Selectivity of Maxi Chloride Channels in the L6 Rat Muscle Cell Line

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Abstract. A method is described for the determination of reversal potentials (E_{rev}) from variance of single-channel currents vs. membrane potential in ramp pulse mode. The variance-voltage relationship is represented by a parabola with a minimum of the best fit curve corresponding to the value of E_{rev}.

The reversal potential of the maxi-Cl channels changes according to the activity of Cl ions at the internal side of the excised (inside-out) patch membrane as expressed by the Goldman-Hodgkin-Katz equation with $P_A/P_{Cl} = 0.15$, indicating the anion nature of the channel.

The relative permeabilities (P_A/P_{Cl}) found for halide anions, were 1.15 for iodide and 1.18 for bromide. The relative permeabilities measured (P_A/P_{Cl}) for other anions were 1.13 for nitrate, 0.59 for bicarbonate, 0.60 for methanesulfonate, 0.40 for SO_4^{2-}, 0.44 for propionate, and 0.10 for glutamate. No significant differences in P_A/P_{Cl} of the investigated anions were observed between proliferating myoballs and quiescent myoblasts. This may mean that the newly formed channels possess full-grown selective filters.

A close correlation ($r = 0.89$) was found between the calculated Stokes diameters of the anions under investigation and their relative permeabilities (P_A/P_{Cl}). The intercept of the best fit line with the abscissa is 7.2 Å (7.2×10^{-10} m), which may correspond to the diameter of the selectivity filter of the maxi-chloride channel in L6 myoblasts.

A similar value of the channel size was obtained from the relationship between the minimum cross-sectional areas of the anions and their relative permeabilities, P_A/P_{Cl}. The best fit line intercepts the abscissa at 27.5 Å^2, indicating a pore size $\simeq 6$ Å. The minimum areas were obtained by computer from molecular models of the anions.

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Introduction

We have shown recently that the occurrence of maxi-Cl channels in proliferating myoballs is substantially higher than in quiescent myoblasts (Hurňák and Zachar 1994). There was also a slight change (increase) in the conductance of the large-conductance chloride channels in myoballs. In addition to the fact that selectivity of the maxi-Cl channels has not been determined in L6 myoblasts, we wanted to establish, whether the selectivity of the maxi-Cl channels has also undergone changes. The selectivity of maxi-Cl channels was determined shortly after their discovery by Blatz and Magleby (1983) in several cells, which show the presence of maxi-Cl channels in their membranes (Gray et al. 1984; Schwarze and Kolb 1984; Hanrahan et al. 1985; Schneider et al. 1985; Soejima and Kokubun 1988; Bosma 1989; Christensen et al. 1989; Hals et al. 1989; Schlichter et al. 1990; McGill et al. 1992; Bettendorff et al. 1993).

The results have shown that the maxi-Cl channel in L6 myoblasts is moderately anion selective with a P_{Na}/P_{Cl} ratio 0.15, and shows permeability to anions comparable to that of large-conductance chloride channels in different cells examined so far. There is no significant difference between quiescent cells and proliferating myoblasts (myoballs) indicating that the newly formed channels in proliferating rounded myoblasts (myoballs) possess selectivity filter typical of quiescent myoblasts. The approximation of the channel size was attempted by two methods: by relating the permeability ratios, P_A/P_{Cl} to Stokes diameter and to minimized cross-sections of the anions determined from computer molecular models. The results of both methods were in a reasonably good agreement.

Materials and Methods

Solutions

The standard bath contained physiological saline consisting of (mmol/l): 145 NaCl; 0.5 $CaCl_2$; 10 HEPES; pH 7.4 at 25 °C. For all experiments the pipettes were filled with the standard solution. For the experiments on the ion selectivity of the maxi-Cl channels, the physiological bath solution was replaced with the tested salt solution. The junction potentials were estimated and subtracted from the instant data of reversal potential measurements (Barry and Lynch 1991; Neher 1992). All experiments were performed with the tip of the patch pipette positioned inside a perfusion capillary with a diameter of about 1 mm. The introduction of the patch pipette into the capillary by means of two micromanipulators was observed on the screen of a TV monitor connected to a microscope video system. This procedure allowed close control and safe as well as thorough exchange

of solutions bathing the intracellular surface of the membrane. Further details are given elsewhere (Zachar and Hurňák 1994).

Cell culture

The rat muscle cell line L6 was purchased from the American Type Culture Collection (A.T.C.C.; Rockville, MD, USA). Cells for experiments were subcultured (maximum 6 ×) at regular time intervals to prevent fusion in culture media, as described elsewhere (Hurňák and Zachar 1994). Cells were seeded in plastic or glass dishes (35 mm in diameter) at required densities, usually at $\approx 5 \cdot 10^5$ cells/Petri dish showing, however, an uneven dispersion. L6 myoblasts were taken for experiments 24–96 hr after plating when the cultures showed required proportions of proliferating rounded myoballs and quiescent bottom-attached myoblasts. The experimental conditions were thus similar to those used for comparison of conductance-voltage relations in quiescent and proliferating cells (Hurňák and Zachar 1994).

Electrophysiology

Currents were recorded in the excised (inside-out) configuration of the patch-clamp technique as described in detail elsewhere (Hurňák and Zachar 1992, 1994). Pipettes were fire polished without coating with Sylgard. All recordings were made with the Axopatch 1C patch-clamp amplifier and CV-4 0.1/100 headstage (Axon Instruments, Foster City, CA, USA). Currents were low-pass filtered (1–2 kHz) by an eight pole Bessel filter. Data were acquired and analyzed using an IBM AT compatible computer with an analog-to-digital interface board (Labmaster DMA, Scientific Solutions Inc., Solon, OH, USA) and pClamp 5.5.1 software (Axon Instruments). The ramp-pulse recordings were evaluated with a software developped in the laboratory (Stavrovský et al. 1992). Fitting of the variance – voltage relationship and determination of the reversal potential, E_{rev} was performed by means of protocols written in MATHEMATICA 2.1 software (Wolfram Research, Champaign, IL, USA).

Permeability ratios

Goldman-Hodgkin-Katz (Hodgkin and Katz 1949) equation (Eq. 1) was used to calculate the relative permeability (P_A/P_{Cl}) of monovalent anion A with respect to Cl.

$$E_{rev} = \frac{RT}{F} \ln \left[\frac{[Cl]_i + \frac{P_A}{P_{Cl}}[A]_i + \frac{P_{Na}}{P_{Cl}}[Na]_o}{[Cl]_o + \frac{P_A}{P_{Cl}}[A]_o + \frac{P_{Na}}{P_{Cl}}[Na]_i} \right] \quad (1)$$

For divalent cations Eq. 2 as derived e.g. by Fatt and Ginsborg (1958) and Lewis (1979), was used:

$$E_{rev} = \frac{RT}{F} \ln \left[\frac{[Cl]_i + 4\frac{P'_A}{P_{Cl}}[A]_i + \frac{P_{Na}}{P_{Cl}}[Na]_o}{[Cl]_o + 4\frac{P'_A}{P_{Cl}}[A]_o e^{\frac{E_{rev}F}{RT}} + \frac{P_{Na}}{P_{Cl}}[Na]_i} \right] \quad (2)$$

Subscripts i and o denote internal and external ion species, respectively; R, T and F have their usual meaning, and $P'_A = P_A/[1 + \exp(E_{rev}F/(RT))]$.

Ion diameter

Ionic size of anions was calculated as Stokes diameter (Valkanov et al. 1991) from the limiting equivalent conductance (Robinson and Stokes 1970). For methanesulfonate and glutamate anions for which data on limiting equivalent conductances were not available the hydrated ion size was taken from Hals et al. (1989).

Cross sectional area

Molecular models were analysed and plotted by means of MOLGEN software (KISOI T Modra Slovakia). Minimum cross section was found by eye and cross-sectional area was derived by protocols written in MATHEMATICA 2.1.

Results

Analysis of the unit current records

Maxi-Cl channel currents in excised (inside-out) membrane patches were recorded in voltage ramp mode usually in the range from −40 mV to +40 mV. Ramp pulses were repeated 5 times (Fig. 1*A*) and variance of the corresponding currents at each voltage point was calculated. The relationship between the variance of the current and the imposed voltage (*variance-voltage relationship*) is shown in Fig. 1*B*. Two main situations are worth of being considered in these recordings. First when the ramp current records consist of both open and closed states and second, when the records find the channel in the open state only.

Variance vs. voltage relationship when both open and closed state currents are present. The following relations are valid in this situation

$$\begin{aligned} i_c &= g_c\,(V - E_{rev}) \\ i_o &= g_o\,(V - E_{rev}) \end{aligned} \tag{3}$$

where i_c, i_o, g_c and g_o are currents and conductances respectively for the open and the closed states, E_{rev} is reversal potential and V is membrane potential. Calculating variance for n currents in Eq. 3 with m currents in closed state and assigning $g = g_o - g_c$ as channel conductance we obtain

$$\mathrm{var}\,(V) = \frac{m\,(n-m)}{n\,(n-1)}\,g^2\,(V - E_{rev})^2 \tag{4}$$

This corresponds to a parabola with a minimum at E_{rev} (Fig. 1*B*). If the focus is on E_{rev} only, Eq. 4 can be simplified

$$\mathrm{var}\,(V) = a\,(V - E_{rev})^2 \tag{5}$$

and parameters a and E_{rev} can be obtained by fitting.

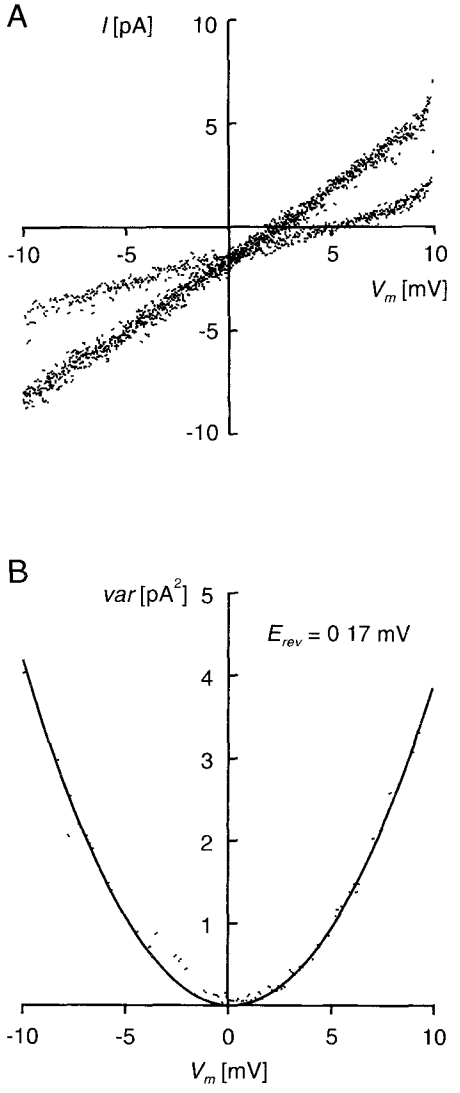

Figure 1. Determination of the reversal potential (E_{rev}) from voltage ramp current records. *A*. Single-channel currents to 10 successive symmetrical *voltage ramp pulses* from −40 to +40 mV applied within 1 s. Only the segments from −10 to +10 mV (V_m) are shown and analysed. *B*. Variance-voltage (var − V_m) relationship determined from the records shown in A. The best fit parabola through the experimental points was calculated by Eq. 3 (*see text*) and yielded a reversal potential, E_{rev} = 0.17 mV corresponding to symmetrical solutions bathing the excised membrane patch (Symmetrical 145 NaCl, 0.5 $CaCl_2$ solutions (in mmol/l)).

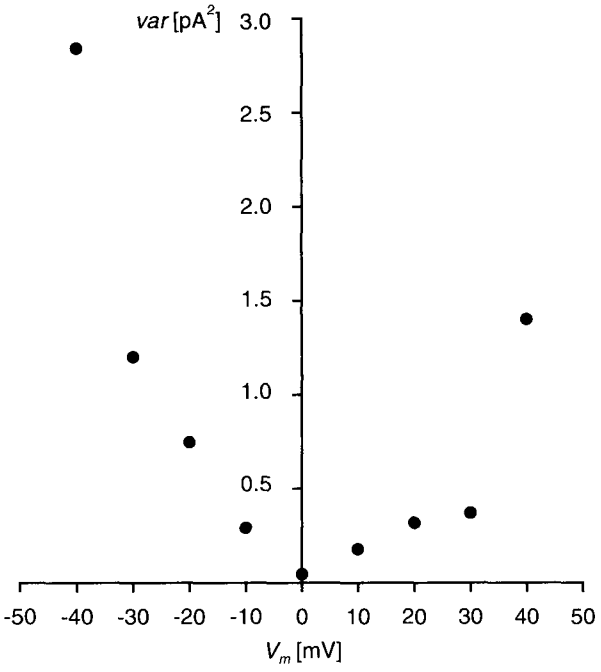

Figure 2. Variance, var (pA^2) of the open state currents at different membrane potentials, V_m (from −40 to +40 mV at 10 mV steps) as measured in the absence of closed states. For explanation see text (Symmetrical 145 NaCl, 0.5 $CaCl_2$ solutions (in mmol/l)).

Variance vs. voltage relationship when open state currents are present only. As shown in Fig. 2 the variance increases with the increasing membrane current (see also Sigworth 1985), the later being a linear function of the membrane potential. It is possible to obtain values of E_{rev} even if points are unavailable for closed state current. A function similar to Eq. 5 can be fitted to experimental data in order to calculate E_{rev} :

$$\mathrm{var}\,(V) = a^2\,(V - E_{rev})^2 \tag{6}$$

Best results were obtained when using region close to the reversal potential to avoid errors due to rectification of the current-voltage relationship.

Reversal potential

Fig. 3 shows the dependence of the reversal potential on the internal concentration of Cl^- ions, $[Cl]_i$. The Cl^- ions were substituted by sucrose. The curve through the experimental points was fitted according to Eq. 1 with $P_{Na}/P_{Cl} = 0.15$ (see

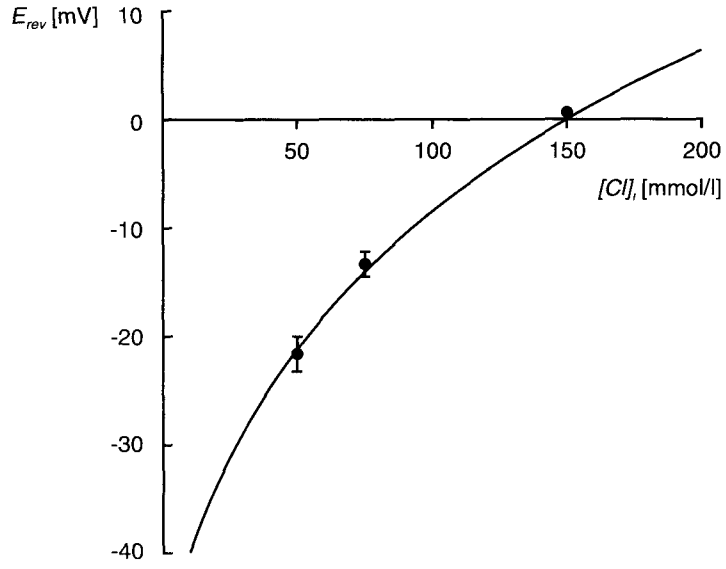

Figure 3. The dependence of the reversal potential, E_{rev} on the internal concentration of chloride ions, $[Cl]_i$. The curve was fitted according to Eq. 1 with $P_{Na}/P_{Cl} = 0.15$.

also Hurňák and Zachar 1994) indicating the anion nature of the channel under examination.

Fig. 4 outlines the method and the results of the reversal potential measurement after the substitution of Cl^- ions on the internal side of the channel with methanesulfonate (A, B) or iodide (C, D) anion. The current records to voltage ramp pulses (five summated successive episodes) clearly show that the determination of reversal potentials, E_{rev} from such records may represent a problem. On the other hand, variance-voltage (var $- V_m$) relationships (B, D) determined from the records shown in A or C point precisely to values of reversal potentials, $E_{rev} = -15.8$ mV for methanesulfonate and $E_{rev} = 3.5$ mV for the iodide anion. It is to be noted that for analysis purposes only linear segments around the reversal potential were chosen from the whole current record (from -40 to $+40$ mV applied within 1 s), i.e. from -20 to -5 mV in A and from -2 to $+10$ mV in C.

The reversal potentials were determined in the same way for several anions, including nitrate (2.6 mV), carbonate (-11.2 mV), sulphate (-3.5 mV), propionate (-16.6 mV), methanesulfonate (-10.9 mV), and glutamate (-38.5 mV), as well as for the halogens bromide (3.7 mV), and iodide (3.0 mV). Reversal potentials measured as shown in Fig. 4 were corrected for junctional potentials (see Materials and Methods). The resulting values are given in Table 1. The values are means from 3–7 measurements and were determined in myoballs as well as in quiescent

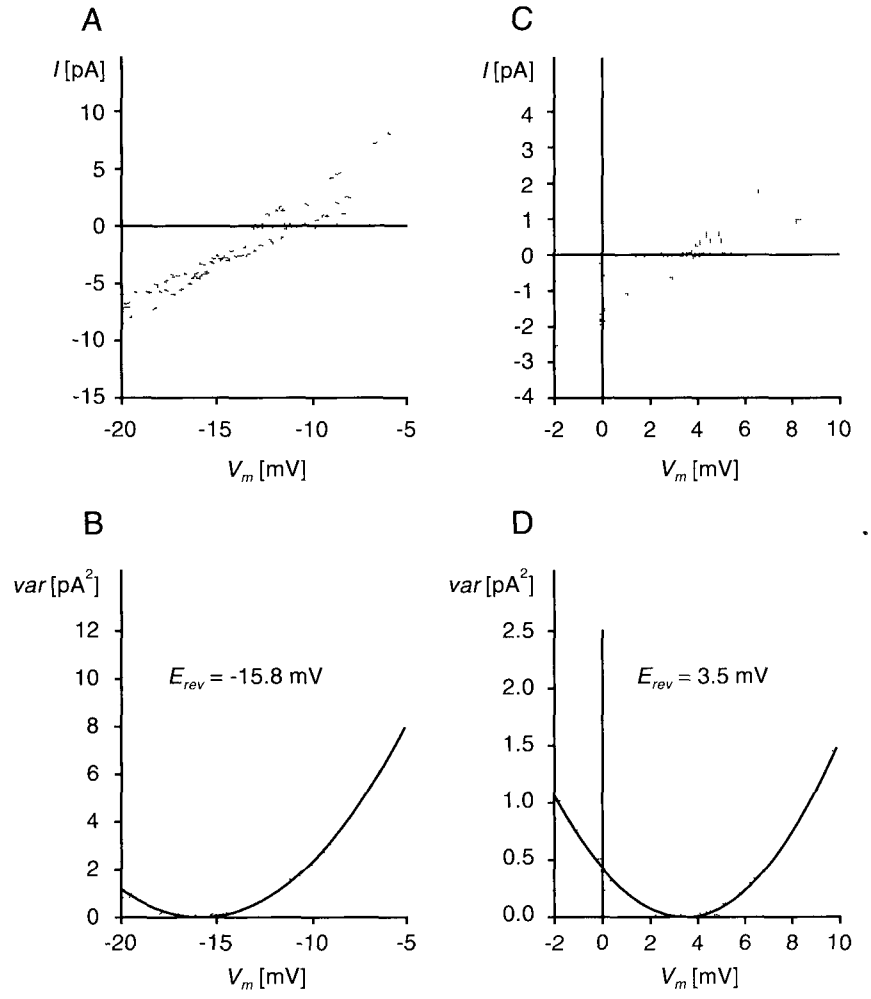

Figure 4. Reversal potentials (E_{rev}) as determined from variance-voltage (var – V_m) relationships in methansulfonate (*A*, *B*) and iodide (*C*, *D*) internal solutions. *A* Segments (−20 to −5 mV) from 5 successive current records to symmetrical *voltage ramp pulses* from −40 to +40 mV applied within 1 s in 145 NaCl//145 Na-methansulfonate solutions. *B*. Variance-voltage (var – V_m) relationship determined from the records shown in *A* with the best fit value of E_{rev} = −15.8 mV. *C*. Segments (−2 to + 10 mV) from 5 successive current records to symmetrical voltage ramp pulses from −40 to +40 mV applied within 1 s in 145 NaCl//145 NaI solutions. *D*. Variance-voltage (var – V_m) relationship determined from the records shown in *B* with the best fit value of E_{rev} = 3.5 mV.

myoblasts. There was no significant difference between these cells as far as the reversal potential shift by the tested anions is concerned.

Table 1. Reversal potentials (E_{eq}) and relative permeabilities (P_A/P_{Cl}) in L6 myoblasts

Anion	z	(E_{eq}) (mV)	P_A/P_{Cl}	Stokes diameter (Å)	Cross-section (Å^2)
Br	−1	3.68	1.18	2.36	10.10
I	−1	3.05	1.15	2.40	13.21
Cl	−1	0.00	1.00	2.41	8.56
NO_3	−1	2.65	1.13	2.58	11.10
HCO_3	−1	−11.22	0.59	4.14	13.00
SO_4	−2	−3.50	0.40	4.60	17.00
Propionate	−1	−16.62	0.44	5.15	17.80
Methanesulfonate	1	−10.91	0.60	5.18	7.20
Glutamate	-1	−38.49	0.10	6.80	27.00

Notes: Values are means from at least 3 measurements. E_{eq} was determined by equimolar replacement of chloride ions with the anions; the junction potentials were subtracted. P_A/P_{Cl} values were calculated by assuming $P_{Na}/P_{Cl} = 0.15$. Molecular cross-sectional areas were calculated by means of the 2D projections of the CPK space filling atomic models generated by the software and oriented so as to give a minimum value of the area.

Relative permeabilities, P_A/P_{Cl}

Relative permeabilities for monovalent anions, P_A/P_{Cl} and for divalent sulphate anion were calculated by means of Eq. 1 and Eq. 2 respectively. The relative permeabilities for halide anions were 1.15 for iodide and 1.18 for bromide. It follows that the permeability of the channel for these anions is higher than for chlorine. The same applies for nitrate, with $P_A/P_{Cl} = 1.13$. The permeability of the channel for bicarbonate is less than for Cl ions, but stil fairly high ($P_A/P_{Cl} = 0.59$), and this may have important consequences for the functioning of the cell. The maxi-Cl channel is fairly well permeable for methanesulfonate ($P_A/P_{Cl} = 0.60$) and moderately permeable even for divalent SO_4^{2-} ($P_A/P_{Cl} = 0.40$) and such large anions as propionate ($P_A/P_{Cl} = 0.44$) or glutamate ($P_A/P_{Cl} = 0.10$). The calculated values of P_A/P_{Cl} for different anions are shown in Table 1.

Relative permeabilities and ionic size

Ionic size of the anions examined was estimated by calculating their Stokes diameters. The Stokes Law, as applied to an ion represented by a sphere, relates its mobility (u) in a hydrodynamic medium to the frictional resistance in terms of the ion radius (r) and the viscosity of the medium (η), i.e.

$$r = \frac{1}{6\pi\eta u} \tag{7}$$

Mobility u can be expressed in terms of limiting equivalent conductivity,

$$u = \frac{N\eta^0\lambda^0}{\eta z F^2}, \tag{8}$$

where F is Faraday constant, N is Avogadro number, z is the valency of the ion, η^0 is the viscosity of the pure solvent, and λ^0 is the limiting equivalent conductivity. The limiting equivalent conductivities were taken from Robinson and Stokes (1965); for methanesulfonate and glutamate hydrated ion the size was taken (see Materials and Methods) in Hals et al. (1989). The Stokes diameters are given in Table 1.

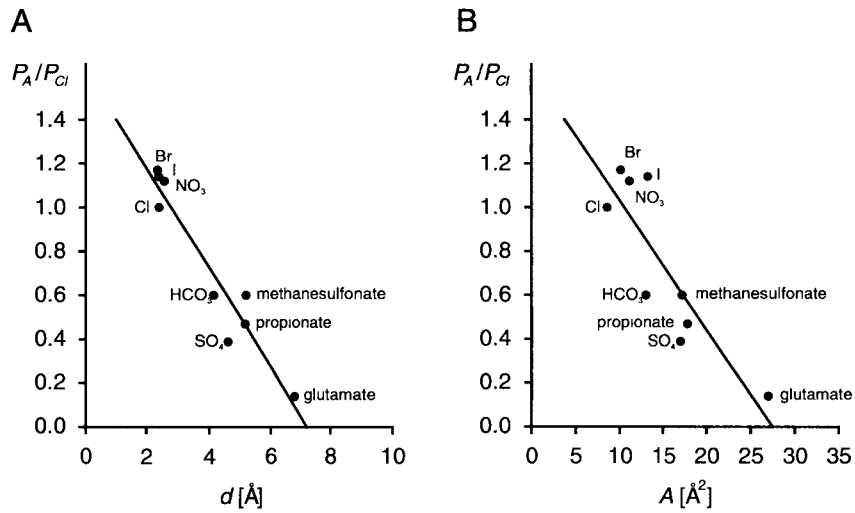

Figure 5. Relationships of relative anion permeabilities P_A/P_{Cl} to Stokes diameters of anions (A) and to cross-sectional areas of the molecules (B). Calibration: 1 Å (10^{-10} m).

Figure 5A shows the relationship between the Stokes radii of the anions and their relative permeabilities. The intercept of the best fit line with the abscissa is 7.2 Å (7.2×10^{-10} m).

Figure 5B shows the relationship between the minimum cross-sectional areas of the anions and their relative permeabilities, P_A/P_{Cl}. The best fit line intercepts the abscissa at 27.5 Å^2, indicating a pore size $\simeq$ 6 Å. The minimum areas were found (as described in Materials and Methods) from the molecular models of the anions drawn to scale in Figure 6.

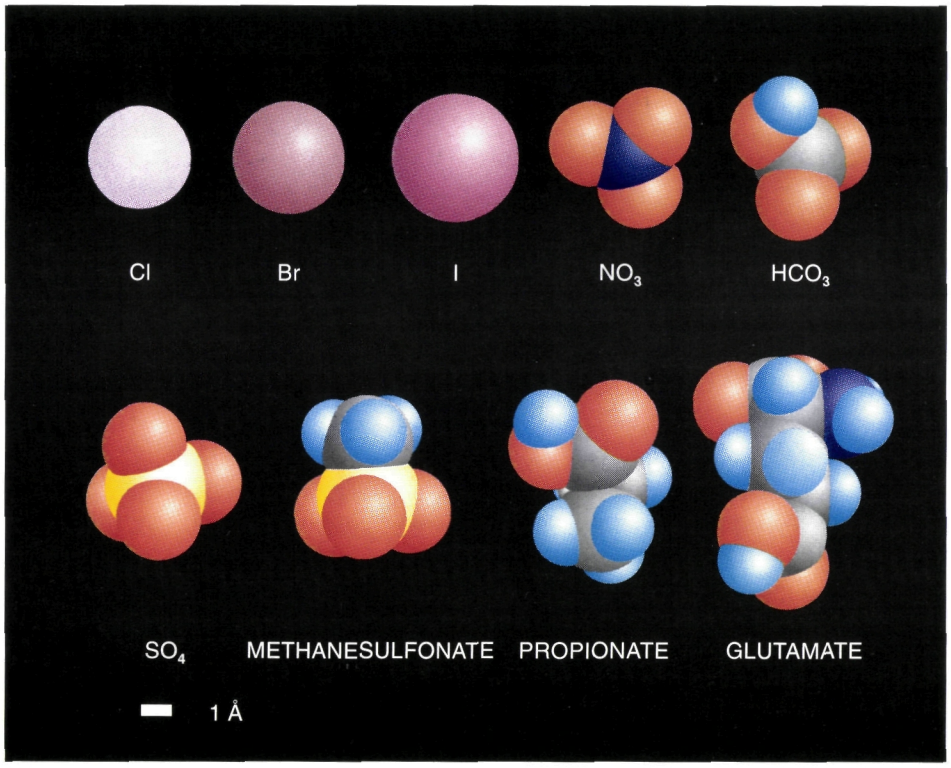

Figure 6. Molecular models of anions which are able to permeate the maxi-Cl channels in proliferating L6 myoblasts. The three dimensional models were used to determine the minimum size ($Å^2$) of two dimensional projections (cross-sectional areas) of the molecules.

Discussion

In spite of their high conductance approaching the theoretical limits (Hille 1992) the maxi-Cl channels show a definite selective permeability, and they pass chloride anions ten times easier than Na^+ ions. The $P_A/P_{Cl} = 0.15$ as found for L6 myoblasts and myoballs is of the same order of magnitude as that found by others for different cell membranes (Table 2). Much higher selectivity was, however, observed for alveolar cells (Schneider et al. 1985) and human T lymphocytes (Schlichter et al. 1990).

The maxi-chloride-channels under investigation discriminate poorly among anions. Not only halide anions, but anions as large as glutamate (Fig. 6) are permeant as well. The later are considered impermeable for common chloride channels of lower conductance (Edwards 1982). As follows from Fig. 5*A* there is a fairly strong

Table 2. Ionic permeability ratios of maxi chloride channels to different anions and Na^+ in different cells

I	Br	Cl	NO_3	HCO_3	mSO_4	SO_4	prop	glut	Na^+	Ref.
1.4	1.2	1.0	–	–	–	0.61	–	0.03	0.20	1
–	–	1.0	–	–	–	–	–	–	0.15	2
1.0	1.0	1.0	–	–	–	0.30	–	–	–	3
1.5	1.02	1.0	0.92	–	–	–	–	–	0.01	4
1.4	1.3	1.0	1.01	–	0.40	0.33	0.32	0.05	0.06	5
1.18	1.07	1.0	0.68	–	–	–	–	–		6
–	–	1.0	1.08	–	–	–	–	–	≤ 0.1	7
1.4	1.0	1.0	1.53	0.96	0.72	0.15	0.27	0.21	0.02	8
1.4	1.04	1.0	1.14	0.56	–	0.49	0.30	–	0.09	9
–	–	1.0	–	0.53	–	–	–	–	0.11	10
–	–	1.0	–	–	–	–	–	–	0.05	11
1.15	1.18	1.0	1.13	0.59	0.60	0.40	0.44	0.10	0.15	12

Notes: mSO_4 – methanesulfonate; prop – propionate; glut – glutamate

Ref.: 1 Gray et al., 1984; 2 Schwarze and Kolb (1984); 3 Hanrahan et al. 1985; 4 Schneider et al., 1985; 5 Soejima and Kokubun, 1988; 6 Bosma (1989); 7 Christensen et al. 1989; 8 Hals et al., 1989; 9 Schlichter et al., 1990; 10 McGill et al. 1992; 11 Bettendorff et al. 1993; 12 Hurňák and Zachar, 1995 (*this paper*)

correlation between the Stokes diameter of the anions investigated and their respective permeability through the maxi-Cl channel. This behavior is also obvious in other preparations containing high-conductance Cl channels, where sufficient data are available. The pore size, as predicted from the P_A/P_{Cl} vs. Stokes diameter relationship (Fig. 5*A*), might be thus around 7.2 Å.

The selectivities of maxi-Cl channels to anions referred to in this paper are compared in Table 2 with selectivities found by others for different cells. As follows from the Table, the maxi-Cl channel in L6 myoblasts shows selectivity sequences similar to other preparations, e.g. cultured rat Schwann cells (Gray et al. 1984), mammalian tight epithelium (Hanrahan et al. 1985), pulmonary alveolar type II cells (Schneider et al. 1985), vascular smooth muscle (Soejima and Kokubun 1988), mouse B lymphocytes (Bosma 1989), chorioid plexus (Christensen et al. 1989), "sarcoballs" from frog skeletal muscle (Hals et al. 1989), human T-lymphocytes (Schlichter et al. 1990) and rat bile duct epithelial cells (McGill et al. 1992). Similar values of channel pore can be obtained from the plotted results of Soejima and Kokubun (1988) for vascular smooth muscle cells (embryonic rat aorta) and of Hals et al. (1989) for "sarcoballs" from frog skeletal muscle, as well as of Gray et al. (1984), who used an anion set different from that shown in Table 2 (i.e. methyl SO_4, acetate, isothionate, aspartate), and found the channel virtually impermeable

for glutamate ($P_A/P_{Cl} < 0.03$). These authors, however, preferred to estimate the channel diameter from the conductance value, and arrived at a value about three times higher (20 Å) and thus similar to the size of channels in the gap junctions (Loewenstein 1981). It is also to be noted that the pore size of maxi-Cl channels, as follows from the present experiments is very close to that (6.5 Å) of Ca-activated Cl channel with high conductance $\gamma = 140$ pS (in symmetrical 140 mmol/l NaCl) described in *Ascaris suum* by Valkanov et al. (1994). The permeability ratios in maxi-Cl channels, however, differ substantially from those of the other classes of Cl channels with much smaller conductances (Edwards 1982; Bretag 1987; Franciolini and Petris 1990).

An independent way of estimation of the channel size is represented by the relationship P_A/P_{Cl} vs. cross-sectional area of the anion as shown in Fig. 5*B*. The best fit line intersects the abscissa at a value of 27.5 Å^2, which is quite close to the value (32 Å^2) found by Soejima and Kokubun (1988) for smooth muscle cells by an analogous method.

It is evident that the selectivity of high conductance chloride channels can be accounted for to a significant degree by the shape of the ions and the pore in addition to assumed energetics of permeation.

One of the aims of the experiments reported in this paper was to find out, if the maxi-Cl channels in newly formed myoblasts (in proliferative myoballs) differ in their permeabilities to anions from quiescent myoblasts. The former were found (Hurňák and Zachar 1994) to increase substantially the number of maxi-Cl channels in their membranes during proliferative stage (i.e. in myoballs before, during or after mitotic division) in comparison with quiescent myoblasts. It follows from the results that the P_A/P_{Cl} ratios did not differ significantly at least as far as the examined anions are concerned. This may mean that the newly formed channels possess full-grown selective filters. It remains, however, to be elucidated what is then the reason for the slight increase of the channel conductance in these newly formed maxi-Cl channels (Hurňák and Zachar 1994).

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