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SIALIC ACID REMOVAL MODULES THE MYOCARDIAL AND VASCULAR ACTIVITY OF CALCIUM CHANNEL LIGANDS*

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Abstract—Selective removal of sialic acid from isolated guinea pig left atrial strips and rabbit thoracic aortic ring segments was performed by neuraminidase prepared from *Clostridium perfringens* and was controlled electron microscopically. Preincubation of these organs (2 units/mL; 2 hr) resulted in enzyme mediated hydrolysis of total tissue sialic acid; 55.2% for atria and 60.9% for aorta. Contractile force of atria and arterial diameter of thoracic aorta were measured isometrically and isotonically by means of a force displacement transducer. Pretreatment of both organs with neuraminidase (2 units/mL; 2 hr) in a carbogen saturated organ bath caused a moderate left-hand shift of the cumulative concentration response curves for the dihydropyridine type calcium antagonist nisoldipine, the phenylalkylamine derivative gallopamil and the benzothiazepine diltiazem. EC_{50} values were significantly lower ($P < 0.05$), particularly in the atrial muscle, when compared to untreated preparations. There was no effect of neuraminidase on the negative inotropic and vasodilator potency of the calcium channel modulator fendiline. Conversely, neuraminidase induced a right-hand shift in the concentration response curves shown by the pure calcium agonist (−)-*S*-Bay K 8644 leading to significantly higher EC_{50} values in both organs. Similarly, the contractile potency of calcium chloride (atria) and potassium chloride (aorta) was attenuated upon neuraminidase treatment. From the results obtained it is concluded that sialic acid removal may modulate the action of calcium channel ligands through an inhibitory effect on transmembrane calcium fluxes and/or by decreasing the external calcium availability. Whether the present results suggest a functional role for sialic acid in the regulation of calcium channels warrants further investigation.

Sialic acid, a family of *N*- and *O*-acyl derivatives of neuraminic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid), is widely distributed in mammals. It usually occurs as a terminal component at the non-reducing end of carbohydrate chains of glycoproteins and glycolipids and is thought to be involved in cell recognition, receptor functions and immunological reactions [2]. Some of these sialylated glycoproteins are called acute phase reactants indicating the onset of an inflammatory reaction [3]. More recently, serum sialic acid concentration has even been discussed as a strong predictor of cardiovascular mortality and may, therefore, reflect the existence of an atherosclerotic process [4].

A few earlier studies have investigated the involvement of sialic acid in the development of membrane surface charge. For example, according to Seaman and Cook [5] sialic acid sensitive to neuraminidase (an enzyme that specifically hydrolyses the glycosidic linkage of sialic acid in glycoproteins and glycolipids [6]), accounts for 70–80% of the total surface negativity in a variety of cell types. Subsequently, a specific binding affinity of sialic acid for ionized calcium under physiological conditions was demonstrated [7]. Moreover, basic control of resting membrane calcium permeability appears to reside at least in part at sites within the glycocalyx containing a large amount of sialic acid [8]. Yee *et al.* [9] reported that neuraminidase-mediated removal of sialic acid led to a marked increment of intracellular calcium in cardiac myocytes. Neuraminic acid did not constitute the surface charge of delayed rectifier potassium channels [10]. Carbohydrates are found in the subunits of the purified calcium antagonist-sensitive skeletal muscle calcium channel to different degrees. L-type calcium channels contain sialic acid residues on both the α_2 and γ-subunit [11]. The presence of sialic acid on the α_1-subunit, which constitutes the channel pore and contains the specific binding domains for different calcium antagonists [12], is yet to be established.

We set out to study whether sialic acid modulated the action of calcium channel ligands in cardiac and vascular smooth muscle, especially since both T- and L-type calcium currents had been altered by sialic acid removal in cardiac myocytes [13]. Moreover, the functional implications associated with sialic acid in the vasculature are unknown and studies in this

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field on the cardiac muscle have been carried out at the single cell level.

MATERIALS AND METHODS

Materials. Salt-fractionated, dialysed, and lyophilized neuraminidase (*N*-acetyl-neuraminate glycohydrolase; EC 3.2.1.18; Type V; prepared from *Clostridium perfringens*) and *N*-acetyl-neuramine lactose were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The Sephadex G_{25} gel column was from Pharmacia (Uppsala, Sweden). Substances and solutions used in the sodium dodecyl sulphate–polyacrylamide gel electrophoresis were obtained from Biometra (Göttingen, F.R.G.). Bovine serum albumin (M_r approx. 66 kDa) was obtained from Sigma. The calcium channel ligands nisoldipine and (−)-*S*-Bay K 8644 were a generous gift from Bayer AG (Leverkusen, F.R.G.). Gallopamil was generously supplied by Knoll AG (Ludwigshafen, F.R.G.). Diltiazem and fendiline (Sigma), the neuropeptide substance P (Fluka, Neu-Ulm, F.R.G.) and N^G-nitro-L-arginine (Serva, Heidelberg, F.R.G.) were obtained as indicated. All other chemicals were purchased from Merck (Darmstadt, F.R.G.) and were of analytical grade.

Neuraminidase activity assay. Since the Sigma specification (1.1 units/mg solid) is based on an assay performed in K-acetate buffered solution (pH 5.0; 30°), it was important to determine the neuraminidase activity under conditions prevailing in the organ bath (pH 7.4; 30 and 37°). Briefly, 0.2 mL of a neuraminidase solution (0.05 mg/mL; the linear relationship between enzyme concentration and sialic acid release rate in this concentration range was confirmed previously) in 0.2% bovine serum albumin was combined with 0.3 mL *N*-acetyl-neuramine lactose (0.67 mg/mL) in carbogen (95% O_2, 5% CO_2)-saturated Tyrode buffer (composition in mM: 136.8 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 23.8 $NaHCO_3$, 0.4 NaH_2PO_4, 10.1 glucose; 30°) as well as in Krebs–Henseleit buffer (composition in mM: 118.1 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 5.1 glucose; 37°). Reaction was stopped after 10 min in boiling water. Release of *N*-acetyl-neuraminic acid (NANA*) was determined according to the thiobarbituric acid procedure of Warren [14] as a percentage of total NANA hydrolysis obtained following 0.1 N H_2SO_4 at 80° for 60 min. Neuraminidase activity is given by the equation:

$$\text{units/mg solid} = \frac{\text{NANA release } (\mu\text{M})}{\text{Incubation time (min)} \times \text{enzyme (mg)}}$$

and refers to a final incubation volume of 0.5 mL. The unit definition is: one unit will liberate 1.0 μM NANA per min at pH 5.0 at 37°. Similar determinations were, therefore, reproduced in *K*-acetate buffered solution (pH 5.0; 37°). Each photometric measurement (546 nm) within the Warren procedure was performed in duplicate.

* Abbreviation: NANA, *N*-acetyl-neuraminic acid.

Sialic acid removal from isolated organs. Male guinea pigs weighing 200–300 g were killed by a blow on the neck. The hearts were quickly removed and strips (6 × 3 mm) were prepared from the left atria. Thoracic aortic vessels were obtained from adult male White New Zealand rabbits (1600–2000 g in weight) showing a considerably higher sensitivitiy to the calcium antagonists/agonist studied than guinea pig vessels. The arteries were excised, dissected free of loose connective tissue and cut into ring segments of 3–4 mm in length. Both organ preparations were allowed to incubate with increasing concentrations of neuraminidase (0.05–4.0 units/mL) for increasing incubation periods (10–240 min) in carbogen-saturated Tyrode (atria; 30°) or Krebs–Henseleit (aorta; 37°)-buffered solution (incubation volume 0.5 mL) in order to adjust optimal conditions for sialic acid removal (defined as a percentage of total tissue sialic acid content following 0.1 N H_2SO_4; see above).

Studies with calcium channel ligands. Isolated guinea pig left atrial strips were fixed vertically between a pair of hook electrodes in an organ bath containing 5 mL of a carbogen-saturated Tyrode solution (pH 7.4; 30° to avoid ectopic pulse generation) of the above described composition. The upper end of each strip was connected to a force displacement transducer (Fleck, Mainz, F.R.G.) by a polyester thread. Isometric tension under a resting force of 0.5 g was displayed on a thermo pen recorder (Hellige Servomed, Freiburg, F.R.G.) after amplification (Fleck). The preparations were electrically stimulated by rectangular pulses of 3 msec duration and an intensity twice the diastolic threshold at a frequency of 2 Hz. The pulses were delivered by an electronic stimulator (Sachs Elektronik, Hugstetten, F.R.G.). After an equilibration period of 60 min during which the atrial strips were washed frequently (contractile force of the strips ranging between 7 and 10 mN), neuraminidase was added in an appropriate concentration, enzyme incubation exactly lasting 120 min. Thereafter, neuraminidase was washed out frequently to avoid further unspecific inhibitory effects of this protein and restitution of resting force was allowed for 1 hr. Subsequently, the action of the calcium antagonists nisoldipine, gallopamil and diltiazem, as well as that of the calcium channel modulator fendiline, was evaluated in cumulative dose–response curves (0.1 nM–100 μM) at 20 min intervals such as to allow steady state of drug action.

The pure calcium agonist (−)-*S*-Bay K 8644 was studied according to a modified experimental procedure shown in Fig. 1. After removal of neuraminidase and equilibration of the atrial strips in Tyrode's medium with an extracellular calcium content of 0.225 mM, calcium concentration was cumulatively increased up to 4.5 mM in the Tyrode solution. Subsequent concentration–response studies with (−)-*S*-Bay K 8644 were performed in the presence of 0.45 mM calcium and were compared to the maximal calcium-induced contraction. Control experiments, in which neuraminidase was omitted from the incubation medium, were conducted in parallel.

Isolated rabbit aortic ring segments were mounted

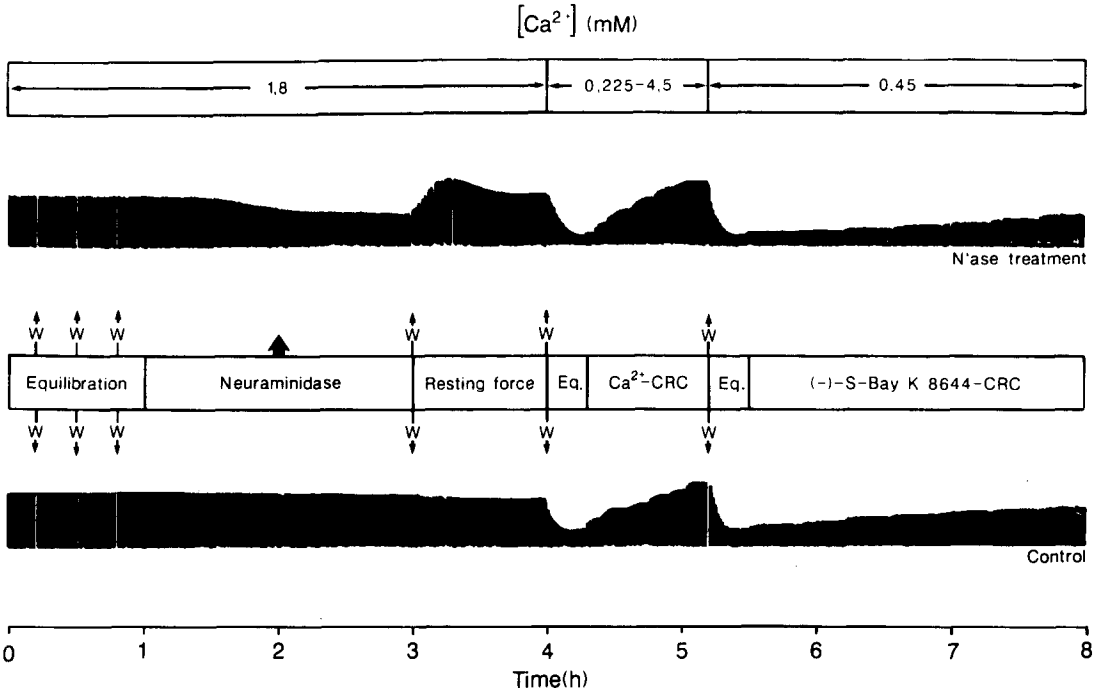

Fig. 1. Experimental protocol used to study the responses of neuraminidase-treated (2 units/mL; upper tracing) and untreated control guinea pig left atrial strips (lower tracing) against cumulative increasing concentrations of extracellular calcium (0.225–4.5 mM) and (−)-*S*-Bay K 8644 (0.1 nM–1 μM). Note the spontaneous reversibility of the neuraminidase effect after removal of the enzyme. The upper panel shows the modulation of the extracellular calcium concentration during the experiment. CRC, concentration response curve; W, wash.

on two stainless steel triangles and incubated in a water-jacketed organ bath (37°) containing 5 mL of a Krebs–Henseleit buffer, of the above described composition, gently flushed with carbogen to give a pH of 7.4. Vascular tone was measured isotonically by means of a strain gauge and was recorded after amplification (Fleck) on a digital point printing recorder (Linseis, Selb, F.R.G.). Equilibration and enzyme incubation, and removal were performed as described above. After precontraction of the arterial vessels with KCl (130 mM; evaluated from separate concentration–response experiments; Fig. 2b), during which contractile work ranged between 20.2 and 25.6 μJ, each calcium antagonist and fendiline were added in a cumulative manner (0.1 nM–100 μM) at 20–30 min intervals. Maximal vasorelaxation was determined from relaxation induced by 200 μM papaverine.

(−)-*S*-Bay K 8644 was studied cumulatively in predepolarized vessels (15 mM KCl) and was compared to an initial KCl (130 mM) induced contraction. Control experiments (omission of neuraminidase) were conducted in parallel.

Electron microscopy studies. These were performed to visually confirm the site of action and ultrastructural alterations mediated by neuraminidase treatment. Rabbit aortic ring segments were immersed in 1% cacodylate-buffered paraformaldehyde-glutaraldehyde fixative for 24 hr at 4° and were postfixed in 1% cacodylate buffered osmium tetroxide. The specimens were stained in 1% uranylacetate in 70% ethanol, dehydrated and embedded in Araldite. Ultra-thin sections were stained with 0.2% lead citrate and were examined in a transmission electron microscope (Zeiss EM 9 A).

Miscellaneous. The solvent for all drugs was dimethyl sulfoxide (stock solution 10 mM, freshly prepared for each experiment) which in concentrations of up to 1% in the organ bath did not affect the measured physiological parameters in control experiments. Studies with nisoldipine were performed under sodium light to avoid photodegradation.

Since vascular endothelium considerably affects vasomotor tone, independence of calcium antagonism from endothelial function was confirmed in depolarized (130 mM KCl) rabbit aorta previously showing substance P (10 nM)-induced relaxations in prostaglandin $F_{2\alpha}$ (1 μM) precontracted (endothelium present) as well as in depolarized arteries previously pretreated with N^G-nitro-L-arginine (100 μM) in order to selectively inhibit endothelium-derived relaxing factor production.

Absence of calcium-complexing reactions in the physiological salt solution during enzyme incubation was verified by a calcium-sensitive electrode (composition: bis(l-butyl-pentyl)adipate/potassium-tetrakis (4-chlorphenyl)borate; reference electrode: ammonium agar).

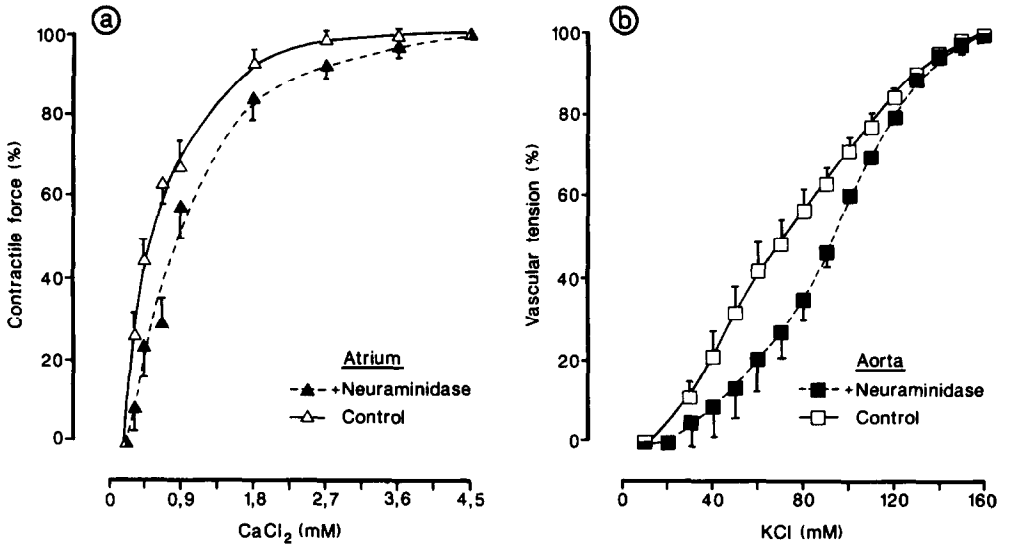

Fig. 2. Influence of cumulative increasing extracellular concentrations of $CaCl_2$ (a) and KCl (b) in neuraminidase (2 units/mL; 2 hr)-pretreated and control guinea pig left atrial strips and rabbit thoracic aortic ring segments. Means ± SEM of 5–6 individual experiments are given as percentages of maximal contraction (see text).

After removal of neuraminidase from the organ bath, there was no restitution of sialic acid content during the organ bath experiment in either tissue. This was ensured by a determination of tissue sialic acid content at the end of each experiment. The enzyme protein was regained by separation from organ bath solution via a dialysing tube or by means of gel filtration (Sephadex G_{25}, medium). A protein concentration of 70% (gel filtration) and of 30–35% (dialysation) was measured according to Lowry *et al.* [15], the regained enzyme protein being completely identical to the commercial product as indicated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Statistical methods. The results of the present experiments were analysed by several statistical methods. All values are expressed as means ± SEM. Concentration for EC_{50} was calculated from each individual concentration–response curve by an appropriate curve fitting analysis (LOGIT transformation). Students *t*-tests for unpaired values were performed; P values less than 0.05 were considered statistically significant.

RESULTS

Enzyme activity

Under optimal conditions (pH 5.0; 37°) the activity of the neuraminidase preparation used was found to range between 0.90 and 1.19 units/mg (N = 9), corresponding well to a value of 1.1 units/mg given by Sigma. Increasing the pH to 7.4 prevailing in the employed carbogen-saturated buffer solutions caused an approximately 4-fold decrease of enzyme activity to a value of 0.27 ± 0.01 units/mg solid for Tyrode's medium (30°) and 0.24 ± 0.01 units/mg solid for Krebs–Henseleit solution (37°; N = 3). Neuraminidase activity was thus considerably influenced by the pH but not by the temperature used.

Tissue sialic acid removal and content

Increasing concentrations of neuraminidase resulted in similar increases in sialic acid removal in both organs studied being maximal at an enzyme concentration of 3 units/mL in left atrial strips (53.6 ± 4.2%) and of a 4 units/mL in aortic rings (61.7 ± 1.9%; Fig. 3a). Similarly tissue sialic acid removal was increased incubation time-dependently. Maximal sialic acid hydrolysis was observed after 4 hr in atrial strips (70.8 ± 1.2%) and after 2 hr in aortic rings (59.2 ± 0.6%; Fig. 3b). From the results obtained it was decided to choose a neuraminidase concentration of 2 units/mL and an incubation time of 2 hr in either tissue leading to mean cleavage rates of 55.2 ± 2.7% (left atria; N = 6) and 60.9 ± 3.7% (aorta; N = 6), which is not significantly different from the maximal values mentioned above (Fig. 3a and b). Total sialic acid content amounted to 1.3 ± 0.03 nM/mg wet wt in left atria (N = 36) and to 1.7 ± 0.1 nM/mg wet wt in aorta (N = 33).

Influence of neuraminidase on contractile force and vascular tension

Incubation of atrial strips with neuraminidase in the organ bath (2 units/mL) led to a suppression of contractile force by 28.4 ± 2.8% after 2 hr whereas arterial tension was almost unaltered in the presence of the enzyme when compared to control organs (Fig. 4a). After removal of the enzyme, contractile force of atrial strips recovered within 40 min (Fig. 1). By contrast, stability of the following precontraction amplitude in the absence of calcium channel ligands was not significantly different in

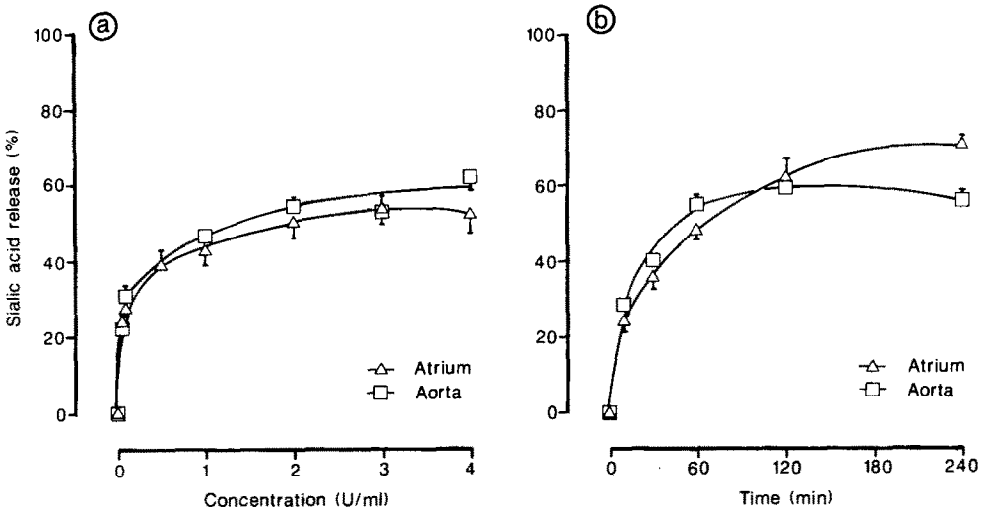

Fig. 3. Concentration- (a) and time- (b) dependent release of sialic acid from guinea pig left atrial strips and rabbit thoracic aortic ring segments by neuraminidase (0.05–4.0 units/mL; 10–240 min). The concentration dependence of sialic acid release was studied at an incubation period with the enzyme of 2 hr, the time course was examined in the presence of 2 units/mL neuraminidase. Means ± SEM of three individual experiments are given as percentages of H_2SO_4 (0.1 N)-sensitive total sialic acid content. The photometrical measurements (546 nm) were each performed in duplicate.

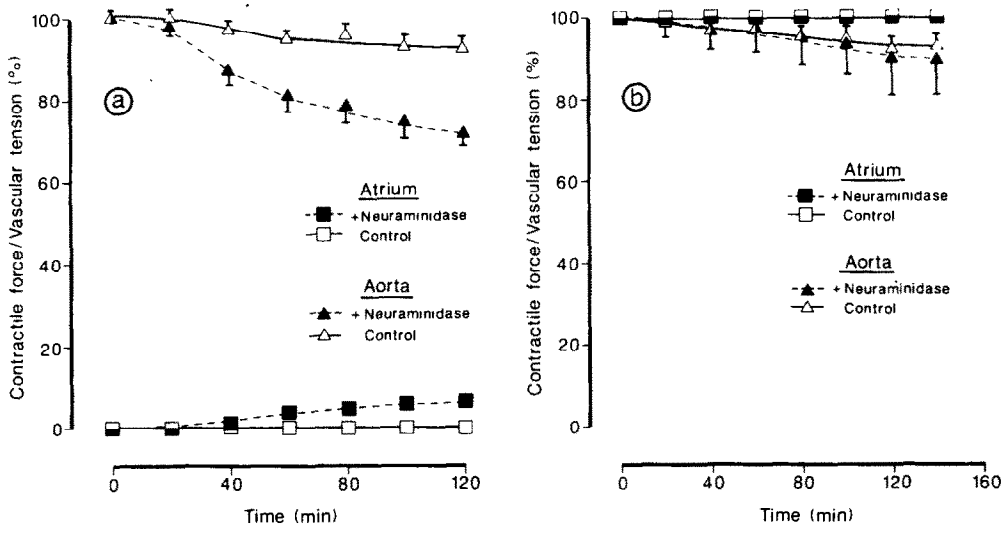

Fig. 4. (a) Alterations in the contractile force of guinea pig left atrial strips (driven at 2 Hz, double threshold voltage) and vascular resting tension of rabbit thoracic aorta during neuraminidase incubation (2 units/mL) lasting 120 min. Control experiments (omission of neuraminidase) are shown for the purpose of comparison. (b) Stability of precontraction amplitudes (atria driven at 2 Hz, double threshold voltage; aorta precontracted by 130 mM KCl) in neuraminidase-treated (2 units/mL; 2 hr) and control organs in the absence of calcium antagonists. Note the lack of influence of neuraminidase treatment. Means ± SEM of four experiments are given as percentages.

enzyme-treated compared to untreated organs (Fig. 4b).

Concentration–response studies with $CaCl_2$ *and* KCl

Regarding the concentration–response curves performed with $CaCl_2$ in guinea pig left atrial strips, as well as with KCl in rabbit aortic rings, a moderate right-hand shift was observed upon neuraminidase treatment compared to control tissues leading to significantly different EC_{50} values in both series of experiments (atria: 0.8 ± 0.1 and 0.5 ± 0.05 mM $CaCl_2$; aorta: 90.2 ± 2.5 and 69.2 ± 4.7 mM KCl; treated and control tissues, respectively). On the other hand, maximal contractility was not affected by

the enzyme when compared to control preparations (atria: 9.4 ± 1.4 and 8.7 ± 1.3 mN; aorta: 21.8 ± 1.0 and 20.1 ± 1.6 μJ; treated and control tissues, respectively; Fig. 2a,b).

Studies with calcium channel ligands

All calcium antagonists studied decreased the contractile force of guinea pig left atria driven at 2 Hz and the vascular tension of rabbit aorta precontracted by 130 mM KCl in a concentration-dependent manner. Compared to untreated organs neuraminidase treatment resulted in an increase of the negative inotropic and vasodilator activity of nisoldipine, gallopamil and diltiazem as demonstrated by a slight to moderate left-hand shift of the respective concentration–response curves (Fig. 5a–c). The cardiovascular activity of the unspecific calcium channel modulator fendiline was not affected by neuraminidase treatment (Fig. 5d). These compounds suppressed vascular tension at considerably lower concentrations than atrial contractile force (Fig. 5a–d).

The dihydropyridine calcium agonist (−)-*S*-Bay K 8644 increased the contractility of atrial strips and the vascular tension of predepolarized (15 mM KCl) aortic rings in a concentration-dependent manner. In contrast to the calcium antagonists, neuraminidase induced a right-hand shift of the cumulative concentration–response curves evoked by (−)-*S*-Bay K 8644 predominantly in guinea pig atrial muscles (Fig. 5e). In the latter tissue (−)-*S*-Bay K 8644-dependent maximal contractions reached only 66.7% (5.8 ± 0.3 mN; N = 6) of the maximal Ca^{++} induced contractions in control experiments. This reduction in maximal contractility was similar to that in atrial muscles subjected previously to neuraminidase treatment (53.1% = 5.0 ± 0.3 mN; N = 6). By contrast, the (−)-*S*-Bay K 8644-induced increase in vascular tension in the aortic smooth muscle was found to exceed considerably the maximal depolarization-dependent (130 mM KCl) contraction in control vessels by 122.1% (44.6 ± 1.6 μJ; N = 5). A similar increase was also observed in neuraminidase-pretreated aortic ring segments leading to a near identical maximal contractile force (43.4 ± 2.4 μJ; N = 5).

The concentrations for half maximal calcium antagonistic and agonistic effects (EC_{50} values) are shown in Table 1. Compared to control organs, following neuraminidase treatment a significant increase in the inhibitory potency was found with nisoldipine and diltiazem in the atrial muscle and with gallopamil in atria and aorta. No such effect was observed with fendiline in either tissue. The enzyme had no influence on the order of negative inotropic and vasodilator potency of the calcium antagonists studied. Both the positive inotropic and vasoconstrictor potencies of (−)-*S*-Bay K 8644 were significantly attenuated by neuraminidase incubation when compared with control organs.

Electron microscopy studies

An electron microscopical survey of the muscular elastic structure of the rabbit aortic ring segments subjected to the physiological experimental, fixation, and staining procedure is shown in Fig. 6a. The typical irregular and oblong shape of a smooth muscle cell attached to elastic fibres (laminae elasticae) is illustrated. A distinct disruption of the sialic acid-containing external glycocalyx at the smooth muscle cell surface was observed in neuraminidase-pretreated specimens (Fig. 6b). A predominantly intact external matrix was seen in control preparations exposed to the same experimental procedure (Fig. 6c).

DISCUSSION

In the present study sialic acid residues of the glycocalyx covering the external cell surface could be effectively released by neuraminidase treatment from the organ preparations used in the physiological experiments. Similar degrees of sialic acid removal have been reported in guinea pig left atria [16], the atrial muscle of rats [17] and rat heart membranes [18], as well as in rat tail arteries [19]. As a reason for the incomplete sialic acid removal found in all these studies, the location of these sugar residues on sites inaccessible to the enzyme protein may be assumed combined with the fact that O-acetylation at positions 4 and 7–9 is present in many biological samples which can render the sialic acid molecules partially or completely resistant to neuraminidase. Conversely, considerable destruction of *O*-acetyl groups occurs when mild acid hydrolysis is used to release sialic acid for analysis [20, 21]. Moreover, we could show an approximately 4-fold decline in neuraminidase activity under physiological pH conditions in the present study which was not considered in previous comparable studies.

In the present examination neuraminidase treatment of aortic rings and atrial strips resulted in an increased sensitivity to calcium channel blocking agents (except fendiline) and in an increased dose level required for the calcium channel agonist (−)-*S*-Bay K 8644, along with a requirement for increased levels of calcium or potassium to contract the muscle. The following evidence suggests that these effects are due to a substantial neuraminidase-mediated sialic acid release from the surface membranes: (1) removal of sialic acid from the tissue preparations studied was verified by a biochemical assay. (2) Electron micrographs revealed a distinct disruption of the external matrix in rabbit ring segments following addition of the enzyme. Similar results were obtained previously in guinea pig left atria according to the lanthanum staining technique [16]. These findings are consistent with the ultrastructural localization of sialic acid residues on human muscle cell surfaces [22]. (3) Preliminary studies with nisoldipine suggested a dose-dependent influence of neuraminidase on the negative inotropic activity of this dihydropyridine compound (unpublished results). (4) Other enzymes (protease, phospholipases, *N*-acetylneuraminic acid aldolase) known to contaminate neuraminidase preparations [23] were shown not to alter force development [19] or failed to mimic the neuraminidase-induced effects [13]. (5) Finally, (a) neuraminidase treatment did not affect the maximal contraction ability in the studies with increasing calcium, potassium, and (−)-*S*-Bay K 8644 concentrations; (b) the stability of precontraction

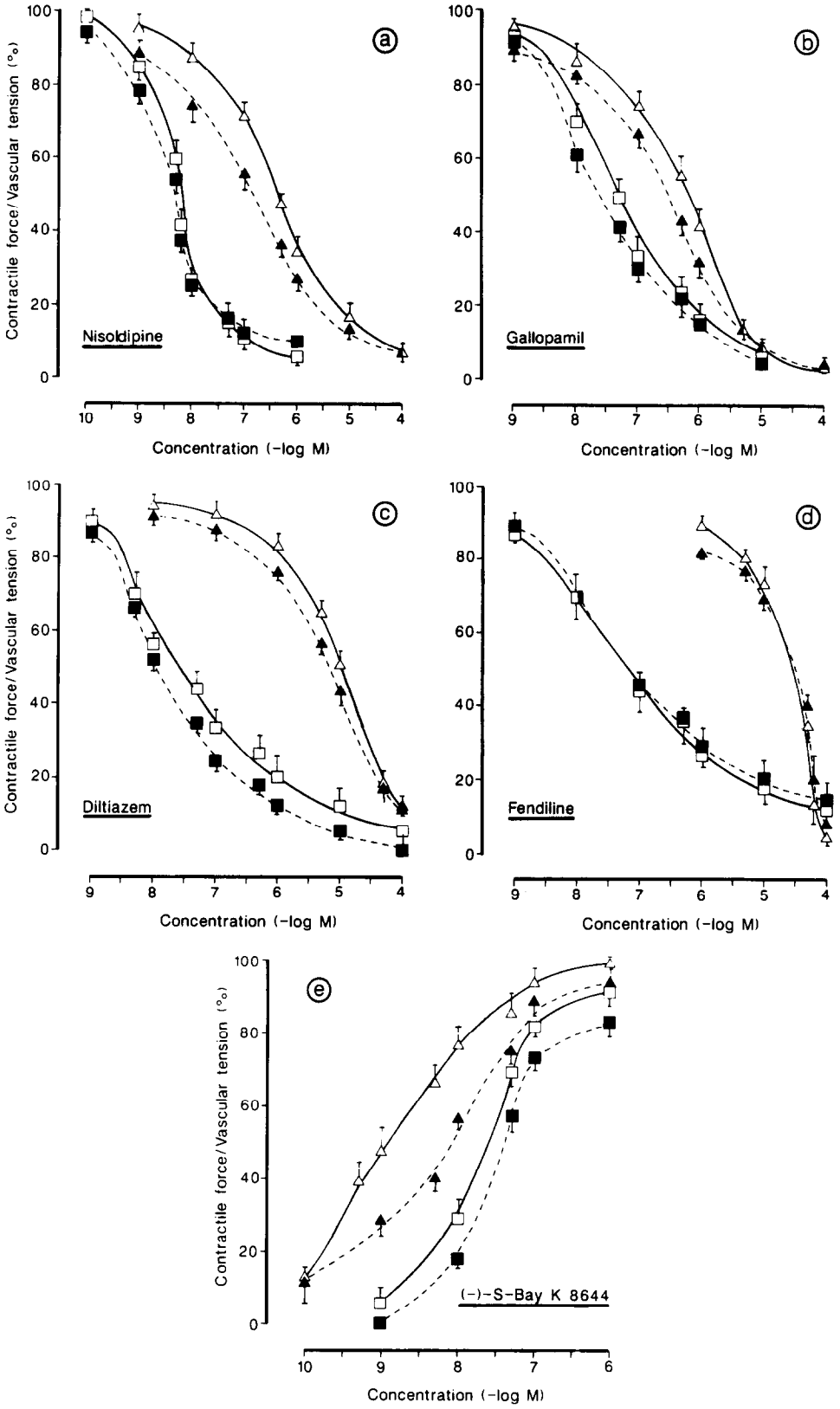

Fig. 5. Influence of cumulative increasing concentrations of different calcium channel ligands (a–e) on the contractile force and vascular tension of neuraminidase-treated (■, ▲; dotted lines) and control (□, △; solid lines) guinea pig left atrial strips (▲, △) and rabbit thoracic aortic ring segments (■, □). Means ± SEM of 6–9 individual experiments are given as percentages. The data for (−)-*S*-Bay K 8644 refer to the maximal positive inotropic and vasoconstrictor effect (= 100%) induced by this drug.

Table 1. Potencies of the calcium channel ligands studied in neuraminidase-treated and untreated organs

	Atrium (nM)		Aorta (nM)	
	Control	Neuraminidase	Control	Neuraminidase
Nisoldipine	460 ± 70	160 ± 40*	6.2 ± 1.1	4.5 ± 0.5
Gallopamil	530 ± 90	290 ± 20*	62 ± 15	28 ± 5*
Diltiazem	8700 ± 1200	5400 ± 800*	30 ± 7	16 ± 2
Fendiline	24,000 ± 300	24,000 ± 3000	110 ± 20	120 ± 30
(−)-*S*-Bay K 8644	1.7 ± 0.5	6.9 ± 1.1*	24 ± 4	40 ± 5*

The biological activity in guinea pig left atrial strips and rabbit thoracic aortic ring segments is expressed as half maximal response (EC_{50}, nM). Means ± SEM of 5–9 individual concentration–response curves are given.

* Denotes significantly different EC_{50} values vs untreated control organs, $P < 0.05$.

amplitudes (atria driven at 2 Hz; aorta prestimulated by 130 mM KCl) did not differ in enzyme-treated and control organs; and (c) the decline in contractile force of electrically paced atrial strips by approximately 28% 2 hr after neuraminidase administration was fully reversible after washing (Fig. 1). We therefore conclude that neuraminidase did not have a detrimental influence on the contractile apparatus of the guinea pig left atria and rabbit aorta.

The reason for the attentuation of contractile force in the atrial muscle during incubation with neuraminidase (Fig. 4a) is unknown; however, since the calcium concentration in the physiological salt solutions remained unchanged in the presence of the enzyme, as determined by a calcium sensitive electrode, the absence of calcium complex binding reactions with the enzyme protein was suggested. One may assume that the bulky enzyme protein non-specifically obstructs the transmembrane ion channels, thereby reducing the contractility of atrial muscles. Such reactions were not observed in the thoracic aorta because the arterial rings were kept at resting tension during enzyme incubation.

The distinct slight to moderate modulation of calcium, potassium, and calcium channel ligand-induced actions by neuraminidase treatment may be explained in different ways. Firstly, the glycohydrolase removes external sialic acid residues screening the calcium channel surface. Since the surface charge of an open-state channel may be different from the surface charge of a closed-state channel [24], an involvement of sialic acid in the determination of distinct calcium channel conformations seems likely, thereby affecting transmembrane calcium fluxes.

Accordingly, as sialic acid contributes to the glycosylation of calcium channel subunits [11], neuraminidase treatment could alter the binding characteristics of the different calcium channel ligands studied. However, whereas Mas-Oliva and Nayler [25] found an increase in the specific binding of radioactive verapamil in isolated sarcolemmal vesicles from rabbit by 21%, Glossmann *et al.* [26] failed to observe an influence of neuraminidase on the specific binding of radiolabelled dihydropyridines in guinea pig brain membranes. Furthermore, if in the present study the augmented negative inotropic and vasodilator potency of nisoldipine upon neuraminidase treatment was due to a facilitation of dihydropyridine binding, this would also apply to (−)-*S*-Bay K 8644, the dihydropyridine calcium agonist. By contrast, a decline in the calcium agonistic potency by neuraminidase was found in the present examination. Moreover, it seems quite unlikely that the enzyme protein permeates into the channel pore thereby reaching the binding domains for calcium channel ligands.

Another possible explanation comes from Recio-Pinto *et al.* [27]. Studying electroplax sodium channels in planar lipid bilayers the authors found a marked shift in the average steady state activation curve toward depolarizing potentials combined with a striking increase in the frequency of reversible transitions to subconductance states in desialidated channels. Additionally, neuraminidase-treated channels could undergo long-lived closures even at depolarizing potentials. From the results obtained the authors concluded that, if sialic acid residues were to play such an electrostatic role in the steady state activation behaviour leading to an increase in the depolarizing stimulus required to achieve a certain level of channel activation, a hyperpolarizing influence on putative channel gating elements within the membrane by a substantial removal of fixed negative charges from the outer surface should be taken into account. However, according to the *modulated receptor hypothesis*, i.e. the membrane potential-dependent binding affinity of calcium antagonists (Sanguinetti and Kass [28]), such an influence of neuraminidase on calcium channels would, in contrast to our study, result in a decrease of calcium antagonistic potency but would explain the observed requirement for increased levels of calcium and potassium in contracting the muscle in desialidated tissues.

Finally, on the basis of the present results it seems reasonable to assume a facilitating influence of sialic acid on transmembrane calcium fluxes and/or calcium channel activity expressed slightly more in guinea pig left atria than in rabbit thoracic aorta. This conclusion is not unequivocally accepted.

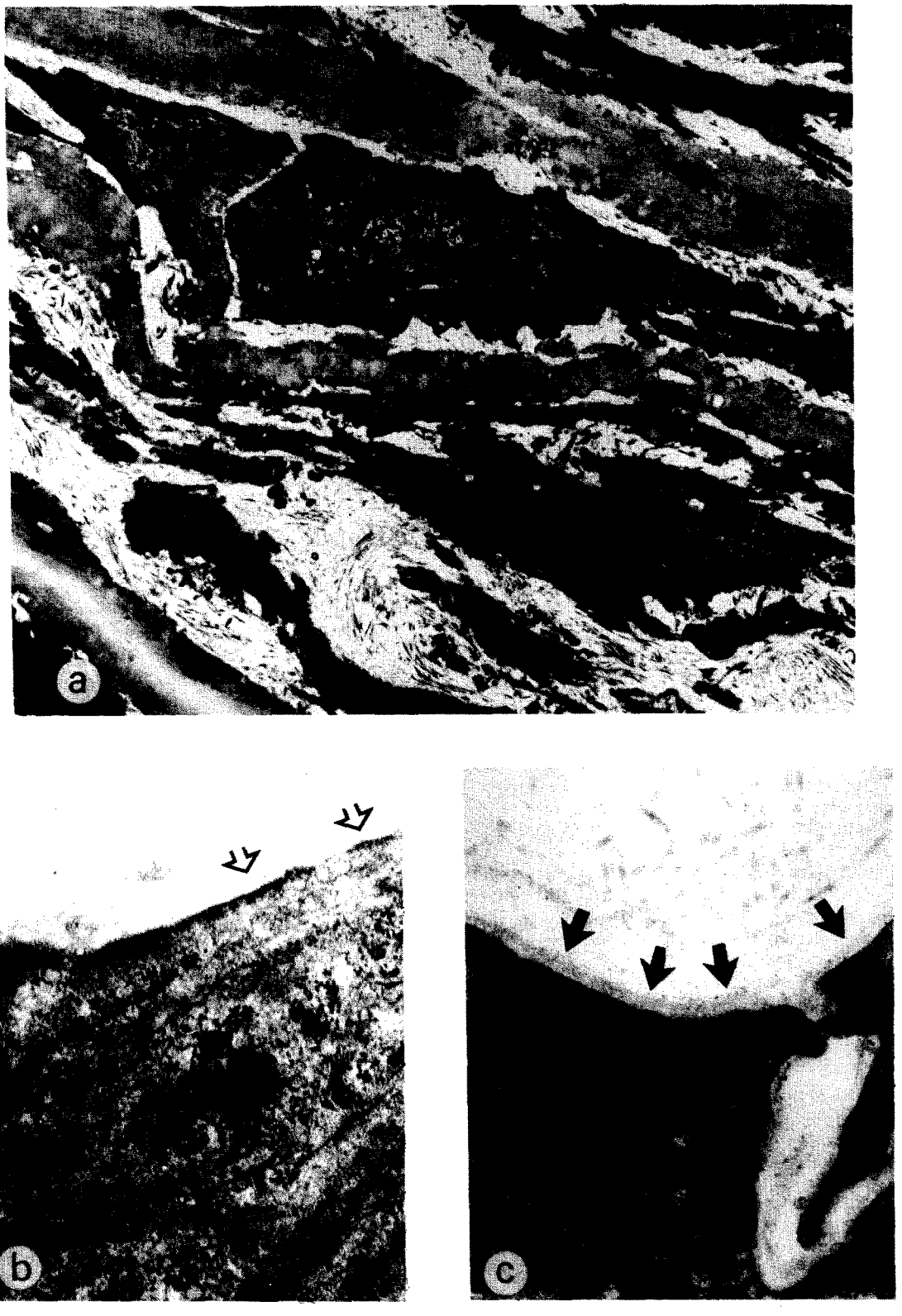

Fig. 6. Electron micrographs illustrating the muscular elastic structure of the rabbit aortic vessel wall media (a; × 7800) and the surface of a single smooth muscle cell derived from a neuraminidase-treated (b; × 36,000) and untreated control (c; ×36,000) ring segment after lead citrate staining. Note the distinct disruption of the external glycocalyx (arrows) in the neuraminidase-treated preparation. The major part of the glycocalyx has been shown to be constituted by sialic acid (see text). M, smooth muscle cell; E, elastic fibre (lamina elastica).

Compared to the present examination in intact organs contrasting results have been presented especially in single heart cells. For example, Bhattacharyya *et al.* [29] reported on initiation of spontaneous firing in the presence of tetrodotoxin, arrhythmic spontaneous activity, depolarization of maximum diastolic potential and significant reduction in the plateau, and duration of the action potential, thought to be due at least in part to an enhanced intracellular calcium content following the release of sialic acid from cultured chick embryonic heart cells by approximately only 25%. In accordance, at high extracellular calcium concentrations (8.1 mM) delayed after-depolarizations were induced only in neuraminidase-treated canine Purkinje fibres, suggesting the sialic acid residues of the glycocalyx to function as a kind of barrier to calcium influx [30]. More recently, neuraminidase treatment of cultured cardiac myocytes was described as specifically increasing cellular ^{45}Ca exchange in proportion to sialic acid removal [9]. On the other hand, even though an increased calcium flux through T-type and L-type channels in five of nine and three of six cardiac myocytes was found after neuraminidase treatment, the enzyme reduced T-type calcium fluxes or had no significant effect on L-type calcium fluxes in cells that did not exhibit such an increase [13]. Moreover, the contractile response developed to calcium concentrations greater than 5 mM and to 50 nM isoproterenol were reduced significantly by incubation of Langendorff-prepared guinea pig hearts with neuraminidase [31]. Finally, consistent with our findings (Fig. 2b) potassium- as well as histamine- but not acetylcholine-induced contractions of guinea pig taenia coli were attenuated in a time-dependent manner by approximately 50% upon neuraminidase administration [32].

As far as the modulation of calcium channel ligand action is concerned in contrast to our results, neuraminidase treatment attenuated significantly the negative inotropic effects of verapamil and diltiazem whereas it had no effect on the inotropic activity of the dihydropyridine compounds nifedipine and Bay K 8644 in whole left atria of rats in an earlier study [17]. In this case, however, the authors used a quite different experimental protocol: the electrical stimulation was interrupted for 90 min in order to transfer the atrial muscles to a neuraminidase-containing incubation medium, before the preparations were resuspended in the organ bath and stimulation was restarted, whereas the present experimental model enabled the force of contraction and vascular tension to be monitored throughout the enzyme incubation. Moreover, Hattori *et al.* [17] used the racemic mixture of Bay K 8644 compared to the pure agonistic (−)-*S*-enantiomer employed in the present study. Another, however, unsatisfactory explanation for the quite conflicting data described above may be that the function of sialic acid could vary with preparation, species or age [31, 33, 34].

Obviously, sialic acid removal affects the action of specific and unspecific calcium antagonists in different ways in that the cardiovascular activity of fendiline was not modulated by neuraminidase pretreatment. The reason for this observation is unknown. However, the action of this unspecific diphenylalkylamine calcium antagonist is not restricted to the modulation of calcium channels: this compound was shown to act additionally at intracellular sites in the rabbit aortic smooth muscle such as in the inhibition of calmodulin [35], a mechanism most probably unrelated to alterations in the peripheral glycocalyx.

In our opinion, the possible influence of neuraminidase treatment on transmembrane calcium fluxes could also be a consequence of a reduction in the membrane calcium binding and/or external calcium availability in the tissues studied since the basal activity of other ion transporting systems such as (Na^+,K^+)ATPase or Mg^{2+}-ATPase was not affected by neuraminidase treatment [31]. In an earlier examination, prostaglandin $F_{2\alpha}$ evoked a biphasic response in feline basilar arteries in calcium-free medium; the major second phase only being abolished by calcium entry blockade (manganese, diltiazem) or by pretreatment with neuraminidase [36]. The author gave evidence for the contribution of sialic acid to a calcium store termed *loosely bound calcium* at the exterior aspect of the cell membrane. Neuraminidase treatment was also shown to reduce the binding capacity and to affect the affinity constant of a high capacity, low affinity calcium pool detected from calcium binding studies on adult guinea pig ventricular myocytes [37].

Thus, sialic acid may contribute to a localized external surface calcium pool. Through its sialic acid-removing influence, neuraminidase may lower the calcium level of this intermediate pool that supplies calcium for voltage activated calcium channels. In the present study, this is manifested in an increased sensitivity of both tissues studied to calcium antagonists. It is also shown by the increased dose level required for the calcium channel agonist (−)-*S*-Bay K 8644 and in the requirement for increased levels of calcium and potassium in contracting the muscle in both tissues. Whether this suggests an additional functional role for sialic acid in the regulation of calcium channels warrants further investigation.

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