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Inhibitors of the $K^+(Na^+)/H^+$ exchanger of human red blood cells

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Abstract

The effect of substances as possible inhibitors of the $K^+(Na^+)/H^+$ exchanger in the human red cell membrane has been tested on the (ouabain + bumetanide + EGTA)-resistant K^+ influx in both physiological (HIS) and low ionic strength (LIS) solution with tracer kinetic methods. It is demonstrated that high concentrations of quinacrine (1 mM) and chloroquine (2 mM) inhibit the residual K^+ influx in LIS solution to 60% and 85%, respectively, but activate it in HIS solution. Thus, chloroquine suppressed the 10-fold LIS-induced activation of the flux nearly completely. Amiloride derivatives were able to inhibit the K^+ influx in both HIS and LIS solution. EIPA (75 μM) reduced the flux by about 20% and 55% in HIS and LIS solution, respectively. Newly developed drugs (HOE 642, 1 mM; HOE 694, 0.5 mM) designed to inhibit Na^+/H^+ exchanger isoforms showed an inhibition of the residual K^+ influx of 40% and 33% in HIS and 65% and 44% in LIS solution, respectively, without haemolysis. The inhibitory effect of HOE 642 persisted in HIS (24%) and LIS (48%) solutions when Cl^- was replaced by $CH_3SO_4^-$. The K^+-Cl^- cotransport inhibitor DIOA (100 μM) stimulated the residual K^+ influx in both solutions. It is, therefore, concluded that the K^+-Cl^- cotransporter does not contribute to the residual K^+ influx both in HIS and LIS media. Okadaic acid decreased the residual K^+ influx by 40% and 25% in HIS and LIS solution, respectively, showing that the residual K^+ influx is affected by phosphatases like other ion transport pathways. The results show that the residual K^+ influx can be decreased further by inhibiting the $K^+(Na^+)/H^+$ exchanger. It remains still unclear to what extent the $K^+(Na^+)/H^+$ exchanger is inhibited by the different substances used. However, the ground state membrane permeability for K^+ is much smaller than assumed so far.
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Keywords: Red blood cells; $K^+(Na^+)/H^+$ exchanger; Ion transport inhibitors; K^+ leak; Membrane permeability

1. Introduction

The problem of how a monovalent cation passes (diffuses) through a biological membrane in the absence of a specific transport system is still a matter of debate. One obvious model system for ion transport studies, including studies on the electrodiffusive mechanism, appeared to be the red blood cell (RBC), which offered many experimental advantages for ion flux measurements. Until quite recently, the best methodological approach to measure the residual ion fluxes, i.e. fluxes persisting after inhibiting and/or not activating existing specific transport pathways (pumps, channels, carriers), across the RBC membrane was to determine the K^+ influx or efflux in the presence of ouabain, bumetanide and EGTA, to suppress the Na^+/K^+ pump, the $Na^+-K^+-2Cl^-$ cotransporter and the Ca^{2+}-activated K^+ channel, respectively [1]. In addition, chloride in the cell suspending solution has to be replaced by nitrate or methylsulphate to inhibit any K^+ flux mediated by the Cl^--dependent K^+-Cl^- cotransport system [2,3]. The K^+-Cl^- cotransport is normally silent under physiological conditions and can be activated by different manoeuvres only [4]. However, nowadays, one has to take into consideration that under these conditions there are still two transport pathways present in the human RBC membrane, which are not affected. This are the non-specific, voltage-activated cation channel [5] and the $K^+(Na^+)/H^+$ exchanger [6,7] both recently identified in the human RBC membrane, and the latter already cloned in humans [8]. The first is activated at positive transmembrane potentials only [5] and, therefore, should not play a substantial role under physiological conditions, i.e. negative transmembrane potentials. The contribution of the second pathway is more complicated to analyse, since no inhibitor is presently available for the

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$K^+(Na^+)/H^+$ exchanger. From previous work, it is known that the $K^+(Na^+)/H^+$ exchanger is remarkably activated in low ionic strength (LIS) solutions [9]. However, at the moment, it remains unclear (i) whether the $K^+(Na^+)/H^+$ exchanger contributes to the residual, i.e. (ouabain + bumetanide + EGTA)-resistant, flux in physiological ionic strength (high ionic strength, HIS) solution at all; and (ii) to what extent it is contributing to the activation of the K^+ flux in LIS solution. The aim of the present work was, therefore, to find inhibitors for the $K^+(Na^+)/H^+$ exchanger and to clarify the possible participation of this pathway in the residual fluxes in both HIS and LIS solutions.

2. Experimental

2.1. Blood and solutions

Freshly drawn blood from healthy human donors was used for the experiments. Red blood cells were separated by centrifugation ($2000 \times g$, 5 min) at room temperature. Plasma and buffy coat were aspirated, and the cells were washed three times with a physiological NaCl (HIS) solution containing (mM): NaCl 145, glucose 10, morpholinoethane sulphonic acid/Tris-(hydroxymethyl)aminomethane (MES/Tris) 10, pH 7.4 at room temperature. The red cells were then washed once in the appropriate medium used for the flux experiment.

To measure fluxes (see below) under LIS conditions, the cells were resuspended in a solution of the following composition (mM): sucrose 200, glucose 10, MES/Tris 10, pH 7.4. The solution that is commonly used to replace 145 mM NaCl contains 250 mM sucrose to maintain the osmolarity. However, the solution used contained only 200 mM sucrose to avoid an initial shrinkage of the cells [7].

Experiments with the phosphatase inhibitors calyculin A and okadaic acid were carried out by a pre-incubation at 37 °C with the substances in physiological HIS solution for 20 min. Then, the cells were washed once in the appropriate medium used for the flux experiment.

In the experiments where Cl^- was replaced by $CH_3SO_4^-$, the cells were treated as described previously [2] in a medium comprising (mM): $NaCH_3SO_4$ 165, glucose 10, MES/Tris 10, pH 7.4. This solution has the same tonicity as the HIS solution [9].

2.2. Measurement of the K^+ influx

Washed erythrocytes were suspended at about 5% haematocrit in a total volume of 1 ml of flux medium (HIS or LIS solution) contained in an Eppendorf 1.5 ml microcentrifuge tube. In all experiments ouabain (100 μM), bumetanide (100 μM) and EGTA (100 μM) were present in the solution during the flux measurement. The cell suspension was equilibrated at the flux temperature of 37 °C for 5 min after which tracer solution ([KCl + ^{86}RbCl]) or ([KCH_3SO_4 + ^{86}RbCl], for Cl^- replacement experiments), 7.5 mM final concentration was added. The duration of exposure of cells to isotope (i.e. the flux time) was 30 min. The isotope uptake was stopped by centrifugation at $15,000 \times g$ (10 s) and the supernatant removed by aspiration. The cells were then washed free of extracellular radioactivity by four successive resuspensions and centrifugations ($15,000 \times g$, 10 s) in ice-cold medium consisting of (mM): $MgCl_2$ 106, *N*-morpholinopropane sulphonic acid (MOPS) 10, pH 7.4. The cell pellet was lysed with 0.5 ml of 1% (v/v) Triton X-100 and the protein precipitated by adding 0.5 ml of 5% (w/v) trichloroacetic acid followed by centrifugation at $15,000 \times g$ for 5 min. The activity of ^{86}Rb in the supernatant was determined by Cerenkov counting in a liquid scintillation analyser (Packard Tri-Carb 2900). Haemoglobin content of red cell suspensions was determined as cyanmethhaemoglobin using Drabkin's reagent. If haemolysis occurred during the flux measurement, the haemoglobin content of the supernatant was also determined.

2.3. Reagents

Inorganic salts, sucrose and glucose were of analytical grade. DIOA, quinacrine, chloroquine, quinine, amiloride, the amiloride derivatives dimethylamiloride (DMA), ethylisopropylamiloride (EIPA), hexamethyleneamiloride (HMA) and benzamil were obtained from Sigma (St. Louis, MO). HOE 694 and HOE 642 (cariporide) are products of Aventis Pharma Deutschland (Frankfurt/M., Germany). Calyculin A and okadaic acid were acquired from QBiogene-Alexis (Grünberg, Germany). MES was purchased from SERVA (Heidelberg, Germany) and Tris from Carl Roth GmbH and Co. (Karlsruhe, Germany). Acridine was obtained from Acros Organics (Geel, Belgium), and $NaCH_3SO_4$ and KCH_3SO_4 were purchased from Merck (Darmstadt, Germany). ^{86}Rb (in RbCl) was produced by Amersham Biosciences (Amersham, UK). Quinacrine and chloroquine were dissolved in deionised water; guinine was dissolved in ethanol; acridine and amiloride and its derivatives as well as HOE 694 and HOE 642 were dissolved in DMSO.

2.4. Statistical treatment of results

Each experiment was repeated at least three times on blood from different donors. The results are presented as the mean ± S.D. of independent experiments. When errors are not shown, they were smaller than the symbols.

3. Results

The strategy of finding substances that act as inhibitors of the $K^+(Na^+)/H^+$ exchanger was to investigate quinacrine, known as inhibitor of the mitochondrial K^+/H^+

exchanger [10], and related substances, as well as amiloride, known as inhibitor of the Na^+/H^+ exchanger isoform 1 (NHE1) [11], and its derivatives. In addition, two newly designed drugs (HOE 694, HOE 642 (cariporide)) have been tested.

In comparison to quinacrine, chloroquine, quinine and acridine has been tested. Fig. 1A and B presents the dose–response curves for quinacrine and chloroquine in HIS and LIS solution, respectively. In HIS solution, both substances increased the (ouabain + bumetanide + EGTA)-resistant K^+ influx with increasing concentration. At 1 mM, a 12-fold or 3-fold activation of the K^+ influx was achieved with quinacrine and chloroquine, respectively. Interestingly, these results were obtained without haemolysis (0.9% for quinacrine, 0.6% for chloroquine). However, in contrary to the results in HIS solution an inhibition is seen in LIS solution. Quinacrine (1–2 mM) inhibited the K^+ influx by about 60% accompanied with a haemolysis of 8.7%. With chloroquine (2 mM) an inhibition of 85% was achieved and the haemolysis was 2.7% only. The IC_{50} values estimated from the curves are 81 and 66 μM for quinacrine and chloroquine, respectively. Quinine showed a small (10%) inhibition of the K^+ flux in LIS solution only. In the presence of acridine, no significant change of the K^+ flux in LIS solution could be measured up to 300 μM, at higher concentrations a significant increase of the K^+ influx was observed (data not shown). The structural comparison of the tested substances let us assume that the side chain $-NH-CH(CH_3)-(CH_2)_3-N(C_2H_5)_2$ causes the inhibitory effect on the (ouabain + bumetanide + EGTA)-resistant K^+ influx in LIS solution.

Together with amiloride, benzamil, DMA, EIPA and HMA have been tested. In HIS solution, amiloride (100 μM) induced a small but significant activation (19%) of the (ouabain + bumetanide + EGTA)-resistant K^+ influx. Benzamil (100 μM) did not show a significant change of the K^+ influx compared to the control. With DMA (100 μM), EIPA (10 μM) and HMA (10 μM) an inhibition of 24%, 20% and 11% was obtained, respectively (data not shown). A 10-fold lower concentration for EIPA and HMA was used because at 100 μM both substances caused a nearly 10% haemolysis of the cells. In LIS solution, all amiloride derivatives (10 or 100 μM) showed an inhibition of up to 30%. With higher concentrations, it was possible to achieve inhibitory effects as far as 55%.

In addition, new substances designed to block Na^+/H^+ exchange (HOE 694 [12] and HOE 642 [13]) have been investigated for their inhibitory effect on the K^+ influx. Dose–response curves are shown in Fig. 2. Interestingly, both substances showed a remarkable inhibitory effect in HIS (Fig. 2A) and LIS (Fig. 2B) solution. For HOE 694 (0.5 mM), an inhibition of 33% and 44% of the (ouabain + bumetanide + EGTA)-resistant K^+ influx was obtained in HIS and LIS solution, respectively. The estimated IC_{50} values are 108 μM in HIS and 74 μM in LIS solution. However, in HIS solution, HOE 694 concentrations higher than 0.5 mM produced no further inhibition but a recovery to control values. The corresponding results for HOE 642 (1 mM) were 40% and 65% inhibition in HIS and LIS solution, respectively. The calculated IC_{50} values are 158 μM in HIS and 285 μM in LIS solution. The haemolysis caused by HOE 694 and HOE 642 was less then 1% in both solutions, except for HOE 694 in LIS solution (1.8%). Thus, in contrast to quinacrine and chloroquine, the amiloride derivatives were able to inhibit the (ouabain + bumetanide + EGTA)-resistant K^+ influx in HIS and LIS solutions. In the case of HOE 694 and HOE 642, the inhibition of the flux in HIS and LIS solutions were nearly identical (in %), and the haemolysis was negligible at high concentrations of these drugs.

Further investigations were carried out to prove whether the $K^+(Na^+)/H^+$ exchanger is regulated by phosphatases.

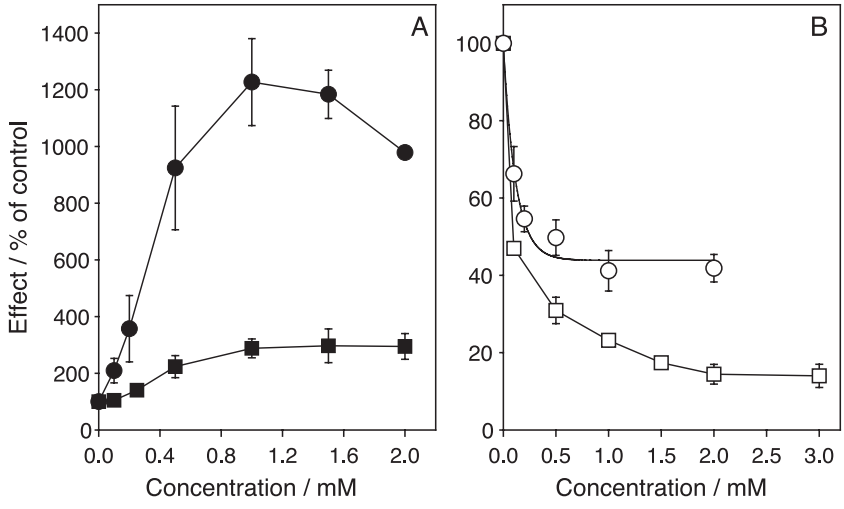

Fig. 1. Dose–response curves for quinacrine (●,○) and chloroquine (■,□) on the (ouabain + bumetanide + EGTA)-resistant K^+ influx in (A) HIS (●,■) and (B) LIS (○,□) solution. Flux values are corrected for haemolysis. For flux solutions and flux measurement, see Section 2.

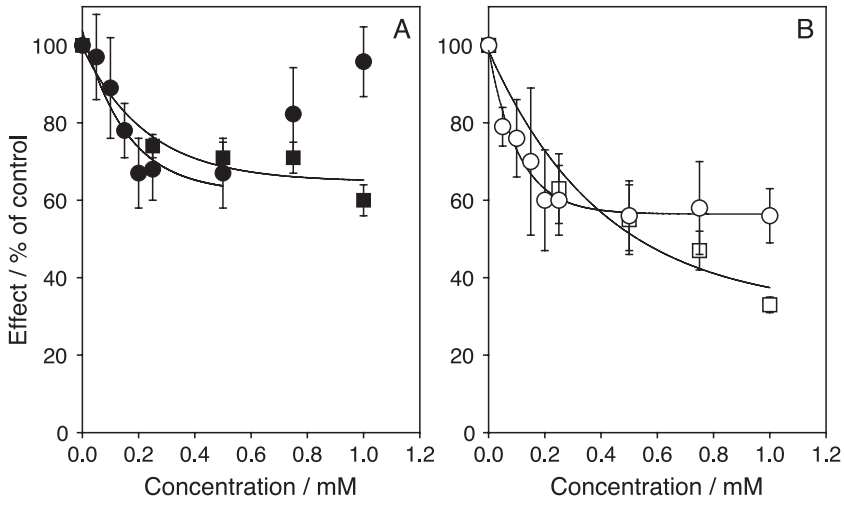

Fig. 2. Dose–response curves for HOE 694 (●,○) and HOE 642 (■,□) on the (ouabain + bumetanide + EGTA)-resistant K^+ influx in (A) HIS (●,■) and (B) LIS (○,□) solution. Flux values are corrected for haemolysis. For flux solutions and flux measurement, see Section 2.

Therefore, the effect of calyculin A as well as okadaic acid, both phosphatase inhibitors, on the (ouabain + bumetanide + EGTA)-resistant K^+ influx was studied (Fig. 3). In HIS solution, calyculin A caused an inhibition of the flux of 57%. The IC_{50} value was 21 nM (Fig. 3A). In LIS media, no significant changes could be observed (Fig. 3A). With the addition of okadaic acid, an inhibition of the (ouabain + bumetanide + EGTA)-resistant K^+ influx of 40% was achieved in HIS solution ($IC_{50} = 94$ nM). Contrary to calyculin A, okadaic acid was able to inhibit the K^+ influx in LIS solution by 25% (Fig. 3B). The optimal concentration for this effect seems to be 0.75 μM. If the concentration of okadaic acid was higher, the inhibitory effect decreased.

Because the estimated IC_{50} value of 21 nM for calyculin A in HIS solution is very close to the IC_{50} value for the K^+–Cl^- cotransporter [14], the effect of DIOA on the (ouabain + bumetanide + EGTA)-resistant K^+ influx was investigated. DIOA is a known inhibitor of the K^+–Cl^- cotransporter [15] that acts optimal in a concentration range of 80–100 μM without affecting other transport systems. However, the addition of DIOA (100 μM) caused a small but significant activation of 27 ± 7% of the K^+ influx in HIS solution (data not shown). This result demonstrates that the K^+–Cl^- cotransporter is not involved in the (ouabain + bumetanide + EGTA)-resistant K^+ influx in HIS solution. Also, in LIS solution, an activation of the K^+ influx caused by DIOA occurred (377 ± 30%). Another attempt to rule out any participation of the K^+–Cl^- cotransporter was made by replacing the Cl^- in HIS and LIS solutions by $CH_3SO_4^-$. In methylsulphate media, the inhibitory effect of HOE 642 (1 mM) on the K^+ influx was 24 ± 7% and 48 ± 8% in HIS and LIS solution, respectively (data not shown).

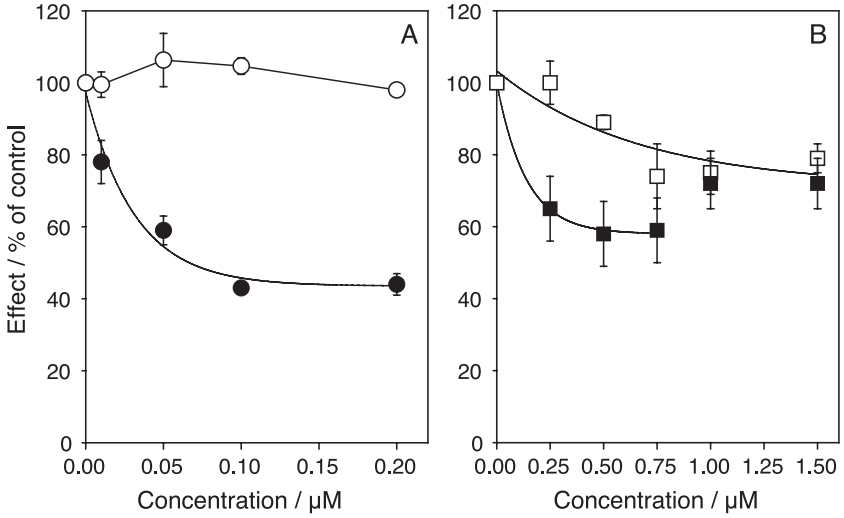

Fig. 3. Dose–response curves for (A) calyculin A (●,○) and (B) okadaic acid (■,□) on the (ouabain + bumetanide + EGTA)-resistant K^+ influx in HIS (●,■) and LIS (○,□) solution. For flux solutions and flux measurement, see Section 2.

4. Discussion

The (ouabain + bumetanide + EGTA)-resistant K^+ influx (residual K^+ influx) of human RBCs accounts for only 5% of the totally measurable K^+ influx in HIS solution. The other 95% are maintained by two transport systems, the Na^+/K^+ pump (about 70%) and the $Na^+–K^+–2Cl^-$ cotransporter (about 25%). However, in LIS solution, 50% of the total K^+ influx contributes to the residual K^+ influx, the other 50% are maintained by the pump (the $Na^+–K^+–2Cl^-$ cotransport is nearly zero at very low outside Cl^- concentrations) [16,17]. It has been shown that the 10-fold activation of the residual K^+ influx in LIS solution, in comparison to HIS solution, is mainly caused by the $K^+(Na^+)/H^+$ exchanger [6,7].

The presented results show for the first time that substances are available to inhibit the residual K^+ influx in HIS solution further, leading to a new level of the ground permeability of the human red blood cell membrane for K^+. In addition, substances have been found that suppress the activation of the residual K^+ influx in LIS solution to a remarkable extent. The classical amiloride derivatives known to prevent Na^+/H^+ exchange inhibit the residual K^+ influx in both HIS and LIS solution. Newly designed drugs for the inhibition of Na^+/H^+ exchange (HOE 694, HOE 642) are more effective in their inhibitory effect on the residual K^+ influx in HIS and LIS solutions. For the human Na^+/H^+ exchanger the IC_{50} values for HOE 694 and HOE 642 are 160 nM [18] and 80 nM [19], respectively. The estimated IC_{50} values for the $K^+(Na^+)/H^+$ exchanger (this paper) are 3 orders of magnitude higher. However, since HOE 694 and HOE 642 reduce the residual K^+ influx in HIS and LIS solution to nearly the same extent, it can be assumed that the $K^+(Na^+)/H^+$ exchanger is active under both conditions.

Quinacrine that has been shown to inhibit K^+/H^+ exchange [10] inhibits the residual K^+ influx only in LIS solution. The same effect is seen for chloroquine indicating the largest inhibition in LIS solution. Interestingly, the residual K^+ influx in LIS solution can be suppressed by chloroquine nearly to the flux value determined in HIS media. The activation of the residual K^+ influx in HIS solution caused by quinacrine and chloroquine is not very surprising taking into account the activation of the K^+/H^+ exchanger in the mitochondria membrane in the presence of quinacrine under certain conditions [10]. However, at the moment, the mechanism of activation remains unclear. It cannot be excluded that both substances directly activate the $K^+(Na^+)/H^+$ exchanger or another transport system, which is silent but not selectively inhibited under the experimental conditions (e.g., $K^+–Cl^-$ cotransport).

The observed inhibition of the residual K^+ influx caused by okadaic acid and calyculin A show that the $K^+(Na^+)/H^+$ exchanger is influenced by phosphatase inhibitors like other cotransporters. It is known from literature that okadaic acid activates the Na^+/H^+ exchanger [20,21] and the $Na^+–K^+–2Cl^-$ cotransporter [22] but inhibits the $K^+–Cl^-$ cotransporter [23].

The different effect of calyculin A and okadaic acid in LIS solution can be explained on the basis of the findings of Bize et al. [14] showing that with decreasing osmolarity (produced by a decrease in the ionic strength of the solution) an increasing amount of the protein phosphatase type 1 is bound to the membrane whereas protein phosphatase type 2A stays in the cytosol. It seems possible, therefore, that okadaic acid does not affect the $K^+(Na^+)/H^+$ exchanger via membrane-bound phosphatases.

One serious problem is the involvement of the $K^+–Cl^-$ cotransporter in the observed effects. It has been shown that the $K^+–Cl^-$ cotransporter is not involved in the residual K^+ influx in HIS and LIS solutions. This was concluded from experiments where Cl^- in the solution and in the RBCs has been replaced by NO_3^- or $CH_3SO_4^-$ by repeatedly washing the cells and demonstrating no significant changes of the flux in the different media [9,17,24]. A further argument against the participation of the $K^+–Cl^-$ cotransporter in the residual K^+ influx is based on the finding that the residual Na^+ influx in LIS solution is similarly stimulated like the K^+ influx [6,9]. In addition, three different manoeuvres to suppress the $K^+–Cl^-$ cotransport in low potassium-type (LK) sheep red cells (replacement of Cl^- by NO_3^-, volume decrease, inhibition by anti-L_1 antibodies) have no effect on the LIS-stimulated residual K^+ influx [25]. However, Bize et al. [14] demonstrated an involvement of the $K^+–Cl^-$ cotransport in the residual K^+ influx by about 50% under physiological conditions on the basis of experiments where Cl^- was replaced by sulphamate (amidosulphonate). The decrease of the Cl^--dependent component caused by calyculin A and okadaic acid described by these authors was explained as inhibition of the $K^+–Cl^-$ cotransporter. However, these results are not consistent with other reports (see above). Furthermore, the estimated flux values for the residual K^+ influx under control conditions, i.e. in physiological solution (0.04 mmol/(l_{cells}·h) [14]), are much smaller than the corresponding values obtained in the present investigations (0.121 ± 0.025 mmol/(l_{cells}·h), $n = 37$) and values reported in the literature [9,24]. That the $K^+–Cl^-$ cotransport does not play a substantial role in the residual K^+ influx is supported by the finding that (i) the inhibitory effect of HOE 642 persists in HIS and LIS solutions when Cl^- is replaced by $CH_3SO_4^-$ and (ii) DIOA, an inhibitor of the $K^+–Cl^-$ cotransport, activates the flux in HIS and LIS solution. The more pronounced stimulation in LIS media is not surprising taking into consideration that many classical inhibitors for other transport pathways also activate the residual K^+ influx, especially under LIS conditions [17]. In addition, if the $K^+–Cl^-$ cotransporter was involved, one would assume that okadaic acid should give nearly the same results as calyculin A in LIS solution, which is not the case.

5. Conclusion

It can be concluded that a remarkable part (about 50%) of the so far supposed residual K^+ influx (representing the ground permeability of the membrane for K^+) in HIS solution is realised by the $K^+(Na^+)/H^+$ exchanger. Taking into account the more pronounced inhibition (about 85%) of the residual K^+ influx caused by some substances in LIS solution, it seems realistic to assume that inhibitors will be found, which reduce the residual K^+ influx in both HIS and LIS solution to a greater than 50% extent.

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