β-Adrenergic Regulation of Voltage-dependent Calcium Currents in Cultured Skeletal Myocytes of the Frog *Rana temporaria*

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Abstract. Effects of β-agonists isoproterenol (Isp) and adrenaline (Adr) and β-adrenoblocker obsidan (Obs) on the voltage-dependent calcium currents in cultured embryonic skeletal myocytes were studied at various stages of development ranging from day 2 to 10, using the whole-cell patch-clamp technique at 19—21 °C.

Adr (or Isp) in concentrations 0.1—10 μmol/l increases the amplitude of both the slow dihydropyridine(DHP)-sensitive calcium current (I_{Ca}) and the fast-activated DHP-insensitive I_{Ca}. From day 2 to 6 after myoblast plating, Adr and Isp did not change the amplitude of I_{Ca} at all or slightly increased it. Obvious strong positive effects (an approximately twofold amplitude increase) on the calcium channels have been observed in 7–10-day-old myocytes only.

β-adrenoblocker obsidan known to abolish the positive β-agonist effect, had a positive effect on membrane calcium currents. It may have been a result of the immaturity of the β-adrenergic regulatory system of the myocytes.

It is concluded that the β-adrenergic regulatory complex can stimulate the activity of the fast and the slow voltage-dependent calcium channels of the frog skeletal myocytes, and that there is a distinct developmental stage at which a functioning β-adrenergic regulatory complex appears in the membrane of skeletal myocytes.

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

Introduction

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Catecholamines are known to modulate the skeletal muscle activity, and in general to increase twitch and tetanic tension (Oota and Nagai 1977; Gonzalez-Serratos et al. 1981; Arreola et al. 1987). Among the possible mechanisms underlying this phenomenon activation of Ca^{2+} channels in the muscle cell membrane has been considered (Arreola et al. 1987). In mature frog skeletal muscle catecholamines were shown to increase the amplitude and to decrease the time constants of the slow dihydropyridine(DHP)-sensitive, and of the fast DHP-insensitive calcium currents (Arreola et al. 1987; Kokate et al. 1993), and of the potassium currents (Muniz et al. 1988). However, these effects have not been studied in developing muscles during myogenesis and in cultured myocytes.

It was of considerable interest to elucidate the adrenergic modulation of Ca-channel activity during early myogenesis, and in particular in skeletal myocytes developing in culture. In cultured frog embryonic myocytes the voltage-dependent sodium, potassium and calcium currents have been described (Moody-Corbett et al. 1989; Lukyanenko et al. 1993, 1994). In general, the currents display characteristics that are similar to those in mature frog skeletal muscle fibres. It is known that during muscle tissue development, the β-adrenergic receptor – adenylate cyclase system which transduces the adrenergic signals, changes its subunit composition (Reddy et al. 1979; Smith and Clarc 1980; Smith 1984; Pertseva 1989, 1991). It has been shown that the protein subunits which make up this system develop heterochronously and independently of each other (Pertseva 1989). These findings arouse interest in the study of adrenergic effects during early development stages of skeletal muscle cells.

In the present study an attempt was made to disclose the effect of adrenergic modulation upon voltage-dependent Ca^{2+} channel activity in muscles during myogenesis in culture.

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 the 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 2 to 10-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 the 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).

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): CsCl 60; TEACl 50; $CaCl_2$ 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. Adrenaline (Sigma), β-adrenoreceptor agonist isoproterenol (Sigma), and β-adrenoblocker obsidan (Germed) were added to the external solution.

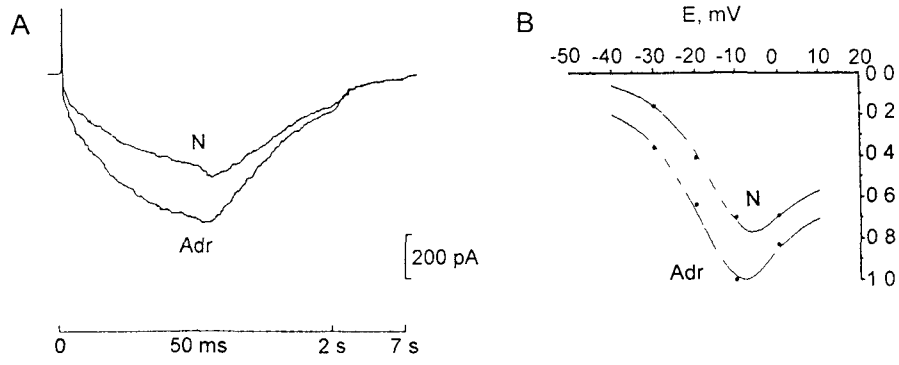

Figure 1. Effect of Adr on slow calcium current in frog myocytes. *A*. Current traces (after leak subtraction) elicited by test potentials to −10 mV before (N) and after the addition of 2 μmol/l Adr to the standard external recording solution. Holding potential (*HP*) −80 mV. Until 50 ms of stimulation the sampling frequency was 10 kHz, between 50 ms and 2 s 100 Hz, after 2 s 10 Hz; accordingly, the time scale is linear below 50 ms, between 50 ms and 2 s, and beyond 2 s. *B*. The peak current-voltage relationships for the experiment. The curves show peak amplitudes of slow calcium current before (N) and after (Adr) application of 2 μmol/l Adr. The ordinate represents peak current amplitude, and the abscissa shows the test potential value (*E*). Seven-day-old culture; myocyte N 24.04.93.5.

Results

In our experiments the adrenoreceptor agonists adrenaline (Adr), and isoproterenol (Isp) in concentrations 0.1–10 μmol/l increased the amplitude of the DHP-sensitive slow calcium current ($I_{Ca,s}$). From day 2 to 6 after myoblast plating Adr ($n = 5$), and Isp ($n = 7$) did not change the amplitude of $I_{Ca,s}$ at all ($n = 5$) or slightly increased it. The average positive effect was 16.3% ($n = 12$) taking into account the rundown for $I_{Ca,s}$. However, from day 7 after plating the reaction of $I_{Ca,s}$ to Adr ($n = 2$), and Isp ($n = 2$) was observed in all cases and was more expressed (average 70.8%). Fig. 1 shows the effect of 2 μmol/l Adr on $I_{Ca,s}$ on a 7-day-old myocyte. The peak current-voltage relationships display amplitude increase but without taking into account the rundown. Fig. 2 shows the peak $I_{Ca,s}$ amplitude changing during the experiment (9-day-old myocyte) and upon the application of Isp and Obs in concentrations 1 μmol/l. The diagram (Fig. 2 *C*) allows account for the rundown and shows approximately twofold amplitude increase.

The fast calcium current ($I_{Ca,f}$) under Adr ($n = 2$), and Isp ($n = 2$) action showed the same changes along the stages of development which were described above for $I_{Ca,s}$ (days 2–10 after plating). Fig. 3 shows the increase of $I_{Ca,f}$ after the addition of 1 μmol/l Isp, and 1 μmol/l Obs to the external recording solution. The diagram of peak $I_{Ca,f}$ amplitude (Fig. 3*C*) allows to appreciate the rundown part. The effects of Adr and Isp increased with the age of the culture from 0–20% (day 2 after plating) to 108% (10-day-old culture).

From the results shown above the unusual effect of the adrenoblocker obsidan is clearly seen (Figs. 2, 3). The application of Obs in concentrations 1–10 μmol/l after Adr or Isp resulted in an additional increase of $I_{Ca,s}$ ($n = 9$) or $I_{Ca,f}$ ($n = 3$) amplitude in all cases and in the same order of magnitude. For $I_{Ca,s}$ the average amplitude increase was 17% before day 7 of culturing and 77% from day 7 to 10.

The speed of activation (half time to peak, $t_{0\,5}$) and inactivation time constant (τ_{in}) for all types of the currents were only slightly changed by all the substances examined (Figs. 1–3) and these changes were negligible.

Discussion

Our data show that the application of adrenergic agents resulted in slight enhancement before day 7 after myoblast plating, and in a marked enhancement of both the fast and the slow calcium currents in 7-10-day-old myocytes. All effects were seen with strong rundown described earlier for $I_{Ca,s}$ and $I_{Ca,f}$ in mature skeletal muscle fibers and embryonic myocytes (Cota and Stefani 1986; Beaty et al. 1987; Garcia and Stefani 1987; Somasundaram and Tregear 1993; Lukyanenko et al. 1994). Because of the rundown the effects could not be accurately estimated. As a rule, before day 7 the amplitude of I_{Ca} after addition of the hormone was

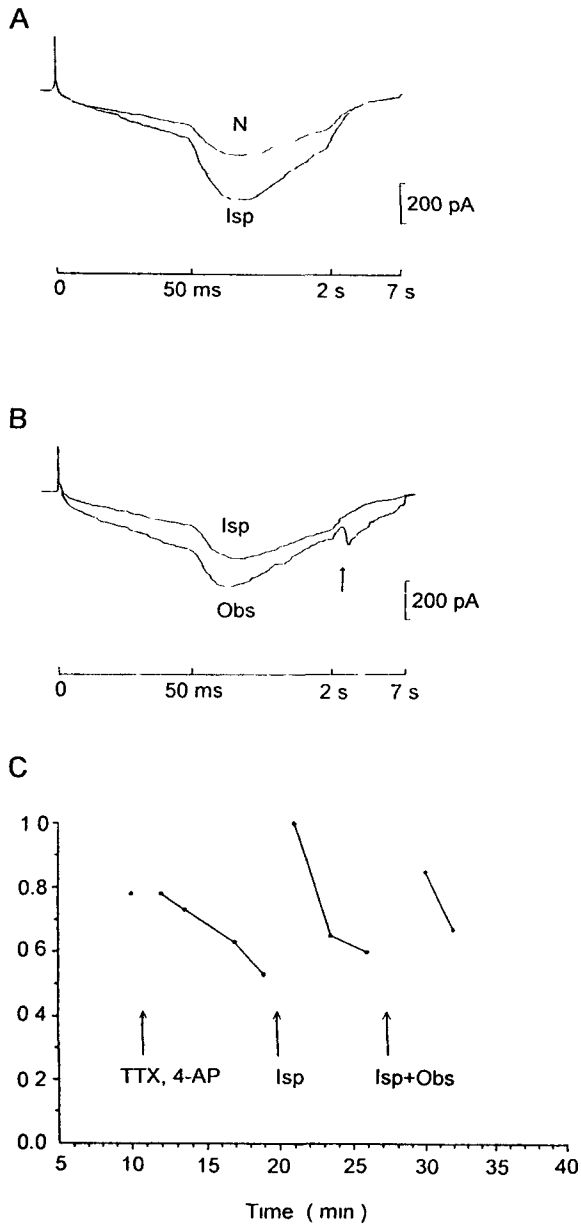

Figure 2. Effect of Isp and Obs on slow calcium current in frog myocytes. *A*, *B*. Current traces (after leak subtraction) elicited by test potentials to −20 mV before (N) and after the addition of 1 μmol/l Isp (*A*: Isp) to the standard external recording solution, and before (*B*: Isp) and after the application of 1 μmol/l obsidan (*B*: Obs). *HP* −80 mV; time scale as in Fig. 1; 9-day-old culture; myocyte N 19.05.93.1. *C*. Diagram of changes in slow calcium current peak amplitude as a function of time, and after the application of Isp and Obs at −20 mV test potential. The ordinate represents peak current amplitude, and the abscissa shows time from the beginning of the experiment.

not higher than before the application, and the effect could only be appreciated roughly assuming linear nature of the rundown process. However even with taking into account the rundown, the increment of I_{Ca} amplitude after the Adr or Isp

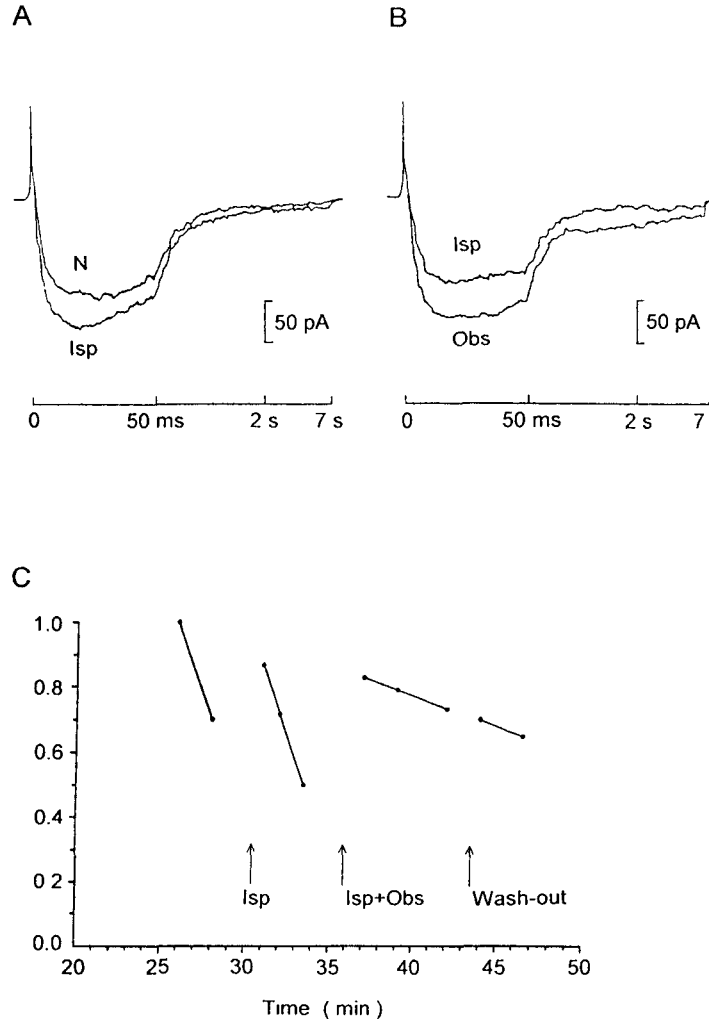

Figure 3. Effect of Isp and Obs on fast calcium current in frog myocytes. *A*, *B*. Current traces (after leak subtraction) elicited by test potentials to −20 mV before (N) and after the addition of 1 μmol/l Isp (*A*: Isp) to the standard external recording solution, and before (*B*: Isp) and after the application of 1 μmol/l obsidan (*B*: Obs). *HP* −80 mV; time scale as in Fig. 1; 10-day-old culture; myocyte N 20.05.93.1. *C*. Diagram of changes in fast calcium current peak amplitude as a function of time, and after the application of Isp and Obs at −20 mV test potential. The ordinate represents peak current amplitude, and the abscissa shows time from the beginning of the experiment.

application in concentrations as above was not seen in 40% of myocytes examined before day 7 after plating.

From day 7 to 10, all cells examined showed large I_{Ca} amplitude increments after Adr or Isp application. Similar positive effects of Adr, Isp, and cAMP were described for $I_{Ca,s}$ and $I_{Ca,f}$ in mature skeletal muscle fibres of frog (Arreola et al. 1987; Garcia et al. 1990; Kokate et al. 1993). However, we did not record any changes in the time constants of I_{Ca} ($t_{0.5}$ and τ_{in}). This is similar to the results reported by Somasundaram and Tregear (1993) in differentiating rat skeletal muscle cells, and is in contrast with that in mature frog muscle fibre (Arreola et al. 1987; Kokate et al. 1993). It may be a result of immaturity of the β-adrenergic regulatory system of the myocytes, although the stage when β-adrenergic stimulation effects were recorded *in vitro* corresponded to *in vivo* developmental stage of mature skeletal muscle fibres (Lukyanenko et al. 1993).

The appearance of voltage-dependent channels and an increase in channel density in the myocyte membrane during myogenesis in culture was shown by several authors (DeCino and Kidokoro 1985; Moody-Corbett et al. 1989; Lukyanenko et al. 1993). The percentages of cells with sodium, calcium and potassium currents increased as well as did the whole-cell current amplitude, with the cell culture age. Our data show an increase of the Adr and Isp effect on calcium channels during myogenesis. Especially strong effect was recorded for all myocytes examined after day 6 of culturing. We can suggest that β-adrenergic receptors appear in the myocyte membrane during days 6–7 after myoblast plating. Our previous data (Lukyanenko et al. 1993) and those of other investigators (Schmid et al. 1984; Amagai and Kasai 1989; Zemková et al. 1989; Arai et al. 1992; etc.) show that on day 6–7 of muscle culturing the protein synthesis reaches maximum intensity, and the process of channel and receptor formation and accumulation is terminated. It is known that the β-adrenergic receptor – adenylate cyclase system is a molecular complex which comprises several subunits. This system develops gradually during the period of muscle maturation, and its subunits arise asynchroneously (Reddy et al. 1979; Smith and Clark 1980; Smith 1984; Pertseva 1989). It can be proposed that the unusual effect of obsidan was a result of the immaturity of the β-adrenergic regulatory complex as a whole, or of the immaturity of the G_i protein complex. The latter differs from the G_s protein by the masses of the respective subunits (β and γ) (Northup 1985), and we can propose that the time of the G-protein complex maturation can vary. Unusual inhibitory effects of G-proteins connected with the β-adrenergic receptor upon the slow calcium channels during early stages of myogenesis were shown by Somasundaram and Tregear (1993) in rat skeletal myocytes.

In conclusion, our results indicate that: 1) the β-adrenergic regulatory complex can stimulate the activity of the fast and the slow voltage-dependent calcium channels of the frog skeletal myocytes; 2) there is a distinct developmental stage at which a functioning β-adrenergic regulatory complex appears in the membrane of skeletal myocytes.

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