

Aldosterone- and testosterone-mediated intracellular calcium response in skeletal muscle cell cultures

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Estrada, Manuel, José Luis Liberona, Manuel Miranda, and Enrique Jaimovich. Aldosterone- and testosterone-mediated intracellular calcium response in skeletal muscle cell cultures. *Am J Physiol Endocrinol Metab* 279: E132–E139, 2000.—Fast nongenomic steroid actions in several cell types seem to be mediated by second messengers such as intracellular calcium ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate (IP_3). We have shown the presence of both slow calcium transients and IP_3 receptors associated with cell nuclei in cultured skeletal muscle cells. The effect of steroids on $[Ca^{2+}]_i$ was monitored in Fluo 3-acetoxymethyl ester-loaded myotubes by either confocal microscopy or fluorescence microscopy, with the use of out-of-focus fluorescence elimination. The mass of IP_3 was determined by radioreceptor displacement assay. $[Ca^{2+}]_i$ changes after either aldosterone (10–100 nM) or testosterone (50–100 nM) were observed; a relatively fast (<2 min) calcium transient, frequently accompanied by oscillations, was evident with both hormones. A slow rise in $[Ca^{2+}]_i$ that reached its maximum after a 30-min exposure to aldosterone was also observed. Calcium responses seem to be fairly specific for aldosterone and testosterone, because several other steroid hormones do not induce detectable changes in fluorescence, even at 100-fold higher concentrations. The mass of IP_3 increased transiently to reach two- to threefold the basal level 45 s after addition of either aldosterone or testosterone, and the IP_3 transient was more rapid than the fast calcium signal. Spironolactone, an inhibitor of the intracellular aldosterone receptor, or cyproterone acetate, an inhibitor of the testosterone receptor, had no effect on the fast $[Ca^{2+}]_i$ signal or in the increase in IP_3 mass. These signals could mean that there are distinct nongenomic pathways for the action of these two steroids in skeletal muscle cells.

steroid hormones; inositol 1,4,5-trisphosphate; calcium waves; nongenomic pathway

STEROID HORMONES ARE CAPABLE of producing rapid (within 2 min) effects in several cell types (12, 22, 36). These rapid responses are not compatible with the classical mechanism of action proposed for these hormones, which involves binding to intracellular receptors, transcriptional processes, and protein synthesis (1, 20). Thus, for rapid steroid effects, the existence of mem-

brane receptors for steroids has been proposed (2, 37). In skeletal muscle, steroid hormones have long been known to modulate gene expression, and hormones like testosterone have been related to both muscle hypertrophy (4, 25) and upregulation of a number of proteins (31). In particular, glucocorticoids and mineralocorticoids have been related to up- and downregulation, respectively, of sodium pumps in skeletal muscle (10), implying the presence of steroid hormone receptors in muscle cells. Because all these effects have been the result of chronic (hours to days) treatments, it would be interesting to know whether exposure to these hormones is also accompanied by fast nongenomic events.

The signal transduction mechanism for the rapid action of steroids in some cellular models could be regulated by second messengers such as intracellular calcium ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate (IP_3). These signal pathways seem to be particularly important in mediating the actions of aldosterone (9, 15, 39), testosterone (2, 19), 17β -estradiol (26), and vitamin D (35). They have also been associated with the fast effects of these hormones in various cells, which are characterized by fast transient increases in free cytosolic calcium levels and in some cases by a rise in IP_3 after exposing the cells to steroids (9, 19). In skeletal muscle cells, $[Ca^{2+}]_i$ regulates contractile activity as well as different energetic pathways. Moreover, it may stimulate cell proliferation and maturation of new fibers (23), and it is suggested that the increase in the muscular mass of sportsmen under constant physical activity is regulated, at least in part, by changes in $[Ca^{2+}]_i$ as well as by plasma steroid levels (32, 34). In chicken skeletal muscle cells, 1,25-dihydroxyvitamin D_3 induces calcium release from intracellular stores, as well as its influx through plasma membrane calcium channels (27, 35).

Skeletal muscle fibers have been shown to possess the basic biochemical machinery for generation and catabolism of inositol phosphates (7, 14, 16, 28), and cultured myotubes have IP_3 receptors associated with cell nuclei (17). Nevertheless, neither the role nor the regulation of this second messenger pathway has been

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elucidated in this tissue. The skeletal muscle responds to exercise with a series of adaptive processes that include hypertrophy, activation of oxidative mechanisms, or metabolic changes (5). The mechanism responsible for these changes is little known; nevertheless, it is known that steroid hormones are important metabolism regulators, some examples of which are growth, reproduction, exercise, and nutrient utilization. These hormones also play roles in metabolic compensation during disease (31). Some steroid hormones, like testosterone, have well-known anabolic effects (25). Little is known about the steroid hormone effects in skeletal muscle, and the fast effects of gonadal steroids (progesterone, estradiol, and testosterone) or adrenal steroids (glucocorticoids and mineralocorticoids) on muscle cells have not been described; neither has a clear role for steroid hormones during muscle development been demonstrated. Skeletal muscle myoblasts are the precursors to skeletal muscle fibers, and during the differentiation process they fuse into multinucleated myotubes that express many skeletal muscle-specific markers (7, 17). Thus we have chosen to study the early effects of different steroid hormones on $[Ca^{2+}]_i$ levels in skeletal muscle cell cultures, analyzing both the spatial and the time distributions of these signals, as well as any possible relation with intracellular IP_3 concentration.

MATERIALS AND METHODS

Hormones. Aldosterone (11 β ,18-epoxy-18,21-dihydroxy-4-pregnene-3,20-dione), 17 β -estradiol (3,17 β -dihydroxy-1,3,5[10]-estratriene), progesterone (4-pregnene-3,20-dione), dexamethasone (9 α -fluoro-16 α -methylprednisolone), testosterone (4-androsten-17 β -ol-3-one), spironolactone (7 α -[acetylthio]-17 α -hydroxy-3-oxopregn-4-ene-21-carboxylic acid γ -lactone), and cyproterone acetate (6-chloro-1 β ,2 β -dihydro-17-hydroxy-3' H -cyclopropa[1,2]-pregna-1,4,6-triene-3,20-dione acetate) were purchased from Sigma Chemical (St. Louis, MO). All steroid hormones were dissolved in ethanol, the final concentration of which never exceeded 0.01%. This ethanol concentration had no effect on $[Ca^{2+}]_i$ concentration or IP_3 mass determination.

Cell cultures. Neonatal rat myotubes kept in primary culture were used for measurement of $[Ca^{2+}]_i$ levels and variations in IP_3 mass, as described previously (18, 30). Briefly, myoblasts were obtained from hindlimbs of 12- to 24-h-old newborn rat embryos. After dissection, the tissue was mechanically dispersed and then treated with 0.2% (wt/vol) collagenase for 15 min at 37°C under mild agitation. The suspension was filtered through nytex membranes or lens tissue paper and spun down at low speed. After 45 min of preplating onto a 150-mm dish to partially eliminate the faster sedimenting fibroblasts, cells were plated onto round coverslips at a density of $\sim 350 \times 10^3$ per dish (35 mm) for cytosolic calcium fluorescence measurements or 9.5×10^5 per dish (60 mm) for IP_3 radioreceptor assay determination. Culture medium was DMEM/F12, 10% bovine serum, 2.5% fetal calf serum, 100 mg/l penicillin, 50 mg/l streptomycin, and 2.5 mg/l amphotericin B. To eliminate remaining fibroblasts, 10 μ M of cytosine arabinoside were added on the third day of culture for 36 h. The medium was then replaced with a cytosine arabinoside-free medium with a lower fetal calf serum concentration (1.8%). Myotubes, some of them spontaneously contracting, with an estimated purity of >90%,

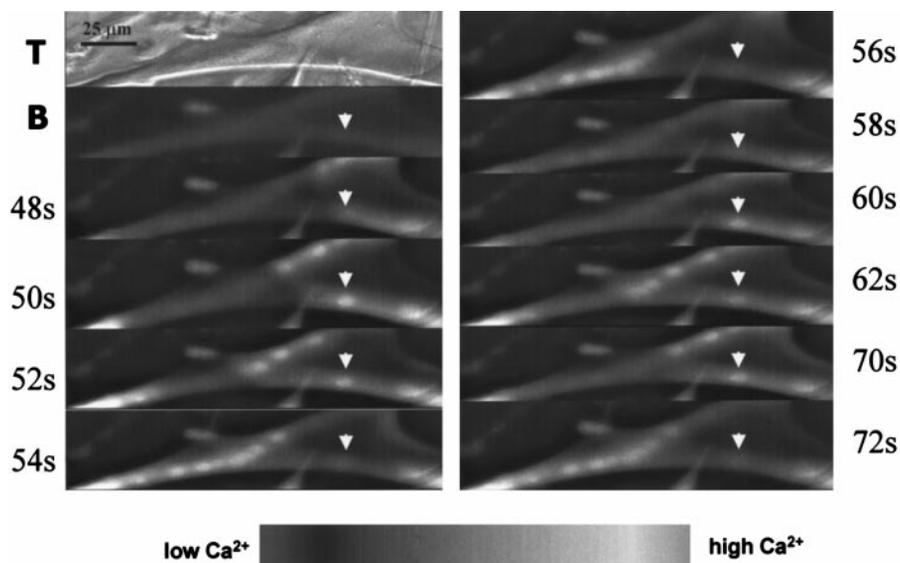
were visible after the fifth day of culture. Unless otherwise indicated, we used 6- to 8-day-old cultures exhibiting a fairly homogeneous population of myotubes with central nuclei and measuring 200–300 μ m in length and 20–40 μ m in width, corresponding to young, not fully differentiated cells.

$[Ca^{2+}]_i$ measurement. Cytosolic calcium images were obtained from single noncontracting myotubes preloaded with Fluo 3-acetoxymethyl ester (Fluo 3-AM; Molecular Probes, Eugene, OR) by means of an inverted confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystems) or a fluorescence microscope (Olympus Diaphot-TMD, Nikon) equipped with a cooled CCD camera and image acquisition system (Spectra Source MCD 600). Myotubes were washed three times with Krebs buffer [(in mM) 145 NaCl, 5 KCl, 2.6 CaCl₂, 1 MgCl₂, 10 Hepes-Na, 5.6 glucose, pH 7.4] to remove serum and were loaded with 5.4 μ M Fluo 3-AM, coming from a stock in 20% pluronic acid-DMSO for 30 min at room temperature. After loading, myotubes were washed with the same buffer and used within 2 h. The cell-containing coverslips were mounted in a 1-ml-capacity plastic chamber and placed in the microscope for fluorescence measurements after excitation with a 488-nm wavelength argon laser beam or filter system. Hormones were added directly, or the solution was fast-changed (1 s) in the chamber. The fluorescent images were collected every 0.4–2.0 s for fast signals and analyzed frame by frame with the image data acquisition program (Spectra Source) of the equipment. An objective lens (PlanApo 60X, numerical aperture 1.4) was generally used. In most of the acquisitions, the image dimension was 512×120 pixels. $[Ca^{2+}]_i$ was expressed as a percentage of fluorescence intensity relative to basal fluorescence (a value stable for ≥ 5 min in resting conditions). The fluorescence intensity increase is proportional to the rise in intracellular calcium level (24).

Digital image processing. Elimination of out-of-focus fluorescence was performed by software. Both the “no-neighbors” deconvolution algorithm and Castleman’s PSF (point spread function) theoretical model were used (8). Complementary to restoration methods, a procedure was created to segment the images. To section an image, an initial contour can be entered manually, and a recursive algorithm that adapts automatically to the region of interest (adaptable contour) can be applied. Each contour is defined by an ordered collection of n points: $V = \{v_1 \dots v_n\}$, $v_i = \{x_i, y_i\}$, $i = \{1 \dots n\}$. For each point, v_i , an energy matrix, is computed: $E(v_i) = \alpha E_{Cont}(v_i) + \beta E_{IntMed}(v_i) + \chi E_{GradMed}(v_i)$. $E_{Cont}(v_i)$ is a “continuity energy” function that influences the contour to take the form of a circle for a closed contour; $E_{IntMed}(v_i)$ is an “average intensity” energy function that influences the contour to move to regions of intensity equal to the average intensity of the initial contour; $E_{GradMed}(v_i)$ is an “average gradient” energy function that influences the contour to move to regions of gradient equal to those of the initial contour. Each v_i is moved iteratively to the point of minimum energy in its surroundings. To quantify fluorescence, the summed pixel intensity was calculated on the section delimited by a contour. As a way of increasing the efficiency of these data manipulations, action sequences were generated. To avoid possible interference in the fluorescence by steroid effects on the cellular volume, the area of fluorescent cell was determined by image analysis with the use of adaptable contour and then creating a binary mask, which was compared with the bright-field image.

Stimulation of IP_3 production. Cells were rinsed and preincubated at room temperature for 20 min with a “resting solution” of the following composition (in mM): 58 NaCl, 4.7 KCl, 3 CaCl₂, 1.2 MgSO₄, 0.5 EDTA, 60 LiCl, 10 glucose and 20 Hepes, pH 7.4. Next, cells were stimulated by fast (1 s)

Fig. 1. Series of Fluo 3 acetoxymethyl ester (Fluo 3-AM) fluorescence images in skeletal muscle cells in primary culture taken at the times indicated, before and after addition of 100 nM of aldosterone. A fast and transient increase in intracellular calcium ($[Ca^{2+}]_i$) in the myotubes loaded with Fluo 3-AM (5.4 μ M for 30 min) is observed. T, transmitted image; B, basal fluorescence. Arrows show fluorescence changes in a single nucleus. Note the oscillatory nature of the signal.



replacement of the resting solution by a solution containing the hormone. At the times indicated, the reaction was stopped by rapid aspiration of the stimulating solution, addition of 0.8 M ice-cold perchloric acid, and freezing with liquid nitrogen. Samples were allowed to thaw, and cell debris was spun down for protein determination. The supernatant was neutralized with a solution of 2 M KOH, 0.1 M MES, and 15 mM EDTA. The neutralized extracts were frozen until required for IP_3 determination.

IP_3 mass determination. Measurements of IP_3 mass were made by radioreceptor assay (6). Briefly, a crude rat cerebellum membrane preparation was obtained after homogenization of tissue in 50 mM Tris \cdot HCl, pH 7.7, 1 mM EDTA, and 2 mM β -mercaptoethanol and centrifugation at 20,000 g for 15 min. This procedure was repeated 3 times, suspending the final pellet in the same solution plus 0.3 M sucrose and freezing it at -80°C until required for use. The rat cerebellum membrane preparation was calibrated for IP_3 binding with 1.6 nM [^3H] IP_3 (Du Pont, Boston, MA) and 2–120 nM cold IP_3 (Sigma) with sample analysis performed in a similar way but replacing cold IP_3 with an aliquot of the neutralized supernatant. [^3H] IP_3 radioactivity, which remained bound to membranes, was measured in a Beckman LS-6000TA liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA).

Statistics. All data are expressed as means \pm SD. Differences between basal and poststimulated points were determined by use of a paired Student's t -test. $P < 0.05$ was considered statistically significant.

RESULTS

Aldosterone and testosterone effects on intracellular calcium. Different concentrations of steroid hormones were used for a systematic study of its effects on $[Ca^{2+}]_i$. For aldosterone, in most experiments, 100 nmol/l were used, because more than one-half of the cells responded to this concentration. Of a total of 56 experiments and by use of 31 different cultures, 64% gave a clear positive response to aldosterone stimulation. A response to lower aldosterone concentrations (50 and 10 nmol/l) was less frequent, and the use of higher concentrations (which did produce a response in

all cases) increases the risk of unwanted nonspecific interactions. The effect of 100 nmol/l of aldosterone on skeletal muscle cells in primary culture was observed as an increase in $[Ca^{2+}]_i$. This increase displayed two different kinetics.

The first was a rapid response, a transient increase in $[Ca^{2+}]_i$ that appeared within the first min of hormone exposure and decayed within 5 min (see Figs. 1 and 2). Part of the fast response is illustrated in Fig. 1. In a representative experiment, aldosterone was added to the incubation chamber 46 s before the acquisition of the first image shown. Increased fluorescence was seen in certain regions of the myotube (48 s, right end, near the plasma membrane), where some nuclei are located. The fluorescence then slowly propagated toward the other end of the myotube (Fig. 1, *left panel*) and the string of nuclei became distinctly bright. After the first wave faded (58 s), a second similar wave developed (Fig. 1, *right panel*). As increased $[Ca^{2+}]_i$ could produce either contraction or changes in the cell volume (33), the latter was monitored by measuring both the position and the diameter of the fluorescence image compared with the bright-field image for each cell. The observed increase in $[Ca^{2+}]_i$ upon addition of either hormone did not produce contraction of able myotubes, nor did it produce any significant area (volume) reduction, indicating that the cytosolic calcium increase must be very low. The increase of $[Ca^{2+}]_i$ was unchanged when the myotubes were previously incubated with 1 mM of spironolactone, an antagonist of the classical intracellular receptor for mineralocorticoids (not shown). A more detailed study of the early response shows that the calcium signals oscillated in a given spot (Fig. 2). In the series of fluorescence images of the multinucleated myotube shown in Fig. 1, a section of cytosol and two adjacent nuclei, all three normal to the wave front, were delimited with "average intensity" and "average gradient" functions. Within each contour, the summed intensity of all pixels in each

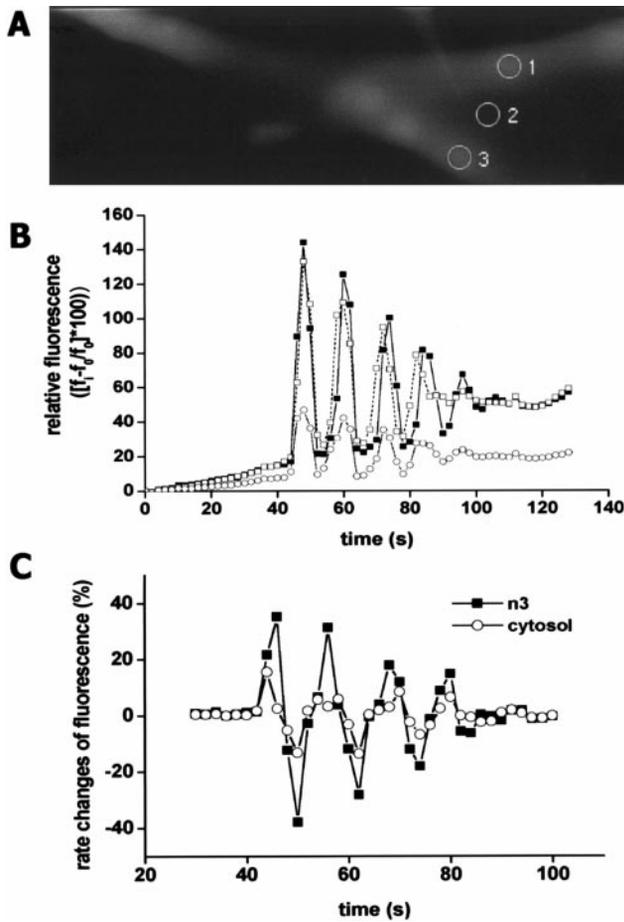


Fig. 2. Fluorescence intensity and spatial distribution of aldosterone-induced calcium oscillations by single-image analysis. *A*: two adjacent nuclei (circles 1 and 3) and one section of cytosol (circle 2) were delimited, and the intensity of all pixels inside these circles was quantified for each image of the acquired series. *B*: observed time displacement between cytosol (○) and nuclear (□ and ■) oscillations. *C*: relative rate of fluorescence changes in the indicated areas for cytosol and one nucleus (circles 2 and 3 from Fig. 2*A*), corresponding to the first time derivative of the fluorescence values.

image of the sequence was calculated. The fluorescence intensity of all pixels inside the preestablished contour was quantified for each one of the images of the acquired series. An oscillation decreasing in fluorescence intensity was observed having an approximate frequency of 0.12 Hz (Fig. 2). A range of 0.1 to 0.2 Hz was obtained for oscillations after aldosterone stimulation. We also observed that relative fluorescence change was much greater in the nucleus than in the cytosol (Fig. 2*B*). Furthermore, the cytosolic and nuclear calcium increases seem to begin simultaneously, but after the cytosolic calcium increase has either reached the fluorescence peak or slowed down significantly, the nuclear signal continues to increase at a high rate, indicating that at least part of the nuclear signal was locally generated. This can be better appreciated if we look at the relative rate of the fluorescence changes in the indicated areas (corresponding to the first derivative from the fluorescence values, Fig. 2*C*). The difference in fluorescence kinetics between these two areas also

indicates some degree of independence between signals making unlikely that either signal could be explained by simple diffusion of calcium from one compartment to the other.

A second response induced by aldosterone was slower and observed as a progressive increase in $[Ca^{2+}]_i$ that reached maximum after 30 min of aldosterone exposure, which was maintained even after 80 min (Figs. 3*A* and 4). Figure 4 shows both the transmitted light and fluorescence images of one myotube 30 min after aldosterone addition, a length of time thus corresponding to the appearance of the slow calcium response. In the transmitted light image (Fig. 4*A*), cell nuclei were seen, which corresponded to discrete zones of observed high fluorescence (Fig. 4*B*), representing increased $[Ca^{2+}]_i$ in these zones. Superimposition of

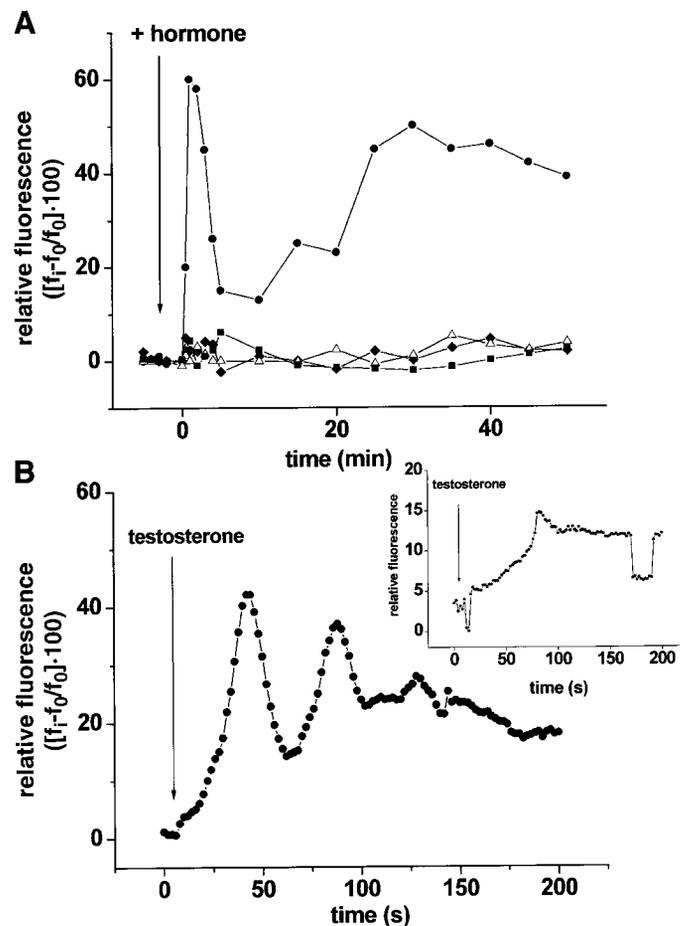


Fig. 3. Time courses of different steroid effects. $[Ca^{2+}]_i$ is expressed as a percentage of fluorescence intensity relative to a basal value of fluorescence. *A*: both the fast transient and the slow calcium responses to aldosterone (●) are shown. After an initial fast response, which peaks at ~3 min, a slow increase in calcium concentration can be seen that reaches maximum 30 min after aldosterone addition and remains high for >20 min. Other steroid hormones, 17β-estradiol (◆, 1 μM), progesterone (△, 1 μM), and dexamethasone (■, 100 nM) do not show an effect on $[Ca^{2+}]_i$ concentration over the time period studied. *B*: testosterone produces an early rapid increase in fluorescence; the response was triggered 15–30 s after testosterone addition and was frequently accompanied by irregular oscillations. *Inset*: the cells were incubated for 2 min with 1 mM EGTA (no calcium added) before adding 100 nM of testosterone.

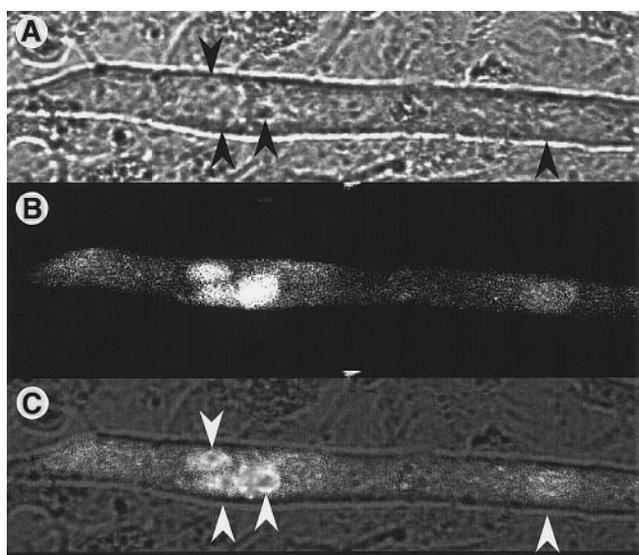


Fig. 4. Myotube localization of the slow calcium rises 30 min after aldosterone addition. The fluorescence image (B) was superimposed (C) over the transmitted light image (A) to show that the presence of high fluorescence, and thus the slow rise in $[Ca^{2+}]_i$, can be localized to the cell nuclei. Arrows indicate the presence of some nuclei visible in the bright-field image. Note that not all the nuclei show a similar increase in the fluorescence.

the bright field and fluorescence images of the same cell (Fig. 4C) clearly localizes the slow fluorescence signal within the cell nuclei. A slow calcium increase, measured as increased fluorescence, was evident in the nuclear regions in 7 out of 11 independent experiments (all presenting a rapid response) in which images were taken over time periods >30 min.

The specificity of this response was investigated by applying several other steroids; testosterone (10–100 nM) also showed an effect on myotubes, characterized by an early increase of $[Ca^{2+}]_i$. The response was triggered 15–30 s after testosterone addition and was frequently accompanied by irregular oscillations with a mean frequency between 0.02 and 0.05 Hz (Fig. 3B). As for the aldosterone-induced responses, the fluorescence increase triggered by testosterone was also evident in both the cytosol and the nuclei (images not shown). No slow responses were observed with this hormone. If the intracellular androgen receptor was responsible for the testosterone-triggered calcium increase, this increase should have been blocked by the antagonist cyproterone acetate. However, the $[Ca^{2+}]_i$ transient triggered by 100 nM of testosterone was not affected by pretreating the myotubes with a high concentration (1 μ M) of cyproterone acetate during 30 min.

The $[Ca^{2+}]_i$ increase was specific to aldosterone and testosterone, not just a response to steroid hormones in general, because other steroids, such as 17 β -estradiol (100 nM to 1 mM), progesterone (100 nM to 1 mM), or dexamethasone (100 nM to 1 mM) had no effect on intracellular calcium levels in myotubes (Fig. 3A). The viability of the myotubes, as well as their capability of calcium release, was determined after hormone treatment by exposure of cells to a high K^+ saline. This

procedure produces both membrane depolarization and release of cytosolic Ca^{2+} and has been described in detail (18), and the magnitude of the relative changes in nuclear and cytosolic fluorescence observed here is similar to those obtained with K^+ depolarization (17).

The increase of $[Ca^{2+}]_i$ can be due to either calcium influx or calcium release from intracellular stores through either ryanodine receptor or IP_3 receptor channels. The effects of aldosterone and testosterone seem to be largely independent of the presence of external calcium. Incubation of myotubes in a calcium-free solution (1 mM EGTA), revealed both the early fast response and the delayed slow response to aldosterone (results not shown). The testosterone-induced response was also still present, although it was diminished in size when the cells were incubated in Ca^{2+} -free medium (Fig. 3B, inset), which could mean that calcium entry plays a role in this particular response. We incubated the cells for 20 min in the presence of 20 μ M ryanodine; the calcium increase produced (not shown) was equivalent to that of the control without ryanodine. This result suggests that ryanodine receptors were not involved in these signals. To explore whether the sensitivity to these hormones was present only during a given developmental period, determinations were made in young (3 days old) and mature (10 days old) myotubes. These cells also show calcium increases in response to these steroid hormones (data not shown).

Effect of aldosterone and testosterone on IP_3 mass. Figure 5B shows the time course of the effect of aldosterone on IP_3 mass in cultured myotubes. The basal value of IP_3 mass (23.6 ± 8.9 pmol/mg protein; $n = 13$) increased significantly after 30 s of hormone exposure (48.2 ± 19.7 pmol/mg protein, $n = 5$; $P < 0.05$). It reached maximum 45 s after hormone addition (53.8 ± 9.7 pmol/mg protein, $n = 4$; $P < 0.01$ vs. basal value). The IP_3 mass then gradually returned to its basal value after 5 min of aldosterone exposure. The increase in the IP_3 mass preceded the fast oscillatory calcium transient (Fig. 5A), which peaks at 50–60 s. Neither the basal value of IP_3 mass nor the increased mass in response to 100 nM of aldosterone was affected when myotubes were incubated for 30 min with 1 mM spironolactone (Fig. 6). Testosterone also produced an increase in IP_3 levels that reached a peak threefold higher than basal level 45 s after hormone stimulation (Fig. 6B). Cultured skeletal muscle cells were incubated for 30 min with cyproterone acetate (1 μ M), an antagonist of classical intracellular receptors for androgens. Preincubation with cyproterone acetate did not modify basal IP_3 mass and did not inhibit the effects of testosterone on intracellular IP_3 levels (Fig. 6B).

DISCUSSION

In this study, we have shown that both aldosterone and testosterone act on cultured skeletal muscle cells, producing fast nongenomic effects that involve intracellular second messengers. Our experimental evi-

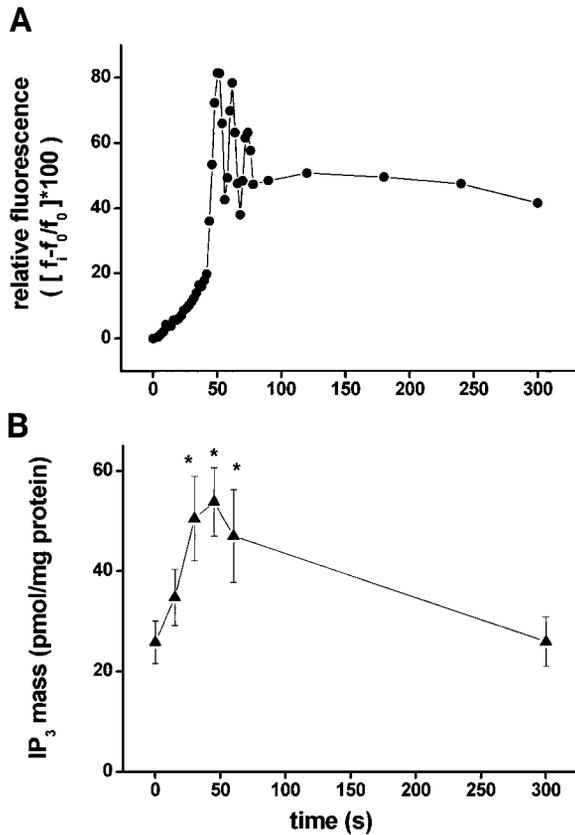


Fig. 5. Time course of aldosterone effects on fluorescence intensity (A) and intracellular inositol 1,4,5-trisphosphate (IP₃) mass (B) in skeletal muscle cell cultures. The IP₃ mass values are means ± SD. The response is significantly different compared with basal values: **P* < 0.05. The rise in the IP₃ mass precedes the fast oscillatory calcium transient.

dence to support this claim includes the following observations. 1) Both aldosterone and testosterone exposure produced a fast but transient increase in intracellular calcium levels in cultured myotubes; the response developed within 1–2 min. 2) Both hormones produced a concomitant increase in the concentration of IP₃. 3) Neither the hormone-induced fast increase in intracellular calcium levels nor the IP₃ increase was changed by antagonists for intracellular steroid hormone receptors.

Aldosterone appears to increase [Ca²⁺]_i in a two-stage process. The classical slow mechanism of action for steroid hormones (genomic pathway) probably involves transcription and subsequent protein synthesis within the cell (1, 20). A slow calcium increase, such as what we see after 30 min of aldosterone exposure, could be produced after early gene expression. However, within the first minute of either aldosterone or testosterone exposure, myotubes exhibit a rapid, transient increase in both cytosolic and nuclear calcium. Because we are not using a ratiometric dye and because the dye may be compartmentalized, higher fluorescence in the nucleus does not necessarily mean higher calcium concentration in this compartment. An increase in [Ca²⁺]_i has been described previously in other cells, including human mononuclear lympho-

cytes (9), smooth muscle cells, endothelial cells (38, 39), and renal epithelial cells (15). Those responses were not blocked by inhibitors of either protein synthesis or transcription (36). The fact that neither spironolactone nor cyproterone acetate was able to block the calcium and IP₃ increases induced by aldosterone or testosterone could indicate that the classical intracellular receptors for these steroids do not participate in these effects. Fast cellular responses not linked to intracellular receptors to steroids have been described in other systems, and the presence of membrane receptors for these hormones has been suggested (2, 9, 19, 36). It is interesting that the effects of either aldosterone or testosterone on skeletal muscle cells seem to be rather specific, because other steroid hormones, such as cortisol, 17β-estradiol, progesterone, or dexamethasone in up to 100-fold concentrations, have no effect on this

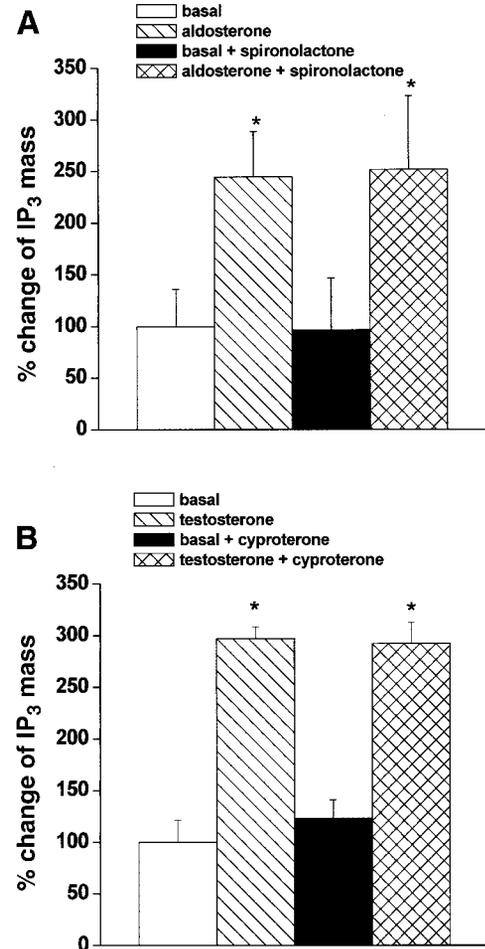


Fig. 6. Effect of steroids and their antagonists on IP₃ mass in rat myotubes. Seven-day-old myotubes were incubated for 30 min with or without the indicated antagonist of the intracellular receptor as indicated. A: bars represent the mass of IP₃ in the control condition (basal), no hormone added; aldosterone (100 nM) was added for 30 s before IP₃ mass measurement in the presence and in the absence of 1 mM of spironolactone. B: a similar experiment, in which bars represent the control and testosterone-stimulated (100 nM) condition both in the presence and in the absence of 1 μM of cyproterone acetate. Bars represent means ± SD of 3 experiments performed in triplicate. **P* < 0.05, Student's *t*-test.

system. Rapid calcium responses to these hormones have nevertheless been described in other cell types (12, 13, 22).

In myotubes, a rapid increase in fluorescence representing increased $[Ca^{2+}]_i$ occurs as oscillations. We have shown that, after potassium depolarization, a row of nuclei frequently oscillates in sequence, originating a propagated wave (17), probably caused by a spread of the response between cell nuclei. It is interesting to note that in the present study, the frequency of oscillations differs in cells stimulated with aldosterone and testosterone. A different oscillatory pattern may mean somewhat different mechanisms of calcium release and recapture, as well as different functions for calcium as intracellular signal.

Some studies using different cell models suggest that steroid hormones could stimulate external calcium entry through voltage-dependent calcium channels such as the L-type channel (19, 35) and thus modulate $[Ca^{2+}]_i$ levels by a mechanism involving calcium entry. On the other hand, Passaquin et al. (29) have shown that aldosterone treatment of C₂C₁₂, a skeletal muscle cell line, causes a decrease in calcium influx. In our experimental system, the hormone-induced calcium transients, although diminished, were not abolished in calcium-free media, meaning that at least part of the hormone-induced signal we see is independent of external calcium.

It has been suggested that in some cases, the early nongenomic mechanism of steroids action involves a transient increase in $[Ca^{2+}]_i$ levels, possibly as a consequence of an increased turnover of inositol phosphates to produce IP₃ (9). Mobilization of Ca²⁺ from intracellular stores is mediated by channel receptors for IP₃ (IP₃R) and ryanodine (3). The transient and rapid increase of $[Ca^{2+}]_i$ levels produced by aldosterone and testosterone in myotubes seems to correlate well with an increase in IP₃, because this messenger reaches maximal levels after 45 s of exposure to the steroids. It is unlikely that contaminant fibroblasts would contribute to the IP₃ change seen in the culture, because their IP₃ content is low (7) and their proportion in the culture highly variable (0 to 10%). Very fast transient (in milliseconds) release of $[Ca^{2+}]_i$ in myotubes probably reflects the physiological excitation-contraction coupling (3, 17). In skeletal muscle, this rapid calcium release during excitation-contraction coupling has been attributed to ryanodine receptors (23); however, for a slower calcium signal (in seconds), a possible role of calcium as an intracellular messenger in processes other than muscle contraction needs to be considered (14, 17, 28). Thus slow calcium waves elicited by steroid hormones via increase in intracellular IP₃ and associated with increased nucleoplasmic calcium may have an important role in the integration and regulation of several processes during muscle function. Our own studies have shown that cultured skeletal muscle cells respond to different stimuli with an increase in perinuclear calcium levels, which are associated with an increase in IP₃ mass (30) and nuclear IP₃Rs (17). The fact that the steroid hormone-induced

calcium response was observed precisely in the nuclear region of the cell suggests that these processes may share a common mechanism of action.

Very little is known of the steroid hormone effects on targets other than those classically described. Early increased plasma aldosterone levels have been found after exhaustive exercise and have been correlated with workload. This should reflect an increased volume demand and the need of fast electrolyte regulation (34). The rapid Ca²⁺ response produced by aldosterone in cultured myotubes suggests some influence of these hormones on this particular cell type, which functions via a cell signaling pathway, with both calcium and IP₃ working as intracellular messengers. Accordingly, a transient increase in calcium should affect certain cellular processes directly (nongenomic pathway); alternatively, they could act through Ca²⁺ binding proteins to regulate slower processes (genomic pathway) via protein synthesis. Recent reports state that both cortisol and aldosterone could alter the Na⁺-K⁺ pump concentration in skeletal muscle (10). Zange et al. (40) observed rapid changes in phosphocreatine levels in response to aldosterone in human calf muscle in vivo, when the individual was subjected to physical exercise. The concentrations of hormones used in this study (particularly aldosterone) are higher than those reported in normal resting conditions; nevertheless, under certain physiological conditions like intense exercise, aldosterone concentrations increase, reaching nanomolar values in humans (34). It is possible, then, that the signals we see could correspond to normal responses of the muscle cell to transient levels of these hormones that may be reached under some particular condition.

It has been suggested that the anabolic androgens could directly mediate cardiac (21) and skeletal (25) muscle hypertrophy. It is known that exercise (5), growth factors (32), and anabolic drugs (4) may induce muscle hypertrophy, although probably by use of different pathways. It is interesting to note that some of these effects appear to be mediated by calcium at the cellular level (11, 32). On the other hand, calcium mobilization by external stimuli may be an important signal mediating some muscle cell functions during development, such as cell proliferation, fiber differentiation, activation of metabolic responses, and contraction (23). In cultured skeletal muscle cells, the steroid hormone 1,25-dihydroxyvitamin D₃ produced a rise in $[Ca^{2+}]_i$ by promoting a nongenomic release of Ca²⁺ from intracellular stores via activation of phospholipases C and D and IP₃ (27) and by calcium influx through L-type and store-operated Ca²⁺ channels (35). Our model system (cultured myotubes), being basically a developing muscle cell, does not allow for distinguishing between responses important for development and those that may be present in adult muscle. Further studies are certainly required to ascertain the exact role of fast responses to both aldosterone and testosterone in muscle cells. The putative processes regulated by $[Ca^{2+}]_i$ as a messenger in both developing and mature muscle cells are yet to be determined.

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