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Cytosolic Ca^{2+} alteration mediates both ryanodine receptor and IP_3 receptor in TE671/RD cells

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ABSTRACT

TE671/RD is a cell line obtained from the rhabdomyosarcoma cells. In the present study, we examined $[Ca^{2+}]_i$ alteration induced by acetylcholine (ACh) in TE671/RD cells with the special attention to ryanodine receptor (RyR) and inositol 1,4,5-trisphosphate receptor (IP_3R). The change of $[Ca^{2+}]_i$ was mediated by muscarinic type 3 (m3) AChR. Both phosphatidylinositol-specific phospholipase C blocker (U73122) and IP_3R blocker (2-APB) inhibited ACh-induced $[Ca^{2+}]_i$ elevation, suggesting that IP_3 pathway is involved in $[Ca^{2+}]_i$ alteration. Ryanodine and FK506 increased ACh-induced $[Ca^{2+}]_i$ elevation, which was decreased by RyR blocker (ruthenium red). These results suggest that RyR and FK506 binding protein 12 kDa (FKBP12) complex was involved in $[Ca^{2+}]_i$ change, and that TE671/RD cell line has a hybrid characteristic of smooth and skeletal muscles.

TE671/RD is a cell line obtained from the rhabdomyosarcoma cells (13). The functional studies and cDNA sequence data showed that TE671/RD cells expressed both nicotinic acetylcholine receptor (AChR) (11) and muscarinic AChR of m3 type (2).

Ca^{2+} is an important intracellular messenger that mediates skeletal muscle contraction (9). At neuromuscular junction, acetylcholine (ACh) is released from the pre-synaptic membrane and opens the nicotinic AChR on the post-synaptic membrane (14). Opening of the receptor induces Na^+ influx and K^+ efflux, followed by release of Ca^{2+} from sarcoplasmic reticulum (SR) as a consequence of depolarization of the cell membrane. In this system, L-type voltage-gated calcium channel (VGCC) on transverse tubules and ryanodine receptor type 1 (RyR1) on SR play an important role. They are combined together on a foot structure, which induces voltage-induced Ca^{2+} release (VICR) (9). In smooth muscles, on the other hand, ACh binds to muscarinic AChR which makes a triplet structure with GTP-binding protein and phosphatidylinositol-specific phospholipase C (PI-PLC) on the cell membrane. PLC converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 receptors (IP_3R) that located on endplasmic reticulum (ER) work as a Ca^{2+} channel. This system is known as IP_3-induced Ca^{2+} release (8). These two systems usually function in skeletal muscle and smooth muscle, respectively.

In human TE671/RD cells, it is reported that muscarinic stimulation increased $[Ca^{2+}]_i$, while nicotinic stimulation did not (5). Electrophysiological experiments revealed, in addition to the nicotinic current, the activation of a K^+-specific current in response to muscarine (4). However, there are only a few reports as to the ACh-induced $[Ca^{2+}]_i$ alteration in TE671/RD cells. In the present study, we exam-

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ined changes of $[Ca^{2+}]_i$ in TE671/RD cells with special attention to RyR and IP_3R.

MATERIALS AND METHODS

Reagents. (±) nicotine, d-tubocurarine, (±) muscarine, atropine, U73122, ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), HEPES, ruthenium red, 2-aminoethoxydiphenyl borate (2-APB), ryanodine and cyclosporine A (CyA) were purchased from SIGMA (St. Louis, MO, USA). FK506 was kindly provided by Fujisawa Pharmaceutical Co. (Tokyo, Japan). Pirenzepine, AF-DX 116, 4-DAMP and tropicamide were purchased from Tocris Cookson Inc. (Ballwin, MO, USA). Fluo-3AM and its solvent Pluronic^R F-127 were purchased form Molecular Probes (Eugene, OR, USA). Nonidet^R P-40 (NP-40) was purchased from Nakalai Tesque (Kyoto, Japan). Dulbecco's Modified Eagle's Medium (DMEM), newborn calf bovine serum (CBS), penicillin and streptomycin were purchased from Gibco BRL, Life Technologies, Inc. (Rockville, MD, USA).

Cell culture. The TE671/RD was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cell line was cultured in DMEM supplemented with 10% CBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere containing 5% CO_2 (v/v). The cells were plated on 96-well plastic plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 5×10^3/mL and cultured for 7 days.

$[Ca^{2+}]_i$ measurement. After the cells were washed 3 times with HEPES-buffered solution (HBS) (140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$, pH 7.3), 50 μL of fluo-3AM diluted in HBS was added to each well (final concentration of 5 μM). Then, the plate was kept in the dark at room temperature. After 1 h, the fluo-3 solution was removed, and then 200 μL of HBS was added to each well. After incubation for 30 min in the dark at room temperature, the buffer was replaced with 50 μL of fresh HBS. $[Ca^{2+}]_i$ in the fluo-3AM loaded cells was fluorometrically determined by Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) at an excitation wave length of 485 nm and an emission wave length of 538 nm. The measuring interval was 1.35 sec. For calibration of the results, the F_{max} signal was obtained by adding 20 μL 1% NP-40 to the wells, which were then measured after a 10-min incubation. The F_{min} signal was obtained by adding 20 μL 0.1 M EGTA to the wells and the plate was measured after 10 min. The estimated $[Ca^{2+}]_i$ was calculated by the formula below (3).

$$Ca^{2+}(nM) = (\Delta F - F_{min}/F_{max} - \Delta F) \times K_d$$

ΔF: observed fluorescence
F_{min}: fluorescence at low Ca^{2+} (EGTA treated)
F_{max}: fluorescence at high Ca^{2+} (NP-40 treated)
K_d: 390 nM for fluo-3

FK506 or other immunosuppressants was added to the cells 24 h before fluo-3AM loading. Atropine, d-tubocurarine, U73122, ryanodine, 2-APB and ruthenium red were added to the cells 10 min before the measurement of $[Ca^{2+}]_i$. Subclass specific muscarinic AChR blocker (pirenzepine, AF-DX 116, 4-DAMP, tropicamide) was also added 10 min before the addition of ACh. ACh was added at a final concentration of 1 μM using a dispenser equipped in Fluoroskan Ascent FL.

Statistical Analysis. For statistical analysis, Student's *t*-test was performed after *F*-test. The *p*-value level of significance was $p < 0.05$. The results are expressed as mean ± S.D.

RESULTS

Characterization of AChR that mediated $[Ca^{2+}]_i$ elevation by ACh

To study $[Ca^{2+}]_i$ response induced by ACh, we measured $[Ca^{2+}]_i$ in TE671/RD cells continuously. The addition of ACh evoked a rapid $[Ca^{2+}]_i$ elevation followed by a slow decay accompanied by oscillatory $[Ca^{2+}]_i$ response, then reached a plateau (Fig. 1A, control). One hundred nM d-tubocurarine had no effect on the $[Ca^{2+}]_i$ elevation, but 100 nM atropine completely suppressed the elevation of $[Ca^{2+}]_i$ (Fig. 1A). Ten μM nicotine had no effect on the $[Ca^{2+}]_i$ level, while 10 μM muscarine evoked a rapid $[Ca^{2+}]_i$ peak (Fig. 1B) without oscillatory response. To further elucidate the muscarinic receptor subtype that mediates $[Ca^{2+}]_i$ elevation, we incubated the cells with muscarinic receptor antagonists (pirenzepine [m1], AF-DX 116[m2], 4-DAMP [m3] and tropicamide [m4]) before the addition of ACh. Only 4-DAMP completely suppressed the $[Ca^{2+}]_i$ spiking evoked by ACh (Fig. 1C, D).

PI-PLC blocker and IP_3R blocker inhibited ACh-induced $[Ca^{2+}]_i$ elevation

We studied the possibility of involvement of the

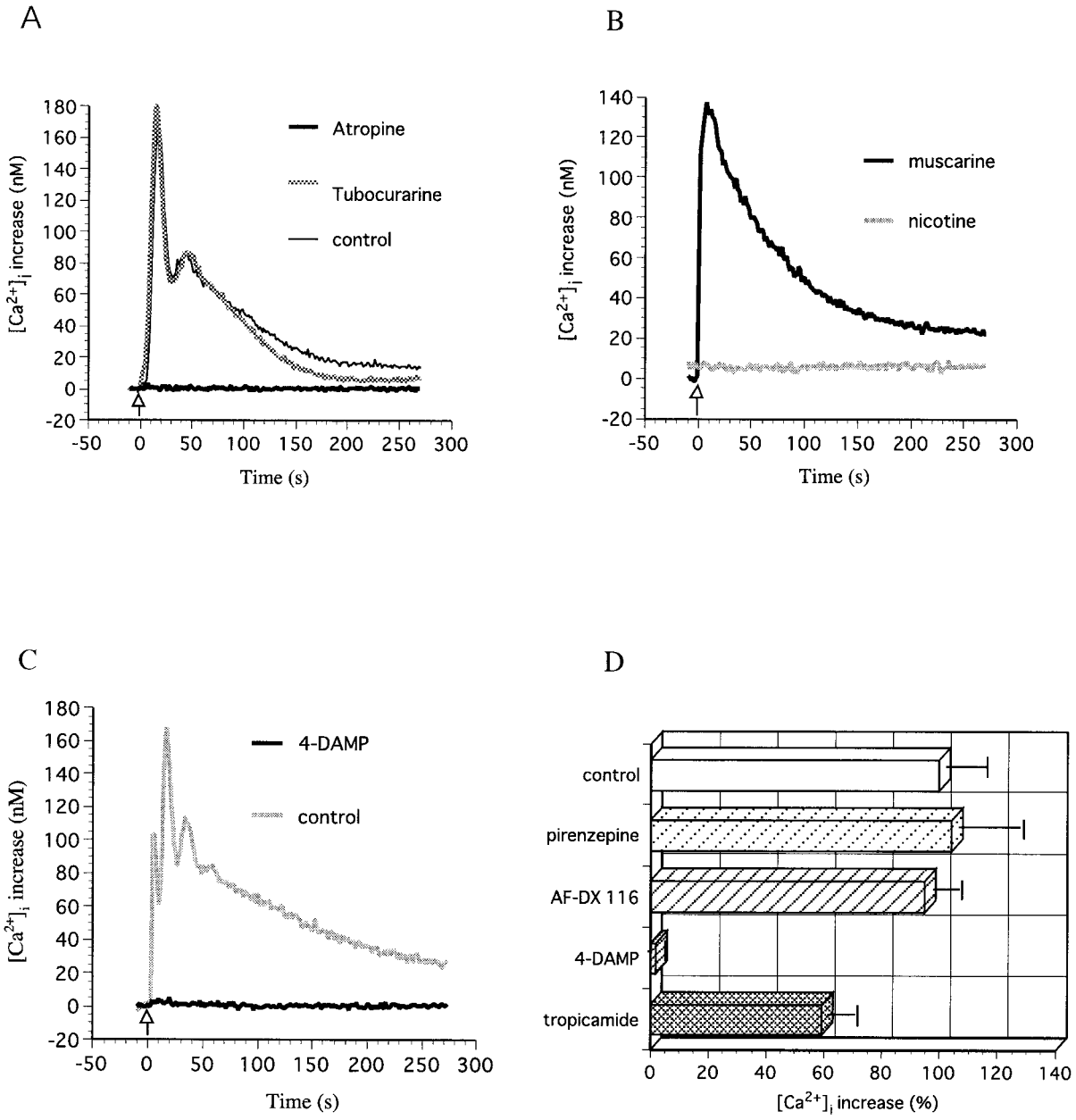

Fig. 1 A. $[Ca^{2+}]_i$ elevation in TE671/RD cells evoked by ACh. TE671/RD cells were loaded with fluo-3AM and $[Ca^{2+}]_i$ was measured at an interval of 1.35 sec. ACh (1 μM in final) was applied at the arrow. Addition of ACh evoked a rapid increase of $[Ca^{2+}]_i$, followed by gradual down and oscillation of $[Ca^{2+}]_i$. Pre-treatment of atropine (100 nM) completely suppressed the elevation of $[Ca^{2+}]_i$. Tubocurarine (100 nM) had no effect on $[Ca^{2+}]_i$ alteration. B. Muscarine (10 μM) mimicked the effect of ACh on $[Ca^{2+}]_i$ change, but oscillatory response was not observed. Nicotine (10 μM) did not raise $[Ca^{2+}]_i$. C. Pre-treatment of 4-DAMP (m3-specific blocker) completely suppressed the elevation of $[Ca^{2+}]_i$. D. Summary of $[Ca^{2+}]_i$ alterations after addition of muscarinic AChR blockers (10 nM). Pirenzepine, AF-DX 116, 4-DAMP, and tropicamide is specific for m1, m2, m3, and m4, respectively. Only 4-DAMP (m3 blocker) completely suppressed the peak of $[Ca^{2+}]_i$.

IP_3 pathway in $[Ca^{2+}]_i$ spiking. The PI-PLC blocker, U73122 (18), significantly inhibited the elevation of $[Ca^{2+}]_i$ in a dose dependent manner (Fig. 2A, B). IP_3R blocker, 2-APB, also significantly inhibited the elevation of $[Ca^{2+}]_i$ in dose dependent manner (Fig. 2C, D). These results indicate that the signal transduction system from m3 AChR, through G-protein, PLC and IP_3R is functional in TE671/RD cells.

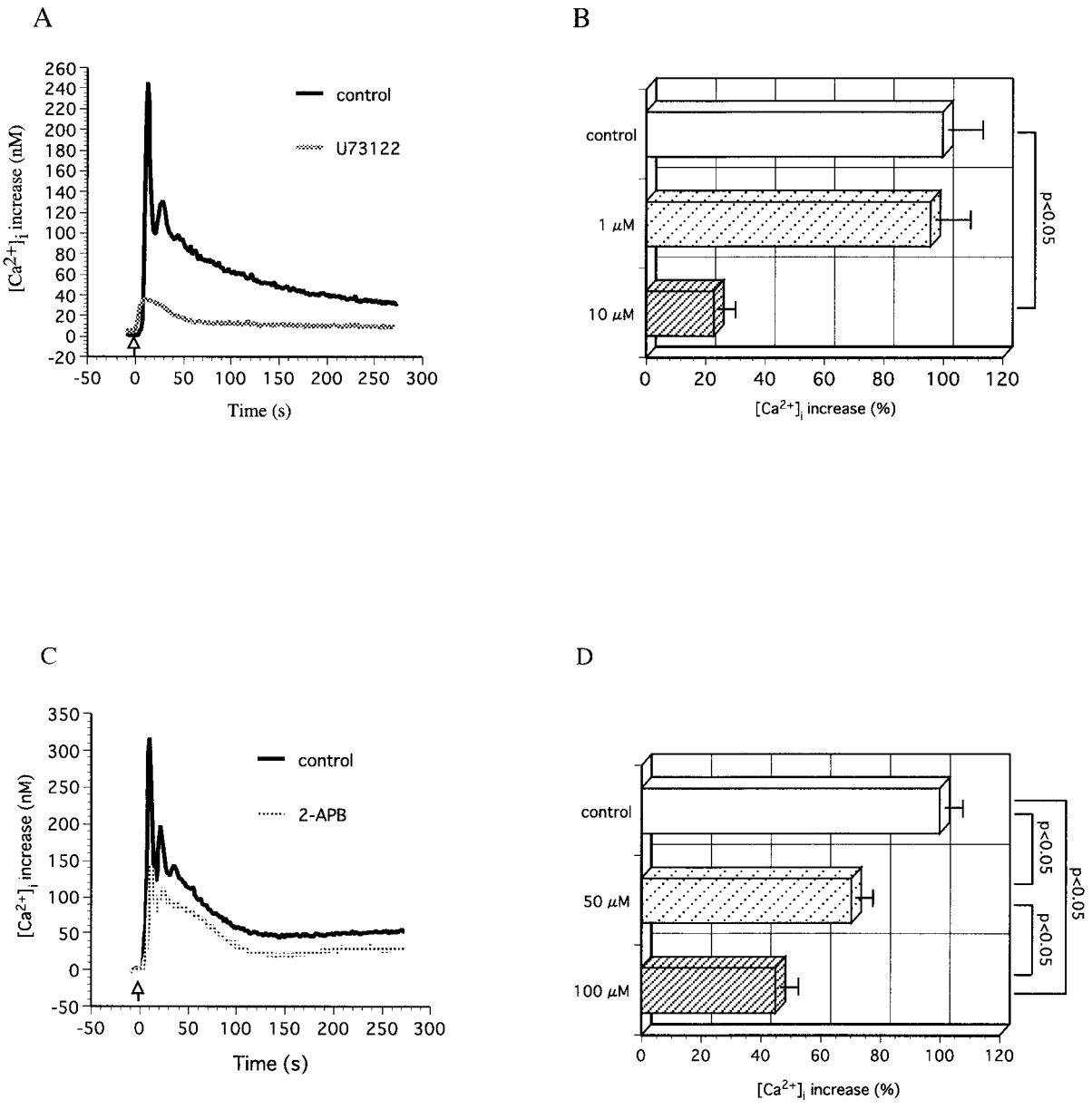

Fig. 2 Effect of PI-PLC blocker, U73122 (A) and IP_3R blocker, 2-APB (C) on $[Ca^{2+}]_i$ alterations in TE671/RD cells. U73122 suppressed $[Ca^{2+}]_i$ elevation (B). 2-APB suppressed $[Ca^{2+}]_i$ elevation in dose dependent manner (D).

Ryanodine and FK506 increased ACh-induced $[Ca^{2+}]_i$ elevation, and ruthenium red decreased it

To determine whether RyR participated in the process of $[Ca^{2+}]_i$ alteration, we examined effects of ryanodine, FK506, CyA and ruthenium red. Pretreatment with ryanodine significantly increased $[Ca^{2+}]_i$ peak evoked by ACh in a dose-dependent manner (Fig. 3A, B). FK506 also increased $[Ca^{2+}]_i$ peak in a dose-dependent manner (Fig. 3C, D). In contrast, CyA had no effect on $[Ca^{2+}]_i$ peak evoked by ACh (Fig. 4A, B). We also tested the effect of ruthenium red, a specific inhibitor for RyR. Ruthenium red decreased $[Ca^{2+}]_i$ peak in a dose-dependent manner (Fig. 4C, D). These results indicated that RyR took a part in ACh induced $[Ca^{2+}]_i$ elevation in TE671/RD, and FKBP12 regulated the function of RyR.

DISCUSSION

We studied the $[Ca^{2+}]_i$ alteration in TE671/RD cells evoked by addition of ACh. $[Ca^{2+}]_i$ elevation was shown to mediated by m3 AChR, PLC and

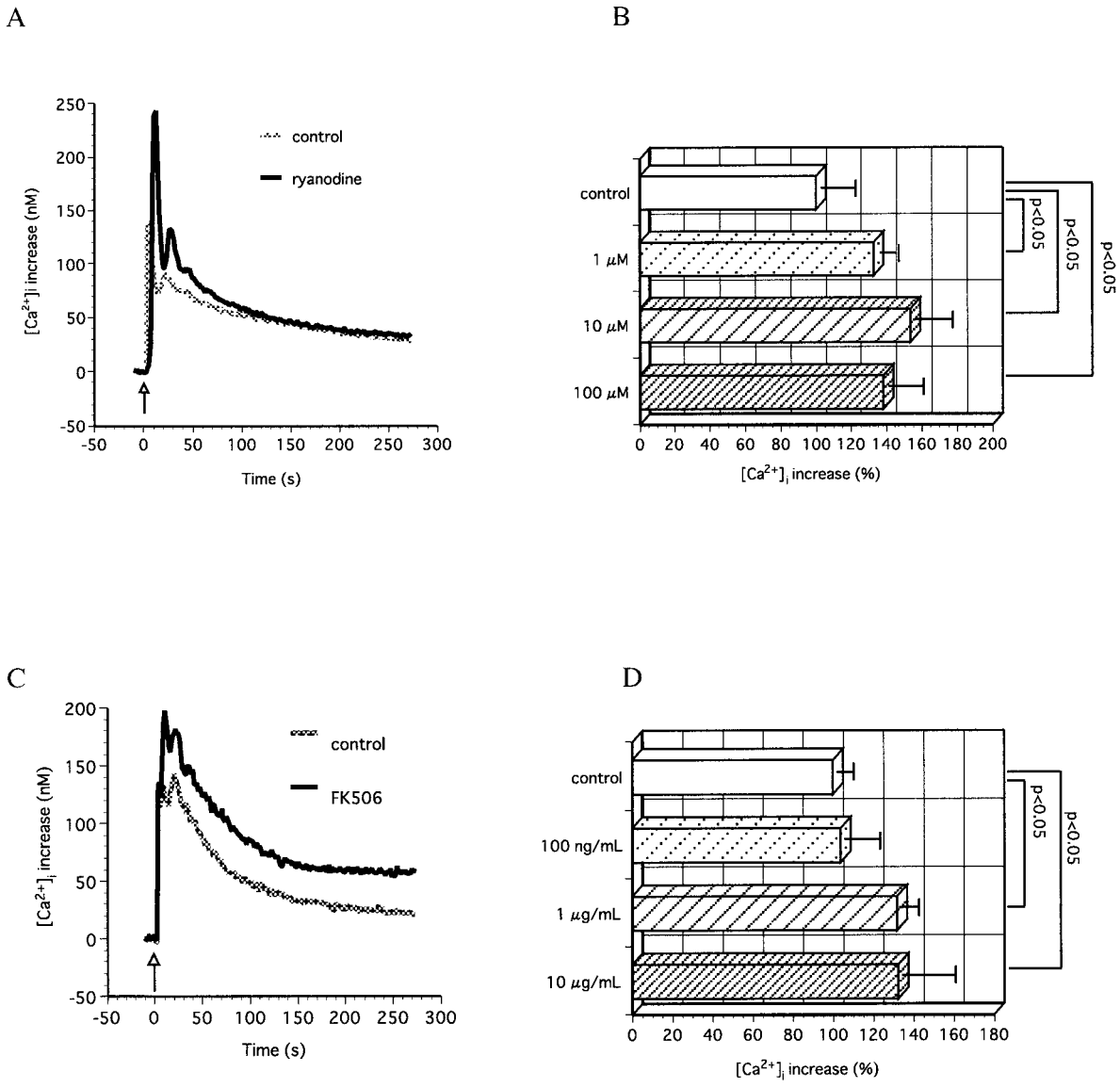

Fig. 3 Effect of ryanodine (A) and FK506 (C) on $[Ca^{2+}]_i$ elevation in TE671/RD cells. Ryanodine increased the peak of $[Ca^{2+}]_i$ in a dose dependent manner (B). FK506 increased the peak of $[Ca^{2+}]_i$ in a dose dependent manner (D).

IP_3R on ER. RyR and FKBP12 on SR were also participated in $[Ca^{2+}]_i$ elevation. As a result, TE671/RD cells have hybrid characteristics of smooth muscle and skeletal muscle. In terms of general consideration, the skeletal muscles exclusively express nicotinic AChRs and also express RyR1 and RyR3 (17), and smooth muscles express m3 AChR, RyRs1, 2 and 3, and IP_3Rs. In TE671/RD cells, the nicotinic AChR has been identified as an embryonic muscle-type receptor, while the muscarinic AChR is pharmacologically characterized as m3 receptor which coupled with the breakdown of phosphoinositides (2). Our results showed that $[Ca^{2+}]_i$ in TE671/RD cells made a sharp peak after ACh stimulation, followed by an oscillatory response, then reached a plateau. Muscarinic stimulation also induced $[Ca^{2+}]_i$ elevation, but it did not induced oscillatory response. We showed that $[Ca^{2+}]_i$ elevation was actually brought by the activation of m3 AChR using a specific channel antagonist. As U73122 and 2-APB suppressed $[Ca^{2+}]_i$ elevation, IP_3 pathway plays a role in $[Ca^{2+}]_i$ alteration. We also showed that ACh-evoked $[Ca^{2+}]_i$ peak was increased by ryanodine and was decreased by ruthenium red, suggesting that RyR play a role in Ca^{2+} regulation in TE671/RD cells. It is well known that FK506 binds

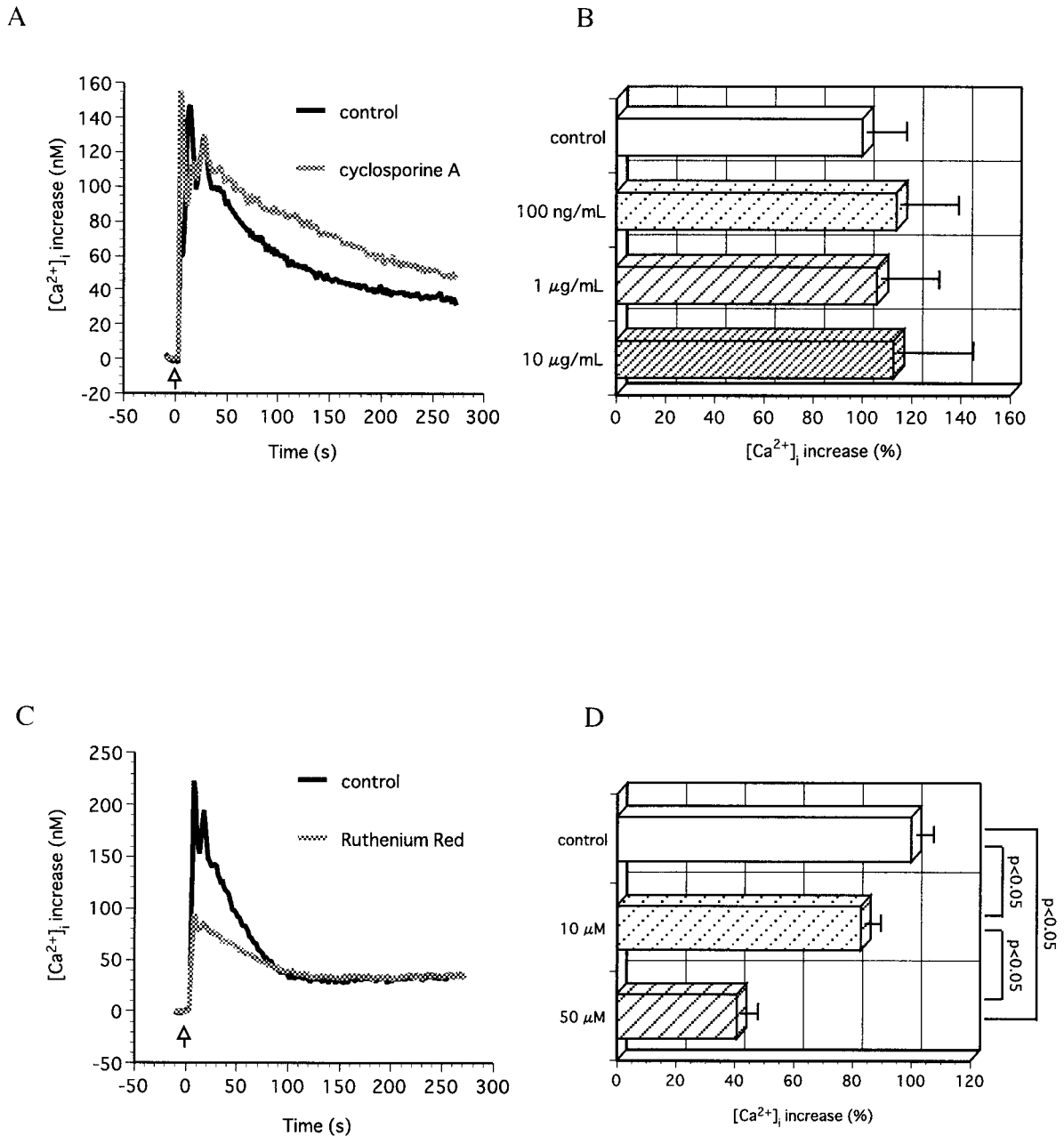

Fig. 4 Effect of cyclosporine A (CyA) (A) and ruthenium red (C) on $[Ca^{2+}]_i$ elevation in TE671/RD cells. CyA did not change the peak of $[Ca^{2+}]_i$ (B). Ruthenium red suppressed the peak of $[Ca^{2+}]_i$ in a dose dependent manner (D).

to a family of intracellular receptors, FKBP12 (6). The binding of FK506 with FKBP12 induces potent immunosuppressive effects in T cells. FKBP12 also exists as a complex with RyR on SR, and stabilizes the closed conformation of the skeletal muscle RyR. FK506 dissociates FKBP12 from RyR (15). We showed that FK506 increased the peak of ACh-evoked $[Ca^{2+}]_i$ elevation, but CyA did not affect it. CyA is a potent immunosuppressant that binds to cyclophilin, and the complex inhibits calcineurin as the same way with the complex of FK506 and FKBP12. Cyclophilin has no direct interaction with RyR. These results indicate that the complex of RyR and FKBP-12 participate in the $[Ca^{2+}]_i$ alteration.

TE671/RD cells are applied for a model of human skeletal muscle, especially in the field of diagnosis and research of myasthenia gravis (MG) and related disorders. TE671/RD based immunoprecipi-

tation assay for detecting nicotinic AChR antibody is widely used for diagnosis of MG (12). Recently, MG was shown to have various autoimmune targets besides nicotinic AChR. For example, autoantibodies for muscle-specific receptor tyrosine kinase (MuSK) (7), RyR (10), titin (1) and neuronal nicotinic AChR (16) were detected. TE671/RD cell line was utilized for detection of these autoantibodies, but its unique characteristics were not discussed closely.

Our results will provide important insight into elucidation of characteristics of TE671/RD cells. This cell line provides us a useful model to study the dynamics of agonist-induced $[Ca^{2+}]_i$ alteration and the effects of reagents on it.

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