

GOLGI ORGANELLE RESPONSE TO THE ANTIBIOTIC X537A

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ABSTRACT

The effects of the ionophoric antibiotic X537A on cell structure were studied with phase-contrast, fluorescence, and electron microscopy. X537A induced selective vacuolation of the Golgi apparatus of vascular and intestinal smooth muscle, epithelium, plasma cells, and cultured chick heart and guinea pig vascular smooth muscle cells. The swelling of the Golgi apparatus induced by X537A was reversible in the systems examined for reversibility: vascular smooth muscle and cultured chick heart. Myelin figures were common in the Golgi apparatus vacuolated by X537A. Fluorescence microscopy of cultured cells incubated with X537A showed the characteristic blue X537A fluorescence associated with lipid globules in the cultured cells. Incubation of cultured chick heart cells with X537A reduced the beating rate and, after 24–72 h, abolished the sarcomere pattern. The swelling of the Golgi membranes produced by X537A in cultured vascular smooth muscle was associated with inhibition of D-[6-³H]glucosamine and [³⁵S]sulfate incorporation into glycosaminoglycans.

The antibiotic X537A, a fermentation product of *Streptomyces* (4, 47), is an ionophore that can act as a carrier of mono- and divalent cations between polar and apolar media (31, 32). Because X537A is an ionophore for calcium, the cation that triggers muscle contraction, its effects on intact muscle and on fractionated components of the contractile system have been studied in several laboratories. It has been shown that X537A has an inotropic effect on the heart (13, 14, 31, 32, 37), can contract vascular smooth muscle preparations (25, 31),¹ and induces the release of calcium from and

inhibits the uptake of this cation by fragmented sarcoplasmic reticulum of striated and cardiac muscle (5, 13, 14, 36).

The original rationale for the studies to be reported here was to correlate the contractile and ultrastructural effects of X537A on smooth muscle. We had hoped to demonstrate calcium release associated with ultrastructural changes in the sarcoplasmic reticulum and thereby further establish the relationship between these organelles and excitation-contraction coupling in vertebrate smooth muscle (11, 41). Preliminary studies¹ showed, however, that X537A had minimal or no contractile effects on vascular smooth muscles¹ when the latter were incubated in calcium-free extracellular media in which there could be only intracellular sources (i.e. sarcoplasmic reticulum)

¹ A. P. Somlyo and A. V. Somlyo, unpublished observations. Portal-anterior mesenteric vein, aorta, and main pulmonary artery of rabbits reserpinized to eliminate effects due to the release of catecholamines were stimulated with X537A up to 300 µg/ml, dissolved in DMSO.

of activator calcium. Furthermore, the antibiotic did not produce detectable changes in the ultrastructure of the sarcoplasmic reticulum except in concentrations that induced severe nonspecific structural damage. During these studies, however, it was observed that relatively low concentrations of X537A produced ultrastructural changes in the Golgi system of vascular smooth muscle.

The Golgi apparatus is a ubiquitous intracellular organelle (for review see reference 1) with distinctive enzymatic properties (16, 17) and plays a major role in protein secretion, lysosome formation, and the transfer of the carbohydrate and sulfate moieties to acid mucopolysaccharides and glycoproteins (3, 33, 34, 46). The presence of glycoproteins in the recognition sites of plasma membranes (48) suggests that the Golgi organelle may have profound effects on other membranous components of cells. In vascular smooth muscle, alterations of the Golgi system may be expected to affect the synthesis of the extracellular proteins of the vascular wall (35, 39) and some of the numerous drug receptors associated with smooth muscle membranes (40). Therefore, we considered it of potential interest to study an agent producing selective ultrastructural changes in the Golgi apparatus of vascular smooth muscle, and to extend these observations to some other tissues.

We shall report here our observations on the ultrastructural effects of X537A on the Golgi system of adult and cultured vascular smooth muscle, cultured beating heart cells, adult intestinal epithelium, plasma cells, and smooth muscle together with some light microscope observations on the cultured tissues. We shall also show that the structural effects of X537A on the Golgi system are accompanied by changes in the incorporation of [^3H]glucosamine and [^{35}S]sulfate into mucopolysaccharides that may be related to changes in the Golgi activity. Finally, we have made some observations on the presumptive localization of X537A in cultured cells through fluorescence microscopy. A preliminary report of some of these findings has been published (19).

MATERIALS AND METHODS

Tissue Preparations

The main pulmonary artery, portal-anterior mesenteric vein, and abdominal aorta were removed from adult white male rabbits (5–7 lb) and the colon from white I.C.R. mice. Strips of tissues were stretched to 1.5 times their excised length and placed in Krebs' solution,

bubbled with 95% O_2 -5% CO_2 , of the following composition (mM): NaCl, 119; KCl, 4.7; CaCl_2 , 1.2; NaHCO_3 , 24.9; KH_2PO_4 , 1.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2; and glucose, 5.6. X537A (Hoffmann-LaRoche, Inc., Nutley, N. J., lot numbers 1804-114 and 2936-85) was added to the incubation media, without diluent unless otherwise noted, as a suspension in concentrations of 0.5–150 $\mu\text{g}/\text{ml}$. Visible suspensions were formed at antibiotic concentrations of 10 $\mu\text{g}/\text{ml}$ or greater, and the concentrations greater than 5 $\mu\text{g}/\text{ml}$ in aqueous solution were indeterminate.

Cell Culture Techniques

Vascular smooth muscle cells were cultured by minor modifications (18)² of the technique described by Ross and Klebanoff (35). Aortas from 1-mo old guinea pigs were removed by sterile technique. The intima and the adventitia were stripped from the aorta, and the remaining media was cut into small segments. These segments were grown as explants in collagen-coated Cooper (Falcon) dishes and fed with minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic (Grand Island Biological Co.). Cells that migrated from these explants were dissociated by incubating the cultures with 0.1% collagenase, 0.25% trypsin, and 0.1% elastase (Nutritional Biochemicals, Cleveland, Ohio) in Ca-Mg-free balanced salt solution (Grand Island Biological Co.) at 37°C in a humidified CO_2 incubator for 30–60 min. After dissociation the cells were collected, centrifuged, washed, and resuspended in nutrient medium. Cells were plated out in collagen-coated Cooper dishes ($2-5 \times 10^5$ cells/60-mm dish). Cells for examination with polarized and UV light were grown on collagen-coated glass cover slips immersed in the medium. Cells for electron microscopy were reared in carbon-coated culture dishes. Cardiac muscle cultures were prepared from 5-day old chick embryonic hearts as described previously (6, 7). X537A was suspended in the culture media in concentrations of 5–20 $\mu\text{g}/\text{ml}$.

Glycosaminoglycan Analyses and

Deoxyglucose Uptake

Cultures were incubated with X537A (10 $\mu\text{g}/\text{ml}$) in the medium. Control and treated cultures were incubated with either D-[6- ^3H]glucosamine (5 $\mu\text{Ci}/\text{ml}$; sp act 3.6 Ci/mmol) or with 10 $\mu\text{Ci}/\text{ml}$ (corrected for decay) carrier-free $\text{Na}_2\text{ }^{35}\text{SO}_4$, when the majority of the X537A-treated cells became vacuolated (1–3 h). X537A was present throughout the incubation with the isotopes. After 20 h of exposure to the label, the medium was decanted and the cells, after being washed with balanced salt solution (Hanks), were scraped off the culture dishes with a rubber policeman and collected separately. The polysaccharide was extracted and analyzed by the

² S. Chacko and S. Blose. Unpublished observations.

method reported by Nameroff and Holtzer (27). The samples of cell and medium were digested by overnight incubation (50°C) with Pronase (Calbiochem, San Diego, Calif., grade B, 0.4 mg/ml) in 0.2 M Tris buffer (pH 8.0). Trichloroacetic acid was added to a concentration of 10%, and the materials were stored at 4°C until precipitation was complete. After centrifugation, the supernate was exhaustively dialysed against 0.01 M unlabeled sodium sulfate or glucose followed by water, and lyophilized. The dry samples were dissolved in a small volume of water, and aliquots were used for scintillation counting or electrophoresis. Samples for electrophoresis were taken with capillary pipettes and spotted on 1 × 12-inch cellulose acetate strips and subjected to 500 V for 90 min at 0°C in 0.1 M pyridinium formate buffer (pH 3.0). The strips were cut into 1-cm segments and counted in toluene with 5 g 2,5-diphenyloxazole and 0.5 g 1,4-bis[2,(5 phenyloxazolyl)] benzene per liter. Strips spotted with a mixture of authentic chondroitin sulfate (Sigma Chemical Co., St. Louis, Mo.), and hyaluronic acid (Nutritional Biochemical Company) were electrophoresed with each group of labeled samples. The known standards were localized after electrophoresis by staining with 0.1% toluidine blue O (pH 6).

The uptake of 2-deoxy-D-glucose (23) was measured by first washing the cells with glucose-free Krebs' solution for 5 min. Control cells were then incubated in the same solution with 1 mM 2-deoxy-D-glucose and traces of [³H]2-deoxy-D-glucose (5 μCi/ml; sp act 5 Ci/mol). Other cultures were incubated simultaneously with X537A (10 μg/ml) and 2-deoxy-D-glucose with the isotope for various intervals from 5 to 30 min or pretreated with X537A for 24 h before 2-deoxy-D-glucose uptake measurement. After completion of the appropriate incubation period, the cells were washed in ice-cold saline, scraped off the dish, and centrifuged. The remaining pellet was dissolved in NCS Solubilizer (Nuclear Chicago Corp., Des Plaines, Ill.) and counted in toluene Liquifluor in a liquid scintillation counter (New England Nuclear, Boston, Mass). The counts were corrected for quenching, with an external standard.

DNA Determination

DNA was determined on aliquots of resuspended cellular pellets by a fluorometric method (9).

Adenine Nucleotide Determination

ATP and ADP were measured in rabbit main pulmonary artery samples quick-frozen between metal plates cooled with liquid nitrogen. The frozen samples were weighed, and the adenine nucleotides were extracted (24) with 0.5 N perchloric acid. The extracts were neutralized with 0.57 M KOH and buffered to pH 7.4 with 0.2 M triethanolamine. The potassium perchlorate precipitate was removed and the samples were assayed with a Varian 4200 liquid chromatograph with Variscan detector (Varian Associates, Palo Alto, Calif.). An NH₂ column was

used with a mobile phase of 0.25 M KH₂PO₄ flowing at 60 ml/h. For quantitative measurements, absorbances of samples and standards at 259 nm were compared.

Electron Microscopy

All tissues were fixed in 2% glutaraldehyde + 4.5% sucrose in 0.075 M sodium cacodylate buffer at pH 7.4 for 2 h, rinsed with buffer, and postfixed in 2% OsO₄ (pH 7.4, in 0.05 M cacodylate buffer) for 2 h. Cell cultures were fixed in the same solutions but for briefer periods: 30 min in glutaraldehyde and 20 min in OsO₄.

Tissues were stained en bloc for 60 min, and tissue cultures for 10 min with saturated aqueous uranyl acetate. All preparations were dehydrated in graded ethanols and embedded in either Epon 812 or Spurr's resin (44). Epon-embedded cell cultures were separated from the plastic dishes by immersion in liquid nitrogen. Gray to silver sections were cut with a diamond knife on a Porter-Blum ultramicrotome (Dupont Instruments, Sorvall Operations, Newton, Conn.), suspended on bare copper grids, stained with lead citrate, and examined in a Hitachi HU 11E (Hitachi Ltd., Tokyo, Japan) or Philips EM 301 electron microscope, Philips Electronic Instruments, Mount Vernon, N. Y.

Light Microscopy

Living cultured cells were examined periodically with a Zeiss microscope equipped with phase optics. For fluorescence studies, the same microscope was used with a UV light source and an excitation filter of 325 nm (UG5, Zeiss) and a barrier filter of 410 nm (range 410 and above).

RESULTS

Effect of X537A on Smooth Muscle, Intestinal Epithelium, and Plasma Cells

The ultrastructure of rabbit vascular smooth muscle has previously been described (11, 42, 43). Fig. 1 is an electron micrograph of control main pulmonary artery (MPA) showing well-preserved mitochondria, thick and thin filaments, sarcoplasmic reticulum, and surface vesicles. The perinuclear location of the Golgi apparatus, consisting of stacked lamellar elements and vesicles, is also evident.

Incubation with X537A (5 μg/ml for 30 min) produced selective dilatation of the Golgi apparatus in smooth muscle of MPA (Figs. 2, 3, 4 B), portal-anterior mesenteric vein and abdominal aorta. Myelin figures were frequent within the dilated membranes (Figs. 3, 4 B). The mitochondria were in the condensed state after treatment and the sarcoplasmic reticulum and rough endo-

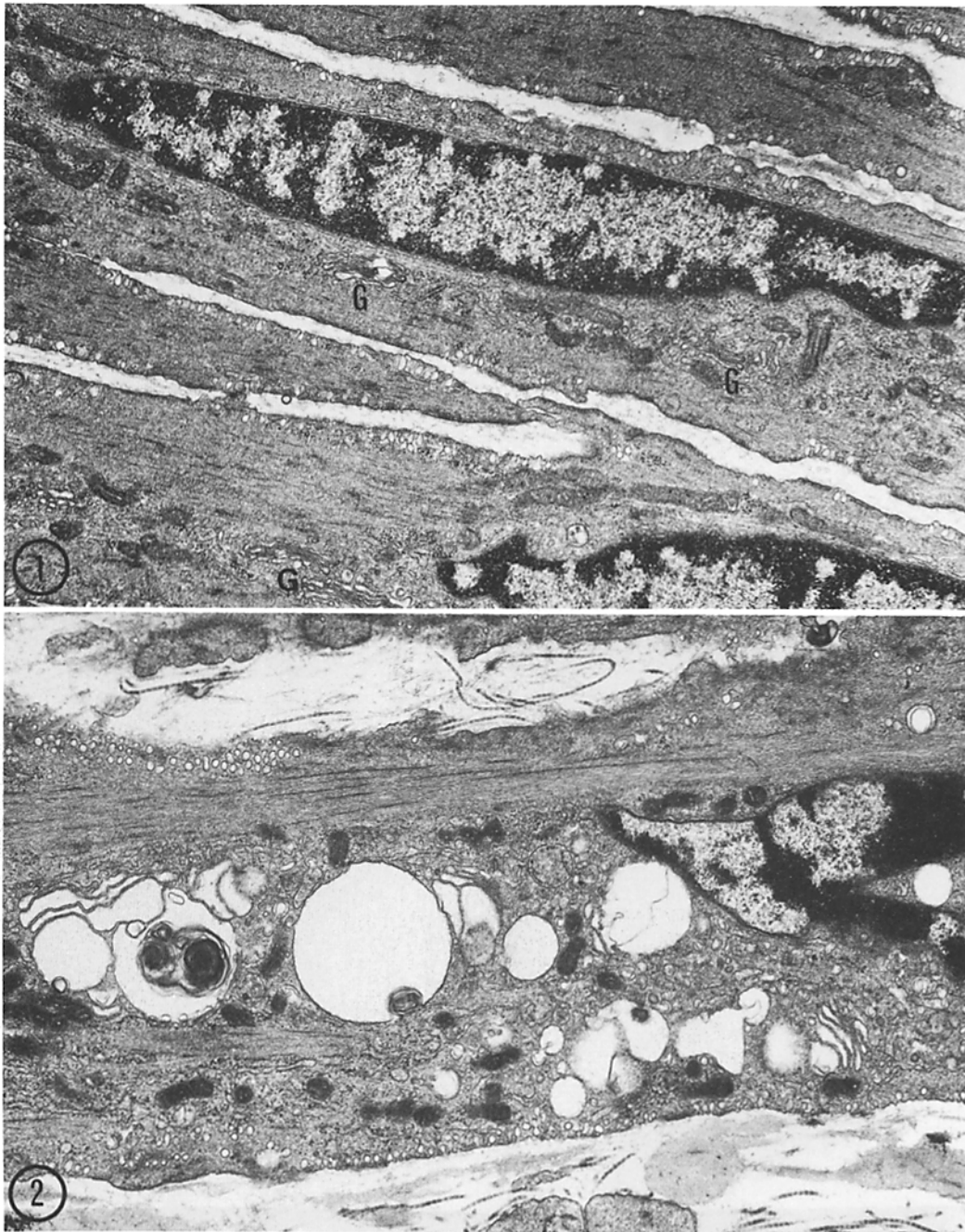


FIGURE 1 Longitudinal section of vascular smooth muscle showing the flattened sacs and vesicles of normal Golgi apparatus (G). Rabbit MPA incubated for 30 min in normal Krebs' solution. $\times 17,500$.

FIGURE 2 Longitudinal section of vascular smooth muscle showing marked swelling of the Golgi system. The vacuolation is at the nuclear pole. Rabbit MPA incubated for 30 min in X537A $5 \mu\text{g/ml}$. $\times 17,500$.

plasmic reticulum were not swollen or dilated (Figs. 2, 3, 4 B vs. Fig. 1). Similarly, $10\text{--}150 \mu\text{g}$ X537A/ml also produced swelling of the Golgi apparatus, but at the higher concentrations some nonspecific damage was also noted. Some tissues (MPA) were treated for 1 h with

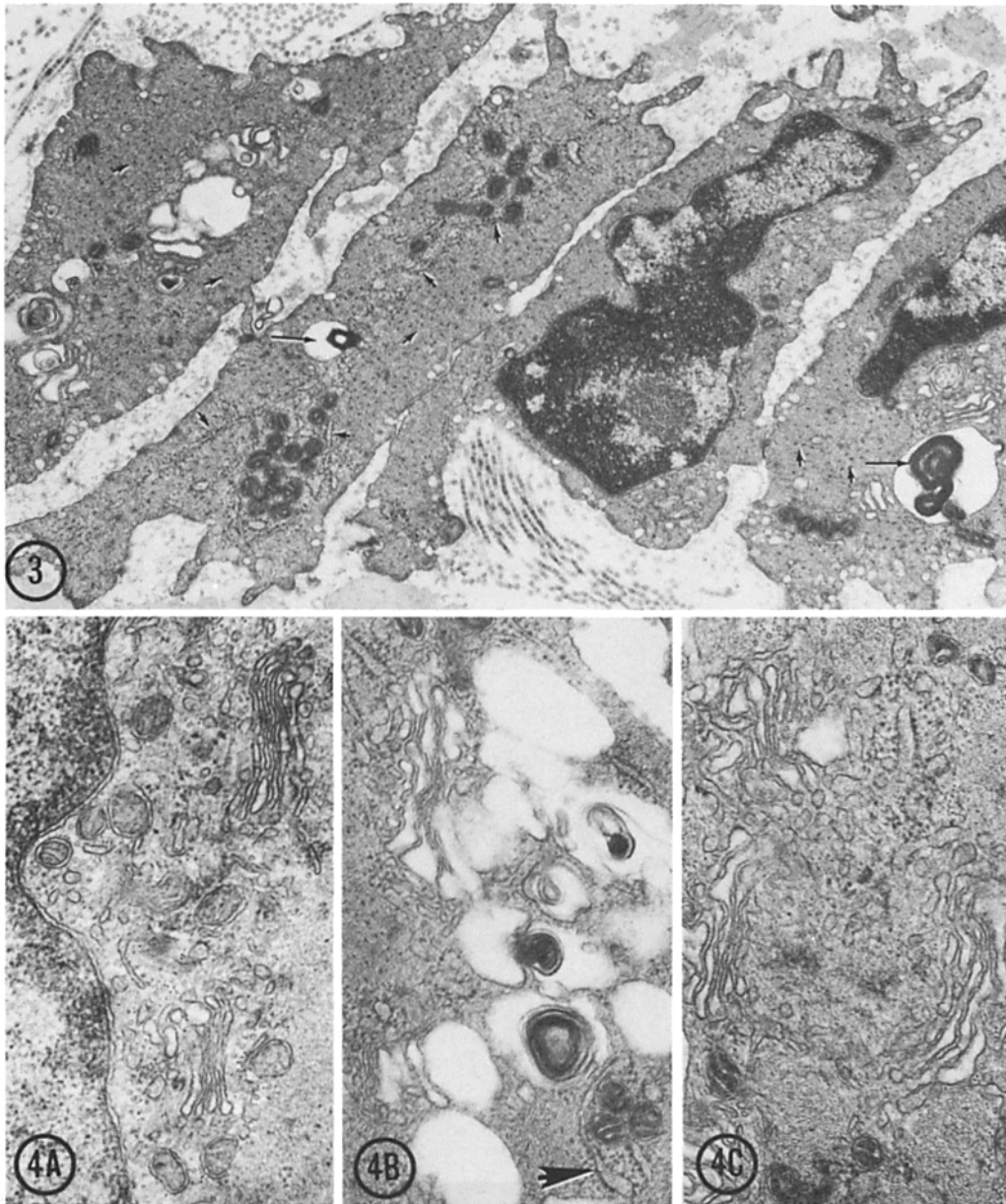


FIGURE 3 Low-magnification transverse section of X537A-treated smooth muscle as in Fig. 2. Some of the markedly vacuolated Golgi membranes contain myelin figures (*arrows*). The thick and thin filament lattice and rough sarcoplasmic reticulum (*arrowheads*) remain normal. Rabbit MPA incubated for 30 min with X537A 5 $\mu\text{g}/\text{ml}$. $\times 19,000$.

FIGURE 4 Reversibility of the Golgi swelling induced by X537A in vascular smooth muscle. (A) Golgi system of control MPA incubated for 30 min in normal Krebs' solution, showing the stacked lamellar Golgi membranes adjacent to the nucleus. (B) Swelling of predominantly the *trans* portion of the Golgi apparatus containing myelin figures, in rabbit MPA incubated for 30 min in Krebs' solution with X537A 5 $\mu\text{g}/\text{ml}$. Note that the rough sarcoplasmic reticulum (*arrowhead*) is not swollen. (C) Relatively normal Golgi apparatus in rabbit MPA smooth muscle exposed for 30 min to X537A 5 $\mu\text{g}/\text{ml}$ (as in B) and then returned to normal Krebs' solution for 1 h. Each micrograph shows a transverse section of MPA from the same rabbit. $\times 40,000$.

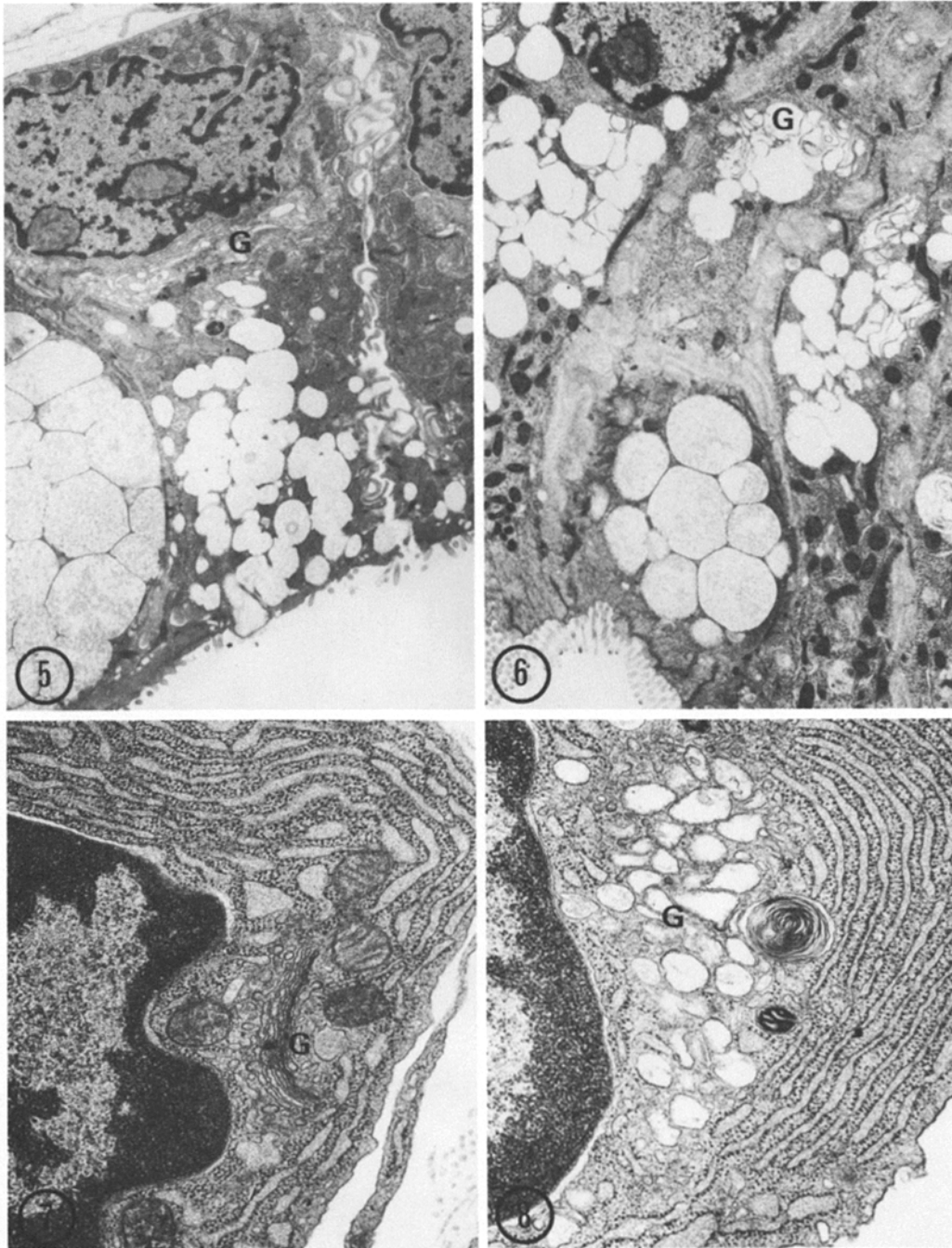


FIGURE 5 Intestinal epithelium in control tissue. Columnar epithelial and goblet cells in mouse colon incubated for 1 h. G, Golgi apparatus. $\times 7,500$.

FIGURE 6 Intestinal epithelium with swollen Golgi apparatus (G). Columnar epithelial and goblet cells in mouse colon incubated with X537A $20 \mu\text{g}/\text{ml}$ for 1 h. Note the marked vacuolation of the Golgi apparatus. $\times 7,500$.

FIGURE 7 Part of a control intestinal plasma cell. Note the flattened Golgi membranes (G) and extensive rough endoplasmic reticulum from mouse colon incubated in Krebs' solution for 1 h. $\times 25,000$.

FIGURE 8 Swelling of the Golgi apparatus (G) in intestinal plasma cell. The Golgi system is vacuolated and contains a myelin figure. Note also the condensed state of the mitochondria and absence of swelling in the extensive endoplasmic reticulum of this plasma cell from mouse colon incubated in X537A, $20 \mu\text{g}/\text{ml}$ for 1 h. $\times 25,000$.

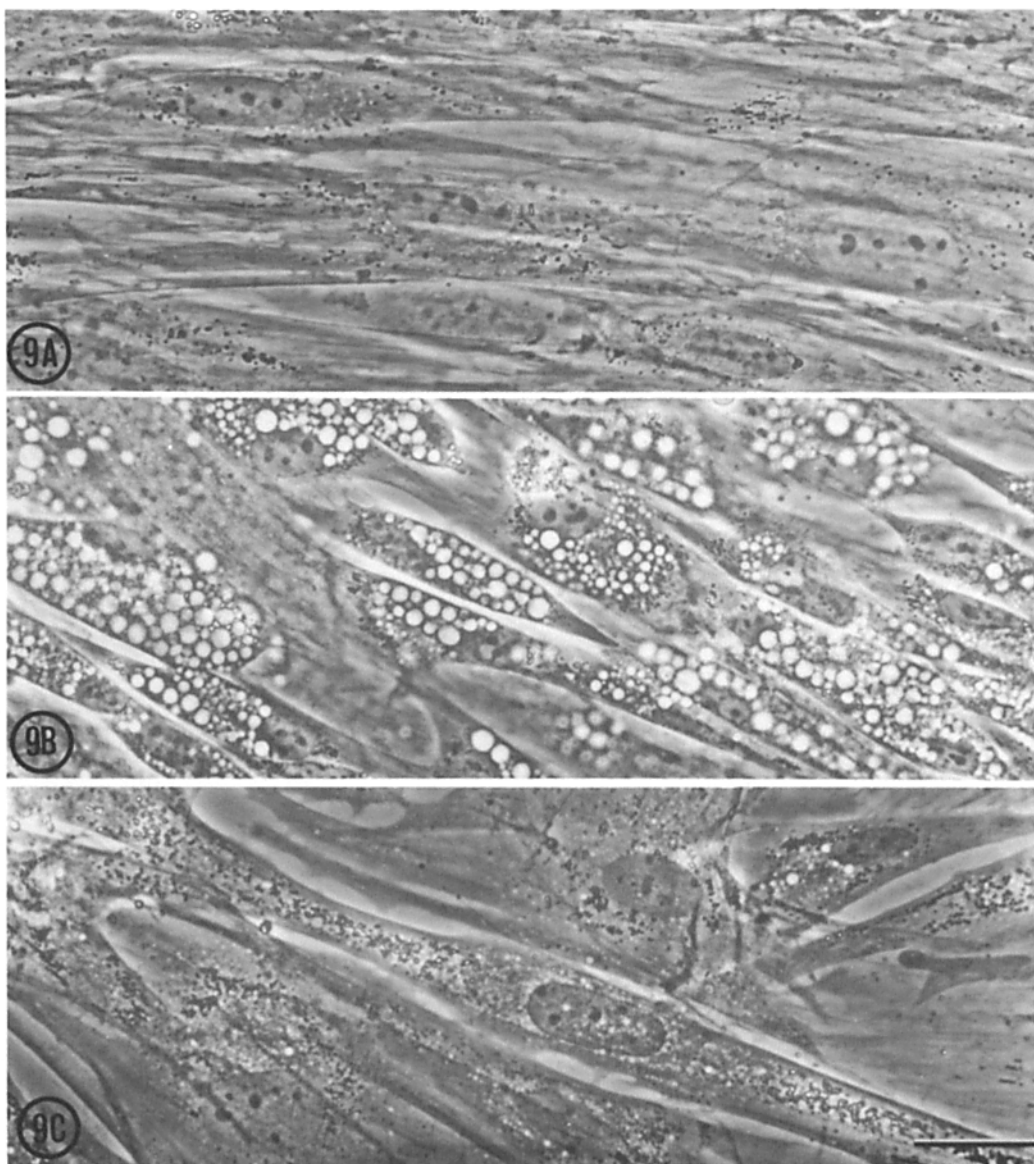


FIGURE 9 Phase-contrast micrographs of the reversal of the vacuolation induced by X537A in cultured vascular smooth muscle. (A) Control guinea-pig aortic smooth muscle cells in culture. (B) Vacuolation after 3-h incubation with X537A 10 $\mu\text{g}/\text{ml}$. (C) Reversal of vacuolation in smooth muscle cells 3 h after removal of X537A from the culture medium. Calibration bar equals 10 μm .

X537A (5 $\mu\text{g}/\text{ml}$) and then incubated for an additional hour in drug-free solution to determine whether the drug effect was reversible. The Golgi apparatus, swollen in tissues for 1 h in X537A (Fig. 4 B), returned to its normal appearance (Fig. 4 A) after incubation of the treated tissues for 1 h in solutions without the drug (Fig. 4 C).

Mouse colon was studied to determine the effect of the antibiotic on epithelial cells known to be

actively engaged in glycoprotein synthesis (3). Figs. 5 and 6 show, respectively, normal controls and the dilatation of the Golgi system in intestinal epithelial cells after X537A treatment (20 $\mu\text{g}/\text{ml}$ for 1 h). In these tissues, the Golgi apparatus of the submucosal plasma cells was also swollen (Figs. 7 and 8), in striking contrast to the unswollen, extensive system of endoplasmic reticulum. The ultrastructure of the colon smooth muscle treated

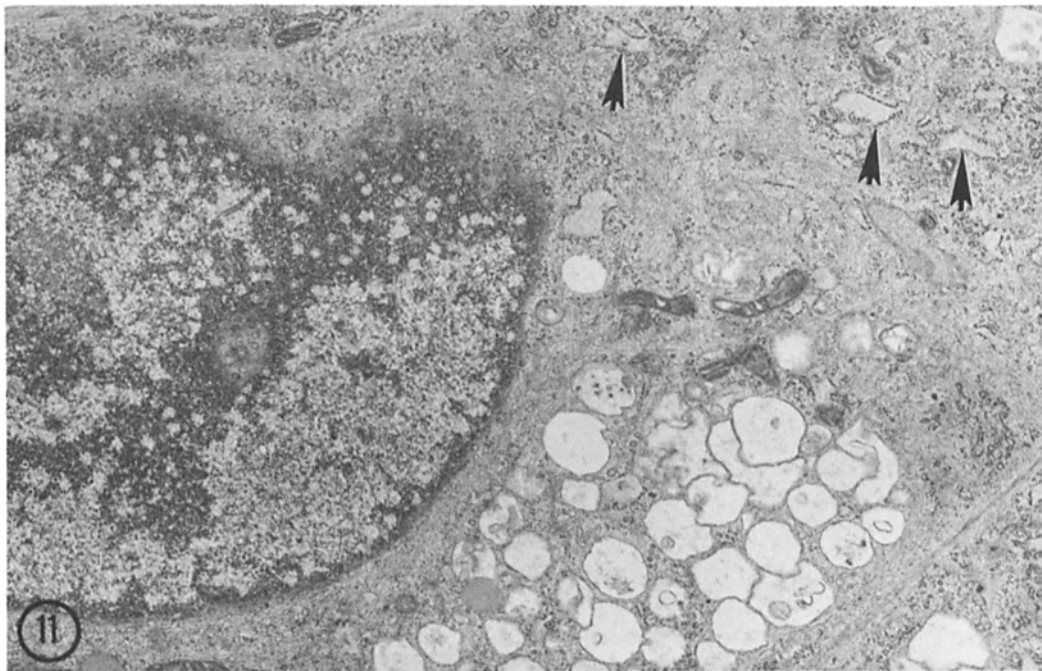
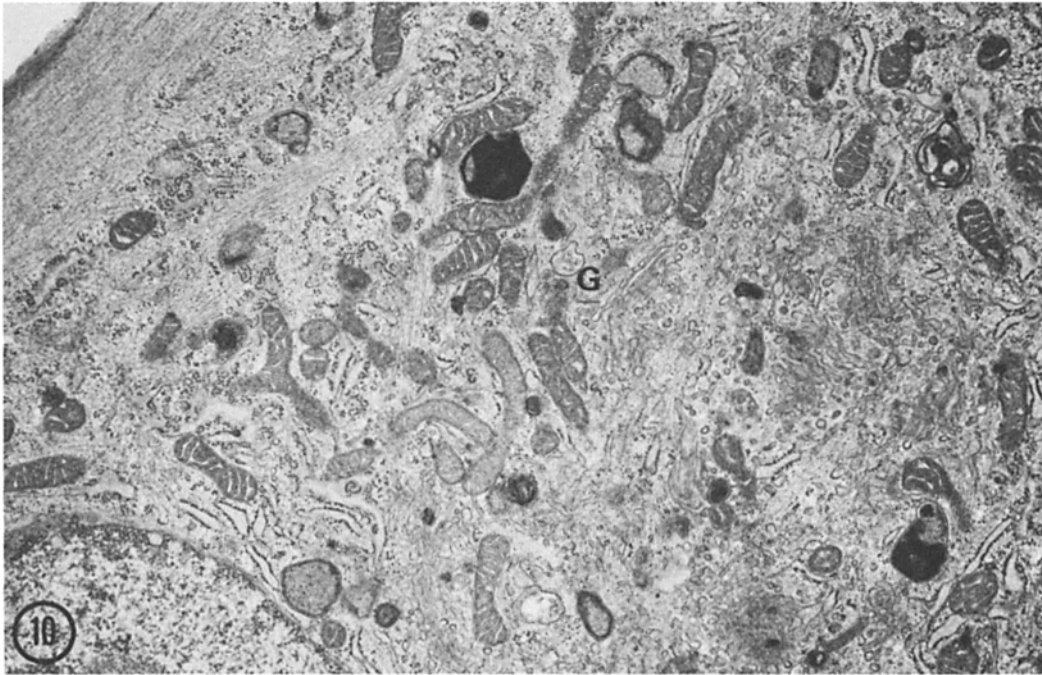


FIGURE 10 Electron micrograph of part of normal cultured vascular smooth muscle cell with Golgi membranes. *G* refers to Golgi apparatus. Cultured guinea pig aorta. $\times 14,500$.

FIGURE 11 Electron micrograph of cultured vascular smooth muscle vacuolated after exposure to X537A. There are no normal Golgi membranes present, but only the vacuolated structures. Note that the rough sarcoplasmic reticulum (*arrowheads*) is not swollen. Guinea pig aorta in culture, incubated with X537A $10 \mu\text{g/ml}$ for 3 h. $\times 14,500$.

with X537A (swollen Golgi system, normal sarcoplasmic reticulum, etc.) resembled that of similarly treated vascular smooth muscle.

Effect of X537A on Cultured Cells

SMOOTH MUSCLE: Treatment of cultured smooth muscle cells with X537A (10–20 $\mu\text{g}/\text{ml}$) caused extensive perinuclear vacuolation as seen with the phase-contrast microscope (Fig. 9). The onset and extent of vacuolation detectable with the light microscope were dependent upon the concentration of X537A suspended in the culture medium. The drug effect first became visible in the light microscope after 1- to 3-h incubation with X537A (10 $\mu\text{g}/\text{ml}$) and could be reversed within 3 h of removing the drug (Fig. 9 C). X537A in concentrations exceeding 20 $\mu\text{g}/\text{ml}$ was lethal to all cells after prolonged incubation (2–3 days).

The ultrastructure of normal aortic cells (Fig. 10) was similar to that of cultured vascular smooth muscle cells described by Ross and Klebanoff (35). Cultured cells treated with X537A (10 $\mu\text{g}/\text{ml}$ for 3 h) showed extensive dilatation of the Golgi system, and normal Golgi membranes were not found in the treated cells (Fig. 11). Other organelles, including the sarcoplasmic reticulum, appeared normal. Mitochondria were predominantly in the condensed configuration.

Localization of X537A within Cells

To localize X537A within cells, we took advantage of the fact that the antibiotic is a fluorophore (10, 13, 31). Light blue fluorescence was characteristic of X537A crystals dry, suspended in minimal essential medium, or in hyperlipemic serum^a when examined in the fluorescence microscope. The blue fluorescence changed to yellow after 10- to 20-min illumination of the crystals.

Cultured smooth muscle cells treated with the antibiotic (10 $\mu\text{g}/\text{ml}$) for 3–24 h were briefly washed in drug-free medium to remove the extracellular drug and examined in the fluorescence microscope. Autofluorescence (yellow and purple) was noted both in controls and in cells treated with X537A. The blue cellular fluorescence characteristic of X537A crystals was seen only in treated cells, but it was extremely variable. Consistent blue fluorescence of most cells was observed when the antibiotic was dissolved in dimethylsulfoxide (0.1

^aSerum was collected from rabbits on high cholesterol diet.

ml DMSO/5 ml medium) before its addition to the medium. Areas of intense blue fluorescence correspond to regions occupied by lipid globules as seen with phase-contrast optics (Figs. 12 and 13). As with the crystals, prolonged UV illumination of the treated cells resulted in disappearance of the blue fluorescence. DMSO without X537A did not induce vacuolation in or modify the autofluorescence of control cells.

Cardiac Muscle

Beating cultured chick embryonic heart cells, examined with the phase contrast microscope after X537A (10–20 $\mu\text{g}/\text{ml}$) treatment for 3 h, showed pronounced vacuolation of the typically perinuclear Golgi organelle (Figs. 14, 15) followed by a decline in the beating rate. After a 5-h incubation with X537A, the beating rate had declined significantly to one-third the rate observed in the same colonies before addition of the drug (Table I). The previously reported (6) variability of the beating rate of normal cultured heart cells was also noted in this study. The drug effects were reversible: the vacuolation disappeared and the beating rate returned to normal after 18 h in a drug-free, normal medium. Other cells treated continuously for longer than 18 h with X537A stopped beating entirely. Light microscopy with polarized light revealed a breakdown of sarcomere pattern (absence of striations) in cells treated for 24–72 h with X537A (Fig. 16), although birefringent material (presumably thick filaments) was still present in these cells.

Electron micrographs of control cultured car-

TABLE I
Effect of X537A on the Beating Rate of Cultured Cardiac Cells

Treatment	Beats/min	Limits of confidence
Control	69 \pm 8.1	
X537A 3 h	59 \pm 6.4	>0.050
X537A 5 h	21 \pm 6.0	<0.001
18 h after washout	56 \pm 10	>0.200

Counts are mean values \pm SE of beats per minute of the same colonies of cells from three separate cultures made before adding X537A (control), after adding X537A 10 $\mu\text{g}/\text{ml}$, and 18 h after washing the drug out of the cultures. Limits of confidence between control and treated cells were determined by the two-tailed, paired Student's *t* test.

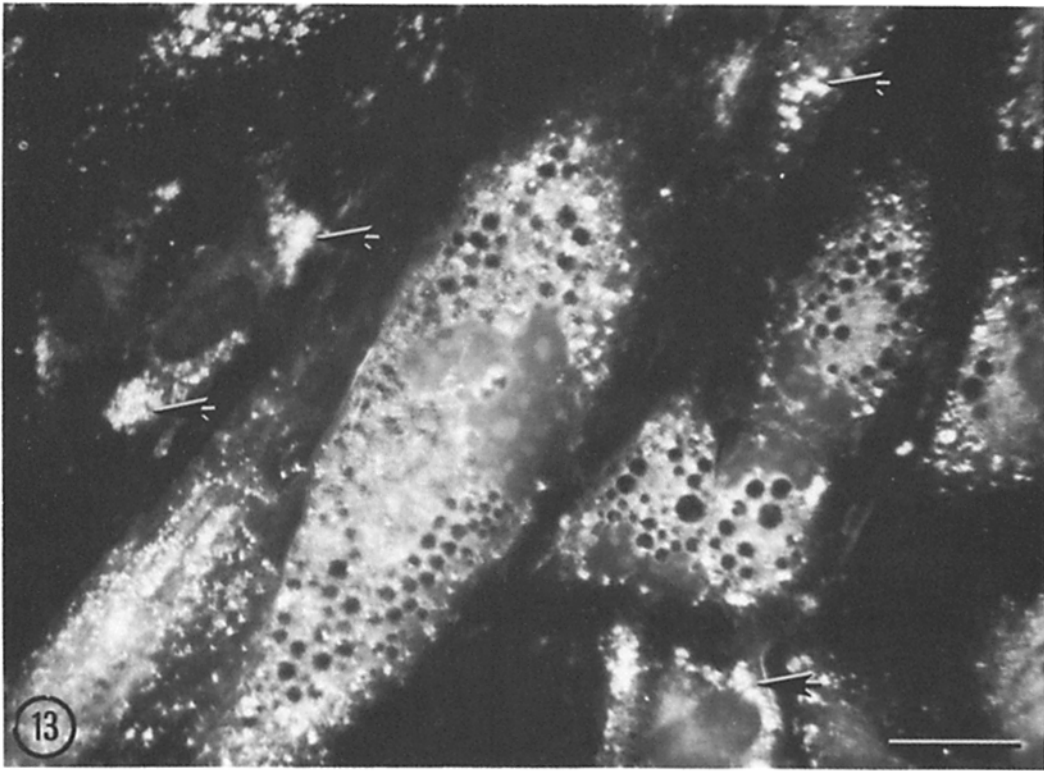
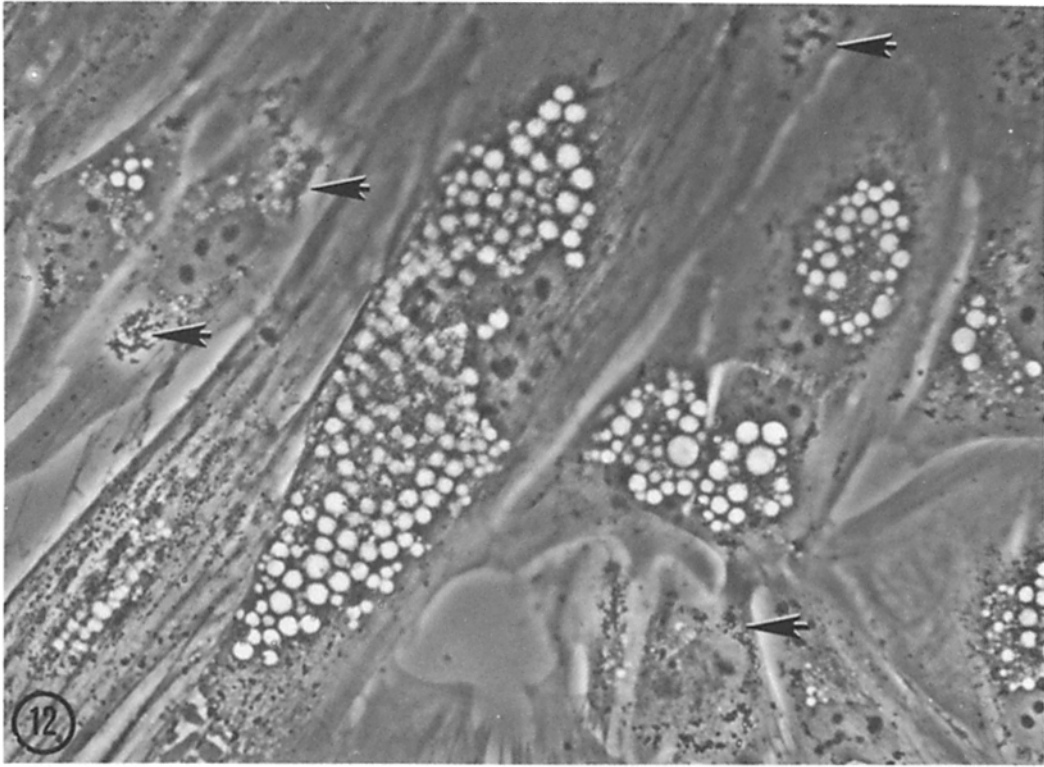


TABLE II
Uptake of [³H]Deoxy-D-Glucose and Incorporation of D-Glucosamine-6-³H by Cultured Vascular Smooth Muscle

Uptake time	Treatment	n	DOG/10 ⁶ cells mean ± SE	[³ H]Glucosamine in GAG
<i>min</i>			<i>nmol</i>	<i>cpm/μg DNA</i>
5	X537A	4	17.33 ± 2.16	
	Control	4	18.44 ± 5.98	
10	X537A	4	23.85 ± 2.87	
	Control	3	36.26 ± 5.10	
15	X537A	4	31.85 ± 3.99	
	Control	4	32.40 ± 2.76	
20	X537A	4	34.61 ± 4.18	
	Control	4	28.66 ± 3.06	
30	X537A	4	37.55 ± 3.42	
	Control	4	35.38 ± 3.98	
30	X537A (24 h)	4	11.63 ± 0.54	
	Control*	5	15.22 ± 1.67	
24(h)	X537A	4		295.10 ± 55.58‡
	Control	5		1025.96 ± 86.26

DOG: [³H]deoxy-D-glucose.

GAG: Glycosaminoglycans determined on cellulose acetate strip electrophoresis. D-glucosamine-6-³H was added as described in Methods.

* A different passage of the cells studied for the short-term (5–30 min DOG uptake) experiments was used, and therefore the control values in the two sets of experiments are not comparable.

‡ Value significantly different from control ($P < 0.01$). The concentration of X537A was 10 μg/ml in each experiment, and, unless otherwise indicated, X537A was added at the same time as the DOG.

diac muscle cells showed thick and thin filaments organized into sarcomeres (Fig. 17). The stacked lamellar membranes of the Golgi system were usually perinuclear.

After 3-h treatment of cultured cardiac muscle with X537A (10–20 μg/ml), the normal Golgi system was replaced by membrane-bounded peri-

nuclear vacuoles (Fig. 18). Thick filaments, but no organized sarcomeres, were present in the treated cells, confirming the light microscope observations. No Z bands were seen in cardiac cells treated for 18 h with X537A (10–20 μg/ml), although some dense bodies with attached thin filaments were still present. In addition, X537A also affected

FIGURE 12 Phase-contrast micrograph of cultured vascular smooth muscle treated with X537A and used as reference for fluorescence microscopy. The predominantly perinuclear vacuoles, characteristic of X537A action, are present: *arrows* point to groups of lipid globules normally seen (without drug treatment) in vascular smooth muscle at this stage of culture. Guinea-pig aorta in culture exposed to X537A 10 μg/ml (dissolved in DMSO) for 3 h. Compare with Fig. 13.

FIGURE 13 Fluorescence micrograph of cultured vascular smooth muscle treated with X537A. This micrograph shows the same group of fibers examined under the phase-contrast microscope shown in Fig. 12. Note that the most intense fluorescence is localized to groups of lipid globules (*arrows*). The contents of the vacuoles are not fluorescent. For further details, see text. Calibration bar equals 10 μm.

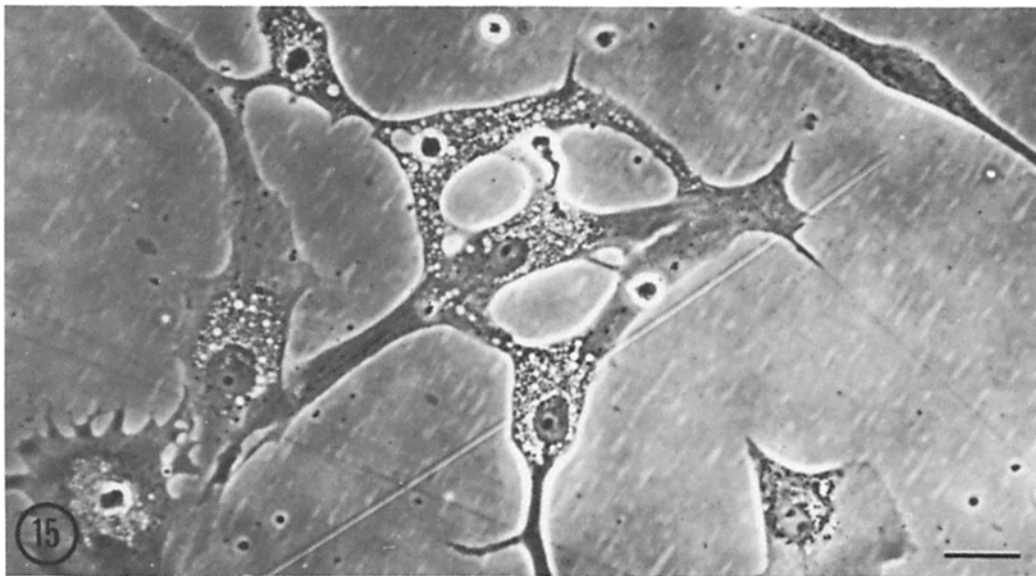
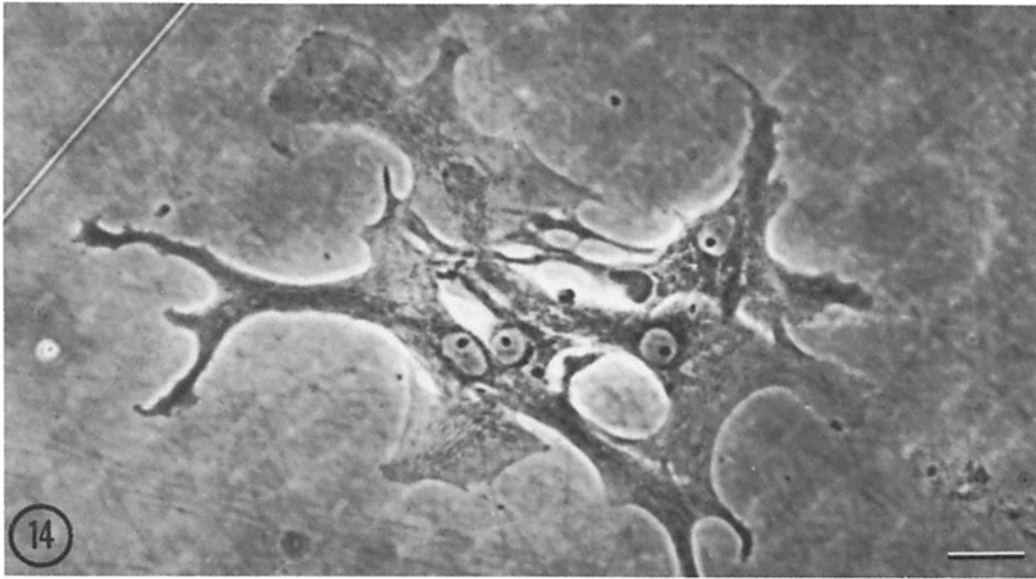


FIGURE 14 Phase-contrast light micrograph of control cultured chick heart cells. Calibration bar equals 10 μm .

FIGURE 15 Phase-contrast light micrograph showing the effect of X537A on cultured chick heart cells. Note the predominantly perinuclear vacuolation typical of the location of the Golgi system. Cultured chick heart cells incubated with X537A 20 $\mu\text{g}/\text{ml}$ for 3 h. Calibration bar equals 10 μm .

the structure of cardiac mitochondria: these contained frequent myelin figures.

Effect of X537A on Glycosaminoglycan Synthesis. [³⁵S]Sulfate and 2-Deoxy-D-Glucose Uptake

To examine the functional effect of X537A on the Golgi system, incorporation of SO_4^- or

D-[6-³H]glucosamine into glycosaminoglycans was studied in 10-day old, high-density cultures prepared from vascular smooth muscle maintained in culture for over 2 mo. Some cultures were treated with X537A (10 $\mu\text{g}/\text{ml}$); others served as controls. Both groups of cultures were incubated either with $^{35}\text{SO}_4^-$ or with D-[6-³H]glucosamine, and the glycosaminoglycans of cells and media were iso-

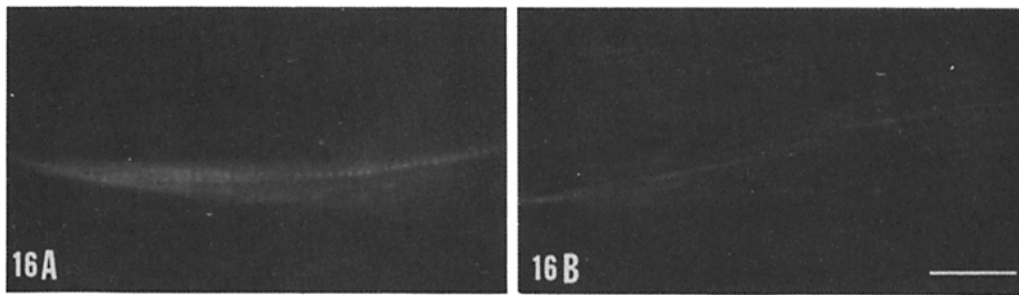


FIGURE 16 The effect of X537A on the sarcomere pattern of cultured chick heart muscle. Polarized light micrographs of (A) control cardiac fiber showing the typical sarcomere pattern and of (B) fiber treated with X537A, 20 $\mu\text{g}/\text{ml}$, for 24 h with consequent disappearance of the striations. Calibration bar equals 10 μm .

lated (see Materials and Methods). The electrophoretic profiles of the mucopolysaccharides synthesized by control and by X537A-treated cells representing examples from one of four experiments are shown in Fig. 19. It can be noted that for the untreated cultures the profiles for cell layer (Fig. 19 A) and the medium (Fig. 19 B) are very similar, with roughly three times the total incorporation being found in the cell layer. The maximum peak both in the cell layer and in the medium corresponds to the electrophoretic mobility of the standard chondroitin sulphate (Fig. 19). X537A reduced the incorporation of [^3H]glucosamine into glycosaminoglycans to approximately one-third net of the control values in the cell layer and nearly completely abolished the glycosaminoglycan peak in the medium [^{35}S]sulfate incorporation into chondroitin sulfate was completely blocked by X537A (Fig. 20).

The uptake of 2-deoxy-D-glucose into cultured vascular smooth muscle cells, at intervals between 5 and 30 min, was not affected by X537A (Table II). In vascular smooth muscle cells pretreated

with the antibiotic for 24 h, the 2-deoxy-D-glucose uptake (30 min) was slightly (but not significantly) depressed when based on cell counts and not depressed at all when the results were normalized to the DNA content of the cultures (1.1 ± 0.25 nmol DOG/ μg DNA in X537A-treated and 1.0 ± 0.19 in controls). However, the incorporation of [^3H]glucosamine into glycosaminoglycans, determined on the same cell passage, was similarly reduced (Table II) as in the cultures illustrated in Fig. 19.

Adenine Nucleotide Content after X537A Treatment and the Action of Metabolic Inhibitors and Ouabain

Because of the changes in mitochondrial configuration induced by X537A (present study) and the previously reported effect of X537A in uncoupling oxidative phosphorylation in isolated mitochondria (15, 26), it could be argued that the ultrastructural effects of the antibiotic on the Golgi system were also secondary to metabolic inhibition.

TABLE III
Effect of X537A and Metabolic Inhibitors on Adenine Nucleotides in Rabbit Main Pulmonary Artery

Treatment	n	ADP	ATP
$\mu\text{mol nucleotide/g frozen tissue} \pm \text{SE}$			
Control	8	0.41 ± 0.01	2.25 ± 0.19
1 h X537A (5 $\mu\text{g}/\text{ml}$)	7	0.41 ± 0.05	2.05 ± 0.14
1 h X537A (10 $\mu\text{g}/\text{ml}$)	2	0.43 ± 0.07	2.28 ± 0.24
1 h IAA (1 mM) + NaCN (10 mM)	6	0.28 ± 0.07	$0.27 \pm 0.09^*$

* Significantly different from control values ($P < 0.01$) using unpaired Student's *t* test.

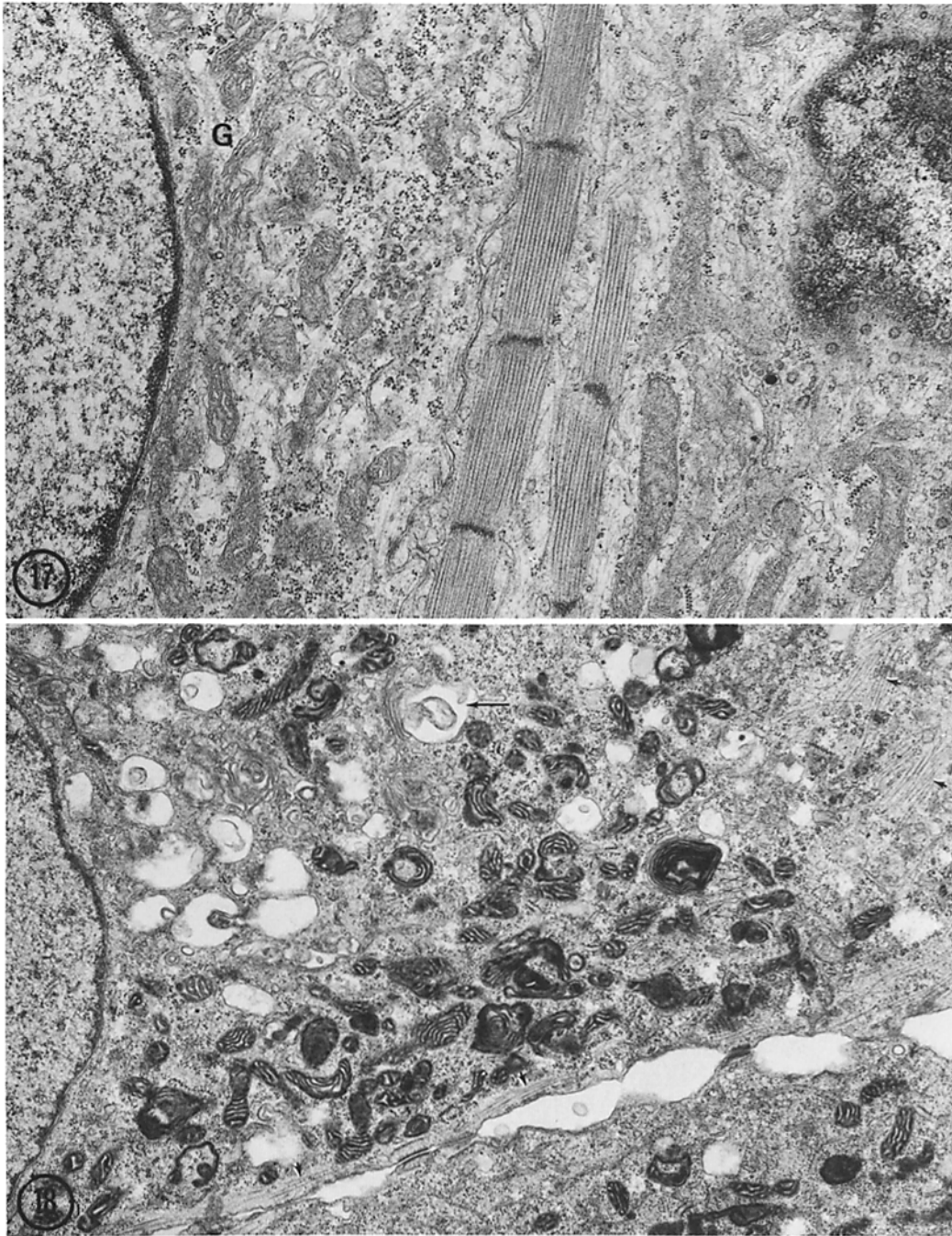


FIGURE 17 Electron micrograph of control chick heart cell. Well-organized sarcomeres and a paranuclear group of Golgi membranes are present. *G* refers to Golgi apparatus. $\times 22,000$.

FIGURE 18 Electron micrograph of vacuolated Golgi system in cultured chick heart cell exposed to X537A. The *cis* portion part of the Golgi system of this fiber remains recognizable while the *trans* portion is represented by a vacuole (*arrow*). No sarcomeres are present although some scattered thick filaments (*arrowheads*) are still seen. The mitochondria are markedly condensed and contain some myelin figures. The rough endoplasmic reticulum is normal. Cultured chick heart cell incubated with X537A, $20 \mu\text{g/ml}$, for 24 h. $\times 22,000$.

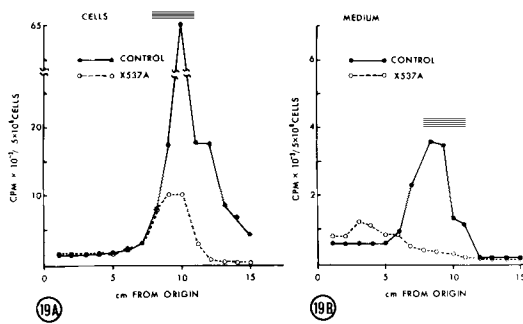


FIGURE 19 A and B The effects of X537A on the incorporation of [³H]labeled glucosamine into glycosaminoglycans in the cell layer (A) and in the medium (B). The counts indicated on the ordinate show the activity of the various fractions migrating on cellulose acetate strips. The horizontal bars above peaks indicate the position of the chondroitin sulphate standard.

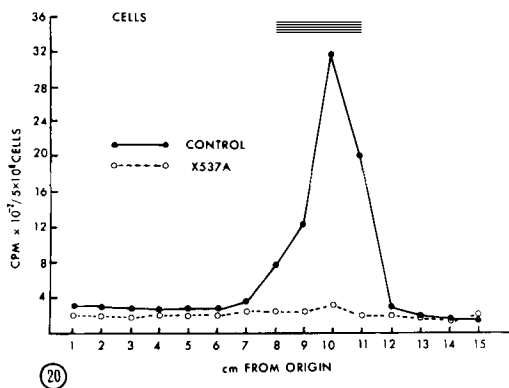


FIGURE 20 The effect of X537A on the incorporation of [³⁵S]sulfate into glycosaminoglycans of cultured vascular smooth muscle cells. The horizontal bars above the peak show the position of the chondroitin sulfate standards on the cellulose acetate strip.

Therefore, we have measured the adenine nucleotide content of rabbit main pulmonary arteries treated with X537A and also determined whether deliberate metabolic inhibition with iodoacetate and cyanide or omission of glucose from the incubation medium can reproduce the ultrastructural effects of X537A on the Golgi system.

Incubation of main pulmonary artery with X537A under conditions that produced pronounced vacuolation of the Golgi system (observed in samples of the same arteries used for adenine nucleotide analyses) had no significant effect on the ATP or ADP content of these preparations (Table III). Conversely, iodoacetate and cyanide in concentrations sufficient to reduce cellular ATP levels to approximately 12% of controls (Table III)

did not produce swelling of the Golgi apparatus (not shown). Nor did incubation for 1 h in glucose-free medium produce swelling of the Golgi system.

Because of the reported vacuolation of the Golgi system of cultured nerve cells by ouabain (49), we also incubated five main pulmonary arteries for 1 h with 10^{-3} M ouabain. This treatment did not produce the type of swelling observed after treatment with X537A. We cannot exclude the possibility that ouabain may produce minimal swelling of Golgi vesicles detectable by quantitative stereological techniques, but any change observed by us in random samples was not greater than that otherwise found due to block-to-block variation in control specimens.

DISCUSSION

X537A, at relatively low concentrations, produced selective ultrastructural changes in the Golgi system of vascular smooth muscle. The characteristic appearance of this organelle in the adult vascular smooth muscle, together with the absence of swelling of the sarcoplasmic reticulum and of mitochondria, enabled us to recognize the ultrastructural specificity of X537A action. Swelling of the Golgi apparatus, most marked in the *trans* portion but also involving the *cis* region in more extremely swollen examples, was the most typical manifestation of X537A effect and was accompanied by the formation of myelin figures. Although little is known about the nature of the latter, their preferential localization to the Golgi apparatus has already been noted by Palade and Claude (28, 29) and, in our experiments, may have represented the production of new Golgi membranes in response to injury. In contrast to normal adult smooth muscle, in which myelin figures are rarely seen, in our normal cultured material myelin figures are relatively common. Therefore, without much more extensive quantitative studies we cannot determine whether X537A also increased their number in cultured cells. In intestinal epithelium, smooth muscle, and submucosal plasma cells, X537A also produced dilatation of the Golgi system, suggesting that the effect of the antibiotic on the Golgi apparatus is not limited to muscle.

Vacuolation of cultured vascular smooth muscle and of chick heart cells incubated with X537A as seen by light microscopy was initiated in the perinuclear region and could also be localized by electron microscopy to the Golgi system. The recognition of the vacuoles as part of the Golgi

system was facilitated by the absence of the normal Golgi apparatus and the lack of swelling of the sarcoplasmic reticulum in X537A-treated cultured muscles. After prolonged exposure to X537A, vacuolation involved nearly the entire cultured cell, but electron microscopy still did not show swelling of the sarcoplasmic reticulum. The extensive vacuolation found after chronic exposure to the antibiotic was limited to a membrane-bounded system and may indicate either a very extensively dispersed (1) Golgi system in developing muscle and/or the new development and overgrowth of Golgi membranes after X537A treatment.

The mechanism of the X537A-induced swelling in the Golgi apparatus cannot be related directly to the antibiotic's functioning as a calcium ionophore across the surface membrane. Incubation of adult vascular smooth muscle in calcium-free, EGTA-containing solutions (or of cultured vascular smooth muscle without added calcium) did not block the swelling of the Golgi system produced by X537A,⁴ and such swelling has never been observed in vascular smooth muscles that have been studied extensively under conditions of calcium influx produced by depolarization with high K solutions (22).

A direct effect of the antibiotic on the Golgi system is supported, although not proven, by the presumptive intracellular localization of X537A through fluorescence microscopy. The predominant localization of the fluorescence ascribed to the ionophore in lipid droplets, rather than in the swollen Golgi membranes, can be readily accounted for by the quantitative aspects of a large lipid pool absorbing this relatively hydrophobic molecule. Indeed, the absence of lipid autofluorescence or X537A fluorescence in the lumen of the Golgi vacuoles suggests that the contents of these vacuoles are relatively aqueous. The aqueous nature of the contents of the normal Golgi apparatus had been inferred from its behavior during ultracentrifugation (2) and from its response to freezing and drying (8, 38).

Swelling of an intracellular organelle can be due to either an increased permeability of its membrane to some normally relatively impermeant intracellular component, the blockade of an outwardly directed pumping mechanism, or the metabolic accumulation of osmotically active particles (e.g., sugars, SO_4) with secondary inward movement of water. Concerning the above three possi-

⁴A. V. Somlyo, R. E. Garfield, and A. P. Somlyo. Unpublished observations.

ble mechanisms, the existence of a pump that transports an aqueous solution of electrolytes from the condensing vacuole to the surrounding cytoplasm has been suggested (20, 21)⁵ while interference with synthetic and conjugating functions of the Golgi system (see below and reference 1) could lead to the accumulation of small, osmotically active carbohydrates, sulphate, etc. In view of the low cytoplasmic concentration of free calcium, an increase in the permeability of the Golgi membrane to the cation, through the ionophoric effect of X537A, is not a likely mechanism of swelling; X537A could, as an ionophore of K, induce swelling of the Golgi apparatus if its normal K concentration were below cytoplasmic levels. Metabolic inhibition cannot account for the ultrastructural effects of the antibiotic, because vacuolation of the Golgi system by X537A was observed without any significant change in tissue ATP and ADP levels and, conversely, deliberate metabolic inhibition with iodoacetate and cyanide did not reproduce the ultrastructural effects of X537A on the Golgi system of vascular smooth muscle. The primary target of X537A, due to its lipophilic nature, may be a membrane lipid or a glycolipid.

The slowing of the rate of beating and the disorganization of the sarcomere pattern in myocardial cells need not be directly related to changes in the Golgi system. The negative chronotropic effect, however, is a direct action on the noninnervated heart cell, unlike *some* of the inotropic effects of X537A on the intact heart that are mediated by catecholamines (32, 51). The disorganization of the sarcomere pattern may be merely a mechanical consequence of the very extensive vacuolation produced by chronic exposure to X537A. Alternatively, it may indicate the involvement of the Golgi apparatus in the organization of the Z bands, a possibility of clearly greater interest.

The finding that serially passed cultured vascular smooth muscle cells synthesize glycosaminoglycans is consistent with recent reports (50). A detailed description of the profiles of the mucopolysaccharides synthesized by cultured vascular smooth muscle cells will be published elsewhere.⁶ The incorporation of [³H]glucosamine and of

⁵More recent studies by Palade and coworkers (45) suggest that the reduction of osmotic activity within the condensing vacuole occurs through complex formation between cationic secretory proteins and sulfated poly-anions.

⁶S. Chacko and J. Gartrell. Manuscript in preparation.

[³⁵S]sulfate into mucopolysaccharides was significantly inhibited in cultures of vascular smooth muscle exposed to X537A. In view of the role of the Golgi apparatus in mucopolysaccharide synthesis (3, 30, 46), it seems reasonable to relate the biochemical changes to the ultrastructurally detectable damage produced by X537A in this organelle.

Although the present study has dealt with those effects of X537A that lead to ultrastructural damage and inhibition of biochemical activity, lower concentrations of the agent may have different, even stimulatory effects on some Golgi functions. Our findings do suggest that X537A may be a useful tool in establishing the functions of the Golgi apparatus, and that further studies of the chronic effects of this antibiotic on glycoprotein behavior and on the formation of deposits in certain storage disorders (12) may be of interest.

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