

FORMATION OF GAP JUNCTIONS BY TREATMENT IN VITRO WITH POTASSIUM CONDUCTANCE BLOCKERS

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ABSTRACT

Gap junctions were regularly seen in thin sections of canine tracheal smooth muscle incubated in vitro. Their number was increased in tissues exposed in vitro to either of two potassium conductance blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP), and at the same time the muscles became mechanically active, with spontaneous contractions. The presence of gap junctions in this smooth muscle may provide one basis for cell-to-cell coupling, and their increase after TEA- and 4-AP-treatment could account for a decreased junctional resistance between cells, contributing to a longer space constant. However, an increase in gap junctions was not sufficient to change the behavior of trachealis smooth muscle from multiunit to single-unit type. Gap junctions in increased numbers persisted after washout of 4-AP, which caused inhibition of spontaneous contractions, and despite inhibition of the contractile effects of 4-AP by atropine. The rapid induction of gap junction formation was not dependent on *de novo* synthesis of protein. The fact that the number of gap junctions can be increased by chemical agents has important implications for control of their formation and provides a tool for analysis of their role in cell-to-cell coupling.

KEY WORDS smooth muscle · gap junctions · potassium conductance blockers

Nexuses have been implicated as sites at which electrotonic spread of current between a variety of excitable cells can occur (9). Between many smooth muscle cells and other cells, nexuses or gap junctions separate the two apposing plasma membranes by a gap of about 2–4 nm (16). Canine tracheal smooth muscle has been described as a multiunit smooth muscle (3), and gap junctions have not been satisfactorily demonstrated to occur in this tissue. Earlier studies showed that in vitro it lacked spontaneous electrical and mechanical activities and did not respond actively to stretch (13, 20). However, on treatment with tetraethylammonium (TEA) ion in vitro, the muscle exhibited spontaneous rhythmic

contractions and oscillations of membrane potential (13, 20). There was also a response to stretch. TEA also depolarized the cells and abolished the marked rectifying property of the smooth muscle cells apparently by virtue of its ability to block potassium conductance (g_K). The increased space constant of the muscle after TEA treatment was attributed to an increase in transmembrane resistance (R_m) after decreased potassium conductance (13). These changes in the electrophysiological properties represent a conversion from multiunit to single-unit type of behavior of this smooth muscle.

The compound 4-aminopyridine (4-AP) had been shown to block selectively potassium channels of the giant axons of cockroach (18) and squid (17, 22, 23) and also to enhance transmitter release at the neuromuscular junction (10) and

sympathetic nerves (12) by inactivating the potassium current. Although the selectivity of action of 4-AP has been established, the site and mode of its action in potassium conductance blockade was shown to differ from those of TEA. The effects of 4-AP on smooth muscle have not been investigated to date. We wanted to test whether 4-AP, presumably by blocking potassium conductance, was, like TEA, capable of bringing about a conversion of canine trachealis muscle from the multi-unit to the single-unit state.

In this quantitative study with electron microscopy, we determined the incidence of gap junctions between smooth muscle cells of the canine trachea and changes in their number in tissues exposed to TEA and 4-AP when single-unit behavior was established. We considered that TEA and 4-AP caused the conversion to unitary activity in this smooth muscle in part by decreasing junctional resistance (R_j) between smooth muscle cells as a result of gap junction formation. We also examined the effects of inhibition of protein synthesis on the TEA- and 4-AP-induced formation of gap junctions. A preliminary account on some of these findings has been presented (11).

MATERIALS AND METHODS

Tracheal smooth muscle was obtained from mongrel dogs by methods previously described (13). The muscle strips 1 cm \times 0.2 cm \times 0.075 cm were mounted vertically in a 20-ml organ bath containing Krebs-Ringer bicarbonate solution through which a mixture of 95% O₂-5% CO₂ was bubbled continuously. An initial 1-g load was applied to all the strips. The Krebs solution had the following composition (in millimolars per liter): NaCl, 121.9; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; KH₂PO₄, 1.2; NaHCO₃, 15.5; and dextrose, 11.5 (pH 7.4). Solutions containing TEA were prepared by replacing equimolar amounts of NaCl with TEA-chloride. 4-AP was dissolved in water, and the pH of this solution was adjusted to 7.4 with 0.1 N HCl to give a final concentration of 0.1 M (stock solution). The Krebs solutions containing the different 4-AP concentrations were prepared by replacing equimolar amounts of NaCl with the stock solution of the drug. Isometric tension was monitored in a Beckman R611 recorder (Beckman Instruments, Inc., Fullerton, Calif.) through an appropriate coupler by attaching one end of the muscle strip to a Grass FT-03 force transducer (Grass Instrument Co., Quincy, Mass.).

Control and TEA- or 4-AP-treated tissues were fixed at appropriate time intervals in 2% glutaraldehyde in 0.075 M cacodylate buffer (pH 7.4) containing 4.5% sucrose and 1 mM CaCl₂ under isometric conditions. After 2 h in fixative, the tissues were rinsed for 1 h in

0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in 0.05 M cacodylate buffer for 90 min. All tissues were stained en bloc with saturated aqueous uranyl acetate for 1 h, dehydrated in graded alcohols, and embedded in Spurr resin. Thin sections, cut on a Porter-Blum MT-2B ultramicrotome (DuPont Instruments—Sorvall, DuPont Co., Wilmington, Del.) with glass knives and mounted on 300-mesh copper grids, were examined in a Philips 301 electron microscope.

Cycloheximide (CHX) Treatment

The tissue strips were incubated in Krebs solution at 37°C containing 0.25 μ mol of [³H]leucine (60 Ci/mmol, sp act) for 1 h. The solution was bubbled with a mixture of 95% O₂-5% CO₂. At the end of the incubation period, an equal volume of Krebs solution containing 5 mM leucine (final leucine concentration, 2.5 mM) was added. The tissues were homogenized by a Polytron tissue homogenizer (Polytron Corp., Elkhart, Ind.) (20-s cycles \times 4) and the homogenate was spun at 4,000 g in a Sorvall (DuPont Instruments—Sorvall, DuPont Co.) centrifuge. The supernate was taken as the starting material. Protein content of the supernate was estimated by the method of Lowry et al. (15).

An equal volume of 10% TCA was added to the supernate and spun in a table-top centrifuge for 20 min. The precipitate was washed twice with TCA and digested with 0.5 ml of 0.4 N KOH at 65°C for 5 min. To this digest, 3 ml of 10% TCA was added and centrifuged for 20 min. The pellet was washed once with 10% TCA and centrifuged. The resulting pellet was washed successively with 1:1 mixture of ethanol:ether, followed by ether, and dissolved in NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). A 0.2-ml aliquot of the NCS-dissolved pellet was plated in a scintillation vial, to which 10 ml of Bray's solution was added, and counted in a Beckman scintillation counter (Beckman Instruments, Inc.).

To study the effect of CHX on [³H]leucine incorporation, the tissue strips were preincubated in Krebs solution at 37°C containing 1 or 5 mM CHX for 30 min and incubated for a further period of 1 h in the presence of [³H]leucine. The tissues were processed for determination of radioactivity in the TCA-insoluble pellet as described above.

Quantitation of Gap Junctions

The numbers of gap junctions in thin sections of smooth muscle cells cut transversely were counted at a magnification of 45,000 in three to five grid squares from each tissue. Cell-to-cell contacts were identified as nexuses or gap junctions if they presented a five-lined or a seven-lined structure with a 2-nm central gap. A series of nonoverlapping photographs of the cells in the scanned grid squares were taken at a magnification of 3,200. The negatives were enlarged three times (9,600, final magnification) and printed on 20.3 \times 25.4 cm

paper. A map measurer (Keuffel & Esser Co., Morristown, N. J.) was used to measure the circumferences of the cells in the photographs.

To ensure that nexuses were not counted from serial sections of tissues, the measurements were made from grid squares occupied by the same section. This section was chosen under the scanning mode of the microscope, with only the criteria that it be very thin and have many cells. The number of such grid squares depended on the section dimensions, and so large sections of tissues were made. The number of gap junctions counted in the scanned grid squares was expressed as number per 1,000 μm length of membrane.

The tissue blocks were assigned different codes. The codes were broken only after the number of gap junctions was determined and the total membrane lengths were measured.

The diameter of the gap junctions counted in the scanned grid squares was measured from high magnification micrographs of cells from control and TEA-treated tissues.

Procedure for Freeze-Fracture

After initial fixation in 2% glutaraldehyde in 0.075 M cacodylate buffer (pH 7.4) overnight, the tissues were rinsed briefly in the buffer and transferred to 30% glycerol in cacodylate buffer for at least 1 h. The tissues were frozen first in liquid Freon-22 cooled by liquid nitrogen, then in liquid nitrogen, and freeze-fractured at -100°C at $\sim 2 \times 10^{-7}$ Torr in a Balzers BA 360M freeze-etch unit (Balzers AG, Balzers Liechtenstein). After Pt-C replication, the specimens were digested in

40% chromic acid overnight and the replicas were washed in bleach for 2 h, rinsed in distilled water three times, and mounted on 200-mesh collodion-coated copper grids to be examined in a Philips-301 electron microscope.

Statistics

The Kruskal-Wallis H test was used for comparing the significance of the mean number of gap junctions in the control, TEA- and 4-AP-treated groups, and the null hypothesis was rejected at $P (H \geq H')$ at 0.05 level. Pairwise comparisons of the data were made using the Mann-Whitney U test.

RESULTS

Gap Junctions in Control Tissues

Control tissues incubated in vitro and fixed for electron microscopy (EM) contained gap junctions. The junctions as seen in thin sections were located on processes connecting two cells. Their mean number was 3.36/1,000 μm membrane length, and the mean diameter, 0.15 μm (Table I). A typical gap junction as seen in thin sections is shown in Fig. 1a and b. The structure is seven-layered with a central gap about 2 nm wide.

Gap Junctions in TEA-Treated Tissues

A significantly greater number of gap junctions was found in thin sections of tissues fixed after

TABLE I
Number and Length of Gap Junctions in Control and 3, 10, and 33 mM TEA-Treated Canine Tracheal Smooth Muscle

	<i>n</i> *	No. GJ†	Distance‡ μm	Junctions/1,000 $\mu\text{m} \pm \text{SD}$	Length $\mu\text{m} \pm \text{SD}$
Control	10	160	49,785	3.36 ± 0.05	$0.15 \pm 0.05\text{¶}$
3 mM TEA**	10	278	54,597	$5.35 \pm 0.20\text{‡‡}$	
10 mM TEA**	10	311	66,251	$5.04 \pm 0.20\text{‡‡}$	
33 mM TEA On-set§§	5	146	28,349	$5.10 \pm 0.16\text{‡‡}$	
1 Hour**	10	248	49,925	$5.69 \pm 0.19\text{‡‡}$	$0.17 \pm 0.09\text{¶}$

* *n* = no. of tissues examined.

† No. of five- or seven-layered gap junctions (GJ) found in three to five grid squares (300 mesh) from each tissue scanned.

‡ Distance = length of membrane (μm) of smooth muscle cells cut in transverse section measured from photographs of scanned grid squares.

|| Mean length (μm) of gap junctions measured from high magnification photographs ($\pm \text{SD}$).

¶ No significant difference in the mean lengths of gap junctions between TEA-treated and control tissues.

** Tissues were exposed to TEA for 1 h and fixed for thin-section EM.

‡‡ Significant differences ($P < 0.05$) in the mean numbers between TEA-treated and control tissues.

§§ Tissues were exposed to TEA and fixed for EM at a time when spontaneous mechanical activity was established (10–15 min).

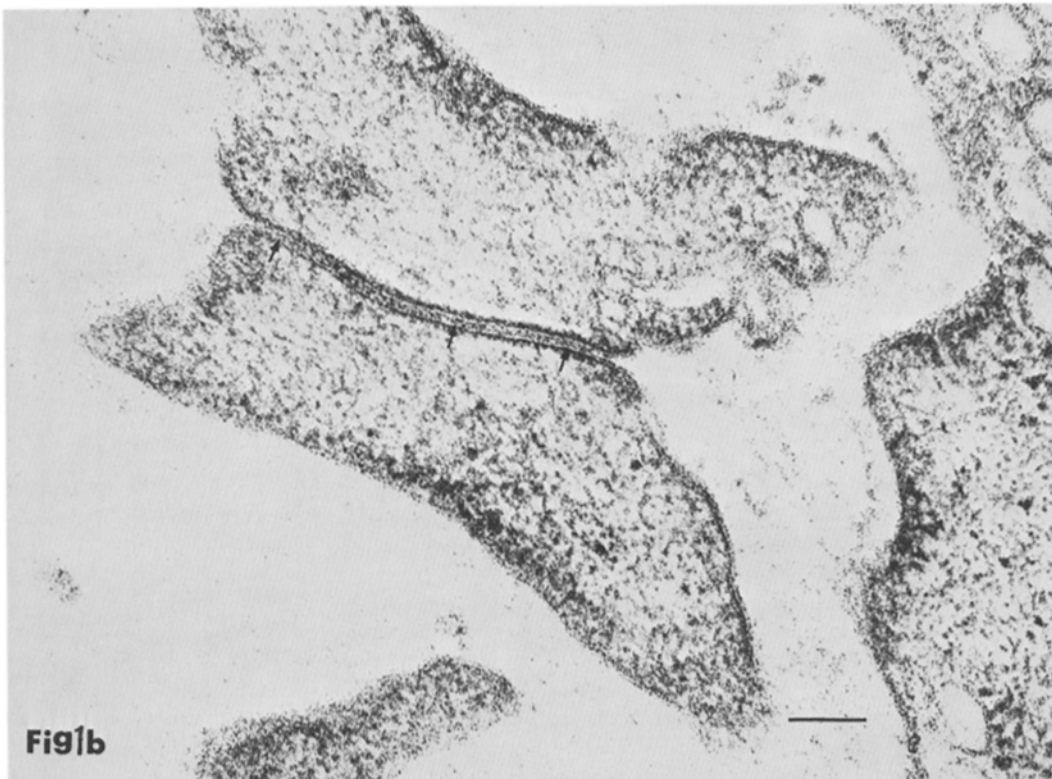
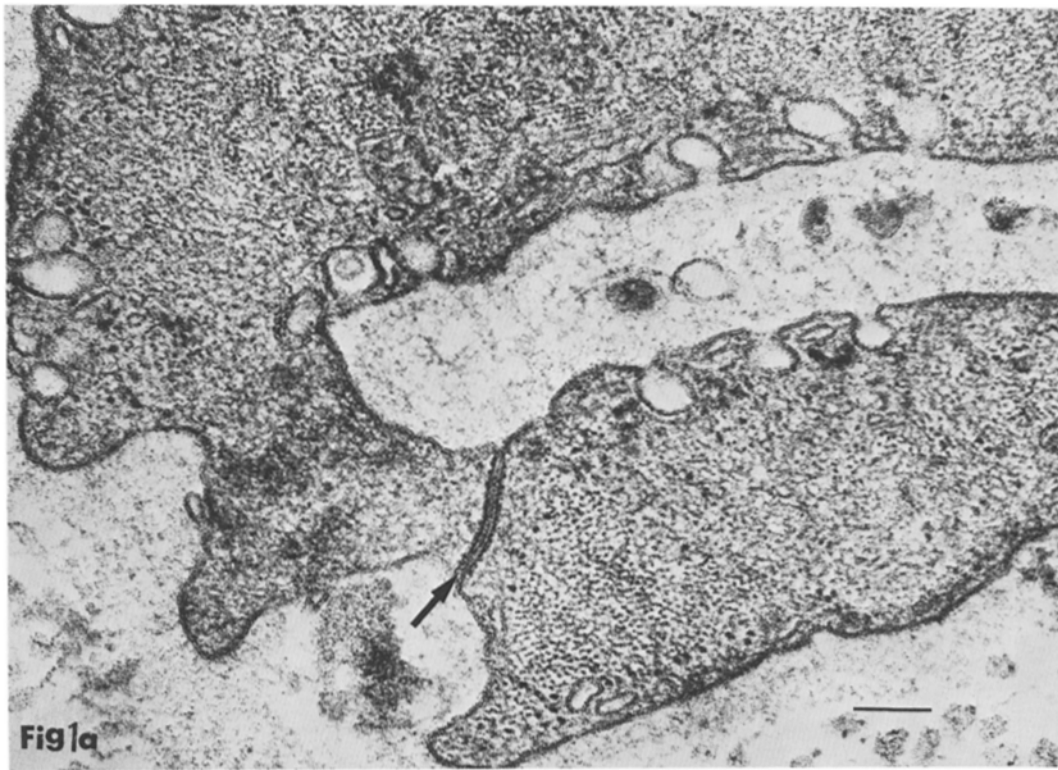


FIGURE 1 Electron micrographs of gap junctions between smooth muscle cells of the canine trachea as seen in thin-sectioned tissue. (a) Low magnification micrograph showing a gap junction (arrow) connecting two cells. Bar, $0.10\ \mu\text{m}$. $\times 100,000$. (b) Higher magnification micrograph showing seven-layered structure of gap junction (small arrows) between processes of two cells. Bar, $0.07\ \mu\text{m}$. $\times 142,000$.

exposure *in vitro* to 3, 10, and 33 mM TEA for 1 h than in control tissues. The TEA-treated tissues, before fixation for EM, showed phasic mechanical activity. Similar increases in the number of gap junctions were noted after 10- to 15-min exposure to 33 mM TEA, at a time when spontaneous mechanical activity was established. The mean number of gap junctions in these tissues ranged from 5.04 to 5.60/1,000 μm membrane length. However, their mean diameter (0.17 μm) was not significantly different from the control value (Table I). As in control tissues, the gap junctions were typically found between cell processes.

Effects of TEA on Isometric Tension in the Canine Trachealis

Our results (16 strips) were in agreement with previous reports (13) which showed that treatment of this smooth muscle *in vitro* with TEA caused spontaneous rhythmic contractions (Fig. 2 A). The maximum isometric tension was related to the concentrations of TEA employed, being higher for larger doses. The increase in the number of gap junctions accompanied the appearance of phasic activity (five strips). However, there was no correlation between the dose of TEA used and the extent of increase in the number of gap junctions. The mechanical responses to TEA were not affected by atropine (10^{-6} M, 12 strips) or tetrodotoxin (TTX) (10^{-7} g/ml, six strips).

Effects of 4-AP on Isometric Tension in the Canine Trachealis

Tension from tissues exposed to 1 or 10 mM 4-AP (10 tissues each) *in vitro* immediately increased, with no clear lag period between addition of the drug and the onset of mechanical response. The tension reached a steady value after 10–15 min and the tissues then contracted rhythmically (Fig. 2 B). The maximum tension attained was related to the dose of 4-AP, the tension being higher for larger doses.

Atropine had no effect on initiation of phasic mechanical activity by TEA (reference 13 and our own experiments), suggesting that the effect is not dependent on the release of acetylcholine (ACh) from the intrinsic nerve endings. However, the phasic activity induced by 4-AP could be either the result of altered release of excitatory transmitter from the nerves or a direct effect on the smooth muscle cell membrane to block potassium conductance, or both.

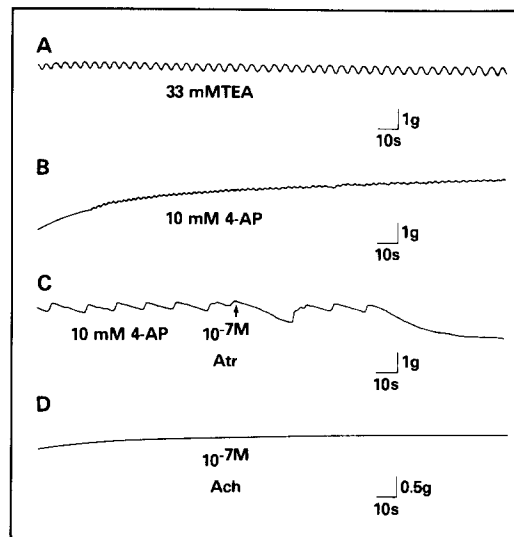


FIGURE 2 Isometric tension responses of canine tracheal smooth muscle strips after treatment with TEA (A), 4-AP (B), 4-AP and atropine (C), and Ach (D). (A) Phasic mechanical activity 15 min after exposure to 33 mM TEA. For this and all other traces, the time marker (10 s) corresponds to position of the tracer with the initial 1-g load placed on the strips. (B) Phasic mechanical activity 2 min after exposure to 10 mM 4-AP. (C) Effect of atropine (Atr) on 4-AP-induced mechanical activity. Atropine was added (at arrow) 15 min after exposing the tissue to 10 mM 4-AP. Note the disappearance of phasic activity and a diminution in tone. (D) Effect of Ach on the mechanical activity of tracheal smooth muscle. The response, 5 min after addition of Ach, consisted of an increase in tone with no phasic activity.

Tissues were exposed to 4-AP (1, 3, and 10 mM), and when phasic activity was established (10–15 min) atropine (10^{-7} to 10^{-6} M) was added to the bath (four strips). The phasic as well as the tonic responses were abolished by atropine (Fig. 2 C). Tissues ($n = 4$) preincubated with the same concentrations of atropine did not show any mechanical activity when later exposed to 4-AP. These findings suggest that the 4-AP-induced mechanical effects require the action of ACh released from the nerve endings. If the effects of 4-AP to produce phasic activity required only the release of ACh from nerves, then application of ACh should result in phasic activity. Tissues ($n = 3$) exposed to Ach (10^{-8} to 10^{-7} g/ml) showed an increase in tone, but no evidence of a phasic component (Fig. 2 D). This rules out such a mechanism. TTX (10^{-7} g/ml) did not have any

effect on 4-AP-induced phasic mechanical activity in six strips.

Gap Junctions in 4-AP-Treated Tissues

The effect of treatment in vitro for 1 h with 1 and 10 mM 4-AP on the number of gap junctions in canine trachealis was studied. The mean number of gap junctions in control tissues was 3.82/1.000 μm membrane length. Tissues treated for 1 h with 1 and 10 mM 4-AP showed a significantly higher number of these contacts (8.11 and 10.8 per 1.000 μm length of membrane for 1 and 10 mM 4-AP-treated tissues, respectively) than control tissues. This increase was evident after only 10- to 15-min exposure to the drug (Table II). Thus, the ability to induce increased formation of gap junctions is shared by both TEA and 4-AP.

Gap Junctions in Tissues in the Absence of Mechanical Activity

After exposure to 3 mM 4-AP for 1 h (when phasic mechanical activity was established), tissues from three animals were washed with Krebs solution until the tension reached the baseline. These tissues were kept in Krebs solution for an additional 1-h period and fixed and examined for the number of gap junctions. The number of gap junctions in these tissues remained higher than in untreated control tissues (10.8 \pm 1.25/1.000 μm membrane length).

Tissues from four animals were treated with 10^{-7} M atropine for 15 min and later exposed to either 1 or 10 mM 4-AP for 1 h and processed for EM. The number of gap junctions in strips treated with 4-AP either in the absence or in the presence of atropine was significantly higher than the number in untreated control tissues and atropine-

treated tissues (four strips each) (Table III). Treatment of tissues ($n = 3$) with Ach (10^{-7} M) for 1 h did not result in an increase in the number of gap junctions (mean 3.50/1.000 μm membrane length). These results and the persistence of gap junctions after washout of 4-AP rule out a dependence of rapid formation of gap junctions on the action of released Ach as well as on tone or shape changes related to contraction.

Freeze-Fracture Studies

The freeze-fracture replicas (15 each) obtained from (13 each) control and TEA-treated tissues revealed extensive membrane fracture faces with the caveolae arranged in linear arrays (Fig. 3). In both tissues, the density of membrane particles was greater in regions containing many caveolae than in other regions. The gap junctions, seen as aggregations of membrane particles, were almost exclusively found on cell processes (Fig. 4) as also

TABLE II
Number of Gap Junctions in Control and 1 and 10 mM 4-AP-Treated Canine Tracheal Smooth Muscle

	<i>n</i>	No. GJ	Distance μm	GJs/1.000 $\mu\text{m} \pm$ SD
Control	7	110	28,944	3.82 \pm 0.12
1 mM 4-AP				
Onset	3	136	17,643	7.71 \pm 0.25*
1 Hour	7	210	25,911	8.11 \pm 0.33*
10 mM 4-AP				
Onset	3	114	11,801	9.72 \pm 0.30*
1 Hour	6	315	29,032	10.80 \pm 0.45*

GJs = gap junctions.

* Values are significantly higher ($P < 0.01$) than values in control tissues.

TABLE III
Effect of Atropine on the Number of Gap Junctions in 4-AP-Treated Canine Tracheal Smooth Muscle

Exp no.	Control	Control + Atr.	1 mM 4-AP	1 mM 4-AP + Atr.	10 mM 4-AP	10 mM 4-AP + Atr.
<i>no. of gap junctions/1.000 μm membrane length</i>						
1	4.1	3.9	6.9	4.6	23.0	7.5
2	6.4	5.2	10.9	8.5	7.5	6.2
3	3.8	2.9	10.9	7.6	19.3	3.8
4	3.0	1.2	10.2	11.5	12.9	6.0
X	4.32	3.30	8.35*	8.08*	15.68*	5.88*

Atr. = atropine.

Consult the text for the experimental design.

* Values are significantly higher ($P < 0.05$) than values in control and control + atropine-treated tissues.



FIGURE 3 Freeze-fracture replica of canine tracheal smooth muscle showing a large area of membrane E face (*EF*). The caveolae (*C*) are arranged in a linear array. No nexuses are seen in this replica or in others examined. Membrane particles are shown by small arrows. Shadow angle is noted by the large arrow in both micrographs. Bar, $0.125 \mu\text{m} \times 80,000$.

FIGURE 4 Freeze-fracture replica of a gap junction (small arrow) on a cell process from tracheal smooth muscle seen as aggregation of membrane particles on the P face (*PF*). Bar, $0.125 \mu\text{m} \times 80,000$.

demonstrated by thin-section EM. They occurred rarely in freeze-fracture replicas, we believe, because of the difficulty in obtaining fracture faces of these processes. Thus, when gap junctions are present on elongate cell processes, the freeze-fracture technique may not provide quantitative information.

CHX Experiments

The extent of [³H]leucine incorporation into TCA-insoluble fraction of canine trachealis strips in the absence and presence of CHX (1 and 5 mM) is given in Table IV. In studies dealing with the effect of inhibition of *de novo* synthesis of protein on TEA- and 4-AP-induced formation of gap junctions, a concentration of 5 mM CHX was used, which resulted in about 95% inhibition of [³H]leucine incorporation. Isometric tension was also recorded in these experiments.

Tissues treated with 20 mM TEA or 10 mM 4-AP for 1 h showed a significantly higher number of gap junctions (8.1 for TEA- and 13.8 for 4-AP-treated tissues/1,000 μm length of membrane) than control or control + CHX-treated tissues (3.7 for control and 2.9 for control + CHX-treated tissues/1,000 μm membrane length (Table V). Phasic mechanical activity was present in tissues exposed to 20 mM TEA or 10 mM 4-AP for 1 h. Tissues preincubated with 5 mM CHX in Krebs solution for 30 min and later exposed to 20 mM TEA or 10 mM 4-AP in the presence of the same concentration of CHX for 1 h also showed phasic mechanical activity. The number of gap junctions in these tissues was significantly higher (6.1 for TEA and 8.9 for 4-AP/1,000 μm membrane length) than in control tissues exposed to CHX alone. These values were slightly less than the values in tissues exposed to the potassium

conductance blockers, but not significantly so (Table V).

DISCUSSION

Multiunit smooth muscles, in general, were initially supposed to lack gap junctions and to be excited by nerves (13). However, Kroeger and Stephens (13) have reported a space constant of 1.6 mm in control tissues, which is many times the length of a smooth muscle cell (4). Our findings, contrary to some reports in the literature, show that gap junctions are regularly present in tracheal smooth muscle (accounting for 0.05% of the total membrane area of cells¹) and may provide one basis for cell-to-cell coupling. The reported (13) increase in space constant in TEA-treated tissues could be a consequence of either an increase in the transmembrane resistance or a decrease in the junctional resistance, or both. Since we observed an increase in the junctional membrane area (to 0.09%) upon treatment with TEA and 4-AP, the latter possibility cannot be ignored.

4-AP brought about single-unit behavior in the canine tracheal smooth muscle as shown by its ability to induce spontaneous phasic activity. There are, however, no electrophysiological data to establish that the underlying mechanism is similar to that seen after TEA treatment. Although induction of spontaneous contractions would suggest such a mechanism, we have provided evidence to indicate that the mode of action of 4-AP differs from that of TEA, in this smooth muscle, in its dependence on the action of Ach.

Retention of gap junctions in tissues where the tone and rhythmic contractions brought about by prior treatment with 4-AP are abolished by returning to normal Krebs solution suggests that the formation of new junction is not an artifact of tissue contraction. The facts that tonic contractions induced by Ach did not elicit gap junction formation and that inhibition of contractile responses to 4-AP by atropine did not abolish the increase in their number also eliminate this possibility. Indeed, the major effect of contraction would be to decrease the probability that gap junctions are being observed. Contraction alters the structure of smooth muscle by markedly increasing the number and length of projecting

¹ The junctional membrane area was calculated by multiplying the mean measured diameter of gap junctions by their mean number per 100 μm length of membrane.

TABLE IV

Effects of CHX on [³H]Leucine Incorporation into the TCA-Insoluble Fraction of Canine Trachealis*

	Control	1 mM CHX	5 mM CHX
	<i>cpm/mg protein</i>		
	12,452	6,000	700
	14,113	8,200	1,150
	20,809	10,100	1,250
	17,231	7,500	550
	6,500	2,570	145
% of Control		47.5 ± 6.9	5.0 ± 2.3

* Consult text for experimental design.

TABLE V
Number of Gap Junctions in CHX-, 4-AP-, and TEA-Treated Canine Tracheal Smooth Muscle

Exp no.	Control	Control + CHX	4-AP	4-AP + CHX	TEA	TEA + CHX
1	3.8	2.8	19.3	11.2	7.2	5.1
2	3.0	2.2	8.3	6.6	6.8	3.7
3	4.3	3.6	—	—	10.4	9.5
\bar{X}	3.7	2.9	13.8*	8.9*	8.1*	6.1*

* Values are significantly higher ($P < 0.05$) than the values in control and control + CHX-treated tissues.

“arms” (7). Since gap junctions tend to occur on these arms which are not oriented in a constant relationship to the long axis of the cells and since a gap junction will not be detectable in thin sections unless the plane of section passes through it nearly at right angles to the plane of the junction, the increase in the number of arms on contraction will diminish the chances that sections at right angles to the long axis of the cell will intersect them appropriately. Thus, the values we obtained for increases in gap junctions during contraction may be underestimates.

The fact that increased numbers of gap junctions can be present without inducing single-unit behavior (after 4-AP and atropine or after wash-out of 4-AP) suggests that their formation may be necessary but not sufficient for single-unit behavior. Also, the difference in effects of TEA and 4-AP in the mechanical response of muscle in the presence of atropine, despite their similarities in forming additional gap junctions, suggests that the formation of gap junctions in muscle is related to a common property of these drugs other than release of Ach, possibly, alteration in g_K ; in contrast, the mechanical response may require some additional action, e.g., increased Ca^{2+} influx. If g_K reduction is related to changes in intracellular Ca^{++} content or binding (2), these may be involved in gap junction formation or in limiting the numbers of gap junctions.

In smooth muscle, the evidence that gap junctions provide the low resistance pathways of current flow between cells is only circumstantial. There have been no direct measurements of the resistance of these junctions, although indirect estimates of the specific resistance of the nexus at the intercalated disk of canine myocardium, a structure resembling the gap junction of smooth muscle, are consistent with their presumptive role as low resistance contacts (19). Direct measurement of junctional resistance by means of intracellular current injection techniques is impossible because current spreads in three dimensions

through interconnections between the smooth muscle cells (21). The passive membrane properties have instead been studied by extracellular polarization and intracellular measurement of electrotonic potentials (21). By this technique, cells in many smooth muscles have been shown to be electrically coupled. However, electrical coupling has been shown in some smooth muscles where gap junctions cannot be demonstrated by either thin-section EM or freeze-fracture techniques (5). Recently, in rat myometrium studied *in situ*, it has been shown that large gap junctions can form rapidly under physiological control (8), but the tissues before gap junction appearance still showed evidence of good electrical coupling as studied *in vitro* (14). Moreover, in recent investigations in our lab (E. Zelcer and E. E. Daniel, unpublished observations), the expected increase in the space constant of the delivering rat uterine smooth muscle could not be demonstrated to be attendant on gap junction appearance. Similar results have been obtained by Kuriyama and Suzuki (14). However, these results must be interpreted cautiously since, in our hands, not all tissues before the appearance of gap junctions showed exponential decay of electrotonic potentials with distance from the source of stimulation. Clearly, the application of modified cable equations to smooth muscle may require reconsideration along with the belief that the space constant determined by the method of Abe and Tomita (1) can reflect changes in the numbers of low resistance contacts between cells.

The increase in the number of gap junctions brought about by TEA and 4-AP could result either from *de novo* synthesis of membrane proteins involved in their assembly or from aggregation of preexisting membrane proteins by accretion into structures recognized as gap junctions. Assembly of small gap junctions (seen with freeze-fracture methods [6] but too small to be evident in thin sections) into larger ones is an unlikely mechanism, since our thin-section data show no

increase in the size of observable gap junctions on TEA treatment. Also, there was no evidence for the occurrence of small gap junctions in the replicas examined.

Our results with CHX show that the TEA- and 4-AP-induced formation of gap junctions is not prevented even in the near absence of protein synthesis, suggesting that some proteins utilized to synthesize extra gap junctions were preformed. The slight decrease in the number of gap junctions by CHX in both control and treated groups could result from a limited quantity of such proteins or from an effect of CHX on the preformed gap junctions.

Thus, the TEA- and 4-AP-induced changes in the biophysical properties of tracheal smooth muscle could result not only from their effect in blocking potassium conductance but also from their ability to decrease junctional resistance due to formation of gap junctions. The ability of these agents to diminish rectification by smooth muscle membranes to depolarizing currents may also play a role. The abilities of relatively simple chemicals (TEA and 4-AP) to increase the number of gap junctions rapidly and reproducibly suggests their use as tools to provide more direct evidence about the role of gap junctions in cell-to-cell coupling in smooth muscle and possibly in other tissues. In this connection, it should be noted that preliminary studies in our laboratory (M. S. Kannan, R. E. Garfield, and E. E. Daniel) show that gap junctions cannot be induced by these agents in tissues apparently without them (e.g., rat myometrium before term) but can increase them in other smooth muscles which possess them (e.g., rat myometrium at term). Thus, there may be programming of genes controlling protein synthesis in some smooth muscle which prevent formation of the necessary connexin protein, while in others the quantity or rate of assembly of these proteins is limited by membrane processes related to K conductance.

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