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Potassium current suppression in patients with peripheral nerve hyperexcitability

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Summary

Acquired neuromyotonia (Isaac's syndrome) is considered to be an autoimmune disease, and the pathomechanism of nerve hyperexcitability in this syndrome is correlated with anti-voltage-gated K^+ channel (VGKC) antibodies. The patch-clamp technique was used to investigate the effects of immunoglobulins from acquired neuromyotonia patients on VGKCs and voltage-gated Na^+ channels in a human neuroblastoma cell line (NB-1). K^+ currents were suppressed in cells that had been co-cultured with acquired neuromyotonia patients' immunoglobulin for 3 days but not for 1 day. The activation and inactivation kinetics of the outward K^+ currents were not altered by these immunoglobulins, nor did the immunoglobulins significantly affect the Na^+ currents. Myokymia or myokymic discharges, with peripheral nerve hyperexcitability, also occur in various neurological disorders such as Guillain–Barré syndrome and idiopathic generalized myokymia without pseudomyotonia. Immunoglobulins from patients with these diseases suppressed K^+ but not Na^+ currents. In addition, in hKv 1.1- and 1.6-transfected CHO (Chinese hamster ovary)-K1 cells, the expressed VGKCs were suppressed by sera from acquired neuromyotonia patients without a change in gating kinetics. Our findings indicate that nerve hyperexcitability is mainly associated with the suppression of voltage-gated K^+ currents with no change in gating kinetics, and that this suppression occurs not only in acquired neuromyotonia but also in Guillain–Barré syndrome and idiopathic generalized myokymia without pseudomyotonia.

Keywords: acquired neuromyotonia; voltage-gated K^+ channel; hKv 1.1 and 1.6; K^+ current suppression; whole-cell patch-clamp

Abbreviations: CIDP = chronic inflammatory demyelinating polyradicuroneuropathy; IGM = idiopathic generalized myokymia; LEMS = Lambert–Eaton myasthenic syndrome; NB-1 = human neuroblastoma cell line 1; NGF = nerve growth factor; VGKC = voltage-gated K^+ channel

Introduction

Acquired neuromyotonia (Isaacs' syndrome) is characterized by the presence of spontaneous and continuous muscle fibre activity (Isaacs *et al*., 1961). Patients have characteristic symptoms of nerve hyperexcitability such as (i) pseudomyotonia (slow relaxation) induced by muscle contraction, (ii) increased cramping and (iii) excessive sweating (Kimura *et al*., 1983). The abnormal activity in acquired neuromyotonia is characterized electromyographically by doublet, triplet or single motor unit discharges that have a high intraburst frequency (40–200/s) (Newsom-Davis *et al*., 1993). These discharges are thought to originate from terminal arborizations of the motor axons (Newsom-Davis *et al*., 1993). In recent reports, acquired neuromyotonia has been described as an autoimmune disorder because anti-voltage-gated K^+ channel (VGKC) antibodies, which are closely associated with the pathomechanism of this disorder, are present in sera from acquired neuromyotonia patients whose symptoms respond to immunotherapy (Newsom-Davis *et al*., 1993; Shillito *et al*., 1995; Watanabe *et al*., 1995; Arimura *et al*., 1997; Hart *et al*., 1997). Results of previous electrophysiological studies suggest that sera from these patients suppress voltage-gated outward K^+ currents in PC-12 cells (Sonoda *et al*., 1996) and induce repetitive firing of action potentials in rat dorsal root ganglion cells (Shillito *et al*., 1995). In terms of electrophysiology, the spontaneous firing that originates in the peripheral nerves may be caused by

VGKC inhibition and the stimulation of voltage-gated Na^+ channels; e.g. prolongation of the open time of the voltage-gated Na^+ channels in ciguatera intoxication (Cameron *et al*., 1991*a*, *b*; Gutmann *et al*., 1996). We have studied previously the involvement of voltage-gated K^+ currents in acquired neuromyotonia patients (Sonoda *et al*., 1996), but that of voltage-gated Na^+ currents has not been investigated.

Symptoms of peripheral nerve hyperexcitability, such as muscle cramp with spontaneous motor unit activities and myokymic discharges, are characteristic not only of acquired neuromyotonia but of a wide range of peripheral neuropathies (Auger *et al*., 1984; Jamieson *et al*., 1994). Jamieson and colleagues defined idiopathic generalized myokymia (IGM) as a clinical syndrome accompanied by generalized continuous motor unit activities or myokymia and having no apparent aetiology (Jamieson *et al*., 1994). IGM has been proposed as the generic name for all disorders with continuous motor unit activity, including acquired neuromyotonia and those with muscle cramp and myokymia without the characteristic symptoms of acquired neuromyotonia (abnormal sweating, pseudomyotonia), e.g. myokymia–cramp syndrome (Jamieson *et al*., 1994). Transient myokymic discharges also occur during the early stage of Guillain–Barré syndrome (Mateer *et al*., 1983). We found that serum from a Guillain–Barré syndrome patient suppressed the K^+ currents in PC-12 cells (Kurono *et al*., 1995).

In the study reported here, we used the patch-clamp method to answer the following questions. (i) Do immunoglobulins from acquired neuromyotonia patients affect K^+ currents in a human neuroblastoma cell line (NB-1) which needs no neurotrophic factor for the expression of ion channels? Our previous findings for the PC-12 cell line, which needs nerve growth factor (NGF) for the expression of ion channels, did not exclude possible effects of the patients' sera on the action of NGF, which affects the expression of VGKCs. (ii) Do immunoglobulins from patients with acquired neuromyotonia affect Na^+ currents? (iii) Do immunoglobulins from acquired neuromyotonia patients alter the kinetics of K^+ currents? (iv) Do immunoglobulins from IGM without pseudomyotonia (all IGM with the exception of acquired neuromyotonia) and Guillain–Barré syndrome patients suppress K^+ currents? (v) Do sera from acquired neuromyotonia patients affect the hKv (human voltage-gated K^+ channel) 1 family expressed in CHO (Chinese hamster ovary)-K1 cells? Hart and colleagues detected autoantibodies to the hKv 1 family expressed in *Xenopus* oocytes in acquired neuromyotonia patients (Hart *et al*., 1997). We therefore examined the electrophysiological effects of patients' sera on hKv 1.1 and 1.6 expressed in CHO-K1 cells. The answers to these questions should help to clarify the pathomechanisms of disorders characterized by peripheral nerve hyperexcitability.

Material and methods

Patients

Immunoglobulins were obtained from four patients with acquired neuromyotonia (patient 1, an 82-year-old man; patient 2, a 44-year-old woman; patient 3, a 35-year-old man; patient 4, an 18-year-old girl), two patients with Guillain–Barré syndrome (patient 5, a 13-year-old girl; patient 6, a 31-year-old man) and two patients with IGM without pseudomyotonia (all IGM with the exception of acquired neuromyotonia: patient 7, a 43-year-old man; patient 8, a 71-year-old man). The diagnosis of acquired neuromyotonia was based on the clinical criteria reported by Kimura (1983) [(i) pseudomyotonia (slow relaxation), (ii) increased cramping and (iii) excessive sweating] and on the EMG criterion of Newsom-Davis that it is characterized by doublet, triplet or multiplet single motor unit discharges that have a high (40–200/s) intraburst frequency (Newsom-Davis, 1993). These abnormal discharges include myokymic and neuromyotonic discharges defined by the AAEE (American Association of Electromyography and Electrodiagnosis) Glossary of terms in Clinical Electromyography. The diagnosis of IGM was based on the criteria of Jamieson (1994): clinical syndromes accompanied by generalized continuous motor unit activities or myokymia and having no apparent aetiology. The patients' clinical features are given in Table 1. Control immunoglobulins were obtained from four healthy volunteers (a 27- and a 30-year-old woman and a 32- and a 55-year-old man), a patient with myasthenia gravis (a 70-year-old woman), a patient with Lambert–Eaton myasthenic syndrome (LEMS) (a 61-year-old man) and a patient with chronic inflammatory demyelinating polyradicuroneuropathy (CIDP) (a 48-year-old man). Informed consent was obtained from all the patients and participants.

Immunoglobulins were isolated from the sera of patients and healthy participants by the 2-ethoxy-6,9 diaminoacridine-lactate (acrinol) method. Each sample was mixed with 0.4% acrinol 1 : 5 then centrifuged at 20 000 *g* for 60 min at 4°C. The supernatant was applied to a desalting column (HiTrap™ desalting column, Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer (0.015 M sodium borate and 0.15 M NaCl, pH 8.5). The eluted solution was monitored in a spectrophotometer at 280 nm, and the peak fractions were collected for further study.

Preparation of cells for recording

NB-1 cells (Miyake *et al*., 1975) obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) were cultured at 37°C in 95% air and 5% CO_2 in standard medium containing 80% MEM (Pharmacia Biotech, Uppsala, Sweden), 10% FBS (foetal bovine serum), 10% horse serum, 100 U/ml penicillin, 50 μg/ml streptomycin and 25 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulphonic acid) (all from Gibco BRL, Rockville, Md, USA) on poly-L-lysine-coated 100 mm culture dishes (Iwaki, Funabashi, Japan). The medium was adjusted to pH 7.4 with NaOH and changed every 2 or 3 days, and cells were passaged weekly. To study the effect of the patients' immunoglobulins on the electrical properties of NB-1, we cultured the cells with 5 μg/ml of the immunoglobulins from the patients or healthy

Table 1 *Clinical features and electromyographic findings*

Case	Diagnosis	Age, sex	Visible myokymia	Cramp	Pseudo-myotonia	Hyper-hydrosis	Weakness	Sensory disturbance	Needle EMG
1	ANM	82, M	−	+	+	−	−	+	Neuromyotonic discharges, myokymic discharges
2	ANM	44, F	+	+	+	+	−	−	Neuromyotonic discharges, myokymic discharges
3	ANM	35 M	−	−	+	+	−	+	Myokymic discharges
4	ANM	18, F	+	+	+	−	−	−	Myokymic discharges
5	GBS	13 F	−	−	−	−	+	+	Not examined
6	GBS	31 M	−	+	−	−	+	−	Not examined
7	IGM	43 M	−	+	−	−	−	−	Neuromyotonic discharges, myokymic discharges
8	IGM	71 M	+	−	−	−	−	+	Neuromyotonic discharges

ANM = acquired neuromyotonia; GBS = Guillain–Barré syndrome; IGM = idiopathic generalized myokymia. The characteristic features of the neuromyotonic and myokymic discharges were defined according to the definition by American Association of Electromyography and Electrodiagnosis (AAEE) Glossary of Terms in Clinical Electromyography.

controls for 1 or 3 days. At concentrations $\geqslant$5 μg/ml there were frequent signs of cell damage; therefore, this concentration was used in all experiments. All the experiments were performed at 32 ± 2°C.

CHO-K1 cells from HSRRB were cultured at 37°C in 95% air and 5% CO_2 in MEM Alpha medium (Nikken BioMedical Laboratory, Kyoto, Japan) supplemented with 5% FBS, 5% horse serum, 100 U/ml penicillin and 25 mM HEPES, on 100 mm polystyrene dishes (Iwaki, Funabashi, Japan). cDNA (complementary DNA) for the VGKC α-subunit of human Kv 1.1 and 1.6 (KCNA1 and KCNA6) were prepared as reported by O. Pongs (Universität Hamburg) (Grupe *et al*., 1990). Cells were split and plated at 2×10^5 cells in 35 mm culture dishes 24 h before transfection, and transfected with plasmid DNA (2 μg/ml) encoding hKv 1.1 or 1.6 using DMRIE-C Reagent (Gibco BRL). Enhanced green fluorescent protein was coexpressed with the channel subunits in order to identify the cells for voltage-clamp analysis. After 5–6 h of exposure, the cells were washed once then incubated for 3 days with growth medium containing 2% serum from the patients or controls. Whole-cell recordings showed typical hKv 1.1 or 1.6 currents in 100% of the cells expressing enhanced green fluorescent protein. Control cells (non-transfected or non-fluorescing cells) did not show these currents.

Patch-clamp recording

Ion current recordings were carried out as described previously (Sonoda *et al*., 1996). In K^+ current recording, cells were held at the holding potential (V_{hold}) of –80 mV, and square pulses (300 ms duration) between –140 and +60 mV (20 mV step) were applied at 15 s intervals. Because the waveform of the elicited current in most NB-1 cells showed decay (Fig. 2A), the peak current and the steady-state current (current at the end of the pulse) during the command pulse were both evaluated. Because the waveform of the elicited K^+ current in transfected CHO-K1 cells showed scarcely any decay, only the peak current was evaluated. For Na^+ current recording, NB-1 cells were held at the same holding potential, and square pulses (30 ms duration) between –100 and 0 mV (20 mV steps) were applied at the intervals used in the K^+ current recording, after which the inward peak currents were evaluated. The linear leak current fraction was calculated by the least-squares method from currents obtained at test potentials of –100, –80 and –60 mV. The membrane capacitance (C_m) of each cell was calculated by measuring the charge transfer during the initial capacitative surge (Q) elicited by a 10 mV depolarizing pulse at the V_{hold} of –80 mV, using the equation $Q = C_m \times V$. Taking into account that the membrane capacitance reflects the membrane area, the ion current was normalized using the equation:

$$\text{ion current density (pA/pF)} = (\text{measured current} - \text{leak current})/C_m$$

Statistics

The F test was used to examine whether the data had a Gaussian distribution. If the P value in the F test was >0.05 the data were analysed using Student's t test, otherwise a non-parametric test (the Mann–Whitney U test) was used.

Table 2 *Electrical properties, K^+ current and Na^+ current in NB-1 cells*

	RPM (mV)	Capacitance (pF)	Measured K^+ current (pA)		Measured Na^+	K^+ current density (pA/pF)		Na^+ current density (pA/pF)
			Steady State	Peak	Peak	Steady state	Peak	Peak
Healthy controls								
Total	–52.7 ± 0.9	47.3 ± 0.9	934 ± 81	1891 ± 129	1549 ± 160	20.0 ± 1.9	40.1 ± 2.8 (40)	32.3 ± 3.1 (43)
1	–50.9 ± 1.1	43.8 ± 1.2	948 ± 123	2163 ± 243	1302 ± 190	21.4 ± 2.5	48.7 ± 5.4 (11)	29.4 ± 3.9 (11)
2	–51.6 ± 2.3	43.7 ± 1.2	1113 ± 158	1675 ± 263	1818 ± 374	26.4 ± 4.8	38.8 ± 5.3 (9)	39.7 ± 7.5 (12)
3	–54.4 ± 2.1	49.2 ± 1.7	943 ± 262	1839 ± 222	1650 ± 406	19.1 ± 5.4	37.1 ± 6.2 (9)	32.9 ± 7.8 (9)
4	–54.4 ± 1.2	53.0 ± 1.5	765 ± 107	2037 ± 228	1418 ± 307	14.1 ± 2.1	35.1 ± 4.1 (11)	26.9 ± 5.8 (11)
Patients								
1	–54.1 ± 2.1	45.5 ± 0.7	312 ± 74	713 ± 131	1015 ± 88	6.3 ± 1.6**	15.0 ± 2.8** (11)	22.4 ± 1.8 (11)
2	–51.9 ± 2.4	47.4 ± 1.1	737 ± 113	1265 ± 191	988 ± 171	14.6 ± 2.1	25.6 ± 3.7* (12)	20.8 ± 3.3 (12)
3	–50.4 ± 1.4	48.0 ± 0.7	427 ± 58	846 ± 115	823 ± 102	8.5 ± 1.1**	17.1 ± 2.2** (12)	17.2 ± 2.1* (12)
4	–51.1 ± 2.9	47.7 ± 2.3	595 ± 125	974 ± 218	1067 ± 230	11.9 ± 2.2*	19.5 ± 4.0** (10)	21.9 ± 3.9 (10)
5	–49.6 ± 2.1	45.8 ± 1.2	542 ± 117	741 ± 126	1031 ± 350	10.7 ± 2.3*	15.1 ± 2.6** (12)	27.3 ± 6.8 (13)
6	–52.5 ± 1.9	46.5 ± 1.3	594 ± 137	1099 ± 239	1209 ± 268	12.0 ± 2.7	22.7 ± 4.7** (10)	25.6 ± 5.4 (10)
7	–46.4 ± 2.1	52.4 ± 5.4	665 ± 158	1301 ± 221	1129 ± 208	12.1 ± 3.0	18.9 ± 4.0** (10)	22.0 ± 4.0(11)
8	–55.0 ± 1.8	46.5 ± 1.1	574 ± 140	938 ± 243	1812 ± 425	10.9 ± 2.3*	18.6 ± 4.4** (12)	34.6 ± 8.2 (12)
Disease controls								
MG	–54.3 ± 1.7	53.3 ± 2.1	1248 ± 247	2213 ± 325	1696 ± 478	22.1 ± 4.1	39.9 ± 5.5 (11)	31.9 ± 9.1 (11)
LEMS	–54.5 ± 1.1	48.8 ± 1.3	826 ± 109	1641 ± 126	1153 ± 114	15.6 ± 2.1	32.1 ± 2.7 (11)	23.6 ± 1.9 (11)
CIDP	–51.3 ± 1.7	48.0 ± 1.3	1094 ± 154	2024 ± 282	1355 ± 217	22.7 ± 3.3	42.2 ± 6.1 (12)	28.8 ± 4.8 (12)

Cells were cultured for 3 days with immunoglobulins from the controls or patients. The test potential of the K^+ current was +40 mV and that of the Na^+ current –20 mV from the holding potential of –80 mV. The numbers of cells are shown in parentheses. Ion current density (pA/pF) = (measured current – leak current)/C_m. MG = myasthenia gravis. *$P < 0.05$, **$P < 0.01$.

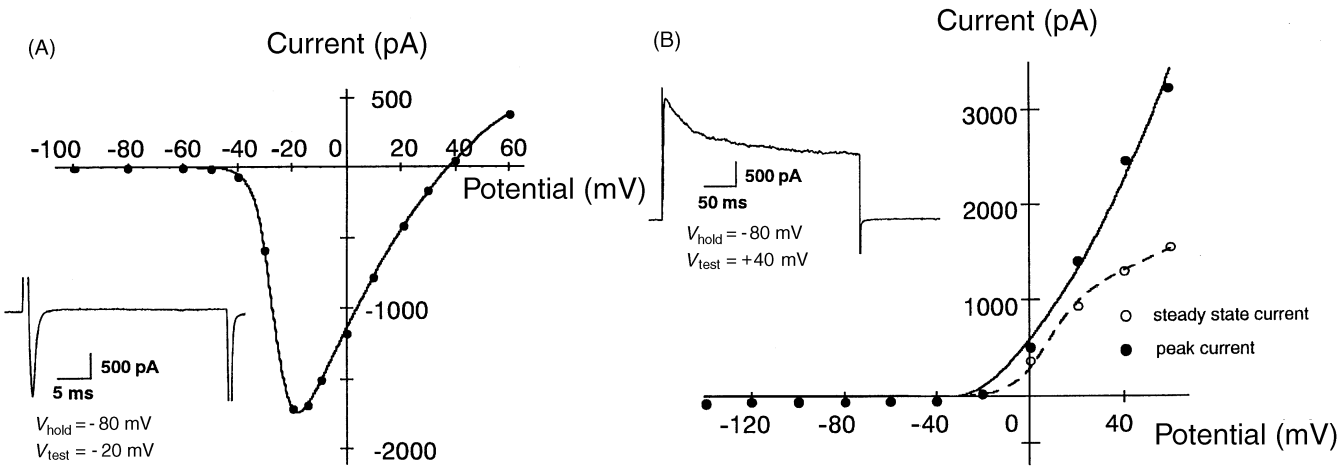

Fig. 1 Current–voltage relationship of Na^+ (**A**) and K^+ (**B**) currents in NB-1 cells. Cells were cultured for 3 days with 5 μg/ml of the control immunoglobulin. The continuous and broken lines are for a linear least-squares fit to the data. In **B**, the continuous line is one fitted for the measured peak K^+ currents, and the broken line is for the measured steady-state currents (currents at the end of the pulse). Current traces were elicited by test pulses of –20 mV (**A**, insert) and +40 mV (**B**, insert) from the holding potential of –80 mV.

Values are given as mean ± standard error unless otherwise specified.

Results

Effects of immunoglobulins from acquired neuromyotonia patients on K^+ currents and Na^+ currents

When NB-1 cells were cultured for 2–3 days, two types of cells, blast and mature cells, were present. The shape of the typical blast type cell is a teardrop with two short, thick processes at each end, whereas the mature cell is round with several long processes. We used the blast type for the patch-clamp experiments because it was better suited to space-clamping. To examine the effects of immunoglobulins from patients with acquired neuromyotonia on the electrical properties of NB-1 cells, the cells were cultured with the immunoglobulins for 3 days. Resting membrane potentials were –46 to –55 mV, and they were not significantly different from those of cells cultured with the control immunoglobulins (–53 mV). Cell capacitances were 46–52 pF, again not significantly different from the control cell value of 47 pF (Table 2).

Figure 1A (insert) shows a typical example of the Na^+ current elicited by a 30 ms depolarizing pulse of –20 mV from the holding potential of –80 mV in an NB-1 cell cultured in medium containing 5 μg/ml of the control immunoglobulin. A transient inward current, elicited just after the positive capacitative current, was abolished when the external solution was changed to Na^+-free solution, and was suppressed by 90% by 10 μM tetrodotoxin, indicating that the inward current was a Na^+ current. The current–voltage relationship of the Na^+ current in the same cell elicited by test voltages of –100 to +60 mV is shown in Fig. 1A. The Na^+ current appeared at about –50 mV, the maximum peak current (–1716 pA) at –20 mV and the reversal potential at +40 mV. Figure 1B (insert) shows a typical example of the K^+ current elicited by a 300 ms depolarizing pulse of +40 mV from the holding potential of –80 mV in a cell cultured under the above conditions. The outward current occurred with decay. No outward current was detected when a K^+-free internal solution was used, evidence that the outward current was a K^+ current. The current–voltage relationship for the K^+ current is shown in Fig. 1B for test voltages from –140 to +60 mV from the holding potential of –80 mV. The continuous line is fitted for the measured peak currents and the broken line for the measured steady-state currents (currents at the end of the pulse). The current was activated at –30 mV and more positive potentials. At 40 mV it was 2451 pA for the peak and 1309 pA for the steady state; therefore, 47% inactivation occurred within 300 ms at this test potential. Na^+ and K^+ currents were present in all the blast-type cells examined (n = 168).

The effects of immunoglobulins from the acquired neuromyotonia patients on the K^+ and Na^+ currents were examined. Figure 2A shows typical K^+ currents elicited by 300 ms test pulses between –140 and +60 mV from the holding potential of –80 mV in a cell cultured with immunoglobulin from a healthy control. Outward rectifying currents with decay were present at potentials more positive than –20 mV. With the control immunoglobulin, the peak K^+ current density at the +40 mV test potential was 48.1 pA/pF and the steady-state current density was 23.4 pA/pF. Figure 2C shows K^+ currents in another cell cultured under the same conditions with the immunoglobulin from patient 1. The respective peak and steady-state current densities were 6.03 and 2.88 pA/pF at +40 mV. Thus, the K^+ currents in cells cultured with the patient's immunoglobulin were considerably less than those in cells cultured with the control immunoglobulin. Similar results were obtained for the immunoglobulins from the other patients with acquired neuromyotonia (patients 2–4). Typical

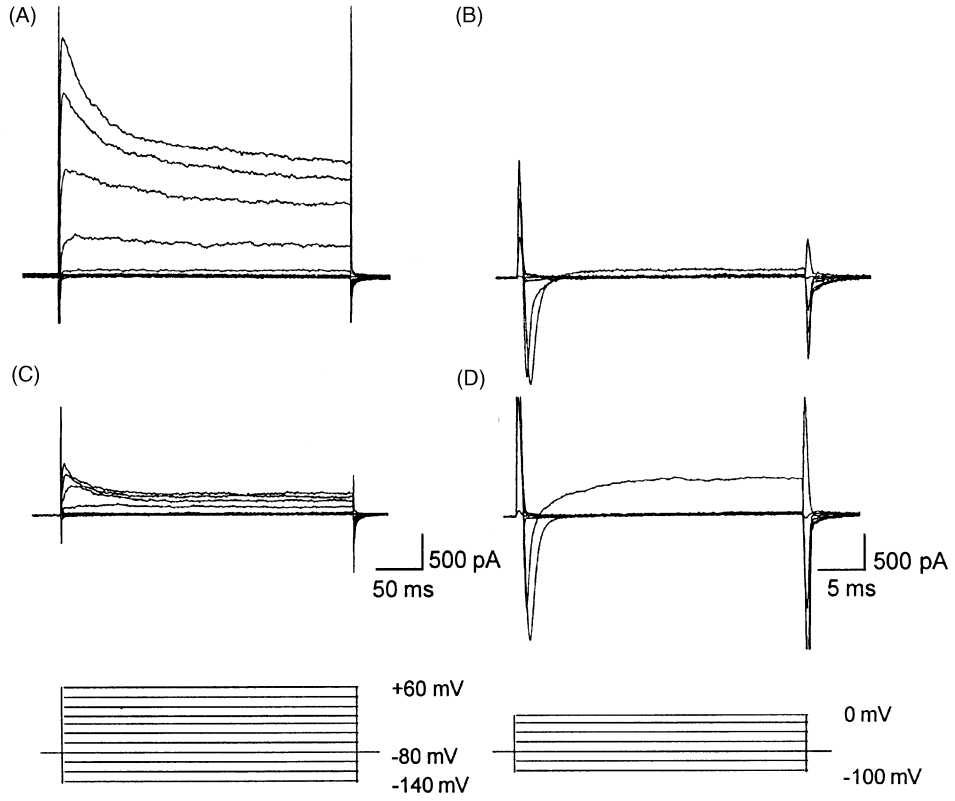

Fig. 2 The K^+ current elicited in NB-1 cells by 300 ms test pulses from –140 to +60 mV from the holding potential of –80 mV. Cells were cultured for 3 days with 5 μg/ml of immunoglobulins from a control (**A**) and patient 1 with acquired neuromyotonia (**C**). Na^+ current was elicited by 30 ms test pulses from –100 to 0 mV from the holding potential of –80 mV under the same conditions from a control (**B**) and patient 1 (**D**).

Na^+ currents are shown in Fig. 2B (with 5 μg/ml control immunoglobulin) and Fig. 2D (with 5 μg/ml immunoglobulin from patient 1). Cells were held at –80 mV and test pulses from –100 to 0 mV were applied for 30 ms. At the test potential of –20 mV, the inward peak Na^+ current density was –39.9 pA/pF in the NB-1 cell cultured with the control immunoglobulin and –36.8 pA/pF in the cell cultured with the patient's immunoglobulin. There was no apparent change in Na^+ current in the NB-1 cells cultured with these two immunoglobulins.

Figure 3A and B give the averages of the K^+ current densities measured at the test potential of +40 mV in cells cultured for 3 days with the immunoglobulins from the four healthy control subjects or immunoglobulins from patients with acquired neuromyotonia (patients 1–4), Guillain–Barré syndrome (patients 5 and 6) and IGM without pseudomyotonia (patients 7 and 8) and patients with other neurological diseases (myasthenia gravis, LEMS and CIDP). The averages of the K^+ current densities for the control immunoglobulins were 20.0 ± 1.9 pA/pF (n = 40) for the steady state and 40.1 ± 2.8 pA/pF (n = 40) for the peak (Table 2). Although the K^+ current amplitude had a large degree of variability within the same treatment group, both the peak and steady-state K^+ current densities were suppressed in NB-1 cells cultured with immunoglobulins from the acquired neuromyotonia patients compared with cells cultured with the control immunoglobulins. In addition, the peak current densities were suppressed more effectively than the steady-state current densities. Figure 3C shows the average Na^+ current densities at the test potential of –20 mV (data shown as absolute values). The mean Na^+ current density in cells cultured with the control immunoglobulins was 32.3 ± 3.1 pA/pF (n = 43). The Na^+ currents in cells cultured with the patients' immunoglobulins did not differ significantly from those in cells cultured with the control immunoglobulins, except in the case of patient 3.

To exclude non-specific effects of the immunoglobulins, we tested those from patients with myasthenia gravis, LEMS and CIDP as the disease controls. The K^+ and Na^+ current densities in NB-1 cells cultured with these immunoglobulins did not differ from those for cells cultured with immunoglobulins from the healthy controls (Fig. 3 and Table 2).

We also examined subacute effects on the K^+ and Na^+ currents of NB-1 cells cultured for 1 day with immunoglobulins from the healthy controls and patient 1. The calculated K^+ current densities for the controls (n = 25) were 18.4 ± 2.0 pA/pF for the steady state and 43.6 ± 3.9 pA/pF for the peak current. In cells cultured with immunoglobulin from patient 1, the steady–state current density was 19.9 ± 3.0 pA/pF and the peak current density 50.4 ± 4.9 pA/pF (n = 10). There was no significant difference between the K^+ current densities in

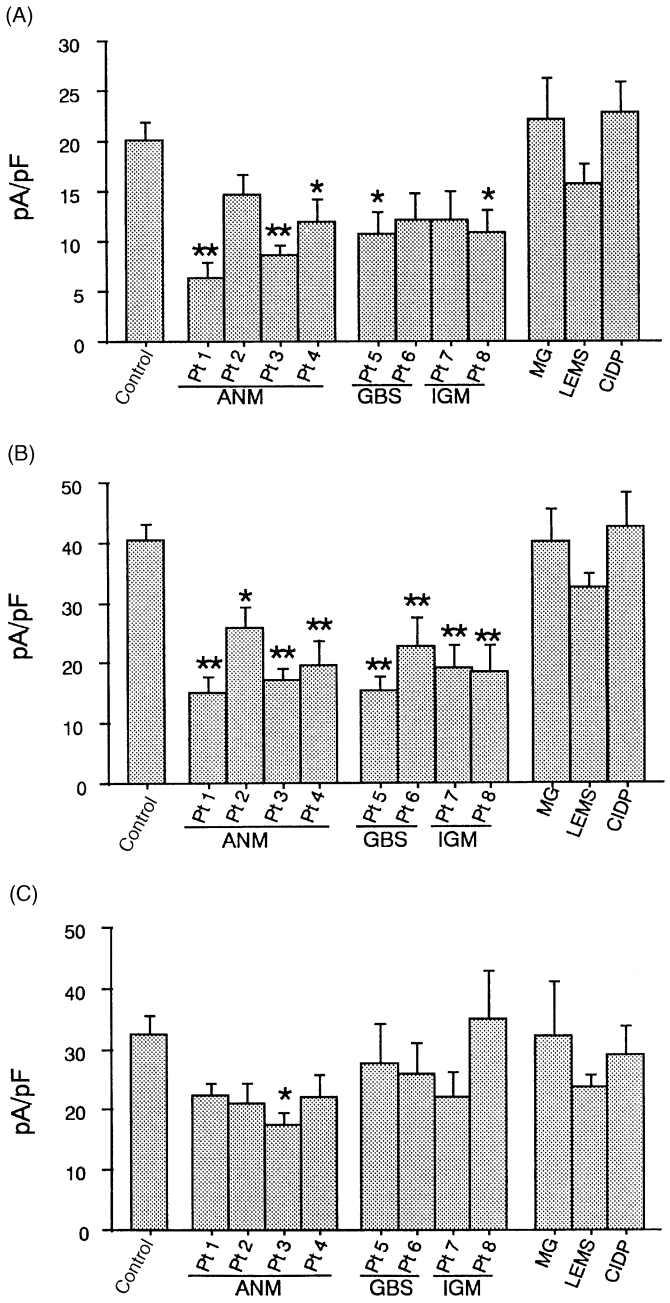

Fig. 3 Chronic effects of immunoglobulins from patients with acquired neuromyotonia (ANM; patients 1–4), Guillain–Barré syndrome (GBS; patients 5 and 6), IGM without pseudomyotonia (patients 7 and 8) and other neurological diseases [myasthenia gravis (MG), LEMS, CIDP] on K^+ and Na^+ currents. NB-1 cells were cultured for 3 days with 5 μg/ml of the immunoglobulins from the patients and controls. Steady state K^+ (**A**) and peak K^+ (**B**) current densities were obtained at the test potential of +40 mV from the holding potential of –80 mV, and Na^+ current densities (absolute values) at the test potential of –20 mV from the same holding potential (**C**). Bars show the mean current density ± standard error. $*P < 0.05$, $**P < 0.01$, compared with four healthy controls.

cells cultured with the immunoglobulins from patients and those from controls. The respective Na^+ current densities of 32.4 ± 4.2 pA/pF (n = 25, control) and 33.2 ± 6.6 pA/pF (n = 9, patient 1) indicated that there was no significant change in the Na^+ currents (data not shown).

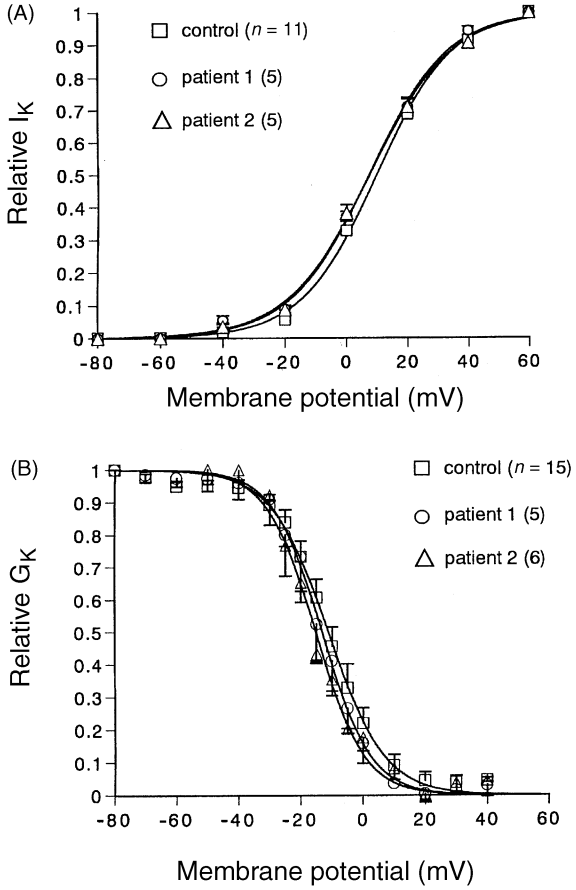

Fig. 4 Voltage-dependence of the activation (**A**) and inactivation (**B**) of the peak K^+ current. In **A**, conductance–voltage relations were determined by non-linear regression fitting to the Boltzmann equation $G_K/G_{K(max)} = 1/\{1 + \exp[(V_t - V_{1/2})/k]\}$, where V_t is the test potential, $V_{1/2}$ the midpotential, k the slope factor and $G_K = I_K/(V_t - E_k)$ (see text). In **B**, voltage-dependent inactivation was calculated from the Boltzmann equation $I_K/I_{K(max)} = 1/\{1 + \exp[(V_t - V_{1/2})/k]\}$. Numbers of cells are shown in parentheses.

Effects of immunoglobulins from acquired neuromyotonia patients on the activation and inactivation of K^+ current

Activation of the peak K^+ current was determined by calculating the peak K^+ conductance from the linear conductance equation: $G_K = I_K/(V_t - E_K)$, where G_K is the conductance, I_K the K^+ current, V_t the test potential and E_K the K^+ equilibrium potential. The E_K value was assumed to be –84 mV in this experimental condition. Figure 4A shows the activation curves of the peak K^+ currents in NB-1 cells cultured for 3 days with immunoglobulins from the controls (n = 11), patient 1 (n = 5) and patient 2 (n = 5). The smooth curves are the least-squares fitted lines for the relative K^+ conductances. The average values of the potential at

which the relative G_K was 0.5 ($V_{1/2}$) were 9.83 mV (control), 6.73 mV (patient 1) and 7.43 mV (patient 2), showing very little difference among them. The average values of the slope factor (k) were –12.49 (control), –15.22 (patient 1) and –13.81 (patient 2), again showing very little difference. Figure 4B shows the inactivation of the peak K^+ current at the test potential of +40 mV, which was examined by altering the holding potential for the controls (n = 15), patient 1 (n = 5) and patient 2 (n = 6). The $V_{1/2}$ averages were –15.40 mV (control), –16.39 mV (patient 1) and –20.54 mV (patient 2). The average k values were 9.09 (patient 1), 7.88 (patient 2) and 7.85 (patient 2). In addition, the respective $V_{1/2}$ averages of the inactivation curves for cells cultured for 6 days with immunoglobulins from the control and patient 1 were –18.27 and –18.30 mV, and the average k values were 5.81 and 4.75 (figure not shown). We therefore concluded that the voltage-dependence for activation and inactivation did not differ significantly between cells cultured with the control or patients' immunoglobulins.

Effects of immunoglobulins from Guillain–Barré syndrome and IGM patients on K^+ currents and Na^+ currents

Effects on K^+ and Na^+ currents in NB-1 cells co-cultured for 3 days with immunoglobulins from patients with Guillain–Barré syndrome and IGM without pseudomyotonia were studied. The resting membrane potential and cell capacitance did not differ from the control values (Table 2). Figure 3A and B shows the K^+ current densities measured in cells cultured with 5 μg/ml of the immunoglobulins from patients 5–8. The steady-state current and the peak K^+ currents were both suppressed, but the suppression was greater for the peak K^+ currents. The Na^+ currents in cells cultured with the patients' immunoglobulins did not differ significantly from those of the controls (Fig. 3C). The effects of the immunoglobulins from the patients with Guillain–Barré syndrome and IGM without pseudomyotonia on both the K^+ and Na^+ currents were similar to those of immunoglobulins from patients with acquired neuromyotonia.

Effects of sera from acquired neuromyotonia patients on expressed hKv 1.1 and 1.6

Figure 5A and B shows the averages of the peak K^+ current densities measured at the test potential of +40 mV in each transfected cell type cultured for 3 days with sera from two healthy participants (controls 1 and 2) and two acquired neuromyotonia patients (patients 1 and 2). Figure 5C and D shows the activation curves of the K^+ currents in these cells. The cells in Fig. 5A and C were transfected with hKv 1.1, and those in Fig. 5B and D with hKv 1.6. The K^+ currents were suppressed significantly by the patients' sera, as shown in Fig. 5A (control, 23.3 ± 2.0; patient 1, 12.2 ± 1.7; patient 2, 10.9 ± 2.6 pA/pF) and Fig. 5B (control, 72.0 ± 8.7; patient 1, 28.4 ± 7.7; patient 2, 32.7 ± 6.1 pA/pF). In Fig. 5C, the $V_{1/2}$ values were –17.36 (control), –16.94 (patient 1) and –19.31 mV (patient 2). The slope factors (k) were –11.53 (control), –12.28 (patient 1) and –12.71 (patient 2). In Fig. 5D, the $V_{1/2}$ values were –14.32 (control), –12.68 (patient 1) and –10.45 mV (patient 2), and the respective k values were –10.37, –11.78 and –12.24. We conclude that the K^+ currents were suppressed significantly by the acquired neuromyotonia patients' sera in CHO-K1 cells transfected with hKv 1.1 or 1.6, whereas there was almost no difference in the activation curves.

Discussion

The pathomechanism of nerve hyperexcitability in acquired neuromyotonia is closely correlated with anti-VGKC antibodies (Sinha *et al*., 1991; Shillito *et al*., 1995; Sonoda *et al*., 1996; Arimura *et al*., 1997; Hart *et al*., 1997). We showed that sera from acquired neuromyotonia patients suppressed K^+ currents in a PC-12 cell line (Kurono *et al*., 1995; Sonoda *et al*., 1996). Because this cell line requires NGF for the expression of VGKCs (Sonoda *et al.*, 1996), the involvement of NGF in K^+ current suppression could not be ruled out. In the study reported here, we used an NB-1 cell line (Miyake *et al*., 1975) that does not require a neurotrophic factor such as NGF for the expression of ion channels. Furthermore, our previous experimental findings using 4-aminopyridine, tetraethylammonium and tetrodotoxin confirmed the existence of voltage-gated K^+ currents and voltage-gated Na^+ currents in NB-1 cells (Y. Horikiri, unpublished observation). The present findings confirm the suppression of voltage-gated K^+ currents in NB-1 cells by immunoglobulins from acquired neuromyotonia patients, indicating that NGF is not involved in the inhibition of K^+ currents.

Our findings also showed that K^+ currents were suppressed only in cells co-cultured with the immunoglobulins from acquired neuromyotonia patients for 3 days, but not in those cultured for 1 day. A similar phenomenon was noted in our previous study of a PC-12 cell line (Sonoda *et al*., 1996). Moreover, the voltage-dependence of the activation and inactivation of the K^+ current did not differ between cells cultured with the control and patients' immunoglobulins, indicating that antibodies to VGKC may not directly suppress its functions or change its kinetics. Interestingly, Meriney and colleagues reported similar finding in the suppression of Ca^{2+} channels by a LEMS IgG (Meriney *et al.*, 1996). They found that the LEMS immunoglobulin required 24 h for maximal effect and proposed that this is consistent with the process of Ca^{2+} channel removal reaching equilibrium with the insertion of new Ca^{2+} channels in the plasma membrane. Taken together, these findings suggest that decreasing VGKC expression or increasing VGKC degradation through some type of intracellular signalling pathway may be involved in the reduction of outward K^+ current.

Nerve hyperexcitability, which is also seen in myokymia, is present in both VGKC downregulation and the prolonged

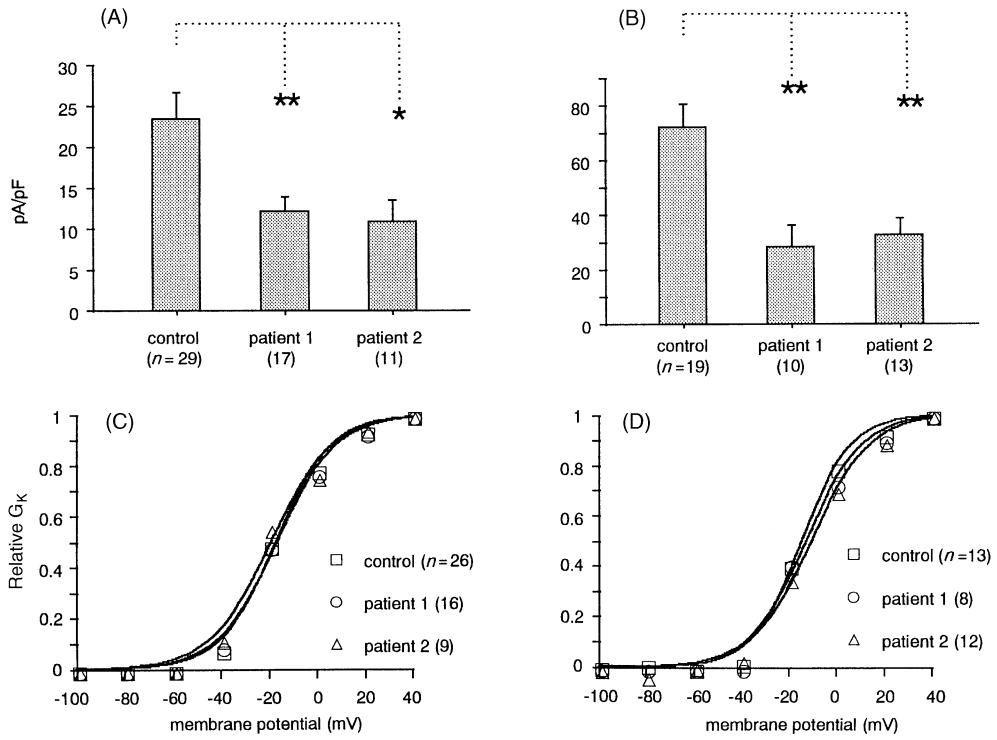

Fig. 5 Effects of serum from patients with acquired neuromyotonia (patients 1 and 2) on hKv 1.1 (**A** and **C**) and 1.6 (**B** and **D**) expressed in CHO-K1 cells. Transfected CHO-K1 cells were cultured for 3 days with 2% serum from the patients and controls. **A** and **B** show peak K^+ current densities obtained at the test potential of +40 mV from the holding potential of –80 mV. **C** and **D** show conductance–voltage relationships by non-linear regression fitting to the Boltzmann equation. Cells in **A** and **C** were transfected with hKv 1.1 and those in **B** and **D** with hKv 1.6. $*P < 0.05$, $**P < 0.01$, compared with two healthy controls. Numbers of cells are shown in parentheses.

activation of voltage-gated Na^+ channels (Cameron *et al*., 1991*a*, *b*; Gutmann *et al*., 1996). We showed that immunoglobulins from patients with acquired neuromyotonia do not affect Na^+ currents, which is evidence that these currents do not function in the pathomechanism of this disease.

Myokymia and muscle cramp caused by nerve hyperexcitability are found in a variety of diseases of unknown aetiology (Auger *et al*., 1984; Jamieson *et al*., 1994). Our patients 7 and 8 had IGM that was characterized by clinical myokymia or muscle cramp with myokymic discharges of unknown aetiology (Auger *et al.*, 1984; Jamieson *et al*., 1994). Because the pseudomyotonia and hypersweating that are characteristic of acquired neuromyotonia were absent in these patients (Table 1), we classified them as patients with IGM but without pseudomyotonia (IGM with the exception of acquired neuromyotonia). The immunoglobulins of these two patients suppressed outward K^+ currents but did not markedly affect Na^+ currents, as was also the case for the immunoglobulins of patients with acquired neuromyotonia. These results suggest that the suppression of K^+ currents, which may induce nerve hyperexcitability, is not specific to acquired neuromyotonia, and that the underlying electrophysiological abnormalities between myokymic discharges and neuromyotonic discharges may represent merely a continuum or spectrum of the same phenomenon.

We believe that the investigation of the suppression of K^+ currents is an important step in establishing the aetiology of nerve hyperexcitability, and that acquired neuromyotonia and IGM without pseudomyotonia (IGM except acquired neuromyotonia) may belong to the same disease spectrum, the former being the more severe.

In our findings, immunoglobulins from patients with Guillain–Barré syndrome suppressed K^+ currents but not Na^+ currents in NB-1 cells. We did not perform an electromyographic study on our Guillain–Barré syndrome patients, and only one patient had muscle cramp. In the early stage of Guillain–Barré syndrome, myokymic discharges are occasionally and transiently detectable by electromyography (Mateer *et al*., 1983), and suppression of K^+ currents may be the pathomechanism of these discharges in Guillain–Barré syndrome.

Western blots showed that NB-1 cells, which we used, have hKv 1.1 and 1.2 but not 1.6 (O. Pongs, unpublished data). Hart and colleagues, in their molecular immunohistochemical assay of *Xenopus* oocytes transfected with the cDNA of human brain VGKCs (KCNA1, KCNA2 and KCNA6), confirmed that all these VGKCs are involved in the pathomechanism of acquired neuromyotonia (Hart *et al.*, 1997). They also showed that acquired neuromyotonia sera vary widely in immunoreactivity to the three VGKCs studied and suggested that the anti-VGKC antibodies in acquired neuromyotonia may be heterogeneous in their fine specificities, binding not

only to the determinants found on one particular VGKC subtype but perhaps to several determinants on the same VGKC subtypes. We therefore examined the electrophysiological effects of sera from acquired neuromyotonia patients on the KCNA1 or KCNA6 expressed in CHO-K1 cells, and found that the K^+ currents through both hKv 1.1 and 1.6 were suppressed without change in the activation kinetics.

Our findings confirm that nerve hyperexcitability is closely related to the suppression of VGKCs without a change in the kinetics. Patch-clamp and immunological studies using cells transfected with other VGKC subtypes should provide information essential for the further investigation of pathomechanisms that involve VGKCs.

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