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Direct Inhibitory Effects of an Antisense Oligodeoxynucleotide upon the Volume-Sensitive Chloride Current in Rat Osteoblast-like (ROS 17/2.8) Cells

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The effects of a 15-mer antisense *c-myc* phosphorothioate modified oligodeoxynucleotide (OdN) upon the volume-sensitive Cl^- current in ROS 17/2.8 cells were investigated using the whole-cell configuration of the patch clamp technique. At 5 μM, the OdN reversibly inhibited the current in a voltage- and time-dependent fashion. This was evident from the reduction in the peak current as assessed at the termination of each voltage pulse and an acceleration of the time-dependent inactivation present at strongly depolarised potentials. The kinetic modifications induced by the OdN suggest it may act by blocking the pore of open channels when the cell membrane potential is depolarised.

The ability of oligodeoxynucleotides (OdNs) to suppress specific cellular protein expression is potentially a source of substantial therapeutic benefit. These effects rely upon the OdN specifically binding to a target sequence of either DNA (antigene effect) or mRNA (antisense effect) within the cell and thus arresting transcription or translation (1). However, relatively fewer studies have addressed the effects of these molecules upon biological systems by other possibly non-selective effects such as direct binding to proteins (aptamer effect).

We have previously reported that the rat osteoblastic osteosarcoma cell line ROS 17/2.8 expresses a volume-sensitive Cl^- current that appears to have a vital role in the volume homeostasis of these cells (2,3). In the present study we show that this current is inhibited by a 15-mer phosphorothioate backbone modified antisense oligodeoxynucleotide directed against *c-myc* expression (15-S-myc).

MATERIALS AND METHODS

Cells. ROS 17/2.8 cells were grown and maintained as previously described (2). For electrophysiological experiments cells were seeded onto uncoated sterile glass coverslips at a density of 1×10^4 cells ml^{-1} and used in experiments 3-5 days later.

Patch-clamp experiments. Experiments were performed using the whole-cell configuration of the patch-clamp technique with Cl^- selective intra- and extracellular solutions as previously described (2,4). Briefly, pieces of coverslip were placed upon the stage of an inverted microscope and superfused at 2 ml min^{-1} with an isotonic bathing solution consisting of (mM): NMDG Cl 140, $MgCl_2$ 0.5, $CaCl_2$ 1.3, Hepes 10, pH 7.4, osmolarity 300 mosmol l^{-1}. Patch pipettes of 2-4 MΩ resistance were filled with an intracellular solution consisting of (mM): NMDG-Cl 140, $MgCl_2$ 1.2, $CaCl_2$ 1.3, EGTA 1, Hepes 10, pH 7.4; osmolarity 300 mosmol l^{-1}. Hypotonic bathing solution (220 mosmol l^{-1}, achieved by reducing the extracellular NMDG Cl concentration to 105 mM) and OdN were applied to the cell under study by means of a multibarrelled fast perfusion system (5). Whole-cell currents were recorded with an Axopatch 1C patch-clamp amplifier and analysed using pClamp 5.5.1 software (Axon Instruments, CA, USA).

Data are expressed as means ± S.E.M. (n, number of observations). Current inactivation was quantified by using a single exponential function Y to yield a time constant value (τ_{inact}), where A_0 and A_1 are the current values at the beginning and end of the fitting region:

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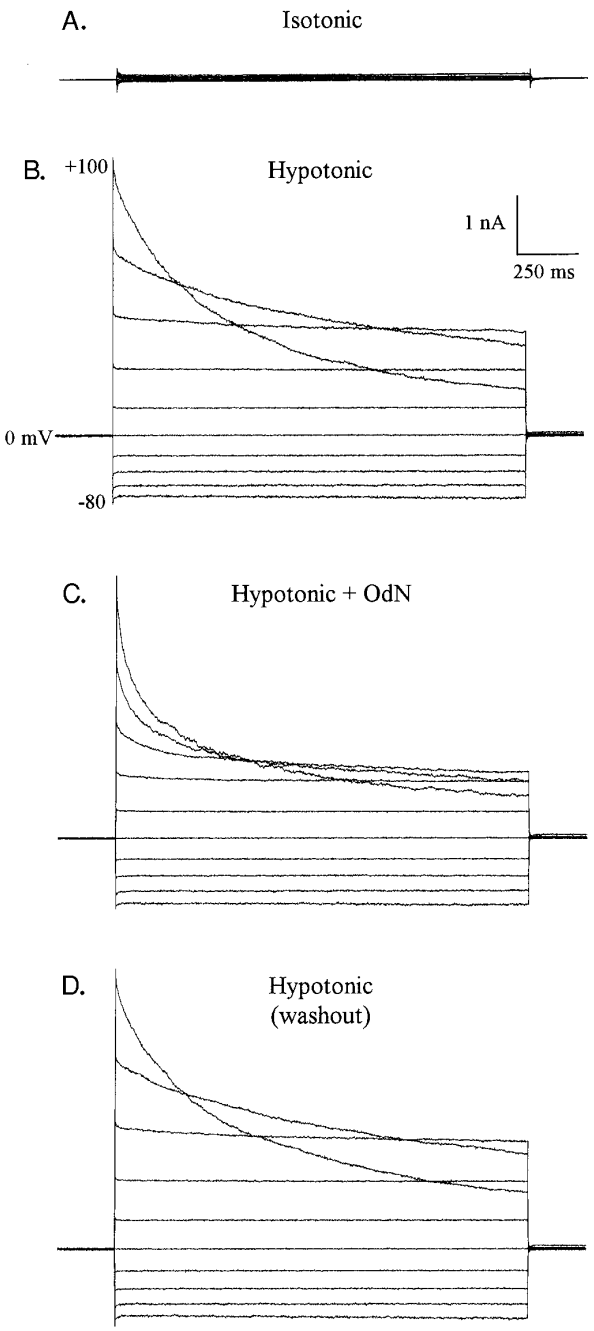

FIG. 1. The effects of OdN (5 μM) upon the volume-sensitive Cl^- current in ROS 17/2.8 cells. Currents responses elicited by voltage-clamp pulses to potentials between −80 and +100 mV (20 mV increments) for 1.6 s under isotonic (A) and hypotonic (B) conditions. C, 30 s after commencing superfusion with 5 μM OdN under hypotonic conditions. D, 1 min after cessation of OdN superfusion (hypotonic conditions). Holding potential was 0 mV and each pulse was preceeded by a 500 ms conditioning pulse to −100 mV to remove any inactivation produced by the previous command step. Data representative of 5 similar experiments.

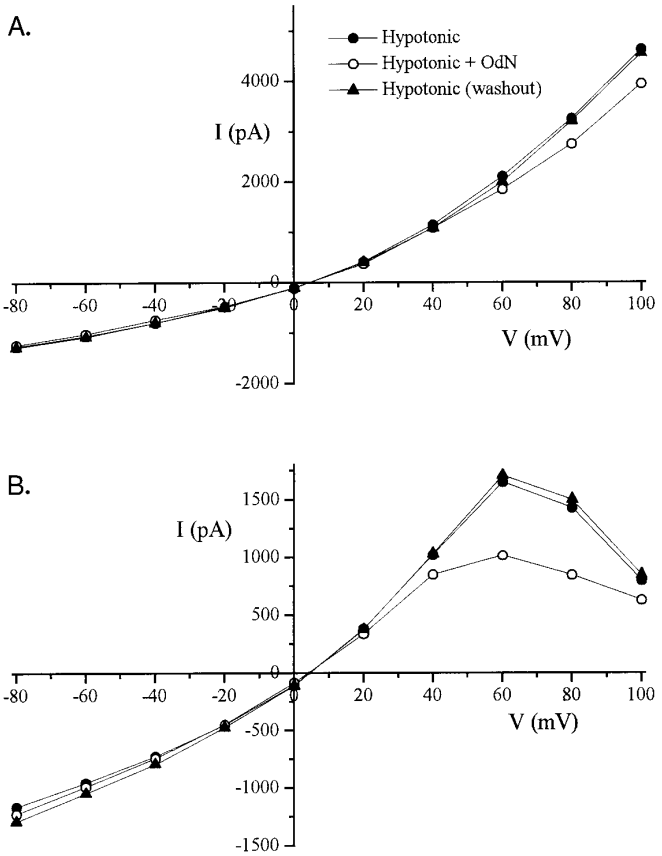

FIG. 2. Current-voltage relationships for currents shown in figure 1. Peak currents were assessed 5 ms after the onset of the voltage clamp pulse (A) and 5 ms prior to it's termination (B). Data representative of 5 similar experiments.

$$Y = A_0 + A_1^{-t/\tau_{inact}}$$

The fitting region commenced 5 ms after the onset of the voltage pulse and continued until 5 ms prior to the end to ensure the fit was free from contamination by any uncompensated capacitance transients.

Oligonucleotide synthesis and purification. Antisense phosphorothioate oligodeoxynucleotides (OdNs) complementary to the AUG initiation codon of the human *c-myc* oncogene exon 2 (3′ TAC GGG GAG TTG CAA 5′) were synthesized on an automated DNA synthesizer (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry and purified as described previously (6). The OdN was dissolved directly into hypotonic bathing solution and used at 5 μM (concentration confirmed by absorbance at 260 nm).

RESULTS

Under conditions designed to isolate Cl^- currents, ROS 17/2.8 cells responded to a hypotonic challenge with large, outwardly rectifying Cl^- currents that exhibited time-dependent inactivation at potentials more positive than +40 mV (fig. 1 & refs 2,4). The effects of extracellular application of 5 μM OdN upon this current are shown in figures 1 and 2. Peak currents at the onset of the voltage pulse were only marginally inhibited, an effect that was not influenced by the clamping potential (inhibition at −80 mV, 6.9 ± 2.0%; +80 mV, 5.9 ± 3.0%, n = 5; see fig 1 and 2A). However, if the currents were assessed 5 ms prior to the cessation of the voltage pulse the inhibition was much more marked and strongly voltage-dependent, occurring only at potentials more depolarized than +20 mV (at −80mV, 7.4 ± 2.3%; +80 mV, 33.8 ±

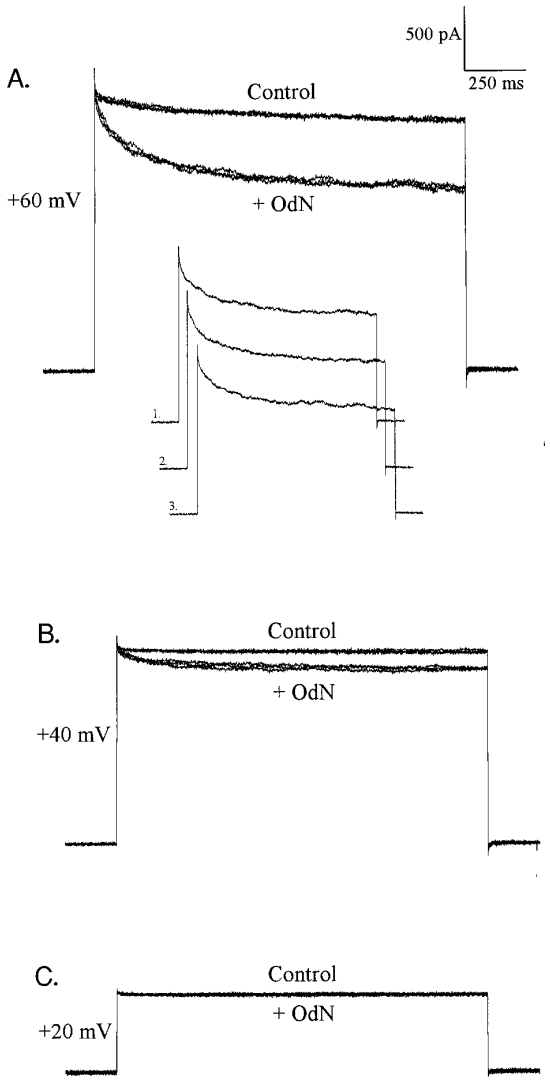

FIG. 3. Effects of 5 μM OdN upon repetetive voltage-clamp pulses. Voltage-clamp pulses were applied to a single osmotically swollen ROS 17/2.8 cell for 1.6 s from a holding potential of 0 mV at 0.2 Hz. 6 pulses were applied at each potential, the first 3 in the absence of OdN, the remaining 3 with 5 μM OdN present (under hypotonic conditions) at +20 (A), +40 (B) and +60 mV (C). Data representative of 2 similar experiments. Inset in A shows the 3 pulses in the presence of OdN in more detail.

6.4%, n = 5; fig. 2B & 3). Interestingly, the inhibitory effect of the OdN at the end of the +100 mV pulse (23.4 ± 4.3%, n = 5) was lower than that at +80 mV. The OdN also elicited kinetic modifications of the current. These were manifest as an acceleration of the inactivation present at strong depolarizations and also the induction of inactivation at depolarized potentials where it was previously absent (e.g. +40 mV; figs. 1 & 3). In 5 cells the monoexponential fit time constant (τ_{inact}) for the +100 mV test step was reduced from a control value of 354 ± 32 to 144 ± 7 ms, and the +40 mV test step from 569 ± 105 to 220 ± 47 ms. All OdN effects were totally reversible upon washout (within 10 s).

Sequential, multiple test steps to +20, +40 and +60 mV (from the holding potential of 0

mV) applied at 0.2 Hz (fig. 3) not only highlighted the voltage-dependence of the OdN effect but suggested that the effect occurred within 5 s of exposure to the cell. As each of the test pulses applied in the presence of OdN commenced with the same peak value this suggested that on returning to the holding potential the OdN dissociated from the channel protein (fig. 3A inset).

DISCUSSION

These results suggest that a 15-mer phosphorothioate OdN reversibly binds to and blocks the volume-sensitive Cl^- current in ROS 17/2.8 cells at depolarized potentials. As this effect can be determined within 5 s of OdN application and is rapidly reversible it is highly unlikely that this effect is mediated via an antigene or antisense mechanism. The most logical explanation of these findings is the direct binding of the OdN to the channel protein (aptamer effect). The exact mechanism by which the OdN inhibits the current is not totally clear, but effects upon both current amplitude and inactivation kinetics suggest this process is both voltage- and time-dependent (figs. 1-3). A number of studies have reported similar effects of the Cl^- channel blocker 4,4′-diisothiocyanatostilbene-2,2-disulphonic acid (DIDS) upon volume-sensitive Cl^- currents and suggested these effects reflect time-dependent, reversible association of the negatively charged blocker with the channel at depolarised potentials (2,7,8). This hypothesis may explain the above results as the OdN used in this study will, at physiological pH, be strongly negatively charged due to the 15 phosphorothioate moieties that form the OdN backbone. The maximal inhibition as assessed at the termination of the voltage pulse occurred at +80 mV, not +100 mV. As the degree and rate of inactivation is much higher at +100 mV than at +80 mV, this strongly suggests that the OdN binds to the channel only when in its open inactivated state (‘open channel block’) and may thus elicit it’s effects via a pore blocking mechanism.

These results suggest that caution should be exercised when interpreting data from electrophysiological assays regarding the efficacy of antisense oligonucleotides against targeted ion channel proteins as inhibition of ion flux may simply be due to a transient aptameric effect rather than a genuine ‘antisense’ mechanism.

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