Short communication

Ionic Requirements for the Activation of Contraction in Embryonic Frog Myoblasts in Culture

G. A. NASLEDOV[1], W. I. LUKYANENKO[1], I. E. KATINA[2] and A. V. LONSKY[2]

1 *Sechenov Institute of Evolutionary Physiology and Biochemistry,*
  Academy of Sciences, St. Petersburg, 194223, Russia
2 *Physiological Institute of the St. Petersburg University,*
  St. Petersburg 199034, Russia

Myoblasts growing in primary culture are known to possess contractile ability already during the first days of development. In chicken cultured myoblasts contractions in response to electrical stimulation have been first observed in the second day of development, and then the amounts of contracting myoblasts proportionally increased (Szepsenwol 1947). In *Xenopus* myoblasts in culture well developed contractile structures and triad complexes could be observed in most cells as early as between days 1 and 2 (Weldon and Cohen 1979; Kidokoro and Saito 1988). Contraction in response to local depolarization and its prevention by external Ca ions removal after one day culturing was described by Moody-Corbett et al. (1989). The aim of the experiments reported here was to characterise the membrane ionic currents necessary for the activation of contraction to occur in frog myoblast in the early stages of development.
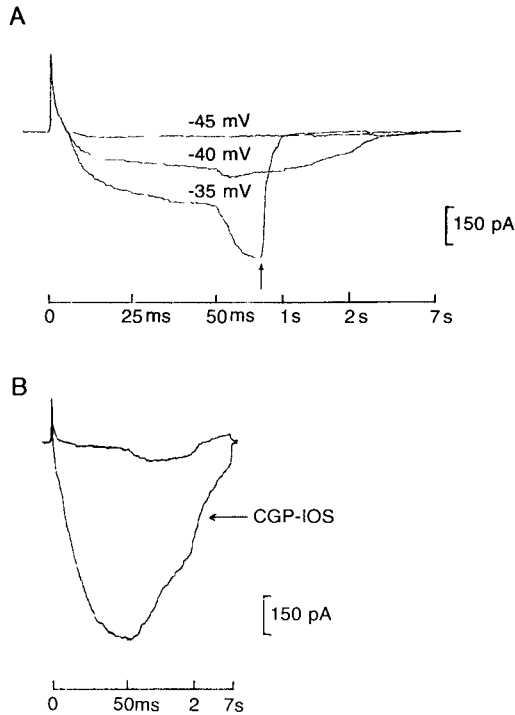
   Muscle cultures were prepared from frog developmental stages 25 (Nieuwkoop and Faber 1967) of *Rana temporaria* embryos. The dorsal part of the embryo was dissected and dissociated during 10 min in solution containing (in mmol/l) NaCl 50.4; KCl 0.67; KH_2PO_4 0.85; $NaHCO_3$ 2.4 (Freed and Mezger-Freed 1970). During the dissociation, the ectoderm was striped and removed, and mesodermal and neural cells were transferred for culturing in a solution containing: Medium 199M (from Institute of Poliomyelitis and Encephalitis, Academy of Medical Sci. of the Russia) 55%; bovine embryonic serum (Ecophond, Russia) 10%; penicillin 50 U/ml and streptomycin 50 mg/ml (Russia). The culture was kept at 20 °C in sterile conditions. The myoblasts plated on the glass bottom of the chamber became spindle-shaped with diameters of 1.2–2 μm and 15–40 μm in length; their size approximately doubled by the last days (5–6) of culturing.

   The experiments were performed in 1 to 6-day-old cultures. The constituents of the culture media as mentioned above almost completely prevent both myoblast

division and fusion. The myoblasts selected for experiments did not show any connections with neuroblasts or with each other. The conventional whole-cell patch clamp method was used for current recording. A standard circuit for voltage clamp experiment (Hamill et al. 1981) was connected to a computer which controlled the stimulation protocol, and recorded, computed and subtracted the linear component of leakage current, and provided for the compensation for the capacitive currents. The experiments were started 10–15 min after establishing whole-cell recording conditions. The basic external solution contained (in mmol/l): NaCl 120; KCl 1.5; $CaCl_2$ 2; HEPES-KOH 8 (pH 7.4). The pipettes were filled with solution containing (in mmol/l): CsCl 110; $CaCl_2$ 1; $MgSO_4$ 1; K_2EGTA 10; HEPES-KOH 8 (pH 7.2). In some cases KCl was replaced by CsCl at the same molar ratio. Dihydropyridine (DHP) agonist CGP-IOS (Institite for Organic Synthesis, Riga, Latvia) was used for identification of DHP-sensitive type of Ca-channels (the agonist effect of this DHP-derivative has been described by Shvinka et al. 1990). Experiments were performed at 18–20 °C.

Myoblast contractions themselves could not be recorded due to the rather small size of the cells Therefore, contractions could be recorded only qualitatively. As a result of contraction in most cases the microelectrode loses its high resistivity contact with the cell membrane, and correspondingly a break of the current recording line is well seen. This kind of recording obviously cannot be used for measurements of contraction parameters or latency. As a rule, the break corresponds to the start of the rapid developing phase of contraction, but it may occur at any moment of the contraction process. It is not sure whether every contraction is associated with the occurrence of a break, however, every break, in standard conditions, can be considered as being the reflection of a contraction, and the latency of the contraction cannot be longer than the time to break.
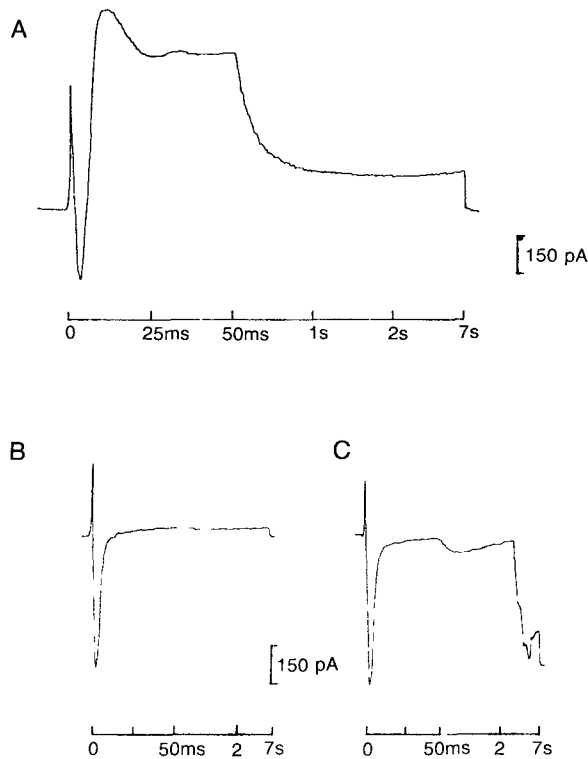
The experiments have shown that contraction of myoblasts in response to depolarizing pulses (0.5–7 s) could be observed as early as on the second day of culturing. At this time the slow Ca^{2+} current could not be recorded from all myoblasts investigated; in cases in which it was present, its amplitude varied to a great extent. Contractions could be observed exclusively in the presence of inward Ca^{2+} current (Fig. 1A). The time to contraction, which is seen as a break of the record, was 1028 ± 457 ms (mean $\pm$ S.E.; $n = 14$). The contractions were elicited by test pulses to -25 ± 3.5 mV from a holding potential of -80 mV. It is important to note that contractions always appeared after the Ca^{2+} current has reached its maximum. The interval between peak Ca^{2+} current amplitude and the contraction averaged 770 ± 464 ms ($n = 14$). Calcium current, recorded from the myoblasts membrane was conducted by slow calcium channels, which are dihydropyridine sensitive. Fig. 1B shows the potentiating action of dihydropyridine agonist CGP-IOS on the Ca^{2+} current, and the appearance of contraction as soon as the current was sufficient for its activation. No contractions could be observed

A

B

Figure 1. Inward ionic current through Ca^{2+} channels from frog myoblasts cultured for 5 days (A) and 3 days (B), in response to 7s depolarizing pulses. A: Superimposed records during the potential shifts from holding level of -80 mV to -45 mV, -40 mV and -35 mV, which results in increase of Ca^{2+} current and finally in contraction appearance (arrow). External solution (in mmol/l): NaCl 120, KCl 1.5, $CaCl_2$ 2, HEPES 8; pipette solution: CsCl 110, $CaCl_2$ 1, $MgSO_4$ 1, EGTA 10, HEPES 8. Myoblast 4.5.06. B: The potentiating effect of DHP-receptor agonist CGP-IOS (50 μmol/l); before and after 10 min (arrow) of CGP-IOS action. External solution (in mmol/l): $BaCl_2$ 2, TEA 111, HEPES 8; pipette solution: CsCl 110, $CaCl_2$ 1, $MgSO_4$ 1, EGTA 10, HEPES 8. Myoblast 6.21.06. Sampling rates: below 50 ms: 10 kHz, between 50 ms and 2 s: 100 Hz; beyond 2 s: 10 Hz; Accordingly, the time scale is linear below 50 ms, between 50 ms and 2 s, and beyond 2 s.

upon substituting Ba^{2+} ions for Ca^{2+} ions in external solution.

Another condition for the activations of contraction to occur is the absence of outward K^+ current. Fig. 2A shows integral current in myoblast membrane obtained in response to a prolonged depolarising pulse in normal solution. Inward fast sodium and outward compound K^+ currents are seen. No Ca^{2+} current and no contraction can be observed in this case. When the K^+ current is abolished

Figure 2. Ionic currents recorded from 4 days (*A*) and 2 days (*B*) cultured frog myoblast in response to 7 s depolarizing pulse. *A*: Inward sodium and outward potassium currents in external solution containing (in mmol/l): NaCl 120, KCl 1.5, CaCl$_2$ 2, HEPES 8; pipette solution: KCl 110, CaCl$_2$ 1, MgSO$_4$ 1, EGTA 10, HEPES 8. Depolarising pulse from holding level of -80 mV to 0 mV. Myoblast 9.22.06. *B*, *C*: Inward calcium current and contraction appearance (arrow) before and 10 min after administration of 4-aminopyridine (0.5 mmol/l) in external solution, containing (in mmol/l): NaCl 120, KCl 1.5, CaCl$_2$ 2, HEPES 8; pipette solution: KCl 110, CaCl$_2$ 1, MgSO$_4$ 1, EGTA 10, HEPES 8. Depolarizing pulse from holding level of −80 mV to −35 mV. Myoblast 2.7.06. Time scale, see legend to Fig. 1.

by the action of the potassium channel blocker 4-aminopyridine, the Ca^{2+} current and a subsequent contraction occurs (Fig. 2*B*, *C*). Due to the presence of Cs ions in the pipette, in most experiments the K$^+$outward current was almost absent. Apparently the sodium current is not involved in activation of contraction, as sodium current was recorded but in 37.5% of our experiments, in which contractions occurred.

It can thus be concluded that the activation of contraction in frog myoblasts

requires: 1) the inward calcium current, and 2) significant reduction of potassium current.

It should be noted that Congard et al.(1988) and Romey et al.(1988) came to similar conclusions concerning the important role of external Ca^{2+} in activation of contraction in cultured rat and mouse myoblasts. These authors have discussed whether calcium enters the cell and activates the contractile proteins, or whether Ca^{2+} act only from outside on dihydropyridine sensitive voltage sensors, the resulting charge movement producing calcium release from the sarcoplasmic reticulum. The latter mechanism has recently been postulated for skeletal muscle fibers (Agnew 1987; Brum et al. 1988; Caswell and Brandt 1989; Dulhunty and Gage 1988).

Our experiments have shown that contractions only occur when preceded by Ca^{2+} current. Although this points to the role of calcium ions which enter the cell, it cannot be considered as strong evidence. Congard et al. (1988) an Romey et al. (1988) have assumed that the Ca^{2+} inward current is not required for activation, as in their experiments contractions appeared although no inward current was recorded at potentials about +50 – +60 mV. However, zero ionic current is no evidence for the absence of Ca^{2+} influx. Inward current may partially be masked by outward currents. In addition, the peak of current-voltage relationship (−25 mV) is identical to the voltage level which elicited maximal contraction. It may well be that in the myoblast, which has a larger ratio of surface area to cell volume, the calcium entry plays a more significant role in contraction than it does in muscle fibers. All this points to the possibility that in myoblasts during the first days of development, the ECC mechanism is to some extent closer to that in smooth muscle cells than it is the case in mature striated muscle fibers.

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