Volume-regulated Cl^- channels in human pleural mesothelioma cells

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Abstract Anion channels in human mesothelial and mesothelioma cell lines were characterized by patch-clamp and biomolecular approaches. We found an outwardly rectifying anionic current which was inactivated at positive voltages and inhibited by extracellular adenosine 5′-triphosphate (ATP). Mesothelial and mesothelioma cells behaved differently concerning current inactivation properties. Inactivation is more pronounced and has a steeper onset in mesothelial cells. Different reversal potentials, in asymmetrical Cl^- solutions, that could be attributed to a different selectivity of the channel, have been observed in the two cell lines. Mesothelioma cell single-channel analysis indicates that the number of the same active anion channel (3–4 pS) increased under hypoosmotic conditions. Immunocytochemistry experiments showed the presence of ICln protein in the cytosol and in the plasma membrane. Western blot analysis revealed an increase of ICln in the membrane under hypotonic conditions, an event possibly related to the activation of Cl^- channels.

Key words: Cl^- channel; Regulatory volume decrease; ICln; Adenosine 5′-triphosphate inhibition; Pleural mesothelium; Immunocytochemistry

1. Introduction

Pleural mesothelial and mesothelioma cells lack an extensive electrophysiological characterization, and up to now there are no data about Cl^- channels. However, the active role of mesothelial cells in water, small solutes and protein transport from the pleural space [1] suggests the presence of anionic conductances. Cl^- channels are known to take part in the transepithelial transfer of solutes and water in epithelia [2]; besides playing a relevant role in ionic homeostasis and in cell volume control (i.e. regulatory volume decrease, RVD) [3]. Channels involved in RVD may participate in proliferation and migration of tumor cells by regulating the cell cycle progress [4] with an active volume modulation. Dysfunctions involving ionic channels, particularly Cl^- channels activated upon hypotonic cell swelling, have been correlated with proliferative and migratory capacity of neoplastic cells [5,6]. RVD channel (RVDC) blockers are potent inhibitors of angiogenesis [7] and they have been shown to stop the proliferation of cervical cancer cells in the G0/G1 stage [8] and the invasive migration of glioma cells [9]. Recent data show an upregulation of anionic mitochondrial channels (VDAC) in mesothelioma cells compared to mesothelial cells [10].

The aim of this study was the characterization of Cl^- channels in mesothelioma and mesothelial cells of human pleura to elucidate their function in solute transport and in cellular ion and volume homeostasis to get some evidences about differences related to the occurrence of the mesothelioma.

2. Materials and methods

2.1. Drugs and chemicals

RPMI 1640 (Roswell Park Memorial Institute) medium, Medium 199 HEPES modification, DMEM (Dulbecco's modified essential medium), PBS (phosphate buffered saline), HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]), TES (*N*-Tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid), NMG (*N*-methyl-D-glucamine), EDTA (ethylenediamine tetraacetic acid), EGTA (ethyleneglycol-bis-[aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid), TEACl (tetraethylammonium chloride), ATP (adenosine 5′-triphosphate), NPPB (nitrophenylpropylamino benzoate) were purchased from Sigma (St. Louis, MO, USA).

2.2. Mesothelioma and mesothelial cell line

Mesothelioma cells were obtained from pleural effusions of patients with histologically demonstrated pleural malignant mesothelioma. Pleural effusions surgically obtained were centrifuged ($200\times g$, at 20°C for 10 min) and cell pellets were treated with erythrocyte lysis buffer (ammonium chloride 0.829%, potassium bicarbonate 0.1%, EDTA 0.0037%) and seeded into 25 cm^2 tissue culture flasks. Cells were cultured in RPMI medium with 15% fetal bovine serum, 2 mM glutamine, 10 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin. Cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C. When cells reached confluence, 1 min trypsin (0.25%)–EDTA (0.03%) treatment was used to detach the cells which were then reseeded at high density. Cell lines were considered established between the 15th and 30th passages. All the cell lines had the immunocytological characteristics of mesothelial cells: coexpression of low molecular weight cytokeratins, vimentin and Calretinin and negative reactivity with antibody to carcinoembryonic antigen (CEA) and thyroid transcription factor (TTF)-1 [11]. Specimens were also examined by electron microscopy for characteristic microvilli and intermediate filaments. The MeT-5A cell line (ATCC-LGC Promochem, UK) was produced from cells in the pleural fluids obtained from non-cancerous individuals. The cells were grown in culture flasks in Medium 199 containing 0.4 μM hydrocortisone, 0.87 μM zinc-free insulin, 3.3 nM epidermal growth factor, 0.05 mg/ml gentamicin, 5% fetal bovine serum. Subcultures were routinely established every third to fourth day by trypsin (0.25%)–EDTA (0.03%) treatment.

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2.3. Solutions for cell maintenance

During the patch-clamp experiments (before the seal formation) cells were maintained in a DMEM solution buffered using 10 mM TES or, when specified, in Krebs–Henseleit solution containing (mM): 110 NaCl; 4.7 KCl; 1.2 $MgSO_4$; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 10 TES; 5 glucose; 50 mannitol (pH 7.4); the quantity of mannitol was changed (0–50 mM) to obtain hypotonic and isotonic solutions, respectively.

2.4. Solutions for whole-cell configuration

The micropipette solution contained (mM): 108 NMGCl, 30 TEACl, 5 glucose, 10 HEPES/NMG (pH 7.2), 4 Mg–ATP, 1.9 $CaCl_2$ and 3 EGTA (free $[Ca^{2+}]$ of 2×10^{-7} M). The symmetrical extracellular solution contained (mM): 113 NMGCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 30 TEACl, 5 glucose, 10 HEPES/NMG (pH 7.4). NMGgluconate replaced NMGCl in Cl^- asymmetrical solution. In experiments for volume regulation the micropipette solution contained (mM): 90 NMGCl, 30 TEACl, 5 glucose, 10 HEPES/NMG (pH 7.2), 4 Mg–ATP, 50 mannitol, 1.9 $CaCl_2$ and 3 EGTA (free $[Ca^{2+}]$ of 2×10^{-7} M). The extracellular solution was (mM): 90 NMGCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 30 TEACl, 5 glucose, 10 HEPES/NMG (pH 7.4) and 0, 50 or 100 mM mannitol was added to obtained hypotonic, isotonic and hypertonic solutions, respectively.

2.5. Solutions for single-channel recording

In cell-attached configuration the bath solution was an iso- or hypotonic Krebs–Henseleit. The micropipette solution (cell-attached and inside-out configurations) contained (mM): 90 NMGCl, 30 TEACl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 HEPES/NMG (pH 7.4), 50 mannitol. In inside-out configuration the bath symmetrical solution contained (mM): 90 NMGCl, 30 TEACl, 1.9 $CaCl_2$, 3 EGTA, 5 glucose, 10 HEPES/NMG (pH 7.2), 50 mannitol. In the bath asymmetrical solution the 90 mM NMGCl was substituted with NMGgluconate.

2.6. Solutions for current-clamp experiments

Bath solution was an iso- or hypotonic Krebs–Henseleit solution. The micropipette solution contained (mM): 20 NaCl, 20 KCl, 100 Kgluconate, 1 $CaCl_2$, 2 EGTA, 10 HEPES/NMG (pH 7.2), 5 glucose, 4 Mg–ATP.

2.7. Patch-clamp technique

Patch-clamp technique and relative statistics were applied as reported [12]. In whole-cell configuration the resistances of the microelectrodes (R_{tel}) and of the seals (R_s) were 2–8 MΩ and 1–2 GΩ respectively. Signals were filtered at 5 kHz with an eight-pole Bessel filter. In single-channel recording the R_{tel} was 15–18 MΩ, and R_s was 15–50 GΩ. Signals were filtered at 0.2 kHz (eight-pole Bessel filter). In whole-cell and cell-attached configurations, potential differences were expressed as overall potential differences, considering the junction potential, the holding potentials and cell membrane potential (pd_{cm}) (cell-attached only). The bath was grounded with an Ag/AgCl electrode immersed in a 3 M KCl agar bridge.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MeT-5A and mesothelial cells by using RNeasy protect kit (Qiagen, Italy), according to manufacturer's instructions. 2–5 μg of total RNA were used for the first strand cDNA synthesis with Superscript II RT (Invitrogen, Italy) according to manufacturer's instruction. The primer pairs used in the PCR reaction were 5′-CGAATTCTCGAGATGAGCTTCCTCAAAAGTTTCCC-G-3′, 5′-GGAATTCGGTCCTCAGTGATCAACATCTGCATCCT-C-3′ for ICln. The PCR fragments were extracted from agarose gels (gel extraction kit, Qiagen, Italy), ligated into pSTblue1 vector (Novagen, Italy) and double strand sequenced in a Li-Cor Gene ReadIR 4200 automatic sequencer (MWG, Germany). Bioedit alignment program and BLAST protein database search program were used for the analysis of sequence similarities.

2.9. Western blot experiments

For total protein extraction cells from a 10 cm plate were lysed in 1 ml of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 10 μg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) and centrifuged at 4°C for 30 min at $10\,000\times g$. Supernatant was recovered and protein concentration measured using the Bradford method.

Cytosol and membrane protein were separated by Triton X-114-based lysis solution as reported by Musch and coworkers [13]. Proteins were resolved on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel, and electrophoretically transferred to Immuno-Blot™ polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Italy). The membranes were quenched in blocking solution overnight (5% non-fat dried milk, 50 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) and incubated with a polyclonal antibody to ICln [14], diluted respectively 1:700 or 1:200 in the blocking solution. The bound antibody was detected by the enhanced chemiluminescence procedure (Amersham Corp.) using horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:20 000.

In the experiments aimed at determining the ratio between the cytosolic and membrane fractions from cells under hypotonic or normal conditions, the densitometric analysis was conducted in a blinded fashion by two researchers who independently chose the regions of interest analyzing Western blot signals of cell fractions obtained from the same cell plate. Chosen areas were numerically integrated and measured with the Image Tool 3.00 software.

2.10. Immunocytochemistry

Cells were grown on coverslips to approximately 70% of confluence, fixed in a PBS (50 mM phosphate buffer, 150 mM NaCl) containing 3% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 3 min at room temperature. Cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and incubated overnight at 4°C with anti-ICln antibody at 1:100 dilution in PBS containing 0.1% BSA. After washing out of the primary antibody, cells were incubated for 1 h with Cy™2-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories). Cells were imaged using a Leica TCS-NT (Leica Microsystems, Heidelberg, Germany) confocal laser scanning microscope, equipped with laser argon/krypton, 75 mW multiline. Focal series of horizontal planes of section were monitored for fluorescein isothiocyanate (FITC) by means of the 488 and 568 nm laser lines, and a FITC band pass 590/30 filter.

2.11. Statistics

The experimental values are expressed as mean ± S.E.M. of n experiments. Student's t-test was used (with unpaired data unless otherwise specified) for the statistical analysis. The value of $P > 0.05$ is indicated by not significant (ns). Best fittings were performed by linear and non-linear regression analyses.

3. Results

We performed whole-cell patch-clamp experiments with symmetrical NMGCl solutions: the currents showed outward rectification and inactivation at voltages over +60 mV (Fig. 1B, E). In asymmetrical NMGCl solutions (Fig. 1C, E) the reversal potential (V_{rev}) shifted from 0.75 ± 0.8 to 34.8 ± 1.8 mV, $n = 4$ ($P < 0.01$, paired data), as expected for a Cl^- selective channel ($P_{gluconate}/P_{Cl} = 0.08 \pm 0.02$, $n = 4$). The current was inhibited by 10^{-4} M NPPB of about 90% at all voltages ($P < 0.05$, paired data, Fig. 1D, E).

Similar results concerning the Cl^- selectivity, the amplitude and the NPPB sensitivity of the measured current were obtained under the same conditions using MeT-5A cells (−0.586 ± 0.095 nA, $n = 9$, at −94 mV; 0.868 ± 0.154 nA, $n = 9$, at 86 mV; 90% of inhibition due to 10^{-4} M NPPB at 86 mV). V_{rev} shifted from 0.8 ± 0.8 to 25.4 ± 2.0 mV, $n = 9$, in asymmetrical NMGCl solutions ($P < 0.01$); this last V_{rev} was lower than that determined in mesothelioma cells ($P < 0.05$). Ratios between the currents values measured at the beginning (I_i) and at the end (I_f) of voltage steps (Fig. 1F) changed with the different cell types. Monoexponential fitting of the currents recorded at voltages of 80 and 100 mV revealed different inactivation time constants (0.82 ± 0.01 s, $n = 9$, vs. 2.05 ± 0.33 s, $n = 4$ at 80 mV, $P < 0.05$, and 0.80 ± 0.01 s, $n = 9$, vs.

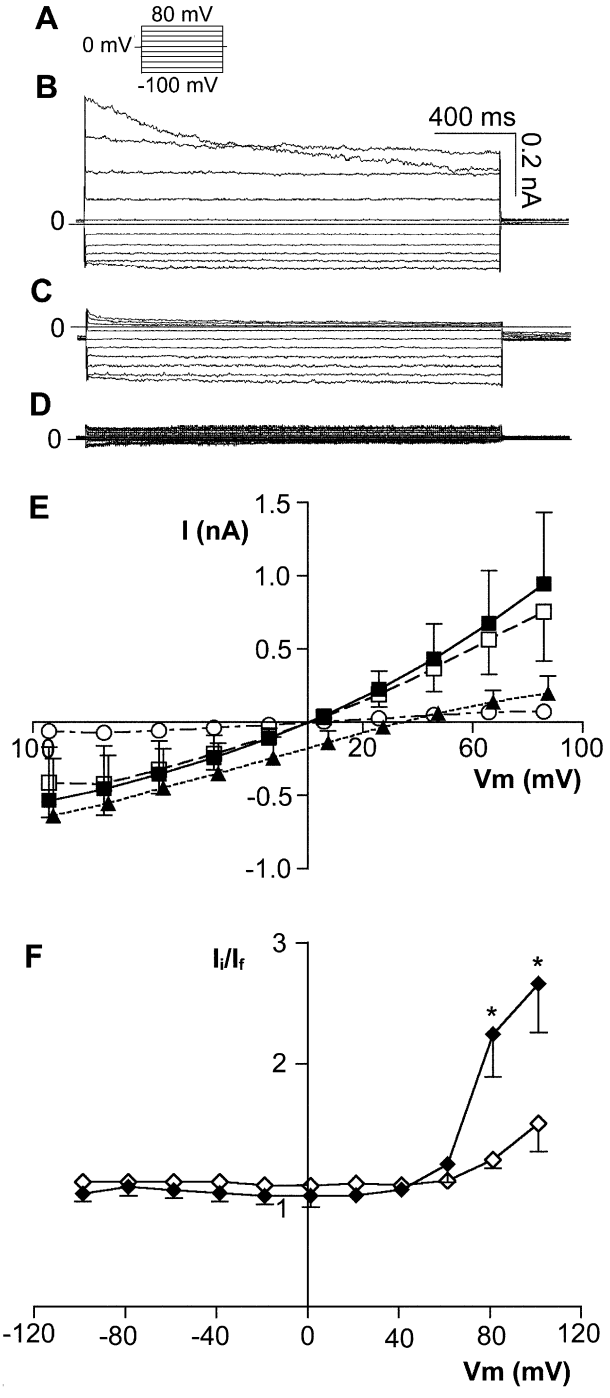

Fig. 1. Cl^- currents in mesothelioma cell line, whole-cell configuration. A: Stimulation protocol. B: Whole-cell currents in symmetrical NMGCl solutions, C: asymmetrical NMGCl solutions (25.6 mM [Cl^-] in the bath) and D: in presence of 10^{-4} M NPPB. E: Current–voltage (*I*/*V*) relationship in NMGCl symmetrical and asymmetrical solutions and NPPB effects: ■ symmetrical solutions, ▲ asymmetrical solutions, ○ 10^{-4} M NPPB, □ recovery in symmetrical solutions (means ± S.E.M., $n = 4$). F: Current inactivation analysis (symmetrical NMGCl solutions): ratio between initial currents (I_i) (at 10 ms to starting of pulse) and final currents (I_f) (at 1980 ms to starting of pulse): ◆ mesothelial cells, ◇ mesothelioma cells; means ± S.E.M. ($n = 9$ and $n = 4$, respectively); $^*P \leq 0.05$ in F.

1.69 ± 0.18 s, $n = 4$ at 100 mV, $P < 0.05$, for mesothelial and mesothelioma cells, respectively).

The tracings (Fig. 2B) and the *I*/*V* relationship (Fig. 2D) show an increase of currents (whole-cell configuration) when the hypotonic solution replaced the isotonic one. 10^{-4} M NPPB in the hypotonic extracellular medium induced a 90% decrease of the current at all voltages (from 5.87 ± 0.51 to 0.31 ± 0.15 nA at 102 mV, $n = 18$, $P < 0.01$). Extracellular ATP (10 mM), usually a voltage-dependent inhibitor of RVDC [15], reduced the swelling-induced current at positive voltages (Fig. 2C, D). In presence of the hypertonic extracellular solution, the current decreased (from 1.16 ± 0.21 nA under isotonic condition, to 0.63 ± 0.16 nA, +82 mV, $n = 9$, $P < 0.05$).

Fig. 3A shows single-channel tracings obtained in cell-attached configuration (isotonic extracellular solution) on human mesothelioma cells. In current-clamp condition the pd_{cm} was -36.2 ± 3.8 mV in isotonic extracellular solution, and -31.2 ± 2.3 mV 3–5 min after the substitution with the hypotonic solution ($P < 0.05$, $n = 8$). The single-channel *I*/*V*

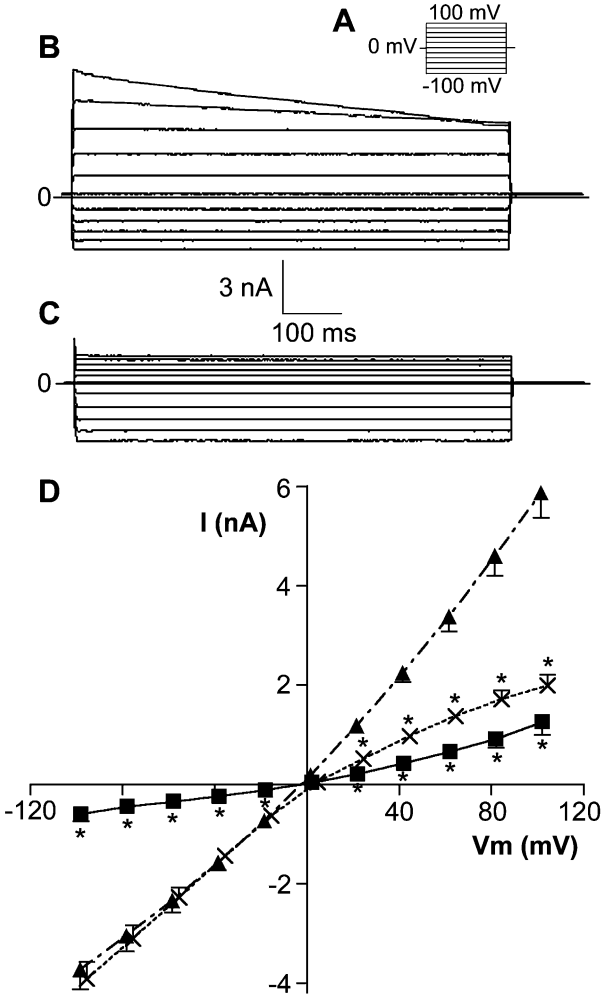

Fig. 2. Osmolarity effect on mesothelioma cell lines, whole-cell configuration. A: Protocol applied. B: Current recorded in hypotonic medium and C: extracellular ATP. D: Current–voltage (*I*/*V*) relationship in hypotonic medium in absence/presence of extracellular 10 mM ATP ($n = 18$). ■ Isotonic solution; ▲ hypotonic solution; × extracellular ATP; means ± S.E.M.; $^*P \leq 0.05$ compared to the hypotonic condition.

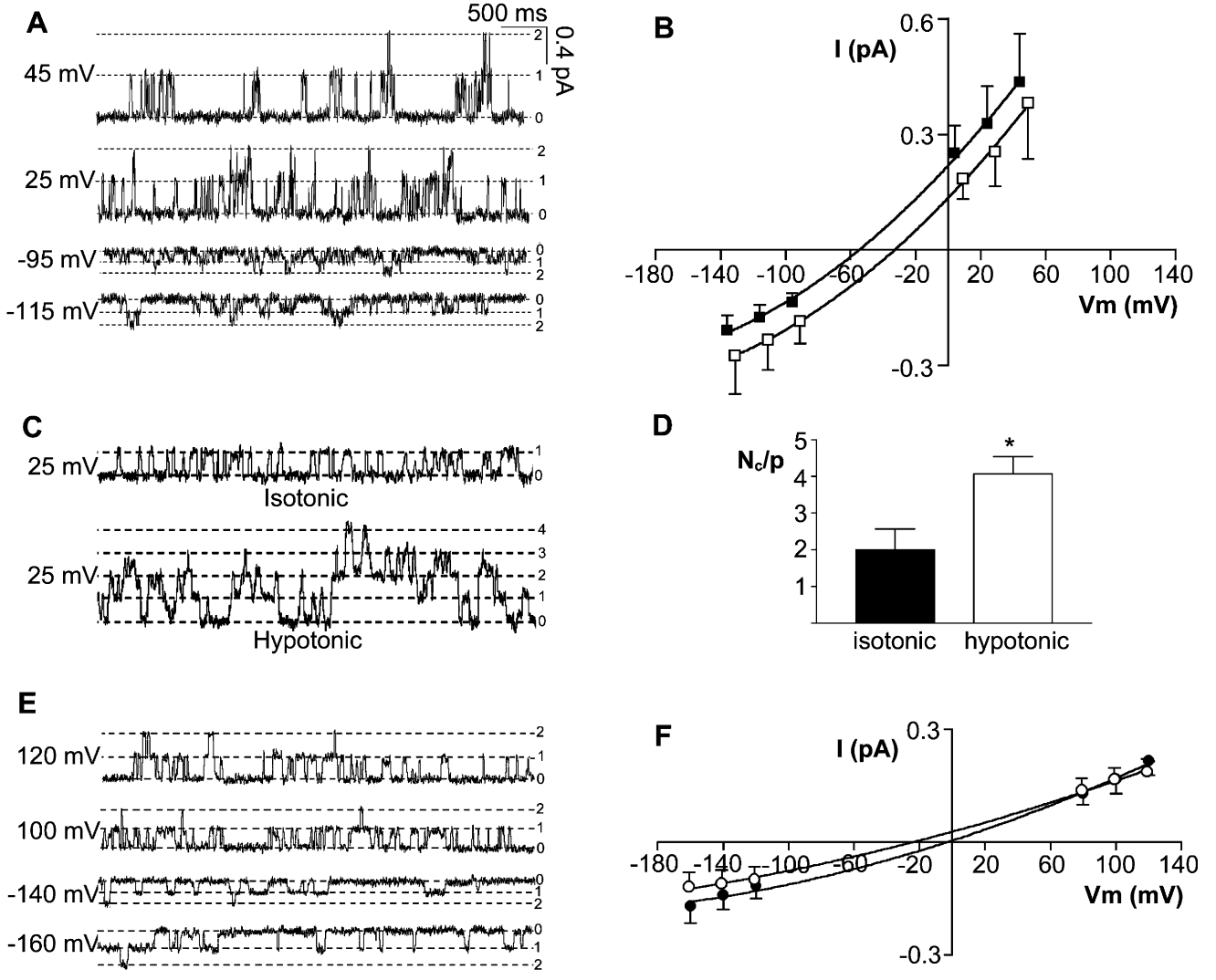

Fig. 3. Single-channel recordings in mesothelioma cell lines, cell-attached and inside-out configuration. A, C, E: Tracings observed at indicated voltages in isotonic or hypotonic solutions and in inside-out configuration (isotonic solutions): individual channels are shown, i.e. 1 and 2, 0 = closed state. B: Current–voltage (I/V) relationship in isotonic (■) and hypotonic (□) condition; means ± S.E.M. ($n = 9$). D: Number of the channels observed/number of patches (N_C/p, $n = 15$, * = $P < 0.05$ compared with isotonic condition). F: Current–voltage (I/V) relationship in inside-out configuration in symmetrical (●) and asymmetrical (○) NMGCl solutions; means ± S.E.M. ($n = 6$).

relationship (isotonic extracellular solution, Fig. 3B) revealed a single-channel conductance of 4.2 ± 1.1 pS (at 45 mV, $n = 9$). The V_{rev} was −56.2 ± 4.1 mV, a value corresponding to an estimated intracellular Cl^- activity of about 15 mM. No significant voltage dependence of P_o was observed (mean value of P_o 0.109 ± 0.018, $n = 9$, between −140 and 45 mV). The I/V relationship in the hypotonic solution (Fig. 3B) showed a different V_{rev} (−37.6 ± 6.0 mV, $n = 9$, $P < 0.05$). The conductance (4.6 ± 1.5 pS at 45 mV, $n = 9$, ns) and the P_o (mean value of 0.137 ± 0.041, $n = 9$, between −140 and +45 mV, ns) were similar to those observed under isotonic conditions, but the number of activated channels in a patch was significantly increased ($P < 0.05$, $n = 15$, Fig. 3C, D). The nature of the channel was confirmed by inside-out experiments (Fig. 3E, F). In presence of symmetrical NMGCl solutions the I/V relationship (Fig. 3F) was outwardly rectifying. The conductance was 1.7 ± 0.4 pS, $n = 6$ (at 100 mV, ns from that in cell-attached configuration) and the V_{rev} was −2.2 ± 2.5 mV, $n = 6$ (ns from 0). Replacing NMGCl with a solution containing NMGgluconate (Fig. 3F) we observed a shift of the V_{rev} (−25.9 ± 5.5 mV, $n = 6$, $P < 0.05$; $P_{gluconate}/P_{Cl} = 0.21 \pm 0.14$, $n = 6$).

We focused our attention on the ICln protein, one of the possible candidates for the swelling-activated current ($I_{Cl,swell}$) [3]. The expression of the protein was confirmed by RT-PCR and by sequencing of PCR products; also Western blot, performed on total cellular lysates kept under isotonic conditions, revealed the presence of the protein (Fig. 4).

By using confocal microscopy and the same antibody used in the Western blot experiments, we performed immunofluorescence tests with tumoral cells kept under isotonic conditions (Fig. 5). In all cells fluorescence was visible in the cytoplasmic region, while almost no immunoreactivity was detectable in the nucleus (Fig. 5A). At a higher magnification a clear signal was detectable also in, or near, the membrane (Fig. 5B). Interestingly, in many cells additional patches of intracellular fluorescence were also observed (Fig. 5C). Presence of the ICln protein both in the membrane and in the cytosol was confirmed also by Western blot experiments per-

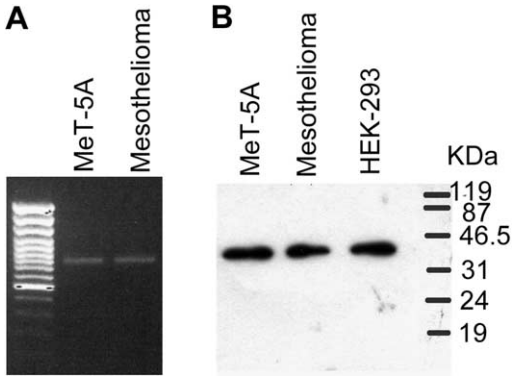

Fig. 4. RT-PCR and Western blot experiments performed on human Met-5A and mesothelioma cells. A: RT-PCR products obtained with ICln-specific primers. A single band of the expected size (714 bp) was found for each cell. B: Western blot of cellular lysates probed with an anti-ICln polyclonal antibody. HEK-293 cells (human embryonic kidney cells) were used as positive control.

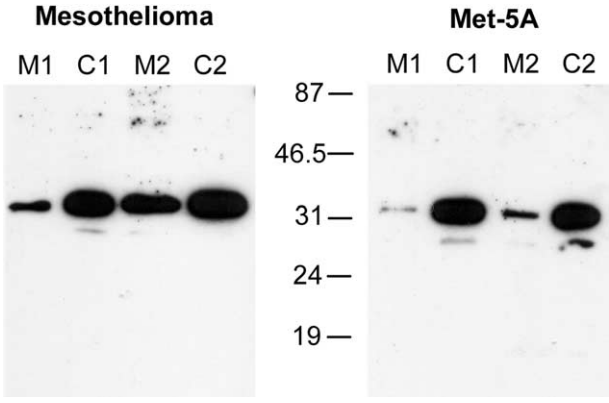

Fig. 6. Western blot performed with an anti-ICln antibody on cytosolic (C) and particulate (M) fractions of human mesothelioma and MeT-5A cells maintained in isotonic solutions (C1, M1) or challenged with a 10 min hypotonic stress (C2, M2).

formed in parallel on protein preparations of the particulate fraction and on the cytosolic fraction of the same cell population (Fig. 6). The signal in the membrane fraction under isotonic conditions increased after stressing the cells with a 10 min hypotonic shock in both cell types (Fig. 6).

4. Discussion

In mesothelioma cells, under isotonic conditions, the observed single-channel current can justify the total Cl^- current measured in whole-cell configuration. The biophysical and pharmacological channel properties resemble those of the anion channels responsible for the swelling-activated anion current ($I_{Cl,swell}$) found in other cell types [16]. In particular, single-channel data recall some characteristics of the ICln protein reconstituted in lipid bilayers [17].

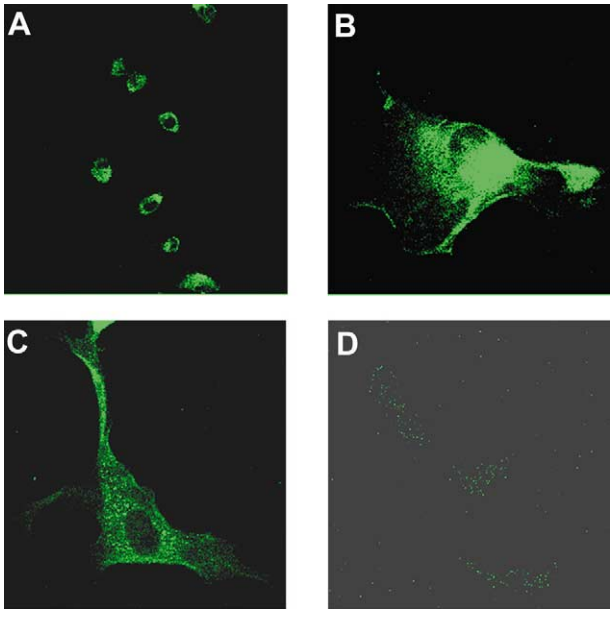

Fig. 5. Localization of ICln in mesothelioma cells by immunocytochemistry. Images were obtained by confocal microscopy. A: 1440× (pinhole 1) magnification showing uniform staining of the cytosol. B, C: 2880× (pinhole 0.43 and 0.7) magnification showing positive signals in correspondence of the cell membrane (B) and of intracellular structures (C). D: Negative control obtained by omitting the primary antibody (2880× (pinhole 0.8) magnification).

In both mesothelioma and mesothelial cells, Cl^- currents inactivated at potentials over +60 mV, but with different kinetics. Inactivation is more pronounced, occurs earlier and has a steeper onset in mesothelial cells. We also observed different V_{rev}, in asymmetrical Cl^- solutions, that could be attributed to a different selectivity of the channel. The physiological meaning of these differences is unclear but could be related to the role of the RVD channel that is thought to be involved in the proliferation and migration process in many cell types. For instance, the proliferation rate of C6 glioma cells, human cervical cancer cells and endothelial cells can be blocked by inhibitors of RVDC [7,8,18] and the migration of human glioma cells depends on the salt and water efflux through the RVDC [9]. In other cell lines differences in ionic conductances (Cl^- in particular) between tumoral and non-tumoral cells have been described and related to their potential in clinical diagnosis and therapy [19,20].

In mesothelioma cells the observed current under hypotonic conditions (whole-cell configuration) increased up to 340% and shared the same features of $I_{Cl,swell}$ [3]. Presence of RVDC was supported also by the extracellular ATP voltage-dependent inhibition of the current [15] and the 50% current decrease in hypertonic conditions [21]. Under hypotonic conditions there was no change of single Cl^- channel properties but for the increased number of active channels in the single patch. All these data suggest that the same channel population sustains the current under iso- and hypotonic conditions.

In both mesothelial and mesothelioma cells RT-PCR and Western blot experiments showed ICln expression; this protein is a candidate for the RVDC [3] as well as for the observed currents in mesothelial cells. Fluorescence confocal microscopy and Western blot experiments revealed presence of ICln in the cytosolic area of mesothelioma cells [14], but a signal was present also at the plasmalemma level, in isotonic conditions. However, these data cannot establish whether ICln protein associates to or is inserted in the membrane. The Western blot experiments also showed that the membrane-associated ICln fraction increases after exposure of the cells to a hypotonic shock, similarly to what has been

reported for rat myocytes [13] and for NIH fibroblasts [14]. The mechanism by which ICln translocates to the membrane has not been yet elucidated; it is believed that no vesicular trafficking event is involved [14,22].

In conclusion, our data show the presence of a RVDC, which is active also in isotonic conditions in mesothelioma cells. The number of active anion channels increases in hypotonic conditions, in coincidence with an enhanced ICln expression in the cell membrane. This last event could be related to the activation of the RVDC. Whether RVDC is due to or regulated by ICln is still in debate [23,24]. Since RVDC may be involved in the regulation of the cell cycle progress, in cell migration and apoptosis [25] further investigations on differences between Cl^- channel properties in mesothelial or mesothelioma cells could prove useful in the diagnosis and/or treatment of mesothelioma, a cancer type particularly resistant to chemotherapy [26].

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