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Physiology

Extracellular matrix regulates smooth muscle responses to substance P

(basement membrane/tachykinin receptors)

CHAUNCEY W. BOWERS AND LISA M. DAHM

Division of Neurosciences, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 91010

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ABSTRACT **Little is known about the extracellular factors that determine a cell's responsiveness to neurotransmitters. This is a particularly important issue for pharmacologically diverse cell types such as neurons and smooth muscle. This report demonstrates that the contractile responses of amniotic smooth muscle to a specific neuropeptide, substance P, is controlled by a molecule(s) intimately associated with the extracellular basement membrane. This molecule(s) normally represses the expression of substance P responsiveness in this tissue. When the amniotic smooth muscle is separated from the basement membrane by dissociation, normally unresponsive cells exhibit a progressive increase in responsiveness to substance P, beginning within the first 24 hr in culture. The induction of substance P responses was completely inhibited when the cells were plated onto isolated amniotic basement membrane rather than onto polyornithine or collagen I. Similar changes in the responsiveness to another agonist, histamine, did not occur. The data demonstrate that extracellular matrix exerts a major instructive influence in determining the responsiveness of avian amniotic smooth muscle to specific ligands. We suggest that similar regulatory mechanisms may operate in other tissues.**

Smooth muscle is a major target of the autonomic nervous system and is capable of expressing a variety of functional receptors *in vivo*. That there is local environmental control over the pharmacological properties of smooth muscle is suggested by the systematic variation of responses between regions of the same vascular bed (1), contiguous regions of gut (2), and contiguous regions of airway smooth muscle (3). At present, the nature of the extracellular signals that control receptor expression in smooth muscle is completely unknown. We have begun an analysis of these extracellular signals by utilizing a smooth muscle-containing system, the avian amnion.

The avian amnion is an anatomically simple tissue, consisting of only two cell types: a simple squamous epithelium and one or two layers of smooth muscle (ref. 4; see also Fig. 4). The muscle is neither innervated nor vascularized (4–6). Despite its lack of innervation, the amnion normally expresses at least 11 different types of functional receptors for 8 different neurotransmitter substances (7). On the other hand, some responses commonly observed in other smooth muscle preparations are not present in amnion; for example, there is no contractile response to substance P (7). This lack of substance P response is part of the unique pharmacological phenotype of amniotic smooth muscle, since most nonvascular smooth muscle, including avian intestine, contracts in response to low concentrations of substance P (7–10).

Because the amnion is thin and avascular, it can be cultured as an intact tissue without dissociation. Results of previous work established that the contractile responses of intact amnion to a variety of ligands develop normally in a defined culture medium (7). Responses normally absent *in ovo*, such as a response to substance P, remained absent in these defined cultures. Because the bulk of its pharmacological properties developed normally and were stable in a defined medium, it was hypothesized that the control of amniotic pharmacology resided primarily within the tissue itself. The studies presented here more rigorously demonstrate that indigenous factors control the pharmacological properties of smooth muscle in the avian amnion. We demonstrate that the major determinant of substance P responsiveness in amniotic smooth muscle resides in the extracellular basement membrane.

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MATERIALS AND METHODS

Culture Media. Dissociated smooth muscle cells from the avian amnion were cultured in a modified N2 medium originally designed for neurons (11). The basal medium was Dulbecco's modified Eagle's medium (DMEM) mixed 1:1 with Ham's nutrient mixture F-12 (F-12) with the following additives (final concentrations): insulin (bovine pancreas; 5 μg/ml), conalbumin (40 μg/ml), sodium selenite (30 nM), progesterone (20 nM), putrescine (100 μM), and ovalbumin (1 mg/ml). The final medium is referred to as N2.

Substrates. Circular glass coverslips (diameter, 13 mm) were coated with either poly(DL-ornithine)hydrobromide (M_r, 3000–15,000) or collagen I (rat tail). For polyornithine, 0.25 mg/ml in 25 mM borate buffer (pH 8.5) was incubated on glass coverslips overnight at 4°C and washed three times with distilled water the next day. For collagen coating, 0.3 mg/ml in 0.17 M acetic acid was air-dried onto glass coverslips and stored in a humidified chamber until use. Both polyornithine and collagen-coated coverslips were sterilized by exposure to ultraviolet light for 20 min.

Cell Dissociation and Culture. Amnions from stage 28–30 embryos (≈6 days incubation) were used for dissociation. Embryos were staged according to the methods of Hamburger and Hamilton (12). For each dissociation, the anterior third of the amnion was removed from three embryos and minced to 1-mm^2 pieces and incubated for 2 hr in 1.5 ml of porcine elastase (1 mg/ml) in DMEM/F-12 with 0.1% ovalbumin. The reaction was stopped by centrifugation of the tissue and replacement of the enzyme solution with fresh medium. The tissue was resuspended in 0.5 ml of nonenzymatic dissociation solution in calcium- and magnesium-free Hanks' solution (Sigma). The minced tissue was gently triturated and passed through 70-μm nylon mesh into 1 ml of N2 medium. The epithelial cells were retained on the nylon mesh and separated from the dissociated smooth muscle because these dissociation conditions did not disrupt the tight junctioned sheets of epithelia. Cells were plated at a density of 8×10^3 cells per cm^2 on either polyornithine, collagen I, or isolated basement membrane (see below) and incubated at 37°C in humidified 5% CO_2/95% air.

Fields were counted using a ×20 phase objective, and exactly the same fields were counted for each time point. Generally, five adjacent fields were counted for each culture well.

BrdUrd Assay. To label cells that were synthesizing DNA, cultures were exposed to 10 μM BrdUrd for 24–48 hr (13). Cells were fixed with 70% ethanol at 4°C, and nuclear DNA was denatured by exposure to 2 M HCl for 20 min. The BrdUrd incorporated into the chromosomes was visualized in the nucleus by using a monoclonal antibody to BrdUrd in combination with a Vectastain avidin-biotin kit following the manufacturer's instructions (Vector Laboratories). The percentage of cells labeled in a culture well was determined from a sample of at least 300 cells. In any given experiment for a given condition, the variation between wells of the percentage of cells labeled was <5%.

Isolation of Amniotic Basement Membrane. To isolate amniotic basement membrane, the anterior third of a stage 28–30 amnion was removed from the eggs and pinned onto Sylgard 184-coated dishes in physiological saline solution (see below for salt concentrations). The smooth muscle cells were lysed with distilled water, and the epithelium was removed as a sheet with forceps. The basement membrane was isolated further by a variation of the method of Meezan *et al.* (14); the remaining muscle nuclei and cellular debris were removed by treating the basement membrane with DNase (500 units/ml) in 1 M NaCl for 1 hr followed by 4% deoxycholate for 1 hr. The basement membrane, still pinned to Sylgard, was rinsed free of detergent for the next 48 hr. To culture dissociated smooth muscle cells on the basement membrane, basement membrane was pressed onto a polyornithine-coated coverslip, epithelial side down, with a cleaned glass coverslip.

Electron Microscopy. Freshly dissected amnions or isolated basement membranes were pinned onto a Sylgard-coated dish and fixed with 1.3% glutaraldehyde and 0.15% ruthenium red in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. Tissues were postfixed in osmium, dehydrated, and embedded in Epon by standard procedures.

Assay of Contractile Responses to Neurotransmitters. Responses to different neurotransmitter-related substances were monitored visually with a ×40 Hoffman objective. Transmitters were applied via pressure ejection from pipettes with a hole in the tip (3–6 μm). The cells were rated solely by whether they contracted in response to the indicated concentrations of drugs. The appearance of these contracting smooth muscle cells was consistent with previous descriptions of other dissociated smooth muscle (e.g., see ref. 15). Visual determination of whether a cell contracted in response to drug application was unambiguous (Fig. 1). Cell cultures were superfused with a physiological saline solution at 1.5 ml/min in a 1.5-ml chamber and kept at 33°C throughout the assay period. The physiological saline contained 127 mM NaCl, 4 mM KCl, 1.6 mM $CaCl_2$, 0.9 mM $MgCl_2$, 10 mM glucose, 5 mM Hepes buffer (pH 7.2), 0.05% bovine serum albumin, penicillin/streptomycin (30 units/ml), and phenol red (0.75 mg/ml). In addition, the perfusing solution contained 3 μM cimetidine to prevent H2 histaminergic responses from inhibiting the H1 contraction (7). Cultured cells were categorized as being either single (not in obvious contact with another cell), paired (physically touching one other cell, usually extensively), or grouped (in an area where several cells touch; groups included 3–10 cells). Although not emphasized in the present report, it was clear that cells in pairs or in groups could act independently in response to a transmitter. For instance, it was not unusual for only 1 of a pair of cells, or for only 2 of a group of 5, to respond to histamine.

Statistics. Statistical significance was analyzed as the difference between two proportions using the equation: $\sigma = [rq(1/n_1 + 1/n_2)]^{1/2}$, where σ is the standard deviation of the difference between the two proportions r_1 and r_2 (the difference between the two proportions is assumed to be zero by the null hypothesis; ref. 16), r is the total proportion of cells responding under the combined conditions, and $q = 1 - r$. The estimate of r, the proportion of the total population responding, was obtained as the average of the two measured proportions $(r_1 \cdot n_1 + r_2 \cdot n_2)/(n_1 + n_2)$, where n_1 and n_2 equal the number of cells assayed under conditions 1 and 2, respectively. From the above equation, $z = (r_1 - r_2)/\sigma$, where z is the number of standard deviations of the actual data from the hypothesized mean (zero). Statistical significance was derived from a table of values for the distribution function for the standard normal distribution.

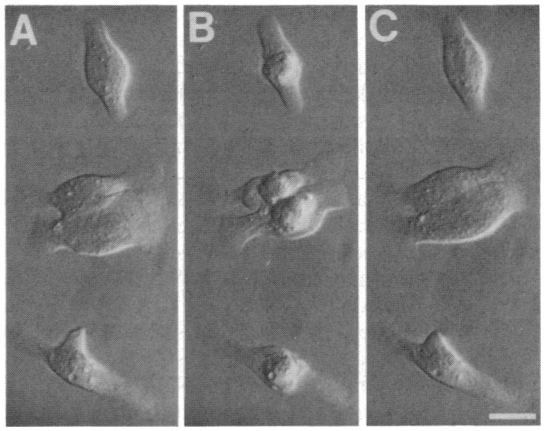

FIG. 1. Dissociated amniotic smooth muscle cells grown in defined medium for 1 week on polyornithine visualized with Hoffman optics. Micrographs show cells before (*A*), 2 sec after (*B*), and 45 sec after (*C*) a 1-sec application of 100 μM carbachol. (Bar = 20 μm.)

Materials. All culture media and additives were purchased from Sigma. Also from Sigma were the polyornithine, collagen, bovine serum albumin, elastase, BrdUrd, carbachol, atropine sulfate, histamine, chlorpheniramine maleate, cimetidine, and ruthenium red. The DNase and Hepes buffer were purchased from Boehringer Mannheim. $MgCl_2$, NaCl, and KCl were from Mallinckrodt; $CaCl_2$ was from Fisher Scientific; glucose was from Aldrich. Nylon mesh filters were obtained from Medical Industries, Los Angeles, and substance P, neurokinin A, and senktide were purchased from Peninsula Laboratories. The monoclonal antibody against BrdUrd was purchased from DAKO (Carpinteria, CA) and fertile eggs from White Leghorn chickens were purchased from Chino Valley Ranchers (Chino, CA).

RESULTS

Effects of Dissociation on Amniotic Pharmacology. The dissociated smooth muscle of the avian amnion attached, grew, and maintained contractility on both polyornithine and collagen type I in defined N2 medium (Fig. 1). The cultures were almost purely smooth muscle cells as evidenced by the fact that, throughout a week in culture, >96% of the cells contracted vigorously in response to 100 μM carbachol.

To assess whether removal of the smooth muscle cells from their extracellular microenvironment would produce changes in their pharmacology, the percentages of cells responding to three substances—carbachol, histamine, and substance P—were monitored after dissociation and plating (see *Materials and Methods*). The initial assay, 12 hr after plating the dissociated muscle, indicated <10% of the cells were responsive to substance P. This was consistent with the previously established lack of responsiveness of the intact tissue (7). However, unlike cultured intact amnion, an increased percentage of dissociated muscle cells began contracting in

response to substance P within 24 hr in culture (Fig. 2). About 90% of the cells responded to substance P after 4 days in culture. Over this same time course, essentially all cells continued to respond to carbachol, while decreasing numbers of cells responded to histamine (Fig. 2). Similar results were obtained when the cells were grown on collagen type I (data not shown).

As with the intact amnion (7), the responses of the dissociated cells to carbachol were completely blocked by 100 nM atropine, and the responses to histamine were blocked by the H1 antagonist chlorpheniramine (100 nM) (this concentration of chlorpheniramine did not block the carbachol responses; data not shown). The newly induced substance P response was maximally activated at 100 nM substance P (Fig. 3). Neurokinin A (substance K), a potent NK-2 receptor agonist, was >2 orders of magnitude less potent than substance P in causing contraction (Fig. 3). In addition, the cells were completely unresponsive to 1 μM senktide, an NK-3-specific agonist (data not shown). As has been reported for other substance P-responsive systems, including intestinal smooth muscle, vascular endothelium, and peripheral neurons (10, 17–19), tachyphylaxis of the substance P responses was observed routinely in the dissociated amniotic cells—i.e., 3 min or more was required for cells to become responsive to a second application of substance P. These observations are consistent with an NK-1-type receptor mediating contraction in dissociated amniotic smooth muscle (see *Discussion*).

The increase in responsiveness to substance P does not require proliferation of the smooth muscle cells. Thus, the total number of cells in culture did not increase significantly over the first 48 hr in culture (4% ± 4%; n = 4 wells), a period during which the percentage of substance P-responsive cells increased from <10% to >60% (Fig. 2). An increase in cell number was noted after 4 days in culture (35% ± 10%). Further evidence that significant proliferation did not occur during the first 48 hr is indicated by assay of the percentage of cells synthesizing DNA by using BrdUrd (see *Materials and Methods*). After 24 and 48 hr of exposure to BrdUrd, 25% and 62%, respectively, of the cultured cells were labeled. BrdUrd did not block the increased substance P responsiveness of the dissociated cells (70% responsive after 48 hr). If the dissociated cells were exposed to BrdUrd for the first 24 hr and then cultured 24 hr in the absence of BrdUrd, only the original 25% of the cells were labeled at the end of the 48-hr period, indicating that proliferation of the cells labeled in the first 24 hr did not occur during the subsequent 24 hr [similar delays in proliferation and slow proliferation rates have been reported for other smooth muscle (e.g., see ref. 20)]. These data demonstrate that cells are not proliferating in the first 48 hr and, therefore, the responsiveness of individual cells is

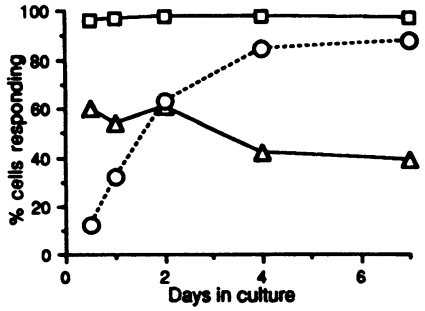

FIG. 2. Percentage of dissociated amniotic smooth muscle cells responding to 100 μM carbachol (□), 100 μM histamine (△), and 100 nM substance P (○) during 1 week in culture on polyornithine. At least 100 cells from the same dissociation are assayed for each drug at each point. Substance P responses on days 0.5, 1, and 2 are each different from all other substance P responses ($P < 0.005$). Histamine response after 7 days is significantly lower than at 1 day ($P < 0.02$).

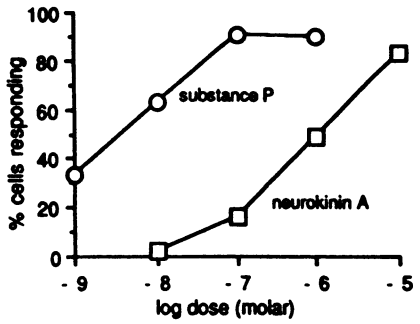

FIG. 3. Dose–response relationships for percentages of cells responding to substance P (○) and neurokinin A (□). Each point represents assay of at least 100 cells that have been cultured for 6 days, all from the same dissociation.

changing. This is in contrast to a mechanism involving a mitogenic stimulation of a subpopulation of cells that is initially sensitive to substance P (i.e., a clonal selection-type mechanism). In addition, analysis of cells occurring in groups, pairs, or isolated indicated no tendency for cells in pairs or groups to be more responsive to substance P than isolated cells. For example, after 4 days in culture, 85%, 80%, and 88% of single, paired, and grouped cells, respectively, contracted in response to substance P (n = 100 for each group).

Effects of Isolated Amniotic Basement Membrane on Smooth Muscle Pharmacology. Because previous data demonstrated that culturing the intact amnion in N2 medium did not result in increased responsiveness to substance P (7), the data on dissociated cells in the present report suggest that removal of the smooth muscle cells from their natural microenvironment induced the expression of substance P responsiveness. This implies that a natural component of the smooth muscle's environment normally inhibits the responsiveness of this tissue to substance P. Electron microscopy of the amnion confirmed that the tissue is as anatomically simple as previously described (Fig. 4*A*). A basement membrane was evident at the electron microscope level, containing fibrillar collagen facing the muscle layer and a relatively undeveloped basal lamina next to the epithelia (Fig. 4*B*). The thickness of the collagen-containing region was variable, but in no case did the smooth muscle directly contact the epithelium. Because the amnion is composed of only two cell types (smooth muscle and epithelia) and because the smooth muscle appears never to make direct physical contact with the epithelium, the relevant microenvironment for the smooth muscle *in vitro* must be either a soluble factor from the epithelium or an interaction with the basement membrane.

Basement membranes containing fibrillar collagen were isolated free of obvious cellular debris at the electron microscope level (Fig. 4*C*). These extracellular matrices retained their anatomical integrity and orientation. Isolated amniotic basement membranes were attached to coverslips with their smooth muscle side facing upward. Smooth muscle cells, dissociated as described above, were cultured onto the basement membranes to determine whether the extracellular matrix would provide a sufficient stimulus to suppress the induction of substance P responsiveness. These basement membrane-containing cultures were compared to sister cultures grown on polyornithine. Amniotic basement membranes completely prevented the induction of substance P responsiveness in cells grown directly on them (Fig. 5). The responsiveness of the dissociated cells to carbachol and histamine was not significantly affected by the basement membranes. Direct contact with the basement membrane was apparently required for the suppression of substance P responsiveness, because cells not in direct contact with the

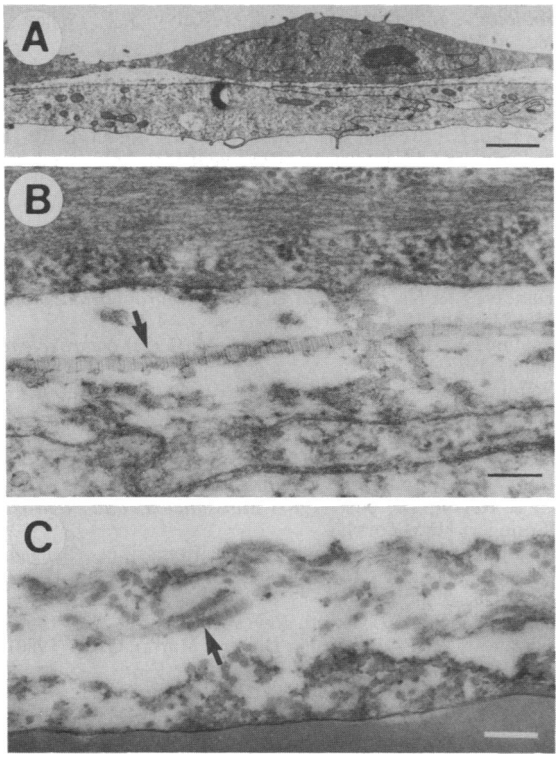

FIG. 4. (*A*) Electron micrograph of intact amnion (stage 29). Tissue contains only two cell types with one layer of each, smooth muscle (above) and epithelium (below). (Bar = 2 μm.) (*B*) Higher-power micrograph of the same amnion as in *A* (same orientation), emphasizing the basement membrane between the smooth muscle and epithelium. Arrow indicates fibrillar collagen within the basement membrane. (Bar = 200 nm.) (*C*) Electron micrograph of basement membrane isolated from avian amnion. Note the lack of cellular debris and the presence of fibrillar collagen (arrow). (Bar = 400 nm.)

basement membrane, but growing in the same well and on the same coverslip to which the basement membrane was attached, showed normal induction of substance P responsiveness (75% responsive after 4 days in culture; $n = 70$ cells).

DISCUSSION

The data presented in this report demonstrate that an extracellular structure, the basement membrane, is a primary factor determining whether smooth muscle from the avian amnion will contract in response to the neuropeptide substance P. Separation of amniotic smooth muscle from its basement membrane induces responsiveness to substance P, while reunion with the membrane prevents induction. This effect is not due to a general increase in the responsiveness to all agonists, as the response to histamine gradually decreased during the same time period. A similar decrease in histamine responsiveness was observed when intact amnions were cultured in the same defined medium (7), indicating that the histamine response may decrease due to removal from fluids in the egg rather than to dissociation from basement membrane.

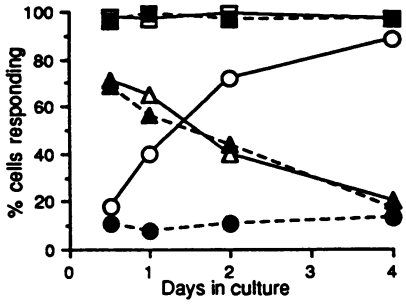

FIG. 5. Effects of amniotic basement membrane on smooth muscle responsiveness to 100 μM carbachol (□, ■), 100 μM histamine (△, ▲), and 100 nM substance P (○, ●). Percentages of cells responding on polyornithine (open symbols) or amniotic basement membrane (solid symbols) are shown over 4 days in culture. Substance P responses on basement membrane are significantly different from those on polyornithine from 1 day of culture onward ($P < 0.005$). Substance P responses on basement membrane do not significantly vary with time in culture ($P > 0.10$).

While the chemical identity of the component of basement membrane relevant to substance P responsiveness remains to be determined, it is noteworthy that the component remains associated with the basement membrane even after treatment with 4% deoxycholate, a routine step in isolation of the membrane. Furthermore, preliminary data indicate that plating amniotic muscle on purified laminin, fibronectin, or collagen IV does not mimic the effect of basement membrane (unpublished observations).

The separation of smooth muscle from the basement membrane appears to directly alter the pharmacology of the cells. The only obvious alternative, that a small subpopulation of substance P-sensitive cells is induced to rapidly divide when separated from the matrix, was not supported by the data. Over a culture period during which the percentage of responsive cells increased 6-fold, significant cell division did not occur.

The measure of responsiveness used in these studies, the percentage of cells responding acutely to an agonist, does not lend itself to direct comparison with more classical equilibrium analyses of pharmacology—e.g., affinities, which have established at least three types of tachykinin receptors, NK-1, -2, and -3. Each of these receptor types is capable of responding to substance P, although at different concentrations (21, 22). The facts that substance P is substantially more potent than neurokinin A and that the response desensitized with repeated administration suggest that the receptor involved in these responses is most similar to the NK-1 receptor (21, 22). That senktide did not induce contraction further supports the notion that the NK-3 receptor is not involved. Clearly, more complete analysis is required to rigorously determine the type(s) of tachykinin receptor functioning in these cells. The mammalian substance P receptor has been cloned and is thought to act physiologically via guanine nucleotide binding protein coupling. Stimulation of the substance P receptor is correlated with increased phosphatidylinositol turnover in several systems (see ref. 23 for a recent review of tachykinin receptors). Whether the basement membrane-dependent induction of substance P responsiveness is due to increased receptor expression or to changes in the cascade of events distal to the receptor is presently unknown.

It is generally accepted that molecules associated with extracellular matrix can influence a variety of cellular processes that are important in development and regeneration. Profound effects of matrix molecules on cell attachment and differentiation *in vitro* have been documented (24–29), while cell migration *in vivo* has been successfully disrupted by techniques that disrupt cell–matrix interactions *in vitro* (for reviews, see ref. 26 and 29–32). Furthermore, the important role of extracellular matrix in regeneration of damaged skeletal muscle, including the ability of motor neurons to return to previously innervated sites, has been elegantly demonstrated *in vivo* (33–35). Although the types of nicotinic receptors expressed by striated muscle appear to be determined primarily by nerve activity (36–38), the location of these receptors in the plasma membrane appears to be determined by components of the basal lamina (39, 40). An appropriately active protein, agrin, purified from cholinergic

synapse-rich basal lamina by McMahan and coworkers (41, 42), appears to be the molecule involved in receptor localization at the neuromuscular junction. As has been recently observed, however, there are probably also roles for matrix-associated factors in regulation of nicotinic receptor types at mature neuromuscular junctions (43, 44). The present report extends these previous observations to smooth muscle and suggests that the extracellular matrix controls the responsiveness of smooth muscle to neurotransmitters. It is possible that such factors determine the local variation in smooth muscle pharmacology already noted (1–3). Furthermore, in analogy with the skeletal system, factors in the extracellular matrix may also enable regenerating smooth muscle to reestablish its appropriate pharmacological responsiveness after injury.

The present report demonstrates that the control of substance P responsiveness in the smooth muscle of the avian amnion is determined by a molecule(s) tightly associated with the basement membrane. It is of interest that the effect of the basement membrane is inhibitory, the removal of which allows the smooth muscle to respond to yet another neurotransmitter. Given that the amnion is neither innervated nor vascularized *in vivo*, and yet normally expresses at least 11 different types of neurotransmitter receptors (7), it may be that tissues containing smooth muscle cells constitutively express a wide variety of receptor types unless specifically instructed otherwise by their environment. The nature of the relevant molecule(s) in the amniotic basement membrane, and the generality of these findings to other pharmacologically versatile cell types (e.g., neurons as well as other smooth muscle), is important since the expression of appropriate functional receptors is a ubiquitous requirement in neural and endocrine systems.

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