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Membrane Voltage Recordings in a Cell Line Derived From Human Ciliary Muscle

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A smooth muscle cell line (H7CM) was established from the ciliary muscle of a 1-day-old human infant. The cultured cells had a normal female karyotype (46 XX) and could be maintained in cell culture for at least 11 generations. A common feature of confluent cultures was the presence of abundant bundles of 6–7 nm microfilaments associated with dense bodies. Both the ultrastructural appearance and the presence of smooth muscle-specific α-isoactin (also present in the human ciliary muscle in situ) support the smooth muscle origin of the H7CM cell line. Continuous membrane voltage (Vm) recordings were obtained in confluent monolayers of H7CM cells using glass microelectrodes. Resting Vm in 105 impalements averaged -66.2 ± 0.7 mV ($\bar{x} \pm$ standard error of the mean). In this system, rapid membrane transients induced by changing of the superfusing test solutions were detectable. Relative K^+ conductance was characterized, and the contribution of electrogenic sodium/potassium adenosinetriphosphatase to Vm was investigated. Under control conditions, H7CM cells were electrically quiescent. However, action potentials could be induced by application of 10 mM barium. Barium-induced action potentials were not abolished by removal of extracellular Na^+ nor were they inhibited by the presence of tetrodotoxin. However, they were blocked by verapamil, fulfilling criteria believed to be typical for smooth muscle cells. Acetylcholine, carbachol, and to a lesser extent pilocarpine induced a reversible Vm depolarization. The effect of acetylcholine was blocked by atropine, implying muscarinic receptor involvement in the Vm response. Collectively, these findings show the potential usefulness of cultured ciliary muscle cells in understanding further the cellular mechanisms underlying drug-induced contraction of the human ciliary muscle. Invest Ophthalmol Vis Sci 31:2420–2430, 1990

The primate ciliary muscle has been classified as a fast, multiunit smooth muscle.[1] The muscle cells show a very dense innervation,[2–5] and a characteristic feature of the ciliary muscle cells is their high concentration of muscarinic receptors, higher than those found in other cholinergic structures.[6,7]

Both direct- and indirect-acting muscarinic cholinergic agonists have been mainstays in the therapeutic treatment of primary open-angle glaucoma for many years due to their efficacy in lowering elevated intraocular pressure.[8] Stimulation of muscarinic receptors induces contraction in human ciliary muscle strips in vitro,[9] and contraction of the ciliary muscle in vivo mediates both accomodation and decreased aqueous outflow resistance in the primate eye.[10]

Little is known as yet about ciliary muscle cellular physiology and function. Electrophysiologic studies of the ciliary muscle may be a useful approach to investigate cellular events involved in the mediation of drug effects used in glaucoma therapy. However, in situ ciliary smooth muscle is not easily accessible for microelectrode studies.[11–13]

Over the last few years cell culture has become a powerful research tool to investigate functional properties of many tissues on a cellular level and under well-controlled conditions. We describe a cell culture model in which human ciliary muscle membrane voltage properties were investigated.

Materials and Methods

Isolation and Cell Culture From Human Ciliary Muscle

Cells used in this study (cell line H7CM) were obtained from the ciliary muscle of an eye of a 1-day-old

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human infant. The enucleated eye was placed in medium 199 containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamicin (PSG) for 15 min before dissection. Dissection was done approximately 24 hr post mortem. The eye was bissected along the equator, and the posterior half was made available to other researchers. The anterior segment was placed on a sterile petri dish cornea-side down. The lens zonule was cut, and the lens removed. The iris was grasped at the pupillary margin and gently pulled away. With the aid of an operating microscope, the ciliary processes were peeled away from the underlying sclera and ciliary muscle. The scleral spur attachment of the ciliary muscle was cut and the ciliary muscle removed. A cell dispersion of the ciliary muscle was then obtained using methods similar to those described by Owens et al.[14] Briefly, the ciliary muscle was incubated at 37°C in Hank's balanced salt solution supplemented with 1 mg/ml collagenase (type CLS II 146 units/mg; Worthington, Freehold, NJ), 0.5 mg/ml elastase (type I 32 units/mg; Sigma, St. Louis, MO), and PSG. Incubation time was 2 hr with trituration at 30-min intervals. The digested tissue was then filtered through a stainless steel mesh (85 μm). Fetal calf serum (FCS) was added to the filtrate at a concentration of 20% to inactivate the enzymes. The filtrate was centrifuged (100 × g, 10 min) and the cell pellet resuspended in 2 ml of medium 199 containing 10% FCS and PSG. The cell suspension was seeded onto a 35-mm uncoated plastic culture dish. Upon confluence the primary culture was subcultured using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid in a Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) to remove the cells from the culture dish and "split" 1:4 each generation thereafter. Cells were maintained at 37°C in a 5% CO_2 atmosphere and culture medium (medium 199 supplemented with 10% FCS and 100 units/ml penicillin and 100 μg/ml streptomycin) was exchanged twice a week. Cell culture media and supplements were purchased from Biochrom KG, Berlin, FRG, or from Sigma. Tissue culture dishes were from Nunc A/S, Roskilde, Denmark, or from Becton Dickinson, Lincoln Park, NJ.

α-Isoactin Immunofluorescence

For these experiments H7CM cells were seeded onto glass cover slips and were used 2–7 days after seeding. Culture medium was removed by rinsing the cells three times with PBS. Subsequently cells were fixed with methanol (−20°C) for 3 min. The methanol was removed by again rinsing the cells three times with PBS. Cells were then incubated for 90 min at room temperature with monoclonal mouse antibody to smooth muscle-specific α-isoactin[15] (Lot No. M415; FA BioMakor, Rehovot, Israel) in a dilution of 1:100 in PBS (primary antibody). After rinsing three times with PBS, the cells were incubated for another 60 min with a fluorescein-conjugated rabbit anti-mouse IgG (Dakopatts, Hamburg, FRG) in a 1:40 dilution in PBS (secondary antibody). Subsequently the cells were thoroughly rinsed with PBS and thereafter embedded in glycerin supplemented with p-phenylenediamine.[16] The cells were evaluated and photographed using a Zeiss photomicroscope equipped for epifluorescence studies (Oberkochen, FRG). Control experiments were done using either PBS or a mouse nonimmune serum instead of the primary antibody. No specific staining was detected in these controls. Furthermore, using the same protocol, immunofluorescence stainings for smooth muscle-specific α-isoactin were done on 10-μm thick frozen sections of the ciliary region of an adult human eye.

Electron Microscopy Studies

For ultrastructural investigation, cells grown on uncoated, plastic petri dishes or tissue culture flasks were used. Preconfluent (5–7 days) cultures of sixth and eighth passages and highly confluent cultures (3–6 weeks) of third, sixth, eighth, and ninth passages were studied. The cells were fixed with Ito's fixative[17] for at least 4 hr. The fixed cells, still in the plastic dish, were postfixed with 1% osmium tetroxide, dehydrated with graded alcohols, and embedded in Epon (Roth, Karlsruhe, FRG). Polymerization was done at 60°C. Tangential and perpendicular sections of the cells were cut on an ultramicrotome. The sections were treated with lead citrate and uranyl acetate. For electron microscopic examination, a Zeiss EM 902 electron microscope (Zeiss, West Germany) was used.

Membrane Voltage Measurements

Membrane voltage was measured using conventional Ling-Gerard microelectrodes. For microelectrode experiments, confluent monolayers of H7CM cells were used usually about 14 days after seeding. Cells used in these experiments were from passages four to 11. The experimental setup has previously been described in detail.[18] In short, the petri dish with attached cells was inserted in a temperature-controlled chamber (37°C). A flow chamber was clamped onto the bottom of the petri dish, isolating a small channel (width, 1.5 mm; length, 30 mm). This channel could rapidly be superfused by up to eight different test solutions with a 90% fluid exchange occurring within about 3 sec, at a perfusion rate of 30 ml/hr. Solution exchanges were done using electro-

magnetic valves (Lucifer type 133 A 54; Geneva, Switzerland). Microelectrodes were drawn from filament borosilicate glass capillaries (outer diameter, 1.0; internal diameter, 0.58 mm; Hilgenberg, Malsfeld, FRG) using a Narishige PD-5 horizontal electrode puller (Tokyo, Japan). Electrodes were filled with 0.5 M KCl solution (resistance in Ringer's solution, 50–120 MΩ). The cells on the bottom of the flow channel were impaled using a microstepping device (Heidelberg Nanostepper; Science Trading, Frankfurt, FRG) which advanced the electrode until a stable membrane voltage recording was obtained. The microelectrode was connected to an electrometer amplifier (WPI model M4-A; World Precision Instruments, Hamden, CT), and the time course of the voltage was continuously recorded on a chart recorder.

Solutions and Source of Chemicals

Control Ringer's solution contained 151 mM Na^+, 5 mM K^+, 1.7 mM Ca^{2+}, 1 mM Mg^{2+}, 130.4 mM Cl^-, 1 mM SO_4^{2-}, 1 mM $H_2PO_4^{1-}$, 28 mM HCO_3^{1-}, and 5 mM glucose; it was aerated with 5% CO_2 in ambient room air, resulting in a pH of approximately 7.4. Solutions with a potassium concentration of 10, 20, 40, or 80 mM contained 146, 136, 116, or 76 mM sodium, respectively. Solutions containing Ba^{2+} were free of SO_4^{2-}. Solutions containing 10 mM Ba^{2+} were nominally calcium free. Sodium-free solutions were obtained by replacing sodium with N-methyl-D-glucamine. Ouabain was obtained from Merck, Darmstadt, FRG. Atropine-free base, pilocarpine HCl, carbamylcholine chloride (carbachol), and acetylcholine chloride were purchased from Sigma. These substances were added from aqueous stock solutions which were prepared the day of the experiment. Acetylcholine esterase inhibitors were not used in our experiments since the cell layer was constantly superfused with fresh test solution. Racemic verapamil HCl was also obtained from Sigma and was added from an ethanol stock solution. In these experiments control solutions contained an equal amount of ethanol (final ethanol concentration 0.5% v/v). Tetrodotoxin (TTX) was from Serva, Heidelberg, FRG, was dissolved in a 5 mM *tri*-sodium citrate solution (pH = 5.3), and then was added to the test solutions. Control solutions contained an equal amount of *tri*-sodium citrate solution (final concentration 0.1% v/v).

Results

Cell Culture and Morphology

Cell culture: With the H7CM cell line approximately 70% of the cells were attached 24 hr after cell dispersion, and confluency was obtained within 3 days. The primary culture appeared to be homogeneous in nature, as were the following generations. At no point in time was there an impression of selected clones of cells "overgrowing" other cell types.

At low density, the cells varied in shape from bipolar to more broad and dendritic. Figure 1A shows a phase-contrast micrograph of proliferating H7CM cells in the ninth passage 1 day after seeding. Confluency was obtained within about 1 week after seeding. At confluency the cells appeared in a monolayer of parallel rows of elongated and spindle-shaped cells. However, proliferation continued even at postconfluency. When cells were allowed to grow to higher densities, many groups of multilayered cells were seen in association with deposits of extracellular material. Eventually, small hillocks were formed, and several smaller oval cells between bands of parallel, elongated cells were seen. Figure 1B shows a postconfluent culture, ninth passage, 20 days after seeding.

Cells could be subcultured at a split ratio of 1:4 up to the 11th passage without showing any obvious

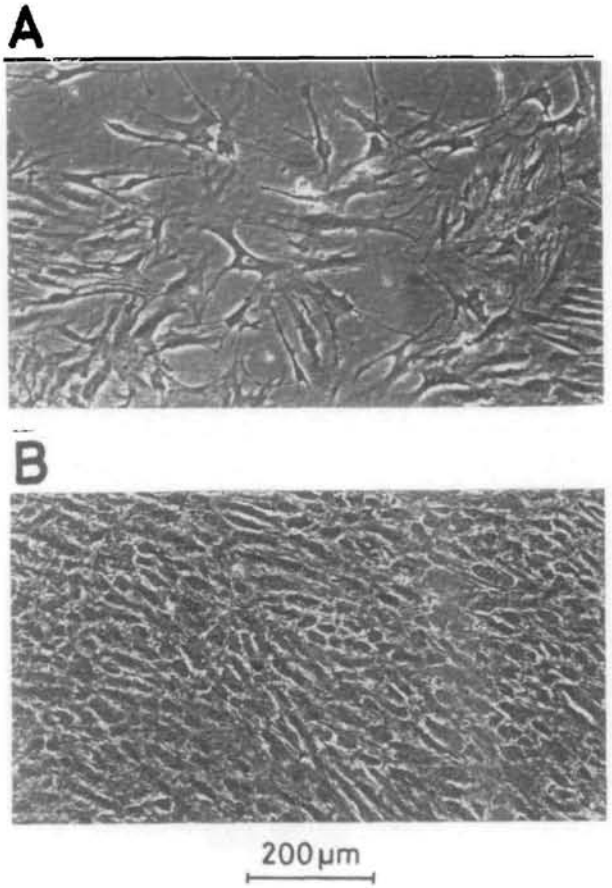

Fig. 1 Phase contrast micrographs of cultured human ciliary muscle cells (H7CM cell line). (**A**) Proliferating cells 24 hr after seeding. (**B**) Confluent cells 20 days after seeding.

morphologic change in phase-contrast microscopy. Vials of H7CM cells from lower passages could be stored under liquid nitrogen using standard techniques for freezing and thawing, enabling experimentation on the same strain of cells on multiple occasions.

Chromosomal analysis of the H7CM cell strain in the fifth passage was kindly done by Dr. V. Wieczorek from the Institut für Humangenetik, Freie Universität Berlin, FRG. A normal human female karyotype (46 XX) was found, using conventional quinacrine staining.

α-Isoactin immunofluorescence: Stainings were done on 5 different days using subconfluent and confluent H7CM cells from passage five to ten. In all experiments smooth muscle-specific α-isoactin filaments were stained as shown in Figure 2. This photograph demonstrates a parallel alignment of intracellular filaments, characteristic of actin filaments.[19] Subconfluent cultures contained some cells in which staining was relatively weak or even absent. However, nearly all cells stained positively in confluent cell layers without indication of a subpopulation of non-smooth muscle cells.

Figure 3 depicts a frozen section of the ciliary region of an adult human eye, demonstrating the presence of smooth muscle-specific α-isoactin in the ciliary muscle in situ. Note that the wall of a scleral vessel seen in this section also strains positively for smooth muscle-specific α-isoactin.

Electron microscopic studies: In preconfluent cultures (Fig. 4), the cells had very irregular borders with both short and long cytoplasmic processes. A large portion of the cytoplasm was occupied by dilated cisternae of rough endoplasmic reticulum (rER), many ribosomes, mainly arranged as polysomes, a large Golgi complex with numerous associated vesicles, and elongated, branched mitochondria. Only scattered bundles of thin (6–7 nm) microfilaments were found throughout the cytoplasm. Perpendicular sections of the cell layer showed the microfilament bundles restricted to a narrow zone just inside the plasma membrane, located toward the bottom side of the cell, ie, parallel to the substratum. In addition, the cells were rich in intermediate-sized (10–11 nm) filaments and microtubules. Cells from sixth and eighth passages showed no differences in ultrastructure.

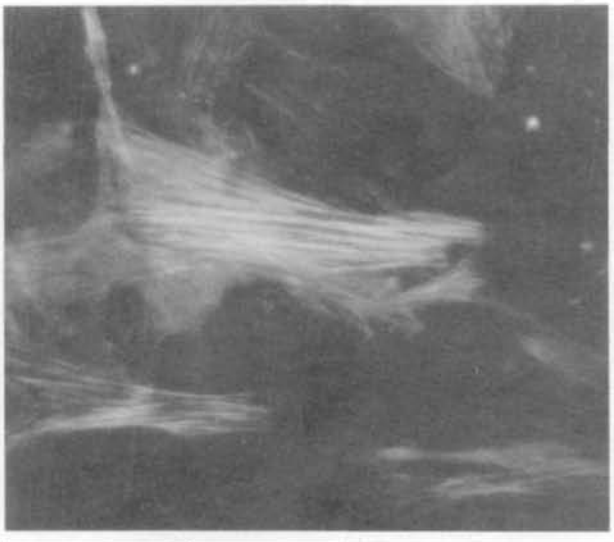

Fig. 2. Immunofluorescence staining of smooth muscle specific α-isoactin filaments in eighth passage H7CM cells 4 days after seeding (original magnification ×550).

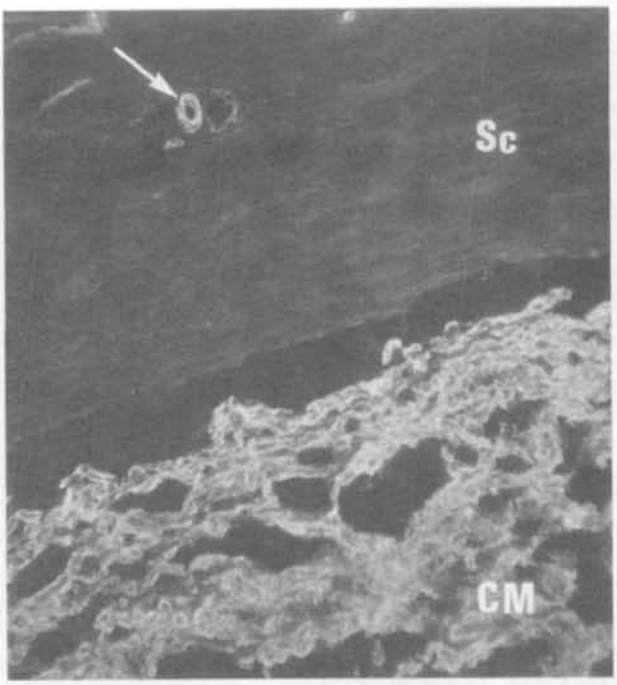

Fig. 3. Smooth muscle specific α-isoactin immunofluorescence in a 10 μm freeze-section of adult human ciliary muscle (CM). Note the absence of specific staining in the scleral tissue (Sc). However, the scleral vessel wall seen in cross section within the scleral tissue (arrow) clearly stains for smooth muscle specific α-isoactin (original magnification ×140).

However, highly confluent cultures had a different ultrastructure (Fig. 5). The cells overlapped each other, thereby forming a multilayer, two to three cells thick. Most of the cells had a more spindle-shaped appearance. The cytoplasm of the cells was filled with microfilament bundles, while rER and Golgi-systems were scarse and restricted to a small area surrounding the nucleus. The microfilament bundles contained many streak-like or ellipsoidal dense bodies and were often flanked by typical surface caveolae and vesicles. The surface vesicles had a diameter of 40–80 nm and were often arranged in rows located between adjacent microfilament bundles. The cells were connected by

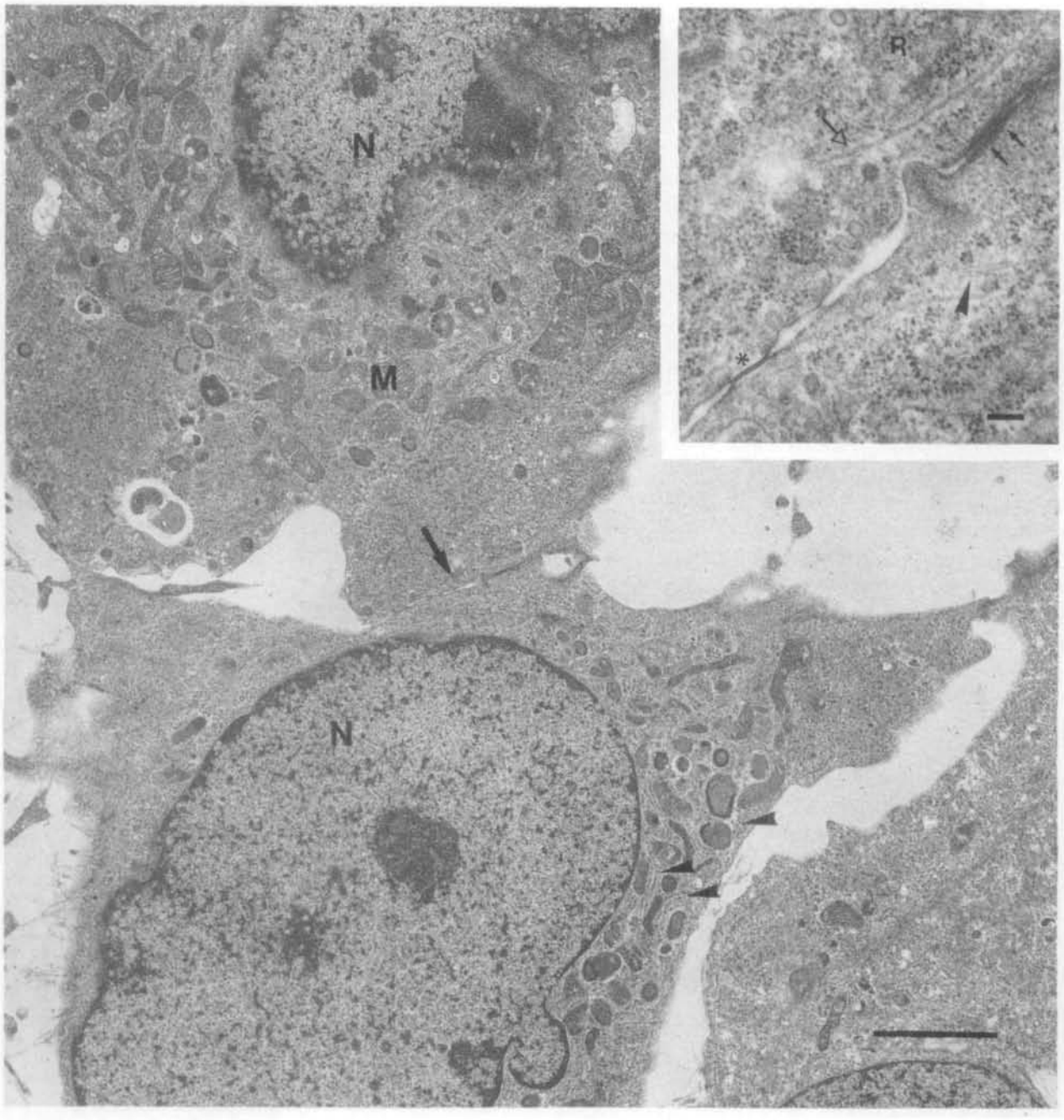

Fig. 4. Electronmicrograph of H7CM cells in preconfluent culture (sixth passage), 3 days after seeding. The cells have a polygonal appearance. Most of the cytoplasm is occupied by mitochondria (M), ribosomes (R in inset), and rough endoplasmic reticulum (arrowheads) (original magnification ×6900; bar: 3.01 μm). The inset shows a higher magnification of the area indicated by arrow; 10–11 nm intermediate filaments (arrowhead) and microtubules (open arrow) can be seen. The cells are connected to each other by a gap junction* and an intermediate junction (arrows) (original magnification of inset: 33,000; bar: 0.21 μm).

intermediate-type and gap junctions. Typical desmosomes were not found. In addition the cytoplasm of the cells was filled with lysosomal structures, autophagic vacuoles, and vacuoles containing myelin-like figures. An incomplete basal lamina and fibrillar extracellular material surrounded the cells. Perpendicular sections revealed that the microfilament bundles were not confined to the cell membrane but were now present throughout the whole cytoplasm. A number of cells (approximately 20%) differed in ultrastructure from the spindle-shaped cells. They were smaller and more oval on tangential sections. Their cell membrane showed many short processes and irregular surface blebs. The cytoplasm contained abundant rER, Golgi systems, and ribosomes, but bundles of microfilaments were not observed. There were no ul-

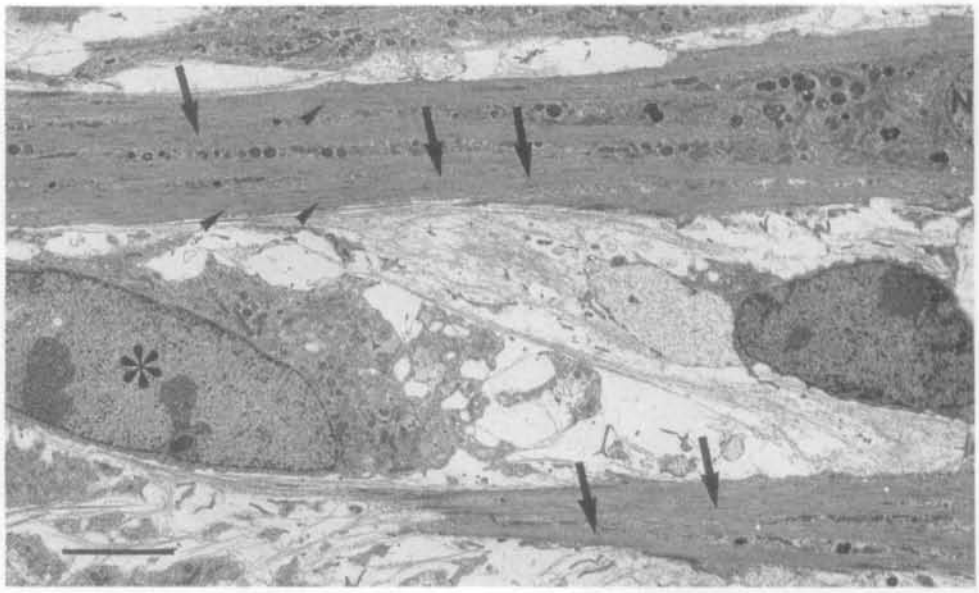

Fig. 5. Electronmicrograph of confluent H7CM cells (eighth passage, 6 weeks after seeding). The cytoplasm of the spindle-shaped cells is filled with dense arrays of 6–7 nm microfilament bundles (arrows) containing dense bands (arrowheads), while rough endoplasmic reticulum and Golgi systems are restricted to the area around the nucleus (N). Between the spindle-shaped cells a smaller, oval cell is seen.* The cytoplasm of the cell contains abundant rough endoplasmic reticulum and Golgi systems, but no microfilament bundles. Between the cells deposits of extracellular material are seen (original magnification ×3300; bar: 6.3 μm).

trastructural differences observed between highly confluent cultures of third, sixth, eighth, and ninth passages.

Membrane Voltage Recordings

Membrane potentials: Successful impalement of a cell led to a steep initial negative deflection of the recorded voltage, which was then followed by a further increase over several minutes until a stable value was reached. Cell impalements were only accepted if the reference potential (baseline) and electrode resistance were stable and had the same value before and after an impalement. The membrane voltage (stable ± 2 mV for at least 3 min) measured in 105 impalements in control Ringer's solution averaged −66.2 ± 0.7 mV (mean ± standard error of the mean, n = 105). We did not detect any difference in the membrane voltage between the passages used in our experiments (four to 11).

Relative K^+ conductance: Increasing extracellular potassium concentration ($[K^+]$) led to a rapid and reversible depolarization of membrane voltage. Figure 6A shows a typical recording of the effect of increasing $[K^+]$ from 5 mM to 10, 20, 40, and 80 mM on membrane voltage. Figure 6B summarizes the results of seven similar experiments shown in Figure 6A. Membrane voltage is plotted against the logarithm of extracellular $[K^+]$. The slope of the curve is steeper at high $[K^+]$ compared with low $[K^+]$. This is reflected by the different transference numbers (t_k) calculated for different $[K^+]$ intervals. Thus t_k was 0.39 for the $[K^+]$ interval 5–10 mM, but t_k was 0.89 for the $[K^+]$ interval 20–40 mM. Application of 1 mM barium, a known blocker of potassium channels, led to a depolarization of 19.9 ± 2.4 mV (n = 11).

Excitability: In control Ringer's solutions H7CM cells were electrically quiescent and membrane-voltage recordings were stable. However, occasionally small abortive "spike-type" membrane voltage deflections with an amplitude of only a few millivolts could be observed under steady-state conditions. As shown in Figure 7A bursts of repetetive and overshooting action potentials could be induced by application of 10 mM barium (n = 23). Barium-induced action potentials were a consistent finding throughout the passages we used (up to passage 11). Action potentials were not abolished in the absence of extracellular sodium (n = 4, Fig. 7B). However, their amplitude was somewhat reduced, probably due to the depolarization induced by the sodium removal itself (not shown).[20] Action potentials were reversibly blocked in the presence of the calcium antagonist verapamil (Fig. 8A) (n = 6), although TTX did not affect the barium-induced action potentials (n = 4, Fig. 8B).

Electrogenic sodium/potassium adenosinetriphosphatase (Na^+/K^+-ATPase): It is now widely accepted that the electrogenic component of Na^+/K^+-ATPase contributes to the resting membrane potential. To determine the contribution of electrogenic Na^+/K^+-ATPase to the resting membrane voltage in H7CM cells we tested the effect of ouabain, a potent inhibitor of Na^+/K^+-ATPase. Application of ouabain 10^{-4} M induced a sudden (ie, within seconds) depolarization of the membrane voltage by 10.6 ± 1.7 mV (n = 8), which is in good agreement with previously reported findings in other smooth muscle preparations.[21–23]

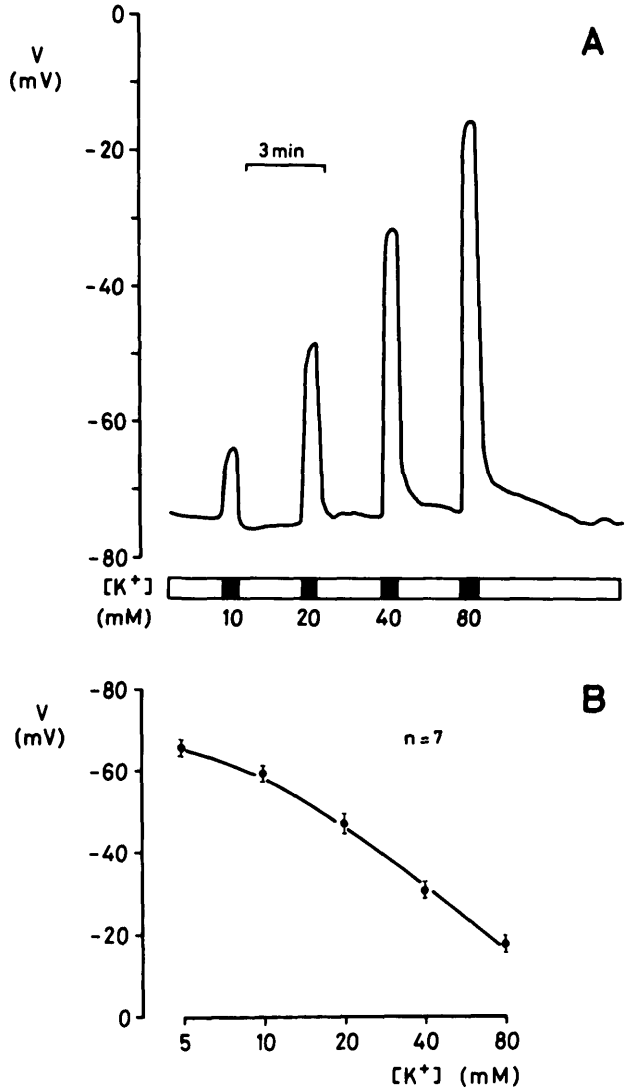

Fig. 6. (A) Typical recording in a H7CM cell demonstrating influence of different extracellular $[K^+]$ on membrane voltage (V). (B) Mean values of V from seven experiments, as shown in A, are plotted as a function of $[K^+]$ on a semi-logarithmic plot. Values were calculated from the maximal depolarization reached within the 30 sec of an extracellular $[K^+]$ increase. Bars indicate SEM values.

Effects of acetylcholine, pilocarpine, and carbachol: As shown in Figures 9 and 10 application of 10^{-4} M and 10^{-5} M acetylcholine led to a sustained depolarization of the membrane voltage by 47.7 ± 4.2 mV (n = 6) and 32.7 ± 3.6 mV (n = 11), respectively. The depolarization was readily reversible after the washout of acetylcholine. In about one half of the experiments spike-like oscillations of the membrane voltage occurred at the depolarized membrane voltage level in the presence of acetylcholine (Fig. 9). The acetylcholine-induced membrane voltage response was reversibly blocked with 10^{-5} M atropine (n = 3, Fig. 10). Pilocarpine in a concentration of 10^{-3} M reversibly depolarized H7CM cells by 27.5 ± 2.6 mV (n = 8) within 3 min (Fig. 11A), while carbachol 10^{-5} M induced a depolarization of 38.0 ± 3.0 mV (n = 7, Fig. 11B).

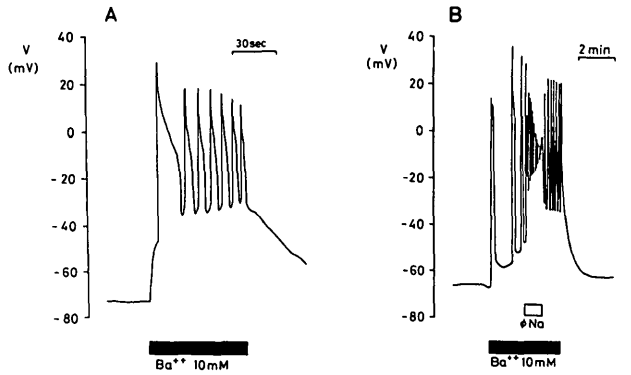

Fig. 7. (A) Repetitive, overshooting action potentials in H7CM cells induced by application of 10 mM Ba^{2+}. (B) Extracellular sodium removal (NMDG) during a burst of barium-induced action potentials in H7CM cells.

Discussion

We described a tissue culture model of human ciliary muscle to investigate some of the electrophysio-

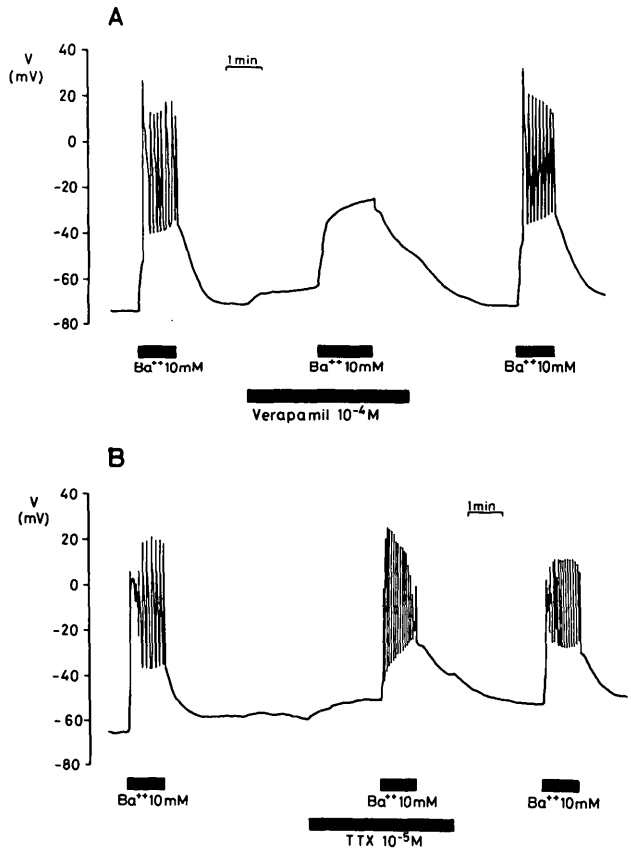

Fig. 8. (A) Effect of verapamil on barium-induced action potentials in H7CM cells. (B) Lack of effect of tetrodotoxin (TTX).

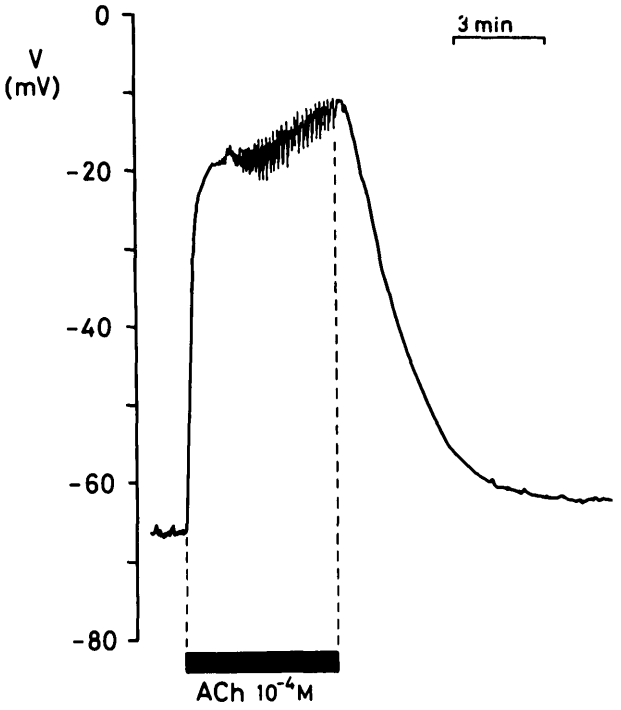

Fig. 9. Membrane voltage response in H7CM cells to application of 10^{-4} M acetylcholine.

logic properties of cultured human ciliary smooth muscle cells. As in all physiologic studies using tissue culture techniques the question has to be asked whether the experimental tissue preserves some of its typical features under tissue culture conditions.

The smooth muscle origin of the H7CM cells seems probable since they express smooth muscle-specific α-isoactin filaments, also present in adult ciliary muscle in situ. Smooth muscle-specific α-isoactin has previously been shown to be present in the medial layer of blood vessels, in cultured rat aortic medial cells, in human leiomyosarcoma cells, in the muscularis and muscularis mucosae of the gastrointestinal tract, in the uterine myometrium, in the mesenchymal components of the prostate, and in myoepithelial cells of mammary and salivary glands.[15,24] Smooth

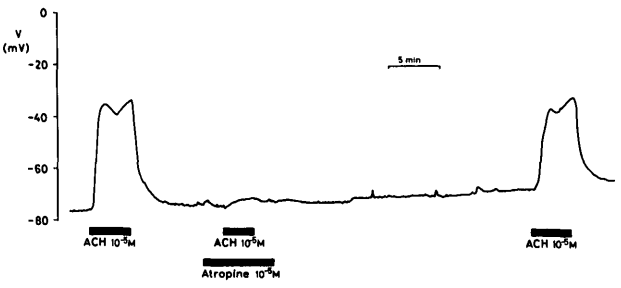

Fig. 10. Effect of atropine on the membrane voltage response induced by application of acetylcholine in H7CM cells.

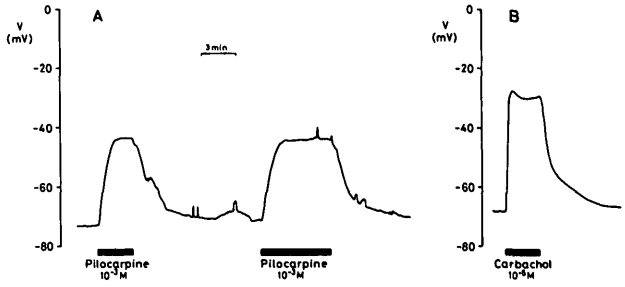

Fig. 11. (A) Effect of pilocarpine on membrane voltage (V) in H7CM cells. (B) Effect of carbachol.

muscle-specific α-isoactin is absent in heart and skeletal muscle, in vascular endothelium, in human and rat dermis fibroblasts, and in a human embryo lung fibroblast cell line.[15,24] Accordingly, using an identical experimental protocol as described in the present study, we previously showed that cultured rat aortic smooth muscle cells stain for smooth muscle-specific α-isoactin, but primary cultures of mouse skin fibroblasts do not (unpublished observation).

Electron microscopy showed that confluent H7CM cells have ultrastructural features commonly considered to be typical for vascular and visceral smooth muscle cells in culture.[25] Such features include a high density of 6–7-nm microfilament bundles, typical surface invaginations, an incomplete basal lamina, and the presence of lysosome-like vesicles.[25–27] It is generally accepted that bundles of intracellular 6–7-nm microfilaments consist mainly of actin filaments.[28]

However, we also found several cells showing a different morphology with abundant rER, Golgi systems, and only a small amount of 6–7-nm microfilaments. A similar morphology was present in most of the cells of preconfluent, growing H7CM cultures. It is well documented for vascular and visceral smooth muscle cells in growing, preconfluent cultures[25,26,29–31] that the cells change their phenotype, a process which includes a relative loss of myofilaments accompanied by an increase of rER and a large Golgi-complex. Ultrastructurally, the smooth muscle cells aquire a fibroblast-like appearance. Furthermore, smooth muscle-specific α-isoactin synthesis and content are known to be low in subconfluent log-phase vascular smooth muscle cells.[14] This could explain the relatively weak staining (or even absence of staining) for smooth muscle-specific α-isoactin in several preconfluent H7CM cells. However, after reaching confluency, most of the cells change back to their in vivo morphology.[25,26]

Our immunohistochemical and ultrastructural findings in H7CM cells indicate that the cell population established in culture was derived from smooth

muscle cells rather than fibroblasts or vascular endothelial cells, which are also present in the ciliary muscle tissue. Neither the α-isoactin staining nor electron microscopy can differentiate between ciliary smooth muscle cells and vascular smooth muscle cells. Thus, it is possible that vascular smooth muscle cells may be present in the H7CM cell line. However, the ciliary muscle contains few blood vessels with a thick smooth muscle cell-rich media,[32] and most smooth muscle cells within the ciliary muscle are ciliary muscle cells. Therefore, the probability is that the H7CM cells were most likely derived from ciliary muscle cells.

The mean resting membrane voltage of −66.2 mV in H7CM cells was in good agreement with the mean membrane voltage of −59.6 mV reported in meridional dog ciliary muscle specimens[13] and is somewhat higher than the membrane voltage usually reported for vascular smooth muscle cells.[23,33] Under resting conditions the H7CM cell membrane was electrically quiescent. This has also been reported for the dog ciliary muscle in which neither spontaneous fluctuations of the membrane potential nor spontaneous action potentials were observed.[13]

In smooth muscle cells an action potential is not an essential requirement for the initiation of contraction,[34] and pharmacomechanical coupling seems to play an important role in the ciliary muscle.[12,13] However, excitability is a prominent feature of cell membranes of muscular origin, and action potentials may be induced in normally quiescent smooth muscle cells by using appropriate maneuvers.

In H7CM cells action potentials could be induced by application of 10 mM barium. Barium-induced action potential in H7CM cells were not abolished in the absence of extracellular sodium and were not inhibited by TTX, a known inhibitor of the fast Na^+ channel.[35] Action potentials in smooth muscle cells are typically insensitive to TTX.[36,37] Barium-induced action potentials have previously been reported in other smooth muscle cells.[23,38,39] Voltage-dependent calcium channels are known to be highly permeable to barium ions and are assumed to be involved in the development of action potentials in smooth muscle cells.[40,41] In H7CM cells barium-induced action potentials were abolished in the presence of the organic calcium antagonist, verapamil. Thus the action potentials in H7CM cells fulfill criteria believed to be typical for smooth muscle cells.[41] Barium-induced action potentials were a constant finding throughout the passages used in the present investigation. This indicates that H7CM cells maintain smooth muscle-like membrane properties even under prolonged cell culture conditions.

In addition we showed that acetylcholine induced a membrane voltage depolarization, which could be blocked by atropine. We concluded that the response is mediated by a muscarinic receptor. Furthermore, carbachol and pilocarpine, direct-acting muscarinic agonists which are widely used in glaucoma therapy, had qualitatively similar effects on membrane voltage as did acetylcholine.

The ionic mechanism(s) underlying the membrane depolarization upon application of acetylcholine and the other muscarinic agonists in H7CM cells need(s) further investigation. Several subtypes of muscarinic receptors have been described and may be coupled to different effector systems.[42,43] Preliminary experiments in our laboratory show that acetylcholine induces an intracellular calcium rise in fura-2-loaded H7CM-cells.[44,45] The increase in the level of intracellular calcium occurs presumably as a result of release from intracellular stores by inositol trisphosphate (IP_3) which is formed by breakdown of phosphatidylinositol via a receptor stimulated phospholipase C.[46,47] Stimulation of phospholipase C by a muscarinic receptor not only increases intracellular calcium concentration via IP_3 but also leads to an activation of protein kinase C via diacylglycerol.[42] Thus it is conceivable that a rise in both intracellular calcium and protein kinase C-related processes could be involved in the activation of depolarizing ion conductances in H7CM cells.[48]

Similar acetylcholine-induced membrane voltage effects as in H7CM cells have previously been reported, eg, in jejunal smooth muscle cells[49] and in guinea pig ileum.[50] The depolarizing component has been attributed to the opening of nonselective cation channels, which are permeable to sodium and calcium, and may play a role for the phasic contraction of smooth muscle cells. An initial transient hyperpolarizing component observed in some experiments in H7CM cells (data not shown) may be due to an activation of Ca^{2+}-activated K^+-channels.[23,51]

In conclusion, we established a human ciliary smooth muscle cell line which has immunohistochemical, ultrastructural, and electrical membrane properties typical of smooth muscle cells and which appears to express a muscarinic acetylcholine receptor. The H7CM cell line may be useful in gaining further insight into the mechanisms underlying ciliary muscle responsiveness to therapeutic agents which mediate accommodation and decreased aqueous outflow resistance.

Key words: human ciliary muscle, tissue culture, membrane voltage, excitability, muscarinic agonists, smooth muscle specific α-isoactin

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