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Antisense oligonucleotides against α_{1E} reduce R-type calcium currents in cerebellar granule cells

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ABSTRACT **Many neurons of the central nervous system display multiple high voltage-activated Ca^{2+} currents, pharmacologically classified as L-, N-, P-, Q-, and R-type. Of these current types, the R-type is the least understood. The leading candidate for the molecular correlate of R-type currents in cerebellar granule cells is the α_{1E} subunit, which yields Ca^{2+} currents very similar to the R-type when expressed in heterologous systems. As a complementary approach, we tested whether antisense oligonucleotides against α_{1E} could decrease the expression of R-type current in rat cerebellar granule neurons in culture. Cells were supplemented with either antisense or sense oligonucleotides and whole-cell patch clamp recordings were obtained after 6–8 days *in vitro*. Incubation with α_{1E} antisense oligonucleotide caused a 52.5% decrease in the peak R-type current density, from −10 ± 0.6 picoamperes/picofarad (pA/pF) (n = 6) in the untreated controls to −4.8 ± 0.8 pA/pF (n = 11) (P < 0.01). In contrast, no significant changes in the current expression were seen in sense oligonucleotide-treated cells (−11.3 ± 3.2 pA/pF). The specificity of the α_{1E} antisense oligonucleotides was supported by the lack of change in estimates of the P/Q current amplitude. Furthermore, antisense and sense oligonucleotides against α_{1A} did not affect R-type current expression (−11.5 ± 1.7 and −11.7 ± 1.7 pA/pF, respectively), whereas the α_{1A} antisense oligonucleotide significantly reduced whole cell currents under conditions in which P/Q current is dominant. Our results support the hypothesis that members of the E class of α_1 subunits support the high voltage-activated R-type current in cerebellar granule cells.**

Individual nerve cells in the vertebrate nervous system express several types of voltage-gated Ca^{2+} channel (1–4), as many as five or six channel types distinguishable in some neurons (5). These channels work together to support fundamental cellular activities such as membrane excitation, neurotransmitter release, neurite outgrowth, and gene expression (6, 7). Considerable advances have been made in the understanding of the relationship between channel types, defined by their biophysical and pharmacological characteristics, and their underlying α_1 subunits isolated by biochemistry and molecular biology (8–12). It is clear that L-type currents are supported by dihydropyridine-sensitive α_{1C} or α_{1D} subunits (13, 14) and N-type currents are generated by ω-conotoxin-GVIA-sensitive α_{1B} subunits (15). Likewise, P- and Q-type currents are likely to arise from ω-Aga-IVA and ω-CTx-MVIIC-sensitive α_{1A} subunits (16–21).

Among the major categories of Ca^{2+} channels uncovered so far, R-type channels were the most recently defined and remain the least well-understood. R-type currents were first identified in rat cerebellar granule neurons (22, 23) and were found to be pharmacologically and kinetically distinguishable from L-, N-, P-, and Q-type currents in the same cells (5). The importance of R-type channels for dendritic Ca^{2+} entry and synaptic transmission has been demonstrated in recent experiments (24–27). In contrast to other high voltage-activated Ca^{2+} channels, the molecular basis of R-type currents is not settled completely. One obstacle has been the lack of a potent and selective inhibitor for R-type current that spares its better-characterized counterparts. The leading candidate for the molecular correlate of R-type currents is the α_{1E} subunit (28–30). When expressed in *Xenopus* oocytes and HEK293 cells, α_{1E} subunits induced a prominently inactivating, fast-deactivating current that was highly sensitive to block by Ni^{2+} (28, 29, 31) and ω-Aga-IIIA (32), similar to R-type current in cerebellar granule neurons (5, 22, 33). However, it also has been suggested that α_{1E} might support a low voltage-activated Ca^{2+} channel instead of R-type currents (34–37).

To test whether α_{1E} underlies the expression of the R-type current in cerebellar granule cells, we turned to an antisense strategy. Here, we show that antisense oligonucleotides against α_{1E} specifically decrease the expression of R-type currents in cultured cells. Thus, α_{1E} subunits support the high voltage-activated R-type current in cerebellar granule cells.

METHODS

Cell Culture. Cerebellar neurons were obtained by using a modification of the procedure described by Malgaroli and Tsien (38). Cerebella were removed from the brains of 2- to 5-day-old rat pups. The cerebella were cut into small pieces and rinsed with Ca^{2+} and Mg^{2+}-free Hank's solution (Sigma) supplemented with 350 mg/ml $NaHCO_3$, 1 mM Hepes, and 10% fetal bovine serum (FBS, HyClone). The tissue was then digested in saline solution containing 13 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_3, 10 mg/ml trypsin (type XI, Sigma), and 5 mg/ml DNase (type IV, Sigma) for 5 min at room temperature. The cells were washed with 10% FBS-Hank's solution and gently dispersed with a fire-polished pasteur pipette in Hank's solution containing 12 mM $MgSO_4$ and 5 mg/ml DNase. Cells were spun down and resuspended two times in 10% FBS-Hanks solution and plated onto coverslips precoated with Matrigel (Collaborative Research). Cell cultures were kept in a 5% CO_2-humidified atmosphere at 37°C in MEM (5.3 mM KCl, GIBCO) supplemented with 5 g/liters glucose, 100 mg/liters transferrin, 25 mg/liters insulin, 300 mg/liters glutamine, 2% B-27 (GIBCO), and 10% FBS. Seventy-five percent of the medium's volume was replaced after 1 day *in vitro* with 4 μM cytosine arabinoside-MEM to a final concentration of 3 μM cytosine arabinoside, 2.5% FBS-MEM. Cells were kept in this medium until recordings were made.

Oligonucleotide Treatment. Oligonucleotides (ON) were diluted in the replacement medium cytosine arabinoside-MEM to a final ON concentration of 4 μM and added to the

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Abbreviations: pA/pF, picoampere/picofarad; FBS, fetal bovine serum; ON, oligonucleotides; P/Q, P- and Q-type currents.

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culture medium after 1 day *in vitro*. In some experiments, the ON medium was replaced every two days to maintain ON concentration.

We used an ON against nucleotides 582–599 of the α_{1E} subunit, a region located at repeat I between the S3 and S4 transmembrane domains (28). This particular region of the protein was chosen for its lack of similarity to L-type Ca^{2+} calcium channel sequences and its low homology with non-L-type channel subunits present in cerebellar granule cells, such as α_{1A} or α_{1B} (27% and 38%, respectively). The antisense ON sequence used was 5′-CGTGGGTGTTGAAATG-3′ and the sense ON was 5′-CATTTCAACACCCACG TG-3′. ON uptake by the cells was monitored with fluorescence microscopy by using antisense ON tagged with fluorescein at the 3′ end of the sequence (data not shown). The α_{1A} antisense ON sequence was 5′-CATCGACTGCTTGTACAT-3′, and the sense sequence was 5′-ATGTACAAGCAGTCGATG-3′; these ONs targeted nucleotides 145–162 of the rat α1A sequence (39).

Electrophysiology. Thirty minutes before recording, single coverslips were removed from the incubator and placed in a Petri dish containing Tyrode solution (in mM: 119 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose, 25 Hepes-NaOH (pH 7.3); 305 milliosmolar) supplemented with 1 μM TTX (Sankyo), 1 μM ω-Conotoxin GVIA (Peninsula Laboratories), 0.5 μM ω-Conotoxin MVIIC (Peninsula), 10 μM nimodipine (Research Biochemicals), and 100 μg/ml cytochrome *c*. The ω-Conotoxin MVIIC was omitted from the solution when P/Q current components were tested. Patch pipettes were made from borosilicate glass with resistance values ranging from 4 to 7 MΩ when measured in the presence of recording solutions. The pipette capacitance was compensated electronically. Cell capacitance and series resistance were measured from the current transient elicited by a hyperpolarizing voltage pulse from −80 to −90 mV and compensated electronically. Mean cell capacitance for each experimental group was (in pF): untreated cells, R-type, 6.1 ± 0.3 (n = 6); untreated cells, P/Q + R-type, 6.1 ± 0.5 (n = 5); antisense α_{1E}-treated, R-type, 6.5 ± 0.4 (n = 11); antisense α_{1E}-treated, P/Q + R-type, 6.2 ± 0.4 (n = 7); sense α_{1E}-treated, R-type, 5.2 ± 1.1 (n = 5); antisense α_{1A}-treated, R-type, 5.1 ± 0.4 (n = 6); antisense α_{1A}-treated, P/Q + R- type, 7.1 ± 0.8 (n = 6); sense α_{1A}-treated, R-type, 4.1 ± 0.7 (n = 5); and sense α_{1A}-treated, P/Q + R-type 4.1 0.7 (n = 5). Mean series resistance was 23 ± 1 MΩ (n = 44). Ba^{2+} currents were recorded by using whole-cell patch clamp technique and elicited from a holding potential (V_{hold}) of −80 mV to various test potentials (V_{test}) from −70 to +50 mV. Test pulse duration was 100 ms with a 3-sec pulse interval. Current traces were corrected for linear capacitive leak with on-line P/4 trace subtraction following the test pulse. Signals were acquired at 10 KHz and filtered at 2 KHz by using an Axopatch 200A patch clamp amplifier (Axon Instruments, CA) interfaced to a personal computer. The recording chamber solution contained (in mM): 160 tetraethylammonium Cl, 10 $BaCl_2$, and 10 Hepes-CsOH (pH 7.3); 305 milliosmolar and supplemented with 1 μM ω-Conotoxin GVIA, 0.5 μM ω-Conotoxin MVIIC, 10 μM nimodipine, and 100 μg/ml cytochrome *c*. The intracellular solution contained (in mM): 108 $MeSO_3^-$ CsOH, 4.5 $MgCl_2$, 9 EGTA, 4 ATP-Mg, 0.3 GTP-Na, and 24 Hepes (pH 7.4); 295 milliosmolar. All experiments were performed at room temperature (22–24°C). Calcium current amplitude did not substantially change in neurons cultured between days *in vitro* 6–8 and thus were analyzed together. When appropriate, data are reported as the mean ± SEM. Statistical significance was tested by using single factor ANOVA, with $P < 0.05$ as the limit for statistical significance.

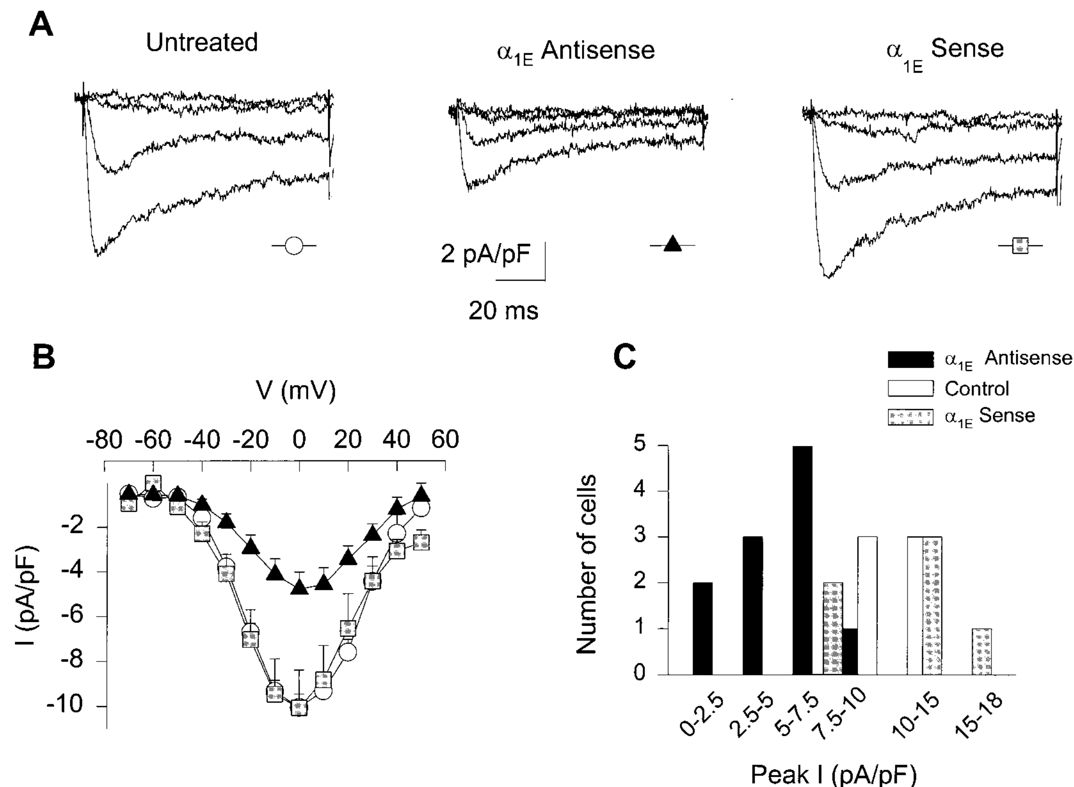

FIG. 1. The presence of α_{1E} antisense ONs in the culture medium decreases the R-type current amplitude. (*A*) Activation of Ba^{2+} currents with various depolarizing pulses (V_{test} = −60, −40, −20, and 0 mV from a V_{hold} = −80 mV) in untreated cells, cells cultured in the presence of 4 μM α_{1E} antisense ON, and cells treated with 4 μM α_{1E} sense ON. Data pooled from 3 to 4 cells. R-type currents were measured in the presence of toxins to block L-, N-, and P/Q-type current components (see *Methods*). (*B*) Current-voltage relationship averages for untreated cells (circles, n = 6), sense α_{1E} ON (squares, n = 5), and antisense α_{1E} ON (triangles, n = 11). Currents from cells treated with antisense ON were significantly smaller than the untreated ($P < 0.01$) and sense-treated controls ($P < 0.03$). (*C*) Peak current distribution in the untreated, antisense α_{1E}, and sense α_{1E}-treated cell groups.

RESULTS

R-type Currents Are Specifically Decreased by α_{1E} Antisense Oligonucleotide. Incubation of the neurons with α_{1E} antisense oligonucleotide caused a significant decrease in the expression of R-type currents, compared with untreated cells or cells grown in the presence of α_{1E} sense ON. Fig. 1*A* illustrates averaged Ba^{2+} current traces obtained from untreated cells, cells cultured in the presence of with 4 μM of α_{1E} antisense oligonucleotide, and cells cultured with α_{1E} sense ON. As evident from the traces and the corresponding peak I–V curves (Fig. 1*B*), treatment with antisense ON in culture significantly reduced the peak amplitude of R-type current. In comparison to the mean peak current value for untreated controls, -10.0 ± 0.6 pA/pF ($n = 6$), peak current in cells treated with α_{1E} antisense averaged -4.8 ± 0.8 pA/pF ($n = 11$), a 52.5% decrease ($P < 0.01$). In contrast, the peak current in granule cells treated with sense ON averaged -11.3 ± 3.3 pA/pF ($n = 5$), not significantly different from the untreated cells ($P < 0.84$), in support of the specificity of the antisense effect. There is considerable variation in the decrease in current induced by the antisense treatment across the entire granule cell population (Fig. 1*C*), consistent with variability in the uptake of antisense ONs as confirmed by examination of the uptake of fluorescein-tagged antisense ONs (data not shown).

α_{1E} Antisense Oligonucleotide Does Not Affect the P/Q Current Component. An additional test of the specificity of the α_{1E} antisense ON for R-type currents was to investigate its effect on the P- and Q-type currents (here abbreviated P/Q because no attempt was made to distinguish between these components). R-type currents were measured in the presence of ω-CTx MVIIC, ω-CTx GVIA, and nimodipine in the bath solution to block P/Q, N-, and L-type currents present in cerebellar granule cells. P/Q + R currents were measured in the presence of ω-CTx GVIA and nimodipine in the solution. Fig. 2 compares the peak current values recorded from untreated cells (*Left*) and in antisense α_{1E}-treated neurons (*Right*). The peak amplitude for P/Q + R current fell from 19.4 ± 1.7 pA/pF ($n = 4$) in untreated granule cells to a value of 13.7 ± 1.1 pA/pF ($n = 8$) in neurons treated with α_{1E} antisense ON, a significant decrease ($P < 0.01$). The reduction was essentially the same as that found when peak R-type current was studied in isolation (decreasing from 10.0 ± 0.6 pA/pF ($n = 6$) in untreated cells to 4.8 ± 0.8 pA/pF ($n = 11$) in antisense ON-treated cells). Thus, the reduction in the aggregate P/Q + R current can be accounted for by a specific decrease in R-type current alone, without any change in the contribution P/Q current.

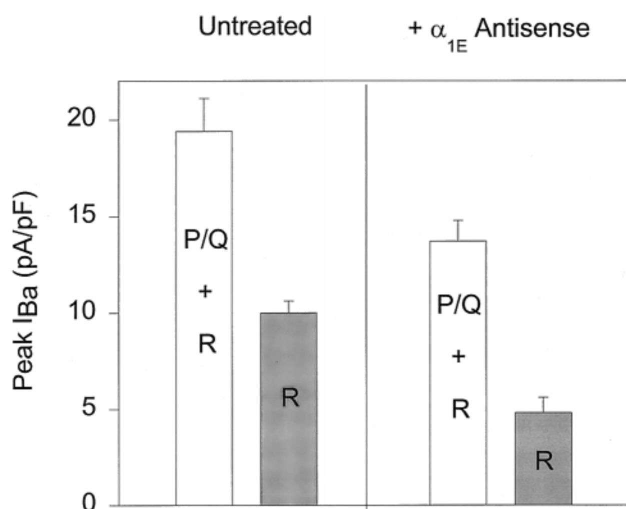

FIG. 2. Effect of α_{1E} antisense ONs is consistent with specific reduction of R-type current. The presence α_{1E} antisense ON did not change the estimated P/Q current amplitude, as shown by the peak Ba^{2+} currents measured in the control group (*Left*) and in the presence of 4 μM α_{1E} antisense ON (*Right*). The P/Q + R current components recorded in the presence and the absence of α_{1E} antisense were significantly different ($P < 0.012$); this reduction α_{1E} antisense (≈30%) can be accounted for by the decrease in R current alone. R-type currents were measured in the presence of 1 μM ω-CTx MVIIC ($n = 5$ for the untreated group; $n = 11$ for antisense-treated cells). P/Q + R components were measured in the absence of the toxin (P/Q + R, $n = 6$ for untreated cells and $n = 7$ for antisense-treated group).

α_{1A} Antisense Does Not Affect R-Type Currents. For a different kind of test of the possible relationship between α_{1E} subunits and R-type currents, we examined the effect of a second set of ONs that targeted the α_{1A} sequence from rat. Because the targeted region of α_{1A} (nucleotides 145–162) lacks any appreciable homology with the α_{1E} sequence, the ON treatment would not be expected to have an effect on the R-type current expression. Fig. 3*A* shows averaged R-type currents from cells cultured in the presence of 4 μM α_{1A} sense ON or the same level of α_{1A} antisense ON. Current traces obtained in the presence of either ON were not different from each other. Peak current amplitude was -11.5 ± 1.7 pA/pF ($n = 5$) in cells treated with α_{1A} antisense ON and -11.7 ± 1.7 pA/pF in cells treated with α_{1A} sense ON ($n = 5$). R-type current expression in either group of cells was not affected when compared with untreated controls (shown in Fig. 1*A*), nor did they change with respect to the cells treated with α_{1E} sense ON (also shown in Fig. 1).

We then corroborated that the lack of effect of the α_{1A} ON in the R-type current was indeed caused by the nonspecificity

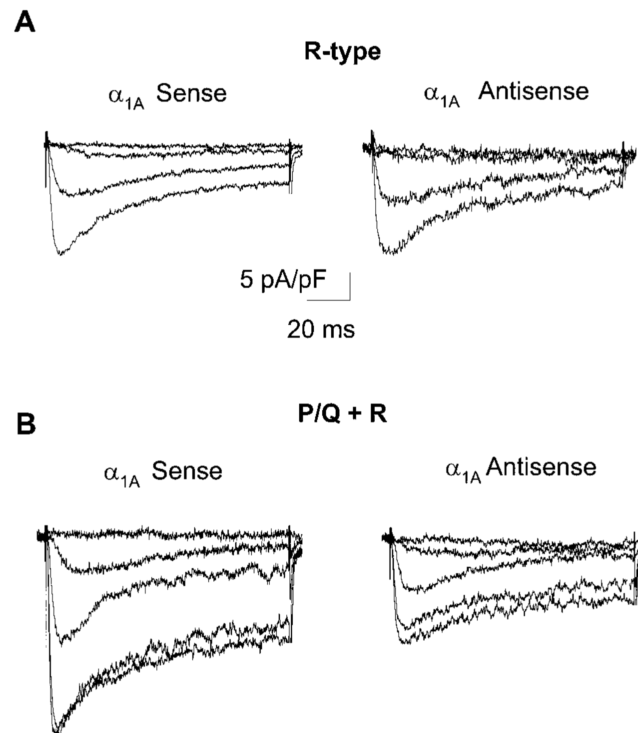

FIG. 3. Antisense ONs against α_{1A} do not affect the expression of R-type currents but decrease the PQ + R components. (*A*) Activation of R-type Ba^{2+} currents with depolarizing pulses from $V_{hold} = -80$ mV to $V_{test} = -60$, -40, -20, and 0 mV in cells cultured in the presence of either 4 μM α_{1A} sense or α_{1A} antisense ON. Data pooled from five cells. (*B*) Addition of α_{1A} antisense ON decreased the P/Q + R component compared with cells grown in the presence of α_{1A} sense ON. Ba^{2+} currents were elicited with depolarizing pulses from $V_{hold} = -80$ mV to $V_{test} = -60$, -40, -20, 0, and $+10$ mV, data pooled from five cells.

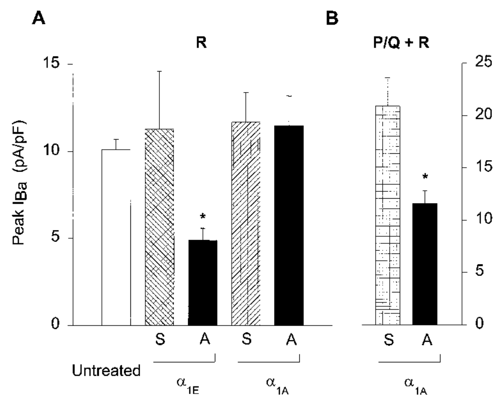

FIG. 4. (*A*) Comparison of R-type peak currents at various culture conditions. Incubation of cerebellar granule cells with antisense oligonucleotides against α_{1E} (n = 11) significantly reduced the expression of R-type currents compared with untreated controls (n = 6)($P < 0.01$). Addition of α_{1A} antisense ON (n = 5), α_{1E} sense ON (n = 5), and α_{1A} sense ON (n = 5) did not affect the current expression. (*B*) Comparison of P/Q + R peak current amplitudes between cells treated with α_{1A} antisense and sense ONs ($P < 0.036$). A, antisense; S, sense.

of the nucleotide sequence and not because of lack of activity of the ON. The efficacy of the α_{1A}-ON set was tested by measuring the P/Q + R current components in cells cultured in the presence of either antisense or sense α_{1A} ONs. Average current traces obtained under these two experimental conditions are shown in Fig. 3*B*. Cells grown in the presence of α_{1A} antisense showed a peak current amplitude of -13.9 ± 3.3 pA/pF (n = 5), significantly smaller than the peak current amplitude from sense-treated cells, -22.1 ± 2.1 pA/pF (n = 5) ($P < 0.036$). The current decrease induced by the antisense ON can be attributed to a decrease in the P/Q current component because these ON did not affect the expression of R-type currents (Fig. 3*A*).

Fig. 4 summarizes the effects of the application of various oligonucleotides on Ca^{2+} channel currents. Only the α_{1E} antisense ON reduced the R-type current ($P < 0.01$), whereas addition of α_{1E} sense, α_{1A} antisense and α_{1A} sense did not alter it relative to untreated controls (Fig. 4*A*). On the other hand, α_{1A} antisense ON significantly decreased the P/Q + R components (Fig. 4*B*), thus demonstrating that its failure to reduce the R current was not caused by ineffectiveness of the compound.

The partial nature of the antisense effect would be consistent with an incomplete turnover of the underlying α_{1E} protein, as is often the case in antisense experiments. As an alternative explanation, we considered the possibility of some heterogeneity in the R-type current, perhaps allowing it to be subdivided by antisense treatment into two distinguishable components. This hypothesis raised the question of whether the current that was abolished by antisense oligonucleotide was somehow different than the current that remained. Accordingly, we compared the properties of currents recorded in neurons treated with antisense or sense ONs or in control cells not exposed to oligonucleotide. As illustrated in Fig. 5, no significant differences emerged in either the voltage-dependence of peak current (Fig. 5*A*), in the activation rate, measured as the 10–90% activation time (Fig. 5*B*) or in the voltage-dependent time constant of inactivation ($\tau_{\text{inactivation}}$) (Fig. 5*B*). The latter measurement showed a trend toward faster values with antisense treatment, albeit not statistically significant. These results are so far consistent with the possibility that the R-type current arises from a single homogeneous population of channels under our particular culture conditions.

DISCUSSION

There has been considerable controversy about the relationship between the α_{1E} subunit and various types of Ca^{2+} channel activity (22, 30, 32, 34). This study addressed the question of whether α_{1E} supports R-type current, a high-voltage activated current resistant to blockers of L-, N-, and P/Q-type channels (5, 32, 33, 40). Our findings with antisense oligonucleotides provided strong support for this hypothesis. The antisense oligonucleotide designed against α_{1E} stood out in its ability to reduce R-type current. None of the control sequences tested, namely a sense α_{1E} ON, an antisense α_{1A} ON, and a sense α_{1A} ON, affected the R-type current. The specific reduction of R-type current by α_{1E} antisense is consistent with previous studies showing that biophysical and pharmacological properties of R-type currents in neurons (5, 40) are in close alignment with the characteristics of α_{1E} expressed in cell lines (29, 32, but see ref. 34). Single channel

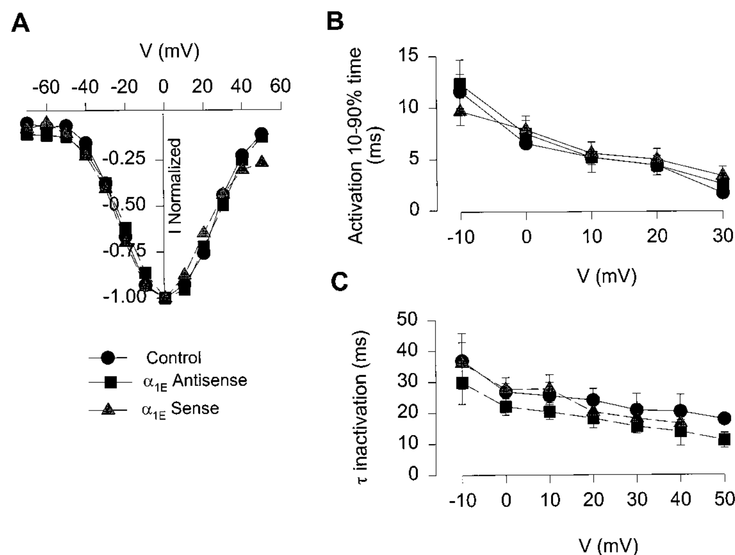

FIG. 5. α_{1E} Antisense treatment does not modify the R-type current properties. (*A*) Voltage-dependence of peak current of untreated cells (circles, n = 6), α_{1E} sense-treated neurones (squares, n = 5), and α_{1E} antisense-treated neurones (triangles, n = 11). Currents were normalized by maximal peak current. (*B*) Time dependence of activation, depicted as time taken to rise from 10 to 90% of peak current. (*C*) Voltage-dependent time constant of inactivation ($\tau_{\text{inactivation}}$) of untreated, α_{1E}-sense, and α_{1E} antisense-treated neurons.

conductances for Ba^{2+} (30, 31, 40) and sensitivity to the neurotoxin ω-Aga-IIIA (32) are some of the key features. Further study of unitary Ca^{2+} conductances of native R-type channels is needed to complete the comparisons with expressed α_{1E} subunits (34).

We did not observe a total elimination of the residual current with the antisense treatment, which is not uncommon when using antisense strategies (21, 35). This partial effect would be expected if the kinetics of α_{1E} turnover within the cell were slow, as often found for membrane channel proteins. Likewise, rates of oligonucleotide uptake and degradation by individual cells also can influence the availability of ON to bind its target. In addition, variability in the ON uptake by individual cells also may be important, as indicated by clear variability in the uptake of fluorescein-tagged oligonucleotide among the population of neurons.

An alternative explanation for the partial effect of the antisense would be the presence of more than one component of R-type current, perhaps including one not supported by α_{1E}. Pietrobon and coworkers (40, 41) have provided evidence for two forms of unitary R-type channel activity, designated G2 and G3, differing by ≈15 mV in their voltage-dependence of activation. We considered the possibility that our antisense sequence affects only one of these subtypes, but we did not observe the expected changes in the voltage-dependence of peak current or in the rate of inactivation. Rather than invoking additional α_1 subunits, we preferred to hypothesize that multiple forms of R-type Ca^{2+} may arise from splice variations in α_{1E} (42) or from association of α_{1E} with diverse ancillary subunits. This kind of explanation also may apply to pharmacological studies with SNX-482, a new peptide neurotoxin that blocks α_{1E} currents in mammalian cell lines and R-type currents in nerve terminals of rat neurohypophysis but fails to inhibit R-type current in rat cerebellar granule cells (43). Interestingly, in cerebellar granule cells cultured under the conditions used by Tottene *et al.* (40), SNX-482 appears able to block a subfraction of R-type current (D. Pietrobon, personal communication). The α_{1E} subunit has been considered for some time as a possible basis for low voltage-activated T-type currents (28, 34–37). The recent cloning and expression of novel subunits labeled α_{1G} and α_{1H} provides a convincing underpinning for T-type channel activity (44). Nonetheless, the possibility remains open that the α_{1E} subunit also may support some form of LVA channel activity (35, 37).

Our study also provides strong confirmation of the generally accepted notion that α_{1A} subunits underlie P/Q-type currents. The most abundant voltage-gated Ca^{2+} channel currents in cerebellar granule cells, P/Q-type currents, are blocked by ω-Aga IVA and ω-CTx MVIIC, like currents generated by α_{1A} cRNA in oocytes and cell lines (16, 20, 45, 46). We found that the antisense oligonucleotide designed against α_{1A} specifically reduced the peak amplitude of the P/Q-type components while leaving the R-type current unaffected. Again, none of the control ONs (α_{1A} sense, α_{1E} antisense, and α_{1E} sense) had any effect on the P/Q components. Based on comparison of pooled data from sense and from antisense-treated neurons (Fig. 4), the component suppressed by the α_{1A} antisense had a prominently decaying time course, as expected if Q-type current were predominant. These results may be compared with α_{1A} antisense experiments in cerebellar Purkinje cells (21), in which P-type currents are strongly predominant (47, 48). α_{1A} antisense reduced P-type current in Purkinje neurons, consistent with previous findings of α_{1A} transcripts and immunoreactivity in these cells. Taken together, these studies leave little doubt that α_{1A} can support both Q- and P-type currents, whatever the explanation for how they differ in pharmacology and inactivation kinetics (see ref. 20).

In summary, treatment of cerebellar granule cells with antisense α_{1E} oligonucleotides induced a specific decrease in R-type current amplitude, consistent with the idea that members of the E class of α_1 subunits engender this high voltage-activated current.

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