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VOLTAGE DEPENDENT CALCIUM CURRENTS IN CULTURED FROG SKELETAL MYOCYTES

Voltage dependent calcium currents (I_{Ca}) in cultured embryonic skeletal frog myocytes at the stages of development ranging from one to six days were studied using the whole-cell patch-clamp recording technique. Fast dihydropyridine (DHP)-nonsensitive I_{Ca} and slow DHP-sensitive I_{Ca} were recorded during the early stages of myogenesis. These currents do not differ in their kinetics from the I_{Ca} found in the adult frog muscle. Two slow voltage-dependent DHP-sensitive I_{Ca} were found. By its activation time, the first slow I_{Ca} is similar to I_{Ca} described in phasic skeletal muscle fibers. The second slow I_{Ca} observed in about 10% of cells examined is similar to I_{Ca} described in tonic skeletal muscle fibers of adult frog. In some cases, two slow currents with different kinetics were recorded in the same myocyte.

INTRODUCTION

A more detailed knowledge of ionic currents in embryonic myocytes developing in culture may assist in further elucidation of the development of ionic channel functions and ionic requirement for the activation of a contraction during the early stages of myogenesis. The aim of our experiments was to characterise the membrane calcium currents at early stages of frog myogenesis, since these currents take part in the activation of a contraction.

Voltage dependent sodium, potassium, and calcium channels have been described in cultured embryonic myocytes. However, only one of the calcium currents (I_{Ca}) described in the frog skeletal muscle [1, 2] has been observed in primary culture of frog myocytes, the current through dihydropyridine (DHP)-sensitive channels [3]. Later, Moody-Corbett and Virgo [4] reported that *Xenopus* skeletal muscle cells in culture develop a transient current which differs from fast noninactivating calcium current in adult frog muscle fibers [1, 5]. This current is similar to the fast transient current described in rat myocytes [6, 7].

Our investigations were performed on frogs in which the ionic currents in mature muscle tissue had been most completely described, providing more information concerning the development of ionic channel functions during myogenesis. Some data obtained have already been published earlier [8].

METHODS

Cell culture. Standard frog embryo muscle cell cultures were prepared from early neurula embryos of *Rana temporaria*. Dorsal portions of the embryos were dissected in 60 % Medium 199M (Institute of Polyomyelitis and Encephalitis, Russian Academy of Medical Sciences) with 2 % fetal calf serum ("Ecofond", Russia), 50 U/ml penicillin, and 50 μg/ml streptomycin (Russia), and washed during 10 min in Ca^{2+} — Mg^{2+}-free solution containing (mM): NaCl, 50.4; KCl, 0.67; KH_2PO_4, 0.86; $NaHPO_4$, 16; $NaHCO_3$, 2.4; EDTA, 1.9 [9]. During the dissociation into single cells the ectoderm was stripped and removed, and mesodermal and nerve cells were transferred for culturing in glass 40 mm Petri dishes separate for every embryo. The growth medium contained 55 % Medium 199M, 10 % fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. These constituents almost completely prevent both myocyte division [10] and fusion. The culture was kept at 20 °C under sterile conditions. The myoblasts plated on the glass bottom of the chamber turned into those spindle-shaped, 1-2 μm in

diameter and 15-40 μm in length; their size almost doubled during the last five-six days of culturing.

The experiments were performed on cells from one- to six-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 technique was used. The voltage-clamp circuit was similar to that described by Hamill et al. [11], with a 5 GΩ head stage feed-back resistor. Linear component of leakage current was subtracted on-line. The fast component of capacity currents associated with capacity of electrode and electrode holder was fully compensated, while the slow component associated with cell capacity could be compensated only partly because of the large size and complex shape of the cells. A resistance of the patch electrodes filled with standard solution ranged between 3 and 7 MΩ. The seal resistance was 5 to 30 GΩ, and the input resistance of the cells ranged between 1 and 5 GΩ. The experiments were started in 10-15 min after the whole-cell recording configuration was established. The membrane potential was held at −80 mV.

The experimentas 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 msec. The 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 (mM): NaCl, 120; KCl, 1.5; $CaCl_2$, 2; HEPES, 8; pH 7.4 was adjusted with NaOH. The pipettes were filled with a solution containing (mM): CsCl, 110; $CaCl_2$, 2; $MgCl_2$, 1; K_2EGTA, 10; HEPES, 8; pH 7.2 was adjusted with KOH. In some cases, CsCl was replased by a mixture of 60 mM CsCl and 50 mM TEACl. The dihydropyridine (DHP) antagonist nifedipine ("Sigma", USA) and agonist CGP-IOS (Institute for Organic Synthesis, Riga, Latvia) were used. The agonistic effects of this DHP derivative described by Shvinka et al. [12]) were used to identify DHP-sensitive type of calcium channels. CGP-IOS and nifedipine were pre-dissolved in dimethylsulphoxide at 10 mM and subsequently dissolved in external solution yielding final concentrations between 20 μM and 100 μM.

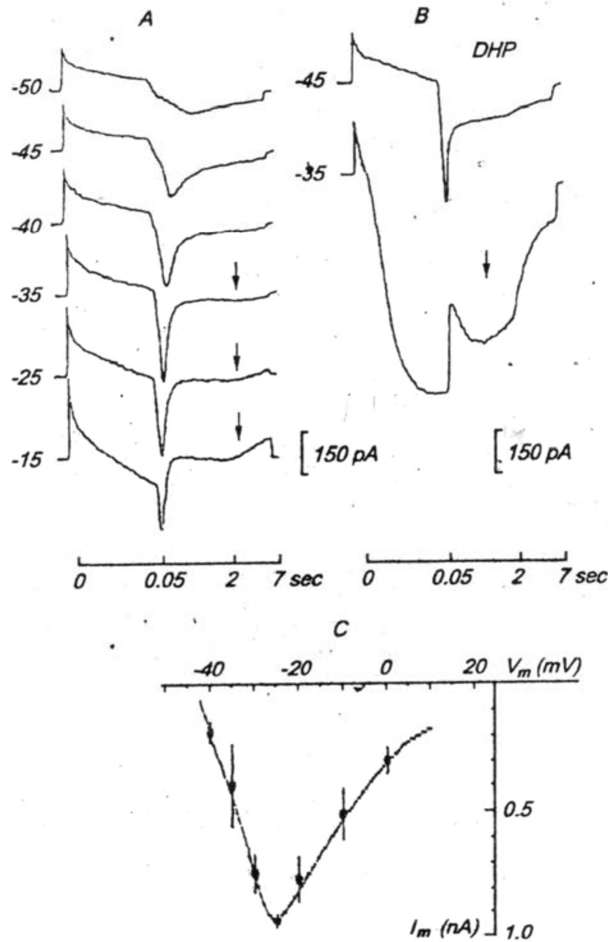
RESULTS

Slow calcium current. Within the first 24 h of plating, about 40% of the cells did not show any detectable I_{Ca}. On sixth day of plating, however, all cells examined exhibited a slow DHP-sensitive I_{Ca}. We recorded two components of slow inward voltage-dependent calcium current. The faster component was enhanced by adding Ca^{2+} or Ba^{2+} , or the DHP agonist CGP-IOS (20-100 μM; n = 24) to standard external solution (Fig. 1). It could be blocked by external application of 2 mM $CoCl_2$ (n = 13), 0.1-0.4 mM verapamil (n = 9), or 0.5-3 μM D-600 (n = 4) (Fig. 2), and removed by the DHP antagonist nifedipine (40—100 μM) (Fig. 3).

Currents through calcium channels were observed in 114 myocytes. I_{Ca} reached its peak amplitude within 402±75 msec at normal extracellular calcium concentration (2 mM Ca^{2+}) and test pulse corresponding to the maximum value of the peak amplitude. In 12 cells examined, a slower component of current through calcium channels was recorded ($P < 0.001$). Under the same experimental conditions the amplitude of this component reached its maximum within 1431±79 msec. This component could be blocked by external application of 2 mM $CoCl_2$ (n = 3) or 3 μM D-600 (n = 3). It was enhanced by external addition of Ba^{2+} (n = 10) or 50 μM CGP-IOS (n=2). In five myocytes, both types of slow DHP-sensitive currents were found in the same cell (Fig. 1), and in two of them the currents were recorded at normal external calcium concentration (2 mM Ca^{2+}). Inactivation time constants (τ_{in}) for both types of I_{Ca} could not be estimated due to the current superposition and the remaining outward current.

The DHP agonist CGP-IOS (20-100 μM) increased both types of slow I_{Ca} in all cases, accelerated the current activation, and shifted the membrane potential of activation by 10—15 mV to more negative values (Fig. 1).

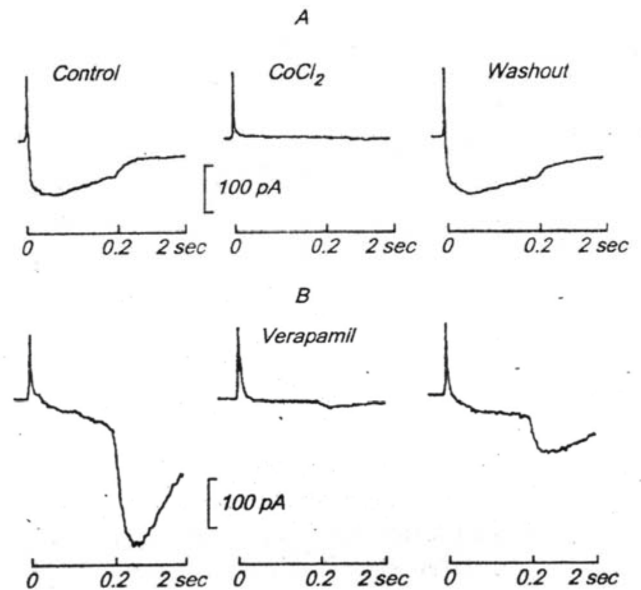
Fast calcium current. The fast component ($t_{0.5}$ = = 8-20 msec) of I_{Ca} (Fig. 3) appeared approximately at −60 mV and reached its maximum amplitude at −30 mV. It did not significantly decline during long-lasting depolarization (τ_{in} = 500-800 msec). The fast component was insensitive to 2 μM TTX and 50 μM nifedipine (Fig. 3, *A, B*), but enhanced after [Ca^{2+}] elevation (to 8 mM) in the standard external solution



F i g. 1. Two types of slow dihydropyridine (DHP)-sensitive calcium currents in cultured myocytes.
The whole-cell current (without leakage subtraction) elicited by test potentials from-50 to-15 mV (indicated in mV near each trace) was recorded from a six-day-old culture myocyte prior to (*A*) and after the addition of 50 μM DHP agonist CGP-IOS (*B*) to tne external solution. The external solution contained (mM): $BaCl_2$, 2; TEACl, 110; HEPES, 8. In the standard internal solution KCl was replaced by CsCl in equimolar amount. Arrows indicate the second slow current. Holding potential (HP) is −80 mV. Data until 50 msec of stimulation were sampled at 10 kHz, between 50 msec and 2 sec — at 100 Hz, and after 2 sec — at 10 Hz; accordingly, time scale is linear below 50 msec, between 50 msec and 2 sec, and beyond 2 sec. *C* is normalized peak current-voltage relationship as averaged for 15 experiments at 2 mM $CaCl_2$ in the external solution. *Abscissa*: the test potential value, mV. *Ordinate*: normalized peak current amplitude. Vertical bars represent S.E.M.

Р и с. 1. Два типи повільних дигідропіридинчутливих кальцієвих струмів в культивованих міоцитах.

(*C, D*). Thus, the fast-activated component of inward current was carried by Ca^{2+} through DHP-nonsensitive calcium channels.



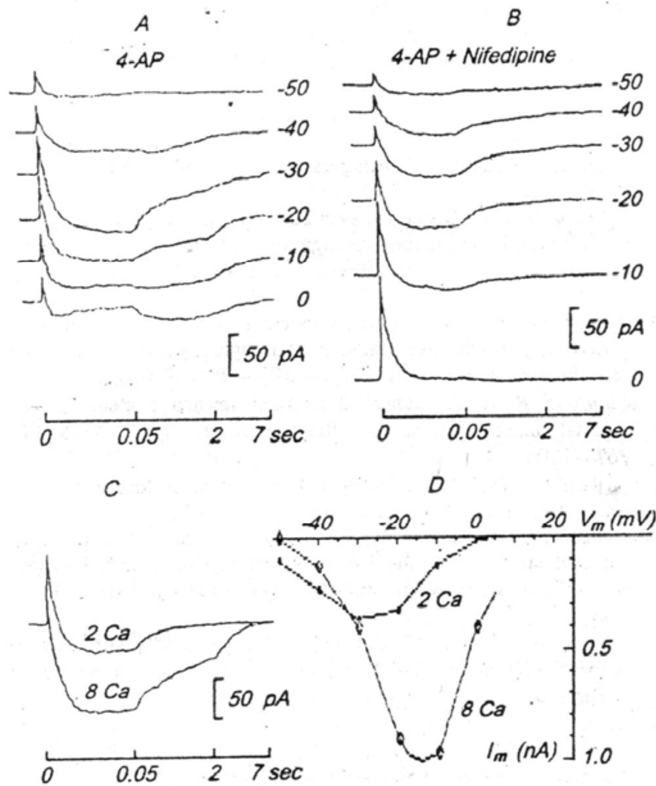
F i g. 2. Effects of blockers on current through calcium channels in five-day-old cultured frog myocytes.
A — effect of 2 mM $CoCl_2$ (test potentialis +5 mV); *B* — effect of 0.3 mM verapamil (test potentialis −20 mV; other myocyte). The blockers have been added to external recording solution containing (mM): $BaCl_2$, 2; TEACl, 110; HEPES, 8 μM and 50 μM CGP-IOS. Holding potential (HP) is −80 mV. Time scale is the same as in Fig. 1.

Р и с. 2. Вплив блокаторів на струми через кальцієві канали в п'ятиденних культивованих міоцитах.

DISCUSSION

We found that myocytes in culture showed two slow inward ionic currents through calcium channels that are voltage-operated and sensitive to DHP, and one DHP-nonsensitive fast I_{Ca}. The first slow current was similar to that through DHP-sensitive calcium channels in twitch skeletal muscle of the adult frog [13-15]. The second slow current was likewise transferred via DHP-sensitive calcium channels (Fig. 1, *B*), however, its characteristics were less clear since it was quite infrequent. By its activation time, the second slow I_{Ca} was similar to I_{Ca} found in tonic skeletal muscle fibers of adult frog [16, 17].

Almers and McCleskey [18] have recorded from frog muscle cells in external solution containing 10 mM Ca^{2+} and 10 mM Ba^{2+} the current traces with two slow components, resulting of anomalous mole-fraction behavior (AMFB). We recorded two slow components not only in a solution with 2-8 mM Ba^{2+},



F i g. 3. Fast calcium current in cultured frog myocyte.
A, B — whole-cell currents (after leakage subtraction) elicited by testing pulse from -50 to 0 mV (indicated in mV near each record) for myocyte in a six-day-old culture recorded after the addition of 2 mM 4-aminopyridine (4-AP) (*A*) and 50 μM nifedipine (*B*) to the standard external solution; *C* — the potentiating effect of Ca^{2+} addition (to 8 mM $CaCl_2$) to the standard external solution with 2 mM 4-AP and 50 μM nifedipine. The current has been elicited by testing pulse to −20 mV (after leakage subtraction). In *A—C*: KCl in the internal standard solution was replaced by 60 mM CsCl and 50 mM TEACl. Holding potential (HP) was −80 mV. Time scale is the same as in Fig. 1. *D* — the peak current-voltage relationships for experiments with 2 mM $CaCl_2$ (filled symbols) and 8 mM $CaCl_2$ (open symbols) in external solution. *Abscissa*: the testing potential level, mV. *Ordinate*: peak current amplitude, nA.

Р и с. 3. Швидкий кальцієвий струм в культивованих міоцитах жаби.

but also in standard solution (2 mM Ca^{2+}; n = 2). The mode of change of extracellular solution used in our experiments allowed us to suggest that co-existence of slow currents was not a result of AMFB.

These results can be interpreted in terms of a hypothesis that in some myocytes the DHP-sensitive calcium channels which are specifically associated with twich and tonic types of adult muscle fibers co-exist. Figure 1 shows two components of DHP-sensitive inward slow current recorded from the same myocyte.

Moody-Corbett et al. [3] and Moody-Corbett and Virgo [4] reported that *Xenopus* embryonic muscle cells grown in culture exhibit DHP-sensitive slow and DHP-nonsensitive fast transient currents through the calcium channels. The former current is similar to the slow current described in the adult frog muscle in the present study. The latter one is similar to the fast transient current described in rat and mouse cultured myocytes [2, 6, 7, 19], and differs from the fast noninactivating current described in the adult frog muscle [1, 5, 16, 20].

We have also recorded fast I_{Ca} similar to fast I_{Ca} described in the adult frog muscle. The amplitude of fast I_{Ca} is known to be n-times smaller than the amplitude of slow I_{Ca}. We have observed fast noninactivated, DHP-nonsensitive I_{Ca} in the experiments with potassium currents (I_K) strongly blocked by TEA^+ and Cs^+ added to intracellular solution.

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ПОТЕНЦІАЛЗАЛЕЖНІ КАЛЬЦІЄВІ СТРУМИ В КУЛЬТУРІ МІОБЛАСТІВ ЖАБИ

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Р е з ю м е

В культивованих ембріональних скелетних міоцитах жаби на стадіях розвитку від однієї до шістьох діб із застосуванням методики петч-клемпу на цілій клітині були досліджені потенціалзалежні кальцієві струми (I_{Ca}). Вже на ранніх етапах ембріогенезу були зареєстровані швидкий, не чутливий до дигідропіридину I_{Ca} та повільний, чутливий до дигідропіридину I_{Ca}. За кінетикою та фармакологічними властивостями ці струми не відрізняються від I_{Ca}, що були описані в м'язі дорослої жаби.

Крім того, були зареєстровані два різновиди потенціалзалежних повільних, чутливих до дигідропіридину I_{Ca}. За своїм часом активації один із них є ідентичним до I_{Ca}, виявленого у фазних скелетних м'язових волокнах. Другий, більш повільний I_{Ca}, зареєстрований приблизно в 10 % досліджених клітин, був подібним до I_{Ca} в тонічних скелетних м'язових волокнах дорослої жаби. В деяких випадках в тому самому міоциті спостерігалися обидва види повільних струмів з різною кінетикою.

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