Contribution of a non-inactivating potassium current to the resting membrane potential of fusion-competent human myoblasts

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1. Using the patch-clamp technique, a new non-inactivating voltage-gated potassium current, $I_{K(ni)}$, was studied in cultured fusion-competent human myoblasts.

2. $I_{K(ni)}$ is activated at voltages above −50 mV and its conductance reaches its maximum around +50 mV. Once activated, the current remains at a steady level for minutes.

3. Reversal potential measurements at various extracellular potassium concentrations indicate that potassium ions are the major charge carriers of $I_{K(ni)}$.

4. $I_{K(ni)}$ is insensitive to potassium channel blockers such as charybdotoxin, dendrotoxins, mast cell degranulating (MCD) peptide, 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP) and apamin, but can be blocked by high concentrations of TEA and by Ba^{2+}.

5. A potassium channel of small conductance (8·4 pS at +40 mV) with potential dependence and pharmacological properties corresponding to those of $I_{K(ni)}$ in whole-cell recording is described.

6. $I_{K(ni)}$ participates in the control of the resting potential of fusion-competent myoblasts, suggesting that it may play a key role in the process of myoblast fusion.

Studies on chick embryonic myoblasts indicated that changes of the membrane potential may be an important factor in the process which leads to myoblast fusion (Entwistle, Zalin, Bevan & Warner, 1988). In the chick embryo, fusion of myoblasts has been found to be promoted by prostaglandin E_1, acetylcholine, or elevated extracellular potassium (Hausman, Dobi, Woodford Petrides, Ernst & Nichols, 1986; Entwistle *et al.* 1988). It has been suggested that these effects are mediated by a depolarization of the cell membrane. It was further suggested that myoblasts, which have a low resting membrane potential (−10 mV in chick: Fischbach, Nameroff & Nelson, 1971; Spector & Prives, 1977; −8 mV in rat: Ritchie & Fambrough, 1975), become more permeable to potassium before they fuse (Entwistle *et al.* 1988). The resulting hyperpolarization would render these cells more sensitive to the depolarizing signals just mentioned, allowing them to turn on the fusion programme. It is interesting to note that resting potentials of young myotubes of rats are more negative than those of myoblasts (Ritchie & Fambrough, 1975). This observation supports the hypothesis that a major change in membrane electrical properties, probably linked to potassium permeability, precedes or accompanies the fusion process.

A precise indication of the type of potassium channel that might be responsible for the increase in resting potential of fusion-competent myoblasts does not exist. In proliferating human myoblasts, only a calcium-activated potassium current ($I_{BK(Ca)}$) is expressed (Hamann *et al.* 1994). However, the intracellular Ca^{2+} concentrations in rat myoblasts and myotubes (120 nM; Cognard, Constantin, Rivet-Bastide & Raymond, 1993) and in human myotubes (180 nM; Sarabia & Klip, 1989) are such that activation of $I_{BK(Ca)}$ is expected to begin only at potentials above 0 mV (Marty, 1983; Blatz & Magleby, 1987). Therefore, $I_{BK(Ca)}$ is not expected to contribute much to the resting potential of human myoblasts and we shall present evidence for this. A similar conclusion was reached regarding the contribution of $I_{BK(Ca)}$ to the resting potential in smooth muscle (Fleischmann *et al.* 1993). Another potassium current, $I_{K(dr)}$, a delayed rectifier current (Trautmann, Delaporte & Marty, 1986), was found to be expressed in cultured human myoblasts that are about to fuse (Widmer, Hamann, Baroffio, Bijlenga & Bader, 1995). This current inactivates

at depolarized voltages (Widmer *et al.* 1995), which makes it too small to affect the resting potential of myoblasts. However, in smooth muscle cells, $I_{K(dr)}$ was found to contribute significantly to the resting potential (Fleischmann, Washabau & Kotlikoff, 1993).

Recently, a novel type of potassium current, $I_{aKv5\cdot1}$, which does not inactivate at depolarized voltages, was described in *Aplysia* neurones (Zhao, Rassendren, Kaang, Furukawa, Kubo & Kandel, 1994). In this paper we describe the properties of a similar non-inactivating voltage-gated potassium current, $I_{K(ni)}$, which we find to be present in fusion-competent human myoblasts. The pharmacological properties of $I_{K(ni)}$ differ from those of $I_{aKv5\cdot1}$ and the elementary $I_{K(ni)}$ current is mediated by a low conductance channel (8·4 pS at +40 mV). $I_{K(ni)}$ contributes to the resting potential of fusion-competent human myoblasts, which we found to be significantly more hyperpolarized than proliferating myoblasts.

METHODS

Dissociation and culture procedures

Samples of human skeletal muscle (576 ± 84 mg) were obtained during corrective orthopaedic surgery of six patients (9 months to 20 years old) without any known neuromuscular disease. Biopsies of muscles were obtained in accordance with the guidelines of the ethical committee of the University Hospital of Geneva, Switzerland (written informed consent was obtained from patients or their legal guardians).

The dissociation procedure used to isolate and prepare clonal cultures from satellite cells was as previously described (Baroffio, Aubry, Kaelin, Krause, Hamann & Bader, 1993). Briefly, the muscle sample was cleaned, minced and incubated for 1 h at 37 °C in 0·05% trypsin. The cells were then centrifuged and resuspended several times in a wash medium (F10 nutrient medium (Gibco) supplemented with 15% fetal calf serum) in order to pellet muscular debris. Tris-ammonium chloride buffer was added to lyse red blood cells.

Single satellite cells were manually collected with a micropipette and cultured individually in wells containing proliferation medium in which they actively divide (clonal culture). The proliferation medium was composed of F10 nutrient medium (Gibco) supplemented with 15% fetal calf serum, 0·5 mg ml^{-1} bovine serum albumin, 0·5 mg ml^{-1} fetuin, 10 ng ml^{-1} epidermal growth factor, 0·39 mg ml^{-1} dexamethasone, 0·18 mg ml^{-1} insulin and 0·1 μg ml^{-1} gentamycin (Ham, StClair, Blau & Webster, 1989). When confluent, cells were replated at a lower density. Clonal cultures of myoblasts can proliferate for several months. Formation of myotubes can be induced by replacing the proliferation medium with a medium that promotes myoblast differentiation and fusion (StClair, Meyer, Demarest & Ham, 1992). The differentiation medium was composed of Dulbecco's modified Eagle's nutrient medium (DMEM; Gibco) supplemented with 0·5 mg ml^{-1} bovine serum albumin, 10 ng ml^{-1} epidermal growth factor, 10 μg ml^{-1} insulin and 1 μg ml^{-1} gentamycin. Half of the culture medium was changed 3 times a week.

Prefused myoblasts

Fusion-competent myoblasts were prepared by seeding proliferating myoblasts in the differentiation medium for 1–5 days at very low density to avoid physical contact between cells (10 000 cells per 35 mm culture dish). In this condition, myoblasts should presumably be in a more mature state, but most of them are prevented from fusing due to the low cell density. To confirm that the recorded prefused myoblasts were actually mononucleated, the relationship between cell capacitance and number of nuclei was studied in prefused myoblasts (Krause, Hamann, Bader, Liu & Baroffio, 1995).

Electrophysiological recordings

Ionic currents were recorded under voltage clamp in either whole-cell or single-channel (outside-out) configurations of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using an Axopatch 200A amplifier. The same amplifier in current clamp mode was used to measure resting membrane potential. Capacitance of the recorded cells were obtained from direct reading of the whole-cell capacitance potentiometer of the amplifier. The resistance of the pipettes was 2–5 MΩ for whole-cell recordings and 5–10 MΩ for single-channel recordings. Currents were recorded at 20–22 °C and low-pass filtered at 0·5–1 kHz. Sampling rates were 2·5 and 5 kHz during whole-cell and single-channel recordings, respectively. To obtain spherical cells which were more easily patched, cells were treated with 0·05% trypsin and replated 1–2 h before recording.

Solutions and materials

Whole-cell recording. The extracellular control solution was composed of (mM): NaCl, 22; *N*-methyl-D-glucamine chloride (NMG-Cl), 100; KCl, 5; $MgCl_2$, 3; Hepes, 5; and glucose, 8. The pH was adjusted to 7·3 with NMG. When TEA-Cl and $BaCl_2$ were added to the solution, NMG-Cl was reduced to keep the osmolarity constant. The extracellular solutions used for reversal potential determination were composed of (mM): NaCl, 125; NMG-Cl, 28, 25, 18, or 0; KCl, 2, 5, 12, or 30; $MgCl_2$, 3; Hepes, 5; and glucose, 8. The intracellular (pipette) solution was composed of (mM): KCl, 130; NaCl, 3; $MgCl_2$, 1; Hepes, 10; EGTA, 20; and glucose, 5. The pH was adjusted to 7·3 with KOH.

Single-channel recording. The extracellular solution was composed of (mM): sodium acetate, 50; NMG-Cl, 100; KCl, 5; $MgCl_2$, 3; Hepes, 5; $CaCl_2$, 0·5; and nifedipine, 0·03. The pH was adjusted to 7·4 with NMG. The intracellular (pipette) solution was composed of (mM): KCl, 70; KF, 80; NaCl, 4; $MgCl_2$, 1; Hepes, 5; and BAPTA, 10. The pH was adjusted to 7·4 with KOH.

Trypsin (from bovine pancreas) was from Boehringer Mannheim. Ham's F-10 nutrient medium, DMEM and gentamycin were from Gibco. Bovine serum albumin, dexamethasone, fetuin, insulin, Hepes, nifedipine (a stock of 50 mM nifedipine was prepared in DMSO and kept at 4 °C), 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP), apamin, 5-hydroxytryptamine (5-HT), muscarine chloride, MgATP, NaGTP and DMSO were from Sigma. Charybdotoxin, α-, β-, γ- and δ-dendrotoxins and mast cell degranulating peptide (MCD peptide) were from Alomone Labs (Jerusalem). Epidermal growth factor was from Collaborative Research (Bedford, MA, USA). Fetal calf serum was from Ready System (Zurzach, Switzerland) or Inotech (Dottikon, Switzerland). α-Bungarotoxin was from Calbiochem. NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, KOH and sodium acetate were from Merck. CsCl, $CdCl_2$, EGTA, BAPTA, NMG and TEA-Cl were from Fluka.

Fusion index

Cultures were fixed for 5 min at −20 °C with 100% methanol and stained with Haematoxylin. Nuclei were counted in twenty randomly chosen microscope fields (2 culture dishes, 10 fields in

each dish) at a magnification of ×400. One microscope field contains usually between 100 and 150 nuclei. In t tests, n refers to the number of microscope fields counted. The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei.

Statistics

Results are expressed as the means ± S.E.M. Statistical comparisons were made using Student's paired t tests.

RESULTS

Presence of a non-inactivating outward current

Figure 1*A* illustrates the non-inactivating potassium current ($I_{K(nl)}$) studied here. In most cells, both $I_{K(nl)}$ and the delayed rectifier $I_{K(dr)}$ were present. In eighty-seven cells, $I_{K(nl)}$ measured at +50 mV represented 55 ± 3% of the total potassium current made up of $I_{K(nl)} + I_{K(dr)}$ (all recordings were made with 20 mM EGTA in the pipette and without calcium in the extracellular solution, so that there was no contribution of $I_{BK,Ca}$ to the total potassium current; see below). In a few cells, however, either $I_{K(dr)}$ or the non-inactivating current were very small or absent. In the cell illustrated in Fig. 1*A* there was no detectable $I_{K(dr)}$. During a depolarizing step from −80 to +40 mV, $I_{K(nl)}$ increased to a level that then remained constant for 11 min. Similar long-lasting recordings were made in five cells in which the ratio of the steady current after 408 ± 72 s (range, 5–11 min) to that observed after 1 s was 1·02 ± 0·04 (mean current density was 21 ± 5 pA pF^{-1}). This indicates that $I_{K(nl)}$ does not inactivate over the time period examined ($P > 0·6$).

Previous studies on human myoblasts indicated that fetal calf serum deprivation induces the expression of $I_{K(dr)}$ (Widmer *et al.* 1995). Typical behaviour of $I_{K(dr)}$ recorded in prefused myoblasts (see Methods section) is illustrated in Fig. 1*B*. During a depolarizing step to +40 mV, an outward current developed rapidly (trace labelled with ◇). When the cell was held steadily at +40 mV (trace labelled with ○), however, the outward current diminished markedly. This reflects the time- and voltage-dependent inactivation of $I_{K(dr)}$ which totally inactivates at +40 mV in myoblasts (Widmer *et al.* 1995). The outward current remaining at +40 mV in this cell, which represents $I_{K(nl)}$, could still be

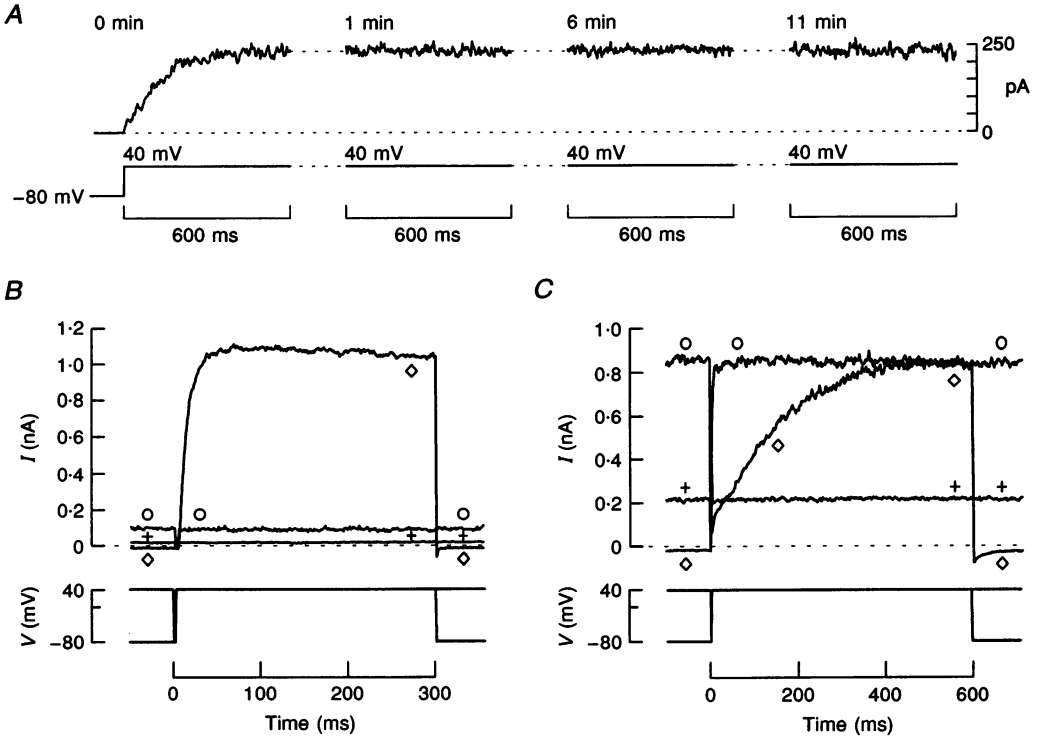

Figure 1. Presence of non-inactivating ($I_{K(nl)}$) and of inactivating ($I_{K(dr)}$) outward currents in prefused myoblasts

A, recordings from a myoblast after 2 days in differentiation medium. Leak current was estimated by adding 30 mM TEA to the bath medium, and was subtracted. The cell capacitance was 10 pF. *B*, recordings from a myoblast after 3 days in differentiation medium. ◇, current recorded from a cell held at −80 mV and then stepped to +40 mV for 300 ms. ○, recording from the same cell held for 30 s at +40 mV; +, recording from the same cell held for 30 s at +40 mV in the presence of 30 mM TEA. Current traces are not corrected for leak current. Cell capacitance was 25 pF. *C*, in another myoblast (1 day in differentiation medium) only $I_{K(nl)}$ was observed. Same protocol as in *B* (except step duration, which was 600 ms). Cell capacitance was 45 pF. Current traces are not corrected for leak current.

reduced substantially by TEA (trace labelled with +). As will be seen in Fig. 3, the TEA-sensitive current is a potassium current.

Figure 1*C* illustrates a recording from a cell in which only $I_{K(nl)}$ was present. It can be seen that during a depolarization from −80 to +40 mV, an outward current developed (◇) with a slower time course than $I_{K(dr)}$ in Fig. 1*B*. When the potential was held steadily at +40 mV for more than 1 min there was no decrease of the outward current in this cell (○). As in Fig. 1*B*, the steady outward current could be reduced by applying 30 mM TEA (+). It should be noted that a linear extrapolation to +40 mV of the small inward leak current at −80 mV (18 pA) in Fig. 1*C* would give a much smaller current (40 pA) than that actually observed in the presence of TEA at +40 mV. This suggests that $I_{K(nl)}$ is not totally blocked by 30 mM TEA.

Some of the basic properties of $I_{K(nl)}$ in prefused myoblasts are illustrated in Fig. 2. The cell studied in Fig. 2*A* expressed this current almost exclusively, with only a minor $I_{K(dr)}$ component. To study the activation properties of $I_{K(nl)}$ the potential of a cell was stepped to a series of voltages from a holding potential of either −80 or +40 mV. The symbols used to identify the various current traces are indicated in Fig. 2*A d*. The trace recorded during a step to +10 mV from a steady holding potential of −80 mV is labelled with squares (□). A brief inward sodium current (see Hamann *et al.* 1994) was recorded first, followed by an outward current. The trace recorded during a steady holding potential of +40 mV is indicated with diamonds (◇). Note that one would expect the relaxing current (◇) to meet the current activated by the voltage step (□) only if there is no inactivation of the outward current at +40 mV. Otherwise, the current relaxing from +40 mV is expected to be of smaller amplitude than that rising from −80 mV (see Fig. 1*B*). When the voltage protocol was repeated from −80 mV in the presence of 130 mM TEA, a marked decrease in the outward current occurred (trace labelled with circles).

In Fig. 2*A a–f*, the protocols just described were applied for a series of 600 ms steps to various voltages (−50, −30,

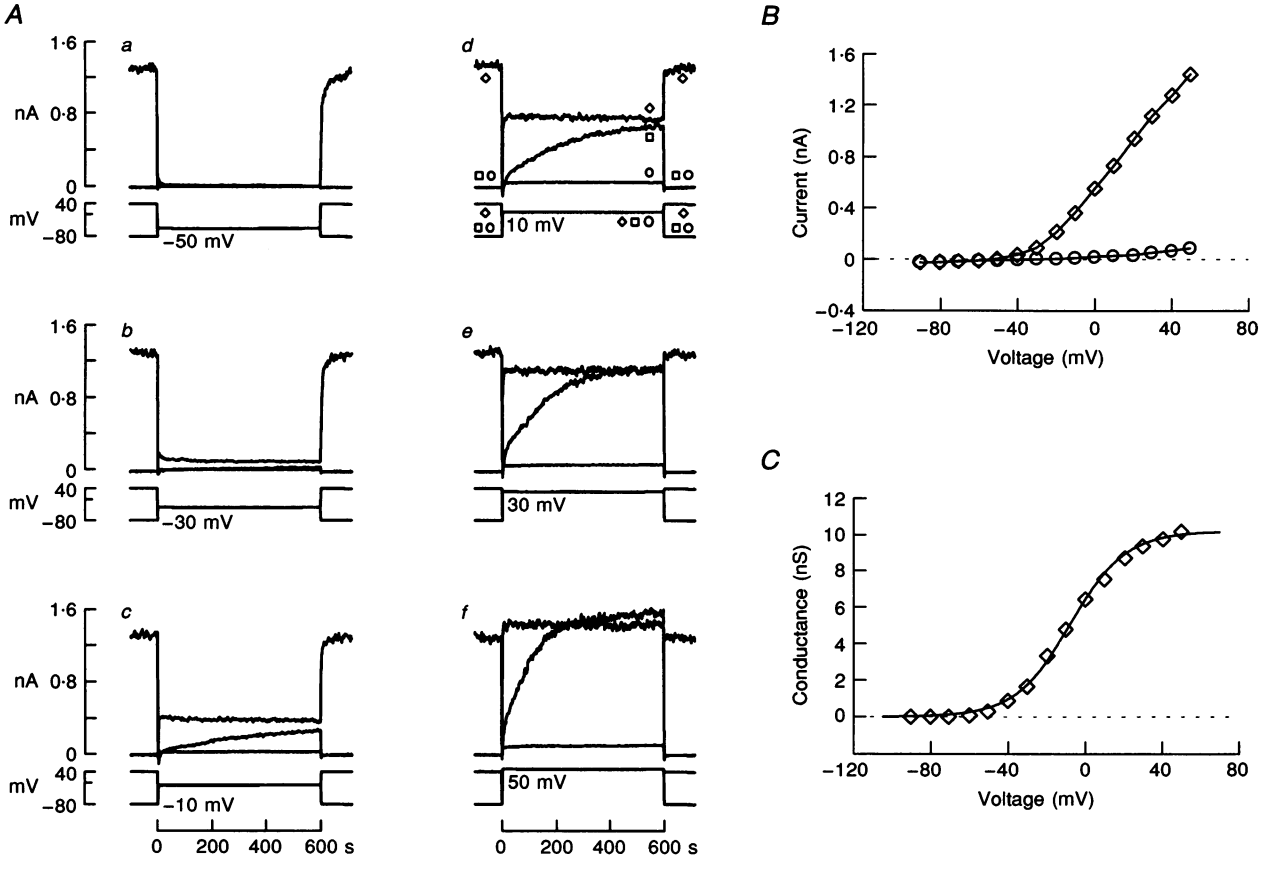

Figure 2. Properties of the non-inactivating outward current ($I_{K(nl)}$)

A, recordings from a myoblast after 2 days in differentiation medium. The cell was held at −80 mV (□; see *A d*) or +40 mV (◇; see *A d*) and stepped to various potentials for 600 ms. Leak current was estimated by adding 130 mM TEA to the superfusion solution (○; see *A d*). Cell capacitance was 35 pF. *B*, steady-state current–voltage properties of $I_{K(nl)}$. ◇, $I_{K(nl)}$; ○, leak current recorded in the presence of 130 mM TEA. Same cell as in *A*. *C*, steady-state conductance as a function of voltage. Leak-subtracted current was measured at the end of the 600 ms step. Same cell as in *A*.

−10, +10, +30 and +50 mV). The interval between steps was 10 s. This long interval was chosen to minimize the effect of each step on the subsequent one. In Fig. 2*Af*, it can be seen that the outward current recorded during the step to +50 mV from a holding potential of −80 mV crosses the current trace recorded during the corresponding step elicited from +40 mV. As explained earlier, this indicates that there is a small inactivating component in the overall outward current. This inactivating component is due to the presence of $I_{K(dr)}$. In this cell, $I_{K(dr)}$ was small and accounted for approximately 10% of the total outward current during the step to +50 mV from a holding voltage of −80 mV.

In Fig. 2*B*, the value of the current at the end of each voltage step from +40 mV is plotted against the voltage during a step (◇). This graph represents the steady-state current–voltage properties of $I_{K(ni)}$. Note that in order to fully inactivate $I_{K(dr)}$, the cell was held at +40 mV for 2 min before initiating the series of steps from that level. In Fig. 2*B*, the currents recorded during the steps in the presence of 130 mM TEA are also illustrated (○). By comparing the two curves it can be seen that $I_{K(ni)}$ is elicited for voltage steps exceeding −50 mV. The steady-state conductance of this current was calculated by dividing, at each membrane potential (V_m), the difference in current (trace labelled ◇ − trace labelled ○) by the driving force (V_m) minus the reversal potential (E_{rev}). E_{rev} of the non-inactivating current was −82 mV (E_K; see below). The steady-state conductance curve is plotted in Fig. 2*C*. It can be seen that it was adequately described by a Boltzmann equation:

$$G_\infty(V_m) = G_{\infty,max}/[1 + \exp(Q(V_0 - V_m)/kT)],$$

where $G_{\infty,max}$ is the maximum conductance, Q is the gating charge, V_0 is the voltage at half-activation and k is the Boltzmann constant. In fourteen cells, the gating charge was 1·6 ± 0·1 times the elementary charge and V_0 was 5·1 ± 2·3 mV. In the eighty-seven cells recorded, the mean capacitance was 26 ± 1 pF and the steady-state conductance at +50 mV (which represents 95% of $G_{\infty,max}$) was 127 ± 11 pS pF^{-1}.

Charge carrier of $I_{K(ni)}$

To determine the nature of the charge carriers of $I_{K(ni)}$, the reversal potential of the current was measured at different extracellular potassium concentrations. Figure 3*A* illustrates the relaxation currents observed in a cell when the potential was stepped from a steady holding potential of 0 mV to a series of more negative voltages at extracellular potassium concentrations of 30 and 5 mM. The currents illustrated are the difference between the currents recorded in the absence and presence of 30 mM TEA. It can be seen that in the presence of 30 mM extracellular potassium, the reversal potential (zero current trace) was −38 mV (E_K = −37 mV) and that it was −79 mV in the presence of 5 mM potassium (E_K = −82 mV).

In Fig. 3*B*, reversal potentials measured in several cells are plotted *versus* the extracellular potassium concentration (semilogarithmic plot). The straight line is the fit to the

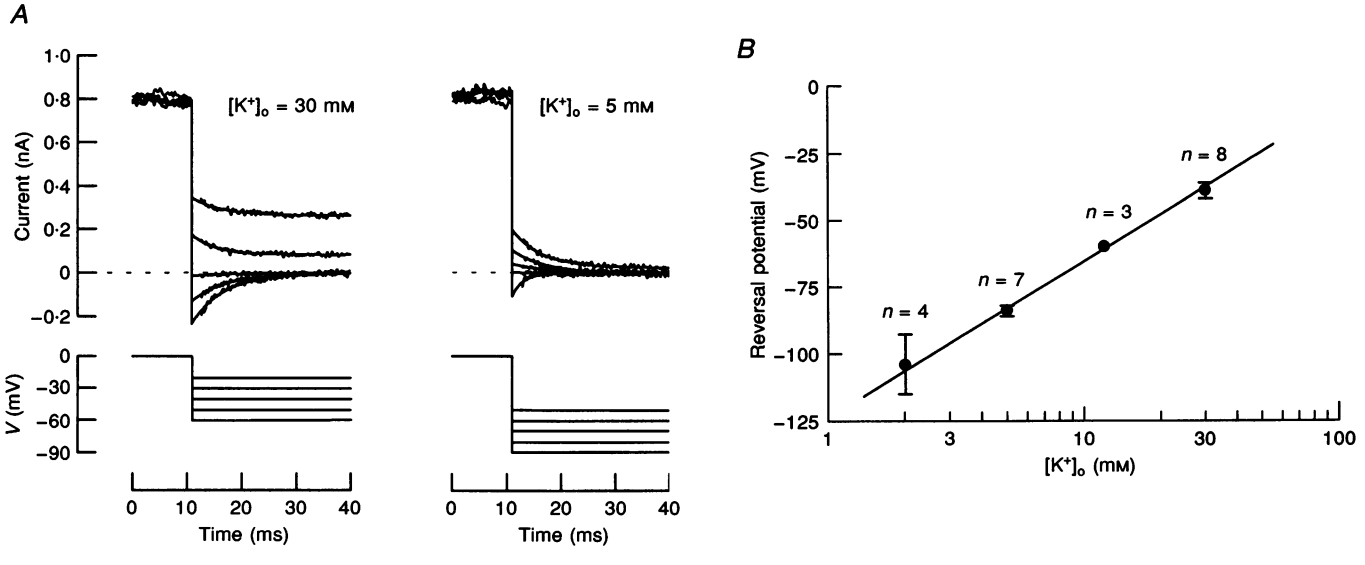

Figure 3. $I_{K(ni)}$ is mainly carried by potassium ions

A, recordings from a myoblast after 1 day in differentiation medium. Currents are leak subtracted. Leak current was estimated by adding 30 mM TEA to the superfusion solution. Decaying exponential functions are fitted to relaxation currents and capacitance artefacts are blanked. Cell capacitance was 19 pF. *B*, reversal potential measured in 2, 5, 12 and 30 mM K_o^+ (12 myoblasts were recorded; *n* refers to the number of measurements at each K^+ concentration). Intracellular K^+ was 130 mM. The straight line is the Nernst equation ($E_K = A \log_{10}([K^+]_o/[K^+]_i)$) adjusted with the '*A*' coefficient as a free parameter. The fit gave an '*A*' coefficient of 58·6 mV.

Nernst equation adjusted for an intracellular potassium concentration corresponding to that in the intrapipette solution (130 mM). The fitted slope is 58·6 mV. This result indicates that $I_{K(nl)}$ is indeed a potassium current.

$I_{K(dr)}$ but not $I_{K(nl)}$ is sensitive to nifedipine

$I_{K(nl)}$ cannot always be studied directly in isolation, as most prefused myoblasts also express the delayed rectifier $I_{K(dr)}$. Figure 4*A* shows the current recorded in a cell that expressed a large $I_{K(dr)}$ in addition to $I_{K(nl)}$. When the voltage of the cell was held at −80 mV and stepped to +40 mV, a large outward current developed (◇). The outward current when the cell was held steady +40 mV was considerably reduced (○).

A useful finding was that the application of 30 μM nifedipine markedly reduced the amplitude of the outward current during a step to +40 mV from a holding potential of −80 mV (□). Interestingly, it can be seen that the nifedipine-reduced current (□) eventually reached the level of current measured when the cell was held steadily at +40 mV in the absence of nifedipine (○). It was also observed that the current at a steady holding potential of +40 mV was insensitive to nifedipine (+). The effect of nifedipine was reversible (see superimposed traces labelled with diamonds). Therefore, we conclude that 30 μM nifedipine totally suppresses $I_{K(dr)}$ in prefused myoblasts without affecting $I_{K(nl)}$. This is confirmed by results shown

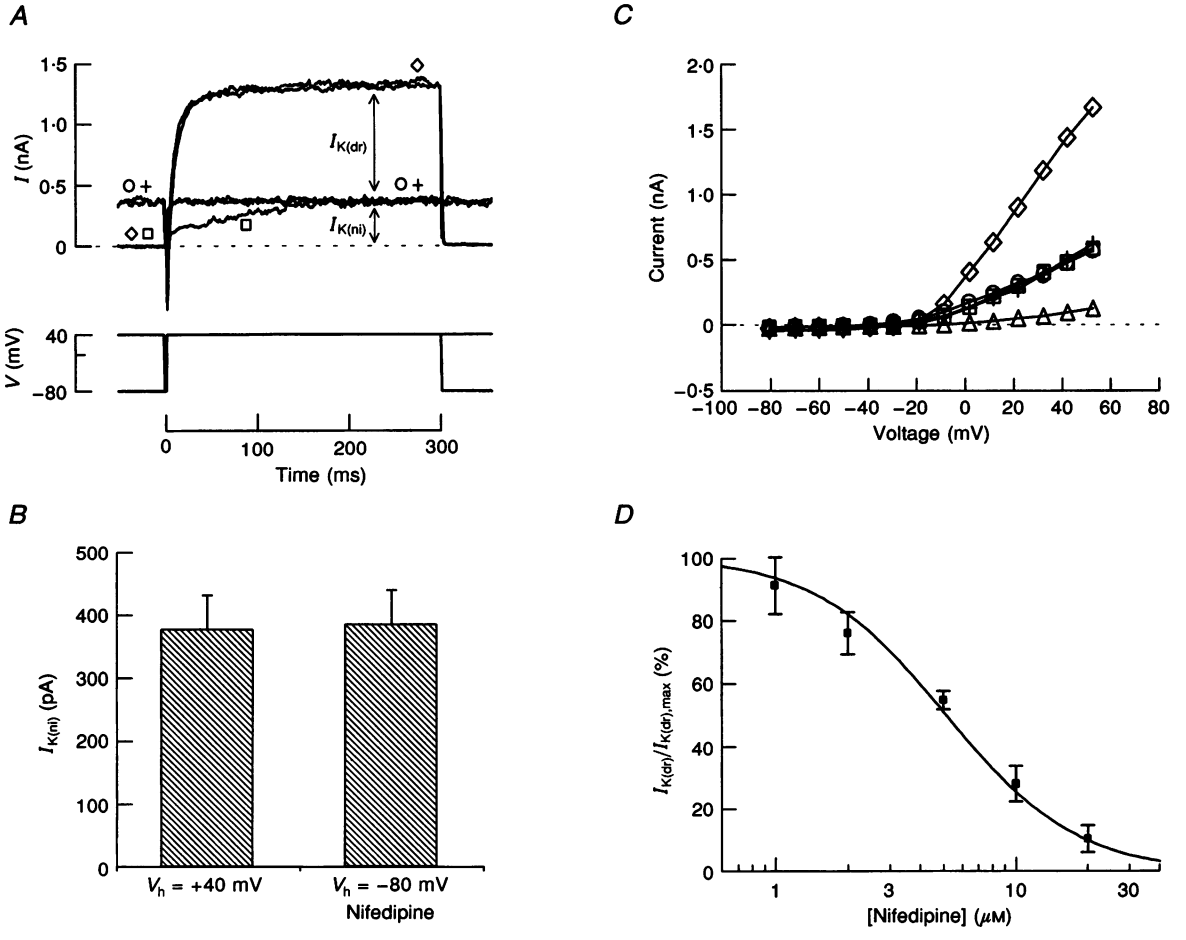

Figure 4. Unlike $I_{K(dr)}$, $I_{K(nl)}$ is insensitive to nifedipine

A, recording from a myoblast after 4 days in differentiation medium. ◇, holding potential, −80 mV; step to +40 mV. ○, holding potential steady at +40 mV. □, extracellular nifedipine (30 μM) with holding potential at −80 mV followed by a step to +40 mV. +, extracellular nifedipine (30 μM) with holding potential steady at +40 mV. Currents are leak subtracted. Leak current was estimated by adding 30 mM TEA to the external medium. Cell capacitance was 19 pF. *B*, left column is the mean $I_{K(nl)}$ isolated by a steady holding potential (V_h) at +40 mV. Right column is the mean $I_{K(nl)}$ during a step to +40 mV from a holding potential of −80 mV in presence of 30 μM nifedipine. *C*, steady-state currents as a function of voltage. Same cell as in *A*. Same symbols as in *A* except for △, which represents the leak current recorded after addition of 30 mM TEA to the superfusion solution. *D*, dose-dependent inhibition of $I_{K(dr)}$ by nifedipine. $I_{K(dr)}$ was defined as the current elicited during a voltage step to +50 mV from a holding potential at −80 mV minus the current remaining during a steady holding potential at +50 mV. Results are means from 5 cells.

in Fig. 4*B*; the current was measured in twenty-seven cells during a steady holding potential of +40 mV in the absence of nifedipine and was compared with the current during a step to +40 mV from a holding level of −80 mV in the presence of 30 μM nifedipine. In all twenty-seven cells, the paired *t* test indicated that the two currents were not statistically different ($P > 0{\cdot}8$). Thus nifedipine provides us with a convenient tool to study $I_{K(nl)}$ in isolation. A stock solution of nifedipine was prepared in DMSO. An application of 30 μM nifedipine corresponded to a concomitant application of 7·6 mM DMSO, and an application of 7·6 mM DMSO alone reduced $I_{K(dr)}$ by $24 \pm 3\%$ ($n = 5$). Thus, a fairly large concentration of DMSO had only a moderate effect on $I_{K(dr)}$ ($P = 0{\cdot}02$).

Figure 4*C* shows the current–voltage relationship of the outward current recorded under the various conditions described above: holding at −80 mV and stepping to +40 mV ($I_{K(nl)} + I_{K(dr)}$, ◇), holding at +40 mV ($I_{K(nl)}$ alone, ○), holding at −80 mV and stepping to +40 mV with nifedipine ($I_{K(nl)}$ alone, □), holding at +40 mV with nifedipine ($I_{K(nl)}$ alone, +) and with 30 mM TEA (△). In Fig. 4*D*, the dose-dependent inhibition of $I_{K(dr)}$ by nifedipine is illustrated (mean from 5 cells). The data points are adequately fitted by an equation of the form $I/I_{max} = 1/(1 + ([\text{nifedipine}]/K_d)^n)$ with $n = 1{\cdot}6$ and a K_d for nifedipine of 5·1 μM.

$I_{K(nl)}$ is distinct from $I_{BK(Ca)}$

The experiments described so far were all perfomed 20 mM intracellular EGTA and no added extracellular Ca^{2+}. This was done to minimize any contribution of the large conductance calcium-activated potassium current ($I_{BK(Ca)}$) which is present in human myoblasts (Trautmann *et al.* 1986; Hamann *et al.* 1994). Another indication that $I_{K(nl)}$ was distinct from $I_{BK(Ca)}$ was given by the different sensitivities of these currents to TEA. $I_{BK(Ca)}$ is decreased by $59 \pm 3\%$ in the presence of 0·5 mM TEA (Hamann *et al.* 1994) whereas even 30 mM TEA does not fully block $I_{K(nl)}$ (see Fig. 1*C*). In addition, charybdotoxin, which reduced the amplitude of $I_{BK(Ca)}$ by $67 \pm 4\%$ (Hamann *et al.* 1994) at a concentration of 100 nM did not significantly affect $I_{K(nl)}$ ($n = 4$, decrease of $3 \pm 6\%$, $P > 0{\cdot}3$).

To exclude completely any contribution of $I_{BK(Ca)}$ to $I_{K(nl)}$, we performed experiments in the presence of the calcium chelator BAPTA, added at 20 mM to the patch pipette and at 0·5 mM to the superfusion solution. Under these conditions, $I_{K(nl)}$ was still observed ($G_{K(nl),40\,mV} = 110 \pm 23$ pS pF^{-1}, $n = 3$) and there was no significant effect following the addition of 0·5 mM BAPTA to the bath medium (decrease of $3 \pm 8\%$, $P > 0{\cdot}7$).

Several drugs were tested by application in the extracellular solution to determine if an efficient blocking agent of $I_{K(nl)}$ could be found. In addition to charybdotoxin and TEA, we tested α-, β-, γ- and δ-dendrotoxins (100 nM), MCD peptide (200 nM), Cs^+ (2 mM), Cd^{2+} (2 mM), 4-AP (2 mM), 3,4-DAP (2 mM), apamin (200 nM) and Ba^{2+} (5 mM). Each drug was tested on three to seven cells and none of them had a significant effect on $I_{K(nl)}$ ($P > 0{\cdot}05$) with the exception of Ba^{2+}. Thus only Ba^{2+} and TEA were effective blockers of $I_{K(nl)}$ (see below).

The modulatory effects of extracellular muscarine (10 μM) and 5-HT (30 μM) were considered, since both compounds are known to inhibit non-inactivating potassium currents (Brown & Adams, 1980; Siegelbaum Camardo & Kandel, 1982). When muscarine and 5-HT were tested, the intracellular solution was supplemented with MgATP (5 mM) and NaGTP (0·1 mM). $I_{K(nl)}$ was not affected by either 10 μM muscarine or 30 μM 5-HT. In addition, the fact that 5 mM intracellular MgATP on its own did not reduce $I_{K(nl)}$ (the amplitude of $I_{K(nl)}$ recorded with and without MgATP in the pipette was not significantly different, $P > 0{\cdot}5$, $n = 6$) suggests that $I_{K(nl)}$ is not modulated by intracellular ATP (Spruce *et al.* 1987).

$I_{K(nl)}$ and the resting potential of myoblasts

The molecular mechanisms controlling the resting potential of myoblasts have not been identified so far. We wondered whether $I_{K(nl)}$, in view of its voltage range of activation and of its non-inactivating property, contributes to the resting potential of prefused myoblasts. We recorded from these cells in the current clamp mode and superfused solutions with various potassium channel blockers. The results of these experiments are given in Fig. 5*A*. In the control solution (no Ca^{2+} added), the mean resting potential of prefused myoblasts was -27 ± 1 mV ($n = 38$). This is significantly more negative than the potential of proliferating myoblasts that do not have voltage-gated potassium currents other than $I_{BK(Ca)}$ (-8 ± 1 mV; Hamann *et al.* 1994). Incidentally, as already suggested in the Introduction, $I_{BK(Ca)}$ does not appear to play a significant role in the resting potential of fusion-competent myoblasts; in six cells in which the intracellular free calcium concentration was set to 310 nM (20 mM BAPTA + 12 mM calcium), application of 3 mM TEA (known to strongly inhibit $I_{BK(Ca)}$; Lerche, Fahlke, Iaizzo & Lehmann-Horn, 1995) did not significantly affect the resting potential of the myoblasts ($P > 0{\cdot}6$).

An important finding was that 30 μM nifedipine, which totally blocks $I_{K(dr)}$, did not reduce the resting potential. This indicates that in the presence of $I_{K(nl)}$ the contribution of $I_{K(dr)}$ to the resting potential of prefused myoblasts is negligible (the small increase is not significant, $P > 0{\cdot}2$). Since under these conditions $I_{K(dr)}$ was not involved in the resting potential, we examined the contribution of $I_{K(nl)}$. Experiments conducted under voltage clamp indicated a sensitivity of $I_{K(nl)}$ to TEA (see Figs 1*B* and 2*A*). In addition, in an experiment in which $I_{K(dr)}$ was suppressed by 30 μM nifedipine (Fig. 5*B*, □) we found that Ba^{2+} was an efficient blocker of $I_{K(nl)}$ (Fig. 5*B*, ○; in 5 cells, 5 mM Ba^{2+}

blocked $I_{K(ni)}$ by 66 ± 7%) and that a combination of Ba^{2+} (5 mM) and TEA (90 mM) was even more efficient than Ba^{2+} alone (Fig. 5*B*, △). Examination of the current–voltage relationship in the presence of the various drugs indicates that in 5 mM Ba^{2+} and 90 mM TEA the relation is nearly linear (Fig. 5*C*, △). This suggests that this combination totally suppresses $I_{K(ni)}$.

When measurements were made under current clamp (Fig. 5*A*), we found that TEA produced a depolarization of prefused myoblasts. The resting potentials in the presence of 30 mM TEA (−17 ± 6 mV) are significantly different from control values (−27 ± 1 mV) but with little confidence ($P = 0{\cdot}04$), probably due to the low number of cells tested ($n = 4$) or some variability in the sensitivity to TEA. However, it can be seen that TEA at concentrations of 90 and 130 mM depolarized prefused myoblasts very significantly to −8 ± 3 mV ($n = 11$, $P < 0{\cdot}001$) and to −9 ± 1 mV ($n = 12$, $P < 0{\cdot}001$), respectively. Ba^{2+} (5 mM) produced a significant depolarization with little confidence (from −27 ± 1 to −15 ± 4 mV, $n = 3$, $P = 0{\cdot}03$).

The combination of 5 mM Ba^{2+} and 90 mM TEA, which totally blocks $I_{K(ni)}$, nearly depolarized prefused myoblasts to 0 mV (Fig. 5*A*). In seven cells, the membrane potential was −2 ± 2 mV. The effects of the drugs, individually or in combination, were fully reversible. The combination of Ba^{2+} and TEA, which totally blocks $I_{K(ni)}$, cannot be used to assess the contribution of $I_{K(dr)}$ to the resting potential *in the absence of* $I_{K(ni)}$ (reverse of the nifedipine experiment described above) as it would also block $I_{K(dr)}$.

Concentrations of Ba^{2+} higher than 5 mM could not be used as the recordings became very unstable. The blocking effect of TEA at 90 mM, however, was maximal, since there was no significant difference ($P > 0{\cdot}8$) between 90 mM TEA ($n = 11$) and 130 mM TEA ($n = 12$). Thus, the fact that the

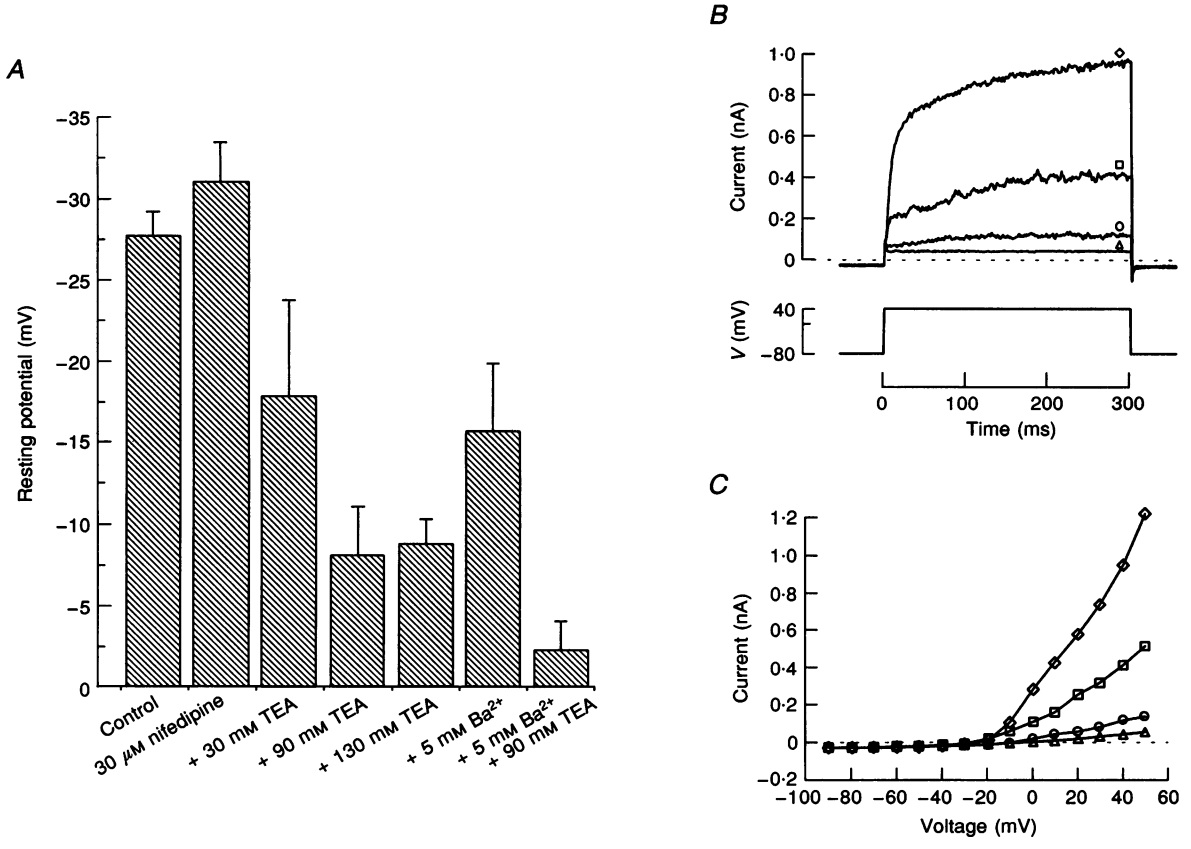

Figure 5. Sensitivity of the resting potential to nifedipine, TEA and barium

A, mean resting potential of myoblasts in control conditions ($n = 38$), 30 μM nifedipine ($n = 20$), 30 μM nifedipine + 30 mM TEA ($n = 4$), 30 μM nifedipine + 90 mM TEA ($n = 11$), 30 μM nifedipine + 130 mM TEA ($n = 12$), 30 μM nifedipine + 5 mM barium ($n = 3$) and 30 μM nifedipine + 90 mM TEA + 5 mM barium ($n = 7$). Drugs were applied in the external medium. *B*, a myoblast (4 days in differentiation medium) was held at −80 mV and stepped to +40 mV for 300 ms in control conditions (◇), in the presence of 30 μM nifedipine (□), in the presence of 30 μM nifedipine + 5 mM barium (○) and in the presence of 30 μM nifedipine + 5 mM barium + 90 mM TEA (△). Cell capacitance was 18 pF. *C*, same cell as in *B*. The amplitude of the current measured at the end of 300 ms steps to various potentials from a holding potential of −80 mV is plotted against the potential during the step. Same symbols as in *B*.

combination of Ba^{2+} (5 mM) and TEA (90 mM) is significantly more efficient than either drug alone, could suggest that the two drugs act on distinct sites of the $I_{K(nl)}$ channel and potentiate each other. Alternatively, the drugs may act on two different potassium channels which do not inactivate at depolarized voltages so that only a combination of both drugs suppresses $I_{K(nl)}$. So far, single-channel recordings of $I_{K(nl)}$ have allowed us to identify only one channel (see below). In conclusion, our results suggest that the resting potential of prefused myoblasts is determined in part by $I_{K(nl)}$ but its precise contribution cannot be assessed as no specific blocker of this current is yet available.

$I_{K(nl)}$ in single-channel recordings

We looked for the presence of a sustained single-channel activity at depolarized voltages in outside-out patches. To exclude the contribution of the BK_{Ca} channels known to be present, 10 mM BAPTA was added to the intrapipette solution and only 500 μM Ca^{2+} was present in the extracellular solution (in the absence of a small concentration of extracellular calcium, the patches rapidly became leaky and stable single-channel recordings could not be obtained). Nifedipine (30 μM) was always added to block K_{dr} channels. In order to compare single K_{nl} channel recordings with whole-cell recordings of $I_{K(nl)}$, we used solutions that were similar to those used in whole-cell recordings (see Methods section). The inset of Fig. 6*A* shows three traces (open channel is up) recorded during a 60 s recording period at 0 mV. The period started 20 s after establishing the holding potential at 0 mV. Trace *a* was the first recording in the period, and traces *b* and *c* were recorded 30 and 60 s later, respectively. There was steady activity of the channel during this sustained depolarization. For comparison with $I_{K(dr)}$ in human myoblasts, note that this run at 0 mV would cause $I_{K(dr)}$ to inactivate by 95% (Widmer *et al.* 1995). In addition, before this run at 0 mV, the patch had been held at −20 mV for 2 min, which would have inactivated $I_{K(dr)}$ by 80%.

The inset of Fig. 6*B* shows, in another patch, that the single-channel current activates in the presence of 30 μM nifedipine during a depolarizing step from −80 to +40 mV (trace *a*). In addition, we found that the channel activity was suppressed by exposure to 30 mM TEA (Fig. 6*B*, inset, trace *c*). The effect of TEA was reversible (trace *d* was recorded after a 1 min wash with control superfusion solution).

To demonstrate that potassium ions were the charge carriers of the elementary outward current, the amplitude of the current was measured with amplitude histograms obtained during recordings at −40, −20, 0, +20 and +40 mV. Mean amplitudes from three patches are plotted against voltage in Fig. 6*A* (■). Sample recordings at each voltage are also illustrated near the corresponding data points. It can be seen that a Goldman–Hodgkin–Katz (GHK) equation (see Hille, 1992, chap. 13) is an adequate description of the data. The GHK equation was adjusted with the single-channel permeability coefficient as the only free parameter ($[K^+]_i$ and $[K^+]_o$ were 150 and 5 mM, respectively). The quality of the fit indicates that the elementary current is a potassium current (single-channel permeability coefficient, $3{\cdot}74 \pm 10^{-14}$ cm^3 s^{-1}). The elementary conductance of the channel at +40 mV ($\gamma_{40\,mV}$) is 8·4 ± 0·8 pS ($\gamma_{40\,mV}$ was calculated by dividing the elementary current measured at +40 mV (1·06 ± 0·1 pA) by the driving force on potassium ions at +40 mV ($E_K = -86$ mV).

The reversal potential of the elementary $I_{K(nl)}$ was not observed directly for several reasons. First, due to the prevailing potassium concentrations, only small currents can be elicited at potentials negative to the reversal potential (see GHK curve near −100 mV in Fig. 6*A*). Second, whole-cell recordings indicate that $I_{K(nl)}$ deactivates rapidly at hyperpolarized potentials (see Fig. 3*A*, $[K^+]_o = 5$ mM, trace at −90 mV). Our attempts to visualize a few inward openings at −120 mV immediately after switching the voltage from +40 to −120 mV were unsuccessful.

To compare the single-channel current with $I_{K(nl)}$ in whole-cell recordings, it was important to show that the single-channel current we describe has voltage-dependent properties. In one of the patches the recording was particularly stable and, in addition to the elementary current (i), the open probability (P_o) of K_{nl} channels at various voltages (−40, −20, 0, +20 and +40 mV) could be measured during 60 s recording periods. As shown in Fig. 2*C*, the steady-state whole-cell conductance, $G_\infty(V_m)$, is adequately described by a Boltzmann equation. $G_\infty(V_m)$ can also be determined from i, P_o and the number of functional channels present in a cell (N): $G_\infty(V_m) = (NiP_o)/(V_m - E_K)$. Note that $i(V_m - E_K)$ is equal to γ, the elementary conductance at each voltage. Thus, ideally it should be possible to describe the behaviour of $P_o \times \gamma$ obtained in single-channel recordings with the same Boltzmann equation used to describe whole-cell results (in whole-cell recordings, the gating charge was 1·6 ± 0·1 times the elementary charges and V_0 was 5·1 ± 2·3 mV). In Fig. 6*B*, the product $P_o \times \gamma$ is plotted as a function of voltage. It can be seen that the data are indeed well described by a Boltzmann equation with a gating charge of 1·8 and a V_0 of 4·5 mV. In two other patches, the same voltage-dependent trend was observed, but transient instabilities of the baseline did not allow us to perform the same extended study over 60 s periods. Thus, we conclude that the elementary potassium current we describe here has properties that resemble those of $I_{K(nl)}$ measured in whole-cell mode and we consider that it corresponds to the elementary $I_{K(nl)}$.

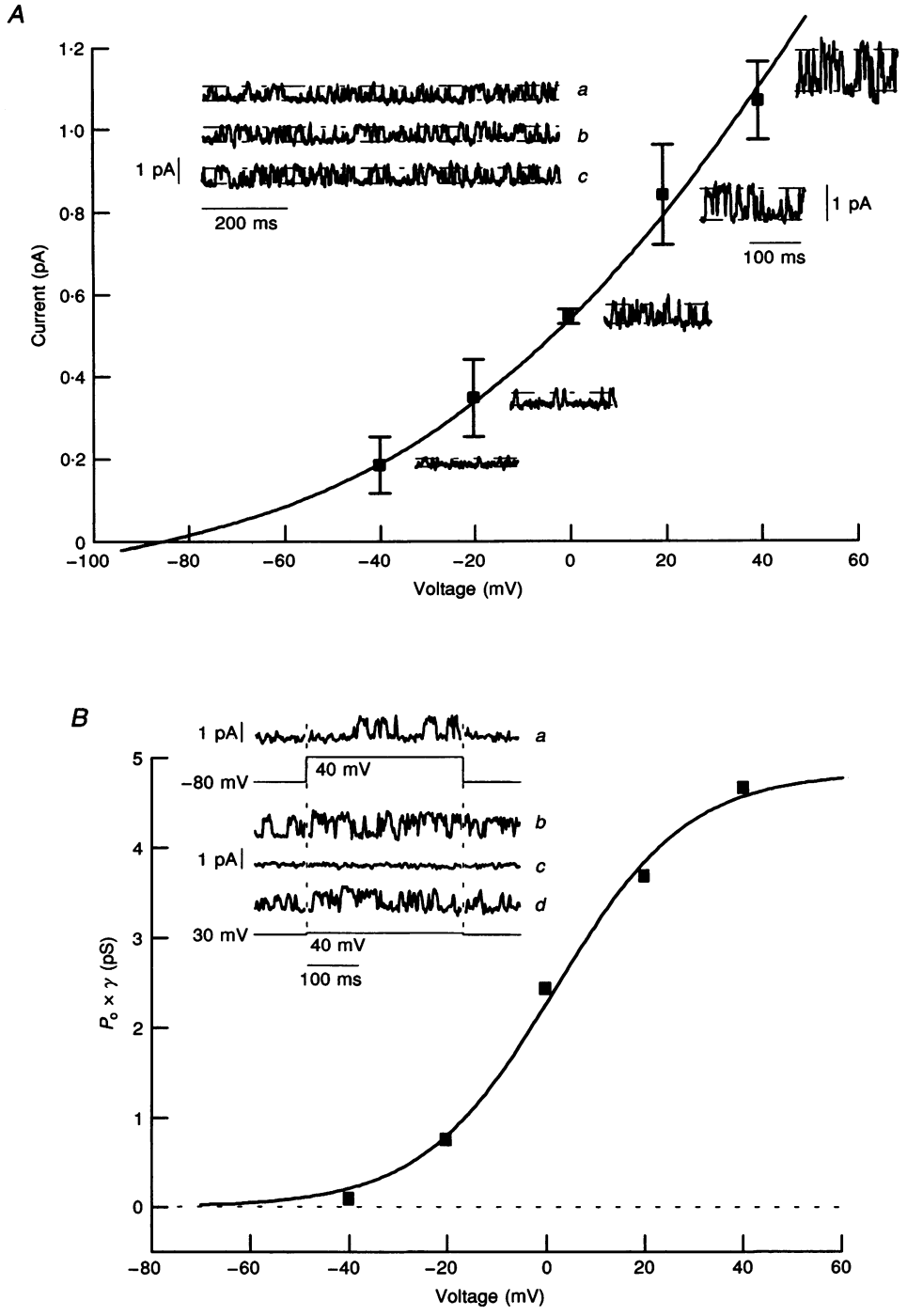

Figure 6. Single K_{ni} channels

Patches of membrane were excised from myoblasts kept for 1–3 days in differentiation medium. In *A*, ■ represents the mean single-channel amplitudes obtained from amplitude histograms computed from current traces recorded at steady holding potentials between −40 and +40 mV ($n = 3$). Examples of single-channel recordings are shown for each potential. The continuous line is a fit to the Goldman–Hodgkin–Katz equation. Inset, current traces recorded during a steady holding potential at 0 mV lasting 60 s. Three samples of 875 ms recorded at time 0, 30 and 60 s are shown. *B*, the open probability (P_o) multiplied by γ, the elementary conductance, is plotted against the holding potential (V_m). $(P_o \times \gamma)_{max}$ is 4·84 pS. Inset, trace *a* was recorded during a voltage step to +40 mV from a holding potential of −80 mV. Holding the patch at +30 mV and stepping to +40 mV did not reduce channel activity (trace labelled *b*). TEA (30 mM) abolished all channel activity (trace labelled *c*), which recovered after washout of the TEA (trace labelled *d*).

DISCUSSION

This paper is the first report of a voltage-gated and truly non-inactivating potassium current in prefused human myoblasts. Newly formed myotubes also possess this $I_{K(nl)}$ current (data not shown) but no similar current was observed in proliferating myoblasts (Hamann *et al.* 1994). This current is activated at potentials more depolarized than −50 mV and does not inactivate for minutes. It is insensitive to potassium channel blockers such as charybdotoxin, dendrotoxins, MCD peptide, 4-AP, 3,4-DAP, apamin but can be blocked by high concentrations of TEA and by Ba^{2+}. In single-channel recordings we identified a small conductance potassium channel with properties corresponding to those of $I_{K(nl)}$ in whole-cell recordings.

We also show that the blocking of $I_{K(nl)}$, but not that of the delayed rectifier $I_{K(dr)}$, affects the membrane potential of prefused myoblasts. Thus, $I_{K(nl)}$ plays an important role in determining the resting potential of these cells.

$I_{K(nl)}$ is distinct from $I_{BK(Ca)}$ and from other sustained potassium currents

A calcium-activated potassium current such as $I_{BK(Ca)}$, which is present in human myoblasts (Hamann *et al.* 1994), would have the necessary properties to remain steadily open at depolarized voltages. $I_{K(nl)}$, however, is distinct from $I_{BK(Ca)}$ as $I_{K(nl)}$ was present when the intracellular Ca^{2+} concentration was greatly reduced and it is insensitive to charybdotoxin. In addition, the single-channel conductance of $I_{K(nl)}$ is 10-fold less than that of $I_{BK(Ca)}$ channels measured in adult human skeletal muscle (Lerche *et al.* 1995). $I_{K(nl)}$ is also distinct from another calcium-activated potassium channel, $I_{SK(Ca)}$, as the activity of this channel is insensitive to voltage (Blatz & Magleby, 1986).

There are other potassium currents which can remain open steadily: $I_{K(ATP)}$, $I_{K(M)}$ and $I_{K(S)}$ (Spruce, Standen & Stanfield, 1987; Brown & Adams, 1980; Siegelbaum *et al.* 1982). These potassium currents are blocked respectively by intracellular ATP, by acetylcholine acting on a muscarinic receptor and by serotonin. $I_{K(nl)}$ was still present when 5 mM ATP was added to the intracellular medium and was not affected by a bath application of 10 μM muscarine or 30 μM 5-HT. In addition, $I_{K(ATP)}$ and $I_{K(S)}$ are voltage insensitive and are thus distinct from $I_{K(nl)}$. $I_{K(M)}$ is voltage dependent, but it is activated at more hyperpolarized potentials than $I_{K(nl)}$, and $I_{K(M)}$ deactivation is much slower than that of $I_{K(nl)}$. Taken together, we conclude from these results that $I_{K(nl)}$ is clearly different from $I_{K(ATP)}$, $I_{K(M)}$ and $I_{K(S)}$.

Selectivity of $I_{K(nl)}$ for potassium

The reversal potential of $I_{K(nl)}$ in whole-cell recordings can be accurately predicted by the Nernst equation, knowing the potassium concentrations on both sides of the cell membrane. As the experiments were all done in the presence of 125 mM extracellular sodium, the results indicate that this potassium channel is highly selective. Similarly, in single-channel recordings, the amplitude of the elementary current as a function of voltage was well described by a GHK equation.

Effect of nifedipine on the delayed rectifier $I_{K(dr)}$

There are several reports to indicate that high concentrations of dihydropyridines (DHP) affect currents other than L-type Ca^{2+} channels. Considering potassium currents, a delayed rectifier in embryonic rat sensory neurones is blocked by DHP at concentrations greater than 5 μM (Valmier, Richard, Devic, Nargeot, Simmoneau & Baldy, 1991). In *Aplysia* bag cell neurones, potassium currents are blocked with a K_d of 3–5 μM (Nerbonne & Gurney, 1987), similar to the K_d of nifedipine for the blocking of $I_{K(dr)}$ in prefused myoblasts. More recently, it has been shown that the A-current of bovine adrenal cells is also affected by DHP (Mlinar & Enyeart, 1994).

$I_{K(nl)}$ in other preparations

Besides the calcium- and transmitter-modulated potassium channels which are different from $I_{K(nl)}$ (see above), there are other sustained potassium currents. Reports on non-inactivating potassium currents are numerous, but they generally refer to delayed rectifiers which inactivate slowly when compared with the A-current. These currents are clearly different from $I_{K(nl)}$. To our knowledge, the only reports of currents resembling $I_{K(nl)}$ are those based on observations in *Drosophila* (Wei, Covarrubias, Butler, Baker, Pak & Salkoff, 1990) and in *Aplysia* neurones (Zhao *et al.* 1994). In *Drosophila*, the potassium current *Shaw* does not inactivate over a period of 10 s (Wei *et al.* 1990). Unlike $I_{K(nl)}$, this current is sensitive to 4-AP and not to TEA. In addition, as discussed by Zhao *et al.* (1994), potassium current homologues of D-*Shaw* in other species are all inactivating potassium currents. In *Aplysia* neurones, the current is a new class of potassium current, $I_{aKv5\cdot1}$, which does not inactivate over a 5 s period. Its overexpression in neurones leads to a hyperpolarization. As for $I_{K(nl)}$, the voltage dependence of the steady-state conductance of $I_{aKv5\cdot1}$ is well described by a Boltzmann equation. For $I_{aKv5\cdot1}$, $V_0 = -21$ mV and the value of the gating charge is 2·3 in the native cell. The sensitivity of $I_{aKv5\cdot1}$ to TEA, however, is considerably higher ($K_d = 0{\cdot}36$ mM) than that of $I_{K(nl)}$ in prefused myoblasts. Single-channel characteristics of $I_{aKv5\cdot1}$ are not yet available.

Single-channel properties of $I_{K(nl)}$

In prefused myoblasts, elementary currents were recorded in the outside-out mode and had properties consistent with those of $I_{K(nl)}$ recorded in the whole-cell mode. The elementary current is a potassium current. It can be suppressed by TEA at high concentration and its voltage domain of activation is the same as that of $I_{K(nl)}$ in the whole-cell mode. In addition, the elementary current does not inactivate during a long depolarization. For these

reasons, we consider that the single-channel current described in this paper corresponds to $I_{K(ni)}$.

Comparison of the maximum conductance of $I_{K(ni)}$ in whole-cell recordings (95% of the G_{max} is 127 pS pF^{-1} and the mean capacitance is 26 pF, $n = 87$) with the maximum $P_o \times \gamma$ of a single channel (4·84 pS; see Fig. 6*B*) indicates that a cell of 26 pF will express about 700 functional channels. With a cell capacitance of 26 pF and assuming a specific capacitance of 1 μF cm^{-2}, we calculated that the density of K_{ni} channels in prefused myoblasts is 0·27 channels μm^{-2}.

Our yield of K_{ni} channel recordings was low but consistent with this density of channels. In a total of 105 tight seals (seal resistance, >10 GΩ) we obtained seven patches with single K_{ni} channel activity. Of these, only four lasted long enough for a complete characterization. Thus, our yield of K_{ni} channel is between 4 and 7 in 105 attempts. Given the surface area at the open end of our electrodes (0·2 μm^2) and the average channel density, we would predict that we should find about six K_{ni} channels in 105 attempts.

Physiological role of $I_{K(ni)}$

The drugs that affect $I_{K(ni)}$ also modify the resting membrane potential of prefused myoblasts. We found that the more effective the block of $I_{K(ni)}$, the more depolarized are the cells. This suggests that $I_{K(ni)}$ plays an important role in the resting potential of prefused human myoblasts.

It is interesting to note that proliferating myoblasts do not have $I_{K(ni)}$ and that these cells have resting potentials (-8 ± 1 mV, $n = 218$; Hamann *et al.* 1994) considerably and significantly more depolarized than prefused myoblasts (-27 ± 1 mV, this study). Thus, the hypothesis raised by Entwistle *et al.* (1988), that the expression of a new potassium current may precede fusion of chick embryo myoblasts, is supported here by our experiments on human fusion-competent myoblasts.

We reported previously that in myoblasts cultured in differentiation medium and exposed to TEA, the fusion index (number of nuclei in myotubes divided by the total number of nuclei; see Methods section) was decreased (Widmer *et al.* 1995). This effect may in fact be due to a block of $I_{K(ni)}$ rather than to a block of $I_{K(dr)}$, the other voltage-gated potassium current present in prefused myoblasts. Indeed, we know now that $I_{K(dr)}$ is probably not directly involved in the fusion process as the fusion index is not affected by 30 μM nifedipine ($P > 0·1$, $n = 40$, 2 experiments, C.-A. Haenggeli, unpublished observations), a concentration which totally blocks $I_{K(dr)}$ (see Fig. 4). Confirmation of the role of $I_{K(ni)}$ as an important element in the sequence of events leading to myoblast fusion will come from experiments in which the expression of this current can be suppressed, either by specific blockers or by anti-sense oligonucleotides.

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