Pharmacological Analysis of Voltage-dependent Potassium Currents in Cultured Skeletal Myocytes of the Frog *Rana temporaria*

V. LUKYANENKO[1], I. E. KATINA[2], G. A. NASLEDOV[1] and D.A.TERENTYEV[1]

1 *Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez Ave. 44, 194223 St. Petersburg, Russia*
2 *Institute of Physiology of St. Petersburg University, University emb. 7/9, 199034 St. Petersburg, Russia*

Abstract. Previously, the existence of nine types of outward potassium current (I_K) was shown. The whole family of I_K may be divided into two groups: fast transient currents (f) with time to peak less than 70 ms (at test potential near 0 mV), and slow (s) components (Lukyanenko et al. 1993). The latter were completely blocked by 4-aminopyridine (4-AP) and the former were more sensitive to TEA than slow I_K. In the present study we analyzed the effects of calcium blockers on different types of I_K using the whole-cell patch-clamp technique. One to seven-day-old myocytes without slow calcium currents and without contact with nerve cells were examined.

Extracellular application of 40–80 μmol/l dihydropyridine (DHP) antagonist nifedipine did not change maximal conductance of K-channels, but induced a parallel shift by 5–10 mV of chord conductance curve along the voltage axis in the direction of more negative potentials. Quinidine in concentrations 30–200 μmol/l caused a reversible block of the fast and the slow I_K ($C_{0.5}$ = 75 μmol/l), and enhanced the current decay (2–3-fold at 150 μmol/l). Verapamil (VP) in concentrations 100–700 μmol/l reduced I_K with dose-dependent effect ($C_{0.5}$ = 200 μmol/l) and changed its kinetic properties. VP 100 μmol/l caused a complete irreversible block of the slow I_K. VP reduced the time inactivation constant of fast I_K with a dose-dependent effect (8–10-fold at 300 μmol/l), and this effect was stronger during depolarizing pulses. The latter points to the possibility that the fast K-channels preferentially bind VP in open state.

An analysis of the effects suggests that K-channels of the frog myocytes could be divided into 2 groups: 1) K-channels which are irreversibly blocked by VP and

Correspondence to: Prof. Dr. G. A. Nasledov, Sechenov Institute of Physiology and Biochemistry, RAS, M.Thorez av. 44, 194223 St. Petersburg, Russia.

4-AP (slow), and 2) those reversibly inhibited by VP and 4-AP (fast potassium channels).

Key words: Frog embryonic myocytes — Skeletal myocyte culture — Potassium currents

Introduction

Previously, we have shown the existence of nine types of outward potassium current (I_K) which most obviously differ in their kinetic properties: the rate of activation, the rate and extent of inactivation. The whole family of I_K may be divided into two groups: fast transient currents (f) with time to peak less than 70 ms (5, 12, 20, 30 and 50 ms at test potential near 0 mV) inactivating completely within 2 s, and slow (s) components (time to peak 190, 700, 2000, more than 7000 (S) ms) with slow or no inactivation (Lukyanenko et al. 1993). As a rule, 2–3 or more components were present simultaneously in the same cell, but the frequency of occurrence and the relative conductance of the components varied depending on the stage and conditions of cultivation. However, none of the I_K could be directly compared to I_K in mature frog muscle fibres, where the existence of two types of delayed rectifier currents (fast and slow) were described (Adrian et al. 1970; Stanfield 1970; Almers and Palade 1981; Lynch 1985). Nevertheless, the properties of the fast and the slow group as a whole are nearly compatible with the properties of the fast and slow I_K in mature skeletal muscle fibers. The components of the fast and the slow group also differ in their pharmacological properties: the fast components were more sensitive to 100 mmol/l tetraethylammonium (TEA) than slow I_K whereas the slow ones were completely blocked by 0.5 mmol/l 4-aminopyridine (4-AP)(Lukyanenko et al. 1993).

In the present study an attempt was made to disclose the additional pharmacological properties of voltage dependent potassium channels of frog cultured skeletal myocytes.

Materials and Methods

Cell culture

Standard embryonic muscle cell cultures were prepared from early neurula embryos of *Rana temporaria*. The dorsal portions of the embryos were dissected in 60% Medium 199M with 2% fetal calf serum and 50 U/ml penicillin and 50 μg/ml streptomycin, and washed during 10 min in calcium-free salt solution containing (in mmol/l): NaCl 50; KCl 0.7; KH_2PO_4 0.9; Na_2HPO_4 16; $NaHCO_3$ 2.4; EDTA 1.9 (Freed and Mezger-Freed 1970). After dissociation into single cells the ectoderm was stripped off, mesodermal and neural cells were transferred for culturing on glass, in 40 mm Petri dishes. The growth medium contained: Medium 199M 55%; fetal calf serum 10%; 50 U/ml penicillin and

50 μg/ml streptomycin. These constituents prevent both myocyte division and fusion. The culture was kept at 20 °C. The myoblasts plated on the glass bottom of the chamber turned spindleshaped, 10 μm in diameter and 100 μm in length. The experiments were performed with cells from 1 to 7-day-old cultures. The myocytes selected for experiments did not show any connections with neuroblasts or with each other.

Patch-clamp recording and data analysis

The conventional whole-cell voltage-clamp recording procedure was used (Hamill et al. 1981). The linear component of the leakage current was subtracted electronically. The fast component of capacity currents associated with electrode and electrode holder was fully compensated, however the slow component associated with cell capacity could be compensated only partly due to the large size and complex shape of the cells. The seal resistance was 5–30 GΩ and the input resistance of cells ranged between 1–5 GΩ. The experiments were started 10–15 min after the whole-cell recording configuration was established. The membrane potential was held at −80 mV. The current signal was sampled at 0.1–10 ms sampling intervals. Experiments were performed at room temperature (19–21 °C). One to seven-day-old myocytes without slow calcium currents were examined. Absence of the calcium current was defined by the absence of large "calcium tails" at the end of the test pulse.

Solutions and chemicals

The basic external solution contained (in mmol/l): NaCl 120; KCl 1.5; $CaCl_2$ 2; HEPES 8; pH 7.4 adjusted with NaOH. The pipettes were filled with a solution containing (mmol/l): KCl 110; $CaCl_2$ 2; $MgCl_2$ 1; K_2EGTA 10; HEPES 8; pH 7.2 adjusted with KOH. DHP antagonist nifedipine (Sigma) was preliminarily dissolved in dimethylsulfoxide at 10 mmol/l and subsequently in external solution yielding a final concentration of 0.1 mmol/l. Nifedipine, quinidine (Sigma), verapamil (Sigma) or 4-aminopyridine (Merck) was added to the external solution.

Results

Extracellular application of 40–80 μmol/l dihydropyridine (DHP) antagonist nifedipine did not change maximal conductance of K-channels, but induced a parallel shift by 5–10 mV of the chord conductance curve along the voltage axis in the direction of more negative potentials ($n = 16$) Fig. 1 shows I_K recorded before (A) and after the addition of 80 μmol/l nifedipine (B) to the standard external recording solution.

Quinidine ($n = 8$) in concentrations 30–200 μmol/l caused a reversible block of the fast and the slow I_K ($C_{0\,5} = 75$ μmol/l), and induced acceleration of current decay (2–3-fold at 150 μmol/l). Fig. 2 shows that current traces before the quinidine application (1) and after the washout (3) do not differ. During depolarizing pulses the I_K amplitude fell step by step but no additional block was recorded between the pulses (Fig. 3). Verapamil (VP) in concentrations 100–700 μmol/l reduced I_K with a dose-dependent effect ($C_{0\,5} = 200$ μmol/l), and changed its kinetic properties ($n = 9$). Fifty μmol/l VP induced a complete irreversible block of the

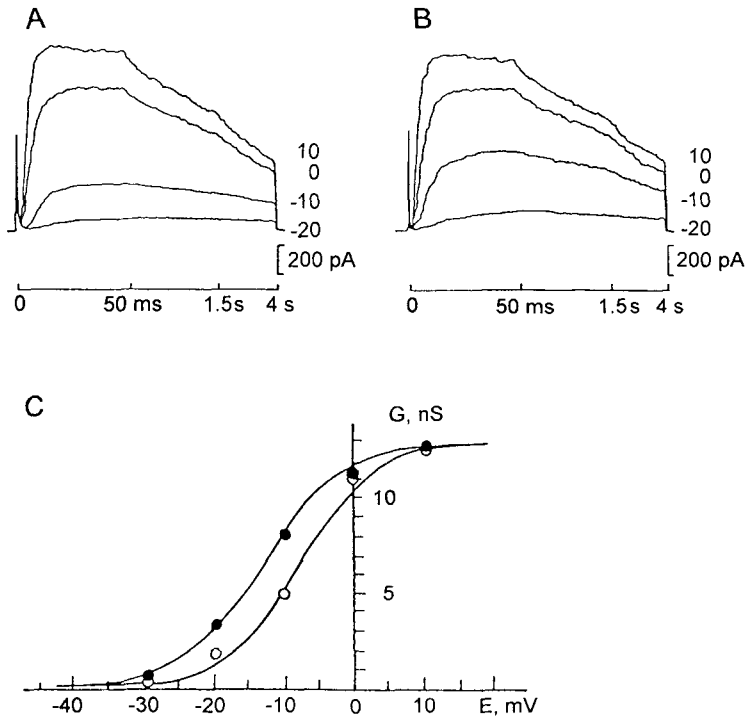

Figure 1. Effect of nifedipine on potassium currents of frog myocytes. *A*, *B*. Current traces (after leak subtraction) elicited by test potentials to −20 – +10 mV (indicated in mV at each recording) for myocyte N5.06.92.4 in a 5-day-old culture before (A) and after the addition of 80 μmol/l nifedipine (*B*) to the standard external recording solution. *HP* −80 mV. Until 50 ms of stimulation the sampling frequency was 10 kHz, between 50 ms and 2 s was done at 100 Hz, and after 2 s at 10 Hz; accordingly, the time scale is linear below 50 ms, between 50 ms and 2 s, and beyond 2 s. *C*. The voltage dependence of potassium peak conductance before (open symbols) and after the addition of nifedipine (filled symbols). The ordinate represents peak potassium conductance (G), and the abscissa shows the test potential value (E). Data for A and B were calculated assuming linear I/V relationship: $G = I/(E - E_R)$, where E_R is the current reversal potential. The smooth lines are the best fit of the peak potassium conductance to Boltzmann function: $G(E) = G_K/\{1 + \exp[(E - E_{0.5})/k]\}$, where G is the conductance at a given membrane potential (E), G_K is the maximal peak conductance, $E_{0.5}$ is the mid-point on the curve, and k is the slope factor of the curve. For standard solution: $E_{0.5} = -7.8$ mV and $k = -5.3$ mV; after nifedipine treatment: $E_{0.5} = -13$ mV and $k = -5.8$ mV (Lukyanenko et al. 1994).

slow I_K (Fig. 4). VP decreased the time inactivation constant of fast I_K in a dose-dependent manner (8–10-fold at 300 μmol/l), and this effect was stronger during depolarizing pulses. The latter points to the possibility that the fast K-channels

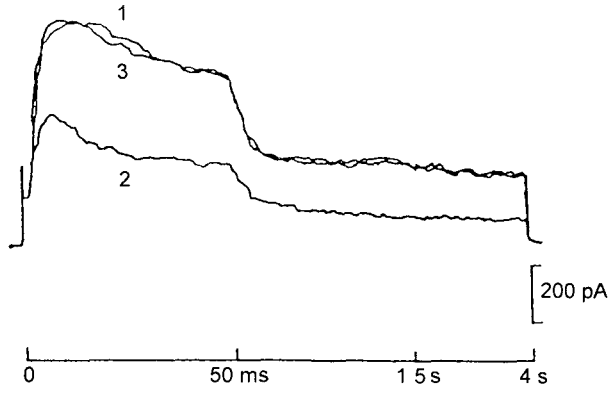

Figure 2. Effect of quinidine on I_K in frog myocytes. Current traces (after leak subtraction) elicited by test potentials to +10 mV before (1) and after the addition of 50 μmol/l quinidine to the standard external recording solution, and after washout (3). *HP* −80 mV; time scale as in Fig. 1; 3-day-old culture; myocyte N8.06.92.2.

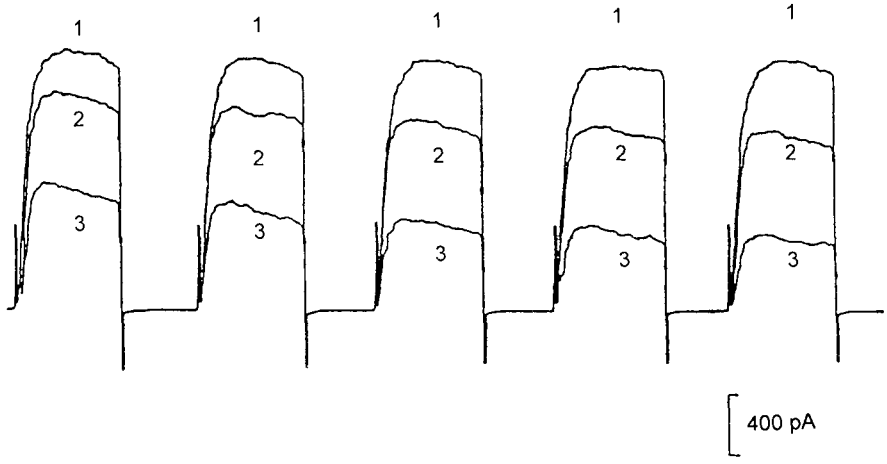

Figure 3. Effect of quinidine on fast I_K in frog myocytes. Current traces (after leak subtraction) elicited by a train of short (50 ms) depolarizing pulses (from −80 mV to 0 mV) at 1 s interval, before (1) and after the addition of 50 μmol/l quinidine (2) and 150 μmol/l quinidine (3) to the standard external recording solution. Five-day-old culture; myocyte N8.05.92.3.

preferentially bind VP in open state. Addition to the standard external solution of 30–80 μmol/l nifedipine before some experiments with verapamil or quinidine did

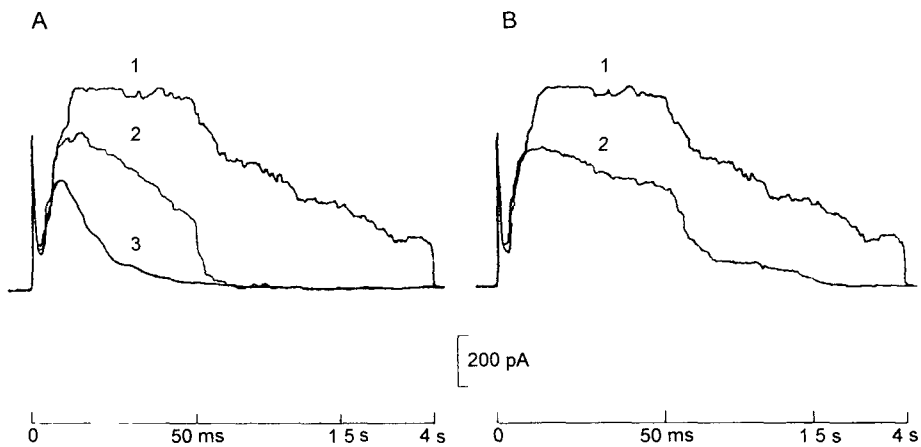

Figure 4. Effect of verapamil on I_K in frog myocytes. Current traces (after leak subtraction) elicited by test potentials to +10 mV before (*A*1 and *B*1) and after the addition of 50 μmol/l verapamil (*A*2) and 140 μmol/l verapamil (*A*3) to the standard external recording solution and after washout (*B*2). Holding potential (*HP*) −80 mV; time scale as in Fig. 1; 1-day-old culture; myocyte N26.05.92.1.

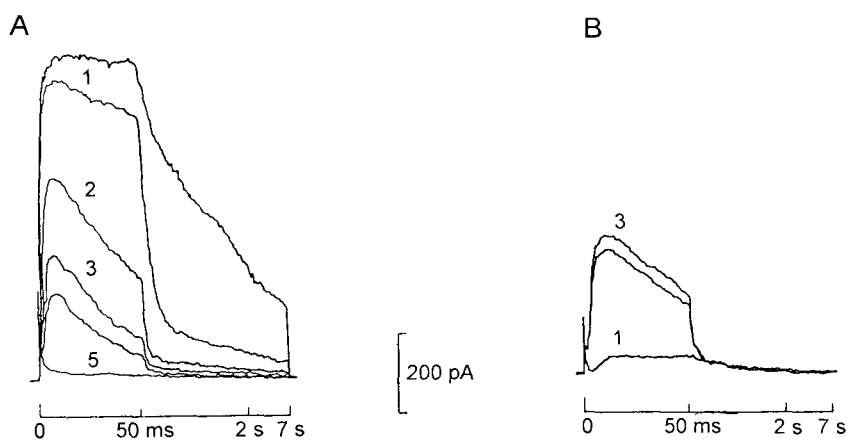

Figure 5. The principal differences in the nature of extracellular action of 2 mmol/l 4-AP on the currents of the slow and the fast group. *A*. Outward potassium currents under depolarization from −80 mV to 0 mV in control solution (the upper trace) and at the first depolarization step in test solution (5 min after the application of 4-AP) and in subsequent 2 min intervals (traces 2 to 6). *B*. Currents after washout of 2 mmol/l 4-AP. The first record was made 5 min after the removal of 4-AP, the following records were made at 2 min intervals. *HP* −80 mV; time scale as in Fig. 1; 5-day-old culture; myocyte N8.05.92.2.

not change their effects on I_K as mentioned above.

Extracellular or intracellular application of 0.05–0.1 mmol/l 4-AP caused irreversible block of the slow I_K, the onset of block appeared to be voltage and state independent (or very fast) ($n = 20$). For the fast I_K, more than 1 mmol/l was needed upon extracellular and more than 0.3 mmol/l upon intracellular application. The onset of the block and its relief after extracellular application of the drug for the fast currents were slow and required (or were much more accelerated by) cell depolarization. Fig. 5 shows the principal difference in the pattern of the extracellular action of 4-AP on currents of the slow and the fast group. The slow currents were nearly completely abolished in the first test trace, the onset of the block on the fast component was slow: the amplitude of the first trace was very similar to that in normal conditions, and then gradually decreased. In the absence of a restoration of the slow current, the amplitude of the fast currents restored gradually, but only partially (to not more than 85%).

Discussion

Nifedipine

It is known, that some types of potassium channel are DHP-sensitive (Valmier et al. 1991). We tried to registrate the effects of nifedipine in frog myocytes. We could not show any effect of nifedipine on the conductance of K-channels, but we registrated the shift of the conductance curve. This shift most probably reflects the influence of nifedipine on negative surface charge density (Gilbert and Ehrenstein 1969; Mozhayeva and Naumov 1970; Enhrenstein and Gilbert 1973). The direction and the value of the shift under the influence of 60-80 μmol/l nifedipine are parallel an e-fold reduction of the extracellular concentration of Ca^{2+}. So, it might be suggested that nifedipine can bind with a high affinity to some sites of the outer surface, but has less effect on the surface charge than the main counterions.

Quinidine

Our data mentioned above suggest that all types of potassium channels in frog myocytes are approximately equally sensitive to the blocking action of quinidine. The acceleration of the current decay allows to suggest that potassium channels are blocked by quinidine preferentially in open state; this is in agreement with the conclusions of other investigators (Bokvist et al. 1990; Oyama et al. 1992).

Verapamil

Our results show that verapamil applied at high concentrations has an inhibitory effect on potassium conductance, independent of its calcium antagonist property. Similar effects of the same concentrations of phenylalkylamines on Ca^{2+}-indepen-

dent outward potassium currents have been shown in experiments in rat ventricular myocytes (Lefevre et al. 1991) and rat hippocampal neurons (Potier et al. 1990).

4-Aminopyridine

For fast K-channels in the frog myocyte the mechanism of interaction is similar to that described earlier for K-channels in T-lymphocytes (Katina et al. 1991) and B-lymphocytes (Choquet and Korn 1992), despite a considerable difference in affinity. We did not find the principal differences in the sensitivity or the pattern of interaction with 4-AP among the components belonging to the fast or the slow group (except, probably, the reversibility of block on different fast I_K).

An analysis of the effects suggests that K-channels of the frog myocytes can be divided in 2 groups: 1) K-channels which are irreversibly blocked by VP (slow), and 2) those which are reversibly inhibited by VP (fast potassium channels). We can suggest that the similarity of pharmacological properties of the 2 groups results from K-channels being formed during myogenesis from the same set of monomers. It is known that potassium channels are heteromultimeric and that the absence of one subunit or a sequence of them may change the functional properties of the channel (McCormac et al. 1990; Pongs 1992, 1993). During skeletal muscle maturation in culture the quantity of membrane channels grows day by day. Our data (Lukyanenko et al. 1993) as well as those of other investigators (Schmid et al. 1984; Amagai and Kasai 1989; Zemková et al. 1989; Moody-Corbett et al. 1989; Moody-Corbett and Gilbert 1990) have shown that the process of channel formation and accumulation is completed after 6-7 days of muscle culturing. Arai et al. (1992) have supposed that induction of protein genes is under the control of common myogenic differentiation. We may suppose that the appropriate genes for proteins of some subunits of potassium channels become expressed later than do genes encoding other subunits.

These results indicate that outward I_K of the frog myocytes: 1) are not DHP-sensitive; 2) are blocked by quinidine; 3) can be divided into 2 groups according to the mechanism of interaction with pharmacological agents: K-channels which are irreversibly blocked by VP and 4-AP (slow), and those which are reversibly inhibited by VP and 4-AP (fast channels).

Acknowledgements. This research was made possible in part by Grant No. NVQ 000 from the International Science Foundation.

References

Adrian R. H., Chandler W. K., Hodgkin A. L. (1970): Voltage clamp experiments in striated muscle fibres. J. Physiol. (London) **208**, 607–644

Almers W., Palade P. T. (1981): Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. J. Physiol. (London) **312**, 159—176

Amagai Y., Kasai Sh. (1989): Calcium action potential and prolonged after hyperpolarization in developing myotubes of a mouse clonal myogenic cell line. Jpn. J. Physiol. **39**, 33—42

Arai M., Otsu K., MacLennan D.H., Periasamy M. (1992): Regulation of sarcoplasmic reticulum gene expression during cardiac and skeletal muscle development. Amer. J. Physiol. **262**, C614—C620

Bokvist K., Rorsman P., Smith P. (1990): Effects of external tetraethylammonium ions and quinine on delayed rectifying K channels in mouse pancreatic b-cells. J. Physiol. (London)**423**, 311—325

Choquet D., Korn H. (1992): Mechanism of 4-aminopyridine action on voltage-gated potassium channels in lymphocytes. J. Gen. Physiol. **99**, 217—240

Ehrenstein G., Gilbert D. L. (1973): Evidence for membrane surface charge from measurement of potassium kinetics as a function of external divalent cation concentration. Biophys. J. **13**, 495—497

Freed J. J., Mezger-Freed L. (1970): Culture methods for anuran cells. In: Methods in Cell Physiology. Vol. 4. (Ed. D. M. Prescot), pp. 19—48, Acad. Press., New York

Gilbert D. L., Ehrenstein G. (1969): Effect of divalent cations on potassium conductance of squid axons: determination of surface charge. Biophys. J. **9**, 447—469

Hamill O. P., Marty A., Neher E., Sakman B., Sigworth F. J. (1981): Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. **391**, 85—100

Katina I. E., Lonsky A. V., Roschina N. G., Tolstikov V. E., Butov S. N. (1991): Kinetical properties of voltage-dependent potassium channels in T-cell line Jurkat. Vestnik LGU., **13**, 73—78 (in Russian)

Lefevre I. A., Coulombe A., Coraboeuf E. (1991): The calcium antagonist D600 inhibits calcium-independent transient outward current in isolated rat ventricular myocytes. J. Physiol. (London) **432**, 65—80

Lukyanenko V. I., Nasledov G. A., Katina I. E., Lonsky A.V. (1993): Voltage dependent ionic currents in cultured frog skeletal myocytes. Gen. Physiol. Biophys. **12**, 231—247

Lukyanenko V. I., Katina I. E., Nasledov G. A. (1994): Voltage dependent fast calcium current in cultured skeletal myocytes of the frog *Rana temporaria*. Gen. Physiol. Biophys. **13**, 237—246

Lynch C. (1985): Ionic conductances in frog short skeletal muscle fibres with slow delayed rectifier currents. J. Physiol. (London) **368**, 359—392

McCormack K., Lin J. W., Iverson L. E., Rudy B. (1990): Shaker K^+ channel subunits form heteromultimeric channel with novel functional properties. Biochem. Biophys. Res. Commun. **171**, 1361—1371

Moody-Corbett F., Gilbert R. (1990): Inward rectifier potassium current on embryonic Xenopus muscle cells at different times in culture. Develop. Brain Res. **55**, 139—142

Moody-Corbett F., Gilbert R., Akbarali H., Hall J. (1989): Calcium current in embryonic Xenopus muscle cell in culture. Can. J. Physiol. Pharmacol. **67**, 1259—1264

Mozhayeva G.N., Naumov A.P. (1970): Effect of surface charge on the steady-state potassium conductance of nodal membrane. Nature **228**, 164—165

Oyama Y., Harata N., Akaike N. (1992): Accelerating action of quinidine on the decay phase of transient outward current in hippocampal pyramidal neurons. Jpn. J. Pharmacol. **58**, 185—189

Pongs O. (1992): Molecular biology of voltage-dependent potassium channels. Physiol. Rev. **72**, (Suppl.), S69—S88

Pongs O. (1993): Structure-function studies on the pore of potassium channels. J. Membrane Biol. **136**, 1—8

Potier B., Rascol O., Lamour Y., Dutar P. (1990): Verapamil has an effect independent of its calcium channel antagonist action in rat hippocompal neurons. C. R. Acad. Sci. Paris, **311**, Serie III:89—94

Schmid A., Renaud J.-F., Fosset M., Meaux J.-P., Lazdunski M. (1984): The nitrendipine-sensitive Ca^{2+} channel in chick muscle cells and its appearance during myogenesis *in vitro* and *in vivo*. J. Biol. Chem. **259**, 11366—11372

Stanfield P.R. (1970): The effect of the tetraethylammonium ion on the delayed currents of frog skeletal muscle. J. Physiol. (London) **209**, 209—229

Valmier J., Richard S., Devic E., Nargeot J., Simonneau M., Baldy-Moulinier M. (1991): Dihydropyridines interact with calcium-independent potassium currents in embryonic mammalian sensory neurons. Pflügers Arch. **419**, 281— 287

Zemková H., Vyskočil F., Tolar M., Vlachová V., Ujec E. (1989): Single K^+ currents during differentiation of embryonic muscle cells *in vitro*. Biochim. Biophis. Acta **986**, 146—150

Final version accepted December 12, 1995