Voltage Dependent Ionic Currents in Frog Cultured Skeletal Myocytes

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Abstract. The voltage dependent ionic currents in cultured embryonic skeletal myocytes at stages of development ranging from 1 to 6 day were studied using the whole-cell patch clamp technique. Sodium (I_{Na}) and calcium (I_{Ca}) inward and potassium (I_K) outward currents were observed at all stages. I_{Na} did not differ from that described in adult frog striated muscle fibres. Slow I_{Ca} was mediated by current through dihydropyridine sensitive Ca channels and it did not differ in its kinetics from corresponing slow I_{Ca} in frog adult twich muscle fibres. In about 10% of cells examined for I_{Ca}, this current was significantly slower and similar to I_{Ca} described in frog tonic muscle fibres. In some cases two slow calcium currents with distinguishable kinetics were recorded in the same myocytes. Fast dihydropyridin-insensitive noninactivating I_{Ca} could also be observed. At least 6 types of I_K were registered, with approximate time-to-peak (at test pulse of −10 mV) 5, 12, 20, 30, 50 ms (fast I_K) and more than 7 s (slow I_K). Three of them (5, 20 and 30 ms) predominated in 3-day cultures and disappeared in 6-day-old cultures. I_K in myocytes did not correspond fully in the kinetics to I_K reported in adult frog skeletal muscles. Channels associated with transient fast and noninactivating slow I_K were shown to be highly sensitive to low temperature (+5 °C).

Key words: Frog embryonic myocytes — Skeletal myocytes culturing — Ionic currents in myocytes

Introduction

Detialed understanding of ionic currents in embryonic myoblasts developing in cul-

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ture may help elucidate the development of ionic channel functions during the early stages of myogenesis. Voltage dependent sodium, potassium and calcium channels have been described in cultured embryonic myocytes. In the course of development of myocytes in culture, sodium channels do not change their major kinetic properties; their characteristics are similar to those in adult frog muscle fibres (DeCino and Kidokoro 1985). Only one of the Ca^{2+} currents described in frog skeletal muscles (Sanchez and Stefani 1978, 1983; Cota and Stefani 1986) has been observed in primary culture of frog myocytes: the current through dihydropyridine sensitive channels (Moody-Corbett et al. 1989). Later, Moody-Corbett and Virgo (1991) have reported that Xenopus skeletal muscle cells in culture develop a transient current, which differs from fast noninactivating calcium current of adult frog muscle fibres (Cota and Stefani 1986; Garcia and Stefani 1987). This is similar to the fast transient current described in rat myocytes (Beam et al. 1986; Cognard et al. 1986).

In frog myocytes developing in primary culture 4 types of outward I_K have been described (Moody-Corbett and Gilbert 1988; Gilbert and Moody-Corbett 1989); however, their characteristics are not fully known as yet, and the kinetic properties of these currents do not directly correspond to delayed rectifier currents in adult frog muscle fibres (Adrian et al. 1970; Lynch 1985). In chicken muscle fibre culture, 7 types of voltage-sensitive K^+ channels have been reported, the relative densities of which changed during the culture development (Zemková et. al. 1989).

The above data indicate the need of more detailed mapping of transmembrane ionic currents appearing during the development of frog muscle cell. The present investigations performed in frogs with the ionic currents in adult tissue being most completely described provide more information on the development of ionic channel functions during myogenesis.

Some of the data have already been published in short form in Russian (Lukyanenko et al. 1992a,b).

Materials and Methods

Cell culture

Standard frog embryo muscle cell cultures were prepared from early neurula embryos of *Rana temporaria*. The dorsal portions of the embryos were dissected in 60% Medium 199M (Institute of Polyomyelitis and Encephalitis, Academy of Medical Sci. of the CIS) with 2% fetal calf serum (Ecophond, CIS), and 50 U/ml penicillin and 50 μg/ml streptomycin (CIS), and washed during 10 min in calcium-magnesium free salt solution containing (in mmol/l): NaCl 50.4; KCl 0.67; KH_2PO_4 0.86; $NaHPO_4$ 16; $NaHCO_3$ 2.4; EDTA 1.9 (Freed and Mezger-Freed 1970). During the dissociation into single cells the ectoderm was striped and removed, and mesodermal and neural cells were transferred for culturing on glass, in separate 40 mm Petri dishes for every embryo. The growth medium contained: Medium 199M 55%; fetal calf serum 10%; penicillin 50 U/ml and strepto-

mycin 50 μg/ml. These constituents almost completely prevent both myocyte division (Teylor-Papadimitriou and Rosengurt 1979) and fusion. The culture was kept at 20 °C under sterile conditions. The myoblasts plated on the glass bottom of the chamber turned spindle-shaped, 1–2 μm in diameter and 15–40 μm in length; their size almost doubled by the last days (5–6) of culturing.

The experiments were performed with cells from 1 to 6-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.

The voltage-clamp circuit was similar to that described by Hamill et al. (1981), with a 5 GΩ head stage feed-back resistor. The linear component of the leakage current was substracted electronically. The fast component of capacity currents associated with electrode and electrode holder was fully compensated, the slow component associated with cell capacity could be compensated only partly due to the large size and complex shape of the cells. The resistance of patch electrodes filled with standard solution ranged between 3 and 7 MΩ. 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 experiments were on-line computer-controlled (voltage pulse delivery and recording of current responses). The current signal was sampled using a ±10 bit A/D converter, at sampling intervals 0.1–10 ms.

The potassium conductance was calculated assuming linear I/V relationship:

$$G_K = I_K/(E - E_R), \tag{1}$$

where E_R is the current reversal potential. The voltage dependency of peak potassium conductance (G_P) and steady-state inactivation were fitted by Boltzmann function (2) using least squares criterion:

$$G_K(E) = \overline{G}_K/\{1 + \exp[(E - E_{0\,5})/k]\}, \tag{2}$$

where G_K is the conductance at a given membrane potential (E), $\overline{G}_K$ is the maximal peak conductance, $E_{0\,5}$ is the mid-point on the curve (the value of E at which $G_K/\overline{G}_K = 0.5$), and k is the slope factor of the curve.

Experiments were performed at room temperature (18–20 °C). Mean ± standard error of the mean are given.

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 (in mmol/l): KCl 110; $CaCl_2$ 2; $MgCl_2$ 1; K_2EGTA 10; HEPES 8; pH 7.2 adjusted with KOH. In some cases KCl was replaced by CsCl in equimolar amounts. Dihydropyridine (DHP) antagonist nifedipine (Sigma) and agonists CGP-IOS (Institute for Organic Synthesis, Riga, Latvia) was used to identify DHP-sensitive type of calcium channels (the agonistic effects of this DHP derivative has been described by Shvinka et al. 1990). CGP-IOS and nifedipine were preliminarily dissolved in dimethylsulphoxide at 10 mmol/l and subsequently in external solution yield final concentrations between 20–100 μmol/l.

Results

Sodium current

In order to record the inward currents the recording pipette was filled with CsCl (110 mmol/l) instead of KCl used for outward current recording. Due to the presence of cesium ions in the pipette, the outward current was almost abolished. As a result, two components of inward current were recorded. The first, fast transient component of inward current could be blocked by 2 μmol/l TTX ($n = 27$) (Fig. 1). This component could not be recorded in sodium free external solution ($n = 11$), but it appeared again after addition of sodium ions ($n = 3$). Hence, the fast component of inward current was identified as I_{Na} ($n = 70$).

I_{Na} was elicited by test pulses to potentials more positive than -40 mV (average). The peak amplitude of I_{Na} approached its maximum value at -16 ± 1.9 mV (Fig. 2). The peak current amplitude in the youngest cultures tested tended to be smaller then those in the older cultures.

It should be noted that within the first 24 h of myocytes plating 85% of cells examined did not develop detectable I_{Na}. With the ageing of the culture, 80% of the cells showed I_{Na} and there was an increase in the current amplitude. A

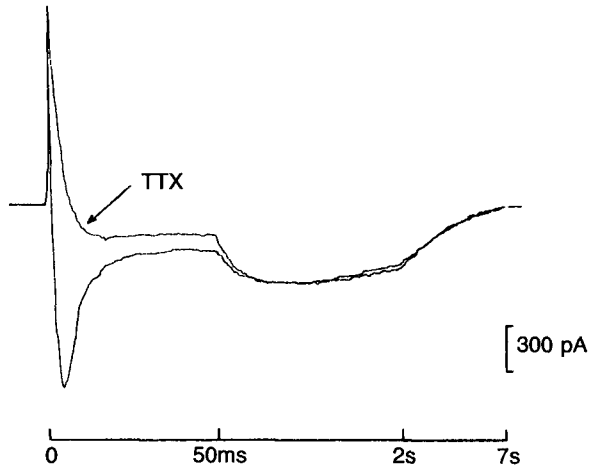

Figure 1. Whole-cell inward currents in a frog cultured myocyte. The current traces elicited by test pulse to -20 mV (after leak subtraction) for a myocyte in a 5-day-old culture; holding potential (HP) -80 mV; myocyte N 1.17.5. Currents recorded in standard extracellular recording solution prior to and after the addition of 2 μmol/l TTX (arrow) to the external solution. In the standard internal solution KCl was equimolarly replaced by CsCl. Data until 50 ms of stimulation were sampled at 10 kHz, those between 50 ms and 2 s at 100 Hz, and those 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.

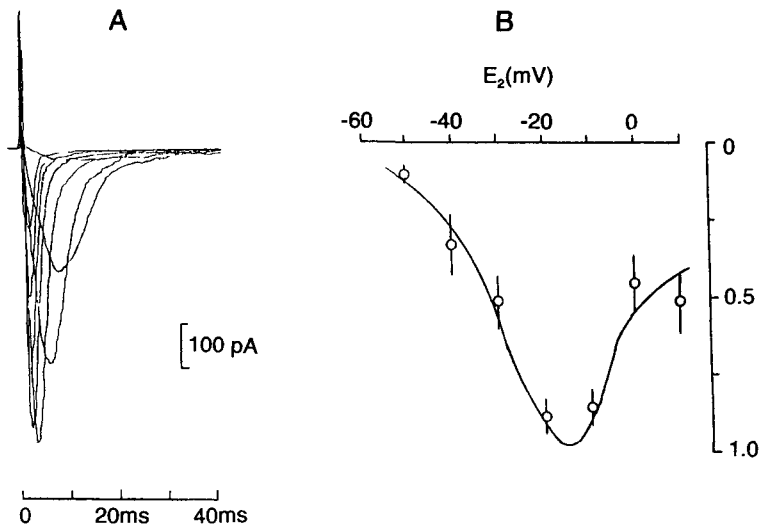

Figure 2. Voltage-dependent sodium current in a frog cultured myocytes. *A.* Superimposed traces of sodium currents elicited by test pulses from −60 to +20 mV for myocyte N 4.25.5 in a 5-day-old culture. Currents recorded in standard extracellular recording solution. In the standard internal solution KCl was replaced by CsCl (equimolar). HP −80 mV. *B.* Normalized peak current-voltage relationship averaged for 10 experiments. Vertical bars represent S.E.M. Ordinate, normalized peak current amplitude; abscissa, test potential values (E_2).

particularly fast increase in percentages of cells with I_{Na} was observed on day 4 of plating.

Calcium current

The slow component of inward current could not be blocked by TTX (Fig. 1). This component was enhanced by adding to standard external solution calcium ($n = 7$) or barium ions ($n = 51$), or the dihydropyridine agonist CGP-IOS (20–100 μml/l, $n = 24$). It could be blocked by external application of DHP antagonist nifedipine (40–100 μml/l, $n = 5$), 2 mmol/l $CoCl_2$ ($n = 13$), 0.1–0.4 mmol/l verapamil ($n = 9$) or 0.5–3 μmol/l D-600 ($n = 4$) (Fig. 3). Within the first 24 h of plating, about 40% of the cells did not show any detectable calcium current. On day 6 of plating however, all cells examined exhibited a slow DHP-sensitive I_{Ca}.

Currents through Ca^{2+} channels were observed in 114 myocytes. I_{Ca} reached peak amplitude within 402 ± 75 ms with 2 mmol/l extracellular Ca^{2+} concentration and a test pulse corresponding to the maximum value of peak amplitude (Fig. 4).

In 12 cells examined a slower component of current through Ca^{2+} channels

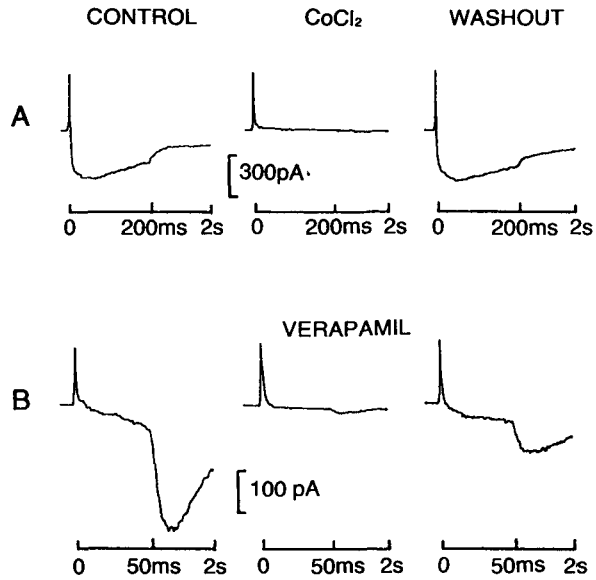

Figure 3. Effects of blockers on currents through Ca^{2+} channels. Effect of 2 mmol/l $CoCl_2$ (*A*; test potential +5 mV; myocyte N 3.16.6), and 0.3 mmol/l verapamil (*B*; test potential −20 mV; myocyte N 1.12.6), added to the external recording solution containing (in mmol/l): $BaCl_2$ 2; TEACl 110; HEPES 8 and 50 μmol/l CGP-IOS. In the standard internal solution KCl was replaced by equimolar CsCl. HP −80 mV; time scale as in Fig. 1.

Table 1. Characteristics of the currents mediated through DHP-sensitive Ca channels of frog cultured myocytes.

Permeable ion	N_f	Time to peak at $E_{2,max}$ (ms), mean ± S.E.M. Faster	Slower	N_s
Ca^{2+}	11	402 ± 76	1431 ± 79	3
Ba^{2+}	12	405 ± 64	1258 ± 474	3
Ba^{2+} plus CGP	11	222 ± 40	267 ± 92	4

N is the number of recordings of faster (N_f) and slower (N_s) currents; $E_{2,max}$ is the test potential which corresponds to maximum value of the current amplitude; permeable ion concentration 2 mmol/l; CGP-IOS concentration 50 μmol/l; room temperature 19–20 °C.

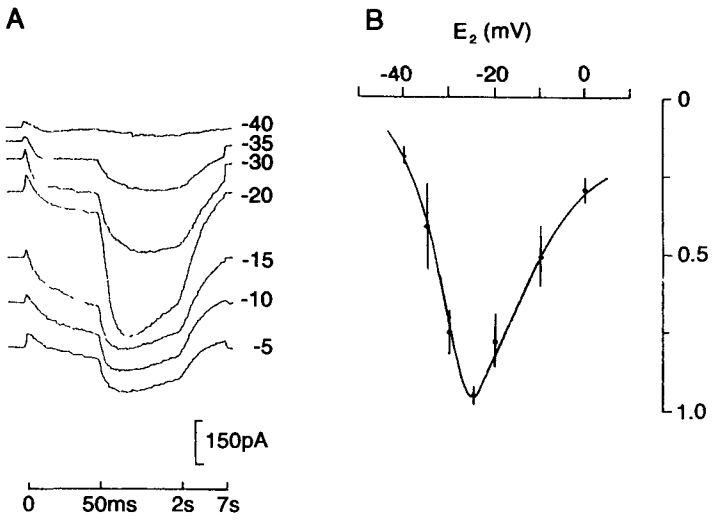

Figure 4. Slow DHP-sensitive calcium current in a frog cultured myocyte. *A.* Whole-cell currents (without leak subtraction) elicited by test potentials from −40 to −5 mV (indicated at each recording) for myocyte N 3.23.5 in a 3-day-old culture recorded with 2 mmol/l $CaCl_2$ in the external recording solution. In the internal standard solution KCl was replaced by equimolar CsCl. HP −80 mV; time scale as in Fig. 1. *B.* The normalized peak current-voltage relationship averaged for 15 experiments with 2 mmol/l $CaCl_2$ in the external recording solution. Vertical bars represent S.E.M. Ordinate, normalized peak current amplitude; abscissa, test potential values (E_2).

was recorded ($P < 0.001$). Under the same experimental conditions the current amplitude of this component reached a maximum value within 1431 ± 79 ms (Table 1). This component could be blocked by external application of 2 mmol/l $CoCl_2$ ($n = 3$) or 3 μmol/l D-600 ($n = 3$). It was enhanced by external addition of barium ions ($n = 10$) or 50 μmol/l CGP-IOS ($n = 2$). Both types of slow DHP-sensitive currents in the same cell were manifested in 5 myocytes (Fig. 5), and in 2 of them both currents were recorded at normal external calcium ion concentration (2 mmol/l Ca^{2+}).

DHP agonist CGP-IOS (20–100 μmol/l) increased both types of inward current in all cases ($n = 25$), accelerated the current activation, and shifted the membrane potential of activation 10–15 mV to more negative values (Fig. 5). Inactivation time constants (τ_{in}) for both types of I_{Ca} could not be estimated due to the currents superposition and to the continuation of outward current. Insensitive to nifedipine and displied high time constant of decay (500–800 ms) fast I_{Ca} also be registered,

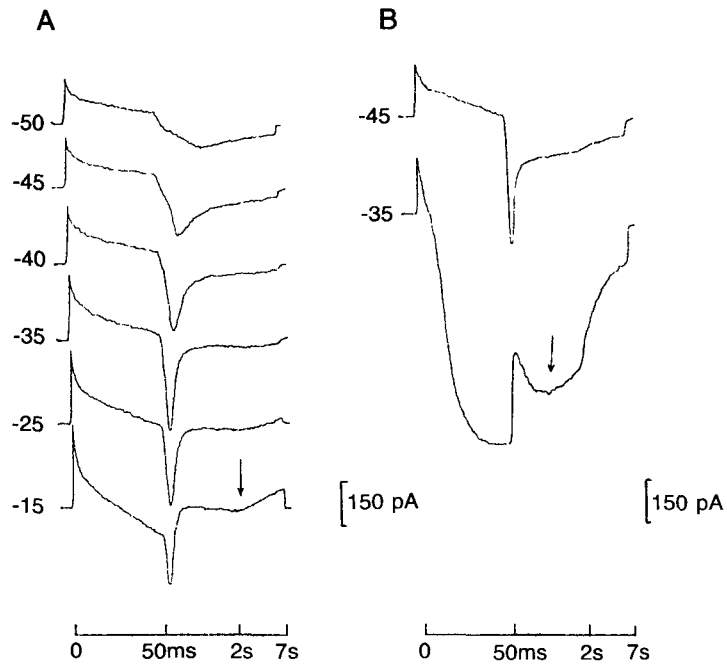

Figure 5. Two types of slow DHP-sensitive calcium current. Whole-cell currents (without leak subtraction) elicited by test potential from −50 to −15 mV (indicated at the recording) for myocyte N 2.16.6 in a 6-day-old culture prior to (*A*) and after the addition of 50 μmol/l dihydropyridine CGP-IOS (*B*) to the external recording solution. The external solution contained (in mmol/l): $BaCl_2$ 2; TEACl 110; HEPES 8. In the standard internal solution KCl was replaced by equimolar CsCl. Arrows indicate the second slow current; HP −80 mV; time scale as in Fig. 1.

but only in the case of strongly blocked I_K by combined action of TEA^+ and Cs^+ in intracellular solution.

Potassium currents

Under voltage-clamp conditions, deporalization to potentials exceeding −40 mV elicited a large outward current (Figs. 6, 7). This current could be almost completely abolished upon replacing internal KCl with an equimolar amount of CsCl (n = 100). Specific potassium channel blockers tetraethylammonium (TEA, 30–100 mmol/l) and 4-aminopyridine (4-AP, 0.2–0.5 mmol/l) also reduced the outward current when applied externally.

The reversal potential (E_R) of I_K in standard ionic conditions was evaluated by

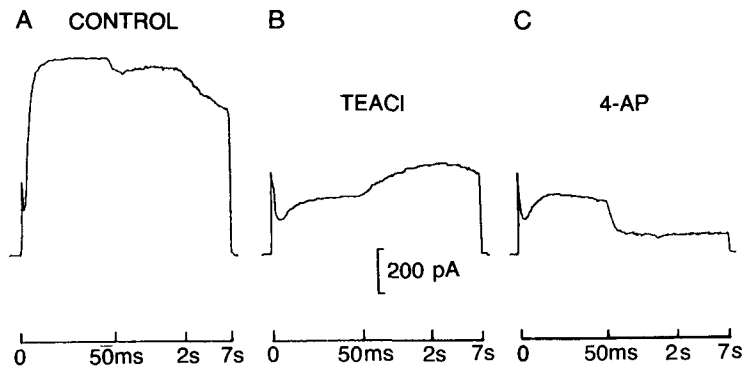

Figure 6. Effects of K^+ channel blockers on outward currents. Whole-cell current traces recorded from myocyte N 4.19.6 in a 1-day-old culture with standard external and internal recording solutions prior to the addition of drugs (control; *A*), after the addition of 110 mmol/l TEACl (*B*) and 0.5 mmol/l 4-aminopyridine (*C*) to the external solution. Test pulse −20 mV; HP −80 mV; time scale as in Fig. 1.

constructing instantaneous $I-V$ relationship (conditioning potential $E_1 = 10$ mV; test potentials, $E_2 = -10-+20$ mV). The value of E_R was practically independent of the duration of E_1 (30 and 150 ms) and averaged -77.5 ± 0.54 mV ($n = 8$). A permeability ratio $P_{Na}/P_K = 0.03$ was calculated from a modified Goldman-Hodgkin-Katz equation.

The time course of outward I_K was complex with usually more than one peak (Fig. 6). It depended both on the test and on the holding potential, and was different for cells at different stages and for different conditions of cultivation.

An analysis of the kinetics and pharmacological properties showed 9 components of outward current which differred one from another by the time courses of activation (Table 2). At test potentials near −10 mV all the components can be divided into two groups: fast components (f) with time-to-peak less than 70 ms ($f5$, $f12$, $f20$, $f30$, $f50$), and slow (s) components with longer time-to-peak ($s190$, $s700$, $s2000$, S). The figures represent the approximate time-to-peak; no steady-state level was observed for component S, even at a duration of E_2 as long as 7 s. The components of the fast and the slow group differ also in their pharmacological properties: the fast components are more sensitive to TEA (30–110 mmol/l) whereas the slow ones are more sensitive to 4-AP (0.2–0.5 mmol/l) (Fig. 6). Three of the four slow components ($s190$, $s700$ and $s2000$) were recorded rather rarely and only at high concentrations of inhibitors (110 mmol/l TEACl and/or 8–30 mmol/l $BaCl_2$), when all other outward current components were completely abolished.

As a rule, 2–3 or more components were present simultaneously in the same

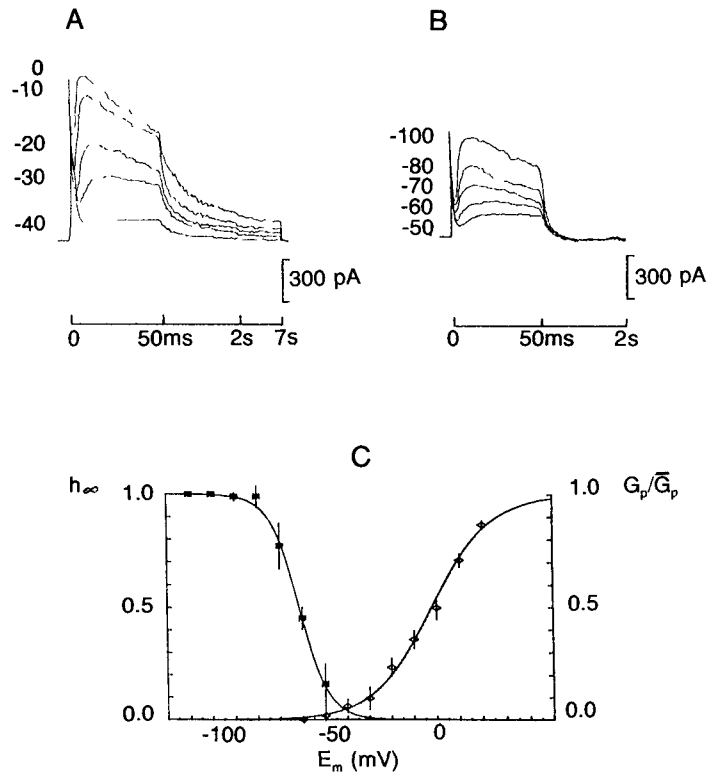

Figure 7. Voltage dependence of peak conductance (G_p) and steady-state inactivation of potassium current (component type $f12$). *A*. Currents (after leak subtraction) elicited by test potentials from −40 to 0 mV (indicated at the recording). HP −80 mV; 6-day-old cultured myocyte N 1.31.5; standard external and internal recording solutions. *B*. Current traces (after leak sutraction) elicited by test potentials of −10 mV at condition potentials (duration 10 s) from −100 to −50 mV (indicated at the recording). HP −80 mV; 2-day-old cultured myocyte N 1.7.6; standard external recording solution with 0.5 mmol/l 4-AP; time scale as Fig. 1. *C*. The right curve corresponds to the voltage dependence of G_p normalized to maximal conductance ($\overline{G}_P$) at large positive potentials ($G_P/\overline{G}_P$) as a function of membrane voltage E_m ($n = 6$) . The left curve reflects steady state inactivation (h_∞); values of peak current at given E_m normalized to maximal currents at $E_m = -100$ mV ($n = 4$). Mean values ± S.E.M (bars); the lines are the best fit to Boltzmann function (2), where for $G_p/\overline{G}_p$: $E_{0\ 5} = -2$ and $k = 13$; and for h_∞: $E_{0\ 5} = -61$ and $k = 7$.

cell, and it was difficult to separate properties of the individual components. In some cases, only one of the fast and/or one of the slow currents was predominant. Fig. 7 illustrates the properties of outward current in cells with predominant $f12$ type component. Currents for various depolarizing test pulses ($E_2 = -40 - 0$ mV)

from holding potential of −80 mV are shown in Fig. 7 *A*, and currents at test potential $E_2 = -10$ mV from various conditioning levels ($E_1 = -100 - -50$ mV) in Fig. 7 *B*. These data allowed to plot peak conductance and steady-state inactivation voltage relations. Averaged data from an *f*12 experiment are shown in Fig. 7 *C*; the right curve corresponds to the voltage dependence of peak conductance G_p normalized to maximum conductance ($\overline{G_p}$) at large positive potentials ($n = 6$). The left curve reflects the steady-state inactivation (values of peak current at a given E_1 are normalized to maximum currents at $E_1 = -100$ mV ($n = 4$). The results of an analogous analysis for some components are summarized in Table 2 (n=3–7, for each component type).

Table 2. Stationary characteristics of the outward current components in frog cultured myocytes.

Component type	N	Time to peak at $E_2 = -10$ mV		Curve parameters (mV)			
		Range (ms)	Mean ± S.E.M. (ms)	Peak conductance		Steady-state inactivation	
				$E_{0\,5}$	k_G	$E_{0\,5}$	k_h
*f*5	25	4 – 8	6.4 ± 0.4	–	–	-55	6
*f*12	58	10 – 15	12.8 ± 0.2	-2	-13	-61	7
*f*20	66	19 – 25	21.4 ± 0.2	-22	-9	-43	9
*f*30	44	29 – 34	30.4 ± 0.2	-14	-8	-39	9
*f*50	20	40 – 70	49.0 ± 1.4	-16	-11	-74	8
*s*190	9	110 – 250	190.0 ± 18.4	–	–	–	–
*s*700	11	450 – 1110	734.0 ± 56.7	-18	-8	–	–
*s*2000	7	1500 – 2550	1865.0 ± 165.0	–	–	–	–
S	33	> 7000	–	-44*	-11*	–	–

N is the number of recordings; *f*, fast; *s*, slow; E_2 is the test potential; $E_{0\,5}$ and k are parameters from the Boltzmann function (2), where $E_{0\,5}$ is the potential at half-maximum conductance, k is the slope factor (these parameters give the best fit to Boltzmann function).

* The curves for this type of current were plotted for values of conductance at the end of long (7 s) depolarizing pulses.

In a few experiments we were able to analyze not only stationary properties of components but also to estimate τ_{in} for individual components or the group as a whole. For three fast components, *f*5, *f*12 and *f*30 , the values of τ_{in} were less than 100 ms (at E_2 near −10 mV), and for components *f*20 and *f*50 they were as high as 300–600 ms. Slow components *s*190 and *s*700 are characterized by τ_{in} of 500–2000 ms, the component *s*2000 being inactivated much slowlier, if at all.

Within the first 24 h of plating, the examined cells displayed the following

current types: $f12$ (48% of myocytes), $f20$ (50%), $f30$ (70%), $f50$ (53%) and S (78%), but not type $f5$. As the age of the culture increased to 3 days, type $f5$ appeared in most cells (80%) and types $f20$ and $f30$ became more prominent (82% each) than other types of outward components. By the 4th day the proportion of cells with detectable currents $f20$ and $f30$ rapidly decreased. By the 5th day the proportion of myocytes with type $f5$ currents also decreased. The percentage of myocytes with I_K types $f12$ and S increased from the second day through sixth day of plating (100%).

It is interesting to note that, in contrast to myocytes kept under conventional conditions at +20 °C, myocytes, kept for 5–7 days at +5 °C before plating, did not exhibit the outward current components $f5$ and S ($n = 24$).

Discussion

Sodium current. Sodium current registered in our experiments exhibits kinetics and pharmacological properties very similar to those described for frog embryonic myocytes (DeCino and Kidokoro 1985) and skeletal muscle fibres of adult frogs (Adrian et al. 1970; Campbell and Hille 1976; Stefani and Chiarandini 1982).

The percentages of cells with sodium current were low on the first day of culturing (15%), increasing rapidly in the course of culture development with a simultaneous increase of the amplitude. This is assumed to be a result of an increase in Na^+ channel density in the myocyte membrane with the cell culture age (DeCino and Kidokoro 1985). Particularly rapid increases of the percentages of cells with I_{Na} occurrence were observed on days 4–5 of culturing. These data point to the existence of a distinct developmental stage at which functioning sodium channels appear in membrane of skeletal myocytes. Similar results were obtained with chicken embryonic muscle cells. Using labelled channel blockers sodium channels were shown to appear after 2–3 days of culturing, reaching maximum amounts by day 5–7 (Frelin et al. 1981; Strichartz et al. 1983).

Calcium currents. We found that myocytes in culture show a slow inward ionic current through Ca^{2+} channels that is voltage operated and sensitive to dihydropyridine (DHP). This current was similar to that mediated by ionic flux through DHP-sensitive Ca^{2+} channels in twitch skeletal muscle of the adult frog (Stanfield 1977; Sanchez and Stefani 1978, 1983; Almers and Palade 1981; Almers et al. 1981; Stefani and Chiarandini 1982; Beaty et al. 1987; Henček et al. 1988; Feldmeyer et al. 1990).

Moody-Corbett et al. (1989) and Moody-Corbett and Virgo (1991) reported that Xenopus embryonic muscle cells grown in culture develop DHP-sensitive slow and DHP-insensitive fast transient currents through calcium channels. The former is similar to the slow current described in the present study and in adult frog

muscle. The latter in turn is similar to the fast transient current described in rat and mice cultured myocytes (Beam et al. 1986; Cognard et al. 1986; Shimahara and Bournaud 1991), and it differs from the fast noninactivating current described in adult frog muscle (Cota and Stefani 1986; Beaty et al. 1987; Garcia and Stefani 1987; Henček et al. 1988).

No fast I_{Ca} was observed in our experiments under described experimental conditions. The amplitude of fast I_{Ca} is known to be n-times smaller than the amplitude of slow I_{Ca}. In our study even slow I_{Ca} had a low amplitude and we had to replace standard extracellular solution by a solution containing Ba^{2+} in order to increase current through DHP-sensitive channels (Almers and McCleskey 1984); also, remains of currents through K^+ channels had be blocked by TEA. However, Ba^{2+} do not increase current through T-type Ca^{2+} channel (Fox et al. 1987), and TEA inhibits it (Beaty et al. 1987; Garcia and Stefani 1987). It is likely that the high concentrations of CsCl and EGTA used in the internal recording solution affected the appearence of the fast current (Beaty et a. 1987). We have observed fast noninactivated, DHP insensitive I_{Ca} in addition experiments with strongly blocked I_K by TEA^+ and Cs^+ in intracellular solution. These results will be described separately.

A second slow current, characterized by slower activation and inactivation and a lower amplitude, was seen in 12 myocytes, on days 4–6 of culturing (Fig. 5*A*). This current also appeared to be mediated via DHP-sensitive Ca^{2+} channels (Fig. 5*B*), however, its characteristics were less clear since it was quite infrequent. By its activation time, the second slow current is similar to one described in tonic skeletal muscle fibres of adult frog. Huerta and Stefani (1986) have shown that the amplitude of slow I_{Ca} in frog tonic muscle fibres reached a maximum value within 1000 ms. Also, Henček et al. (1988) have reported that it took the amplitude of the slow current in tonic fibres more than 1 s to reach a maximum value, whereas in twitch fibres time-to-peak was only 200–700 ms (Stanfield 1977; Almers and Palade 1981; Stefani and Chiarandini 1982; Henček et al. 1988).

Our results can be interpreted in terms of a hypothesis that in some myocytes DHP-sensitive Ca^{2+} channels specifically associated with twitch and tonic types of adult muscle fibres coexist. Fig. 5 shows two components of DHP-sensitive inward slow current in the same myocyte. Almers and McCleskey (1984) have reported current traces with two slow components in external solution containing mixtures of 10 mmol/l Ca^{2+} and 10 mmol/l Ba^{2+}, as a result of anomalous mole-fraction behavior (AMFB). We recorded two slow components not only upon substituting standard external solution by a solution with 2–8 mmol/l Ba^{2+}, but also in standard solution (2 mmol/l Ca^{2+}; n=2). The mode of substitution of extracellular solution used in our experiments allowed us to suggest that in our case the coexistence of slow currents was not a result of AMFB. Moody-Corbett et al. (1989) and Moody-Corbett and Virgo (1991) did not record a second slow current in Xenopus

myocytes. Presumably, the reason may have been relatively short depolarizing pulses (only 500 ms), whereas 7 s were used in our experiments.

Potassium currents. In our experiments frog embryonic muscle cells in culture exhibited 9 components of voltage operated potassium current. Three of them, slow components $s190$, $s700$ and $s2000$, had low frequencies of occurrence and were evident only in the presence of high concentrations of potassium channel blockers (TEA^+ and/or Ba^{2+}), when other outward current components were suppressed. The components $f5$, $f12$, $f20$, $f30$, $f50$, and S can be considered as separate types of currents through corresponding potassium ionic channels, all of them being sensitive to specific K-channels blockers. This conclusion is based on the differences in time of activation of the currents, as well as on some other differences. Namely, S-type of I_K exhibits the most negative range of activation ($E_{0.5} = -44$). Types $f5$, $f20$ and $f30$ of I_K disappeared on day 6 of culturing, but $f12$ and S were registered at that time in 100% of myocytes. Types $f20$ and $f30$, in spite of their similarity in time to peak value, are characterized by different parameters of conductance, different stationary inactivation curves (Table 2), and by different speeds of inactivation. Current $f50$ differs from other fast I_K by its range of steady-state inactivation curve being the most negative one ($E_{0.5} = -74$ mV), which is close to the reversal potential (-77.5 mV).

The absence of $f5$ and S currents after preincubation of myocytes at $+5$°C during 5–7 days also points to the existence of separate types of potassium channels. It might be suggested that K^+ channels $f5$, which did not display any activity at the beginning of normal cell incubation, could not develop under low temperature. Activity of S type of channels was observed in 80% of myocytes on the first day of normal culturing, but in none of them ($n = 24$) after cold preincubation. It means that the already developed S-type activity of I_K can be supressed by low temperature.

In recent years several types of I_K were described in embryonic muscle cells. Moody-Corbett and Gilbert (1988) and Gilbert and Moody-Corbett (1989) registered 4 voltage dependent outward I_K in embryonic myocytes of Xenopus using whole-cell configuration. By their kinetic and pharmacological properties, these currents appear to be similar to our currents $f5$, $f12$, $f50$ and S. Zemková et al. (1989) examined single K^+ channel currents during differentiation of chicken embryonic muscle cells *in vitro* and identified 7 types of K^+ channels. These authors described also differences between myoblasts and myotubes concerning the percentages of the individual channel types; however muscle cells with blocked fusion, showed the same change in channel population as shown in the present paper.

None of the I_K components found in the present study could be directly compared to currents in adult frog muscle fibres, where the existence of two types of delayed rectifier currents (fast and slow) were described (Adrian et al. 1970; Stan-

field 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 adult frog muscle. It is likely that the fast and the slow delayed rectifier currents in adult skeletal muscle fibres are a result of a coexistence of several I_K types.

In terms of conventional classification (Rudy 1988), three fast components ($f5$, $f12$ and $f30$) could be considered as fast transient type, which differs from typical "A" currents by its low sensitivity to 4-AP. Components $f20$ and $f50$ are closely related to fast delayed rectifier, and the S-type may be refferred to as a slow delayed rectifier.

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