Voltage Dependent Fast Calcium Current in Cultured Skeletal Myocytes of the Frog *Rana temporaria*

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Abstract. Voltage dependent calcium currents in cultured frog embryonic skeletal myocytes at stages of development ranging from 2 to 9 days were studied using the whole-cell patch clamp technique at 19–21 °C. Membrane currents were recorded in the presence of 2 mmol/l Ca^{2+} (outside), and 60 mmol/l CsCl and 50 mmol/l TEACl (inside).

In the absence of sodium current two components of inward current were observed in response to depolarization already during the early stages of myogenesis: the well-known slow dihydropyridine (DHP)-sensitive calcium current ($I_{Ca,s}$), and a fast-activated current. Both components persisted in the presence of 2 μmol/l tetrodotoxin. The fast-activated component was enhanced upon addition of 6 mmol/l Ca^{2+} or Ba^{2+} to the external recording solution and was decreased when the standard external solution was replaced by Ca^{2+} free solution. Thus, the fast component of the inward current was also carried by Ca^{2+} ($I_{Ca,f}$). Unlike $I_{Ca,s}$, it was not blocked with 30 – 150 μmol/l DHP nifedipine. During 7 s depolarization $I_{Ca,f}$ was detected at approximately −50 mV, 20 mV more negative than the membrane potentials at which $I_{Ca,s}$ appeared. At various test potentials $t_{0.5}$ for activation of $I_{Ca,f}$ was 8–20 ms, and the current declined during depolarization with τ_{in} of 500 – 800 ms. These results indicate the existence of two types of voltage-dependent Ca^{2+} channels in early stages of development of frog myocytes, both known in mature frog skeletal muscle fibres.

Key words: Frog embryonic myocytes — Skeletal myocyte culture — Ca^{2+} currents

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Introduction

Voltage dependent sodium, potassium and calcium channels have been described in cultured frog embryonic myocytes (DeCino and Kidokoro 1985; Gilbert and Moody-Corbett 1989; Moody-Corbett et al. 1989; Moody-Corbett and Gilbert 1990; Moody-Corbett and Virgo 1991; Lukyanenko et al. 1993) These currents display characteristics that are in general similar to those in mature frog skeletal fibres (Adrian et al. 1970; Campbell and Hille 1976, Stanfield 1977, Sanchez and Stefani 1978, 1983, Almers and Palade 1981; Stefani and Chiarandini 1982, Huerta and Stefani 1986, Beaty et al 1987, Henček et al. 1988). However, fast calcium current ($I_{Ca\,f}$) described for developing muscle cells of *Xenopus laevis* displays fast inactivating dynamic properties (Moody-Corbett et al. 1989, Moody-Corbett and Virgo 1991) in contrast to "non-inactivating" $I_{Ca\,f}$ for twitch and tonic mature frog skeletal fibres (Cota and Stefani 1986, Henček et al. 1988) The transient $I_{Ca\,f}$ was similar to the transient $I_{Ca\,f}$ known from adult skeletal muscle fibres of *Xenopus laevis* and *Rana temporaria* (Henček et al. 1988).

The present study shows that cultured frog myocytes display $I_{Ca\,f}$ with slow inactivation process known to be present in adult frog muscle fibres already from the second day after plating. It provides more information about ionic currents in embryonic myocytes developing in culture and may assist in further elucidation of the development of ionic channel functions, and of excitation processes on the membrane

Some of the data have recently been published elsewhere (Lukyanenko et al 1994).

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 Sciences of Russia) supplemented with 2% fetal bovine serum (Ecophond, C I S) and 50 U/ml penicillin and 50 μg/ml streptomycin (C I S), 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 Na_2HPO_4 16, $NaHCO_3$ 2 4, EDTA 1 9 (Freed and Mezger-Freed 1970) During dissociation into single cells the ectoderm was stripped off, mesodermal and neural cells were transferred for culturing on glass in 40 mm Petri dishes separately for every embryo The growth medium contained Medium 199M 55% fetal bovine serum 10% penicillin 50 U/ml and streptomycin 50 μg/ml These constituents almost completely prevent both myocyte division (Taylor-Papadimitriou and Rosengurt 1979) and fusion The cultures were kept at 20 C under sterile conditions The myocytes plated on the glass bottom of the dish turned spindle-shaped 1 2 μm in diameter and 15 40 μm in length their size almost doubled by the last days of culturing

The experiments were performed with cells from 2 to 9-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 subtracted 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 had been 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 of 0.1–10 ms.

Experiments were performed at room temperature (19–21 °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): CsCl 60; TEACl 50; $CaCl_2$ 2; $MgCl_2$ 1; K_2EGTA 10; HEPES 8; pH 7.2 adjusted with KOH. Dihydropyridine (DHP) antagonist nifedipine (Sigma) was preliminarily dissolved in dimethylsulfoxide at 10 mmol/l and subsequently in external solution yielding final concentrations between 30–150 μmol/l.

Results

In the absence of sodium current (I_{Na}) two components of inward voltage-dependent currents were observed in response to depolarization: the well-known slow calcium current ($I_{Ca,s}$) and a fast-activated current of small amplitude ($n = 17$). Fig. 1 shows whole-cell currents elicited during 7 s depolarization prior to (*A*) and after the addition of 50 μmol/l DHP antagonist nifedipine (*B*) to the external recording solution. The fast DGP-insensitive component of the inward current is seen in Fig. 1 *B*. This was insensitive to 30–150 μmol/l nifedipine ($n = 11$) and was also insensitive to 10 μmol/l TTX ($n = 12$) (Fig. 1 *B* and Fig. 2), which completely blocked the I_{Na} at this concentration (Lukyanenko et al. 1993). Note that we took for Fig. 1 results of experiments where the $I_{Ca,s}$ amplitude was relatively small. In most cases the $I_{Ca,s}$ amplitude was 2–3-fold higher than for the fast component. In our previous paper (Lukyanenko et al. 1993) we showed that within the first day

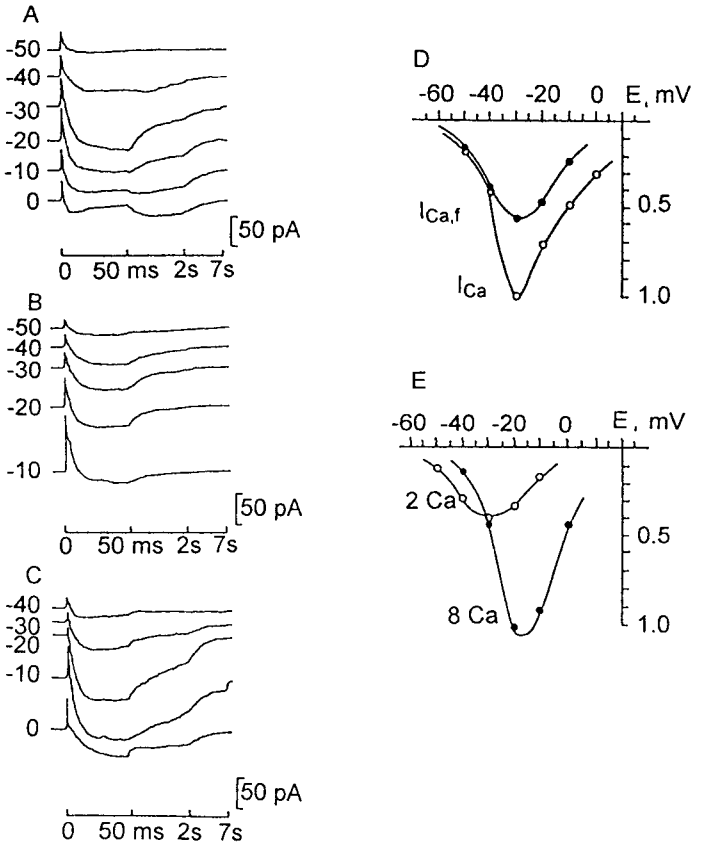

Figure 1. Fast calcium current in frog cultured myocyte. *A*, *B*, *C* – Whole-cell currents (after leak subtraction) elicited by test potentials from −50 to 0 mV (indicated in mV near each recording) for myocyte in a 7-day-old culture recorded before (*A*), and after the addition of 50 μmol/l nifedipine (*B*) to the standard external recording solution with 2 mmol/l 4-aminopyridine (4-AP), and after the replacement of this recording solution (2 mmol/l $CaCl_2$, 2 mmol/l 4-AP and 50 μmol/l nifedipine) by the equimolar recording solution of 8 mmol/l $CaCl_2$ (*C*). Holding potential (*HP*) of −80 mV. Data below 50 ms of stimulation were sampled at 10 kHz, between 50 ms and 2 s at 100 Hz, 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. Myocyte N° 23.05.92.4.; *D*, *E* – The peak current-voltage relationships for the experiment (myocyte N° 23.05.92.4) normalized to maximal peak current amplitude in compared files: (*D*) for peak current amplitudes of inward currents from A (open symbols) and B (filled symbols); (*E*) for the fast-activated component peak amplitudes from *B* (open symbols) and *C* (filled symbols). The ordinate represents peak current amplitude, and the abscissa shows the test potential value – *E*.

of plating about 40% of the examined myocytes did not show the slow current at all.

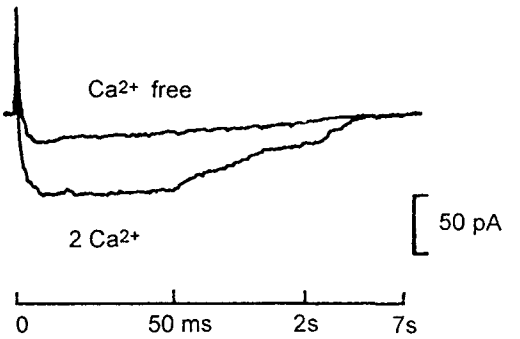

Figure 2. Effect of Ca^{2+} free solution on fast component of inward current in frog cultured myocyte. Whole-cell currents (after leak subtraction) elicited by test potential of −20 mV for myocyte N° 20.04.93.1 in a 3-day-old culture recorded before and after the replacement of the standard recording solution (with 10 μmol/l TTX and 80 μmol/l nifedipine) by the equimolar recording solution nominally Ca^{2+} free (upper trace). *HP* of −80 mV; time scale as in Fig. 1.

The amplitude of the fast component was enhanced with elevation of calcium or barium ion concentration to 8 mmol/l in the standard external recording solution ($n = 7$) (Fig. 1 *C*, *E*), and decreased when the external solution was replaced by Ca^{2+} free solution (Fig. 2). Fig. 2 shows whole-cell currents recorded prior to and after the replacement of the standard recording solution by the equimolar recording solution nominally Ca^{2+} free (upper trace). The GΩ pipette contact with myocyte membrane was destroyed very fast during exposition of solutions containing chelates. It is known that the contact of cell membrane with glass needs Ca^{2+}. Hence, we could use only a nominally Ca^{2+} free solution (without chelates) and only approximately 30 s. Fig. 2 shows the $I_{Ca,f}$ and residual current after removing the standard recording solution for nominally free solution. The figure illustrates a principal dependence of $I_{Ca,f}$ amplitude on calcium concentration. All these findings indicate that the fast-activated component of inward current was also carried by Ca^{2+} as is the case for the slow component.

The fast calcium current ($I_{Ca,f}$) was observed in 17 out of 22 examined myocytes and always together with slow DHP-sensitive $I_{Ca,s}$. Within 2 to 4 days after plating, approximately 50% of the examined cells displayed $I_{Ca,f}$, and from day 5 of plating, however, all myocytes displayed this current. Fig. 3 shows the normalized peak current-voltage relationship of the $I_{Ca,s}$ (*A*) and the $I_{Ca,f}$ (*B*) averaged for 15 (*A*) or 7 (*B*) experiments with 2 mmol/l $CaCl_2$ in the external recording solution. During 7 s depolarizations in standard recording solution the $I_{Ca,f}$ was detected near −50 mV (Figs. 1 and 3 *B*) and reached maximal peak amplitude at −20 mV (time to peak approximately 20 ms) that is about 20 mV more negative than the membrane potentials at which $I_{Ca,s}$ appeared and reached their maximum value (Fig. 3 *A*).

The peak amplitude of $I_{Ca,f}$ tended to be lower during experiment as for the $I_{Ca,f}$ of frog mature skeletal muscle fibres (Cota and Stefani 1986; Garcia and Stefani 1987). At test potentials from −40 to −10 mV $t_{0.5}$ of $I_{Ca,f}$ changed from −20 ms to −7 ms, and inactivation time (τ_{in}) value changed from 600 to 300 ms respectively. However, for depolarization larger than to −30 mV, the time course of $I_{Ca,f}$ could not be accurately estimated because the superposition of ionic current remained (first of all $I_{Ca,s}$ which could not be abolished completely at test pulse of −20 mV with 80–150 μmol/l nifedipine) and since its onset was masked by the capacity transient.

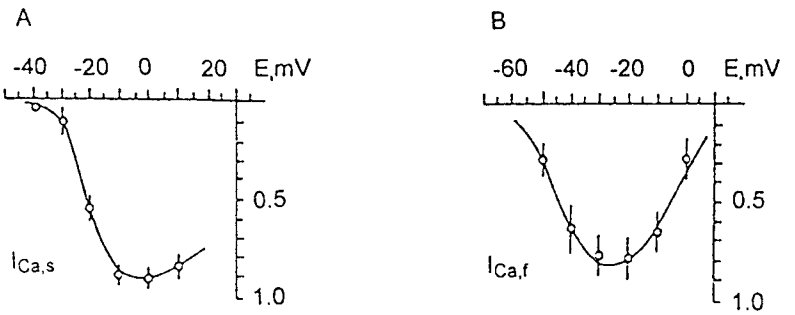

Figure 3. Current-voltage relationships for the two types of calcium current in cultured frog skeletal myocytes. The normalized peak current-voltage relationships of the slow DHP-sensitive (*A*) and the fast-activated DHP-insensitive (*B*) calcium currents averaged for 15 (*A*) or 7 (*B*) experiments with 2 mmol/l $CaCl_2$ in the external recording solution. Vertical bars represent S.E.M. The ordinate represents normalized peak current amplitude, and the abscissa shows the test potential value − E.

Discussion

Our results indicate that in early stages of frog myocyte development two types of voltage-dependent Ca^{2+} channels exist: fast and slow, both of them known also in adult frog skeletal muscle fibres.

In our previous experiments (Lukyanenko et al. 1993) we have shown that cultured frog myocytes display two types ("twitch" and "tonic") DHP-sensitive slow calcium currents ($I_{Ca,s}$) having time to peak 402 ± 76 ms and 1431 ± 79 ms respectively (2 mmol/l Ca^{2+} in the external recording solution, and 18–20 °C). The $I_{Ca,s}$ were similar to DHP-sensitive $I_{Ca,s}$ in twitch and tonic, respectively, skeletal muscle fibres of the adult frog (Cota and Stefani 1986; Huerta and Stefani 1986; Beaty et al. 1987; Garcia and Stefani 1987; Henček et al. 1988). However, we could not find $I_{Ca,f}$ when used potassium blockers outside (Henček et al. 1988) because

they did not abolish I_K completely (Lukyanenko et al. 1993). The experiments in which potassium currents are strongly blocked by TEA^+ and Cs^+ from the intracellular side show that myocytes in culture also display the fast-activated ionic currents through Ca^{2+} channels that are voltage operated and insensitive to DHP. The $I_{Ca,f}$ current was similar to "non-inactivating" $I_{Ca,f}$ known in twitch and tonic skeletal muscle fibres of the adult frog (Cota and Stefani 1986; Beaty et al. 1987; Garcia and Stefani 1987; Henček et al. 1988) and differs from the DHP-insensitive transient $I_{Ca,f}$ known from *Xenopus laevis* embryonic muscle cells grown in culture (Moody-Corbett et al. 1989; Moody-Corbett and Virgo 1991) and described also in adult frog muscle fibres (Henček et al. 1988). The latter are similar to the current described in rat and mouse cultured myocytes (Beam et al. 1986; Cognard et al. 1986; Shimahara and Bournaud 1991).

In our experiments almost all examined myocytes display $I_{Ca,f}$ and the contribution of its amplitude ranged from 15 to 60% in the total I_{Ca} at various test potentials (Fig. 1 *D*, open symbols show $I - V$ for common trace of I_{Ca} and filled ones show $I_{Ca,f}$). This may be the main reason why $I - V$ relationship for $I_{Ca,s}$ shown in our previous paper (Lukyanenko et al. 1993) differs from that in this article (Fig. 3 *A*). We could not eliminate $I_{Ca,f}$ in our experiments, however for $I - V$ shown in Fig. 3 *A* we selected the myocytes that did not display $I_{Ca,f}$ at test potentials from −50 to −30 mV and then did not display this current after nifedipine application at the end of the experiment. This $I - V$ differs by its threshold and peak from that in Fig. 1 *D* and in our previous paper of approximately 20 mV, as well as $I - V$ for $I_{Ca,f}$ differs from $I - V$ for $I_{Ca,s}$ in Fig. 3. Moreover, it was found that nifedipine can shift $I - V$ of $I_{Ca,f}$ 5–10 mV to $I - V$ of $I_{Ca,s}$. For separation of the $I_{Ca,f}$ we used DHP-antagonist (nifedipine) treatment, whereas in adult muscle fibres fast component did not separate and it was seen only at potentials when $I_{Ca,s}$ was not seen. Nifedipine blocked $I_{Ca,s}$ almost completely at the concentrations used but the effect of nifedipine on some characteristics of $I_{Ca,f}$ can not be excluded, especially because we have found that nifedipine changes the characteristics of potassium currents (I_K) by nonspecific mechanisms. Fig. 4 shows I_K recorded before (*A*), and after the addition of 80 μmol/l nifedipine (*B*) to the standard external recording solution. Nifedipine does not change maximal potassium conductance but induced a parallel shift of chord conductance curve along the voltage axis in the direction of more negative potentials by 5–10 mV ($n = 16$). This effect most probably reflects the effect of nifedipine on negative surface charge density (Gilbert and Ehrenstein 1969; Mozhayeva and Naumov 1970; Ehrenstein and Gilbert 1973). The direction and value of shift are equal to *e*-fold reduction of the extracellular concentration of Ca^{2+}. So it might be suggested that nifedipine can bind to some sites of outer surface with high affinity, but has less effect on surface charge than the main counterions. If the nature and density of negative surface charge near the potassium and the fast calcium channels are equal, a similar

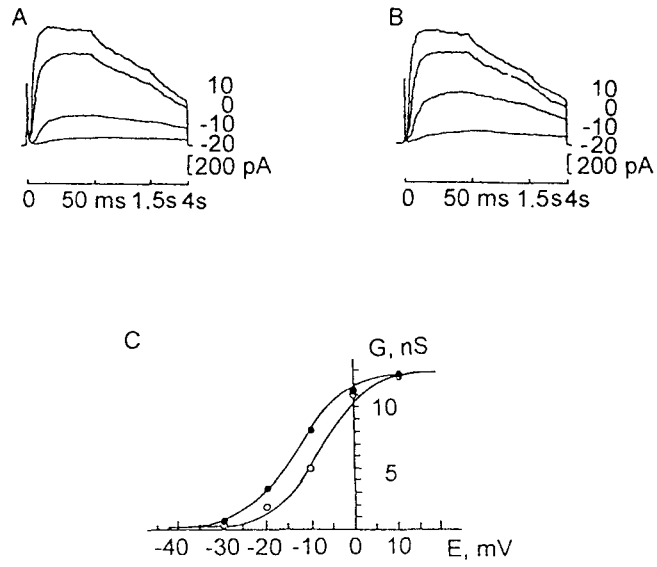

Figure 4. Effects of nifedipine on potassium currents in myocytes. A, B – Potassium currents (after leak subtraction) elicited by test potentials from −20 to +10 mV (indicated in mV near each recording) for myocyte in a 5-day-old culture recorded before (A), and after the addition of 80 μmol/l nifedipine (B) to the standard external recording solution. The pipette solution (in mmol/l): KCl 110; $CaCl_2$ 2; $MgCl_2$ 1; K_2EGTA 10; HEPES 8; pH 7.2 adjusted with KOH. HP of −80 mV. Time scale is linear below 50 ms, between 50 ms and 1.5 s, and beyond 1.5 s. Myocyte N° 5.06.92.4. C – 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). For data represented on A and B the potassium conductance was 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: $G(E) = \overline{G}_K/\{1+\exp[(E-E_{0.5})/k]\}$, where G 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 and k is the slope factor of the curve. For standard solution: $E_{0\,5} = -7.8$ mV and $k = -5.3$; after nifedipine treatment: $E_{0\,5} = -13$ mV and $k = -5.8$.

shift of $I_{Ca,f}$ characteristics may be expected. Hence, the real difference in thresholds and peaks of $I - V$ for $I_{Ca,s}$ and $I_{Ca,f}$ is not more than 10–15 mV (Fig. 1 A), whereas it is 30 mV in adult frog muscle fibres (Cota and Stefani 1986; Beaty et al. 1987; Garcia and Stefani 1987; Henček et al. 1988).

Small range of potentials between thresholds for fast and slow I_{Ca} in standard solution and residual $I_{Ca,s}$ in the presence of nifedipine did not allow to get precise biophysical characteristics of $I_{Ca,f}$ for the whole range of test potentials.

Nevertheless, the existence of fast type of the I_{Ca} with kinetic and pharmacological properties which differ from the properties of $I_{Ca\,s}$ was shown We consider that the $I_{Ca\,f}$, can probably take considerable part in the activation of frog myocyte contraction (Nasledov et al 1992)

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