Low Single Channel Conductance of the Major Skeletal Muscle Chloride Channel, ClC-1

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ABSTRACT We expressed the skeletal muscle chloride channel, ClC-1, in HEK293 cells and investigated it with the patch-clamp technique. Macroscopic properties are similar to those obtained after expression in *Xenopus* oocytes, except that faster gating kinetics are observed in mammalian cells. Nonstationary noise analysis revealed that both rat and human ClC-1 have a low single channel conductance of about 1 pS. This finding may explain the lack of single-channel data for chloride channels from skeletal muscle despite its high macroscopic chloride conductance.

INTRODUCTION

Skeletal muscle has a high chloride conductance accounting for about 80% of the total membrane conductance at rest (Bretag, 1987). Its physiological importance for stabilization of the membrane potential is emphasized by the phenotype associated with myotonia (Rüdel and Lehmann-Horn, 1985), a hereditary disease resulting from mutations in the gene coding for the major skeletal muscle chloride channel, ClC-1 (Steinmeyer et al., 1991b; Koch et al., 1992). Macroscopic chloride conductance has been characterized in some detail by several investigators (Palade and Barchi, 1977). However, no convincing single channel data exist of chloride channels from intact skeletal muscle preparations (Chua and Betz, 1991). Several chloride channels have been described in cultured muscle cells with conductances above 20 pS (Blatz and Magleby, 1985; Fahlke et al., 1993). For several reasons it remained in doubt if these channels correspond to the channel mediating the large chloride conductance in intact skeletal muscle. First, chloride conductance is relatively low in myoballs. Second, data from several groups demonstrate that ClC-1 is down-regulated after denervation (Camerino and Bryant, 1976; Conte Camerino et al., 1989) and cannot be detected in myotubes by Northern analysis (Wischmeyer et al., 1993).

Localization of the chloride channel in the T-tubular membrane could be one reason why it has evaded single-channel recording (Palade and Barchi, 1977; Heiny et al., 1990). Another possibility is that it has a small single channel conductance, rendering it unresolvable from noise in patch-clamp measurements.

Cloning of the major skeletal muscle chloride channel, ClC-1, from rat (rClC-1, Steinmeyer et al., 1991a), and human (hClC-1, Koch et al., 1992) opens the possibility for biophysical characterization of ClC-1 in heterologous expression systems. ClC-1 can be expressed in *Xenopus* oocytes and exhibits a voltage dependence similar to that found for macroscopic skeletal muscle chloride conductance (Steinmeyer et al., 1991a) with an open probability increasing at positive voltages, and an inwardly rectifying open channel I–V.

In the present paper we investigate the conductance of ClC-1 expressed in a mammalian cell line using nonstationary noise analysis. We find that ClC-1 has a low single channel conductance of about 1 pS.

EXPERIMENTAL PROCEDURES

Transfection of cells

Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO), penicillin (100 units ml^{-1}), and streptomycin (100 units ml^{-1}; Biochrom, Berlin, Germany). 24 h before transfection, cells were replated and then transfected with 1 μg ml^{-1} of hClC-1 cDNA which had been cloned into the pcDNAI vector (Invitrogen, San Diego, CA) using the calcium phosphate precipitate method (Chen and Okayama, 1987). The Ca^{2+} phosphate-DNA mixture was added to the cell culture medium and left for 9–12 h. The cells then were washed with fresh medium. Electrophysiological measurements were started 36 h after transfection.

Electrophysiological methods

Patch-clamp experiments of 293 cells were done in the on-cell, whole cell, and outside-out configurations (Hamill et al., 1981) at room temperature. The bath solution contained 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.2. Patch pipettes were pulled from borosilicate glass, coated with Sylgard, and filled with 140 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM EGTA, pH 7.3 ("symmetrical solution") or a low chloride solution containing 100 mM potassium glutamate, 40 mM CsCl, 1 mM $MgCl_2$, 10 EGTA, 10 Hepes, pH 7.3 ("asymmetrical solution"). We corrected for liquid junction potentials which were measured to be about +3 mV for the high chloride pipette solution and +9.5 mV for the low chloride solution. Currents were measured with an Axopatch 200 amplifier (Axon Instruments), filtered at 5 kHz and stored on a computer using pCLAMP software (Axon Instruments). For the experiment shown in Fig. 1 *b*, the bath solution was exchanged by a solution in which 135 mM NaCl was replaced by 135 mM NaI.

Nonstationary noise analysis was performed according to the method of Heinemann and Conti (1992) with the exception that no correction for leak or capacitive currents was performed for the calculation of mean current and variance. Briefly, variance was obtained by averaging the squared difference

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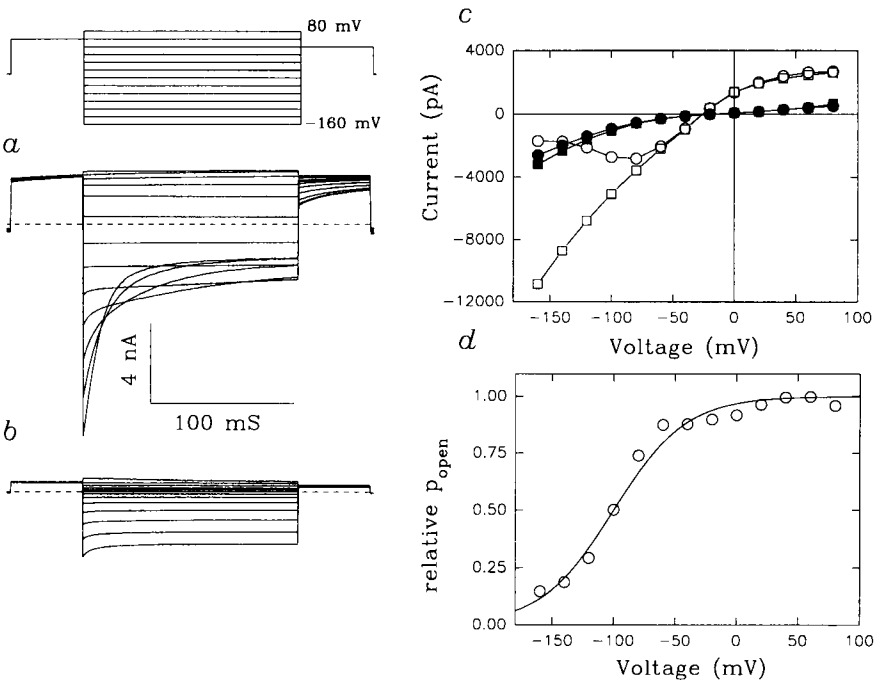

FIGURE 1 Electrophysiological analysis of hClC-1 expression in HEK293 cells. (*a*) Voltage-clamp traces in a whole cell patch of a transfected HEK293 cell in asymmetrical chloride solutions evoked by the stimulus protocol as indicated above. (*b*) Currents after exchanging 135 mM NaCl for 135 mM NaI in the bath. Effects were fully reversible. (*c*) Steady state (*circles*) and instantaneous currents (*squares*) obtained from *a* (*open symbols*) or *b* (*filled symbols*) as a function of membrane potential. Instantaneous currents were obtained by fitting an exponential function to the initial part of the decaying current and back-extrapolating it to the start of the voltage step. (*d*) Ratio of steady state and instantaneous currents as a measure of open probability. The ratio reflects the steady state open probability relative to the maximum open probability. From noise analysis it can be inferred that the maximum open probability is smaller than one (see Fig. 2). The solid line represents the fit of a simple Boltzmann equation to the activation curve yielding an apparent gating valence of 0.86 and half-maximal activation at −100 mV.

of consecutive records and appropriate scaling. Fitting a parabola of the form $\langle\sigma^2\rangle = i\langle I\rangle - \langle I\rangle^2/N$ to the variance-current plot (see Fig. 2 *c*) with the free parameters i (single-channel current) and N (number of channels) was performed using the nonlinear curve-fitting routine of SigmaPlot (Jandel Scientific, Corte Madera) without weighting points by their standard error. Conductance values were calculated assuming a linear I–V. This is appropriate in the negative voltage range where the open channel I–V is linear (see Fig. 1 *c*). Whole cell recordings were used for noise analysis only if series resistance was below 4 MΩ. Macroscopic currents could also be measured in cell attached and outside-out patches. Results from noise analysis were similar in cell free and whole cell recordings.

RESULTS

Both rClC-1 and hClC-1 could be transiently transfected in 293 cells with high efficiency. Macroscopic currents could be measured in cell attached, whole cell, and outside-out patches. Fig. 1 *a* shows typical whole cell voltage-clamp traces from a cell transfected with hClC-1. Currents are evoked by the pulse protocol shown on top. Holding potential was chosen to be close to the reversal potential in order to avoid excessive current flow. Reversal potential was about −30 mV for the asymmetrical solution and about 0 mV for the symmetrical solution. Both values are close to the calculated Nernst potentials for chloride, and thus demonstrate the chloride selectivity of the current. We never observed similar currents in nontransfected cells.

After maximal activation at 60 mV, currents deactivate within about 50 ms when the voltage is stepped to values more negative than −100 mV (Fig. 1 *a*). Deactivation of ClC-1 expressed in 293 cells is considerably faster than that observed for channels expressed in *Xenopus* oocytes (Steinmeyer et al., 1991a). The reason for this kinetic difference remains to be determined.

Fig. 1 *c* shows the steady state (*open circles*) and the instantaneous (*open squares*) I–V obtained from the currents shown in Fig. 1 *a*. The inward rectification of the open channel I–V and the steady state voltage dependence are very similar to results obtained from ClC-1 expressed in *Xenopus* oocytes (Steinmeyer et al., 1991a).

The relative open probability obtained by dividing steady state and instantaneous I–V is shown in Fig. 1 *d*. It is well fitted by a Boltzmann function with an effective gating valence of 0.86 and half-maximal activation at −100 mV. Thus gating of ClC-1 has a voltage dependence very similar to the fast activation gate of the structurally homologous ClC-0, the voltage-dependent chloride channel from the electric organ of *Torpedo* (Jentsch et al., 1990; Bauer et al., 1991; Miller and White, 1980). The saturating value of the open probability at positive voltages cannot be determined by the experiments shown in Fig. 1, but can be estimated by noise analysis as described below.

Another distinctive feature of ClC-1 expressed in *Xenopus* oocytes is its strong blockage by extracellular iodide, despite the rather high permeability of iodide as assessed by reversal potential measurements.[4] The same is true when the channel is expressed in 293 cells as is shown in Fig. 1 *b*. The replacement of most extracellular chloride by iodide resulted in only a small shift of the reversal potential in the positive direction, but both outward and inward currents were largely reduced (Fig. 1 *c*, *filled symbols*). Gating seems to be affected as well, since channels hardly deactivate at negative voltages.

The main purpose of the present study was to measure the single channel conductance of ClC-1. Because the channel changes open probability drastically and with resolvable kinetics when the membrane potential is stepped from positive to negative voltages, nonstationary noise analysis is well suited to estimate its conductance properties (Sigworth, 1977).

Fig. 2 shows the results of a typical noise-analysis experiment recorded from the same cell used for Fig. 1. Fig. 2 *a* shows the mean current obtained by averaging 290 individual current traces. Fig. 2 *b* shows the corresponding variance. In Fig. 2 *c* mean variance is plotted versus mean current (*circles*). Fitting the data with a parabola yielded a single channel conductance of 0.8 pS. Values ranging from 0.4 to 1.2 pS were obtained from several whole cell, cell attached, and outside-out patches at voltages negative to −100 mV (mean 0.9 ± 0.4 pS, $n = 17$, mean ± S.D.). Almost identical values were obtained for the rat homologue rClC-1 (0.9 ± 0.4 pS, $n = 7$, mean ± S.D.). In control experiments we expressed the *Torpedo* channel ClC-0 in 293 cells and measured a conductance of 9.0 ± 2 pS ($n = 4$) using noise analysis. This value is close to the one reported for *Torpedo* chloride channels in bilayers (Hanke and Miller, 1983) and to the conductance of ClC-0 expressed in *Xenopus* oocytes (Bauer et al., 1991).

Apart from conductance, noise analysis yields information about open probability. No noise should be generated at an open probability of one. It can be seen in Fig. 2, *b* and *c*, that a considerable variance can be measured at the very start of the test pulse to −140 mV. This means that, at the holding potential of −30 mV, open channel probability is well below one. On the other hand, Fig. 1, *c* and *d*, show that open probability is almost saturated at −30 mV. Therefore, it seems likely that even when the channel is maximally activated by voltage it gates between open and closed states.

DISCUSSION

We have expressed the major skeletal muscle chloride channel, ClC-1, in HEK293 cells and investigated it with patch-clamp techniques. Macroscopic properties are similar to results obtained from expression in *Xenopus* oocytes, apart from faster kinetics observed in mammalian cells. In addition, from nonstationary noise analysis we have determined that ClC-1 has a small single-channel conductance of about 1 pS.

It may be argued that the heterologous expression system used in this study does not reproduce the "physiological" conductance of the channel. We have no stringent evidence against this. However, as opposed to kinetic properties, conductance is a robust property of ion channels: values similar to physiological conductances were measured in different heterologous expression systems for voltage-dependent sodium (Stühmer et al., 1987), potassium (Stühmer et al., 1988), and calcium (Mori et al., 1991) channels, transmitter-activated chloride (Verdoorn et al., 1990) and cation channels (Imoto et al., 1988), and cyclic nucleotide-gated cation channels (Kaupp et al., 1989). In no case has a drastic change of channel conductance been reported. Thus we feel confident that ClC-1 has a low conductance also in vivo.

The low conductance of ClC-1 provides a simple explanation for why it has not yet been detected by patch-clamp measurements from intact skeletal muscle preparations. Several chloride channels have been proposed to represent the main skeletal muscle channel (Weber-Schürholz et al., 1993; Blatz and Magleby, 1985; Fahlke et al., 1993). Most of these have been measured in myoballs or myotubes and have a different voltage dependence than ClC-1, with conductances at least one order of magnitude higher than the value reported here. We now can rule out that these channels are mediating the large macroscopic chloride conductance of skeletal muscle. Thus any changes observed in

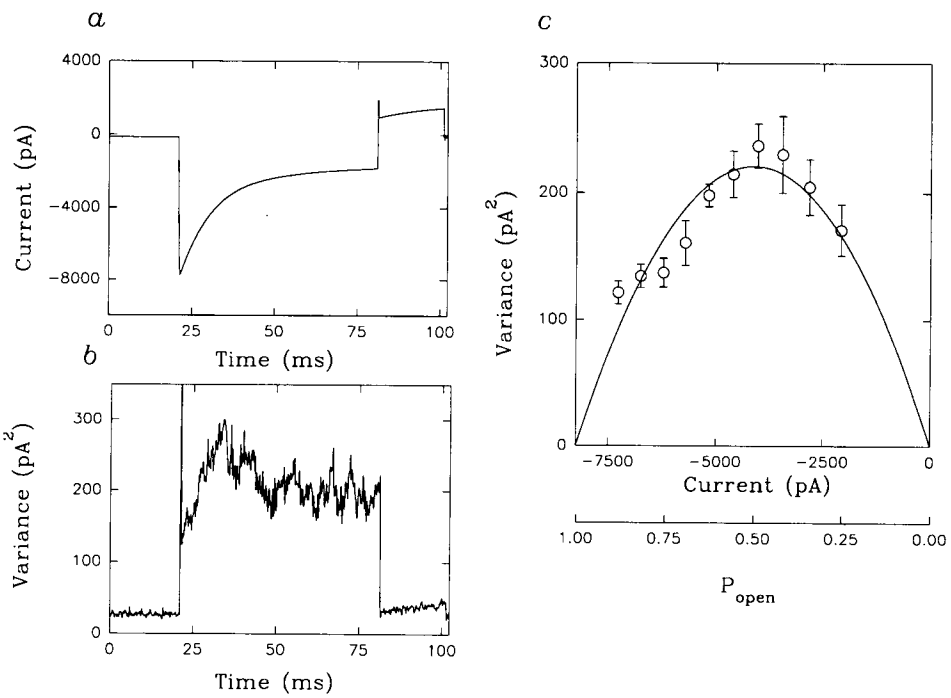

FIGURE 2 Noise analysis of hClC-1 expressed in HEK293 cells. (*a*) Mean of 290 records measured from the same cell shown in Fig. 1 obtained by stepping the membrane voltage repeatedly to −140 mV. In *b* the variance of the 290 records is shown. (*c*) Variance-current plot of the data shown in *a* and *b*. The current range was divided into 10 bins and the corresponding current and variance values were averaged. The data were fitted by the equation $\langle\sigma^2\rangle = i\langle I\rangle - \langle I\rangle^2/N$, were i is the single channel current and N represents the number of channels (Sigworth, 1977; Heinemann and Conti, 1992). The fit (*solid line*) yielded in this case $N = 7.7 \cdot 10^4$ and $i = 0.1$ pA corresponding to a conductance of 0.8 pS assuming a linear current-voltage relationship. The scale below the current axis in *c* corresponds to the open probability predicted by the fit.

these channels (Fahlke et al., 1993) cannot be the primary cause of myotonia.

Low-conductance chloride channels have been reported, e.g., in mast cells (Penner et al., 1988), chromaffin cells (Doroshenko et al., 1991), and lymphocytes (Lewis et al., 1993). Any structural relationship of these channels with the ClC family of voltage-dependent chloride channels remains an open question.

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