Negative inotropic activity of antilaminin IgG: participation of cholinergic mechanisms

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- 1 Antilaminin IgG decreased the dF/dt of mouse isolated atria and inhibited the mechanical effect of acetylcholine in a non-competitive fashion.
- 2 Inhibitors of nicotinic and muscarinic cholinceptors impaired the negative inotropic action of antilaminin IgG in mouse isolated atria. Hemicholinium and tetrodotoxin also reduced the response while the antihistamine, pyrilamine was without effect.
- 3 These results suggest that antilaminin IgG modulates cholinergic function in mouse isolated atria. Possible mechanisms are discussed.

Introduction

It is known that laminin is a non-collagenous glycoprotein present in all basement membranes, with multiple biological activities that have been related to the different components of the molecule. By rotary shadow electronmicroscopy, laminin revealed a characteristic cross-shape structure composed of three short arms and a long arm held together by disulphide bonds. Globular structures are present in the ends of the long and the short arms (Timpl *et al.*, 1983).

Laminin mediates the attachment of cells to basement membranes and probably links the components of basement membranes (type IV collagen, heparin-sulphate proteoglycans, intactib, nidogen) to one another to form a supramolecular structure. Heparin binding is located at the globular end of the long chain whereas type IV collagen attaches to the globular ends of the short chains. Moreover, the binding to the cells has been ascribed to the region where the short chains interact (Yamada, 1983).

It has been demonstrated, *in vitro*, that laminin plays an important role in cell adhesion, migration, growth and differentiation (Kleinman *et al.*, 1985).

These biological activities include a role of laminin in the phenomenon of metastasis (Lissitzky *et al.*, 1986). Another property of this glycoprotein is the promotion of neurite outgrowth activity (Baron Von Evercooren *et al.*, 1982); this has been related to the long chain of the molecule (Engvall *et al.*, 1986).

It is thought that the receptor on laminin to which cells bind is located in the region where the short chains interact (Horan-Hand *et al.*, 1985; Lesot *et al.*, 1983). Direct evidence for the role of the laminin receptor in the binding of cells to laminin was provided by a monoclonal antibody which inhibited almost completely the binding of radiolabelled laminin MCF-7 breast carcinoma (Rogers *et al.*, 1983).

Recently Mollenhauer & Von der Mark (1986) demonstrated that the laminin receptor is associated with or related to a 5'-nucleotidase (AMPase). This enzyme is present in many cells such as smooth muscle, liver cells, glial cells and tumour cells (Rogers *et al.*, 1983). It catalyzes the cleavage of AMP and GMP into nucleotides and phosphate on the cell surface. The physiological role of this enzyme is still unknown and it is possible that this enzyme initiates developmental changes by activating adenylate and guanylate cyclase systems.

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On the other hand, previous studies (McMahan *et al.*, 1978; Vigny *et al.*, 1983) have indicated that the asymmetrical form of acetylcholinesterase is localized in the basement membrane zone of the neuromuscular junction.

It is important to note that each skeletal muscle fibre is ensheathed by a basement membrane. This structure plays an important role in neuromuscular function and reinnervation. In fact, acetylcholine receptors present in regenerating myotubes following injury, cluster at synaptic sites on the basement membrane. Laminin is present in synaptic as well as extrasynaptic regions of the muscle fibre basement membrane while other components of the extracellular matrix are confined to only one of these areas (Sanes & Cheney, 1982).

The aim of the experiments described here was to obtain evidence for pharmacological and molecular actions of the antilaminin IgG on presynaptic and postsynaptic acetylcholine receptors in mouse heart. We conclude that antilaminin IgG is able to exert a pharmacological effect acting on nicotinic and muscarinic receptors in the isolated heart of the mouse, behaving as a cholinergic agonist and decreasing myocardial contractility.

Methods

Preparation of antilaminin IgG

Laminin from an Engelbreth Holm Swarm (EHS) murine sarcoma (Bethesda Res. Lab., Maryland, U.S.A.) was used as antigen. Rabbits of about 2.5 kg weight were injected with an emulsion prepared of equal parts of laminin (1 mg ml^{-1}) and Freund's complete adjuvant. Injections of 0.5 mg were repeated 3 times more at intervals of 15 days. Rabbits were bled after the last injection. IgG was isolated from the rabbit antiserum by precipitation with ammonium sulphate and passage through a DEAE-cellulose column. IgG from a normal rabbit serum was also obtained in order to use it in control experiments.

Titers of antilaminin antibodies from the total rabbit serum and from the isolated IgG were detected by the enzyme-linked immunosorbent assay (ELISA) mainly following the method described by Avila *et al.* (1984). Wells of a microtiter plate were coated with $200 \mu\text{l}$ of laminin ($1 \mu\text{g ml}^{-1}$). Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma Chemical Co, U.S.A.) 1:1000 was used. Enzyme activity was measured at 405 nm in a Dynatech photometer. Titer of antibodies was determined from the serum dilution that reveals a

ten fold difference in the absorbance values between the sera obtained from the rabbits before and after immunization.

By ELISA, a titer of 6.4×10^5 was detected either in the total rabbit antiserum or in the isolated IgG. Antilaminin antibodies were not detected in normal rabbit serum. The molecular weights of the normal and antilaminin IgG were 150,000.

Mouse isolated atria preparation

Male BALB/c mice (20–40 g) were decapitated and the entire heart was quickly removed. Atria were separated from ventricles, carefully dissected and placed in a glass chamber containing Krebs-Ringer-Bicarbonate (KRB) solution gassed with 5% CO_2 in O_2 and maintained at pH 7.4 and 30°C . The ionic composition of KRB was described by Borda *et al.* (1984a). After 1 h for equilibration, initial control values for inotropy of the isolated atria were recorded with a force transducer coupled to an ink writing oscillograph (Borda *et al.*, 1986). The preparations were paced with a bipolar electrode and Grass Stimulator. Stimuli were of 2 ms duration and 10% above threshold voltage. A preload tension of 350 mg was applied to the atria.

Inotropic effects (dF/dt) were assessed by recording the maximum rate of tension development during electrical stimulation at a fixed frequency of 300 beats min^{-1} , that is close to the resting heart rate of mice and consequently inotropy was tested within the physiological range of the force-frequency relationship.

Control values (=100%) refer to the dF/dt before the addition of antibodies and drugs. The mean absolute value for dF/dt at the end of the equilibrium was $4.1 \pm 0.3 \text{ gs}^{-1}$. After a period of equilibration, each preparation was exposed to graded concentrations of antilaminin IgG and acetylcholine. Cumulative dose-response curves were obtained by the method of Van Rossum (1963). Single doses were administered in volumes of 0.01 to 0.025 ml of KRB solution. The total volume added to the bath never exceeded 0.1 ml. The volume of the organ bath was 15 ml. Atria were exposed for 15 min to each concentration of the IgG to obtain the maximal effect.

Drugs

Acetylcholine chloride, atropine sulphate, hexamethonium bromide, hemicholinium-3, pyrilamine maleate and tetrodotoxin (Sigma Chemical Company, U.S.A.) were used. Stock solutions of all the drugs were dissolved in distilled water. The drugs were diluted in the bath to achieve the final concen-

trations shown in Table 3. At these concentrations the drugs had no effect *per se* before the addition of the antibodies and acetylcholine. Mice isolated atria were incubated for 30 min with cholinceptor antagonists before adding increasing amounts of antilaminin IgG, normal IgG and acetylcholine.

Statistics

Experimental results were compared by means of Student's *t* test. Differences between means were considered significant if $P < 0.05$.

Results

Antilaminin IgG induced a concentration-dependent decrease in atrial dF/dt whereas normal IgG was without effect (Figure 1). The inhibitory effect of antilaminin IgG developed gradually reaching a plateau at approximately 12–18 min and for this reason atria were exposed for a period of 15 min to each antilaminin or normal IgG concentration.

Figure 1 Effect of antilaminin or normal IgG on dF/dt of mouse isolated atria. Increasing amounts of antilaminin IgG (●) or normal IgG (○) were added to the bath leaving each concentration 15 min to produce its maximal effect. Values are means with s.e.mean (vertical bars) from 12 experiments.

Figure 2 Comparative effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon dF/dt of mouse isolated atria in the presence (○) or absence (●) of 3×10^{-7} M atropine. Values are means with s.e.mean (vertical bars) from 8 experiments.

Cholinoceptor participation in the effect of antilaminin IgG

The degree of inhibition induced by exogenous acetylcholine was directly proportional to its concentrations. Atropine antagonized in a competitive manner the action of acetylcholine shifting the dose-response curve for the cholinceptor agonists to the right (Figure 2b). The negative inotropic effect of antilaminin IgG was antagonized by atropine but in a non-competitive manner (Figure 2a).

The nicotinic antagonist, hexamethonium, failed to modify the dose-response curve for exogenous acetylcholine (Figure 3b) but the inhibitory action of high concentrations of antilaminin IgG was attenuated by the presence of hexamethonium (Figure 3a).

Figure 3 Effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon dF/dt of mouse isolated atria. Increasing amounts of antilaminin IgG or exogenous acetylcholine were added in the presence (○) or absence (●) of 1×10^{-6} M hexamethonium. Values are means with s.e.mean (vertical bars) from 6 experiments.

Figure 4 Inhibitory effect of atropine (○) (a) or hexamethonium (□) (b) on the antilaminin IgG action (●). The values were obtained by fitting data of Figures 2 and 3 to Lineweaver-Burk plots. A typical non-competitive inhibition is shown.

Lineweaver-Burks plots of the data presented in Figures 2a and 3a confirm the non-competitive nature of the inhibitory effects of atropine or hexamethonium on the negative inotropic action of antilaminin IgG (Figure 4).

Figure 5a shows a significant reduction of the inhibitory action of antilaminin IgG in the presence of the acetylcholine synthesis inhibitor hemicholinium. Hemicholinium did not modify the dose-response curve to exogenous acetylcholine (Figure 5b). Tetrodotoxin (5×10^{-7} M) impaired the negative inotropic action of antilaminin IgG but did not modify that of exogenous acetylcholine (Figure 5).

Antilaminin IgG (10^{-9} M), but not normal IgG, decreased the maximum effect of acetylcholine,

Figure 5 Effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon dF/dt of mouse isolated atria. Increasing amounts of antilaminin IgG (●) ($n = 10$) or exogenous acetylcholine (○) ($n = 10$) were added in the presence of 2×10^{-5} M hemicholinium (○) ($n = 5$) or 5×10^{-7} M tetrodotoxin (□) ($n = 5$). Values are means with s.e.mean (vertical bar); n = number of preparations.

Table 1 Inhibitory effect of antilaminin IgG on the negative inotropic action of exogenous acetylcholine (ACh): comparison with normal IgG

Additions	$E_{\max}$ ($g\ s^{-1}$)	K_d (10^{-8} M)	n
ACh	$2.26 \pm 0.18^*$	19.6 ± 2.51	7
ACh + antilaminin IgG	$3.21 \pm 0.22^*$	17.8 ± 1.9	8
ACh + normal IgG	2.30 ± 0.20	18.2 ± 2.3	6

Atria were exposed to different concentrations of acetylcholine (ACh) in the absence or presence of 1×10^{-9} M antilaminin or normal IgG. $E_{\max}$: maximum effect of ACh. K_d : effective concentration of ACh causing 50% of the maximum response. Basal value: $4.21 \pm 0.28\ g\ s^{-1}$. Values are means $\pm$ s.e.mean. n : represents the number of experiments. $^*P < 0.01$ between ACh vs ACh + antilaminin IgG.

increasing the $E_{\max}$ without affecting the K_d of the dose-response curve for acetylcholine (Table 1). Neither antilaminin IgG action nor exogenous acetylcholine action were modified by the histamine receptor blocking agent pyrilamine (Table 2).

None of the pharmacological interventions used in this study modified the magnitude of the resting dF/dt of mouse isolated atria nor altered the action of normal IgG (Table 3).

Discussion

A negative inotropic action of antilaminin IgG on isolated atria of the mouse is described which

Table 2 Action of pyrilamine on the negative inotropic effect of antilaminin IgG and acetylcholine (ACh)

Additions	$E_{\max}$ ($g\ s^{-1}$)	K_d (10^{-8} M)	n
Anti-L IgG	2.73 ± 0.16	3.5 ± 0.2	5
Anti-L IgG + Pyrilamine	2.69 ± 0.14	3.7 ± 0.3	5
ACh	2.28 ± 0.20	18.3 ± 1.9	5
ACh + Pyrilamine	2.26 ± 0.18	19.4 ± 2.0	5

Atria were exposed to different concentrations of antilaminin IgG (Anti-L) or acetylcholine (ACh) in the absence or presence of pyrilamine (1×10^{-6} M). Values are means $\pm$ s.e.mean; n : number of experiments.

Table 3 Influence of the antagonist agents on the effect of normal IgG upon dF/dt of mouse isolated atria

Incubation (30 min) of atria with:		dF/dt (gs^{-1})*		n
		without normal IgG	with normal IgG	
KRB alone		4.5 ± 0.5	4.1 ± 0.4	12
KRB + atropine	$(3 \times 10^{-7} M)$	4.7 ± 0.6	3.8 ± 0.6	5
KRB + hexamethonium	$(1 \times 10^{-6} M)$	4.4 ± 0.4	3.7 ± 0.5	6
KRB + hemicholinium	$(2 \times 10^{-5} M)$	4.2 ± 0.7	3.3 ± 0.6	5
KRB + pyrilamine	$(1 \times 10^{-6} M)$	4.5 ± 0.4	3.7 ± 0.6	5
KRG + tetrodotoxin	$(5 \times 10^{-7} M)$	4.6 ± 0.8	3.2 ± 0.2	5

* Mean $\pm$ s.e.mean. Normal IgG added to the organ bath at a final concentration of $1 \times 10^{-7} M$. n: number of preparations.

KRB: Krebs Ringer bicarbonate solution.

appears to be mediated via cholinergic mechanisms. Thus the antilaminin IgG action was impaired by the nicotinic antagonist hexamethonium, the muscarinic antagonist atropine and the acetylcholine synthesis inhibitor hemicholinium, but not by the antihistamine pyrilamine. Hexamethonium impairs the negative inotropic action of antilaminin IgG but not that of exogenous acetylcholine, implicating nicotinic receptors in the action of antilaminin IgG. Nicotinic receptors on the postganglionic vagal fibres may be activated directly by antilaminin IgG or indirectly by the antibody acting on the preganglionic nerves to induce a release of endogenous acetylcholine, as hemicholinium opposes the action. However, the action of hemicholinium may be exerted at the postganglionic nerve endings. Antilaminin IgG induces firing of propagated action potentials in the postganglionic neurones as tetrodotoxin impairs its activity, as does atropine by blocking the action of the neuronally-released acetylcholine on the postsynaptic muscarinic receptors. However, antilaminin IgG decreases the maximal negative inotropic effect of acetylcholine with the implication that the antibody interacts also with the muscarinic receptor or its transduction mechanism.

Immunohistochemical studies showed (Sanes & Cheney, 1982) that acetylcholinesterase is present at synaptic sites of muscle fibre basal lamina, where it constitutes the main non-collagenous glycoprotein. It is still a matter of discussion if the collagen subunit of this enzyme interacts with laminin or other components of basement membranes. Vigny *et al.* (1983) as well as Brandan & Inestrosa (1987) demonstrated that the asymmetrical form of acetylcholinesterase binds to heparin sulphate proteoglycan and to a lesser extent to laminin and fibronectin contained in the basal lamina present at the neuromuscular junction. However, Grassi *et al.* (1983) were not able to find any binding of the enzyme to basement membrane components.

The antilaminin IgG is able to mimic the biological effects of a cholinergic agonist such as acetylcholine. However, there are some important differences between the action of acetylcholine and that of the antibody: (1) atropine inhibited in a non-competitive manner the inotropic action of antilaminin IgG while the same drug inhibited in a competitive way the action of acetylcholine and (2) hexamethonium and hemicholinium attenuated the action of the antibody while they were unable to modify the effect of exogenous acetylcholine. In addition none of the pharmacological agents used was able alone to block completely the action of antilaminin IgG.

We suggest that the antibody directly or indirectly stimulates nicotinic cholinergic receptors that are located on the parasympathetic postganglionic nerves. Activation of these receptors excites the postganglionic neurone and causes the release of acetylcholine which then stimulates the postsynaptic muscarinic receptors, culminating in a negative inotropic response. Post-junctional muscarinic receptor activation by antilaminin IgG is also possible.

We have already described other antibodies with specificity for cardiac membranes antigen that can also trigger the pharmacological effect in isolated myocardium. In fact, we observed that alloimmune IgG against histocompatibility antigen is able to induce a positive inotropic effect on mouse isolated atria (Sterin-Borda *et al.*, 1984). The IgG fraction from sera of Chagas' disease patients also exerted a positive inotropic effect (Borda *et al.*, 1984b). Both type of antibodies (alloimmune and chagasic) bind to β -adrenoceptors of myocardium. The sera of patients with Chagas' disease have been reported to contain antibodies to laminin (Szarfman *et al.*, 1982; Avila *et al.*, 1984). These antibodies recognize a carbohydrate epitope present on mouse laminin, which was assigned to a terminal galactosyl (α 1-3)-galactose group (Towbin *et al.*, 1987).

The mechanism whereby antilaminin IgG modulates cholinergic function is uncertain. Whether components of the extracellular matrix are related to the anchorage of asymmetric acetylcholinesterase to the synaptic basal lamina or to the anchorage of acetylcholine receptors to the plasma membrane is still unknown. Laminin is present in practically all tissues. It may be postulated that the binding of a

ligand to laminin antigen may induce directly or indirectly the activation of cholinergic receptors. Further studies will be required before any mechanism can be proposed.

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