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Sialic Acid and the Surface Charge Associated with Hyperpolarization-Activated, Inward Rectifying Channels

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Summary. The whole-cell configuration of the patch-clamp technique was used with cultured pacemaker cells from the rabbit sinoatrial node to test the hypothesis that sialic acid residues (NANA) constitute much of the negative surface charge associated with hyperpolarization-activated, inward rectifying channels. Activation-voltage relationships (between −70 and −140 mV) were determined for hyperpolarization-activated (inward rectifying) current (i_f). Addition of 10 mM Ca^{2+} shifted the half-activation potential ($V_{1/2}$) from −89.5 ± 0.9 mV to −77.9 ± 2.6 mV ($P < 0.01$), confirming the presence of negative fixed charges on the myocytes after 3 to 5 days in culture. Addition of 20 mM dimethonium, an organic divalent cation that "screens" but does not bind to negative surface charge, shifted $V_{1/2}$ from −86.8 ± 1.4 mV to −75.0 ± 1.7 mV ($P < 0.001$) without affecting the amplitude of the current. In contrast, 10 mM Ca^{2+} reduced the amplitude of i_f significantly. Incubation of cells with a highly purified preparation of neuraminidase (0.1–2.0 U/ml, 1 hr, 37°C), an enzyme that selectively removes NANA from glycoproteins and glycolipids, failed to alter $V_{1/2}$ or the amplitude of i_f significantly. Pretreatment of cells with neuraminidase (1.0 U/ml, 1 hr, 37°C) failed to alter the positive shift of $V_{1/2}$ produced by dimethonium. The results suggest that NANA does not constitute the negative surface charge associated with hyperpolarization-activated, inward rectifying channels.

Key Words surface charge · sialic acid · sinoatrial node · neuraminidase

Introduction

It is well known that divalent cations can alter the surface potential near (or on) ion channels and thereby shift their conductance-voltage curves. Since the pioneering work of Frankenhaeuser and Hodgkin (1957), these effects have been explained by electrostatic attraction of the cations to fixed negative charges on the surface of the membrane. Although much is known about the function of these fixed charges, their identify has yet to be determined. In theory, they could arise from (i) the phosphate groups of the phospholipids, (ii) negatively charged groups of anionic phospholipids such as phosphatidylinositol and phosphatidylserine, (iii) acidic carbohydrates such as sialic acid and (iv) the anionic side chains or carboxy-terminal end of proteins.

The purpose of the present study was to test the hypothesis that sialic acid residues (N-acetylneuraminic acid; NANA) constitute much of this surface charge. We investigated whether the presence of NANA alters the voltage dependence or amplitude of hyperpolarization-activated current (i_f). This current, which is also referred to as the "inward rectifier" (i_h or i_Q), has been observed in both cardiac and neuronal cells. Its function is to promote pacemaker activity in the sinoatrial node, cardiac Purkinje fibers, hippocampal neurons and cerebellar Purkinje cells and to limit the amount of hyperpolarization a membrane can experience, e.g. in photoreceptors and sensory ganglion neurons (*see* DiFrancesco, 1985, for references).

A highly purified preparation of neuraminidase, an enzyme that selectively catalyzes the hydrolysis of the glycosidic linkage of NANA in glycoproteins and glycolipids (Drzeniek, 1973), was used to test the hypothesis. If NANA contributes to the surface charge, such a treatment would be expected to reduce the negative surface potential and shift the activation-voltage relationship for i_f in the positive direction. A preliminary report of this work has been published (Fermini & Nathan, 1988).

Materials and Methods

Cell Isolation and Culture

Single pacemaker cells were isolated from the sinoatrial node as described previously (Nathan, 1986*a*). Briefly, male albino rab-

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bits (0.8–1.5 kg) were anesthetized by cervical dislocation, and their hearts were removed rapidly and immersed in a low Ca^{2+} (50 μM) N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered balanced salt solution (HBSS) at room temperature. The ventricles were pinned to a silicon-coated petri dish (Sylgard; Dow Corning, Midland, MI), and the right atrium was removed. Then a region bounded by, but not including, the crista terminalis, the superior vena cava and the interatrial septum was excised and cut into small pieces. The minced tissue was incubated in 6 ml of nominally Ca^{2+}- and Mg^{2+}-free phosphate-buffered salt solution (PBSS, pH adjusted to 7.4 using 1.0 N NaOH) that contained 0.2% collagenase (type CLS II; Worthington Biochemical, Freehold, NJ), 5.5 μg/ml deoxyribonuclease (D-1001; Sigma Chemical, St. Louis, MO), and 0.1% bovine serum albumin (BSA: A-2153; Sigma). After the tissue had been incubated in this enzyme solution (and stirred with a 3 × 10 mm magnetic stir bar) for 10 min (37°C), the supernatant was discarded and 6 ml of fresh solution was added. After an 8-min period without stirring and a 2-min period with stirring, the supernatant was removed and added to 16 ml of HBSS containing 50 μM Ca^{2+} and 0.1% BSA (room temperature). Fresh enzyme solution was added and, after each of three 10-min incubations with stirring, the supernatant was added to the HBSS. The cell suspension was centrifuged at 180 g for 15 min, and the resulting pellet was resuspended in 0.5 ml of HBSS containing 50 μM Ca^{2+} and 0.1% BSA. The Ca^{2+} concentration was then increased in a step-wise fashion to 0.1, 0.2, 0.4, 0.8 and 1.8 mM, each after 5 min of equilibration. Finally, after centrifugation (180 g, 15 min), the cells were resuspended in a culture medium containing 1.8 mM Ca^{2+}, counted on a hemacytometer, and plated in 35-mm plastic culture dishes (Grand Island Biological, GIBCO, Grand Island, NY) at densities between 3 and 6 × 10^4 cells/dish. The cells were maintained in a CO_2 incubator (humidified atmosphere of 95% air and 5% CO_2; 37°C) for periods of 1–7 days. The experiments described below were performed at 36–37°C after 3–5 days in vitro.

Electrophysiology

We used the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Microelectrodes were pulled from 1.0-mm o.d. square-bore glass (Glass Company of America, Bargaintown, NJ) or borosilicate capillary glass (Sutter Instrument, San Rafael, CA) on a Flaming and Brown horizontal puller (model P80/PC; Sutter Instrument) and filled with the following solution (in mM): 130 KCl; 5 EGTA; 5 Na_2ATP; 1 $MgCl_2$; 10 HEPES; pH adjusted to 7.3 with KOH. Assuming that contaminant $[Ca^{2+}]$ was less than 50 μM, we calculated the pCa of this pipette solution to be >9.03 (Fabiato, 1988). The resistances of these electrodes ranged from 5–10 MΩ. The electrode potential measured in the bath was zeroed, but the data were not adjusted for the liquid-junction potential between the cytoplasm and pipette. A correction of approximately −5 mV (Désilets & Baumgarten, 1986) was not applied because this potential declined at variable rates, which depended on the size of the tip (Marty & Neher, 1983), during the course of the experiments. Whole-cell currents were recorded at 1 kHz bandwidth using a patch-clamp amplifier (model 8900; Dagan, Minneapolis, MN) with a 10-GΩ feedback resistor. Series resistance compensation was performed in some experiments but was unnecessary because of the current's slow kinetics. Currents were normalized for cell surface area, which was determined from microscopic measurements of the major and minor axes ($2a$ and $2b$) assuming that each cell was an oblate spheroid:

$$A = 2\pi a^2 + \pi b^2 \ln[(1 + \varepsilon)/(1 - \varepsilon)]/\varepsilon \quad (1)$$

where

$$\varepsilon = (a^2 - b^2)^{1/2}/a. \quad (2)$$

The membrane capacitance was determined from the capacitive current that flowed in response to voltage steps of ±5 mV from a holding potential of −60 mV. Under this condition, the amplitudes of time- and voltage-dependent currents were small compared with the capacitive current, as indicated by the almost mirror images of the capacitive surges in response to negative and positive clamp pulses. Because the capacitive transients could be well fit by a single exponential (correlation coefficient = 0.97 ± 0.01; n = 10), the input capacitance was approximated by

$$C = I_0\tau/\Delta V \quad (3)$$

where I_0 is the amplitude of the capacitive current at time zero, τ is the time constant of the decay, and ΔV is the amplitude of the voltage step. Both I_0 and τ were determined using *pClamp* software from Axon Instruments, Burlingame CA (Kegal et al., 1985).

Solutions

The bathing medium consisted of (in mM): 130 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 1.0 $MgCl_2$; 5.0 dextrose; 10 HEPES; pH 7.3 with NaOH. Tetrodotoxin (TTX; 30 μM, Sigma) was present in all experiments. The HBSS contained (in mM): 126 NaCl; 1.8 KCl; 0.8 $MgCl_2$; 5.5 dextrose; 25 HEPES; pH 7.4 with NaOH. The PBSS consisted of (in mM): 116 NaCl; 5.4 KCl; 0.4 NaH_2PO_4; 1.0 Na_2HPO_4; 5.6 dextrose. The culture medium contained 20% Medium 199 (GIBCO), 4% fetal bovine serum (GIBCO), 2% donor horse serum (GIBCO), 0.5% gentamicin sulfate (GIBCO), and 73.5% of a balanced salt solution composed of (in mM): 116 NaCl; 1.8 $CaCl_2$; 0.8 $MgSO_4$; 0.9 NaH_2PO_4; 26.0 $NaHCO_3$; 5.6 dextrose. Sera were heat inactivated at 56°C for 30 min. Neuraminidase (type X, Sigma) was dissolved in distilled water to yield a stock solution of 100 U/ml. This solution was frozen and, when needed, an appropriate volume was added to cells incubated in HBSS containing 1.8 mM $CaCl_2$. The amount of NANA released by neuraminidase was unaffected by such freezing and thawing (J.C. McDonagh and R.D. Nathan, *unpublished results*). Dimethonium bromide was provided by Dr. Stuart McLaughlin, State University of New York, Stony Brook.

Data Analysis

Membrane potentials or currents were stored on magnetic tape (3.75 in/sec; model 3968; Hewlett-Packard, Palo Alto, CA) and simultaneously digitized (model TM 80, Scientific Solutions, Solon, OH) and stored on the hard disk of an IBM PC/AT computer with the help of *pCLAMP* software. Hyperpolarization-activated current was taken to be the difference between current measured at the end of a 10-sec voltage step and that at the onset of the step. Isochronal (10-sec) activation-voltage relationships were determined from normalized current tails that were elicited at a holding potential of −60 mV and that followed voltage steps between −70 and −140 mV. The continuous curves in the figures were calculated from the relationship

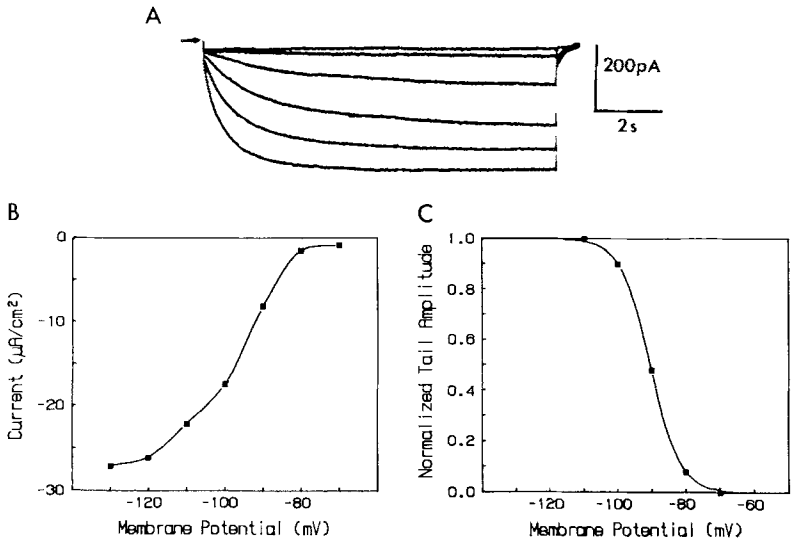

Fig. 1. Hyperpolarization-activated current (i_f) recorded from a pacemaker cell after 3 days in culture (*A*), the current-voltage relationship (*B*), and an isochronal activation-voltage relationship (*C*). The activation curve was determined from current tails in *A* that were elicited at a holding potential of −60 mV and that followed 10-sec steps (0.07 Hz) at potentials between −70 and −120 mV. The half-activation potential ($V_{1/2}$) was −90.4 mV, and the Boltzmann equation slope factor (k) was 4.3 mV. Arrow in *A* indicates 0 pA

$$I/I_{max} = 1/(1 + \exp[(V - V_{1/2})/k]). \quad (4)$$

These curves and values for the half-activation potential ($V_{1/2}$) and slope factor (k) were determined from a nonlinear least-squares curve-fitting program written for the IBM PC by W.N. Goolsby, Department of Anatomy and Cell Biology, Emory University, Atlanta, GA. Data are expressed as mean ± SEM. Student's t tests for paired or grouped data were used to evaluate the statistical significance of differences between means.

Results

Properties of the Cultured Pacemaker Cells

All recordings were performed on type II pacemaker cells (Nathan, 1986*a*) after 3–5 days in vitro. There were no obvious differences in electrical properties with time in culture. For ten cells, the average major and minor axes were 21.7 ± 1.1 and 16.0 ± 0.6 μm, and the surface area was 1,446 ± 143 μm². The input capacitance was 19.4 ± 2.6 pF and, when calculated for each cell and then averaged, the membrane capacitance was 1.40 ± 0.18 μF/cm², which is consistent with the value obtained previously for freshly isolated nodal cells (1.30 ± 0.24 μF/cm²; Nakayama et al., 1984). All of the 52 cells investigated in the present study exhibited rhythmic contractions and a hyperpolarization-activated current that could be blocked by Cs^+ (Nathan, 1986*a*). Figure 1 illustrates time-dependent currents elicited by 10-sec hyperpolarizing pulses applied from a holding potential of −60 mV (*A*) and the resulting current-voltage relationship (*B*). An *isochronal* (10-sec) rather than a steady-state activation curve is illustrated in *C*, since some of the currents in *A* did not reach a steady state. These and all other experiments were performed in the presence of 30 μM TTX to prevent contamination of the decaying tails by sodium current (i_{Na}) activated upon return to the holding potential (Nathan, 1986*a*); transient (T-type) calcium current is not activated at this potential (Hagiwara, Irisawa & Kameyama, 1988). For 10 cells, the mean current amplitude at −70 mV (and at 10 sec) was −21.3 ± 6.9 pA, $V_{1/2}$ was −86.8 ± 1.4 mV and k was 7.2 ± 0.5 mV. These values are similar to results obtained from some freshly isolated pacemaker cells (Nakayama et al., 1984; DiFrancesco et al., 1986) and multicellular sinoatrial node preparations (Yanagihara & Irisawa, 1980).

Confirmation of the Presence of Negative Surface Charge

In order to confirm that negative fixed charges on or near hyperpolarization-activated channels had not been permanently removed during the cell isolation procedure, we added 10 mM $CaCl_2$ to the bath. As would be expected for a reduction of the negative surface potential if Ca^{2+} were to "screen" and bind to the surface charge, the activation curve for i_f was shifted to more positive potentials (Fig. 2*A*). For five cells in which continuous recordings were performed, $V_{1/2}$ was shifted from −89.5 ± 0.9 to −77.9 ± 2.6 mV ($P < 0.01$, paired t test), while the slope factor remained unchanged (6.2 ± 0.7 and 6.5 ± 1.1 mV, $P > 0.05$). The magnitude of this shift, 4.8 mV/e-fold change in $[Ca^{2+}]$, is consistent with calcium's effect on the activation of i_{K2} (now known as i_f) in cardiac Purkinje fibers (*see* Table 1 in Nathan, 1986*b*, for references). In addition to shifting the activation curve, 10 mM Ca^{2+} significantly reduced

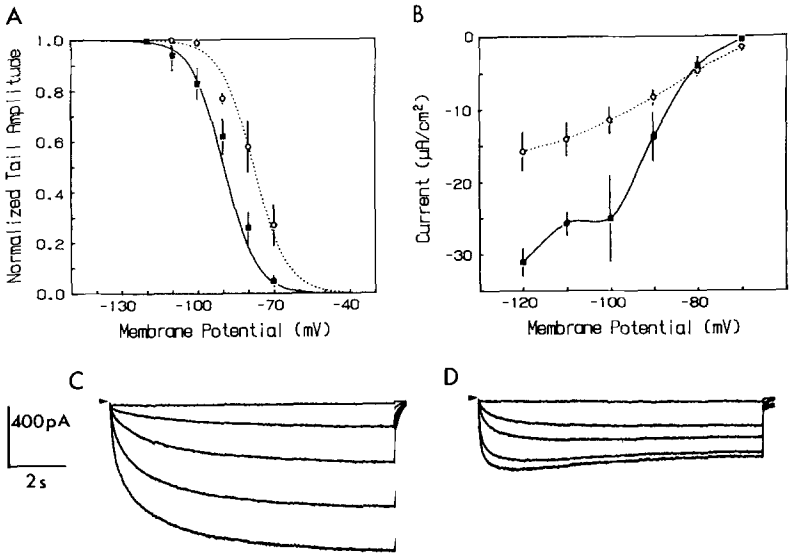

Fig. 2. Effect of 10 mM $CaCl_2$ on activation (*A*) and amplitude (*B*) of i_f recorded 5 min after its addition. (*A*) $V_{1/2}$ was shifted from -89.5 ± 0.9 to -77.9 ± 2.6 mV ($P < 0.01$, paired t test), while k was unchanged (6.2 ± 0.7 and 6.5 ± 1.1 mV; $P > 0.05$). (*B*) High calcium reduced the mean current significantly ($P < 0.05$, paired t test) at -110 and -120 mV. Filled squares: controls. Open circles: addition of 10 mM $CaCl_2$. Each point represents the mean ± SEM for five cells. (*C* and *D*) Voltage-dependent "relaxation" of i_f induced by high external calcium. Membrane potentials were -80, -100, -110, -120, -130 mV before (*C*) and -80, -100, -110, -130, -140 mV, 5 min after (*D*) external Ca^{2+} was increased from 1.8 to 11.8 mM. In this cell, relaxation of the current was seen at potentials negative to -120 mV. Arrows indicate 0 pA. Holding potential was -60 mV

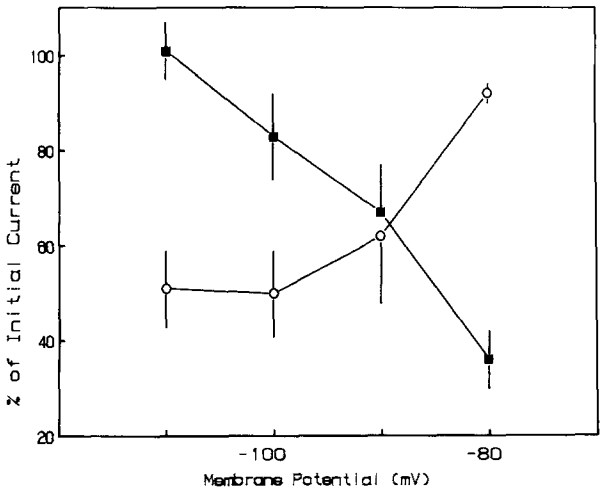

Fig. 3. Comparison of the reduction of i_f 5 min after addition of 10 mM $CaCl_2$ (open circles; $n = 5$) and the reduction of i_f in 1.8 mM $CaCl_2$, 20 min after penetrating the membrane patch (filled squares; $n = 6$)

the amplitude of i_f (Fig. 2*B*) and, in three of five cells, produced "relaxation" of the current at potentials negative to -120 mV (*compare* Fig. 2*C* and *D*).

As displayed by other ionic currents (e.g., Ca^{2+}; Belles et al., 1988), i_f exhibits "rundown," which is characterized by a progressive decline in the amplitude of the current and a rather rapid shift of the activation curve to more negative potentials (DiFrancesco et al., 1986). Thus, we compared the voltage dependence of the current decline in the presence of high Ca^{2+} with the rundown of i_f in 1.8 mM Ca^{2+} (Fig. 3). The two were very different: the reduction associated with rundown decreased at more negative potentials, whereas that due to high Ca^{2+} *increased* at more negative potentials. While we cannot rule out the possibility that Ca^{2+} enhances the rundown of i_f, it is unlikely that the elevation of extracellular Ca^{2+} would enhance *intracellular* Ca^{2+}, since the pCa of the pipette solution was buffered to >9.0. A more likely explanation is that elevated concentrations of external Ca^{2+} can block the current (*see* Discussion).

Effect of Neuraminidase

Having shown that negative surface charge is indeed present on or near hyperpolarization-activated channels, we proceeded to remove most of the sialic acid residues on the membrane by using a highly purified preparation of neuraminidase. When the cells were incubated with this enzyme for 1 hr (0.1–2.0 U/ml; 37°C), neither the half-activation potential nor the slope factor was changed significantly (Table). Moreover, lengthening the incubation in 1.0 U/ml to two hours, which is twice as long as the period for asymptotic release of NANA (Bhattacharyya, Nathan & Shelton, 1981), also failed to have an effect. To rule out the possibility of a transient effect, we added neuraminidase (1.0 U/ml) to the bath while recording i_f, but saw no effect for the duration of the recordings (10–30 min). These results do not support the hypothesis that sialic acid residues constitute the negative surface charge that is closely associated with the gating "sensor" of hyperpolarization-activated channels. Finally, following 1-hr incubations in four different concentrations of the enzyme (0.1–2.0 U/ml), none

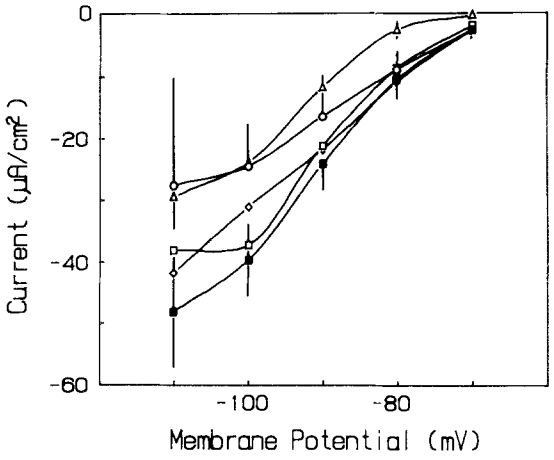

Fig. 4. Average current densities measured in cells treated with neuraminidase for 1 hr (37°C). Filled squares: controls (n = 10). Open circles: 0.1 U/ml (n = 5). Open triangles: 0.2 U/ml (n = 5). Open squares: 1.0 U/ml (n = 7). Open diamonds: 2.0 U/ml (n = 5). As determined by Student's t test for grouped data, none of the values measured after treatment with neuraminidase were significantly different from the controls ($P > 0.05$)

Table. Neuraminidase has no significant effect on i_f activation parameters

Condition	n	Half-activation potential (mV)	Slope factor (mV)
Control	10	−86.8 ± 1.4	7.2 ± 0.5
Neuraminidase (0.1 U/ml)	5	−84.2 ± 2.0	6.8 ± 1.0
Neuraminidase (0.2 U/ml)	5	−88.1 ± 1.9	5.6 ± 0.8
Neuraminidase (1.0 U/ml)	7	−85.9 ± 3.0	6.9 ± 0.6
Neuraminidase (2.0 U/ml)	5	−83.8 ± 1.1	7.7 ± 0.8

Cells were treated for 1 hr at 37°C. Values are mean ± SEM and n = number of cells. As determined by t tests for grouped data, none of the differences are statistically significant.

of the current amplitudes differed significantly from the controls ($P > 0.05$) at any of the potentials investigated (Fig. 4).

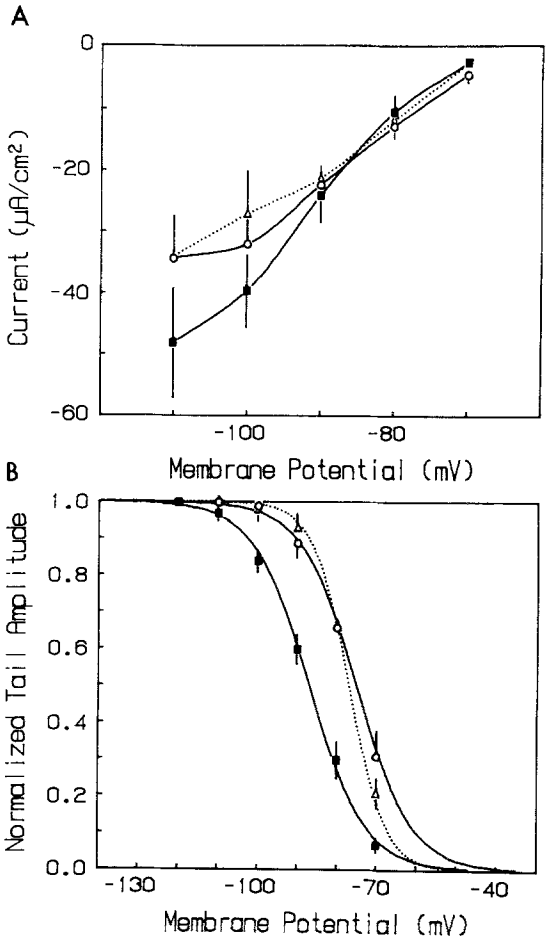

Fig. 5. Effect of 20 mM dimethonium bromide on the amplitude of i_f (A) and its activation (B) in controls and cells pretreated for 1 hr (37°C) with neuraminidase (1.0 U/ml). Changes in mean current density were not statistically significant in either type of experiment ($P > 0.05$); however, the shifts of $V_{1/2}$ from −86.8 ± 1.4 mV (controls) to −75.0 ± 1.7 mV (dimethonium) or to −76.9 ± 1.5 mV (neuraminidase pretreatment followed by dimethonium) were highly significant ($P < 0.001$; t test, grouped data). Neuraminidase failed to alter the shift of the i_f activation curve produced by dimethonium; neither the half-activation potentials (above) nor the normalized tail amplitudes were significantly different. The slope factor for cells pretreated with neuraminidase (k = 4.6 ± 0.5 mV) was significantly smaller than the ones for control (k = 7.2 ± 0.5 mV) or dimethonium-treated cells (k = 7.0 ± 0.8 mV). Filled squares: controls (n = 10). Open circles: 20 mM dimethonium (n = 6). Open triangles: neuraminidase pretreatment followed by 20 mM dimethonium (n = 6)

Neuraminidase Fails to Alter the Shift in Activation Produced by Dimethonium

Dimethonium, an organic divalent cation that has been shown to screen but not bind to negative surface charge (McLaughlin et al., 1983), was used to avoid the unwanted effects of Ca^{2+} on the amplitude of the current. In the presence of 20 mM dimethonium bromide, i_f decreased in amplitude at both −100 and −110 mV (Fig. 5A). However, such changes were not statistically significant ($P > 0.05$), and we failed to observe any voltage-dependent relaxation of the current. On the other hand, dimethonium did produce a significant positive shift of the activation curve for i_f (Fig. 5B), providing additional evidence for the presence of negative fixed charges

on or near hyperpolarization-activated channels. Normalized current tails recorded from six cells in the presence of 20 mM dimethonium bromide gave an average $V_{1/2}$ of -75.0 ± 1.7 mV, an 11.8-mV shift from the control value ($P < 0.001$); the slope factor was not changed significantly (7.2 ± 0.5 and 7.0 ± 0.8 mV, $P > 0.05$). Pretreating cells with neuraminidase (1.0 U/ml) for 1 hr (dotted curves in Fig. 5) failed to alter either the amplitude of i_f or the voltage shift produced by dimethonium; $V_{1/2}$ was -76.9 ± 1.5 mV ($n = 6$). These results indicate that negatively charged moieties *other than* sialic acid must be screened by dimethonium to produce the shift of the activation curve.

It is interesting that after cells had been treated with neuraminidase, the slope factor was reduced significantly ($P < 0.01$) by the addition of dimethonium (dotted curve, Fig. 5*B*; $k = 4.6 \pm 0.5$), whereas it was unchanged by neuraminidase (Table) or dimethonium alone (Fig. 5*B*). One explanation for this effect is that neuraminidase might have removed NANA residues that had prevented dimethonium from binding to the surface charge. Because of the discrete nature of membrane surface charge, such binding can increase the slope of activation/inactivation curves when the fraction of time the charge is bound exceeds 0.5 (Attwell & Eisner, 1978). Our hypothesis assumes that calcium ions, unlike dimethonium, had access to the surface charge both before and after the removal of NANA. Thus, neuraminidase, alone, would have had little effect on Ca^{2+} binding and the shape of the activation curve. Moreover, without neuraminidase pretreatment, addition of dimethonium would not have altered the shape either, since this cation usually screens but does not bind to surface charge (McLaughlin et al., 1983), and *binding* is required to change the shape of the activation curve.

Rundown of i_f

DiFrancesco et al. (1986) have shown that in freshly isolated sinoatrial node cells, the size of i_f tends to diminish in the course of an experiment, and this decrease is accompanied by a negative shift of the activation curve along the voltage axis. It was our concern that the lack of effect observed in cells treated with neuraminidase could have resulted from competition between a negative shift produced by rundown and a positive shift produced by the removal of negative surface charge. We assessed the importance of rundown in our preparation by measuring i_f immediately after initiating the whole-cell clamp and then 20 min later. Significant rundown was seen in three of six untreated cells. Within 20 min, the half-activation potential had shifted an average of -7.1 mV, and i_f had declined in two of the cells. In contrast, after a 1-hr treatment with neuraminidase (0.2–2.0 U/ml), only four of 11 cells displayed any rundown. When all 11 of these cells were taken into account, the mean values of $V_{1/2}$ and k did not change significantly, even after 20 min of recording. These results indicate that rundown of i_f also occurs in *cultured* pacemaker cells from the sinoatrial node. However, when these cells are treated with neuraminidase, they exhibit less rundown than do freshly isolated cells (DiFrancesco et al., 1986). More importantly, the absence of a positive shift of the activation curve in neuraminidase-treated cells cannot be explained by an equal and opposite shift due to rundown.

Discussion

Effect of Ca^{2+} on i_f

When cultured pacemaker cells from the sinoatrial node were exposed to a 10-mM increase in external Ca^{2+}, the activation curve for i_f was shifted 12 mV in the positive direction. The magnitude of this shift is similar to that observed previously in cardiac Purkinje fibers, suggesting that the surface-charge densities of the two pacemaker preparations are similar. For example, Kass and Tsien (1976) found a 4-mV positive shift of the steady-state activation curve for i_{K2} (i_f) in calf Purkinje fibers when external Ca^{2+} was increased from 1.8 to 7.2 mM, whereas 8-mV (Brown & Noble, 1978) or 10-mV (DiFrancesco & McNaughton, 1979) shifts were found for sheep Purkinje fibers.

The results showing that Ca^{2+} affects the amplitude of i_f are interesting, considering the fact that Ca^{2+} itself is not a charge carrier for this current (Maylie & Morad, 1984). Rundown of calcium current (i_{Ca}) is accelerated by processes that increase intracellular Ca^{2+} (Belles et al., 1988); therefore, the observed decrease in i_f in the presence of high Ca^{2+} (Fig. 2*B* and *D*) might also be explained by increased rundown. However, several findings argue against this: (i) the voltage-dependent reductions of i_f in 1.8 mM Ca^{2+} and in 11.8 mM Ca^{2+} are completely different (Fig. 3); (ii) the positive shift of the i_f activation curve induced by 10 mM Ca^{2+} (Fig. 2*A*) is opposite to the shift usually seen with rundown (DiFrancesco et al., 1986); and (iii) a dependence on intracellular Ca^{2+} is unlikely, since the pCa of the pipette solution was buffered to >9.0, and elevation of intracellular Ca^{2+} would have *enhanced* i_f (Hagiwara & Irisawa, 1989). Considering that the decline in current amplitude with dimethonium was not statistically significant (Fig. 5*A*), it would appear that

calcium's effect on i_f did not derive from a reduction of the negative surface potential and, therefore, the local concentration of cations near the mouth of the channel. Instead, Ca^{2+} probably was acting as a channel blocker. In fact, high external Ca^{2+} has been shown to block sodium channels in single cardiac Purkinje cells (Sheets et al., 1987) and guinea pig ventricular myocytes (Nilius, 1988). As was observed for i_f, the block of i_{Na} by Ca^{2+} was stronger at more negative potentials.

The idea that Ca^{2+} blocks i_f is also consistent with the "relaxation" of i_f at potentials negative to −120 mV (Fig. 2*D*). Such a decay is similar to the inactivation of inward rectifying potassium current (i_{K1}) during hyperpolarizing steps negative to −100 mV. Biermans, Vereecke and Carmeliet (1987) have shown that, together with Na^+ and Mg^{2+}, even physiological concentrations of extracellular Ca^{2+} can block these channels. In the absence of both Na^+ and divalent cations, i_{K1} was much larger and showed virtually no decay in comparison with solutions containing 1.8 mM Ca^{2+}. Although we did not see voltage-dependent relaxation of i_f in the presence of 1.8 mM Ca^{2+}, it could be a dose-related phenomenon unmasked by higher concentrations of Ca^{2+} that compete with Na^+ for entry into the channel.

Sialic Acid Residues and the Surface Charge

Our results show that sialic acid residues do not influence the gating or permeation of hyperpolarization-activated channels in cultured pacemaker cells from the rabbit sinoatrial node. Using a highly specific preparation of neuraminidase to cleave NANA from the cell surface, we were unable to see any significant effect of this removal on: (i) the activation of i_f (i.e., the half-activation potential and slope factor); (ii) the amplitude of i_f at the end of 10-sec voltage steps; or (iii) the positive shift of the half-activation potential produced by 20 mM dimethonium. It could be argued that the absence of an effect was due to partial or complete loss of the glycocalyx during the cell isolation procedure (Schanne, Bkaily & Fermini, 1983; Lee & Hsu, 1987). However, sialic acid residues on the cell surface regenerate in culture within 24 hr (Kraemer, 1966; Collins, Holland & Sanchez, 1973; B. Fermini, *unpublished results*) and can be detected on cardiac myocytes after several days in culture (Frank et al., 1977; Barron, Markwald & Nathan, 1982). In the present study, positive shifts of the activation curve following the addition of Ca^{2+} (Fig. 2*A*) or dimethonium (Fig. 5*B*) confirmed that negative surface charge was indeed present on or near the gating "sensor" of hyperpolarization-activated channels when the pacemaker cells had been in culture for 3 to 5 days.

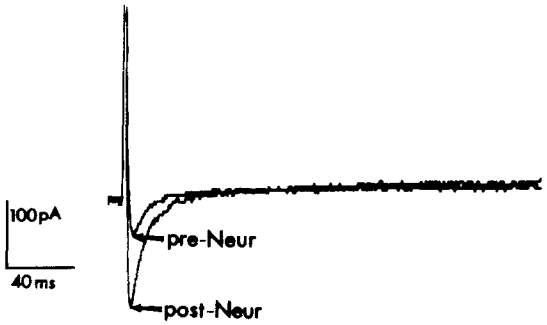

Fig. 6. Neuraminidase enhances calcium current. Superimposed current traces recorded from a sinoatrial node cell after 3 days in culture. Voltage-clamp pulses to −40 mV (450-msec duration) were elicited from a holding potential of −90 mV, before and after a 10-min exposure to neuraminidase (1.0 U/ml; 36°C). In this example, the current increased by 129%; similar changes were seen in four other cells. The bathing medium consisted of (in mM): 126 NaCl; 5.4 KCl; 20 CsCl; 0.8 $MgCl_2$; 2.5 $CaCl_2$; 0.33 NaH_2PO_4; 5.5 dextrose; 5.0 HEPES; and 0.03 TTX; pH adjusted to 7.3 with NaOH. The pipette was filled with (in mM): 120 CsCl; 20 tetraethylammonium chloride; 1.0 $MgCl_2$; 5.0 MgATP; 5.0 Na_2 creatine PO_4; 10 EGTA; and 5.0 HEPES; pH adjusted to 7.3 with CsOH

Is it possible that NANA *does* account for much of this charge but that neuraminidase failed to remove it? Although we cannot rule out this possibility, it is unlikely because the same preparation of neuraminidase used in the present study (1.0 U/ml, 37°C) removed more than 85% of the surface NANA on embryonic chick ventricular myocytes during 1-hr treatments (McDonagh & Nathan, 1988). Two other results support the idea that the enzyme was effective: (i) pretreatment with neuraminidase increased the slope of the i_f activation curve following the addition of dimethonium (Fig. 5*B*); and (ii) neuraminidase (1.0 U/ml) enhanced i_{Ca} by more than twofold during 10-min treatments (Fig. 6). Such an increase in i_{Ca}, which ranged from 96–403% in five pacemaker cells, has also been seen in guinea pig ventricular myocytes. (Yee, Weiss & Langer, 1989).

Because surface potentials influence the gating and permeation of ion channels, functions essential for pacemaker activity of the heart, it is important to identify the fixed charges that generate these potentials. Our results suggest that sialic acid residues do not influence hyperpolarization-activated channels. The data do not, however, exclude the possibility that these residues are present on the sarcolemma. Rather, the results imply only that NANA either is not associated with this particular type of

channel or is too distant from the gating "sensor" to influence it. Until hyperpolarization-activated channels have been purified and reconstituted in lipid bilayers, it will be difficult to distinguish between these two possibilities. On the other hand, NANA *is* known to constitute 10–12% of the total weight of the sodium channel α subunit (Barchi, 1988). When eel electroplax or rat brain sodium channels are treated with neuraminidase and then incorporated into lipid bilayers in the presence of batrachotoxin (BTX), they exhibit reduced conductance states, suggesting that NANA might help to stabilize the normal conductance state of BTX-activated sodium channels (Recio-Pinto et al., 1987; Scheuer et al., 1988). Thus, sialic acid residues might be important for the function of some types of ion channels.

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