ELECTROPHYSIOLOGICAL RECORDINGS FROM SPONTANEOUSLY CONTRACTING REAGGREGATES OF CULTURED SMOOTH MUSCLE CELLS FROM GUINEA PIG VAS DEFERENS

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ABSTRACT

Smooth muscle cells were enzymatically dispersed from vasa deferentia of adult male guinea pigs (250–400 g). These cells reassociated in vitro to form monolayers and small spherical reaggregates (0.05–0.3 mm in Diam). Within 48 h of being placed in culture, cells in both types of preparation began to contract spontaneously. The contractions were rhythmic and slow. Cells in the monolayers stopped contracting after ~1 wk in vitro, but the reaggregates continued to contract spontaneously for at least 3 wk. Electron microscopy of the reaggregates revealed the presence of thick and thin myofilaments. Overshooting action potentials were recorded in many of the cells penetrated (primarily in reaggregates), and were accompanied by visible contractions of the aggregate or monolayer. Quiescent cells could often be excited by intracellularly applied depolarizing and hyperpolarizing (anodal-break) current pulses. The resting potentials had a mean value of -58 ± 2 mV. The action potentials were usually preceded by a spontaneous depolarization. The action potentials had slow rates of rise (1–4 V/s) which were unaffected by tetrodotoxin (TTX, 1 μg/ml), a known blocker of fast Na^+-channels. Verapamil (1 μg/ml) blocked the action potentials. The mean value of input resistance was 6.9 ± 0.5 MΩ ($n = 12$). These electrophysiological properties are similar to those of intact adult vas deferens smooth muscle cells. Thus, the cultured adult vas deferens smooth muscle cells retain their functional properties in vitro even after long periods.

KEY WORDS   cultured smooth muscle cells · electrophysiology of smooth muscle · differentiation in vitro · vas deferens smooth muscle · reaggregate culture · electrogenic pump · ultrastructure of smooth muscle

The properties of smooth muscle cells in culture have been reported to be either similar to, or quite different from, those of the cells in their in situ environment. The reasons for these differences in the functional state of the cultured cells is not known, but may be related to the type of culture (i.e., explant vs. enzyme-dispersed), the culture medium (i.e., presence of "conditioning" factors), and cell associations (i.e., monolayer vs. reaggregates). For example, cells dispersed from great vessels near the heart and from mesenteric

vessels (mixed arteries and veins) of chick embryos possess similar properties when induced to reaggregate into small spheres in vitro, and also retain thick and thin contractile myofilaments (25). Kimes and Brandt (19) also reported that enzymatically dispersed cells from thoracic aortic media of embryonic rats contract spontaneously and fire overshooting action potentials in monolayer cell culture, even after many passages in vitro. However, cells which emigrate from medial explants of adult rat aorta only rarely contract either spontaneously or in response to drugs (23).

Although cells which grow out from such explants lose their thick myofilaments, they retain the ability to synthesize proteoglycans typically found in atheromatous lesions of blood vessels (3, 28, 29). While some selection of cells may have occurred during emigration from the explants, it appears that cells derived by division of smooth muscle cells in the explant may alter their functional state, i.e., partially dedifferentiate,[1] much as cells in blood vessels *in situ* do in response to injury (see reference 31 for review).

Conditions in vitro, then, may determine whether the altered cells can return to their original functional state or further dedifferentiate. For example, Chamley and Campbell (8) reported that smooth muscle cells from guinea pig vas deferens contract spontaneously for several days in monolayer cell culture, and then cease contracting. If the monolayers reached confluence within ~10 d, contractions reappeared; however, if confluence was not reached within that time contractions never reappeared, even if confluence was ultimately reached. They also found that cells in clumps which had not been dispersed never lost contractile ability. Simultaneous with the loss of contractility, the cells lost their ability to bind antimyosin antibody, but retained the ability to bind anti-smooth muscle actin antibody; when contractions reappeared in the confluent monolayers, the ability to bind antimyosin antibody also returned (15). Similarly, vascular smooth muscle cells from adult human, monkey, and rabbit blood vessels initially contracted in response to pharmacological and mechanical stimuli and could bind both anti-smooth muscle actin and myosin antibodies (10). Upon intense proliferation, antimyosin binding capacity was lost and was subse-

quently regained upon achievement of confluence. This partial dedifferentiation, or modulation, could be slowed by the addition of neurons to the cultures (9), by the addition of cyclic AMP to the medium, or by the presence of a layer of fibroblasts (10). Cells that migrated from explants of vascular muscle resembled the dedifferentiated cells at all times in their inability to bind smooth muscle antimyosin antibodies. Thus, it appears that culture conditions, e.g., cell associations, are important in determining the expression of normal phenotypic properties, i.e., the degree to which the cultured cells resemble cells in the intact tissue.

One factor which Chamley et al. (9) found to be important was the plating density of the cells. One possible explanation offered by these investigators is that at higher plating density the number of divisions necessary to achieve confluence was less and the cells could thereby retain or regain their original properties; however, at low density and with prolonged proliferation, this plasticity might be lost. Alternatively, some factor related to cell association may be important. We have previously shown that enzymatically dispersed chick embryonic myocardial cells induced to reaggregate into small (0.1–1.0 mm) spheres in culture retain many adultlike electrophysiological and pharmacological properties for long periods in vitro, i.e., they do not dedifferentiate (34).

We set out here to determine whether functional properties of the intact tissue could be retained by cultured vas deferens smooth muscle cells, without dedifferentiation (if the cells are allowed to reaggregate into small spheres), by using for comparison the electrophysiological properties of intact vas deferens recently described (4, 5, 14). We have found that smooth muscle cells, derived from adult guinea pig vas deferens, retained their musclelike functional properties if allowed to reaggregate in culture.

MATERIALS AND METHODS

Cell Culture

Male guinea pigs (250–400 g) were killed by a blow to the base of the skull and the abdomen was opened under sterile conditions. The vasa deferentia were dissected bilaterally by cutting free the mesentery and then transecting the vasa at the junction with the epididymis and close to the prostate gland. The isolated vasa were placed in cold culture medium (40% Puck's N-16 nutrient solution, 50% Tyrode's balanced salt solution [144.0

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[1] This process has been termed "modulation" by Ross (personal communication).

mM Na^+, 2.7 mM K^+, 1.8 mM Ca^{++}, 1.0 mM Mg^{++}, 145.2 mM Cl^-, 1.1 mM $H_2PO_4^-$, 6.0 mM HCO_3^-, and 5.5 mM glucose], and 10% fetal calf serum) until 6–12 vasa had been collected. They were subsequently washed with Ca^{++}- and Mg^{++}-free Tyrode's solution and then placed in a 50-cc Erlenmeyer flask, which contained a magnetic stirring bar, for trypsinization. They were then stirred at room temperature for 20 min in 10 ml of Ca^{++}- and Mg^{++}-free Tyrode's containing trypsin (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. 1:300, 0.0125 g/100 ml) to remove adventitial connective tissues. At the end of the 20 min, the supernate which contained connective tissue cells was discarded and fresh enzyme solution was added, and the trypsinization process was repeated. After this, the trypsin solution was replaced with Ca^{++}- and Mg^{++}-free Tyrode's containing collagenase (Worthington Biochemical Corp., Freehold, N. J., Grade III; 0.1 mg/ml of 480 U/mg) and hyaluronidase (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml). After each period of stirring in the second enzyme solution, the supernate which contained dispersed muscle cells was decanted into 3 vol of ice-cold culture medium in 50-ml centrifuge tubes. Cells were collected at 10-min intervals until digestion was complete (total of 6–8 collections). The cells in each centrifuge tube were pelleted by centrifugation for 10 min at low speed (500 rpm; ~50 g) in an IEC model UV centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). The cells were resuspended in culture medium at a density of ~10^6 cells/ml, and ~3 ml were plated in Falcon plastic culture dishes (No. 1,007; Falcon Plastics, Division of Bioquest, Oxnard, Calif.) containing sterile glass microscope cover slips. It was important that sufficient numbers of cells were added to each culture dish to obtain optimal viability and function. Cells which settled on the glass cover slips adhered and spread out. After 48 h in vitro, small clusters of cells formed and these began to spontaneously pulsate slowly. Cells which settled onto the plastic, to which they adhered poorly, formed freely floating spherical reaggregates 0.05–0.3 mm in Diam. These reaggregates also pulsated spontaneously. The cultures were maintained in a moist-air incubator bubbled with 95% air-5% CO_2 for 5–30 d before experimentation.

Electrophysiological Recordings

Experiments were performed after culture periods of 5–30 d. For electrophysiological studies of the cultured smooth muscle cells, two types of preparations were employed: (a) Monolayers of cells attached to glass cover slips were studied by placing the cover slip in an uncirculated bath containing culture medium equilibrated with 95% O_2-5% CO_2 (pH 7.3) at 37°C on the stage of a trinocular microscope. A television camera was attached to the third ocular of the microscope and connected to a remote monitor to aid penetration of the single cells at × 500-1,000. A De FonBrune pneumatic

micromanipulator (Beaudouin Co., Motrouge, France) was used to make the microelectrode impalements. (b) Reaggregates were transferred to a bath containing culture medium or balanced salt solutions of various compositions at 37°C. The solution was circulated by a jet of gas (95% O_2, 5% CO_2), and the air over the experimental bath was humidified to minimize evaporation. The microelectrodes were mounted on a Zeiss sliding micromanipulator (Carl Zeiss, Inc., New York, N. Y.).

For intracellular potential measurements, glass microelectrodes filled with 3 M KCl (40–100 MΩ) were used. Ag:AgCl half-cells were used, and the preamplifier was a W-P Instruments model WP-1 (W-P Instruments, Inc., New Haven, Conn.) for the monolayers, or a Dagan model 8500 preamplifier (Dagan Co., Minneapolis, Minn.) for the reaggregates. A bridge circuit was used to allow simultaneous passage of polarizing current while recording voltage. Use of the bridge circuit and its limitations have been described in detail elsewhere (32). The preamplifier signals were amplified and displayed on a Tektronix 565 oscilloscope (Tektronix, Inc., Beaverton, Oreg.). Maximal rates of rise of the action potentials were measured from photographs of tracings taken at fast sweep speeds and with a passive RC differentiator. Grass Kymograph cameras (Grass Instrument Co., Quincy, Mass.) were used to photograph the oscilloscope tracings.

Drugs were added directly to the experimental bath from concentrated stock solutions. To determine the effects of extracellular K^+ concentration on membrane potential, equimolar substitution of K^+ for Na^+ in the Ringer's solution was made.

Microscopy

Still photomicrographs of the various preparations were taken with a Leitz Orthomat camera system (E. Leitz, Inc., Rockleigh, N. J.). Motion pictures were taken with a 16-mm movie camera.

For electron microscopy, the reaggregates or monolayers were fixed for 4 h in 4% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4. The tissues were rinsed in cacodylate buffer, and postfixed in 1% OsO_4 for 4 h. They were then stained en bloc in uranyl acetate, dehydrated through a graded series of acetone concentrations, passed through propylene oxide, and embedded in Epon 812. Thin sections were cut with diamond knives on a Reichert thermal-advance ultramicrotome (C. Richert. Sold by American Optical Corp., Buffalo, N. Y.). They were mounted on copper grids, stained with lead citrate, and examined with a Zeiss EM-9A transmission electron microscope.

RESULTS

Morphology

LIGHT MICROSCOPY: Cells which adhered to glass coverslips reassociated into monolayer

patterns (Fig. 1 C) and small clusters (Fig. 1 D) within ~24 h after plating. Small spherical reaggregates (50-300 μm in Diam) also were formed spontaneously, apparently from cells which did not adhere well to the plastic substrate (Fig. 1 B). Some reaggregates were attached to the substrate (glass or plastic) by an apron (outgrowth) of cells. Fig. 1 A shows the general arrangement of the various types of cell groups (including monolayers, clusters, and spherical reaggregates) at low magnification. Most cells in the monolayers and clusters were elongated and spindle-shaped, characteristic of smooth muscle cells. Some cells began to contract spontaneously after ~48 h. Although cell division occurred, the cells were plated at a high density (>6 × 10^5 cells/ml) to allow rapid attainment of confluence (within 5 d). Upon attainment of confluence, the monolayers and clusters ceased contracting spontaneously and could not be induced to contract by electric-field stimu-

lation. In contrast, reaggregates continued to contract spontaneously for up to 3 wk in vitro. Contractions of single cells in the reaggregate aprons could be readily observed for up to 9 d in culture.

The spontaneous contractions of the cells in monolayers and small cell clusters occurred either as single contractions or repetitively. When repetitive, the cycle was ~3-6 s. Many cells remained quiescent for long periods and suddenly began to contract rhythmically or gave single contractions. In the case of the spherical reaggregates, various regions could be seen to contract asynchronously at one moment, and then the whole reaggregate would contract synchronously. It was difficult to alter the rate of the spontaneous contractions by extracellular electric field stimulation (brief rectangular pulses delivered through platinum plate electrodes). Sustained contractions were also elicited by irritation of clusters or reaggregates with
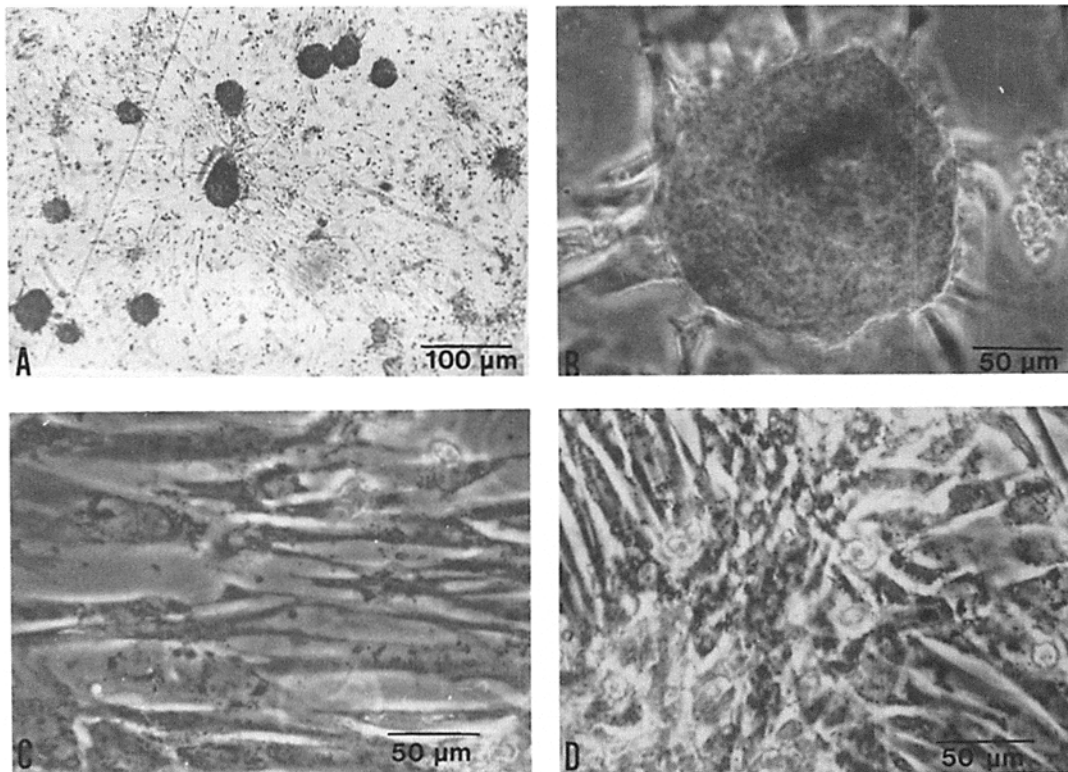

FIGURE 1 Light micrographs of cultured vas deferens smooth muscle cells. (A) Low power micrograph showing spherical reaggregates interspersed among monolayer cells. Most of the reaggregates in this field were spontaneously contracting. (B) Higher power phase-contrast micrograph showing a spherical reaggregate after 5 d in vitro. (C and D) Phase-contrast micrographs of monolayer cells which were contracting after 5 d in vitro.

the microelectrode which resulted in contraction of many cells for several minutes, followed by slow relaxation. This type of contraction may have been induced by cell injury (34).

ELECTRON MICROSCOPY: Ultrastructural study of cells in the spherical reaggregates (2 wk in culture) revealed bundles of thick cytoplasmic myofilaments ($\sim$174 $\pm$ 9 Å (n = 27) in Diam) (Fig. 2). In some cells, the myofilaments were not aligned in parallel, but instead were oriented in perpendicular directions (Fig. 2A). Abundant thin filaments ($\sim$73 $\pm$ 5 Å (n = 16) in Diam) ran parallel to the thick filaments. The myofilaments radiated towards "dense bodies", which are structures characteristic of smooth muscle cells (Figs. 2 and 3). The cells also had sarcolemmal caveolae along extensive lengths of membrane (Figs. 2A and C, and 3). Such caveolae are also characteristic of smooth muscle cells. The cells abutted in close apposition for considerable lengths, but no gap junctions were observed. All cells in the reaggregates appeared to be homogeneous in type, i.e., they all seemed to be smooth muscle cells. Almost all the cells appeared to be healthy, as evidenced by the intact mitochondria, normal-looking nuclei, and absence of vacuolization.

Examination of confluent monolayers/multilayers of cells which had been cultured for 2 wk and had ceased to contract revealed absence of thick myofilaments (Figs. 4 and 5). Many cells had dense subsarcolemmal fascicles of thin filaments (e.g., Figs. 4B and 5). However, these cells retained their sarcolemmal caveolae (Fig. 4C). Appearance of dilated cisterns of rough endoplasmic reticulum (Fig. 5B) and extracellular fibrillar material (some with a periodicity similar to that of collagen) (Fig. 4C) suggested that these cells were manufacturing and secreting proteins, as described by Ross (28). That is, the cells in such monolayers/multilayers cultured for 2 wk or more seemed to be in an altered functional state, i.e., partially dedifferentiated, and characterized by synthesis and secretion of proteins rather than contractility.

Electrophysiology

RESTING POTENTIAL: In spherical reaggregates, the mean resting potential obtained by the intracellular microelectrode impalements in normal culture medium (5.4 mM K^+) was -58 ± 2 mV (n = 60). In the monolayers, lower values of resting potential were recorded (-38 ± 1 mV, n

= 12), probably because of the greater difficulty of obtaining adequate stable impalements.

In spherical reaggregates, the resting membrane potential (E_m) was also recorded as a function of the external K^+ concentration ($[K]_o$). The results of these experiments are given in Fig. 6. As can be seen, the cells became depolarized in elevated K^+ concentrations. The slope of the straight line between 3 and 10 mM $[K]_o$, obtained by linear regression analysis, was 23 mV/decade (filled circles) (correlation coefficient of 0.99). However, the extrapolated intercept of this straight line to the abscissa (zero potential) was 516 mM, a value much too high for $[K]_i$.

Therefore, to test for a possible contribution of an electrogenic pump potential, the curve was repeated in the absence (filled squares) and in the presence (unfilled squares) of ouabain (10^{-4} M) to inhibit the (Na, K)-ATPase and Na-K pump, and the results of these experiments are also shown in Fig. 6. (For further explanation of the symbols, see the legend for Fig. 6). All measurements of E_m in the presence of ouabain were made between 2 and 7 min after ouabain addition, to minimize any run down of the ionic gradients. The slope of the straight line between 6 and 100 mM $[K]_o$, obtained by linear regression analysis, was 28 mV/decade (correlation coefficient of 0.99). Extrapolation of this straight line to the abscissa (zero potential) gave a value of 187 mM, which is a much more realistic value for $[K]_i$. The electrogenic pump potential contribution to the measured E_m varied from 8 to 15 mV, depending on the $[K]_o$ level, with a mean value of 10.5 mV (between 5.7 and 100 mM $[K]_o$). Therefore, a substantial electrogenic pump potential is present throughout the various $[K]_o$ levels and is responsible for the high extrapolated value in experiments performed without inhibition of the ion pump.

Lowering of $[K]_o$ below 3 mM, namely to 1.0, 0.5, and 0.1 mM, also produced depolarization to a mean value of ~ -40 mV at 0.1 mM, as shown in Fig. 6. This depolarization may be because of two factors: (a) inhibition of an electrogenic pump potential contribution to E_m in low $[K]_o$, and (b) a decrease in K^+ conductance (G_K) in low $[K]_o$ caused by less K^+ ions available to carry current. In addition, there is evidence that the K^+ permeability (P_K) is lowered in low $[K]_o$ (6). Thus, G_K is lowered for two reasons: a low P_K, and less ions available to carry current. Two experiments were
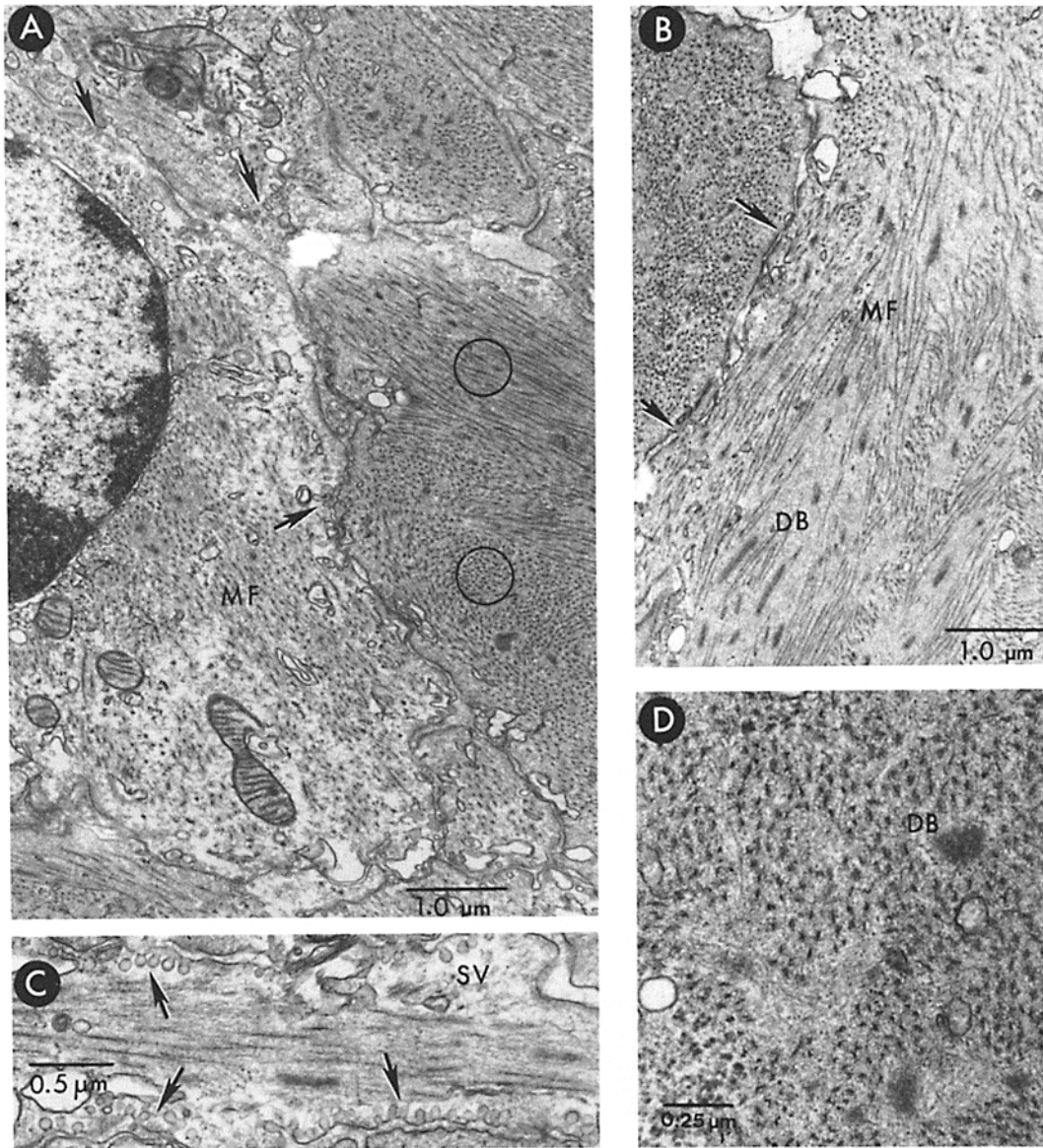
FIGURE 2   Electron micrographs of reaggregated vas deferens smooth muscle cells in culture for 9 d. (A) Low power view showing thick myofibrils (MF) running in different directions in the cytoplasm of several neighboring cells. Arrows point to sarcolemmal vesicles. Some myofibrils in longitudinal section and in cross section have been circled. (B) Micrograph showing two adjacent cells (arrows indicate apposition of cell membranes) containing myofibrils in cross section and longitudinal section, and showing the characteristic dense bodies (DB) among longitudinally cut myofibrils. (C) Higher power electron micrograph showing rows of characteristic sarcolemmal vesicles (SV and arrows) along borders of a muscle cell. (D) Cross section of myofibrils at high power showing thick ($\sim$174 Å) and thin ($\sim$73 Å) filaments and dense bodies.
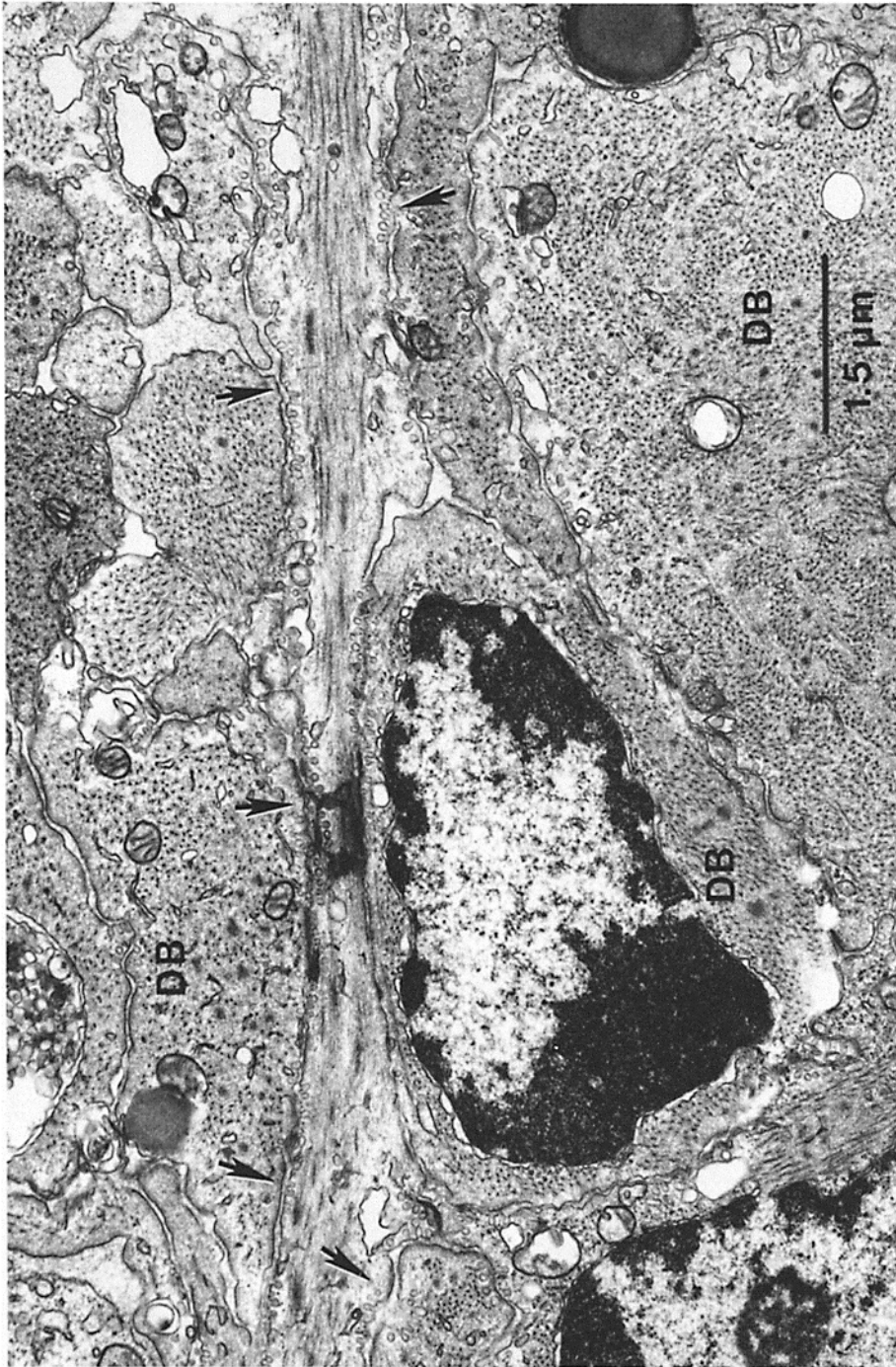
FIGURE 3 Electron micrograph of vas deferens smooth muscle cells in a reaggregate (cultured for 9 d) at low power to show that all cells in the field contain myofibrils. Arrows point to some of the sarcolemmal vesicles. Dense bodies are also evident in the cells.
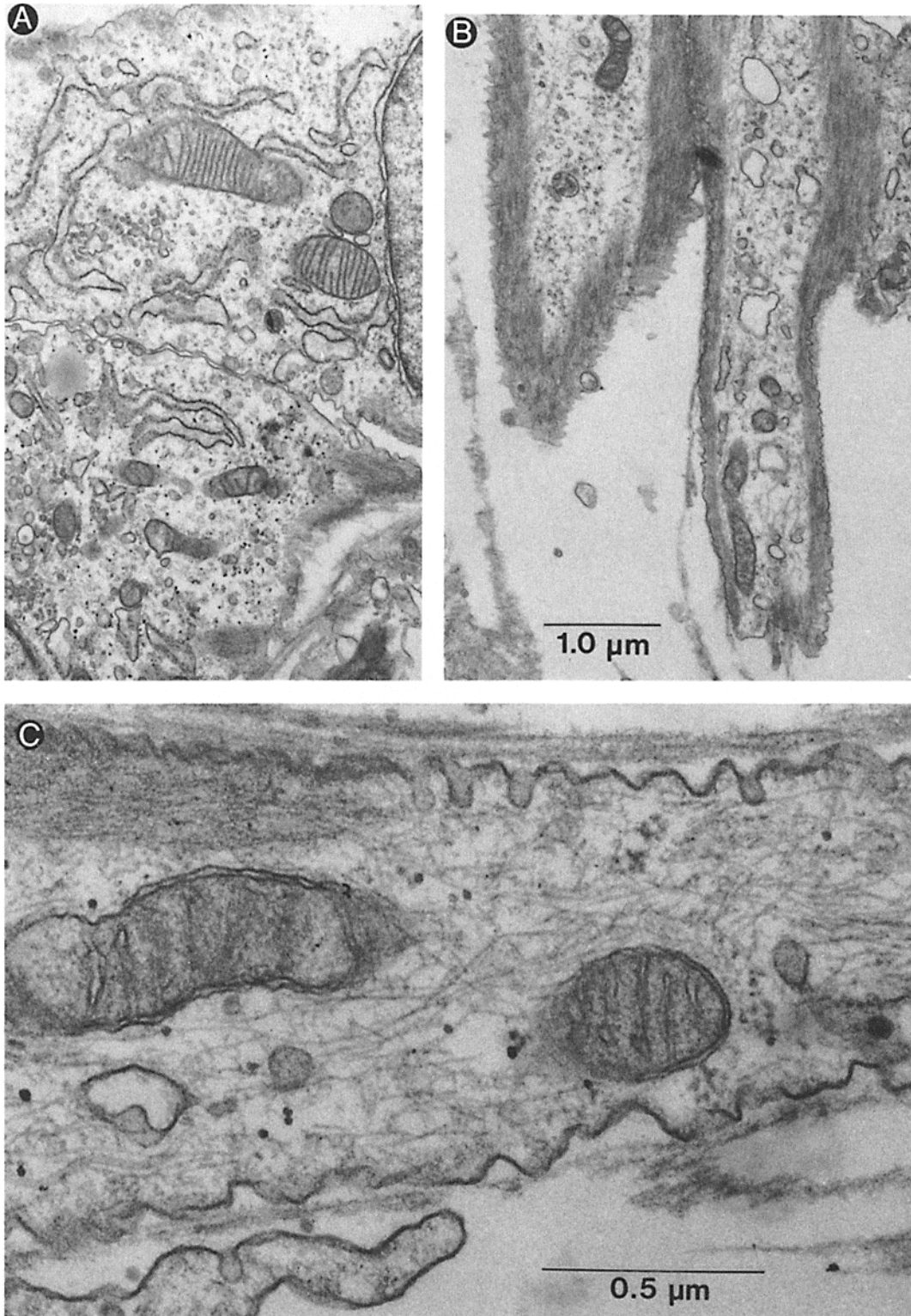
FIGURE 4   Electron micrographs of cells in monolayer culture after 14 d in vitro. (*A*) Low power view of area of abutment between three cells. Note dilated cisterns of rough endoplasmic reticulum and well-preserved mitochondria. (*B*) Low power micrograph that illustrates the subsarcolemmal localization of a dense matting of thin filaments. (*C*) Higher power view of a cell with subsarcolemmal aggregation of filaments and sarcolemmal vesicles (caveolae). Note also the presence of extracellular fibrillar material. Calibration in Fig. 4*B* applies also to Fig. 4*A*.

performed in which ouabain was added in 2.7 and 1.0 mM $[K]_o$. As shown in the figure, the cells became depolarized to ~ -35 mV. These results are reasonably close to the expected results based on the depolarization to -40 mV produced in 0.1 mM $[K]_o$. That is, in 0.1 mM $[K]_o$, there still could be a residual small electrogenic pump potential.

The presence of a substantial electrogenic pump potential may be an important factor in the suppression of automaticity and contraction in the "normal" condition found in the in vitro cultures. For example, the experiments described above dealing with ouabain were performed on quiescent cells, but the depolarization produced by the ouabain led to spontaneous generation of action potentials in many cases (Fig. 7). In Fig. 7A, ouabain $(10^{-4}$ M) was added to a reaggregate which was quiescent and exhibited a large stable resting potential. Within 20 s, a burst of spikes occurred in the impaled cell, probably caused by propagation from another cell depolarized by the ouabain because of inhibition of its electrogenic pump potential. A second burst of spikes occurred ~ 40 s after the first burst (Fig. 7B). The right-hand side of Fig. 7B shows that the impaled cell itself became suddenly depolarized, leading to a sustained train of impulses. Continuation of the train is shown in Fig. 7C. The rapid depolarization produced by the ouabain was ~ 8 mV in this case. These ouabain-induced action potentials usually did not overshoot or even reach the zero potential, as depicted in Fig. 7.

ACTION POTENTIALS: Only a small percentage of cells impaled in spherical reaggregate cultures (e.g., $\sim 10\%$) generated spontaneous action potentials (and contractions). Such a small percentage was found between 3 d and 4 wk in culture. Most of the quiescent cells, however, could be excited by intracellular or external electrical stimulation. After 4 wk in culture, all cells impaled were quiescent and inexcitable to electrical stimulation (both intracellular and external). The monolayer/multilayer cultures behaved in a similar fashion, except that they lost automaticity and excitability considerably earlier, namely after 5 d in culture.

Illustration of the spontaneous action potentials obtained in spherical reaggregate cultures is given in Fig. 8. As can be seen, an impalement gave a sharp drop in potential (Fig. 8A), concomitant with the appearance of overshooting action potentials. The amount of overshoot varied between 10 and 20 mV. The duration (at 50% repolarization)

of the action potential spikes was ~ 10–30 ms. The frequency of the spontaneous action potentials varied from ~ 1 to 3/s. The action potential trains were sometimes erratic, the spikes occurring in groups of two or three, as illustrated in Fig. 8B. In some instances, the contractions either completely dislodged the microelectrode or led to a partial injured state with a sustained partial depolarization (Fig. 8C). Some of the cells exhibited action potentials which possessed prominent depolarizing afterpotentials (Fig. 8D) (27). In the cases with rhythmic firing, application of a long-duration hyperpolarizing current pulse through the recording microelectrode (by means of the bridge circuit) slowed the frequency of discharge (Fig. 9A), as expected of pacemaker cells. Clear examples of pacemaker prepotentials may be seen in Figs. 8, 9A, and 10A and B.

The cells in spherical reaggregates which were quiescent upon impalement usually possessed a high and stable resting potential. However, as mentioned above, intracellular application of depolarizing (Fig. 9B) or hyperpolarizing (Fig. 9C) current pulses gave rise to overshooting action potentials accompanied by contractions. In the case of depolarizing pulses, the response occurred on the "make" (i.e., during the initial part of the pulse), whereas in the case of hyperpolarizing pulses, the response occurred on the "break" (i.e., anodal-break excitation).

The action potentials were not sensitive to tetrodotoxin (TTX). That is, their rate of rise and overshoot were not affected (Fig. 10B). The control maximal rate of rise $(+\dot{V}_{max})$ was generally ~ 1–4 V/s, and TTX $(10^{-6}$ M) did not affect this parameter. These results indicate that the channels which pass the inward current for the action potentials are insensitive to TTX (a specific blocker of fast Na^+-channels).

However, the action potentials were abolished by the addition of verapamil $(10^{-6}$ M), an agent which blocks slow current and slow channels (20, 30). This is illustrated in Fig. 10C. Thus, in conjunction with the TTX experiments, these results demonstrate that the channels which pass the inward current during the action potentials in the cultured smooth muscle cells have pharmacological properties similar to those of channels found in noncultured smooth muscles of various types.

MEASUREMENT OF INPUT RESISTANCE: Input resistance (R_{in}) was determined from voltage changes in response to intracellularly applied

A

1.0 μm

B

0.5 μm
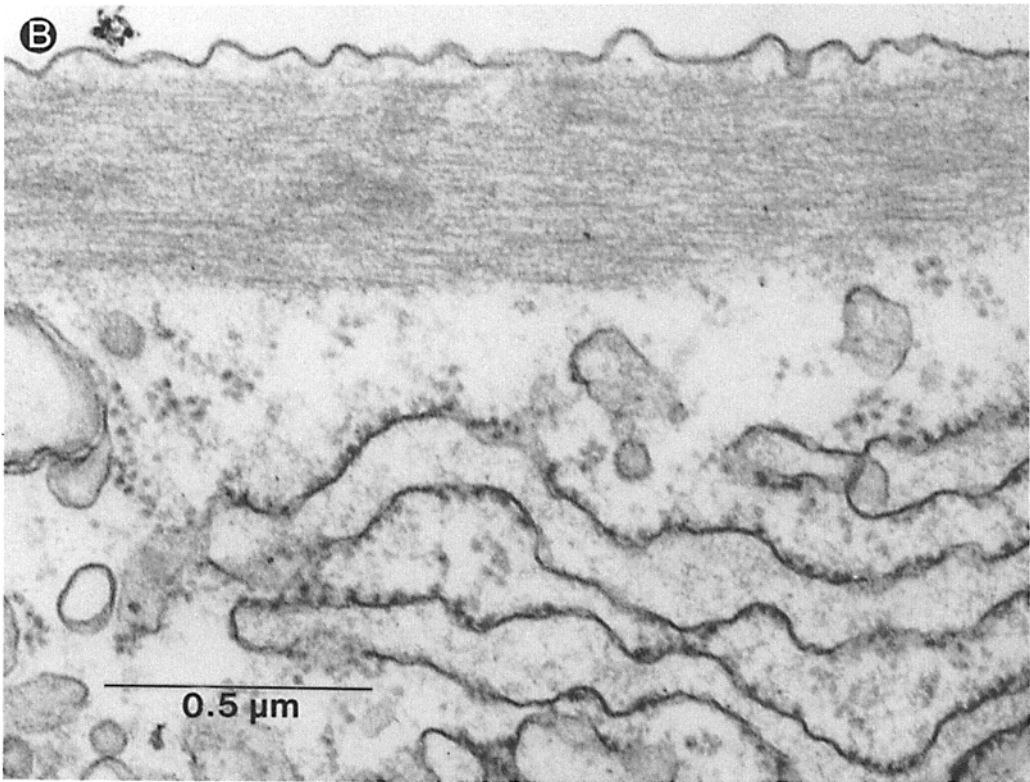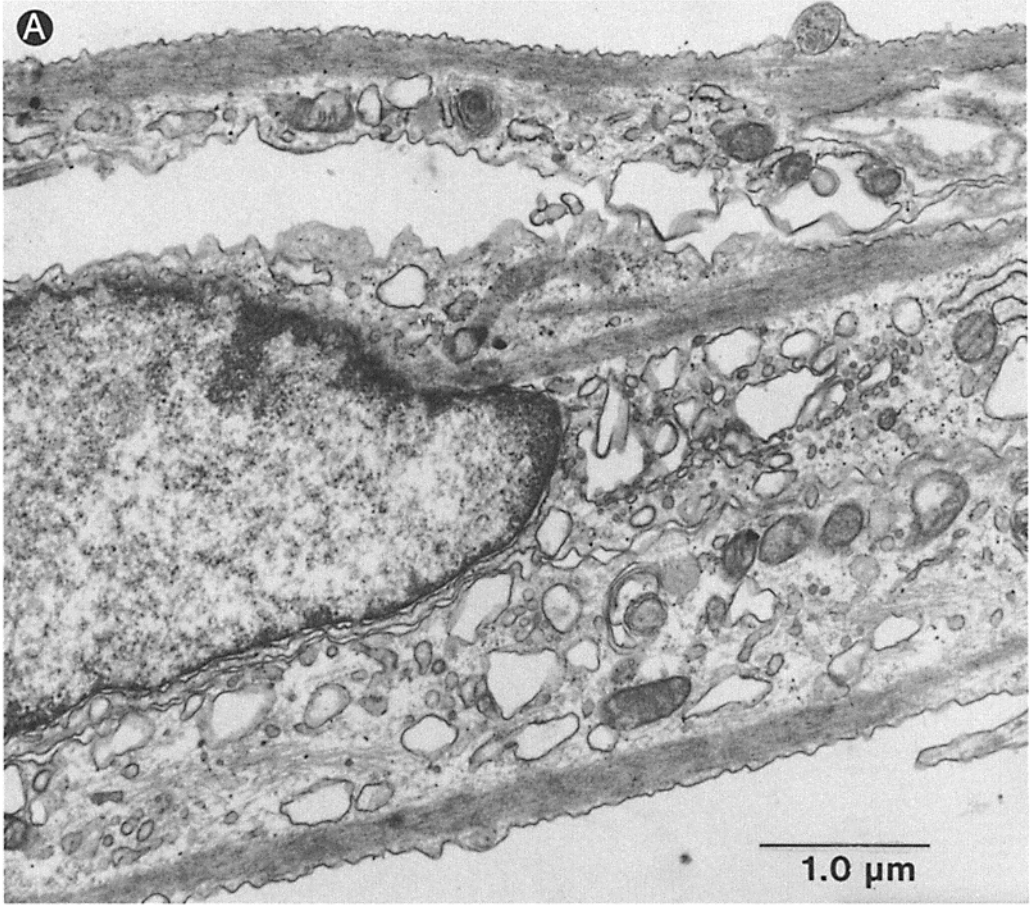
(through the microelectrode) polarizing current pulses. The mean R_{in} was 6.9 ± 0.5 MΩ ($n = 12$), and was nearly the same for cells in monolayers and in spherical reaggregates. Because the degree of cell coupling (low resistance) and the cell dimensions and geometry are not known in
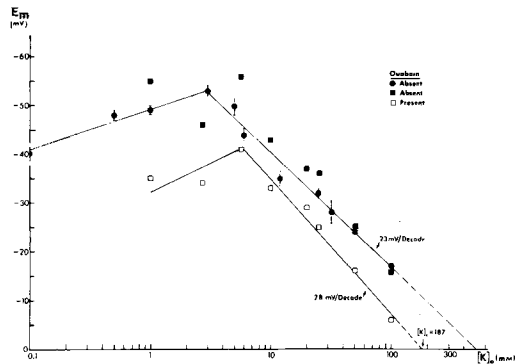

FIGURE 6   Plot of resting membrane potential (E_m) versus extracellular K^+ concentration ([K]$_o$, on a logarithmic scale) for the cultured reaggregates of guinea pig vas deferens smooth muscle cells. Data were collected in the absence (filled symbols) and in the presence (unfilled squares) of ouabain (1×10^{-4} M). The pair of square symbols at each |K|$_o$ value represents a single experiment; the control E_m before the addition of ouabain is given by the filled square. After this sequence in the protocol, several additional impalements were made in other cells in the presence of ouabain, and the experiment was accepted only if the value of E_m (in the presence of ouabain) was not significantly different statistically from the mean value of these extra penetrations. The filled circles give the mean $\pm$ 1 SE for a separate set of experiments performed only in the absence of ouabain; the SE bars at 50 and 100 mM |K|$_o$ are too small to be visible (n varied between 9 and 35 impalements). Exposure to ouabain was for short periods (2-7 min) only to suppress the electrogenic Na$^+$-pump potential without causing the ionic gradients to become significantly dissipated. The curve in the presence of ouabain was extrapolated to zero potential (dashed line) to give an approximation of the internal K^+ concentration ([K]$_i$). Extrapolation of the curve in the absence of ouabain gives an erroneously high value for [K]$_i$ because of the error (potential offset) introduced by the electrogenic pump potential.

FIGURE 7   Ouabain induction of automaticity in quiescent cultured smooth muscle cells (spherical reaggregates of guinea pig vas deferens). (A) Impalement of a quiescent cell with a large (−60 mV) stable resting potential; ouabain (10^{-4} M) was added at the arrow. Within 20 s, a burst of spikes occurred. (B) A second burst occurred ~20 s after the first burst, and this was followed within another 20 s by a sustained depolarization of 8 mV and a sustained train of impulses. (C) Continuation of the train. The three records depicted are consecutive sweeps of the oscilloscope.
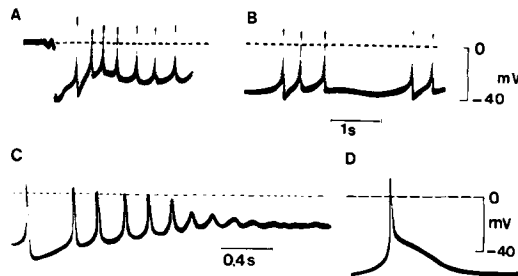
FIGURE 8   Intracellular electrical recordings from cultured spherical reaggregates of vas deferens smooth muscle cells which spontaneously contracted up to 3 wk in vitro. (A) Recording obtained upon impalement of a cell in a reaggregate cultured for 5 d, showing a sustained train of spontaneous overshooting action potentials. Note presence of pacemaker potentials. (B) Periodic bursting of action potentials in a cell in another reaggregate cultured for 2 wk. (C) Decrementing of action potentials recorded as the microelectrode became partially dislodged from a cell during contraction (reaggregate cultured for 2 wk). (D) Illustration of an action potential which possessed a prominent depolarization afterpotential. Time and voltage calibrations in Fig. 8B apply to Fig. 8A and B. Time calibration in Fig. 8C also applies to Fig. 8D. Horizontal broken line represents zero potential.

FIGURE 5   Electron micrographs of cells in monolayer culture after 14 d in vitro. (A) Cross section through thickness of monolayer to illustrate the overlapping (layering) of at least three cells. (B) Higher power view of an area containing subsarcolemmal filaments to illustrate the dilated cisterns of rough endoplasmic reticulum. The cell surface contains sarcolemmal vesicles.
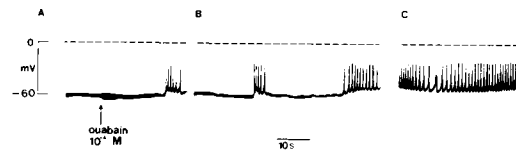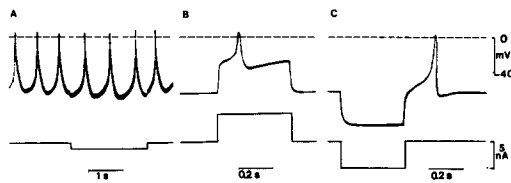
FIGURE 9 Effect of intracellularly applied current pulses on cultured spherical reaggregates of vas deferens smooth muscle cells. The current pulses were applied through the recording microelectrode by using a bridge circuit. (A) A 1-nA hyperpolarizing current pulse applied in a spontaneously firing cell produced hyperpolarization, an increase in action potential amplitude, and a decrease in the rate of firing, indicating that this cell was a pacemaker cell. (B) An action potential was triggered on the make of a depolarizing pulse (5 nA) (in a different reaggregate). (C) Anodal-break excitation occurred in response to a hyperpolarizing current pulse (5 nA). Voltage and current calibrations in Fig. 9C apply throughout.
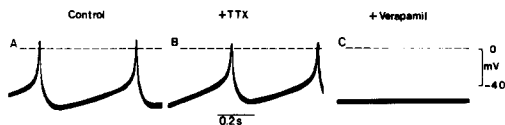

FIGURE 10 Effects of tetrodotoxin (TTX) and verapamil on the action potentials of cultured reaggregates of vas deferens smooth muscle cells. (A) Control action potentials with slow rates of rise and preceded by pacemaker potentials. (B) TTX (4 μg/ml) addition did not affect the action potential rate of rise. (C) Verapamil (1 μg/ml), a known antagonist of slow current, rapidly abolished the action potentials and contractions.

this preparation, calculation of membrane resistivity (R_m) was not done.

DISCUSSION

The present results demonstrate that functional smooth muscle cells can be maintained in primary cultures prepared from adult guinea pig vas deferens. (It should be noted that the cultured smooth muscle cells were readily prepared from adult tissues, i.e., embryonic or neonatal tissue was not required.) The conclusion that the cultured cells remain differentiated as functional smooth muscle cells is based on the following criteria: (a) electrophysiological recordings of spontaneous and stimulated action potentials, (b) the presence of visible spontaneous contractions,

and (c) ultrastructural detection of thick and thin myofilaments. When the cultured smooth muscle cells are prepared as monolayers, they lose their musclelike properties within ~1 wk in vitro, i.e., they appear to dedifferentiate: they lose their contractility and their excitability. In contrast, when the cultured smooth muscle cells are prepared as reaggregates, they continue to contract spontaneously and fire action potentials during observation periods of up to 3 wk. We do not know the reason why the reaggregated cells retained their state of differentiation for longer periods than the monolayer cells. Several possibilities include: (a) considerably reduced rate of cell division in the reaggregated cells, (b) trapping of some essential factor secreted by the smooth muscle cells within the interstitial space of the reaggregate, and (c) the three-dimensional apposition of cells and cell-to-cell contacts.

The persistence of highly differentiated functional activity in reaggregates for extensive periods in culture has been noted not only in the present study of vas deferens smooth muscle, but also in reaggregates of cultured embryonic heart cells (11, 33) and in vascular smooth muscle (25; Harder & Sperelakis, unpublished observations). Chamley et al. (9) have previously shown that vas deferens smooth muscle cells from neonatal guinea pigs contract spontaneously for ~1 wk in vitro, but then dedifferentiate while proliferating intensely. Our present findings with the monolayer cultures are similar to theirs. Chamley and Campbell (8) pointed out that division of cultured smooth muscle cells resembles the ability of the smooth muscle in intact tissues to dedifferentiate, i.e., alter their functional state, and undergo cell division in wound healing. Chamley et al. (9) reported that if confluence of the monolayers were achieved within a certain period of time, the original musclelike properties could be regained; but after this time, the cells remained dedifferentiated, even though confluent. Thus, the relationship of cell division to the state of differentiation remains unclear.

In many respects, the propertis of the cultured vas deferens smooth muscle cells resemble those of the intact vasa, as described by Goto et al. (14). For example, membrane potential parameters were very similar, including: (a) resting potentials (−58 mV both in the cultured cells and in the intact tissue), (b) action potential rate of rise (1–4 V/s in the cultured cells compared with 4 V/s in the intact tissue), and (c) action potential

duration (80 ms in the cultured cells versus 100 ms in the intact tissue). In addition, in agreement with the observations of Goto et al. (14), we found only a small percentage of cells (~10%) to be spontaneously firing. When the impalements were held for long periods some cells which were initially quiescent became spontaneously active. However, we did not observe junctional potentials as recorded by Goto et al. (14), because the cultured smooth muscle cells were denervated. The cultured cells were not sensitive to TTX as expected, because smooth muscle cells are well known not to possess fast Na^+-channels (2, 21). On the other hand, the action potentials were blocked by verapamil, a known blocker of slow current (20, 30).

The present results also demonstrated the presence of a relatively large electrogenic pump potential, averaging ~11 mV, in the cultured cells. Similar findings of an electrogenic pump potential in intact smooth muscles were reported by several laboratories (see review by Thomas [36], and summary in a recent review by Kumamoto [21]). For example, Hendrickx and Casteels (16) concluded that the electrogenic Na pump plays an important role in maintaining the membrane potential of arterial smooth muscle cells. Evidence for Na-pump potential in the rat myometrium was reported by Taylor et al. (35) and in the smooth muscle of guinea pig portal vein by Kuriyama et al. (22).

The inhibition of the electrogenic Na-K pump by ouabain (12, 17, 18, 26) caused a rapid depolarization which was followed by the initiation of spontaneous discharge in cells which were previously quiescent (see also reference 24). That is, the larger resting potential caused by the presence of the electrogenic pump suppresses excitability.

The results of the E_m vs. log $[K]_o$ curve in the absence and presence of ouabain were unexpected. First, the extrapolation of the curve in the absence of ouabain gave a value for $[K]_i$ that was much too high (516 mM). Therefore, the curve was repeated in the presence of ouabain (1×10^{-4} M) to inhibit any electrogenic pump potential (V_{pump}) contribution to the E_m which could be the cause of the erroneously high extrapolated value. When this was done, the curve did extrapolate to a more reasonable value for $[K]_i$, namely 187 mM (see Fig. 6). This value is close to values reported for intact smooth muscles (see summary in Kumamoto [21] and Casteels [7]), and demonstrates

that the cultured smooth muscle cells must maintain their normal Na-K pumping ability. The results clearly showed that ouabain rapidly produced a large depolarization of ~10 mV in 100 mM $[K]_o$, consistent with the presence of a large V_{pump} even at high $[K]_o$. We have no explanation for this strange behavior, because in most tissues, the V_{pump} contribution decreases in high $[K]_o$ (presumably because of the decrease in membrane resistance in high K^+), such that the two curves (in the absence and presence of ouabain) converge in high $[K^+]_o$ (e.g., see reference 13). On the other hand, a similar high extrapolated value was reported for smooth muscle by Kuriyama (22). In addition, it has been calculated by Anderson (1) that, for vascular smooth muscle, although there is some convergence between the two curves in high $[K]_o$, they do not completely converge to the same point, and there was a large difference in potential of ~6.5 mV in 100 mM $[K]_o$.

Another problem with the extrapolation method is that the slopes of the curves are much shallower than the theoretical 60 mV/decade, and therefore extrapolation is less accurate. Thus, the extrapolation method for estimation of $[K]_i$ at best provides an approximation of the real value.

In conclusion, we have identified smooth muscle cells in a primary culture preparation from adult vas deferens by several functional properties which are also present in intact vas deferens. That the cultured cells and the cells in the intact tissue have similar properties makes the cultured cells useful for many types of investigations. For example, the smooth muscle cells can be studied in the absence of neural influences. Other advantages of the cultured smooth muscle cell reaggregates include: (a) the reaggregates are easier to impale with one or two microelectrodes than are the intact strips because of the absence of connective tissue, (b) the cultured cells can be maintained in vitro for long periods in a carefully controlled environment, and (c) chemical microanalyses can be performed on pure smooth muscle cells (in the absence of other cell types).

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