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Inotropic effects of Ca^{2+} channel agonist and antagonists in neuraminidase-treated left atria of rats

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1 The effects of removal of sialic acid from cardiac sarcolemma on contractile functions and on inotropic responses to Ca^{2+} channel agonist and antagonists were investigated in rat left atria.

2 About 64% of the total sialic acid content of the left atria was removed during a 90 min exposure to neuraminidase (2 u ml^{-1}).

3 The removal of sialic acid neither affected the development of twitch tension induced by stimulation at a frequency of 0.5 Hz, nor altered the interval-dependent changes in contractility such as the force-frequency relationship and post rest contractions.

4 The positive inotropic effects produced by isoprenaline, and by an increase in extracellular Ca^{2+} concentration were the same in the neuraminidase-treated preparations as those in the untreated preparations. Bay K 8644, a Ca^{2+} channel agonist, induced an increase in conractility in the neuraminidase-treated preparations comparable to that in the untreated ones.

5 Neuraminidase treatment significantly attenuated the negative inotropic effects of verapamil and diltiazem, whereas it had no effect on that of nifedipine.

6 The results indicate that sialic acid removal modifies neither the basal contractile functions nor the positive inotropism which is associated with an enhancement of the slow inward Ca^{2+} current. However, sialic acid, which constitutes the glycocalyx of the cardiac sarcolemma, may be involved in the mechanism of the Ca^{2+} channel antagonistic actions of verapamil and diltiazem, but not that of nifedipine. Thus, our results provide pharmacological evidence that verapamil and diltiazem behave differently from the dihydropyridine compounds.

Introduction

Sialic acid is a major constituent of the glycocalyx covering the surface of cardiac cells (Persons & Subjeck, 1972; McNutt & Fawcett, 1974) and sialic acid residues account for a considerable part of the superficial Ca^{2+}-binding sites in the cardiac tissue (Frank *et al.*, 1977). Langer and his co-workers have found that removal of sialic acid from cultured myocardial cell surface by treatment with neuraminidase produces an enhancement of the Ca^{2+} permeability of the membrane (Frank *et al.*, 1977; Langer *et al.*, 1979; 1981). This observation suggested that the glycocalyx might in some way regulate the calcium ions which participate in the contractile response. In accord with this theory are the findings that depression of cardiac contractility and manifestation of cardiac failure were associated with a decrease in the sialic acid content of sarcolemma in cardiomyopathic hamsters (Bailey & Ma, 1980), and that neuraminidase treatment of guinea-pig isolated hearts prevented the positive inotroic effects of ouabain (Bailey & Fawzi, 1980). However, conflicting results have been obtained by Harding & Halliday (1980); they demonstrated that removal of sialic acid had no effect on the contractile functions of guinea-pig atria. Furthermore, the positive inotropic effect of ouabain has been reported to be unaltered by neuraminidase treatment (Grupp *et al.*, 1980).

Removal of sialic acid by neuraminidase has been shown to increase the binding of [^{14}C]-verapamil in cardiac sarcolemmal preparations of rabbits (Mas-Oliva & Nayler, 1980). However, little is known about the role of sialic acid in the pharmacological responses to Ca^{2+} channel antagonists.

Structurally diverse Ca^{2+} channel antagonists have recently been classified according to their binding characteristics (Glossmann *et al.*, 1983; Murphy *et al.*, 1983) or pharmacological profiles (Rodenkirchen *et al.*, 1982; Spedding & Berg, 1984). Thus, it would be of interest to compare the cardiodepressive effects of structurally different Ca^{2+} channel antagonists in

neuraminidase-treated myocardial cells, even though Isenberg & Klöckner (1980) have reported that the slow inward Ca^{2+} current in cardiac cells of adult rats is not altered by cell coat removal.

The experiments were undertaken with the electrically paced isolated left atrium of the rat in order to assess whether (1) the contractile function of this preparation is altered when sialic acid is removed from cardiac sarcolemma by neuraminidase treatment, (2) sialic acid removal has different effects on the negative inotropic actions of three structurally different Ca^{2+} channel antagonists and modifies the positive inotropic effect of the Ca^{2+} channel agonist Bay K 8644, a novel synthetic dihydropyridine compound (Schramm *et al.*, 1983a,b).

Methods

Experimental procedure

Wistar rats of either sex weighing 300–500 g were killed by a blow on the head. The hearts were quickly removed and the left atria prepared for monitoring of tension development as previously described (Hattori & Kanno, 1984). The muscle was mounted vertically in a water-jacketed bath containing 50 ml of Krebs-Henseleit solution of the following composition (mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10.0. The solution in the bath was gassed with 95% O_2 and 5% CO_2 and its temperature maintained at 30 ± 1°C. The lower end of the left atrium was pinned on a pair of hook-electrodes for stimulation and the other end connected by a silk thread to a force transducer (Nihon Kohden TB-612T). The isometric tension, under a resting tension of 2 g, was displayed on a pen recorder (Nohon Kohden RJG-3026) through a preamplifier (Nihon Kohden RP-5). The preparations were electrically driven by rectangular pulses of 3 ms duration and an intensity twice the diastolic threshold at a frequency of 0.5 Hz, unless otherwise stated. The pulses were delivered by an electronic stimulator (Sanei-Sokki 3F46) through an isolation unit (Sanei-Sokki 5361).

After a 60 min period of equilibration at a stimulation rate of 0.5 Hz, the developed tension of the preparations was recorded, and then the muscles were transferred to an incubation vessel containing 1 ml Tris-HCl buffer solution (NaCl 143.9, $CaCl_2$ 1.0, KCl 6.0, $MgSO_4$ 1.2, Tris 10.0, glucose 3.0 mM, pH7.4) plus 2 u ml^{-1} neuraminidase (Sigma, Type X). The incubation medium was continuously bubbled with oxygen and its temperature kept at 30°C. The neuraminidase used in the present study is specified to be devoid of protease and N-acetylneuraminic acid aldolase activities. Control tissues were also treated as stated above, except that neuraminidase was omitted from the incubation medium. After an incubation period of 90 min, the preparations were resuspended in the organ bath and stimulation was restarted at 0.5 Hz. Then the muscles were further equilibrated for 45–60 min before the commencement of the experimental procedures described in the text.

Measurement of sialic acid

The measurement of sailic acid content was performed in all control and neuraminidase-treated left atria used in the experiments. Total sialic acid content of the left atria was determined by the thiobarbituric technique of Warren (1959) after incubating the tissues in 0.1 N H_2SO_4 at 80°C for 60 min.

In preliminary experiments we found that sialic acid release from the rat left atria reached its maximum after 90 min of incubation in the Tris buffer containing neuraminidase (2 u ml^{-1}). It was also confirmed that the mean percentages of the amount of sialic acid released from the atria by treatments with neuraminidase at 1, 2 and 4 u ml^{-1} in the 90 min period were not significantly different. Therefore, we decided to incubate the atria in the buffer containing 2 u ml^{-1} neuraminidase for 90 min in order to remove effectively the sialic acid from the atria.

Statistical analysis

All values are expressed as the mean ± s.e. The statistical significance of the differences was evaluated by means of Student's t tests for unpaired values. The criterion for statistical significance was $P < 0.05$.

Drugs

Bay K 8644 methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate and nifedipine were generous gifts from Dr B. Garthoff of Bayer AG (Leverkusen, FRG). Verapamil hydrochloride was a gift from Eisai Pharmaceutical Co. Ltd (Tokyo, Japan) and diltiazem hydrochloride was a gift from Tanabe Pharmaceutical Co. Ltd (Osaka, Japan). Neuraminidase (Type X) and (±)-isoprenaline hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All drugs except Bay K 8644 and nifedipine were dissolved in distilled water. Bay K 8644 and nifedipine were dissolved in ethanol prior to diluting in distilled water; the final ethanol concentration was less than 0.1%, and ethanol at this concentration was confirmed to have no effect on the developed tension of the rat left atria. Ascorbic acid (10^{-4} M) was added to the isoprenaline solutions.

To avoid light-induced oxidation of the dihydropyridine ring, the experiments with

Bay K 8644 and nifedipine were carried out in a dark room.

Results

Sialic acid content in neuraminidase-treated atria

The results obtained from untreated (control) and neuraminidase-treated preparations are summarized in Table 1. The sialic acid content of untreated and neuraminidase-treated left atria was 0.97 ± 0.04 and 0.35 ± 0.01 nmol mg^{-1} wet weight, respectively. About 64% of the total sialic acid residue was removed from the left atria by exposure to neuraminidase 2 u ml^{-1} for 90 min.

Effect of sialic acid removal on contractile functions

Exposure of the rat left atria to neuraminidase (2 u ml^{-1}) for 90 min did not affect the contractile tension developed at a stimulation rate of 0.5 Hz compared with that of control atria (Table 1). The developed tension of control atrial preparations after incubation in Tris buffer without neuraminidase declined to $68 \pm 5\%$ of that recorded before the incubation. Treatment with neuraminidase resulted in the same reduction of atrial contractions ($68 \pm 4\%$).

The rat left atrial muscle has a peculiar force-frequency relationship which shows a negative correlation between the developed tension and the stimulation frequency. The neuraminidase treatment neither potentiated nor attenuated this frequency-response curve (Figure 1).

Post rest contraction, i.e. the developed tension of the first contraction following a rest period of 3 min after steady stimulation of 1 Hz (Hattori & Kanno, 1981), was not affected by treatment with neuraminidase. In untreated and neuraminidase-treated preparations, the post rest contractions were $156 \pm 10\%$ ($n = 10$) and $168 \pm 13\%$ ($n = 10$) of the steady-state contractions obtained before the rest period, respectively. No significant difference between the two groups was found.

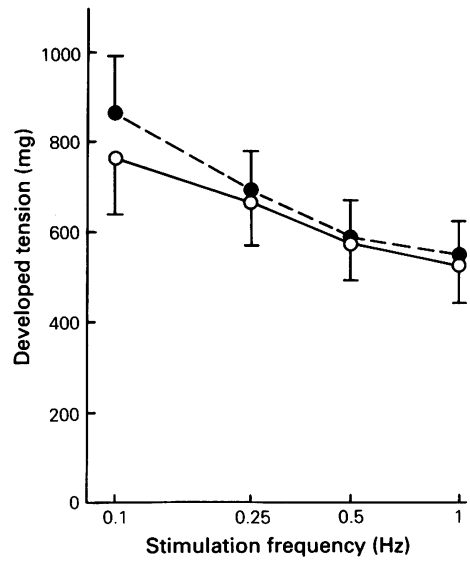

Figure 1 A comparison of the force-frequency relationships in control (○) and neuraminidase-treated (●) left atria of the rat. Values represent the mean response of 10 preparations; vertical lines indicate s.e.

Effect of neuraminidase treatment on the positive inotropic responses to an increase in $[Ca^{2+}]_o$ and to isoprenaline

Inotropic responses to an increase in extracellular calcium were determined. Five pairs of control and neuraminidase-treated left atria, driven at 0.5 Hz, were subjected to increasing calcium concentrations, in a cumulative manner from 2.5 to 7.0 mM. However, no distinct differences in the responses to calcium were observed between the two groups (Figure 2).

In addition, the neuraminidase treatment did not produce a significant change in the cumulative dose-

Table 1 Effect of neuraminidase treatment on sialic acid content and contractile force in rat left atria driven at 0.5 Hz

	Sialic acid content (nmol mg^{-1} wet weight)	*Contractile force* (mg)	*Number of experiments*
Control	0.97 ± 0.04	620 ± 47 ($68 \pm 5\%$)	53
Neuraminidase-treated	0.35 ± 0.01*	597 ± 43 ($68 \pm 4\%$)	51

All values are mean ± s.e. Numbers in parentheses indicate % of the contractile force recorded immediately before incubation with Tris buffer either with or without neuraminidase.
*$P < 0.001$ vs. the control value.

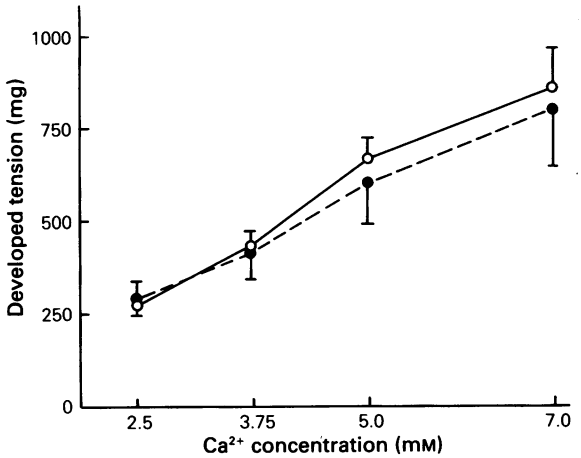

Figure 2 Effect of increasing the extracellular concentration of calcium (in a cumulative manner) on the developed tension, of rat left atria driven at 0.5 Hz, in control (○) and neuraminidase-treated (●) preparations. Values represent the mean response of 5 preparations; vertical lines indicate s.e.

response curve for the positive inotropic response to isoprenaline in the left atria driven at 0.5 Hz. Thus, the pD_2 values were 9.16 ± 0.11 ($n = 5$) and 9.22 ± 0.17 ($n = 5$), and the maximal increases in tension were approximately 130% and 135%, in control and neuraminidase-treated preparations, respectively.

Effect of neuraminidase treatment on the negative inotropic responses to Ca^{2+} channel antagonists

Verapamil, diltiazem and nifedipine all produced a concentration-dependent negative inotropic effect in the control left atria driven at 0.5 Hz. The negative inotropic effects of verapamil and diltiazen were both significantly attenuated by the treatment with neuraminidase (Figure 3a,b), but the responses to diltiazem were inhibited to a smaller extent, by the treatment than those of verapamil. However, the neuraminidase treatment did not affect the negative inotropic response to nifedipine, a dihydropyridine Ca^{2+} channel antagonist (Figure 3c).

Effect of neuraminidase treatment on the positive inotropic response to a Ca^{2+} channel agonist

Bay K 8644, designated as a Ca^{2+} channel agonist (Schramm *et al.*, 1983a,b), produced a positive inotropic effect in rat left atria driven at 0.5 Hz which was concentration-dependent. However, no significant

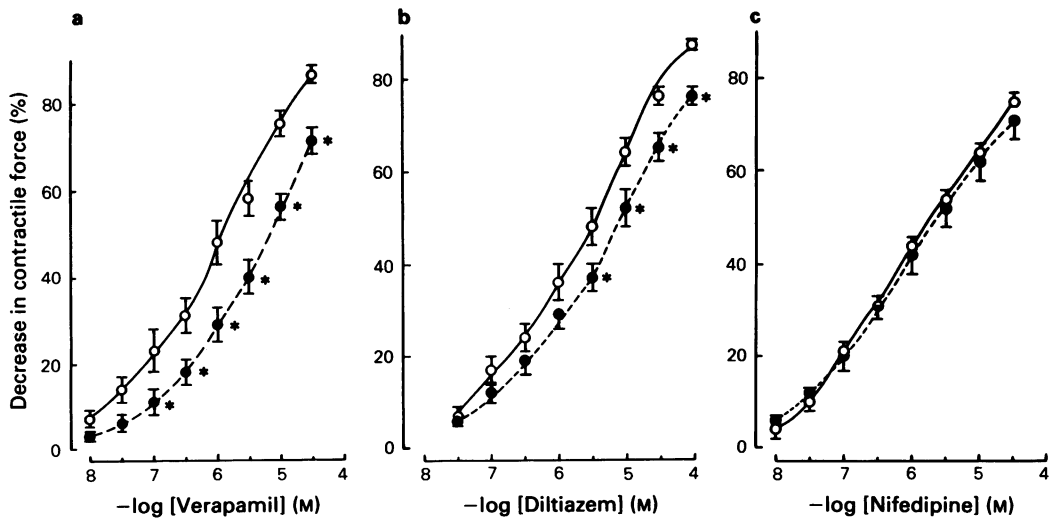

Figure 3 The concentration-response curves for the negative inotropic effects of (a) verapamil, (b) diltiazem and (c) nifedipine, in rat left atria driven at 0.5 Hz, in control (○) and neuraminidase-treated (●) preparations. Values represent the mean of 7–8 experiments; vertical lines indicate s.e. $^*P < 0.05$ vs. the corresponding values obtained in control preparations.

difference in the positive inotropic response to the compound was detected between control and neuraminidase-treated preparations (Figure 4).

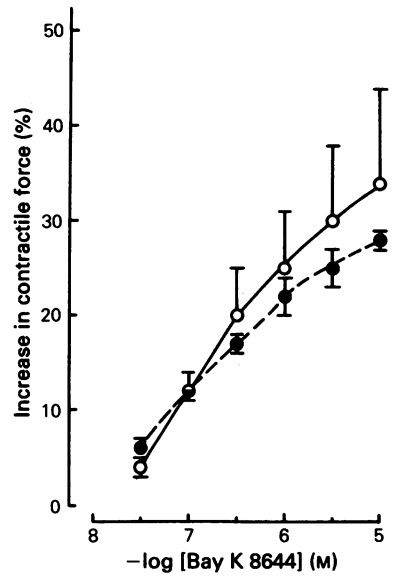

Figure 4 The concentration-response curves for the positive inotropic effect of Bay K 8644, in rat left atria driven at 0.5 Hz, in control (○) and neuraminidase-treated (●) preparations. Values represent the mean response of 5 preparations; vertical lines indicate s.e.

Discussion

In the present study treatment with neuraminidase ($2\,u\,ml^{-1}$) removed up to 64% of the total sialic acid content of the glycocalyx of rat left atria. A similar degree of sialic acid removal has been reported in guinea-pig left atria (Harding & Halliday, 1980) and cultured cells of the rat heart (Frank *et al.*, 1977). In these studies, all the membrane structure, except the glycocalyx, has been shown to be electromicroscopically intact even when treated with neuraminidase. Thus, sialic acid residues of the glycocalyx covering the external surface can be effectively removed by neuraminidase treatment of the whole atria.

We found that neuraminidase treatment did not change the contractile tension of electrically driven (0.5 Hz) left atria of the rat. Harding & Halliday (1980) also found that sialic acid removal had no effect on developed tension in electrically stimulated (3 Hz) guinea-pig left atria. Rat atria are known to exhibit a negative relationship between the stimulation frequency and the developed tension, the relationship being quite opposite to the positive one observed in guinea-pig atria (Koch-Weser & Blinks, 1963). We previously reported that the peculiar force-frequency relationship in rat atria was differently affected by α- and β-adrenoceptor stimulation, especially at a higher range of stimulation rates (Hattori & Kanno, 1984). Complexly intertwined processes, sometimes acting in opposite directions, determine the actual tension developed at a constant stimulation rate (Chapman, 1979), and changing the stimulation interval may unmask a fundamental step of cellular Ca^{2+} dynamics (Allen *et al.*, 1976; Edman & Jóhannsson, 1976). Therefore, in the present study, we further examined whether the interval-dependent contractile responses of isolated atrial muscles were affected by sialic acid removal from cardiac sarcolemma. However, treatment with neuraminidase exerted no influence on the interval-dependent changes in contractility such as force-frequency relationship and post rest contractions. These findings indicate that the calcium-binding sialic acid residues in the external glycocalyx of the cardiac cells may play only a minor role in regulating the cellular Ca^{2+} dynamics which determine the beat to beat contractions in the intact atrial muscles.

In addition, it was shown that neuraminidase treatment had no effect on the positive inotropic actions induced by isoprenaline and an increase in the extracellular Ca^{2+} concentration. Enhancement of the slow inward Ca^{2+} current has been proved to be causually related to the positive inotropic response resulting from β-adrenoceptor stimulation by isoprenaline (Reuter, 1974). The results thus suggest that sialic acid removal by neuraminidase does not functionally modify voltage-dependent Ca^{2+} channels in rat left atria. Further support for this postulate is provided by our observation that neuraminidase treatment did not affect the positive inotropic response to the Ca^{2+} channel agonist, Bay K 8644 (Schramm *et al.*, 1983a,b). Our interpretation concurs with that of Isenberg & Klöckner (1980); they showed that the slow inward Ca^{2+} current in adult rat heart cells was unchanged when the glycocalyx was destroyed by treatment with collagenase and hyaluronidase.

The present study also showed that the negative inotropic effects of verapamil and diltiazem were significantly attenuated in the neuraminidase-treated atria, though the negative inotropic action of diltiazem was less affected than that of verapamil. This attenuation indicates a decrease in their efficacy of blocking voltage-dependent Ca^{2+} channels. However, neuraminidase treatment did not change the inotropic response to nifedipine, a dihydropyridine Ca^{2+} channel antagonist. Recently, binding studies with [^{3}H]-nifedipine and [^{3}H]-nitrendipine have indicated that verapamil and diltiazem interact at a site(s) different from, but allosterically linked to, the dihydropyridine binding sites which are closely associated with Ca^{2+}

channels (Holck *et al.*, 1982; Murphy *et al.*, 1983; Janis *et al.*, 1984). In more recent studies, pharmacological interactions of Bay K 8644 with Ca^{2+} channel antagonists have shown that the sites of action of verapamil and diltiazem are different from the dihydropyridine sites (Spedding & Berg, 1984; Ishii *et al.*, 1985). In view of these results, it appears reasonable to assume that the differential effects of neuraminidase treatment on the negative inotropic effects of Ca^{2+} channel antagonists reflect the differences in their mode of interaction with the sites of action. In other words, sialic acid constituting the glycocalyx of cardiac sarcolemma may be involved in the mechanism of the Ca^{2+} channel antagonistic actions of verapamil and diltiazem, but not that of the dihydropyridine Ca^{2+} antagonist.

The present results are difficult to reconcile with those of Mas-Oliva & Nayler (1980), who observed an increase in [^{14}C]-verapamil binding in rabbit myocardial sarcolemmal preparations treated with neuraminidase. This discrepancy cannot easily be explained, but differences in experimental protocol and conditions could be the cause. In addition, it has been suggested that the functions of sialic acid may vary with different preparations, species or age (Langer *et al.*, 1981; Woods *et al.*, 1982). However, further experiments are necessary to ascertain the mechanism of the attenuating effect of neuraminidase treatment on the negative inotropic responses of rat left atrial muscles to verapamil and diltiazem.

In conclusion, removal of sialic acid from cardiac sarcolemma by treatment with neuraminidase does not affect the contractile functions in rat left atria. It also does not functionally modify voltage-dependent Ca^{2+} channels. However, the treatment attenuates the negative inotropic effects of verapamil and diltiazem without changing the inotropic responses to the dihydropyridine compounds, which provides pharmacological evidence that verapamil and diltiazem have a different mode of action from the dihydropyridines.

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