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Antisense Oligonucleotides Discriminating between Two Muscular Na^+ Channel Isoforms

H. Brinkmeier,* B. Schu,* H. Seliger,† L.L. Kürz,* Ch. Buchholz,* and R. Rüdel*
**Abt. für Allgemeine Physiologie, †Sektion Polymere, Universität Ulm, D-89069 Ulm, Germany*

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Various 15-mer antisense oligodeoxynucleotides (aODNs) were constructed against RNAs coding for two closely related isoforms of the voltage-dependent Na^+ channel, i.e. those of human heart (hH1) and skeletal (hSkM1) muscle. When translated *in vitro,* either RNA yielded a 220 kDa band on polyacrylamide gels, indicating that the translation product had full length. Of six different aODN constructs developed against hH1 RNA, two each inhibited translation completely, moderately or not at all, depending on the target position. The specificity of the effect (no cross reaction at 10 μM) was confirmed by incubation with 15-mer aODNs against hSkM1 RNA. The most effective aODNs were those hybridizing between bases 3840 and 3880 of hSkM1 RNA and the homologous segment of hH1 RNA. When either of the RNAs was co-injected with its most effective (phospho rothioate-capped) aODN into *Xenopus* oocytes, the production of Na^+ channels was strongly suppressed (relative I_{Na} for hSkM1: 0.08 ± 0.05 times control, n = 14; for hH1: 0.11 ± 0.08, n = 11). We conclude that aODNs are able to discriminate between closely related RNAs. The efficacy of an aODN depends strongly on its RNA target position.

Precise functioning of the voltage-dependent Na^+ channels is a prerequisite for correct excitability of nerve and muscle cells. Na^+ channel function impaired by changed gene expression (1), defective regulation, the action of toxins (2) or by genetic mutations (3,4) may lead to cellular hyperexcitability or paralysis. In fact, such defects are the cause of many diseases in man and animals. Apart from alterations of excitability, Na^+ channel dysfunction may even induce neurodegeneration (5,6). The therapy of these "sodium channelopathies" is usually based on pharmaceutical agents that bind to the Na^+ channel protein, thus normalizing its function. Unfortunately, the specificity of both Na^+ channel agonists and antagonists is often low, as they interact likewise with the Na^+ channel isoforms of skeletal muscle, heart and nervous tissue (7,8). At present, eight genes, SCN1A-SCN8A, are known that encode the different α subunits of human voltage-dependent Na^+ channels (4). The adult skeletal muscle Na^+ channel, hSkM1, is encoded by SCN4A. The cardiac Na^+ channel, hH1, is encoded by SCN5A. The latter is also expressed in immature and regenerating skeletal muscle.

As to the influence on the function of the protein, an alternative strategy to using drugs is the manipulation of the lifespan of its RNA. This can be achieved by the use of antisense oligodeoxynucleotides (aODNs) that interact with mRNAs (9-11). The action of an aODN involves hybridization to its target RNA by which the latter is either arrested from being translated (12) or is degraded by the endogenous RNase H (13). Antisense oligonucleotides have been found effective when directed against the 5′ untranslated region of the RNA, when spanning the AUG, when binding within the coding region, or at intron-exon junctions. They can exert their effects both within the nucleus and the cytoplasm (14). It is still impossible to predict generally the best target site for an antisense ODN. Therefore, the search for an effective aODN is an experimental one.

We have followed a step-wise approach to develop effective antisense ODNs for the selective elimination of RNAs coding for the two voltage-dependent Na^+ channels of human heart (hH1) (15) and skeletal (hSkM1) muscle (16). As both channels are regularly expressed in human skeletal muscle cultures (17,18), selective knock-out of either channel by aODNs would indicate a specific action.

The aim of the present work was to test whether aODNs (15-mers) can discriminate between the two forms of Na^+ channel RNA with high homology (75% sequence identity) and whether the aODN target positions are crucial for their action, i.e. inhibition of translation. We first studied inhibition of RNA translation *in vitro,* and then tested the such determined optimal aODNs in a cellular system, injecting them into *Xenopus* oocytes and measuring the amplitude of the Na^+ currents.

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MATERIALS AND METHODS

Plasmids and preparation of cRNA. Plasmids (pSP64T) containing the full length cDNAs of the human hH1 and the hSkM1 Na^+ channels (α subunits) were obtained from Dr. A.L. George, Vanderbilt University, Nashville, TN, USA (15,16). The plasmids were linearized with EcoR1 (hSkM1) or Spe1 (hH1). Transcription was performed using 0.5 μg cDNA and an *in-vitro* transcription kit (#1581 040, SP6 Cap Scribe, Boehringer Mannheim, Mannheim, Germany) in the presence of the methylated 5′-cap analogue (m^7GpppG).

RNAs were separated with TAE (tris/acetic acid/EDTA) agarose gels (0.5%) to which about 5 mg/l iodoacetic acid (Fluka, Neu Ulm, Germany) was added. Molecular weight markers were from Sigma (# R-3137, Sigma Chemie, Deisenhofen, Germany).

Oligonucleotides. Standard oligodeoxynucleotides (15-mers) and phosphorothioate-capped oligonucleotides (phosphorothioate-modified nucleotides at positions 1 and 15) were obtained from Interactiva (Ulm, Germany). As to the designation of our constructs, the number corresponds to the start nucleotide of the target sequence of the published cDNAs (15,16) and "a" and "sen" indicate whether the ODN is an antisense or a sense construct, respectively. The used aODNs against hH1 RNA were (sequences from 5′ to 3′ end): GGTAATAGGAAGTTT (156a), TTTGGGTGCTATAGA (407a), TCAGGTTCACCAGGT (1358a), TAATGAGACCACCCC (1945a), CTCTTCATACCCCCT (4444a), TCAGTGGGAATACAA (4464a). The aODNs against hSkM1 RNA were: CGGGAGAAGGAGGAG (3846a) and GCAGTACGAGGTGAA (3866a). In all cases the complementary sense constructs were used as controls. A 20-mer oligonucleotide that is not related to either RNA (sequence GAGGCTGAGGATCCCTATTC) was used for controls.

In-vitro translation and SDS gel electrophoresis. In-vitro translation was carried out with a rabbit reticulocyte lysate system (# L4960, Promega Corp., Madison, WI, USA) in the presence of canine pancreatic microsomal membranes (# Y4041, Promega) and the ribonuclease inhibitor RNasin (# N2511, Promega). The components were stored at −70 °C and thawed prior to use. The reactions were carried out in 50 μl samples containing 1 μl of the supplied amino acid mixture without methionine, 40 μCi of ^{35}S-labeled methionine (Amersham, Braunschweig, Germany), 33 μl of the rabbit reticulocyte lysate, 80 U/50 μl RNasin, 3 μl of the canine pancreatic microsomal membranes and 0.4 to 0.8 μg of the prepared hSkM1 or hH1 RNAs. For controls, 2 μl of the supplied luciferase RNA solution were added instead of the Na^+ channel RNAs. The final volume of the samples was adjusted to 50 μl with diethylpyro carbonate (DEPC)-treated H_2O. The samples were incubated for 2 h at 33 °C, centrifuged and mixed 1:1 (v/v) with the sample buffer used for application to the SDS gels. The sample buffer consisted of 31.25 mM Tris/HCl, pH 6.8, 5% glycerol, 1% SDS, 2.5 % mercaptoethanol and 0.000625% bromphenol blue. All components were obtained from Sigma except bromphenol blue (Fluka).

The translation products were separated on a 4-12% acrylamide/bisacrylamide gradient gel (SDS gel electrophoresis). A stacking gel of 4% acrylamide/bisacrylamide was used. Acrylamide and bisacrylamide were ob tained from Sigma and mixed in a ratio of 37.5:1 (v/v). Protein electrophoresis was carried out with the mini protean II electrophoresis system (Bio-Rad, München, Germany) using the standard protocols suggested by the producer. Molecular weight markers, myosin heavy chain, β-galactosidase, phosphorylase b, bovine serum albumin and ovalbumin were also from Bio-Rad (# 161-0303). After electrophoresis, the gels were stained with coomassie brilliant blue R-250, dried for 2 h at 80 °C and exposed to a phosphor storage screen (Molecular Dynamics, Krefeld, Germany). After 10-20 h of exposition, a phosphoimager-system with appropriate software (Molecular Dynamics) was used for evaluation.

Translation of Na^+ channel cRNA in Xenopus oocytes and electrophysiology. Both hH1 and hSkM1 cRNAs were injected into groups of oocytes in amounts of about 1.25, 6 or 12 ng per cell. After 3 d of incubation (for details see (19)), Na^+ inward currents could be recorded from all groups, except those injected with 1.25 ng hH1 RNA. To obtain Na^+ inward currents with a maximum amplitude of 5-10 nA, 1.25 ng of the hSkM1 RNA and 6 ng of the hH1 RNA were required, 12 ng seemed to be in surplus, and was therefore not used in attempts to suppress RNA translation.

For electrophysiology, single oocytes were placed in a modified ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES-NaOH, pH 7.5. The two-microelectrode voltage-clamp technique (CA-1 amplifier, Dagan, Minneapolis, MN, USA, microelectrode resistance 0.5-2 MΩ) was used to elicit Na^+ inward currents (with square voltage pulses going from −80 mV to between −70 and +40 mV in 10-mV steps; each test pulse preceded by a 100-ms prepulse to −100 mV). The maximum of each current trace was plotted against the applied test potential (current-voltage relation), and the absolute current maximum was registered.

To investigate the efficacy of an aODN in suppressing RNA translation, 6 ng hH1 RNA or 1.25 ng hSkM1 RNA, dissolved in a 100 μM aODN solution, were coinjected into the oocyte. For controls, oocytes were coinjected either with the corresponding sense ODN or with RNA alone. All injected oocytes had normal resting potentials.

Calculation of RNA secondary structure. RNA structures were calculated with a com mercially available software (Genetics Computer Group, Madison, WI, USA) according to an algorithm of Zuker (20).

RESULTS

Effect of the aODNs on the in-vitro translation of hH1 RNA and hSkM1 RNA. Separation of the cDNA transcripts on an 0.5% TAE agarose gel (Fig. 1A) reproducibly yielded two bands for the hH1 RNA, indicating the presence of a smaller and a larger RNA molecule (lane 1). The larger one is probably the full-length transcript, because functional Na^+ channels were expressed when the prepared cRNA was injected into *Xenopus* oocytes. For hSkM1 RNA, agarose gel separation always yielded a single band at the expected position (lane 3). Both full-length transcripts showed a higher mobility in the TAE gel as expected from their sequences (7.5 kB for hH1 and 6.5 kB for hSkM1). This may be due to secondary structures attained by the RNA molecules under running conditions.

In-vitro translation of the hH1 cRNA yielded two bands of about 220 (calculated molecular weight 227) and 170 kDa on an SDS gel (Fig. 1B). This is in agreement with the above assumption that transcription yields two RNAs of different size.

Of the six different aODN constructs developed against hH1 RNA (see Methods), two each inhibited translation completely (4464a and 4444a), moderately (1358a and 1945a) or not at all (407a and 156a), depending on the target position. For example, Fig. 1B shows the effects of three selected aODNs at 10 μM on the quantity of the translation products (lanes 2,4,6; the controls obtained with the corresponding sense ODNs are in lanes 1,3,5). The most effective aODN found in this study was construct 4444a which completely suppressed the

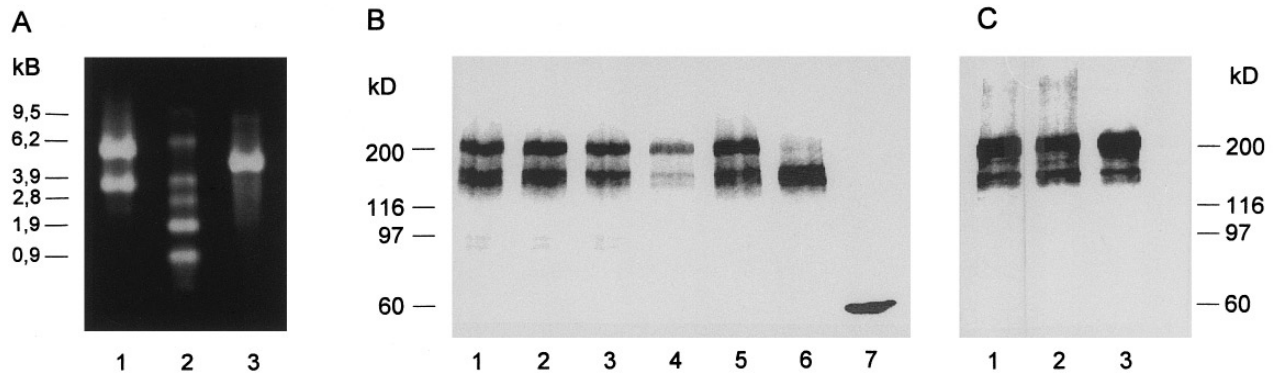

FIG. 1. Electrophoresis plots of cDNA transcripts and their in-vitro translations products for heart (hH1) and skeletal muscle (hSkM1) voltage-dependent Na^+ channels (α subunits). A, Separation of cDNA transcripts on an 0.5% TAE agarose gel; lane 1: hH1 RNA, lane 3: hSkM1 RNA, lane 2: molecular weight standard (the size of the RNA fragments in kB is indicated on the left). B, *In-vitro* translation products of the hH1 cRNA, synthesized in the presence of different 15-mer oligodeoxynucleotides (ODNs). Translation in presence of ^{35}S methionine and in presence of antisense ODNs 156a, 1945a and 4444a (lanes 2,4,6) or their corresponding sense ODNs (lanes 1,3,5). All ODNs at 10 μM. Lane 7 shows the 60 kDa protein derived from luciferase RNA. Positions of molecular weight standards, indicated on the left (in kDa), transferred from coomassie stain of same gel. C, Control experiment showing the ineffectivity of sense ODNs applied at 10 μM (lane 1, 4444sen; 2, 1945sen; 3, no ODN). Positions of molecular weight standards determined as in B.

larger band, whereas it slightly increased the smaller band (lane 6). The corresponding 4444sen construct was ineffective (lane 5).

The finding that ODN 4444a only blocked the upper band is easily understandable. Only the RNA resulting in the 220 kDa protein, i.e. the supposed full-length transcript, contains position 4444. In contrast, ODN 1945a suppresses both bands.

Another control is illustrated in Fig. 1C. The effects of the sense ODNs 4444sen (lane 1) and 1945sen (lane 2) are compared with a run without addition of any ODN (lane 3). The three lanes do not differ, indicating the ineffectivity of the sense ODNs in our test system. Also the non-related 20-mer ODN had no effect on hH1 RNA translation.

Having gained experience with constructs against hH1 RNA we designed two constructs against hSkM1 RNA. The most effective construct, 3866a, completely blocked translation when tested as described above. Next, we tested the concentration dependence of the effect of the two most effective aODNs (Fig. 2). Translation of hH1 RNA was well inhibited when the construct 4444a was at 1 μM or 0.32 μM (lanes 1,2). At 0.1 μM inhibition was weak (lane 3). The concentration dependence of construct 3866a, directed against hSkM1 RNA, was similar (lanes 4-6). For both aODNs the concentration required for half maximum inhibition was between 0.1 and 0.32 μM.

Lanes 7 and 8 illustrate a test for cross reaction of the aODNs. The construct 3866a (10 μM), designed against hSkM1 RNA, had no effect on hH1 RNA translation and, vice versa, 4444a, designed against hH1 RNA, had no effect on hSkM1 translation. To test whether the ODN homologous to 4444a against hH1 RNA is also very effective in the case of the hSkM1 RNA, we tested the effect of ODN 3846a on translation of hSkM1 RNA. A considerable decrease of translation was observed at 10 μM, but not a complete suppression.

Effects of aODNs on functional expression of hSkM1 and hH1 channels in Xenopus oocytes. Coinjection (10 nl) of hSkM1 cRNA and the 3866a construct (final concentration: 100 μM) showed no effect on functional expresssion in a first experiment. Thus, phosphorothioate capped aODNs were used in the following experiments to reduce intracellular ODN degradation. Three groups of oocytes were tested, (i) a control group injected with 1.25 ng hSkM1 cRNA only, (ii) a group coinjected with

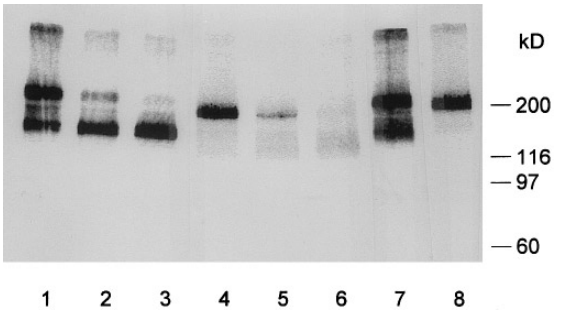

FIG. 2. Concentration dependence of the effects of two selected antisense oligo nucleotides against hH1 and hSkM1 RNAs. *In-vitro* translation as in Fig. 1. Lanes 1–3: hH1 RNA translated in the presence of 0.1 μM (1), 0.32 μM (2) and 1 μM (3) aODN 4444a. Lanes 4–6: same experiment carried out with hSkM1 RNA and 0.1 μM (4), 0.32 μM (5) and 1 μM (6) aODN 3866a. Lanes 7 and 8: results of a test for cross reactions between the aODNs with the non-fitting RNAs. Lane 7: hH1 RNA and 10 μM of 3866a (ODN designed for hSkM1); 8: hSkM1 RNA and 10 μM of 4444a (ODN designed for hH1).

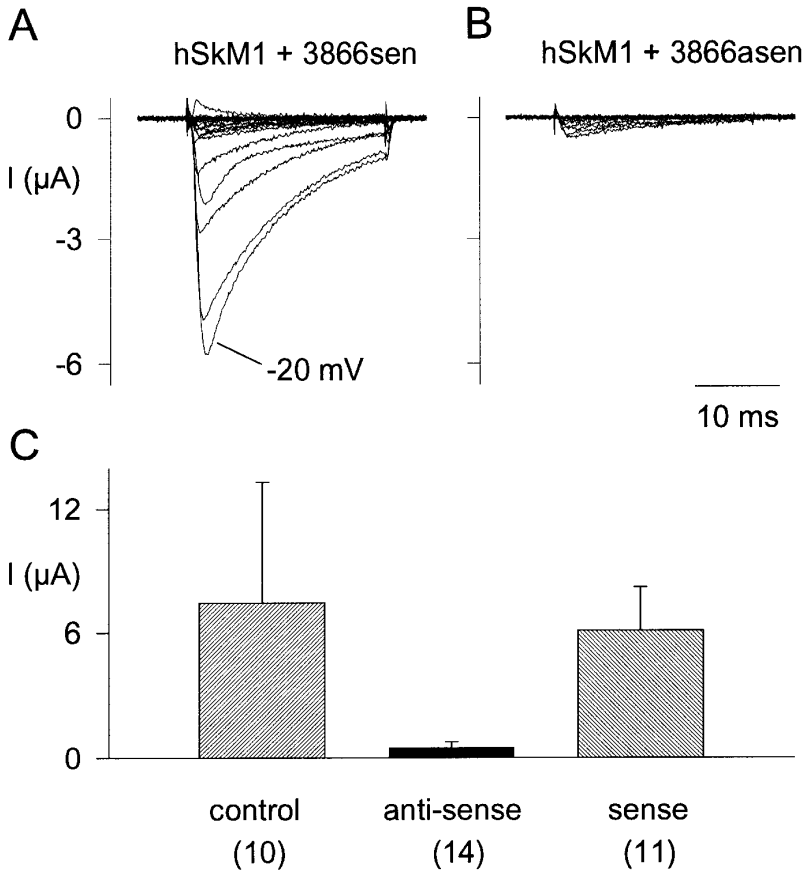

FIG. 3. Suppression of translation of the hSkM1 Na^+ channel cRNA in *Xenopus* oocytes. About 1.25 ng (in 10 nl) of the hSkM1 cRNA injected per oocyte. RNA (0.25 $\mu g/\mu l$ in H_2O) mixed 1:1 (v/v) with aODN 3866a (200 μM in H_2O), aODN 3866sen or pure H_2O (control). First and last nucleotides of the ODNs replaced by phosphorothioate analogs. A, Family of Na^+ inward currents induced by square voltage pulses going from a holding potential of −80 mV to test potentials varying between −70 and +40 mV in 10-mV steps (test pulses preceded by a 100-ms prepulse to −100 mV; response to test pulse of −20 mV indicated). B, Corresponding family of Na^+ inward currents from oocyte injected with mixture of RNA and ODN 3866a. C, Peak current amplitudes derived from current-voltage curves resulting from experiments shown in A and B. Means ± SD for controls: cells injected with hSkM1 cRNA only; anti-sense: cells inject ed with mixture of RNA and the aODN 3866a; sense: cells injected with a mixture of RNA and ODN 3866sen. Data from 10–14 oocytes (as indicated in parenthesis).

RNA and the phosphorothioate-capped aODN 3866a and (iii) a group coinjected with RNA and the corresponding sense ODN, 3866sen. Fig. 3A shows a family of membrane currents in response to depolarizing voltage pulses of different size. The currents must be interpreted as Na^+ currents because of the extracellular ion composition (mainly NaCl) and because of their kinetics, direction of flow and equilibrium potential. The maximum amplitude occurred with test pulses to between −20 to −10 mV. The slow inactivation of the current transients, compared to recordings from skeletal muscle cells, can be explained with of the lack of the β-subunit (21).

Oocytes injected with a mixture of cRNA and the 3866a ODN (100 μM) had only very small Na^+ currents (Fig. 3B). Cells coinjected with the corresponding sense construct showed Na^+ currents in the normal range. To quantify the effect of 3866a, the maxima of the determined current-voltage curves were plotted for many recorded cells from two independent experiments (Fig. 3C). In the 3866a group the average amplitude of the current-voltages curves was only 0.08 ± 0.05 times control, i.e. an inhibition of about 90% was achieved by the aODN. The experiments were carried out with hH1 RNA and the 4444a ODN in the same way as shown for hSkM1 in Fig. 3, except that more RNA (6 ng in 46 nl instead of 1.25 ng in 10 nl) had to be injected to obtain Na^+ currents in the μA range. From the oocytes injected with both, hH1 cRNA and the 4444a ODN (100 μM), only very small Na^+ currents could be recorded (Fig. 4B) while cells coinjected with the corresponding sense construct and the RNA showed Na^+ currents in the range of several μA (Fig. 4A). Evaluations of current-voltage curves from many oocytes resulted in an average reduction of the current maximum to 0.11 ± 0.08

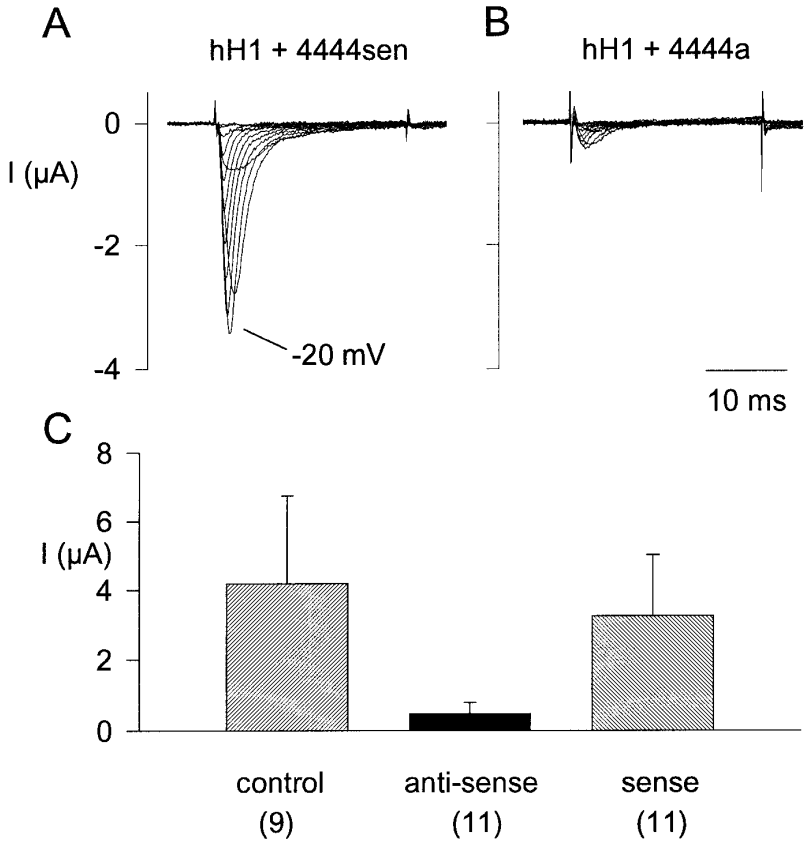

FIG. 4. Suppression of translation of the hH1 Na^+ channel cRNA in *Xenopus* oocytes. About 6 ng (in 23 nl H_2O) of hH1 cRNA injected. Application of the ODNs 4444a and 4444sen and electrophysiological recordings as described for Fig. 3. A, Family of Na^+ inward currents from oocyte coinjected with 4444sen ODN. B, Corresponding family of Na^+ inward currents from an oocyte coinjected with aODN 4444a. C, Peak currents derived as in Fig. 3. Means ± SD are given for three groups of oocytes; control: cells injected with hH1 RNA only; anti-sense: cells injected with a mixture of RNA and ODN 3866a; sense: cells injected with a mixture of RNA and ODN 3866sen; data from the evaluation of 9–11 oocytes (as indicated in parenthesis).

times control, i.e., also for hH1 an inhibition of about 90% was achieved (Fig. 4C).

DISCUSSION

To our knowledge, this is the first report of a successful *in-vitro* translation of voltage-gated Na^+ channels with the full-length channel proteins documented by SDS gel electrophoresis. It was not a matter of course that this is possible with membrane proteins the size of more than 2000 amino acids.

Our attempts to develop effective and specific aODNs started with 15-mer constructs directed against the cardiac isoform, therefore, we tested more different target positions against hH1 RNA than against hSkM1 RNA. At a concentration of 10 μM, the aODN close to the translation start (156a) was ineffective, whereas several aODNs located within the coding region effectively inhibited RNA translation. For *in-vitro* translation, one could have expected that aODNs directed against target sites well within the coding region of the RNA result in truncated proteins. This was, however, not the case. All these constructs resulted in either complete or incomplete generation of the full-length product, indicating that hybridization led to degradation of the RNA.

The most effective aODN against hH1 RNA showed marked inhibition of translation even when applied at no more than 0.32 μM. In most cases, 10 μM were required to see an effect (Fig. 1). These results show that, in contrast to short aODNs (22), for aODNs as large as 15-mer the efficiency is strongly dependent on their RNA target position. This finding was also reported by others (23) and is generally explained with the RNA secondary structure. The latter may consist of double- and single-stranded regions, of which the latter are supposed to be sites of good access for aODNs. Interestingly, the regions of good access were homologous in both hH1 and hSkM1 RNA (Fig. 5C), indicating structural similarity of the two RNA molecules. Calculation of the secondary structures (performed after conduction of the experiments) showed that the binding sites for effective aODNs were in single-stranded re-

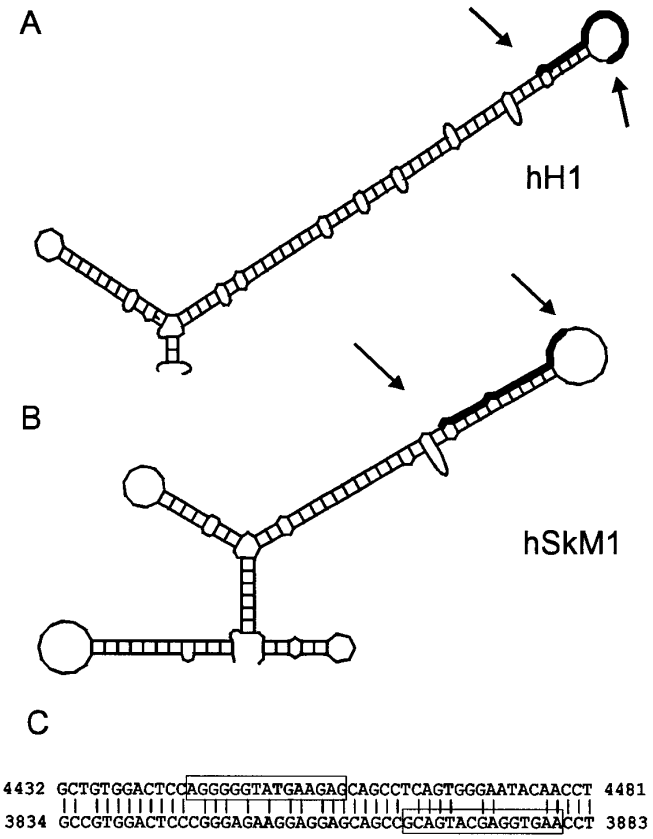

FIG. 5. Sequence alignment and models of secondary structures of parts of hH1 and hSkM1 RNAs. A, Predicted secondary structure of a segment of the hH1 RNA calculated using the method of Zuker (see Methods). The position of the most effective aODN against hH1 RNA found in this study, 4444a, is drawn as a thick line covering putative loop. B, Secondary structure of a segment of the hSkM1 RNA determined in the same way. The hSkM1 RNA segment is homologous to hH1 RNA segment in A. The position of the most effective aODN, 3866a, drawn as a thick line. C, Part of an alignment of the hH1 cDNA (upper line) and the hSkM1 cDNA (lower line) covering the target sequences of the two most effective oligodeoxynucleotides. Boxes are plotted around the target intervals of the two 15-mer aODNs shown in A and B. The nucleotide sequences of C cover loops and parts of their stems drawn on the right in A and B.

gions (Fig. 5A,B). We suggest that the high effectivities of ODNs 4444a (against hH1 RNA) and 3866a (against hSkM1 RNA) are the consequence of their good access to these loop structures.

The efficacy of the six hH1 aODNs to suppress *in-vitro* translation differed by more than a factor of 30 (Figs. 1,2). This shows, that such *in-vitro* tests are helpful steps for generating optimal constructs. Calculation of the aODN/RNA ratio showed that the constructs were in 1500-fold molar excess when used at 10 μM. That means, that the most potent aODNs, 4444a against hH1 RNA and 3866a against hSkM1 RNA, exert their half-maximum effect at 50-fold molar excess (0.32 μM). Compared to other studies (23), this value is quite low.

In the oocyte expression system, the mechanism of action of the aODNs involves cleavage of the RNAs by RNase H, and further RNA degradation by endogenous enzymes (24). We have no indication that application of the aODNs resulted in translation of incomplete Na^+ channel proteins with abnormal function, as kinetics of the Na^+ current transients and their voltage dependence of activation and inactivation were very similar in the aODN and control groups. Sense ODNs did not interact with RNA translation, indicating the specificity of the aODN effects, and had no effect on functional Na^+ channel expression in oocytes (Figs. 3,4). The ODN-injected oocytes did not differ in their resting potentials from the controls. From these results it may be concluded that, in the concentrations used, the phosphorothioate-capped ODNs are not toxic. The estimated intracellular concentration of the ODNs in the tested oocytes was below 5 μM.

We regard our results are an encouraging step in the development of antisense oligodeoxynucleotides as therapeutic tools.

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