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Elimination of potassium channel expression by antisense oligonucleotides in a pituitary cell line

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ABSTRACT The clonal rat pituitary cell line GH_4C_1 expresses the genes for several voltage-dependent potassium channels including Kv1.5 and Kv1.4. Dexamethasone, a glucocorticoid agonist, induces a slowly inactivating potassium current in these cells but does not alter the amplitude of a rapidly inactivating component of potassium current. We have found that the induction of the slowly inactivating current can be blocked by an antisense phosphorothioate deoxyoligonucleotide to the Kv1.5 mRNA sequence. In contrast, antisense deoxyoligonucleotides against Kv1.4 mRNA specifically decrease the expression of the dexamethasone-insensitive rapidly inactivating current. These results demonstrate the usefulness of antisense oligonucleotides in correlating potassium currents with specific potassium channel proteins in the cell types in which they are naturally expressed.

A large number of different members of the Shaker superfamily of potassium channels have been cloned (1–3). When they are expressed in heterologous expression systems, such as *Xenopus* oocytes, some of the clones produce fast-inactivating (A-type) currents, and some produce delayed-rectifier-type currents. It has, however, been difficult to attribute a specific potassium current to any specific potassium channel gene *in vivo*. There are several reasons for this. First, several different potassium channels with similar characteristics can coexist in one cell (4). Moreover, some channels are able to form heteromultimers that have characteristics different from those of homomultimers (5–9). In addition, cell-specific posttranslational modifications, such as protein phosphorylation, may make currents recorded in their native cells differ from those of the same channel protein expressed in a heterologous expression system. Finally, potassium channel α subunits may associate with other proteins such as the β subunit to change their kinetic behavior (10).

The expression of potassium channels in pituitary cell lines, such the GH_3 and GH_4C_1 cell lines, is subject to modulation by the glucocorticoid agonist dexamethasone. It has been reported that dexamethasone increases the levels of mRNA for the potassium channel Kv1.5 in these cells (11). Following exposure to the hormone, Kv1.5 mRNA levels increase about 3-fold within 4 hr. This has been shown to be accompanied by an increase in Kv1.5 protein levels and by an increase in the amplitude of voltage-dependent potassium current (11, 12). Because, however, the kinetics of inactivation of the induced current do not match those of Kv1.5 expressed in *Xenopus* oocytes, it has not been possible to attribute directly the increase in potassium current to the Kv1.5 channel.

Antisense deoxyoligonucleotides have been used to manipulate gene expression (13–16). By introducing DNA complementary in sequence to a portion of the target mRNA, translation of the mRNA can be specifically inhibited. In this report we have used antisense deoxyoligonucleotides against Kv1.5 to eliminate the induction of delayed rectifier potassium current by dexamethasone. Our findings confirm that the increase in the potassium current in GH_4C_1 cells following dexamethasone exposure is indeed caused by the induction of Kv1.5 channels. We also show that a major part of the rapidly inactivating, dexamethasone-insensitive current in these cells can be eliminated by antisense deoxyoligonucleotide against Kv1.4 mRNA.

MATERIALS AND METHODS

Cell Culture. A stock line of GH_4C_1 cells was maintained by growing cells in a 10-cm culture dish in 10 ml of Dulbecco's modified Eagle's medium supplemented with 15% horse serum. Culture dishes were kept in a 95% air/5% CO_2 humidified atmosphere at 37°C. Growth medium was replaced twice a week and the cells were split (1:3 to 1:5) into subcultures once a week. At each pass some cells were plated onto 2.5-cm culture dishes (Corning) for electrophysiological measurements. Electrophysiological recordings were made 4–6 days after plating of the subcultures. To investigate the effects of dexamethasone on membrane currents, cells were incubated with 1 μM dexamethasone for 8 hr before recordings. To study the effects of deoxyoligonucleotides on membrane currents, cells were incubated with 1 μM of each of the deoxyoligonucleotides for 48 hr before currents were recorded. After 24 hr of incubation, the medium was exchanged for fresh medium containing the same deoxyoligonucleotides. Dexamethasone (1 μM) was added 8 hr before recording. All tissue culture media and serum were obtained from GIBCO. Dexamethasone and other reagents were purchased from Sigma.

Antisense Deoxyoligonucleotides. All deoxyoligonucleotides were deoxyoligophosphorothioates, synthesized on an Applied Biosystems model 380B synthesizer, gel-purified, precipitated in ethanol, and then dissolved in the extracellular medium. The antisense deoxyoligonucleotides were designed to be complementary to the 5′ sequences of Kv1.5 (17) and Kv1.4 (4) and flanked the initiator methionine sequences (5′-GAGATCTCCATGGTCCGGGG-3′ for Kv1.5 and 5′-GCCACCTCCATGGTGGTAGT-3′ for Kv1.4). As a control for the antisense deoxyoligonucleotides, a 20-mer deoxyoligonucleotide of random sequence was synthesized (5′-GCCCCGTATGACCGCGCCGG-3′). This oligonucleotide would not be expected to hybridize to the mRNA for any sequence in the GenBank data base.

Electrical Measurements. For electrophysiological recordings, culture dishes on which cells were growing were rinsed thoroughly with bath solution (see below) so as to minimize the amount of extracellular debris at the surface of the dish. Recordings were carried out on isolated cells not in contact with neighbors.

Because the amplitude of currents recorded in GH cells can vary according to batch of cells and time in culture, all

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Abbreviation: TEA, tetraethylammonium.

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experiments were carried out by recording equal numbers of control and experimental cells prepared from the same culture and recorded on the same day (11, 18). With this protocol it was possible to record four to seven control cells and an equal number of experimental cells in 1 day.

Conventional whole cell recordings were performed using 3- to 5-MΩ resistance electrodes (TW 150-4; World Precision Instruments, Sarasota, FL). The pipette solution contained 150 mM KCl, 3 mM NaCl, 10 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.4). The bath solution contained 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM tetraethylammonium (TEA) chloride, 10 mM glucose, and 10 mM Hepes (pH 7.4). The calcium-dependent potassium currents were suppressed by adding 10 mM EGTA to the pipet solution and by using a nominally calcium-free bath solution containing 10 mM TEA chloride (18–20). The Kv1.5 channel is insensitive to 10 mM TEA although this concentration of the blocker does eliminate the TEA-sensitive component of delayed rectifier current (17, 18). A two-pulse protocol was used to separate the delayed rectifier current from the rapidly inactivating current (21). The cell membrane was held at −80 mV. To isolate the delayed rectifier current, a 100-ms depolarizing prepulse to −30 mV was applied before the test pulse to inactivate the rapidly inactivating currents. In experiments in which the rapidly inactivating currents were measured, the total voltage-dependent potassium currents were first activated by a 500-ms test pulse from −80 mV to +20 mV. The delayed rectifier current was then evoked using the prepulse to −30 mV, and the rapidly inactivating current was measured as the difference current between these two voltage-clamp protocols.

The current signals from voltage-clamp experiments were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized at 2 kHz and stored on an MS-DOS-compatible computer. The computer was equipped with a Labmaster analog/digital board coupled to an Indec IBX instrumentation interface and used Indec BASIC-FASTLAB software (Indec Systems, Sunnyvale, CA).

RESULTS

Induction of Potassium Current by Dexamethasone. The delayed rectifier current in GH_4C_1 cells can be separated into TEA-sensitive and TEA-insensitive components. Fig. 1*A Left* shows superimposed traces of the TEA-insensitive delayed rectifier current evoked by voltage steps to +20 mV in the presence of 10 mM TEA in seven different control GH_4C_1 cells. The delayed rectifier currents were isolated from the rapidly inactivating potassium currents by a 100-ms prepulse to −30 mV, as described in *Materials and Methods*. As described previously, cells that have been treated with 1 μM dexamethasone for 8 hr have higher current densities than control cells (11, 12). This is shown in Fig. 1*A Right*, which displays superimposed traces from seven dexamethasone-treated cells. The average peak current density of control cells was 14.5 ± 5.1 pA/pF (±SD; n = 25). The average of hormone treated cells was 27.5 ± 11.1 pA/pF (±SD; n = 25). This represents a 90% increase over controls, and the difference between these two current densities is statistically significant (unpaired Student t test, $P < 0.01$).

In control and dexamethasone-treated groups there is variability in the kinetics of the current. In particular, individual currents in both groups undergo differing degrees of inactivation over the course of the 500-ms pulse. After treatment with dexamethasone, however, inactivating currents are more evident. Individual current traces in Fig. 1*A* were added together and the resulting average currents from these cells are shown in Fig. 1*B*. After treatment with dexamethasone, not only is the total current increased but also the kinetics of the induced current differ from those of the average control

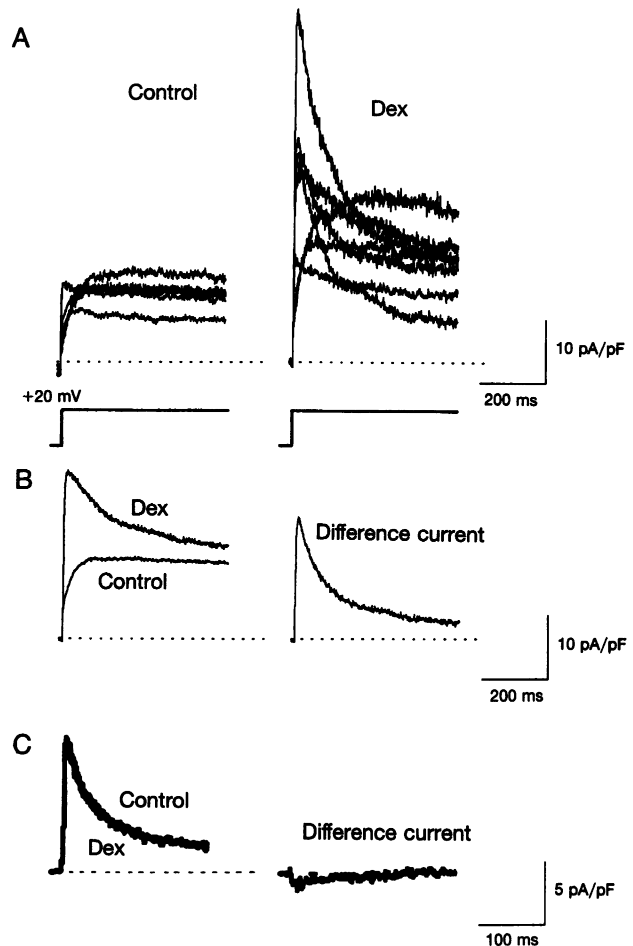

FIG. 1. Dexamethasone induces delayed rectifier currents but does not affect rapidly inactivating currents. (*A*) Delayed rectifier current traces from seven different control cells (*Left*) and seven dexamethasone-treated cells (*Right*). Cells were held at −80 mV, and the 500-ms test pulses were given after a −30 mV, 100-ms prepulse to inactivate the rapidly inactivating currents (see text). Cells were treated with 1 μM dexamethasone for 8 hr prior to measuring the currents. (*B Left*) Averaged current from control and hormone-treated cells. Each trace was obtained by averaging the currents in *A*. The difference current between the two averaged currents is shown in *B Right*. (*C Left*) Averaged rapidly inactivating currents from seven control and seven dexamethasone-treated cells. The rapidly inactivating currents were obtained by subtracting the delayed rectifier current from the total voltage-dependent current (see text). The difference between the averaged currents is shown in *C Right*. In all traces the size of the current traces has been normalized to the cell capacitance.

current. In particular, most dexamethasone-treated cells have a major component of current that inactivates slowly, with a time constant of 50–150 ms. The average current induced by the dexamethasone was obtained by subtracting the two traces in Fig. 1*B Left* and is shown in Fig. 1*B Right*.

The dexamethasone-induced current differs from the rapidly inactivating current that can be recorded in control GH_4C_1 cells. The rapidly inactivating currents decay with a time constant of 15–30 ms and are fully inactivated by a 100-ms prepulse to −30 mV. Moreover, the amplitude of the rapidly inactivating currents was not altered by dexamethasone treatment. The peak current density of the rapidly inactivating current in control cells was 19.1 ± 5.8 pA/pF (±SE, n = 8), and that of the hormone-treated cells was 17.6 ± 5.8 pA/pF (±SE, n = 8), representing a small decrease in current that was not statistically significant. The averaged traces of the rapidly inactivating current of seven control and seven dexamethasone-treated cells are shown in Fig. 1*C*. The two traces overlap,

as emphasized by the difference current shown on the right of the figure.

Elimination of the Dexamethasone-Induced Current by Kv1.5 Antisense Deoxyoligonucleotides. To test whether the dexamethasone-induced current is a direct result of the increase in Kv1.5 mRNA that occurs on treatment with this agent (11), we incubated GH_4C_1 cells in a medium containing 1 μM dexamethasone and an antisense deoxyoligonucleotide that is complementary to the start of translation of Kv1.5 mRNA. We measured the voltage-dependent potassium currents in these cells and compared them to those in cells treated with dexamethasone in the presence of a nonsense oligonucleotide, which would not be expected to block synthesis of the Kv1.5 protein. Fig. 2*A* shows the delayed rectifier current traces from five different cells treated with nonsense deoxyoligonucleotide and five cells treated with the antisense deoxyoligonucleotide. Dexamethasone-treated cells in the presence of nonsense deoxyoligonucleotide had an average peak current density of 24.8 ± 5.5 pA/pF (±SE, $n = 20$). In the presence of antisense deoxyoligonucleotide, current density was reduced to 15.7 ± 3.2 pA/pF (±SE, $n = 20$). The difference between the current densities in the two groups of cells was statistically significant (unpaired t test, $P < 0.05$). The averaged currents obtained by adding the current traces in Fig. 2*A* are presented in Fig. 2*B*. The difference current in Fig. 2*B* represents the dexamethasone-induced current that could be blocked by antisense deoxyoligonucleotide against Kv1.5. The kinetics of the current in Fig. 2*B* resemble the dexamethasone-induced current in Fig. 1*B*, in that both currents inactivate with time constants of <100 msec (90 msec in Fig. 1*B* and 65 msec in Fig. 2*B*), indicating that the induced current is indeed the result of the increase in the synthesis of Kv1.5 channels. Fig. 3 summarizes

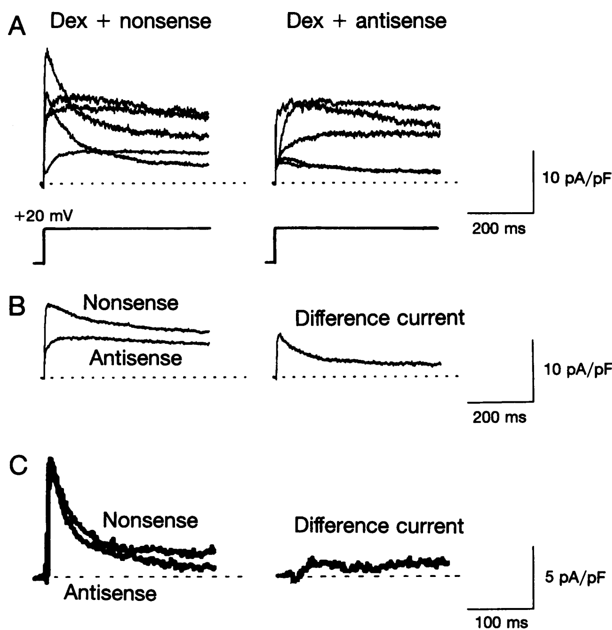

FIG. 2. Kv1.5 antisense deoxyoligonucleotide eliminates the induction of the delayed rectifier current by dexamethasone. Cells were treated with deoxynucleotides for 48 hr and with 1 μM dexamethasone for 8 hr. (*A*) Superimposed delayed rectifier current traces from five cells treated with nonsense deoxyoligonucleotide (*Left*) and five cells treated with 1 μM Kv1.5 antisense deoxyoligonucleotide (*Right*). Currents were measured as in Fig. 1. (*B*) Averaged currents from nonsense and antisense deoxyoligonucleotide-treated cells. Each trace (*Left*) was obtained by adding the five different currents in *A*. The difference between the averaged currents is shown in *B Right*. (*C Left*) Averaged rapidly inactivating currents from five nonsense and five antisense deoxyoligonucleotide-treated cells. The difference current is shown in *C Right*. In all traces, currents have been normalized to the cell capacitance.

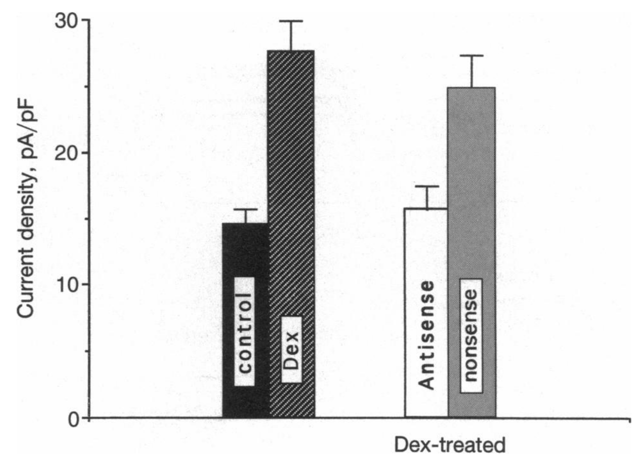

FIG. 3. Kv1.5 antisense deoxyoligonucleotide reduces the induction of the delayed rectifier current by dexamethasone. The left pair of histograms show the mean peak delayed rectifier current in control cells ($n = 25$) and in cells treated with 1 μM dexamethasone ($n = 25$). The right pair of histograms shows the mean delayed rectifier current density in dexamethasone-treated cells preincubated with antisense oligonucleotide ($n = 20$) or with nonsense oligonucleotide ($n = 20$). Error bars represent SE.

the current densities of the delayed rectifier currents in the different experimental conditions and demonstrates that the current induced by dexamethasone is almost completely eliminated by the antisense deoxyoligonucleotide against Kv1.5.

In contrast with these results, the Kv1.5 antisense deoxyoligonucleotide did not affect the rapidly inactivating currents. The average peak current density of the rapidly inactivating current in the presence of antisense deoxyoligonucleotide was 27.6 ± 8.6 pA/pF (±SE, $n = 5$), whereas in the presence of the nonsense deoxyoligonucleotide the current density was 24.3 ± 5.5 pA/pF (±SE, $n = 5$). Rapidly inactivating currents from the antisense and nonsense deoxyoligonucleotide-treated cells are averaged in Fig. 2*C Left*, with the nonsense deoxyoligonucleotide-treated cells having the slightly higher density. Again, the difference current in Fig. 2*C Right* indicates that this component of current is very similar in the presence of either of the deoxyoligonucleotides.

Attenuation of the Rapidly Inactivating Current by Kv1.4 Antisense Deoxyoligonucleotides. To probe further the specificity of the Kv1.5 antisense deoxyoligonucleotides, we tested antisense oligonucleotides against Kv1.4, another potassium channel gene that is expressed in these cells (4). When expressed in *Xenopus* oocytes, Kv1.4 mRNA induces rapidly inactivating currents that resemble the dexamethasone-insensitive currents in GH_4C_1 cells.

As for the Kv1.5 antisense deoxyoligonucleotides, cells were incubated with Kv1.4 antisense deoxyoligonucleotide for 48 hr prior to measuring currents. Fig. 4*A* compares the delayed rectifier currents in cells treated with nonsense deoxyoligonucleotide with those in cells treated with Kv1.4 antisense deoxyoligonucleotide. The mean peak delayed rectifier current in the cells treated with nonsense deoxyoligonucleotide (49.5 ± 4.3 pA/pF; ±SE, $n = 16$) was not significantly different from that in cells treated with antisense deoxyoligonucleotide (53.8 ± 4.1 pA/pF; ±SE, $n = 16$). Moreover, there was little kinetic difference between the two groups. The averaged current traces are shown in Fig. 4*B*. The difference current indicates that Kv1.4 antisense deoxyoligonucleotides produce little change in density or kinetics of delayed rectifier currents.

In contrast to its effect on delayed rectifier currents, the Kv1.4 antisense deoxyoligonucleotide produced a significant attenuation of the rapidly inactivating current. Cells treated with nonsense and antisense deoxyoligonucleotide had average peak current densities of 25.0 ± 6.3 pA/pF (±SE, $n = 8$)

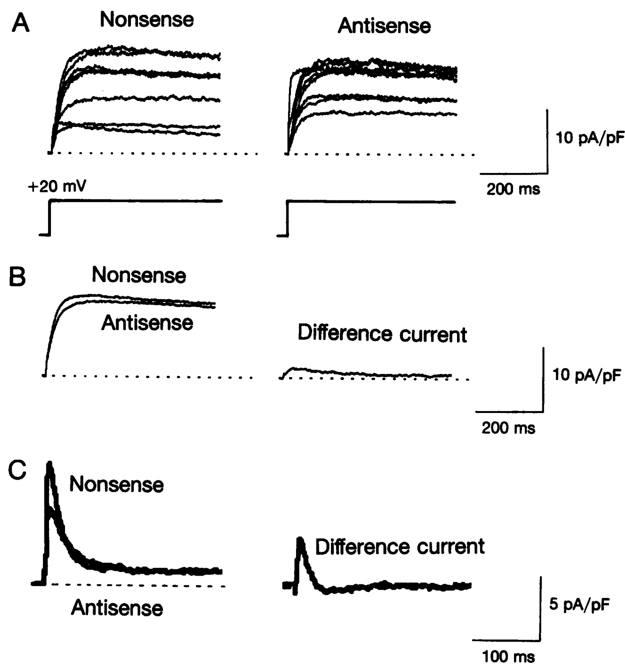

FIG. 4. Antisense deoxyoligonucleotide against Kv1.4 diminishes the amplitude of the rapidly inactivating current. (*A*) Superimposed delayed rectifier current traces from different cells that were treated either with nonsense deoxyoligonucleotide (*Left*) or with the Kv1.4 antisense deoxyoligonucleotide (*Right*). Currents were measured as in Fig. 1. (*B Left*) Averaged delayed rectifier current traces from the nonsense and antisense deoxyoligonucleotide-treated cells. The difference current is shown in *B Right*. (*C Left*) Averaged rapidly inactivating currents from the nonsense and antisense deoxyoligonucleotide-treated cells. The difference current is in *C Right*. The size of the individual current traces was normalized to the cell capacitance.

and 11.6 ± 2.1 pA/pF (±SE, n = 8), respectively. This represents a 54% decrease in the amplitude of the rapidly inactivating current (unpaired t test, $P < 0.05$). Fig. 4*C* shows the averaged rapidly inactivating current for eight different cells treated with either nonsense or Kv1.4 antisense deoxyoligonucleotide. The difference current isolates the current that is eliminated by the antisense deoxyoligonucleotide and shows that its kinetics match those of the rapidly inactivating current.

DISCUSSION

The varied electrical activities of neurons and other excitable cells can largely be attributed to the diversity of the potassium channels expressed in such cells. Each cell may express several different potassium channel subunits that can interact with each other and with other proteins, such as ancillary subunits or second messenger systems, to generate its electrical phenotype. For this reason it has been difficult to ascribe a specific component of potassium current to a given gene product. By using antisense deoxyoligonucleotides, we have been able to demonstrate that the current induced by dexamethasone in the pituitary cell line GH_4C_1 is dependent on the synthesis of Kv1.5 potassium channels.

When Kv1.5 mRNA is expressed in *Xenopus* oocytes, it gives rise to a current that generally matches the dexamethasone-induced current in GH_4C_1 cells in its voltage dependence but differs significantly in its kinetics of inactivation. In particular, the mean dexamethasone-induced current in GH_4C_1 cells inactivates with a time constant of 50–150 ms, and there is variability in the kinetics of inactivation even between cells in a single culture dish. In contrast, the current produced by Kv1.5 mRNA in oocytes undergoes very little inactivation over periods of a second or more (17). Nevertheless, when the Kv1.5 gene is expressed in mammalian cells such as a fibroblast cell line or human embryonic kidney (HEK) cells, the current does inactivate with a time constant comparable to that of the dexamethasone-induced current (22). There are several possible explanations for this. For example, the mammalian cells may normally express a protein that modifies the kinetics of inactivation. The β subunits of potassium channels, which are known to modulate the rate of inactivation, are plausible candidates for such a factor. Alternatively, the rate of inactivation may be influenced by differences in post-translational modifications. In particular, it has been shown that the inactivation rate of the Kv1.5 channel in HEK cells is accelerated by activation of the cyclic AMP protein kinase (22). This raises the possibility that the variability of the rate of inactivation of Kv1.5 current in the GH_3 or GH_4C_1 cells may result from different levels of activity of protein kinases.

We found that the rapidly inactivating (A-type) potassium current in GH_4C_1 cells is not affected either by dexamethasone or by the antisense deoxyoligonucleotide against Kv1.5. A significant reduction in this component of current could, however, be achieved by exposure of the cells to an antisense deoxyoligonucleotide against Kv1.4, which produced no change in the amplitude of delayed rectifier currents. When expressed in *Xenopus* oocytes, Kv1.4 mRNA induces rapidly inactivating currents that provide a good match to the A-type current in GH_4C_1 cells, in terms of both kinetics and pharmacology (4). Treatment with the Kv1.4 antisense deoxyoligonucleotide did not produce a complete loss of the rapidly inactivating current but resulted in an ≈50% decrease in current amplitude. As the turnover rate of these channels is not known, it is possible that preincubation of the cells with the oligonucleotides for 48 hr was insufficient to allow basal turnover to eliminate expression fully. Alternatively, GH_4C_1 cells may express other potassium channel genes that encode rapidly inactivating currents. For example, use of the polymerase chain reaction suggests that the genes for at least four voltage-dependent potassium channels are expressed at some level in the related GH_3B6 cell line (4). One of these, the Kv4.1 gene, like the Kv1.4 gene, gives rise to relatively rapidly inactivating currents in *Xenopus* oocytes (23).

Our results have indicated that the Kv1.5 and Kv1.4 potassium channels contribute to two different components of current in GH_4C_1 cells and are consistent with recent work indicating that the dexamethasone-induced current in GH_3 cells belongs to the Shaker family (24). The antisense deoxyoligonucleotide approach may also prove useful in attributing the products of ion channel genes to specific currents recorded in other cells *in vivo*.

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