SARCOPLASMIC RETICULUM AND THE

TEMPERATURE-DEPENDENT CONTRACTION OF SMOOTH

MUSCLE IN CALCIUM-FREE SOLUTIONS

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ABSTRACT

The contractile response of turtle oviduct smooth muscle to acetylcholine after 30 min of incubation of muscles in Ca-free, 4 mM ethylene (bis) oxyethylenenitrilotetraacetic acid (EGTA) solutions at room temperature was greater than the contractile response after 30 min of incubation in the Ca-free medium at 37°C. Incubation in Ca-free solution at 37°C before stimulation with acetylcholine in Ca-free solutions at room temperature also reduced the contractile response, suggesting that activator calcium was lost from the fibers at a faster rate at higher temperatures. Electron micrographs of turtle oviduct smooth muscle revealed a sarcoplasmic reticulum (SR) occupying approximately 4% of the nucleus- and mito-chondria-free cell volume. Incubation of oviduct smooth muscle with ferritin confirmed that the predominantly longitudinally oriented structures described as the SR did not communicate with the extracellular space. The SR formed fenestrations about the surface vesicles, and formed close contacts (couplings) with the surface membrane and surface vesicles in oviduct and vena caval smooth muscle; it is suggested that these are sites of electromechanical coupling. Calculation of the calcium requirements for smooth muscle contraction suggest that the amount of SR observed in the oviduct smooth muscle could supply the activator calcium for the contractions observed in Ca-free solutions. Incubation of oviduct smooth muscle in hypertonic solutions increased the electron opacity of the fibers. A new feature of some of the surface vesicles observed in oviduct, vena caval, and aortic smooth muscle was the presence of approximately 10 nm striations running approximately parallel to the openings of the vesicles to the extracellular space. Thick, thin, and intermediate filaments were observed in turtle oviduct smooth muscle, although the number of thick filaments seen in the present study appeared less than that previously found in mammalian smooth muscles.

The anatomical source of the calcium that activates smooth muscle contraction has not been established. In contrast, it is now generally accepted that in fast-striated muscles the sarcoplasmic reticulum is a storage site of activator calcium (e.g. Weber and Herz, 1968; Winegrad, 1970; for review see Bianchi, 1968; Ebashi and Endo, 1968; Peachey, 1968; Sandow, 1970), and contractures of these muscles can be elicited even in the absence of extracellular calcium (Axelsson and Thesleff, 1958; Frank, 1962).

Bozler (1969) recently reported and our laboratory (Somlyo and Somlyo, 1970) confirmed that the aortic smooth muscle of turtles can contract

even after vigorous removal of extracellular calcium with the chelating agent EGTA. We attempted to correlate the ability of reptilian smooth muscle to contract in the absence of extracellular calcium with the amount of the sarcoplasmic reticulum in this muscle, but our initial studies indicated that the ultrastructure of aortic smooth muscle was not suitable for even a semiquantitative evaluation of the internal membrane system. We therefore investigated the contractile properties of turtle oviduct smooth muscle, and, having found that this muscle can also contract in calcium-free media, we proceeded to evaluate its ultrastructure. The correlation of the electron microscopic and physiological lines of investigation led to the observation that the ability of smooth muscle to contract after incubation in calcium-free solution is significantly affected by temperature.

In this report we shall characterize the relationship of the sarcoplasmic reticulum to the surface membrane and to the surface vesicles, and present a semiquantitative estimate of the extent of the sarcoplasmic reticulum in oviduct smooth muscle, for comparison with similar measurements on the sarcoplasmic reticulum in striated muscle (Peachey, 1965) and on mammalian smooth muscles currently investigated in our laboratory (Devine and Somlyo, 1970). A limited number of observations were also made on smooth muscle incubated in hypotonic and hypertonic solutions before fixation for electron microscopy, primarily to ascertain that the observed configuration of the membrane systems was not grossly distorted by osmotic changes during fixation.

A fortuitous finding in this study was the presence, in surface vesicles of smooth muscle, of a special structure that does not appear to have been previously described in other so-called "pinocytotic" vesicles (e.g. Caesar et al., 1957; Palade and Bruns, 1968; Cornell, 1970).[1]

METHODS

Female diamondback turtles (*Malaclemys* sp.) from New Jersey were collected throughout the year (in-

[1] Preliminary reports of some of the findings have been published in: Somlyo, A. P., S. R. North, C. E. Devine, and A. V. Somlyo. 1971. *Fed. Proc.* 30:436. (Abstr.); and Somlyo, A. P., C. E. Devine, S. R. North, and A. V. Somlyo. 1971. Proceedings 25th International Congress Physiological Science. German Physiological Society, Munich. 9:526.

cluding stages when eggs were in the oviduct), and the oviduct, aorta, and vena cava were obtained for electron microscopy.

Physiological Studies

Strips consisting of opened rings of the oviduct were suspended in turtle Ringer's (Isojima and Bozler, 1963; millimolar composition: NaCl, 116.0; KCl, 1.5; $CaCl_2$, 1.0; $NaHCO_3$, 2.0; NaH_2PO_4, 2.0) bubbled with 95% O_2, 5% N_2, or in (modified) Krebs' bicarbonate solution (millimolar composition: NaCl, 118.9; KCl, 4.7; $CaCl_2$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 5.6) bubbled with 95% O_2, 5% CO_2 at room temperature (unless otherwise stated), and the tension was recorded through a FTA100 or FTO3 transducer. During experiments at 37°C the muscle chamber was immersed in a water bath held at constant temperature with a Tecam Tempunit (Tecam Instruments Techne Inc., Princeton, N. J.). Calcium-free turtle Ringer's or Krebs' bicarbonate solution was prepared by omitting calcium and adding 4 mM EGTA (ethylene(bis)oxyethylenenitrilotetraacetic acid, Geigy Chemical Corp., Ardsley, N.Y.). EGTA was brought to a pH of 7.3 with NaOH. The osmolality of turtle serum determined in preliminary experiments on two animals was approximately 320 mosmols, that of turtle Ringer's was 220 mosmols, and that of mammalian Krebs' was 290 mosmols. Osmolalities were measured with a Fiske osmometer Model G-66 (Fiske Associates, Inc., Uxbridge, Mass.)

Electron Microscopy

Tissues were fixed immediately after removal from the animal or after being placed in turtle Ringer's or mammalian Krebs' bicarbonate. The effects of osmotic changes on the smooth muscle were investigated on oviducts incubated for 10 or 30 min in hypotonic (diluted with distilled water to 172 mosmols), "isotonic" (220 mosmols), moderate hypertonic with sucrose added (454 mosmols), and extreme hypertonic (812 mosmols) turtle Ringer's before fixation. The tissues were fixed in:

(*a*) 3% glutaraldehyde in 0.1 M cacodylate buffer, turtle Ringer's (isotonic or the same osmolality as the incubation solution) or Krebs' bicarbonate solutions, pH 7.4.

(*b*) 3% glutaraldehyde + 4.5% sucrose in 0.1 M cacodylate buffer, pH 7.4.

(*c*) 3% osmium tetroxide in 0.1 M collidine buffer, pH 7.4 (1.5 hr fixation).

(*d*) 0.5% potassium permanganate in barbital–sodium acetate buffer (1 hr fixation).

(*e*) Some oviducts were stored for 2 wk in 0.1 M cacodylate-buffered, 3% glutaraldehyde.

Cadmium-free, crystalline horse spleen ferritin

(2 X crystallized, Nutritional Biochemicals Corporation, Cleveland, Ohio) was centrifuged at 40,000 rpm (100,000 g) for 120 min at 6°C in a Beckman model L-2 ultracentrifuge. The pellet was resuspended twice in Krebs' bicarbonate solution with a final ferritin concentration of approximately 20–30% by volume. Thin longitudinally stretched strips of turtle oviduct with the endometrium removed were placed in the ferritin solution bubbled with 95% O_2, 5% CO_2 at room temperature for 30–90 min and fixed in 0.1 M cacodylate-buffered 3% glutaraldehyde.

The tissues were fixed for 2 hr (except c and d), rinsed in buffer (in some instances, 6% sucrose was used in the buffer wash), and postfixed in 2% osmium tetroxide for 2 hr in the same buffer (except for c and d), stained en bloc with saturated aqueous uranyl acetate for 30 min, dehydrated in ethanol, and embedded in Spurr's embedding medium (Spurr, 1969). Thin sections were stained with uranyl acetate and/or alkaline lead citrate and viewed in a Hitachi HU11E electron microscope.

Sarcoplasmic Reticulum Volume

Estimates of the sarcoplasmic reticulum (SR) volume were made by cutting out and weighing on a Mettler balance (Mettler Instrument Corp., Princeton, N. J.) the areas occupied by the SR from a total of 34 montages (at final magnification of 47,400) and by expressing the SR areas in terms of the total cell area minus the area occupied by nucleus and mitochondria. The uniformity per weight of area of photographic prints was sufficient for integration by weight, as ascertained through direct comparison with planimetric measurements. The identification of SR was facilitated by the association of glycogen-like particles similar to those described in turtle atrium (Fawcett and Selby, 1958; Revel et al., 1960), and also found in association with SR of tortoise striated muscle (Page, 1968).

RESULTS

Contraction of Turtle Oviduct in Ca-Free Solutions

The contractile responses of oviduct strips to supramaximal acetylcholine (5 or 10 µg/ml) were first recorded in turtle Ringer's solution, and the tension developed was designated as 100%. The strips were then placed for half an hour in calcium-free, 4 mM EGTA turtle Ringer's solution with three washes at 10-min intervals. At this time the response to acetylcholine was again tested and expressed as per cent of the initial response. In the majority of experiments the

TABLE I

Contractile Response of Turtle Oviduct to Maximal Stimulation with Acetylcholine

Oviduct No.	In 4 mM EGTA, Ca-free Ringer's for 30 min (% tension of control)*	Returned to Ringer's for 30 min (% tension of control)*
1	50	—
	37	—
	42	—
2	100	—
	75	—
	83	—
3	60	18
	67	41
	64	28
4	13	67
	9	63
	10	60
5	28	74
	29	52
	32	72
	13	18
	9	18
	10	22
6	120	114
	120	100
7‡	31	70
	29	50
8‡	63	140
	61	120

* The initial maximal contractile response to acetylcholine in Ringer's solution = 100%.
‡ Krebs' solution substituted for Ringer's solution.

strips were then returned for half an hour to normal turtle Ringer's, and the response to acetylcholine was again determined and expressed as per cent of the initial control response: (Table I) considerable contractile responses were observed in the virtual absence of extracellular calcium. Furthermore, the reduction of the contractile response in calcium-free solution cannot be entirely ascribed to lack of activator calcium, since it was also seen in a number of strips after re-equilibration in calcium-containing, normal

turtle Ringer's solution (Table I, column 3) perhaps due to some persistent desensitization of the membrane to cholinergic stimulus. In any event, there was a substantial contractile response of oviduct smooth muscle in the virtually calcium-free solution. Because the compositions of mammalian and turtle salt solutions are different, for example, the former contains magnesium (the serum Mg concentration in two turtles was 2.0 mM/liter), the possibility could not be excluded that differences between reptilian and mammalian smooth muscles, such as the absence of contractile activity of certain mammalian smooth muscles in Ca-free solutions containing chelating agents (Hurwitz and Suria, 1971; see Discussion), was due to the composition of the respective experimental solutions. But the results of experiments on oviduct strips in mammalian Krebs' solution (Table I, asterisked experiments; and next section) were identical to those observed with turtle

Ringer's solution: up to 63% of the control responses were obtained after half an hour incubation in calcium-free, 4 mM EGTA mammalian Krebs' solution. More recently (see Discussion), contractions of mammalian smooth muscles have also been observed under similar (Ca-free) conditions.

Spontaneous, rhythmic contractions (Fig. 1) were more frequently observed when strips of oviduct smooth muscle were placed, under tension, in Krebs' solution than in turtle Ringer's solution. Spontaneous contractions persisted in three strips for 30 min in Krebs' solutions without added calcium and for an additional 30 min in calcium-free solution including 4 mM EGTA.

The strips transiently contracted when the solution was changed from Ringer's or Krebs' solution to Ca-free 4 mM EGTA solution. Acetylcholine-induced contractions were generally more tonic in normal (calcium containing) than in Ca-free solutions.
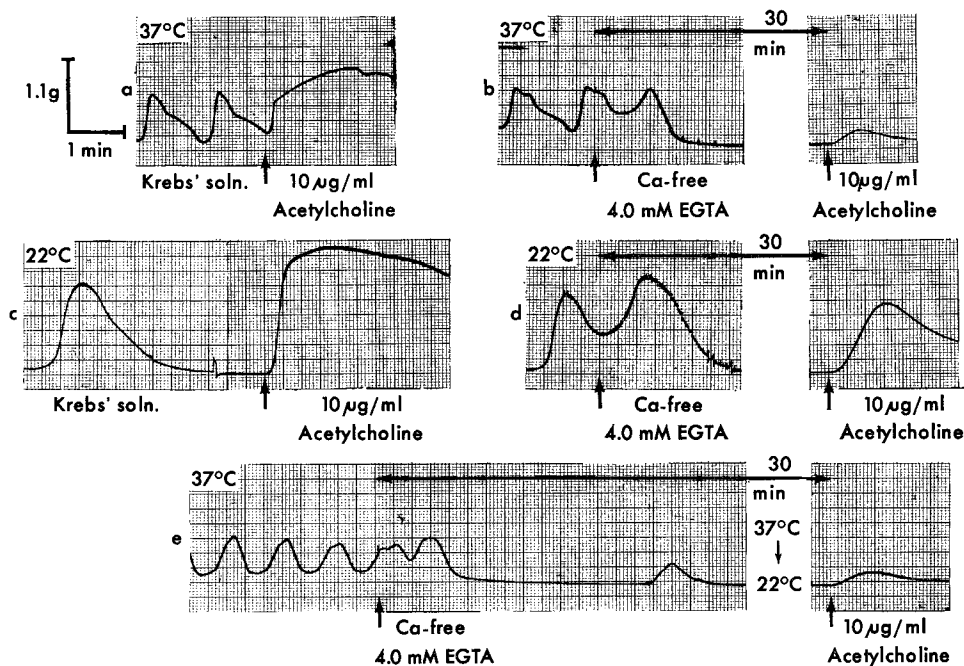

FIGURE 1 Temperature dependence of turtle oviduct responses to acetylcholine in Ca-free media. Contractile response of turtle oviduct at 37°C and 22°C in Krebs' solution (1.2 mM Ca) and in Ca-free solution containing 4.0 mM EGTA. a and c: spontaneous contractions and the response to acetylcholine (arrow) at 37°C and 22°C in 1.2 mM Ca-Krebs' solution. b and d: the responses to changing the bathing solution (arrow) to Ca-free, 4.0 mM EGTA Krebs' solution at 37°C and at 22°C, followed 30 min later by contractile responses to acetylcholine (arrow). In e the strip was changed (arrow) to Ca-free 4.0 mM EGTA solution at 37°C and 15 min later the temperature was decreased to 22°C for 15 min before the addition of acetylcholine (arrow). Contractions not marked by arrows are spontaneous.

Tension Developed by Turtle Oviduct in Response to 10.0 µg/ml Acetylcholine

Temperature	1.2 mM Ca Krebs' tension (mg)	4.0 mM EGTA Ca-free Krebs' tension (mg)
°C	mean ±SE	mean ±SE
37	(a) 4146 ± 898	(c) 394 ± 105
23	(b) 5889 ± 1165	(d) 1528 ± 216
*37 → 23 ± 1		(e) 577 ± 185

* Strips were placed in 4 mM EGTA, Ca-free Krebs' solution for 15 min at 37°C and for a further 15 min at 23°C. Stimulated with acetylcholine at 23°C.
The probability (P) of the difference being due to chance is: a vs. c, b vs. d, and d vs. e: $P < 0.01$; c vs. d: $P < 0.001$; a vs. b: not significant.

Effect of Temperature on Contraction in Calcium-Free Media

The ultrastructural studies (see below) indicated that the sarcoplasmic reticulum of turtle oviduct, while comparatively well developed for a smooth muscle, did not match in volume the SR found in fast-striated muscles. We therefore searched for another mechanism that may contribute to the relatively large contractions developed by turtle smooth muscle in calcium-free media. The different composition of physiological solutions used, as seen in the preceding section, did not alter the experimental results. Another difference between the experiments conducted on reptiles and those on mammals is that the former are conducted at lower temperatures. We therefore explored the effect of temperature on the ability of oviduct smooth muscle to contract in calcium-free media.

A typical experiment is illustrated in Fig. 1. The upper panels show the spontaneous contractions and responses to supramaximal concentrations of acetylcholine, the contraction occurring upon changing the normal Krebs' solution to a calcium-free 4 mM EGTA solution and finally, after half an hour incubation in Ca-free solution,

the response to acetylcholine in the absence ($< 10^{-8}$ M) of extracellular calcium. These experiments were done at 37°C. The panels in the middle row illustrate the experiments done on the same strip at 22°C: the most startling difference between the two sets of experiments is the much greater response in Ca-free solution at the lower temperature. The bottom panels indicate that the effect of incubation in Ca-free solution, rather than the stimulus-contraction relationship itself, is subject to a temperature-dependent process: after 15 min in Ca-free solution at 37°C the strip, even though cooled to 22°C for 15 min before and also during stimulation, still responded with only a small contraction comparable to the one observed when the entire sequence (incubation and stimulation) was conducted at 37°C.

The results of a series of similar experiments (Table II) clearly show that contractions in Ca-free media are significantly ($P < 0.001$) greater when the muscles are maintained at the lower temperatures. Furthermore, preincubation in the Ca-free solution at 37°C significantly ($P < 0.01$) reduces the subsequent contraction at 23° ± 1°C. In normal Krebs' solution the maximal contractile responses to acetylcholine are not signifi-

FIGURES 2–22 The tissues are from smooth muscle of the turtle oviduct, aorta, and vena cava. The incubation times, fixatives, and buffers used are indicated for each figure. Unless otherwise shown (e.g., Figs. 2–5), the calibration lines on each micrograph are 0.1 µm.

Abbreviations

COL, collagen
DB, dense body
E, extracellular space
EL, elastic tissue
G, glycogen

N, nucleus
SM, smooth muscle
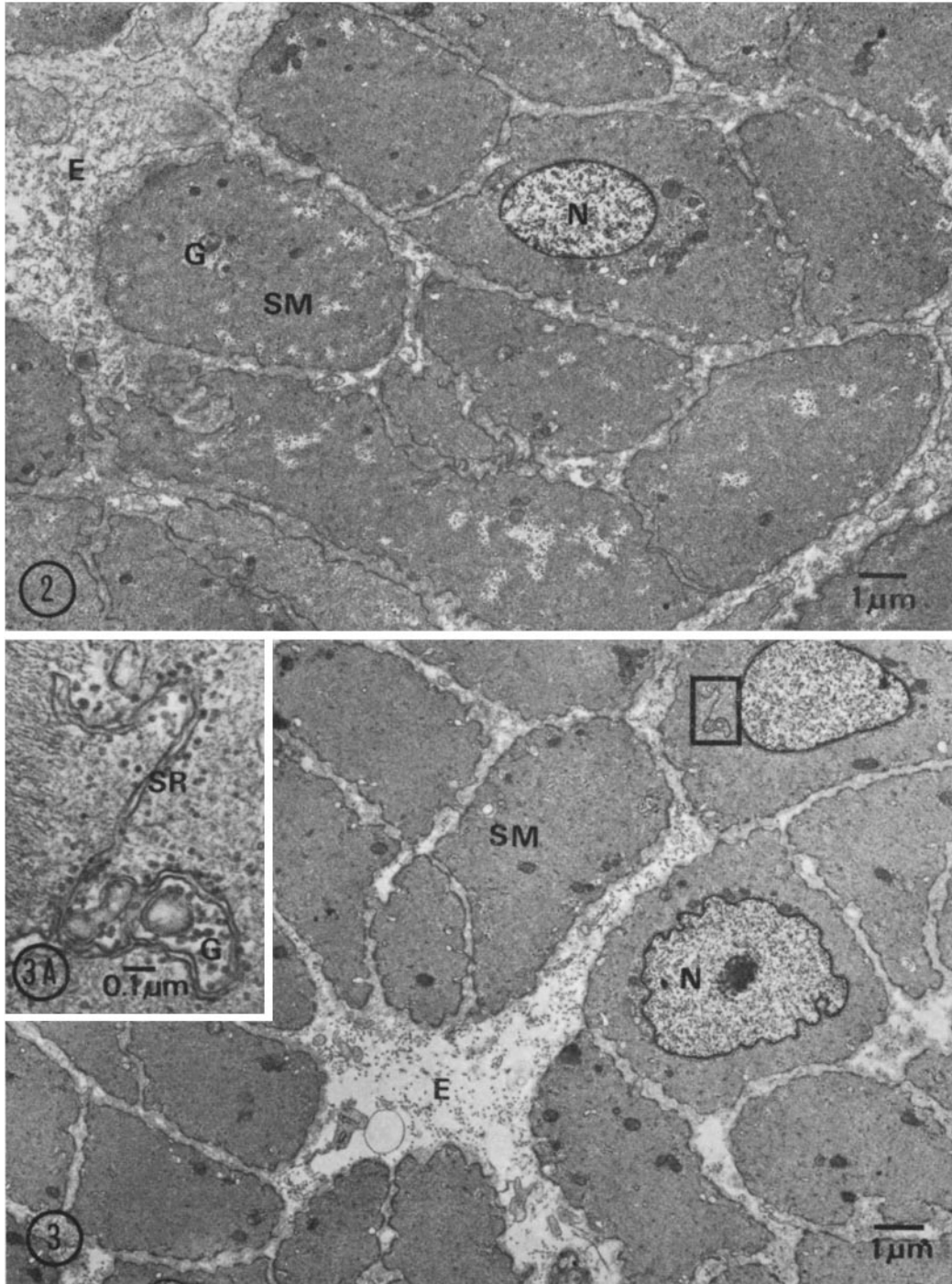SR, sarcoplasmic reticulum
V, surface vesicle

FIGURE 2   Transverse section through a bundle of smooth muscle cells in the turtle oviduct showing the general arrangement of the smooth muscle cells at low magnification. Electron-lucent regions contain glycogen particles. Only portions of the sarcoplasmic reticulum can be seen at this magnification. Oviduct, 10 min incubation in turtle Ringer's (220 mosmols); cacodylate-buffered, 3% glutaraldehyde fixative. × 6600.

FIGURE 3   Transverse section of turtle oviduct from same animal as in Fig. 2 after hypotonic incubation, showing little change from isotonic incubation. The *insert* (Fig. 3 A) shows, at higher magnification, coiled sarcoplasmic reticulum with associated glycogen particles. Fig. 3, oviduct, 10 min incubation in hypotonic (175 mosmols), turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. × 6600. Fig. 3 A, × 46,500.

cantly ($P > 0.05$) different at the two temperatures.

The Effect of Fixatives and the Osmolarity of Incubating Solutions on Ultrastructure

The appearance of oviduct smooth muscles incubated for 10 or 30 min before fixation in isotonic (Fig. 2) or in hypotonic (Fig. 3) turtle Ringer's did not differ greatly. The cells were approximately 3–6 μm in diameter at the nuclear region (measurements from 22 cells), e.g. Figs. 2 and 3, but this measurement will obviously vary with the degree of stretch. At low magnification the smooth muscle incubated in hypotonic solution presented perhaps a slightly more scalloped outline, and at higher magnifications tubular (see below) rather than more dilated sarcoplasmic reticulum may have been more frequent, but these differences were not sufficient to enable an uninformed observer to distinguish between the two pretreatment schedules. There was sufficient variability within a single block examined that observations on freshly fixed, hypotonically and isotonically incubated solutions can be grouped together. Short (10 min) incubation in moderately (454 mosmols) hypertonic solution produced a slight increase in electron opacity but no gross abnormality of smooth muscle cells. Mitochondrial damage appeared to be somewhat more common in fresh tissues fixed in glutaraldehyde buffered with cacodylate without preincubation in a Ringer's solution.

Prolonged incubation in hypertonic solutions (Fig. 4) produced a clear-cut increase in electron opacity of the smooth muscle cells that were shrunken, with an increase in the relative extracellular space. These changes increased with duration of incubation and tonicity of the incubation medium. In the hypertonically shrunken dark cells there were occasional regions of hugely ballooned membrane-limited spaces, presumably representing a very much swollen sarcoplasmic reticulum, but through many regions of these dark cells no clearly definable sarcoplasmic reticulum was present. This may have been due to rupture of swollen sarcoplasmic reticulum outside the plane of section; some regions of broken cell areas were indeed seen, but no further attempt was made to quantitate the volume of sarcoplasmic reticulum in these preparations. The variations in electron opacity of different cells within the same

region (Fig. 4) suggest some nonuniformity of the time course of osmotic response in different fibers.

In tissues fixed for 2 wk in glutaraldehyde buffered with cacodylate, dark cells and light cells were present. Since similar mixtures of dark and light cells can be produced through incubation of turtle (Fig. 4) and mammalian (Somlyo et al., 1971) smooth muscle in hypertonic solutions, we assume these to be due to an osmotic effect of the glutaraldehyde/cacodylate fixative that, for unknown reasons, does not affect all cells within a given preparation in the same manner.

The smooth muscle cells in the aorta and vena cava had a much more irregular outline than those in the oviduct (Fig. 5). There were numerous cellular processes, resulting in apparent inclusions (invaginations) of extracellular space into the muscle. The presence of large masses of glycogen and associated electron-lucent spaces (presumably due to partial removal of glycogen during preparation) further interfered with precise identification and quantitation of the sarcoplasmic reticulum. Furthermore, large regions of pinocytotic vesicles were sometimes seen within the smooth muscle–cell processes without apparent communication with the extracellular space. While we believe that these vesicles, like those of mammalian smooth muscle (Devine and Somlyo, 1970; Somlyo and Somlyo, 1970), communicate with the extracellular space outside the plane of section, this could not be established without the extensive use of tracers. These were the major reasons why we did not attempt to quantitate the sarcoplasmic reticulum in vascular smooth muscle, but concentrated on examining the appearance of oviduct smooth muscle.

Glycogen Deposits and Sarcoplasmic Reticulum

Tissues spaces mixed in with glycogen were often adjacent to sarcoplasmic reticulum, as illustrated in the turtle aorta (Fig. 5). In some experiments (stained for 20–30 min en bloc, during the summer months) no such difficulties of preservation were encountered with oviduct smooth muscle, but in other experiments (stained for 30–90 min, experiments conducted during January through April) large spaces devoid of tissue were present inside smooth muscle cells, leading to difficulties in attempting to evaluate the sarcoplasmic reticulum. Fixation with potassium permanganate (Revel et al., 1960) revealed masses
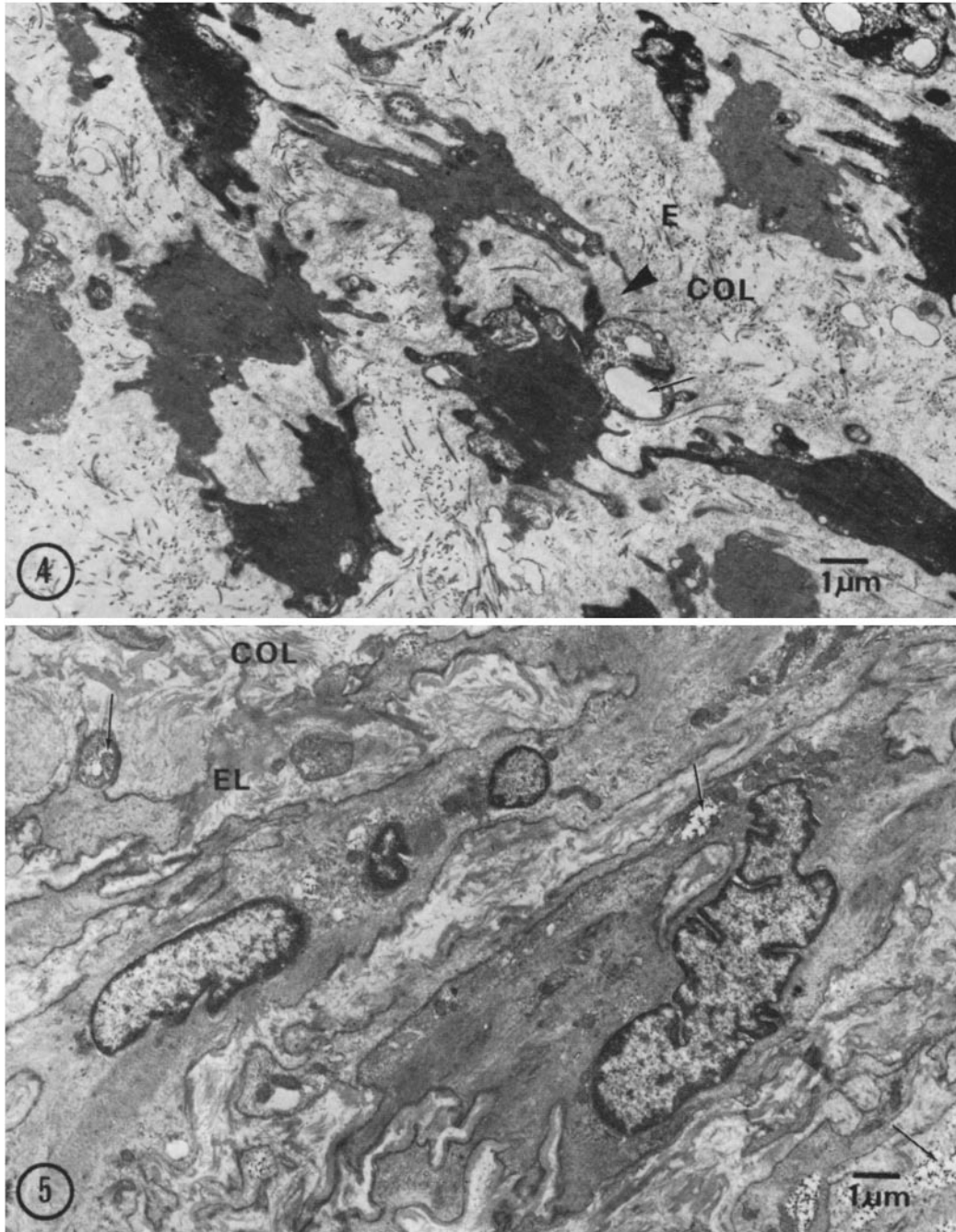
FIGURE 4 Oblique section through smooth muscle cells of the turtle oviduct from the same animal as in Fig. 2 showing changes induced by moderately hypertonic incubation for 30 min. The extracellular space has increased and the cytoplasm of the cell is dense and shrunken with finger-like processes present. Some cells are more electron opaque than others, indicating a varying osmotic response of the cells. Individual cytoplasmic components of the very dark cells are unrecognizable; ballooned, membrane-lined spaces are associated with glycogen (small arrow). Surrounding each cell there is a wide band of amorphous matrix with little or no collagen present within it (arrowhead), although collagen is present outside this region. Oviduct, 30 min incubation in hypertonic (454 mosmols) turtle Ringer's, followed by (454 mosmols) turtle Ringer's buffered, 3% glutaraldehyde fixative. × 6600.

FIGURE 5 Longitudinal section of smooth muscle cells in turtle aorta showing large processes of the smooth muscle cells, elastic tissue, and collagen. Electron-lucent spaces (arrows) indicate that some of the glycogen has been removed during the fixation and block-staining procedures. Aorta, perfused with turtle Ringer's-buffered 3% glutaraldehyde fixative. × 6600.

of glycogen within these cells. These massive glycogen deposits had been presumably removed from glutaraldehyde/osmium tetroxide-fixed tissues due to the acid pH of aqueous uranyl acetate (Manasek, 1969; Vye and Fischman, 1970). Tubular elements of SR often formed rings, in transverse section, enclosing glycogen masses (Figs. 3 A and 6). Incubation in hypertonic solution sometimes revealed swollen spaces containing glycogen and "honeycombed" by membranes of SR, suggesting that, without high magnification studies of ultrathin sections, a proportion of SR may be obscured by glycogen masses. The association of glycogen with the sarcoplasmic reticulum was one of the most characteristic and common findings.

The General Distribution and Cell Volume Occupied by the Sarcoplasmic Reticulum

Tubules of sarcoplasmic reticulum were often seen running predominantly along the longitudinal axis of the cell (Figs. 7–9, 11–12) and approaching the surface membrane (Figs. 9, 10, 12–13), where cisternal dilations were fairly common (Figs. 9–10, 17–21). Radially oriented sarcoplasmic reticulum was also seen connecting the centrally located with the peripheral sarcoplasmic reticulum. Circularly arranged SR tubules surrounded masses of glycogen in transverse section (Figs. 3 A, 6), and glycogen accumulations were also seen surrounded by loops of SR tubules oriented in a longitudinal direction (Figs. 7–8). This region of the sarcoplasmic reticulum sometimes expanded into large sack-like structures that were either entirely electron lucent or appeared to be slightly electron opaque, presumably due to tangential sectioning of the membrane (Fig. 8). Fenestrations of a lacy network of sarcoplasmic reticulum seen in tangential sections often surrounded the surface vesicles (Figs. 11, 13–15). When cells were fortuitously sectioned tangentially, a characteristic arrangement of longitudinally running SR tubules expanding into a lacy network fenestrated about the groups of surface vesicles was seen alternating with dense bodies on the plasma membrane (Fig. 11). Much of the sarcoplasmic reticulum, particularly at the periphery, consisted of longitudinally oriented tubules, but a more detailed description would require spatial reconstruction.

Volume estimates were made from 34 montages (eight animals: six prefixed with glutaraldehyde and two fixed directly with osmium tetroxide) in transverse (10), oblique, and approximately longitudinal orientation. We did not obtain perfectly oriented sections, and consequently the majority of these micrographs included somewhat obliquely sectioned material. Both complete and incomplete cell profiles were included (complete profiles of longitudinally sectioned cells would not be included at the original magnification of 7900), and we excluded from sampling some preparations in which the SR seemed very much collapsed (the two SR membranes touching) or exceptionally dilated. Inclusion of this material would have extended the range of determinations even further. We are not certain whether uncontrollable changes due to variable fixation, differences in the physiological states, or intraspecies variations were responsible for the considerable variation in SR volume encountered. The contractile re-

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FIGURE 6 Transverse section through portion of a smooth muscle cell showing a region of coiled sarcoplasmic reticulum associated with glycogen particles. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde-fixative. × 62,000.

FIGURE 7 Longitudinal section through a portion of a smooth muscle cell showing longitudinal branching elements of the sarcoplasmic reticulum surrounding glycogen particles. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. × 40,000.

FIGURE 8 Portion of longitudinal sarcoplasmic reticulum almost completely surrounding glycogen particles. The broad regions (arrow) of sarcoplasmic reticulum may represent tangential sections of the membrane of large, sack-like expansions. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. × 40,000.

FIGURE 9 Longitudinal section through two smooth muscle cells, showing longitudinally running sarcoplasmic reticulum and portions coming close to surface vesicles (small arrow) and the cell membrane (large arrow). Oviduct, 30 min incubation in turtle Ringer's; collidine-buffered, 2% osmium tetroxide fixative. × 62,000.
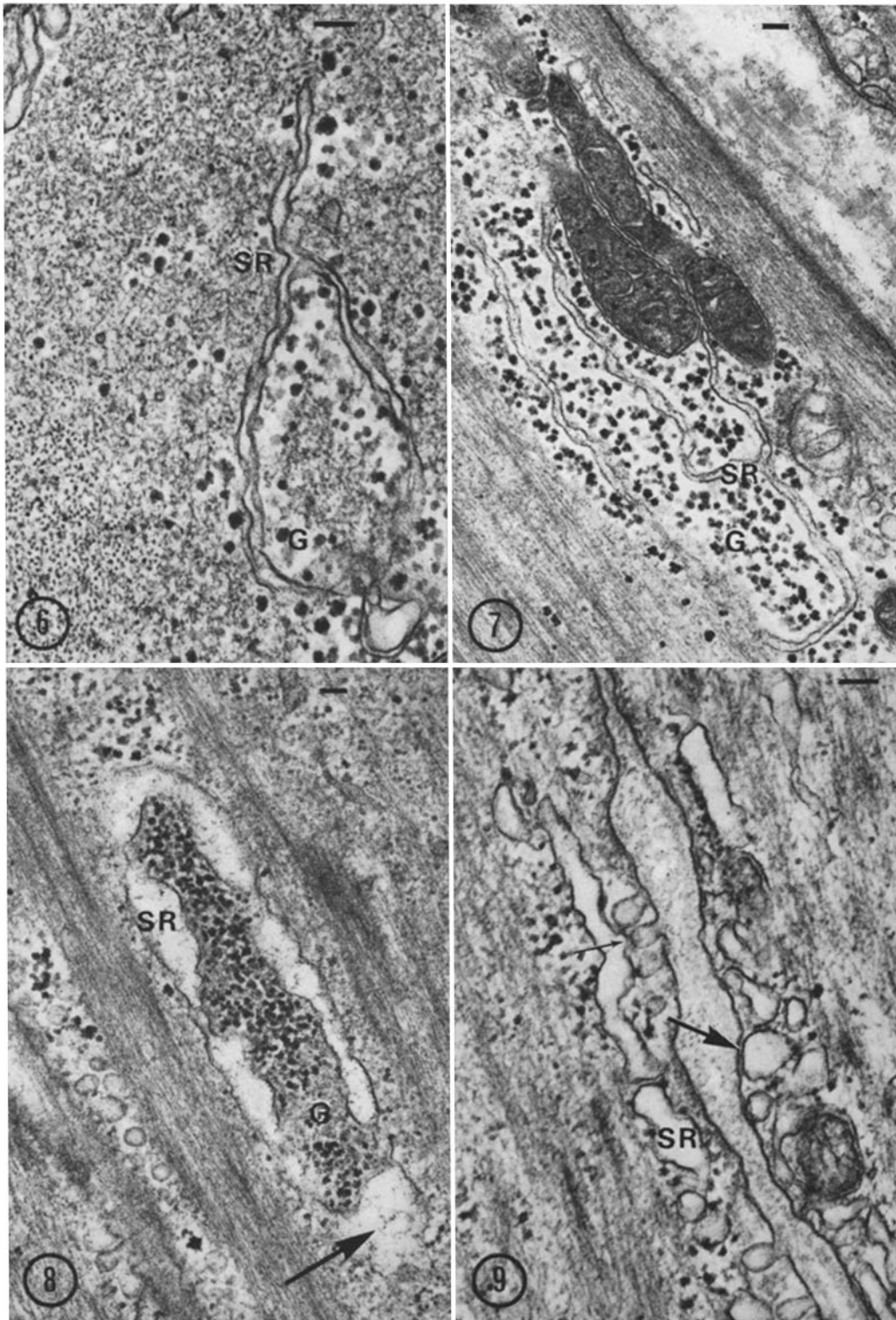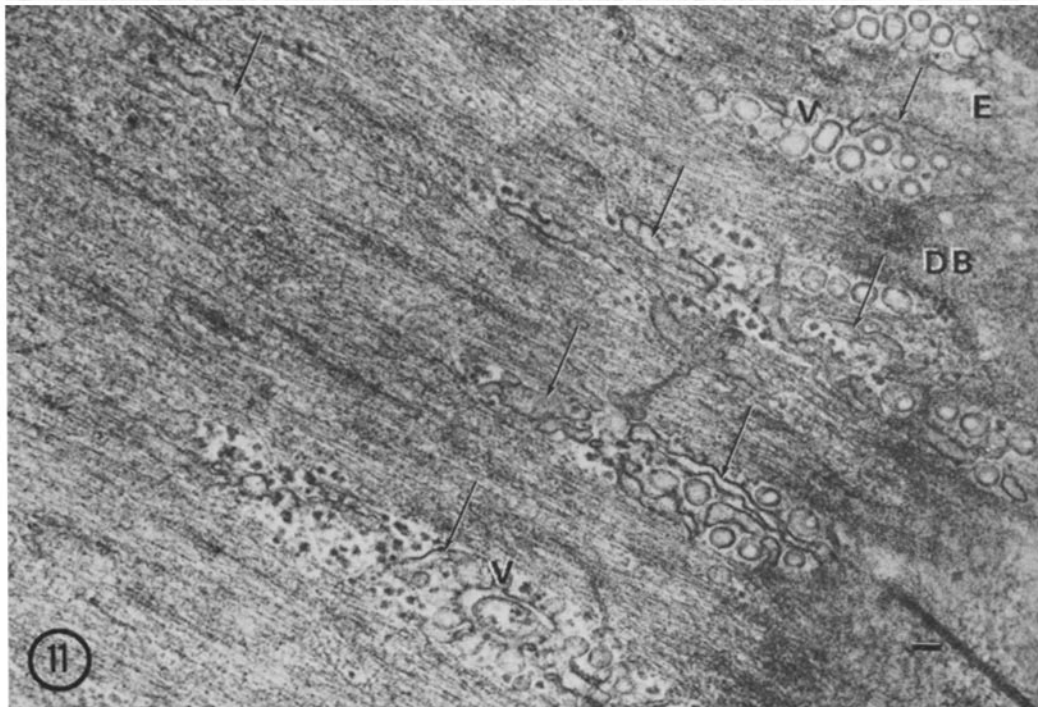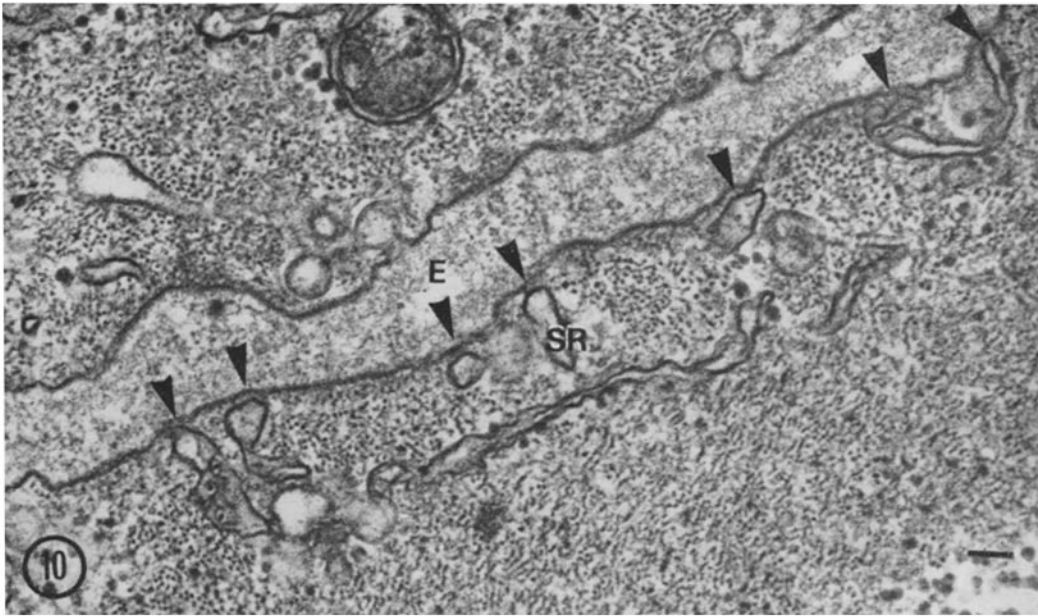
FIGURE 10 Transversely sectioned smooth muscle cells showing transversely sectioned sarcoplasmic reticulum approaching the cell membrane (arrowheads). A longitudinally sectioned element of SR is also present. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. × 62,000.

FIGURE 11 Longitudinal section of a smooth muscle cell through grouped surface vesicles and the sarcoplasmic reticulum (small arrows) which threads between the vesicles. A predominantly longitudinal arrangement of the sarcoplasmic reticulum and surface vesicles can be seen. Dense bodies are present between the rows of surface vesicles. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. × 40,000.

sponses of oviduct in Ca-free solutions were also variable (Table I), but since the tissues used for physiological experiments and for electron microscopy were not identical, we cannot correlate the two observations. The SR volumes established on profiles of approximately 100 fibers from eight animals were considered to be representative of the much larger number of sections examined, and ranged from 1.2 to 4.9% of the nucleus- and mitochondrion-free cell volume, with a mean of 2.8%. The highest value observed in a single fiber of these random samples was 8.7%, but in exceptional instances (nonrandom) fibers with SR volumes as high as 16% could be selected. Assuming a 50% correction factor for tangentially sectioned SR (Loud, 1968), a correction that is especially appropriate if much of the SR is composed of tubules with small diameters, the average values of turtle oviduct SR may range from 1.8 to 7.4% of cell volume, with a mean of 4.2%. We have confirmed, in tilt stage examinations of mammalian vascular smooth muscle (C. E. Devine, and A. P. Somlyo, unpublished observation), the validity of applying a correction factor to the SR of smooth muscle. While, strictly speaking, a correction factor should not be necessary when estimating circular profiles obtained in perfect transverse sections of longitudinally oriented tubules, we believe that the degree of obliqueness of most of these sections warrants the application of a correction factor to our entire material. Averaging uncorrected volumes obtained from the best transverse sections with the corrected values obtained from oblique or longitudinal sections yields a mean SR volume of 3.9%.

Surface Vesicles

The surface vesicles of turtle smooth muscles varied from the characteristic flask-shaped structures to tubular invaginations and multilobed vesicles (Figs. 9-10, 12, 16-18, 20-21).

A new feature, not described in previous classifications of surface vesicles, was the presence of approximately 10 nm electron-opaque striations on some of the surface vesicles in the vena cava (Figs. 16-18), aorta, and oviduct (Fig. 15) smooth muscle, approximately parallel to the openings to the surface and at right angles to the longitudinal axis of the tubular structures. Some of the vesicles were elongated and changed direction within the plane of section. The striations (6-12 nm wide)

were separated by electron-lucent spaces of somewhat variable (8-14 nm) width. In vesicles completely contained within the section, the striations appeared to completely encircle the vesicles (Figs. 15-17). Electron-opaque regions on the rims of vesicles that had an electron-lucent center, presumably due to exclusion of one surface from the section, corresponded to the striations (Figs. 16-18) of whole vesicles. In transversely sectioned vesicles, no electron-opaque areas corresponding to a diaphragm could be found, and, after ferritin incubation, particles of ferritin were also found throughout the whole vesicle (Figs. 20-21).

Relationship of Surface Vesicles and Surface Membrane to the Sarcoplasmic Reticulum

Close contacts occurred between the sarcoplasmic reticulum and either the surface vesicles (Figs. 9, 12-13, 15, 17-18, 20-21) or the nonspecialized surface membrane (Figs. 9-10, 12, 18-21). The space between the sarcoplasmic reticulum and the surface membrane was approximately 8-10 nm and, although it was occasionally traversed by small bridges of electron-opaque material, thus far no periodic connecting structures have been resolved.

Actual physical contact between the surface vesicles and the sarcoplasmic reticulum is suggested by the fact that the latter frequently followed the contour of the adjacent surface vesicles (Figs. 15, 17-18). The couplings between the sarcoplasmic reticulum and the nonspecialized surface membrane were sometimes slightly scalloped, with the sarcoplasmic reticulum appearing to exert some traction in some places on the surface membrane.

Thick and Thin Filaments

The best visualization of thick filaments was possible under conditions found to be useful in mammalian smooth muscle (Devine and Somlyo, 1971): incubation in Krebs' solution of stretched muscles before fixation, and inclusion of some sucrose in the buffered glutaraldehyde fixative. Both thick filaments and intermediate filaments (Somlyo et al., 1971) adjoining amorphous electron-opaque areas (possibly dense bodies) were seen (Fig. 22), and in one embedding large, regular arrays of thin filaments were present (Fig. 22 A). Even in the best preserved preparations,

the thick filament density in the turtle oviduct was much lower than that in the mammalian smooth muscles that we have previously studied. It is possible that the relative paucity of thick filaments reflected the hormonal state of the oviduct, since the preparatory procedures suitable for demonstrating thick filaments were used on specimens obtained during the winter and early spring months, when the oviducts were rather small and did not contain eggs. It is also possible, however, either that we have not obtained the optimal preparatory procedure for preserving thick filaments in the reptile or that the thin-to-thick filament ratio is higher in this species than it is in mammals (Devine and Somlyo, 1971; Rice et al., 1970; 1971).

DISCUSSION

We have found that the smooth muscle of the turtle oviduct can contract, when stimulated with acetylcholine, even after the virtually complete removal of extracellular calcium with high concentrations of a chelating agent. This behavior in calcium-free media resembles that of the turtle aorta (Bozler, 1969; Somlyo and Somlyo, 1970) and indicates that drug-induced contractures of these reptilian smooth muscles are mediated by calcium released from a site not readily accessible to extracellular chelating agents. The persistence

of spontaneous contractions for up to 60 min in Ca-free solution suggests that twitches triggered by action potentials can also be activated by calcium from a similar or the same site: most probably the variable, but often well-developed SR of this muscle. The possibility that acetylcholine can release bound calcium in rat uterine muscle was suggested some time ago (Edman and Schild, 1962), and this bound calcium may be within the sarcoplasmic reticulum that also makes close contacts with the surface membranes (Fig. 4, Somlyo et al., 1971) in mammalian uterine smooth muscle.

The amount of sarcoplasmic reticulum required as a calcium storage site for activating contraction can be estimated (Peachey, 1965). The amount of *total* calcium required for activating the contractile proteins of striated muscle is about 1.2×10^{-4} M for threshold and 2×10^{-4} M for a maximal contraction (Weber, Herz, and Reiss, 1964; Sandow, 1970). The calcium requirements of the contractile proteins of smooth muscles are of the same order of magnitude as those of striated muscle (Bozler, 1968; Murphy, 1971; Sparrow et al., 1970; for review, see Somlyo and Somlyo, 1968 a). The calcium bound to the SR of striated muscle ranges from 80 to 150 μM/g SR protein (Ebashi and Endo, 1968; Carvalho, 1968) or a concentration of some 13–25 mM, assuming

FIGURE 12 Longitudinal section through a smooth muscle cell showing the close relationship of the predominantly longitudinally running sarcoplasmic reticulum to the cell membrane and to the surface vesicles (arrowheads). At the regions of close contact, small electron-opaque bridges are present. Oviduct, 30 min incubation in normal Krebs'; cacodylate-buffered, 3% glutaraldehyde fixative containing 4.5% sucrose. × 93,000.

FIGURE 13 Tangential section of the smooth muscle cell membrane showing surface vesicles and an associated lace-work of sarcoplasmic reticulum (large arrow) surrounding the vesicles. Large circles are interpreted as sections through complete vesicles, and the small circles as sections through the narrow neck portion of vesicles (small arrows). The sarcoplasmic reticulum network around the vesicles forms several close contacts with the vesicles and cell membrane (arrowheads). Oviduct, 10 min incubation in turtle Ringer's; turtle Ringer's-buffered, 3% glutaraldehyde fixation. × 93,000.

FIGURE 14 A group of surface vesicles at the tangentionally sectioned, smooth muscle–cell membrane. An element of the sarcoplasmic reticulum lies between the vesicles and forms a loop around one of the vesicles at the neck region (large arrow) although it does not form a close contact with the vesicle at this point. The closest contact of the vesicles with the sarcoplasmic reticulum occurs at the widest vesicle diameter. Oviduct, 30 min incubation in turtle Ringer's; turtle Ringer's-buffered, 3% glutaraldehyde fixative. × 62,000.

FIGURE 15 A tangential section through the cell membrane between two dense bodies showing a group of surface vesicles with portions of sarcoplasmic reticulum lying between them, in close relationship with some of the surface vesicles (arrowheads). Two striated vesicles are present (large arrows). Oviduct, 30 min incubation in normal Krebs'; cacodylate-buffered, 3% glutaraldehyde fixative containing 4.5% sucrose. × 93,000.
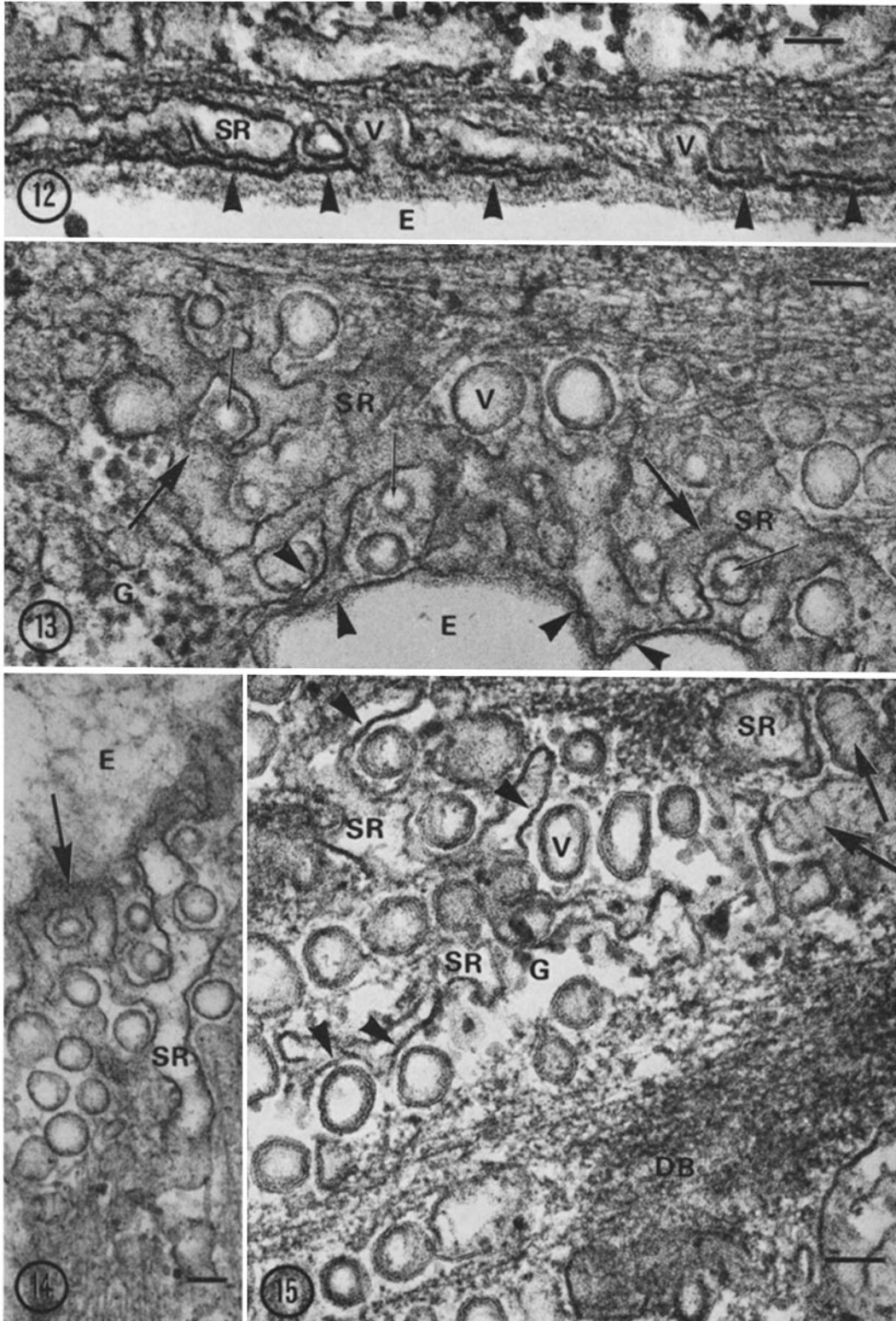
an SR volume/weight of 6 ml/g protein (Ebashi and Endo, 1968). The total amount of SR required to activate contraction will also depend on the fraction of stored calcium that can be released. Assuming that the total calcium is 15 mM/liter SR volume and that, of this, 20% (3 mM/liter) can be released, the SR volume would have to be about 4% for a threshold and 7% for a maximal contraction. Comparison of the contractile responses of the turtle oviduct with the amount of SR (1.8–7.4% of cell volume) suggests that, within the very limited accuracy of these calculations and volume estimates, the SR of this smooth muscle is sufficiently well developed to serve as a calcium store for contractile activation of the muscle in the absence of extracellular calcium. The terminal cisternae of the frog sartorius, the source of activator calcium of this muscle (Winegrad, 1970), comprise approximately 5% (uncorrected) of cell volume (Peachey, 1965). Our calculations assume that in smooth muscle the entire SR (since its central and peripheral portions are connected), rather than only the fraction in direct contact with the surface membrane alone, can serve as a calcium source. It should also be noted that our estimates of the calcium-binding capacity of the SR and of the calcium requirements for contraction are higher than previously assumed (Carsten, 1969). The relatively low calcium-binding activities of isolated smooth muscle SR may

be due to the low yield, high contamination, and preparatory damage to these preparations. It has been suggested that the calcium-accumulating activity of isolated SR preparations is proportional to yield (Baskin, 1971). In recent studies (Batra and Daniel, 1971), uterine microsome preparations containing 72 μM Ca/g protein have been obtained. It may be noted that the amount of myosin (10^{-4} M/kg cell) estimated from electron microscope studies of (mammalian) smooth muscle showing 200 thick (18 nm in diameter) filaments per μm^2 (Devine and Somlyo, 1971; Rice et al., 1971) and a molecular weight of 530,000 (Wachsberger and Kaldor, 1971), exceeds considerably the yields of current biochemical isolation procedures (Murphy, 1971).

It is difficult to assess how much of the variation in the volume of SR is due to uncontrollable day-to-day differences in preparatory techniques, and how much of it is real variability among the different animals. The association of glycogen with the sarcoplasmic reticulum in turtle smooth muscle was consistently observed, supporting similar observations on other tissues (Andersson-Cedergren and Muscatello, 1963; Luciano, Junger, and Reale, 1968) and the suggestion (Andersson-Cedergren and Muscatello, 1963) that the smooth-surfaced sarcoplasmic reticulum plays a role in glycogen metabolism, although the possibility that glycogen merely occupies "available space"

FIGURE 16   Section through a portion of the smooth muscle–cell membrane of the vena cava showing several striated vesicles. In some longitudinally sectioned vesicles the striations extend completely across the cell (large arrow). Vena cava, no incubation; turtle Ringer's-buffered, glutaraldehyde fixative. X 93,000.

FIGURE 17   A close relationship between striated vesicles and sarcoplasmic reticulum of a smooth muscle cell, with a separation of approximately 3 nm between the two membranes (arrowhead). This distance is closer than that more commonly found (cf. Fig. 19) and may be due to overlapping of the images of the two structures. Some dense regions are present on the vesicle membrane, possibly corresponding to portions of the striations sectioned transversely. Vena cava, no incubation; turtle Ringer's-buffered, glutaraldehyde fixative. X 93,000.

FIGURE 18   Section through a cisternal dilatation of the sarcoplasmic reticulum with an extension in close contact with the smooth muscle cell membrane (large arrow) and surface vesicles (arrowhead). Complete striated vesicles and obliquely sectioned striated vesicles (with electron-opaque regions around the perimeter) are present. The cisternal dilatations are connected to tubular regions of the sarcoplasmic reticulum (small arrows). Vena cava, no incubation; turtle Ringer's-buffered, glutaraldehyde fixative. X 93,000.

FIGURE 19   Close relationship of a portion of three sarcoplasmic reticulum profiles with the smooth muscle–cell membrane (arrowheads). The distance between the two membranes is approximately 8 nm at the region where both leaflets of the plasma membrane can be resolved. Some electron-opaque material is present between the sarcoplasmic reticulum and the cell membrane. Oviduct, 10 min incubation in turtle Ringer's; cacodylate-buffered, 3% glutaraldehyde fixative. X 153,000.
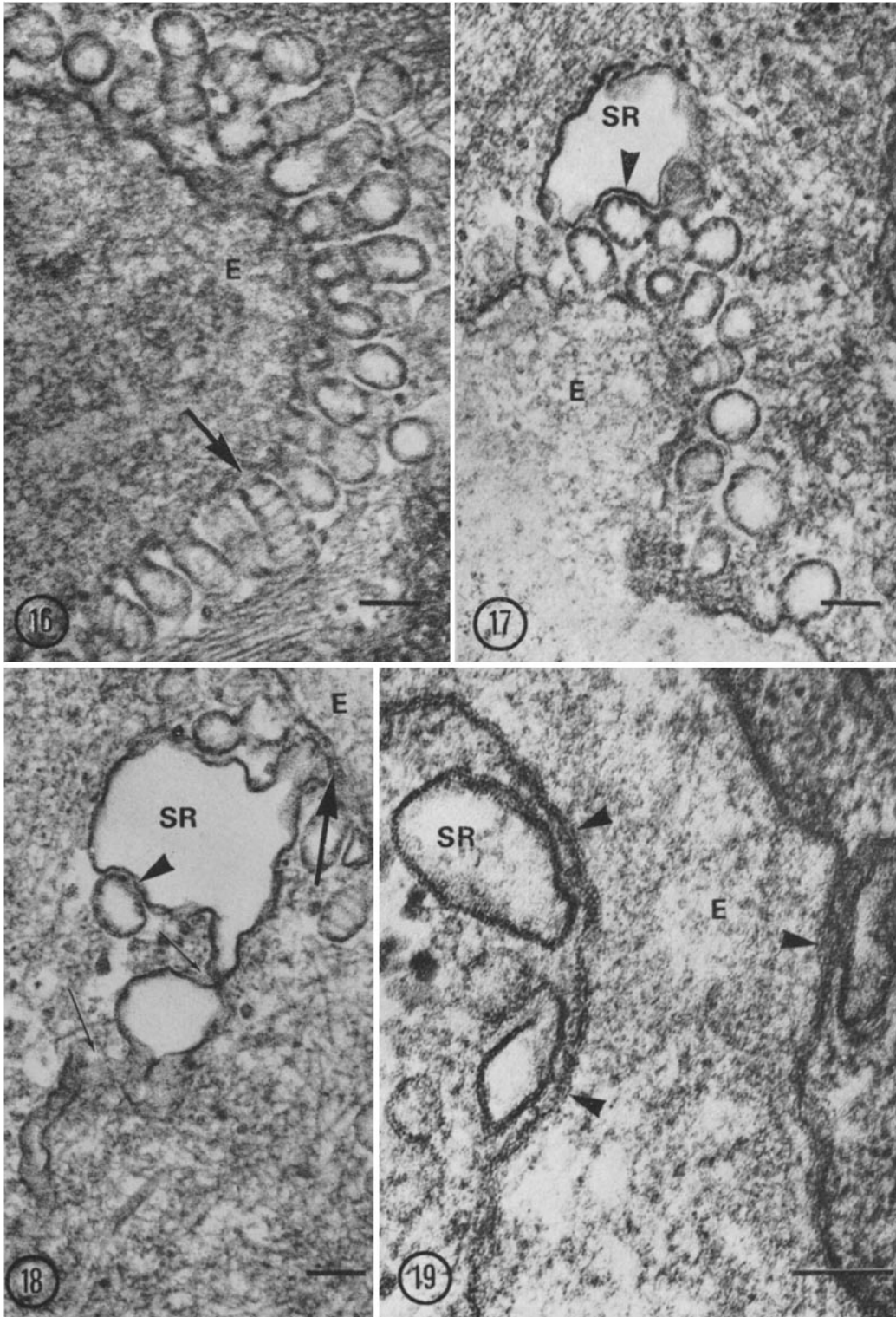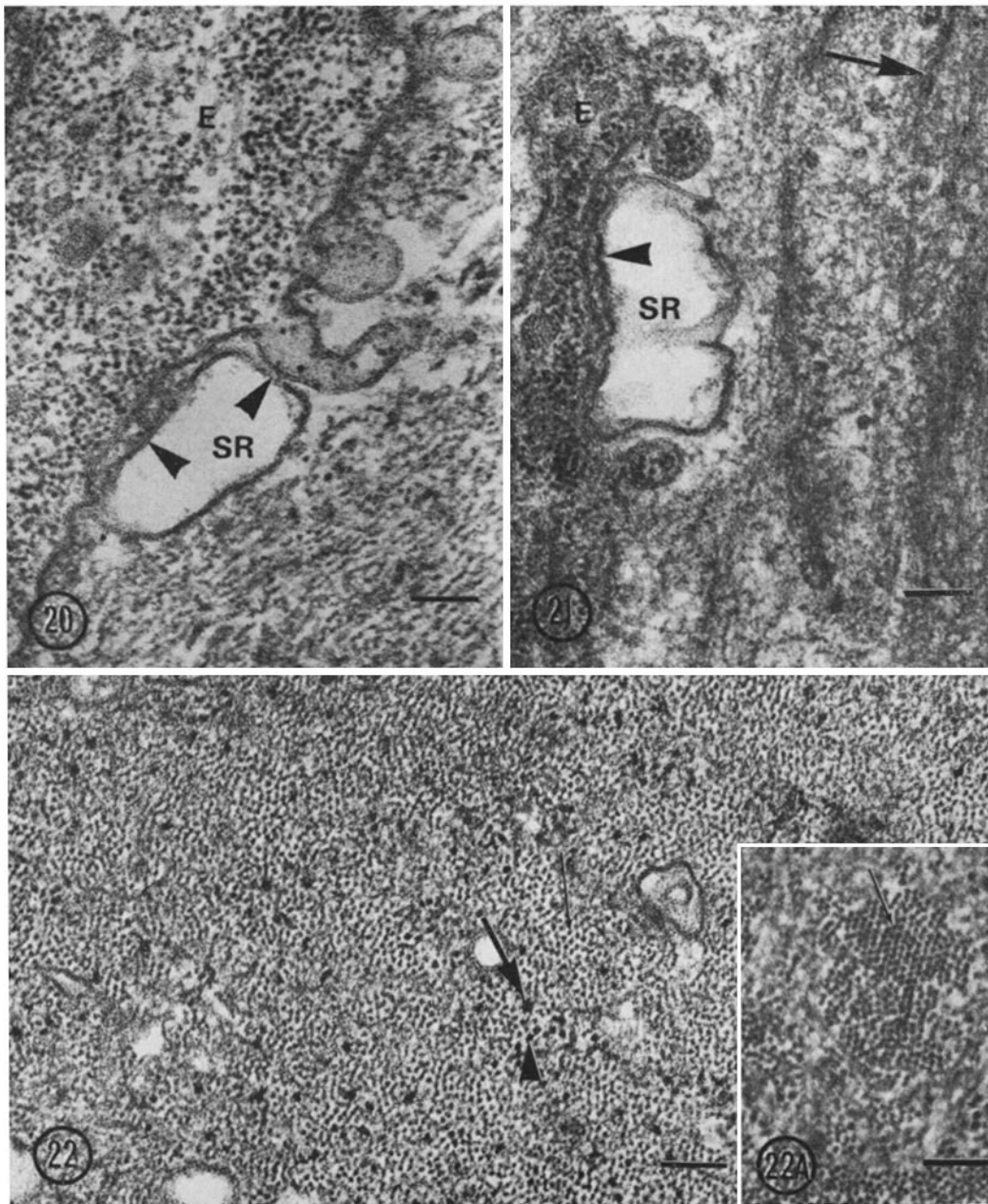
FIGURE 20 Section at the periphery of a smooth muscle cell showing ferritin particles in the extracellular space, in a surface vesicle, and in an elongated surface vesicle (tubule), but not in the sarcoplasmic reticulum. Note that the sarcoplasmic reticulum is in close relationship with the cell membrane and the surface vesicle (arrowheads). Oviduct, incubated for 30 min in Krebs' solution containing 20–30% ferritin by volume. Cacodylate-buffered, 3% glutaraldehyde fixative containing 4.5% sucrose. × 93,000.

FIGURE 21 Dense deposits of ferritin particles are present in the extracellular space and in the surface vesicles of the smooth muscle, but are absent from the sarcoplasmic reticulum which is in close relationship with the cell membrane (arrowhead). Thick myofilaments are present in a longitudinal orientation (large arrow). Oviduct smooth muscle, incubated for 30 min in Krebs' solution containing 20–30% ferritin by volume. Cacodylate-buffered, 3% glutaraldehyde containing 4.5% sucrose. × 93,000.

FIGURE 22 Transverse section through myofilaments showing thick (15–20 nm in diameter; large arrow), intermediate (11–13 nm in diameter; arrowhead), and thin myofilaments (6–7 nm in diameter; small arrow). Portions of transversely sectioned sarcoplasmic reticulum are present. The thin filaments appear to be in a predominantly hexagonal array, but there does not appear to be a regular array of thick or intermediate filaments. An insert (Fig. 22 A) shows a region of very regular hexagonal packing of the thin filaments (small arrow). Oviduct, 30 min incubation under stretch in Krebs'; cacodylate-buffered, 3% glutaraldehyde fixative containing 4.5% sucrose. × 93,000. Fig. 22 A, × 93,000.

between myofilaments (Fawcett and McNutt, 1969) cannot be excluded. In tissue cultures of choriocarcinoma the presence of high glucose concentration in the medium is accompanied by increased amounts of cellular glycogen as well as an increased content of the smooth sarcoplasmic reticulum (Knoth et al., 1969). It is tempting to speculate that an increased metabolic need for glycogen synthesis in smooth muscle may, incidentally, result in an increased calcium-storing capacity due to the increased production of sarcoplasmic reticulum membranes.

A relatively well-developed SR of turtle smooth muscle is not the only factor that can be implicated in the ability of this muscle to contract in Ca-free media. The temperature at which the incubation in Ca-free media was conducted had a very major effect on the ability of the muscles to contract when subsequently stimulated. These observations suggest that at 37°C calcium either is more rapidly lost from the muscle or is sequestered into some site unavailable for release by acetylcholine. The first possibility seems more likely, and has been shown to occur in cardiac muscle (Reuter and Seitz, 1968), and a preliminary report (Seidel and Sparks, 1970) suggests its occurrence in vascular smooth muscle. In rabbit pulmonary artery–smooth muscle the ratio:contraction in Ca-free/contraction in 1.2 mM Ca solution is also higher at 23°C than at 37°C (unpublished observations). Thus the presence of a sarcoplasmic reticulum that can serve as a calcium storage site and experimental conditions that prevent excessive calcium loss from this site into Ca-free solutions (i.e., low temperature) appear to be the major factors contributing to a muscle's ability to contract in Ca-free media. Our experiments do not rule out the possibility, however, that other calcium stores (e.g., mitochondria, plasma membrane, the space between the plasma membrane and basement membrane) may also play a role in activation of smooth muscle contraction.

It is clear that intracellular as well as extracellular sources of calcium can contribute to activation of contraction in smooth muscle. It has been previously suggested (Somlyo, Vinall, and Somlyo, 1969; Somlyo and Somlyo, 1970) that the relative contribution of extracellular and intracellular calcium sources will vary with the amount of SR in a given smooth muscle and the mode of excitation. The volume of the sarcoplasmic reticulum in the turtle oviduct does not necessarily exceed that found in certain mammalian smooth muscles. In the rabbit main pulmonary artery–smooth muscle the sarcoplasmic reticulum occupies an even greater percentage of cell volume than in the turtle oviduct (although these determinations invariably include a fraction of rough–sarcoplasmic reticulum continuous with the smooth–sarcoplasmic reticulum system), and this smooth muscle (but not the rabbit portal-anterior mesenteric vein that has a less extensive sarcoplasmic reticulum) can also contract when stimulated after half an hour exposure to 4 mM EGTA, Ca-free media.[2] A number of mammalian smooth muscles do not contract after similar Ca-depletion (Hurwitz and Suria, 1971) and, until recently, the possibility that the occasionally reported contractile activity of some mammalian smooth muscles in nominally Ca-free solutions was due to trace contamination by unchelated calcium could not be ruled out (Somlyo and Somlyo, 1968 a; 1970). With the more recent use of appropriate chelating agents[2] (Imai and Takeda, 1967; Keatinge, 1968), this objection can be excluded.

The close association of the sarcoplasmic reticulum with surface membrane is compatible with a functional unit comparable to the couplings described in cardiac muscles (Sommer and Johnson, 1968; Fawcett and McNutt, 1969). Both surface vesicles and nonspecialized surface membranes were found in close association with SR, and either or both of these junctions may represent sites of excitation-contraction coupling. The separation between the surface and SR membranes (8–10 nm) is comparable to that found at the striated muscle triad (Franzini-Armstrong, 1970). Electron-opaque projections are also present between the two types of membrane, although we have not been able to find a periodicity of these projections comparable to that in the frog sartorius (Franzini-Armstrong, 1970). The couplings observed could mediate twitch contractions triggered by action potentials in turtle smooth muscle (Roddie, 1962), in a manner analogous to the excitation-contraction coupling process in twitch-striated muscles. The finding is compatible, at least in reptilian smooth muscle, with the view (Somlyo and Somlyo, 1968 a, b) that action potentials trigger contractions through translocation of intracellular calcium, rather than through the influx of extracellular calcium during the action potential. The presence of couplings has also been noted as an unpublished observation

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[2] Devine, C. E., A. V. Somlyo, and A. P. Somlyo. Manuscript in preparation.

by Bozler (1969), although no illustrations or details of the type of preparation investigated were given.

An incidental finding was the presence of electron-opaque striations (approximately 10 nm wide) of the surface vesicles and occasional tubules in turtle smooth muscle. We do not know whether this striation represents some functional, perhaps enzyme-associated structure, or whether it is a residue of a diaphragm similar to that observed at the necks of endothelial vesicles described by Palade and Bruns (1968). In mammalian smooth muscle, however, lanthanum enters even what appear to be "free-floating" vesicles (Devine and Somlyo, 1970; Somlyo and Somlyo, 1970), and in the present study we found no evidence of ferritin being excluded from vesicles by a surface diaphragm.

Note Added in Proof: We are pleased to acknowledge that, since the acceptance of this manuscript, W. R. Keatinge (1971, Proceedings of the 25th International Congress of Physiological Sciences, German Physiological Society, Munich, 9:295) has independently reported the temperature sensitivity of calcium depletion in a study of sheep carotid arteries, and concluded that this smooth muscle can also be activated in the absence of extracellular calcium.

Strontium accumulation by junctional and central sarcoplasmic reticulum of vertebrate smooth muscle *in situ* has been recently demonstrated (Somlyo, A. V., and A. P. Somlyo. *Science (Washington).* In press.), and further supports the functions ascribed in the present manuscript to the sarcoplasmic reticulum.

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