consistent with VACCs. The currents increased approximately sixfold \((P < 0.01)\) when the cells were exposed to hypotonic solution, and the currents returned to the basal level \((P < 0.01\) vs. hypotonic, \(P > 0.05\) vs. isotonic solution control).

**Effect of Replacing Extracellular Chloride with Gluconate on Volume-Activated Current**

To determine the role of chloride in the observed membrane currents during the hypotonic challenge, the effect of replacing extracellular chloride with gluconate was examined (Fig. 3). Figure 3A shows the current traces and \(I-V\) relationship, and Fig. 3B shows the time course of currents. Under basal conditions in isotonic solution, the voltage step protocol elicited a small current with a reversal potential near zero \((2.1 \pm 5.6 \text{ mV}\),

\[ 5.4 \pm 2.9 \text{ mV}, n = 5, \text{ vs. calculated } 6.8 \text{ mV} \]
n = 4, vs. calculated −1 mV), but in hypotonic solution, these voltage steps again induced a large increase of whole cell current (P < 0.01 vs. control isotonic), and the reversal potential shifted rightward (14.0 ± 5.2 vs. calculated 6.8 mV). However, when the cells were exposed to the hypotonic solution with gluconate replacing chloride, the currents were decreased significantly (P < 0.01 vs. hypotonic solution) by ~70% and the reversal potential further shifted significantly rightward (42.5 ± 4.8 mV at extracellular [Cl⁻] = 10 mM, n = 4) but not as much as expected (calculated 67 mV). This discrepancy is most likely due to some permeability of gluconate via the chloride channel. When the cells were reexposed to hypotonic solution containing chloride, the currents returned promptly to the previous high level and the reversal potential also shifted leftward. Again, when the cells were exposed to isotonic solution, the currents returned to the basal level (P < 0.01 vs. hypotonic solution, P > 0.05 vs. control isotonic solution) and the reversal potential also returned to near zero.

Figure 3C is of the mean values of the effect of replacing extracellular chloride with gluconate in the bathing solution on the outwardly rectified chloride currents, showing that the currents were reduced ~70% when extracellular chloride was replaced with gluconate (P < 0.01 vs. hypotonic solution), indicating the significant role of chloride in the volume-activated currents observed.

Effect of Varying Extracellular Chloride Concentration on Volume-Activated Current

To further examine the dependence of the volume-activated currents on chloride, the effect of varying extracellular chloride concentrations by isomolar substitution with glutamate on the volume-activated currents and on the changes in the E_rev were studied as shown in Fig. 4. When the MBDCs were exposed to five different concentrations of external chloride from 110 to 10 mM with isomolar substitution with glutamate, the volume-
activated current amplitude decreased corresponding to the change in extracellular chloride concentration (Fig. 4A). Figure 4B shows the relationship between extracellular chloride concentration and $E_{\text{rev}}$, of volume-activated currents obtained from a number of MBDCs ($n = 5$). The line with solid circles represents a theoretical line of $E_{\text{rev}}$, calculated from the Nernst equation assuming that chloride is the only permeable ion, whereas the line with open circles represents the actual $E_{\text{rev}}$ measured at the different extracellular chloride concentration. The experimentally obtained $E_{\text{rev}}$ closely follows the predicted $E_{\text{rev}}$ for changes in extracellular chloride concentration of $>30$ mM but deviates slightly from the predicted $E_{\text{rev}}$ at extracellular chloride concentrations $<30$ mM, likely suggesting that glutamate may have small and limited permeability through these channels.

In addition, the relative anion permeability was determined by replacing 91% of chloride with other anions in the hypotonic solutions, then calculating the shifts in the reversal potential as described in the MATERIALS AND METHODS (29). As shown in Fig. 4C, the relative permeabilities ($P_x/P_{Cl}$) of 91% NaCl in the bath solution was replaced entirely by the same concentration of NaSCN, NaI, NaF, sodium acetate, sodium glutamate, and sodium gluconate. D: representative current traces with Cl$^-$ and glutamate in the bath solution. $^*P < 0.05$, $^{**}P < 0.01$; values are presented as means $\pm$ SD, $n = 4$.

**Effect of Chloride Channel Blockers on Chloride Currents**

To further identify the outwardly rectified current, the effect of various chloride channel blockers, NPPB (40 $\mu$M), DIDS (250 $\mu$M), glybenclamide (100 $\mu$M), and tamoxifen (2 $\mu$M),
on the volume-activated currents were studied as shown in Fig. 5. Figure 5A shows representative traces and mean value of the effect of 40 μM NPPB on the outward currents. The currents decreased significantly (*P < 0.01 vs. control) when the cells were perfused with hypotonic solution containing NPPB (40 μM), but the currents returned to high levels when the cells were perfused with hypotonic solution without NPPB (*P < 0.01 vs. NPPB, P > 0.05 vs. control). Figure 5B shows representative traces and the mean value of the effect of DIDS (250 μM) on the outward chloride currents; the currents were significantly (**P < 0.05 vs. control) inhibited by 250 μM DIDS, and the currents returned to normal levels when using hypotonic solution to wash (**P < 0.01 vs. DIDS, **P < 0.01 vs. control). DIDS blocked VACC almost exclusively at positive potentials, whereas the inhibition of VACC by NPPB had no significant voltage dependence (Fig. 6). These findings indicate that NPPB is a voltage-independent inhibitor of VACC, whereas DIDS is a voltage-dependent inhibitor. Figure 5C shows representative traces and the mean value of the effect of glybenclamide on outward chloride current (means ± SD, n = 4–6, *P < 0.05, **P < 0.01 vs. NPPB, DIDS, glybenclamide, or tamoxifen).
tonic challenge had a marked inhibition on the volume-activated current, providing other convincing evidence that the observed volume-activated current is, in fact, a VACC.

**DISCUSSION**

As previously shown (24), the MBDC cell line used for the present study is phenotypically normal murine cholangiocytes, which are conditionally immortalized by SV40 virus, and had the expected protein expression for bile duct-specific cytokeratin-19, confirming its biliary origin, and formed ductlike structures when grown in high density. A previous electron microscopic study (24) also indicated that these cells are well differentiated and polarized. Therefore, apart from their ability to proliferate at a permissive temperature, the MBDCs exhibit normal, differentiated phenotypes of bile duct epithelial cells. Thus, considering the known limitations working with mouse cholangiocytes due to difficulty in cell isolation and culture, this well-differentiated, polarized MBDC line provides an invaluable cell model to study murine bile duct biology and physiology.

Specifically, this study presents the first detailed electrophysiological characterization of the VACCs in mouse cholangiocytes. Recently, we have shown that normal mouse cholangiocytes have an intact RVD, which is dependent on both Cl⁻ and K⁺ conductances (6), as previously reported in other cell types (2, 12, 20, 26) as well as in Mz-ChA-1 cholangiocarcinoma cell lines (Table 1) (25). However, the chloride conductances mediating the RVD seen in mouse cholangiocytes are not well characterized. In the present study, the electrophysiological characterizations of MBDCs using whole cell patch-clamping technique indicate that the MBDCs have VACCs with similar electrophysiological characteristics to those reported in the literature (22, 23, 25). The VACCs in many cell types have a number of typical electrophysiological characteristics (22). Their currents are low in amplitude in isotonic conditions but are activated by hypotonic challenge as shown in the MBDC in Fig. 1. In addition, the VACC currents show an outward rectification with a varying degree of time-dependent inactivation at depolarizing potentials depending on cell types (17, 22). Likewise, the volume-activated currents in MBDCs show expected outwardly rectified currents but have a minor degree of time-dependent inactivation as shown in Fig. 1. As reported in the literature (15, 33), they have an anion permeability sequence of SCN⁻ > I⁻ ≥ Cl⁻ > F⁻ ≥ acetate ≥ glutamate ≥ gluconate as shown in Fig. 4. These electrophysiological characterizations of the observed chloride channels are consistent with VACCs.

Furthermore, the inhibitor pharmacology of the VACC also confirms that they are VACCs. Conventional chloride channel blockers such as NPPB and DIDS significantly inhibited the
In addition, our present findings indicate that the results from this conditionally transformed MBDC are quite comparable with those from a human cholangiocarcinoma cell line. As shown in Table 1, the electrophysiological characteristics of VACCs in MBDCs are quite comparable with those from Mz-ChA-1 cholangiocarcinoma cell line (25). As expected, the VACCs in both human cholangiocarcinoma cell and MBDC are activated with hypotonic challenge and exhibit outwardly rectified chloride current, which has little time-dependent inactivation but is inactivated at high depolarization voltages. The reversal potentials for both cell lines in isotonic solutions are 0 mV, whereas those in hypotonic solution are slightly different from those reported for the human cholangiocarcinoma cells in the literature (25). However, in our hands, the results from the same human cholangiocarcinoma cells obtained from the same investigators are quite similar to those from MBDCs.

The chloride inhibitor profiles are also consistent with those of VACCs reported in the literature. Therefore, the present study provides the first confirmation and detailed characterization of VACCs in mouse cholangiocytes involved in RVD. The results showed that the MBDCs contain an outwardly rectified VACC, which is activated by hypotonic challenge, has characteristic reversal potential and permeability sequence of VACC, and is inhibited by chloride replacement in the perfusion solution and by classic chloride channel inhibitors such as NPPB, glybenclamide, DIDS, and tamoxifen. Considering various vital functions of the VACC in cholangiocytes in cell volume regulation, bile secretion, pH regulation, etc., the present study provides an important framework to further understand this important chloride channel. Moreover, recent findings of an impaired cell volume regulation in murine cholangiocytes from cystic fibrosis knockout mouse livers (8) impart further importance and impetus to study the role of cell volume regulation and biliary secretion. Such study is necessary to understand and treat various biliary liver diseases such as cystic fibrosis liver disease by potentially providing a method to modulate the VACCs in the biliary epithelium to provide an alternative chloride conductive pathway to enhance biliary secretion and/or help compensate for the impaired cell volume regulation necessary for correcting pathological alterations in cholangiocytes resulting from the CFTR defect.

**ACKNOWLEDGMENTS**

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**GRANTS**

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**Table 1. Comparison of volume-activated chloride currents in immortalized mouse cholangiocyte cell line and human cholangiocarcinoma cell line Mz-Cha-1**

<table>
<thead>
<tr>
<th>Reversal potential</th>
<th>MBDC</th>
<th>Mz-Cha-1</th>
<th>Mz-Cha-1 (25)</th>
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<tr>
<td>Isotonic 100 mV</td>
<td>247</td>
<td>285</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Hypotonic 100 mV</td>
<td>2,834±888.9 pA</td>
<td>3,453±419.8 pA</td>
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<tr>
<td>Outwardly rectifying</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>NPPB (40 μM)</td>
<td>49%</td>
<td>63%</td>
<td>80% (100 μM)</td>
</tr>
<tr>
<td>DIDS (250 μM)</td>
<td>78%</td>
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</tr>
<tr>
<td>Glybenclamide (100 μM)</td>
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<tr>
<td>Tamoxifen (2 μM)</td>
<td>73%</td>
<td>94%</td>
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Values for hypotonic amplitude are means ± SD. MBDC, mouse bile duct cell; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate.
REFERENCES


