Characters of Hemichannel-Mediated Substrate Transport in Human Retinal Pigment Epithelial Cells under Deprivation of Extracellular Ca$^{2+}$

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Retinal pigment epithelial (RPE) cells form the outer blood–retinal barrier (BRB) and regulate drug/compound exchange between the neural retina and blood in the fenestrated blood vessels of retinal choroid via membrane transporters. Recent studies have elucidated that RPE cells express hemichannels, which are opened by extracellular Ca$^{2+}$ depletion and accept several drugs/compounds as a transporting substrate. The objective of this study was to elucidate the hemichannel-mediated compound transport properties of the outer BRB. In human RPE cells, namely ARPE-19 cells, time-dependent uptake of fluorescent hemichannel substrates, such as Lucifer Yellow, sulforhodamine-101 (SR-101), and propidium iodide (PI) was promoted under Ca$^{2+}$-depleted conditions. The uptake of these substrates under Ca$^{2+}$-depleted conditions exhibited saturable kinetics with a Michaelis–Menten constant ($K_m$) of 87–109 µM. In addition, SR-101 and PI uptake by ARPE-19 cells was dependent on extracellular Ca$^{2+}$ concentration, and that under Ca$^{2+}$-depleted conditions was significantly decreased by typical substrates and/or inhibitors for hemichannels. Moreover, Ca$^{2+}$-depleted conditions promoted the efflux transport of calcine from ARPE-19 cells, and the promoted calcine efflux transport was significantly inhibited by a typical hemichannel inhibitor. These results suggested that hemichannels at the outer BRB were involved in the influx and efflux transport of drugs/compounds.

**Key words** calcium; hemichannel; outer blood–retinal barrier; connexin 43; retinal pigment epithelial cell

INTRODUCTION

Retinal pigment epithelial (RPE) cells are located in the posterior of the eye, and they form the outer blood–retinal barrier (BRB). In the outer BRB, paracellular transport is restricted through formation of tight junctions between RPE cells. Previous reports have shown that several kinds of influx and efflux membrane transporters are expressed in RPE cells, and thus are involved in the selective exchange of compounds/drugs across the outer BRB. For example, hydrophilic vitamin B2 and C are reported to be transported into rat RPE cells via solute carrier (SLC) 52A and SLC23A, respectively. Kennedy and Mangini have reported that P-glycoprotein, an adenosine triphosphate-binding cassette efflux transporter, is expressed on the apical and basolateral membrane of human RPE cells. It has been known that the outer BRB separates the neural retina from the vascularized choroid, which provides four-fifth of blood supply to the retina. Therefore, it is conceivable that membrane transport systems at the outer BRB play an important role in the exchange of drugs/compounds between the retina and circulating blood, and are involved in retinal pharmacology and drug toxicology.

Hemichannels have been known as the membrane transport systems that are activated under pathological conditions. Hemichannels are formed as a hexamer of pannexin (Px) and/or connexin (Cx). In the pyramidal neurons, it has been reported that Px1 is opened under oxygen/plus.oxidative stress conditions. In a human cell line of brain microvascular endothelial cells, influx and efflux transport of various anionic and cationic fluorescent dyes via Px1 and Cx43 has been evaluated. With regard to the expression and function of hemichannels at the outer BRB, functional expression of Px has been implied since it was reported that Panx, known as an inhibitor for Px, inhibited promotion of several genes which relate to inflammatory responses in the RPE cells under several pathological conditions. In addition, Cx43 is reported to be expressed in RPE cells. Our previous study demonstrated that extracellular Ca$^{2+}$ depletion promotes influx transport of anionic fluorescent dyes, namely Lucifer Yellow (LY) and SR-101, in a human RPE cell line, ARPE-19 cells. In addition to the attenuation of the uptake of LY and SR-101 in the presence of typical hemichannel inhibitors under extracellular Ca$^{2+}$-free conditions, it is suggested that Cx43 is a major contributor to LY uptake under conditions of extracellular Ca$^{2+}$ depletion from the studies of RNA interference for Cx43 in the previous study. Taken together, hemichannel-mediated transport of drugs/compounds under pathological conditions is considered to occur in RPE cells. In in vitro and in vivo models of retinal ischemia and age-related macular degeneration, Cx hemichannels including Cx43 were reported to be opened. Hence, it is expected that characteristics of hemichannels in the RPE cells lead to deep understanding of pathophysiology and pharmacotherapy for retinal diseases.

It has been reported that hemichannels have been considered to nonspecifically accept organic and inorganic compounds under a molecular mass of 1000 g/mol. However, several reports showed the compound selectivity of hemichannel-mediated compound transport. The characterization of hemichannels in the RPE cells is expected to improve understanding of pathophysiology and pharmacotherapy for retinal diseases.

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teristics of both anionic and cationic drug/compound transport via hemichannels including Cx43 have been a crucial issue in understanding drug/compound transport at the outer BRB under pathological conditions. So far, the information of hemichannel-mediated influx and efflux transport of compounds is not enough although our previous study elucidated that Cx43 plays a major role in influx transport of LY via hemichannels at the outer BRB. Therefore, the objective of this study was to elucidate hemichannel-mediated compound transport properties at the outer BRB. It has been reported that extracellular Ca\(^{2+}\) depletion, which is known to partially reflect cerebral ischemia, induces the activation of hemichannel-mediated compound transport in *in vitro* several cell lines. Therefore, the objective of this study was to elucidate hemichannel-mediated compound transport properties at the outer BRB. Moreover, the drugs which are interacted with hemichannels at the outer BRB were examined by measuring the alteration of hemichannel-mediated transport in ARPE-19 cells.

**MATERIALS AND METHODS**

**Reagents** Carbenoxolone (Cbx) disodium salt, PI, and SR-101 were obtained from Merck (Darmstadt, Germany). Calcium chloride and L-glutamate (L-Glu) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient Mixture F-12 (DMEM/F12) and LY were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Calcein-acetoxyethyl ester (calcein-AM) and *O*,*O*-bis(2-aminoethyl)-ethylenglycol- *N*,*N*,*N*,*N*'-tetraacetic acid (EGTA) were purchased from Dojindo (Kumamoto, Japan). All other chemicals, such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and sodium valproate, were of analytical grade and purchased from Dojindo, FUJIFILM Wako Pure Chemical Corporation, Merck, MP Biomedicals (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Thermo Fisher Scientific.

**Cell Culture** ARPE-19 cells (American Type Culture Collection; Manassas, VA, U.S.A.) were cultured at 37°C in DMEM/F12 supplemented with 100 U/mL benzylenicillin potassium, 100 μg/mL streptomyacin sulfate, 20mM sodium bicarbonate, and 10% fetal bovine serum, which was purchased from Moregate (Bulimba, Australia), in a humidified atmosphere of 5% CO\(_2)/air.

**Dye Uptake** ARPE-19 cells were seeded onto a collagen I-coated 24-well plate at a density of 7.0 \(\times\) 10\(^4\) cells/cm\(^2\) for 37°C and 2d. The cells were washed at 37°C with regular retinal interstitial fluid (RIF) buffer at pH 7.4 containing (in mM) 1.4 CaCl\(_2\), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–NaOH, 3 KCl, 0.4 K\(_2\)HPO\(_4\), 1.2 MgSO\(_4\), 122 NaCl, 25 NaHCO\(_3\), and 10 sucrose, and then preincubated with several cell lines. In this study, influx transport of SR-101 and PI, in addition to LY, and PI at a concentration of 20, 10, and 50 μM, respectively, was started at 37°C by applying 200 μL of regular RIF buffer, CaCl\(_2\)-removed (Ca\(^{2+}\)-free) RIF buffer, or MgSO\(_4\)-removed (Mg\(^{2+}\)-free) to ARPE-19 cells were prepared by solubilizing the cells as well as measurements of protein concentration and fluorescent intensity (FI) derived from calcein (\(\lambda\_\text{em} = 490 \text{ nm}, \lambda\_\text{ex} = 515 \text{ nm}\)) were performed as described in the previous subsection. PI in the incubated buffer and cell-solubilized solution was normalized by the cellular protein amount (FI/mg protein), and outflow of calcein was calculated according to Eq. 1.

\[
\text{Outflow} (\%) = \frac{\text{Extracellular calcein (FI/mg protein)}}{\text{Intracellular calcein (FI/mg protein)} + \text{extracellular calcein (FI/mg protein)}} \times 100
\]

**Statistical Analysis** To obtain the kinetic parameters, namely maximum influx transport rate (\(V_{\text{max}}\)) and Michaelis–Menten constant (\(K_m\)), the data of concentration-dependent dye uptake were fitted to Eq. 2:

\[
V = V_{\text{max}} \times S / (K_m + S)
\]

where \(S\) and \(V\) are the dye concentration and the influx transport rate at \(S\), respectively. Half-maximal effective concentration (EC\(_{50}\)) of extracellular Ca\(^{2+}\) was obtained using Eq. 3:

\[
CL = CL_{\text{min}} + (CL_{\text{max}} - CL_{\text{min}}) / (1 + [\text{Ca}^{2+}] / EC_{50})
\]

where [Ca\(^{2+}\)], \(CL\), \(CL_{\text{max}}\), and \(CL_{\text{min}}\) are the extracellular Ca\(^{2+}\) concentration, the activity of cellular dye accumulation at [Ca\(^{2+}\)], the maximum of the cellular dye accumulation activity, and the minimum of the cellular dye accumulation activity, respectively. Nonlinear least-squares regression analysis was utilized for the curve fitting, as described previously. These kinetic parameters were represented as mean ± standard deviation.

The other data were expressed as mean ± standard error of the mean (S.E.M). Statistical differences between the means of two groups were determined using unpaired two-tailed Student’s *t*-test, whereas those between the means of more than two groups were analyzed using ANOVA followed by the Tukey–Kramer method or Dunnett’s test.
RESULTS

Uptake Manner of LY, SR-101, and PI in ARPE-19 Cells under Extracellular Ca$^{2+}$-Free Conditions

Figure 1 shows the time-profile of LY, SR-101, and PI uptake by ARPE-19 cells under normal (open circles) or Ca$^{2+}$-free conditions (closed squares). Uptake of LY (Fig. 1A) and SR-101 (Fig. 1B) by ARPE-19 cells under Ca$^{2+}$-free conditions significantly increased for 45 and 60 min by at least 1.84- and 2.30-fold, respectively, compared with that under normal conditions. PI uptake (Fig. 1C) under Ca$^{2+}$-free conditions was significantly elevated for the tested times, namely 15, 30, 45, and 60 min, by 3.58-, 3.44-, 4.34-, and 9.98-fold, respectively, compared with that under normal conditions. In the absence of extracellular Ca$^{2+}$, the uptake of LY (Fig. 2A), SR-101 (Fig. 2B), and PI (Fig. 2C) by ARPE-19 cells comprised of a single saturable component. The obtained $K_m$ and $V_{max}$ values for the uptake are summarized in Table 1.

![Fig. 1. Time-Course of Hemichannel Substrate Uptake by ARPE-19 Cells](image1)

Uptake of LY (A, 20μM), SR-101 (B, 10μM), and PI (C, 50μM) by ARPE-19 cells was tested at 37°C in the presence (open circles) or absence (closed squares) of extracellular Ca$^{2+}$ (1.4mM). Each point represents mean ± S.E.M. ($n = 3–6$). * and ** express statistically significant differences from the data at the respective uptake time in the presence of extracellular Ca$^{2+}$ at $p < 0.05$ and $p < 0.01$, respectively.

![Fig. 2. Concentration-Dependent Uptake of Hemichannel Substrates by ARPE-19 Cells](image2)

Uptake of LY (A), SR-101 (B), and PI (C) by ARPE-19 cells was tested at the indicated concentration for 60min (A) or 45min (B, C) at 37°C in the absence of extracellular Ca$^{2+}$. Obtained data were subjected to Michaelis–Menten and Eadie–Scatchard (inset) analyses, and fitted by nonlinear least-square regression analysis (solid line). The unit of $V/S$ is μL/(mg protein·60 min) (A) or mL/(mg protein·45 min) (B, C). Each point represents mean ± S.E.M. ($n = 3$).

![Table 1. $K_m$ and $V_{max}$ Values for the Uptake of Hemichannel Substrate by ARPE-19 Cells under Ca$^{2+}$-Free Conditions](table1)

| Substrate | $K_m$ (μM) | $V_{max}$ (pmol/(mg protein·60 min)) |
|-----------|------------|------------------------------------|
| LY        | 95.1 ± 21.5 | 713 ± 121                         |
| SR-101    | 87.2 ± 28.8 | 4.03 ± 0.83                       |
| PI        | 109 ± 48    | 1310 ± 420                       |

Each value represents mean ± standard deviation.

Extracellular Divalent Cation-Dependent Promotion of SR-101 and PI Uptake by ARPE-19 Cells

Figure 3 shows the effect of extracellular Mg$^{2+}$ or Ca$^{2+}$ depletion on the uptake of SR-101, which is an anionic hemichannel substrate as well as LY, and PI (a cationic hemichannel substrate) by ARPE-19 cells. SR-101 uptake by ARPE-19 cells under Ca$^{2+}$-free conditions was significantly 2.35- and 2.53-fold greater than that under normal and Mg$^{2+}$-free conditions, respectively (Fig. 3A). In contrast, SR-101 uptake under Mg$^{2+}$-free conditions was not significantly altered compared with that under normal conditions (Fig. 3A). In addition, extracellular Ca$^{2+}$ depletion increased PI uptake by ARPE-19 cells compared with that under normal conditions and under extracellular Mg$^{2+}$ depletion by 18.2- and 16.8-fold, respectively (Fig. 3B). On the contrary, there was no significant difference in PI uptake between normal and Mg$^{2+}$-free conditions (Fig. 3B).

The increase in SR-101 and PI uptake by ARPE-19 cells was inversely dependent on the extracellular Ca$^{2+}$ concentration (Figs. 4A, B). The EC$_{50}$ of extracellular Ca$^{2+}$ concentration for the inverse dependency of SR-101 and PI uptake was calculated to be 106 ± 85 and 104 ± 38 μM, respectively.

Inhibition of SR-101 and PI Uptake by ARPE-19 Cells in the Presence of a Substrate/Inhibitor of Hemichannels

Figure 5 shows the effect of a substrate and inhibitor of hemichannels on SR-101 and PI uptake by ARPE-19 cells under Ca$^{2+}$-free conditions. i-Glu is widely known as a typical substrate of hemichannels including Cx43.

$^{[22,23]}$ Cbx is known to be an inhibitor of Px1, Px2, Cx26, Cx38, and Cx43.$^{[18,24,25]}$ In our previous study, uptake of SR-101 by ARPE-19 cells under
extracellular Ca\(^{2+}\)-free conditions was significantly decreased in the presence of 1 mM Cbx and 2 mM 1-octanol.\(^{14}\) As shown in Fig. 5A, increased SR-101 uptake by ARPE-19 cells under extracellular Ca\(^{2+}\)-free conditions compared with the SR-101 uptake under normal conditions was significantly decreased by 60.0 and 45.1% in the presence of Cbx and L-Glu, respectively, at 200 \(\mu\)M. PI uptake under Ca\(^{2+}\)-free conditions was significantly decreased by 79.1 and 31.9% compared with that in the presence of extracellular Ca\(^{2+}\) (Fig. 6B). In the presence of 200 \(\mu\)M L-glutamate (L-Glu) in the same as that under normal conditions, which is indicated as Ca\(^{2+}\)-free (closed square). Each point represents mean ± S.E.M. ** expresses a statistically significant difference from the data at the respective uptake time in the presence of extracellular Ca\(^{2+}\) at \(p < 0.01\). (A) Outflow of calcein from ARPE-19 cells was tested at 37°C after incubation with regular RIF buffer (open circle) and Ca\(^{2+}\)-free RIF buffer with 2 mM EGTA (closed square). Each point represents mean ± S.E.M. ** expresses a statistically significant difference from the data at the respective uptake time in the presence of extracellular Ca\(^{2+}\) at \(p < 0.01\). (B) The outflow of calcein at 37°C for 60 min was tested after incubation with regular RIF buffer (Ca\(^{2+}\)-free) of 1.4 mM extracellular Ca\(^{2+}\) in the absence or presence of 1 mM carbonoxolone (Cbx). Each point or column represents mean ± S.E.M. (\(n = 3–6\)). **, significant difference according to Tukey–Kramer test at \(p < 0.01\).

**Fig. 3.** Effect of Extracellular Divalent Cation on the Uptake of SR-101 and PI by ARPE-19 Cells

Uptake of SR-101 (A, 10 \(\mu\)M) and PI (B, 50 \(\mu\)M) was tested at 37°C for 45 min in the presence of extracellular Ca\(^{2+}\) and Mg\(^{2+}\), or absence of extracellular Mg\(^{2+}\) (1.2 mM) or Ca\(^{2+}\) (1.4 mM). Each column represents mean ± S.E.M. (\(n = 3\)). **, significant difference according to Tukey–Kramer test at \(p < 0.01\).

**Fig. 4.** Concentration-Dependent Effect of Extracellular Ca\(^{2+}\) on the Uptake of SR-101 and PI by ARPE-19 Cells

Uptake of SR-101 (A, 10 \(\mu\)M) and PI (B, 50 \(\mu\)M) was tested at 37°C for 45 min under normal conditions and with that in the presence of 1 mM Cbx and L-Glu, respectively (Fig. 6A). In addition, this calcein activity of calcein, which is metabolized from calcein-AM by intracellular esterases, was evaluated. The outflow of calcein, after 30-min incubation with calcein-AM, the efflux transport activity of calcein, which was metabolized from calcein-AM by intracellular esterases, was evaluated. The outflow of calcein to the extracellular compartment for 40 and 60 min under extracellular Ca\(^{2+}\)-free conditions significantly increased to 1.27- and 1.35-fold, respectively (Fig. 6A). In addition, this calcein outflow for 60 min was significantly decreased by 18.9% in the presence of 1 mM Cbx (Fig. 6B).

**Fig. 5.** Effect of Typical Hemichannel Inhibitors on the Uptake of SR-101 and PI by ARPE-19 Cells

Uptake of SR-101 (A, 10 \(\mu\)M) and PI (B, 50 \(\mu\)M) was tested at 37°C for 45 min in the presence (Ca\(^{2+}\)-+) or absence (Ca\(^{2+}\)-free) of 1.4 mM extracellular Ca\(^{2+}\) with or without 1 mM carbonoxolone (Cbx) or 200 \(\mu\)M L-glutamate (L-Glu). Each column represents mean ± S.E.M. (\(n = 3–9\)). * and **, significant differences from the control according to Dunnett’s test at \(p < 0.05\) and \(p < 0.01\), respectively. N.S., no significant difference from the control.

**DISCUSSION**

Our results showed that the influx and efflux transport of fluorescent dyes, such as LY, SR-101, PI, and calcein, in human RPE cells were dependent on extracellular Ca\(^{2+}\) levels (Figs. 1–6, Table 1), suggesting hemichannel-mediated trans-
and PI was not identical to that in the blood–brain barrier. This non-linearity of uptake by ARPE-19 cells under Ca\(^{2+}\) depleted conditions was 3.7- and 4.5-fold greater than that by hCMEC/D3 cells, respectively. Hence, it is considered that compound transport in the outer BRB were different from those in the blood–brain barrier.

It has been reported that the depletion of several divalent cations, such as Mg\(^{2+}\), also stimulates the opening of hemichannels. Verselis and Srinivas have reported that the function of Cx46 hemichannels is changed by alterations in extracellular Mg\(^{2+}\) concentration, in addition to extracellular Ca\(^{2+}\) concentration. Spray et al. have shown that LY uptake by cultured astrocytes in rat hippocampus affects the extracellular levels of Mg\(^{2+}\) and Ca\(^{2+}\). In contrast to these results, the uptake of both SR-101 and PI by ARPE-19 cells was not altered in the absence of extracellular Mg\(^{2+}\), although the uptake of SR-101 and PI significantly increased under depleted extracellular Ca\(^{2+}\) conditions (Fig. 3). This result suggested that hemichannels at the outer BRB were specifically opened by alterations in extracellular Ca\(^{2+}\) level, unlike those in Cx46-overexpressing cells and rat cerebral astrocytes. In addition, uptake of SR-101 and PI by ARPE-19 cells was inversely dependent on extracellular Ca\(^{2+}\) concentration, with an EC\(_{50}\) of 106 and 104\(\mu\)M, respectively, suggesting that the responsiveness of extracellular Ca\(^{2+}\) in hemichannels at the outer BRB was not different between anionic and cationic compounds. In addition, extracellular Ca\(^{2+}\)-dependent uptake of SR-101 and PI by a human blood–brain barrier model has been also reported with an EC\(_{50}\) of 22 and 0.795\(\mu\)M, respectively. As the EC\(_{50}\) value in ARPE-19 cells was greater than that in the human blood–brain barrier model, the opening of hemichannels at the outer BRB may be more sensitive to extracellular Ca\(^{2+}\) alteration than that at the blood–brain barrier. Taken together, these findings suggested that extracellular level of Ca\(^{2+}\) as a divalent metal cation was a main effector of hemichannel-mediated transport of drugs/compounds at the outer BRB.

In addition to the promotion of influx transport of SR-101 and PI (Figs. 1, 3, 5), efflux transport of calcein, an anionic fluorescent dye, increased under Ca\(^{2+}\)-depleted conditions (Fig. 6A). However, the effect of a typical inhibitor of hemichannel, namely Cbx, on the promoted influx and/or efflux transport of these fluorescent dyes was different. The influx and efflux transport of anionic fluorescent dyes, such as SR-101 and calcein, respectively, in ARPE-19 cells under extracellular Ca\(^{2+}\)-depleted conditions significantly decreased in the presence of 1\(\mu\)M Cbx (Figs. 5A, 6B). In contrast, the inhibitory effect of Cbx at 1\(\mu\)M on the uptake of PI, a cationic dye, by ARPE-19 cells under Ca\(^{2+}\)-free conditions was not significant, although the influx transport of both SR-101 and PI was significantly inhibited in the presence of L-Glu, another typical hemichannel substrate (Figs. 5A, B). This result implied that the characteristics of hemichannel-mediated transport of anionic compounds were different from those of cationic compounds. It has been shown that Pxl/Px2, Cx26, and Cx38 are inhibited by Cbx, with IC\(_{50}\) of 5, 21, and 34\(\mu\)M, respectively. Cbx is known to inhibit Cx43, which is expressed in the outer BRB, with an IC\(_{50}\) of 3–100\(\mu\)M. In contrast, a recent report has shown that PI uptake by Cx32-overexpressing cells under Ca\(^{2+}\)-free conditions is not affected.

**Table 2. Effect of Various Compounds/Drugs on SR-101 Uptake by ARPE-19 Cells under Ca\(^{2+}\)-Free Conditions**

| Condition       | n | Conc. (\(\mu\)M) | Percentage of control |
|-----------------|---|-----------------|----------------------|
| Control         | 12| 100 ± 7         |                      |
| CaCl\(_2\) (Ca\(^{2+}\) (+)) | 11| 1400            | 60.0 ± 4.5**         |
| Cbx             | 6 | 200             | 62.4 ± 5.0**         |
| Valproate       | 3 | 200             | 40.6 ± 4.7**         |
| Pyruvate        | 3 | 200             | 57.7 ± 3.0*          |
| Diclofenac      | 3 | 200             | 60.7 ± 5.8           |
| Indoprofen      | 3 | 200             | 63.9 ± 6.1           |
| ATP             | 3 | 200             | 64.6 ± 8.0           |
| Ibuprofen       | 3 | 200             | 69.0 ± 14.5          |
| Nicotinate      | 3 | 200             | 80.3 ± 6.0           |
| Flurbiprofen    | 3 | 200             | 81.0 ± 6.3           |
| Procainicidin   | 3 | 200             | 102 ± 13             |
| Hydrocortisone  | 3 | 200             | 129 ± 15             |
| Loxoprofen      | 3 | 200             | 144 ± 4.9            |
| Estrone         | 3 | 200             | 164 ± 2**            |
| Cilnidipine     | 3 | 200             | 166 ± 24**           |
| Memantine       | 3 | 50              | 211 ± 17**           |
| Amantadine      | 3 | 200             | 216 ± 28**           |

Uptake of SR-101 (10\(\mu\)M) was tested at 37°C for 45 min in the absence (control) of 1.4\(\mu\)M extracellular Ca\(^{2+}\) with or without the compounds/drugs at the indicated concentration. Each value represents mean ± S.E.M. * and **, significant differences from the control in Dunnett’s test at \(p < 0.05\) and \(p < 0.01\), respectively. Cbx, carbencoumole.
by the presence of 100 µM Cbx.

Taking these reports into consideration, it is implied that Cx43 participates in influx and efflux transport of the anionic fluorescent dyes, such as SR-101 and calcine, in addition to LY. On the other hand, it is also considered that the contributing molecules to hemichannel-mediated transport of cationic drugs/compounds including PI at the outer BRB may not be Pxs and Cxs, as described above; moreover, complete understanding of the expression of Pxs/Cxs in RPE cells and the characteristics of drug/compound transport in Pxs/Cxs-overexpressing cells is needed to explain the different inhibitory effect of hemichannel inhibitors on hemichannel-mediated transport at the outer BRB. Nevertheless, the findings of our study suggested that Ca²⁺-depletion induced hemichannel-mediated influx and efflux transport of anionic/cationic compounds in the RPE cells. In addition, it is implied that Cx43 plays an important role in the transport of anionic compounds at the outer BRB.

Several monocarboxylates, namely valproate and pyruvate, also inhibited hemichannel-mediated uptake of SR-101 by ARPE-19 cells (Table 2). Karagiannis et al. have reported that hemichannels mediate the release of t-lactate, a substrate of monocarboxylate transporters/SLC16As, from rat hippocampal slices. Monocarboxylate endogenous compounds and drugs, such as valproate, pyruvate, ibuprofen, and nicotinate, are reported to be substrates/inhibitors for SLC16As. Although the protein expression of MCT1 and 4 in human RPE cells have been indicated, SR-101 uptake under normal conditions was not inhibited by the substrates of SLC16As, such as valproate and pyruvate (Supplementary Table 1). Therefore, the inhibitory effect of valproate and pyruvate on SR-101 uptake under Ca²⁺-free conditions was suggested to be derived from hemichannel inhibition in ARPE-19 cells (Table 2). Regarding valproate, which showed strong inhibitory effect on hemichannels in ARPE-19 cells, it has been reported that retinal damage in rats after ischemia–reperfusion is reduced by continuous subcutaneous administration of valproate. Decreased mitochondria-mediated apoptosis has been proposed as one of the mechanisms of retinal damage reduction by valproate. Cx43 is a potent contributor to hemichannel-mediated transport of anionic compounds in ARPE-19 cells, and is reported to be localized on the apical membrane of the outer BRB. Reigada et al. have reported that Cx43 is involved in the release of ATP. Furthermore, ATP is reported to be released from RPE cells, inducing the death of photoreceptors, which contact RPE cells.

Considering these lines of evidence, inhibition of ATP release from RPE cells via Cx43 hemichannels is proposed as another protective effect of valproate. In summary, it is possible that the functional change of hemichannels at the outer BRB by the drugs is related to the retinal pharmacology.

In conclusion, the characteristics of hemichannel-mediated transport of anionic and cationic drugs/compounds at the outer BRB under Ca²⁺-depleted conditions was revealed in this study. Since it has been suggested that retinal hemichannels are opened under the conditions which reflect several retinal diseases, our results are expected to improve the fundamental understanding of drug/compound transport via hemichannels at the outer BRB in the retinal diseases.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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