J. Membrane Biol. 56, 43–48 (1980)

The Journal of
Membrane Biology

Action of Glucosamine on Acetylcholine-Sensitive Channels

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Summary. The action of glucosamine was studied on voltage clamped neurones of *Aplysia*, presenting an excitatory response to acetylcholine. Noise and relaxation experiments show that glucosamine increases the mean channel open time and reduces the amplitude of the elementary current associated with the acetylcholine response. Both effects are enhanced by hyperpolarization of the cell membrane. The results are interpreted by a model assuming glucosamine binding to open channels. This binding impedes the flow of permeant ions and decreases the closing rate of the channels.

The excitatory response to acetylcholine (ACh) at the vertebrate neuromuscular junction, at the eel electroplaque, or an *Aplysia* neurones results from the opening and closing of membrane channels selective for small cations, mainly Na and K (Fatt & Katz, 1951; Takeuchi & Takeuchi, 1960; Lassignal & Martin, 1977; Ascher, Marty & Neild, 1978*a*). A standard procedure in the study of such channels is to replace part or all of the external Na ions by "inert" monovalent cations. Such substitutes for Na should ideally neither cross the channels, nor interact in any way with channel gating or permeation of the remaining ions (e.g., intracellular K ions). However, such requirements may be very difficult to meet. Many small inorganic or organic cations cross the ACh sensitive channels of vertebrate muscle cells (Koketsu & Nishi, 1959; Maeno, Edwards & Anraku, 1977; Huang, Catterall & Ehrenstein, 1978). Other cations such as TEA (Koketsu, 1958; Parsons, 1969; Ascher et al., 1978*b*) or Tris (Ascher et al., 1978*a*) block the cholinergic response, perhaps by interacting with the ACh sensitive channel.

We report here a study of the effects of another widely used substitute for Na: glucosamine. This compound was recently shown to be slightly permeant in eel electroplaques (Lassignal & Martin, 1977) and almost impermeant at the frog neuromuscular junction (Maeno et al., 1977; Dwyer, Adams & Hille, 1979). After replacing part of the extracellular Na with glucosamine, we observed marked modifications of the mean channel open time and of the elementary current. The results suggest that glucosamine blocks open channels, but has little or no interaction with closed channels.

Materials and Methods

The set up and the main experimental procedures have been described previously (Ascher et al., 1978*a*) and will only be briefly summarized here. Cells of an identified family of neurones of the right pleural ganglion of *Aplysia californica* were voltage clamped. ACh was applied ionophoretically in long (~2 min) pulses. The resulting current was analyzed with noise or relaxation techniques.

Two-State Model

It was previously shown that the results of noise and relaxation experiments obtained in normal seawater can be explained by a "two-state model" (see Ascher et al., 1978*a*) similar to the model put forward at the frog endplate (Katz & Miledi, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1975):

$$R \underset{q}{\overset{p}{\rightleftharpoons}} R^*. \tag{1}$$

Here R and R^* represent the closed and open configurations of the receptor channel complex, respectively. p and q are the rate constants governing the opening of closed channels and the closing of open channels, respectively.

Provided that the "low concentration limit" is valid (i.e., that $p \ll q$: *see* Anderson & Stevens, 1973), the mean open time of the channels is given by the relation

$$\tau = \frac{1}{2\pi f_c} = \frac{1}{q} \tag{2}$$

where f_c is the cut-off frequency of the noise power spectrum. Under the same assumption, τ is equal to the time constant measured in relaxation experiments. We took care in the present work to stay in the experimental conditions where the relation $p \ll q$ was previously found to hold true (Ascher et al., 1978*a*; Marchais & Marty, 1979).

Noise Analysis

Each noise power spectrum was an average of 12 spectra calculated from 512 points samples. The control spectra were small enough to be neglected in the frequency range studied (0–50 Hz or 0–100 Hz). The spectra of the ACh-induced current were fitted by eye with a Lorentzian curve to yield the cut-off frequency f_c. The variance increase associated with the ACh-induced current was determined with an analog variance reader. The elementary cur-

0022-2631/80/0056-0043 $01.20

rent, i_{el}, was then calculated as the ratio of the variance over the mean of the ACh-induced current.

Relaxation Experiments

In relaxation experiments the background current obtained by changing the potential in the absence of ACh was usually subtracted before analysis. We assumed that the change of the ACh-induced current observed just after the voltage jump simply reflects the variation of the elementary current with voltage. (This point will be considered again in the Discussion). As previously (Marchais & Marty, 1979), we will call *amplitude* of the relaxation the ratio

$$A=\frac{I_{ss}(V)}{I_{in}(V)}$$

where $I_{in}(V)$ is the "instantaneous" ACh-induced current value obtained just after a jump from the holding potential H to a new voltage V, and $I_{ss}(V)$ the steady-state ACh-induced current at the voltage V. It was previously shown that under the assumptions of the two-state model:

$$A=\frac{p(V)}{p(H)}\times\frac{\tau(V)}{\tau(H)} \tag{3}$$

and that in normal seawater,

$$A=\frac{\tau(V)}{\tau(H)} \tag{4}$$

so that p does not depend on V (Ascher et al., 1978*a*).

Control and Test Solutions

The control solution contained (in mM): NaCl, 480; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer at pH 7.8, 5 mM. In our usual test solution, called 1/4 glucosamine seawater hereafter, 1/4 of the Na ions were replaced by 120 mM glucosamine (D-glucosamine hydrochloride, Sigma). Glucosamine was first titrated to pH 7.8 with NaOH and the quantity of NaCl was adjusted to obtain a total of 360 mM Na ions. At pH 9.6, the solutions were buffered with 10 mM Na tetraborate instead of Hepes.

As the Cl concentration was different from one extracellular solution to another, changing solutions induced slight potential changes at the level of the Ag-AgCl ground electrode. These shifts were measured with the voltage recording electrode and the appropriate correction (always less than 6 mV) was made on the holding potential during the course of the experiment.

Surface Potential Changes

Because a fraction of glucosamine molecules is neutral at pH 7.8, the ionic strength of the glucosamine substituted seawater was less than that of the control seawater. Calculations of the kind previously used (Marchais & Marty, 1979) indicate, however, that the corresponding surface potential change was probably quite small (less than 5 mV in $\frac{1}{2}$ glucosamine seawater). Surface potential changes were therefore neglected in the interpretation of the results.

Results

Relaxation Experiments

Figure 1 illustrates the result of a relaxation experiment performed before and after substitution of 1/4

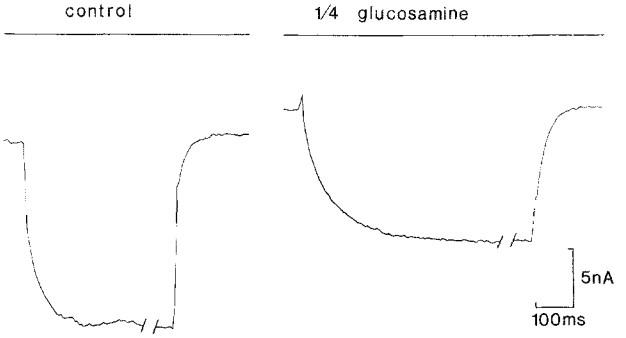

Fig. 1. Relaxation experiment in the presence of glucosamine. −60 to −100 mV voltage jumps. The voltage was held during 1 sec at −100 mV, but only the relaxations following the voltage changes are shown (notice the gaps in the records at −100 mV). The background current (obtained in the absence of ACh) has been subtracted. Its value was not affected by glucosamine. Lines indicate 0 current level. *Left:* control seawater. $\tau(-100)=36$ msec; $\tau(-60)=23$ msec. Amplitude of the relaxation (*see* Methods): $A=1.8$ measured at −100 mV; $A=1.8$ measured at −60 mV. *Right:* 1/4 glucosamine seawater. $\tau(-100)=80$ msec; $\tau(-60)=26$ msec. $A=3.0$ measured at −100 mV; $A=3.3$ measured at −60 mV. The amplitude of the relaxation is larger in the presence of glucosamine than in the control. τ is much larger in the presence of glucosamine at −100 mV, while the values at −60 mV are comparable. It is possible to obtain the instantaneous current at the onset of a voltage jump by extrapolation of the relaxation to the starting time of the jump. This allows one to calculate an instantaneous current ratio (−100/−60 mV) of 1.5 (control seawater) and 0.9 (1/4 glucosamine seawater). (The ratios at −100 and −60 mV were in agreement.) This result suggests that, in the presence of glucosamine, the elementary current is slightly *smaller* at −100 than at −60 mV. *Note* that the noise of the ACh-induced current is much more prominent in control seawater than in the presence of glucosamine. The current was filtered by two second-order filters at 100 and 300 Hz. Temperature 12 °C. Neostigmine 5×10^{-5} M. All effects were fully reversed when returning to control seawater (not illustrated).

of the extracellular Na ions by glucosamine. The current traces shown were obtained for voltage jumps from −60 to −100 mV and back. The relaxations follow single exponentials both in control and in 1/4 glucosamine seawater. At −100 mV, the time constant of the exponential, τ, is much larger in the presence of glucosamine than in the control. τ is also increased at −60 mV in 1/4 glucosamine seawater, but this effect is much less pronounced than at −100 mV.

The relaxation amplitude (*see* Methods) is larger in the presence of glucosamine ($A=3.0$) than in the control ($A=1.8$). The corresponding τ ratios are $\frac{\tau(-100)}{\tau(-60)}=3.1$ and 1.6, respectively. Thus, the values found for A are close to the values of the ratios $\frac{\tau(-100)}{\tau(-60)}$. If one refers to Eq. (3), this indicates that the opening rate p is not voltage sensitive both in the control (as previously observed: Ascher et al., 1978*a*) and in the presence of glucosamine.

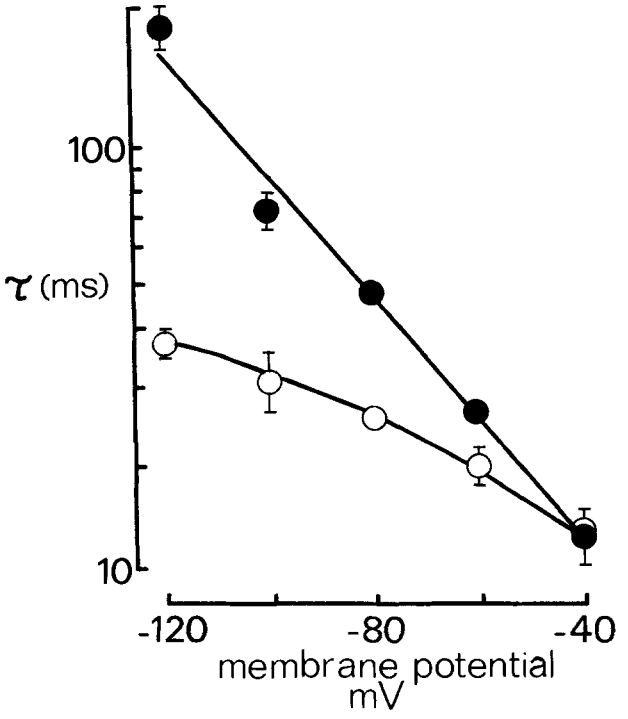

Fig. 2. Relaxation time constant as a function of membrane potential. (○): control seawater. (●): 1/4 glucosamine seawater. Error bars: ±SEM ($n=3$ (○) or 4 (●)); when smaller than the symbols, error bars are not indicated. The slope of the curve is stronger in 1/4 glucosamine seawater (*e*-fold/32 mV) than in control seawater. Temperature, 12 °C.

Other relaxations recorded in 1/4 glucosamine seawater gave a similar agreement between the value of A and the ratio $\frac{\tau(-100)}{\tau(-60)}$ (4 experiments). However, in some experiments, we found that A was larger than the corresponding τ ratio. Occasionally, the same applied also to the control relaxation in normal seawater. Because of the inconsistency of these results, they were attributed to some technical flaw (perhaps a space clamp defect) rather than to a genuine departure from Eq. (4).

Figure 2 gives average results on the variation of τ with membrane potential at 12 °C in control and in 1/4 glucosamine seawater. At hyperpolarized potentials, glucosamine causes a clear increase of τ. On the other hand, τ is not prolonged by glucosamine at −40 mV.

Comparing Fig. 1 *A* and *B*, it can be seen that the instantaneous current changes are strongly affected by glucosamine. Instead of the large instantaneous current increase seen in normal seawater for a −60 to −100 mV voltage jump, one observes a slight *decrease* in Fig. 1 *B*, suggesting (*see* Methods) that in 1/4 glucosamine seawater the elementary current decreases somewhat when the membrane is hyperpolarized beyond −60 mV.

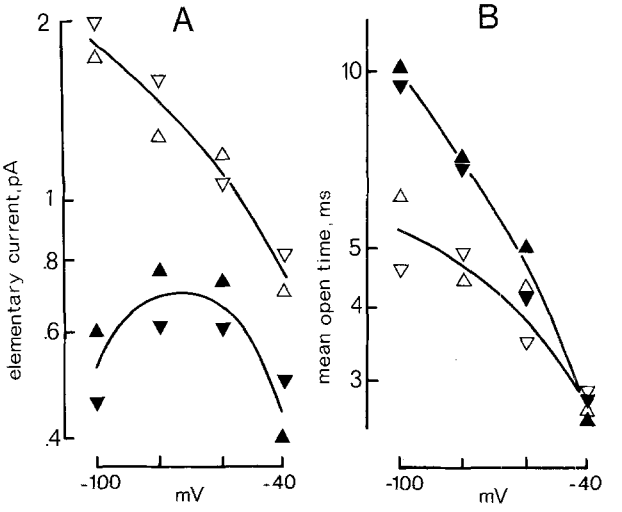

Fig. 3. Effects of glucosamine on i_{el} and τ. The elementary current (*A*) and the mean channel open time (*B*) were determined by noise analysis on two different cells between −40 and −100 mV, in control (△, ▽) and 1/4 glucosamine seawater (▲, ▼). Temperature, 20 °C. Curves were drawn by eye.

Noise Characteristics in the Presence of Glucosamine

Figure 3 shows the results of two noise analysis experiments performed at room temperature in control and 1/4 glucosamine seawater. Substitution of 1/4 of the Na ions by glucosamine induces at all potentials a reduction of the elementary current (Fig. 3 *A*). This reduction is about twice stronger at −100 mV than at −40 mV (factor 3.6 at −100 mV and 1.7 at −40 mV); as expected from relaxation experiments, the elementary current decreases from −60 to −100 mV in the presence of glucosamine. Noise power spectra were Lorentzian both before and during application of glucosamine. (At 12 °C, no significant deviation from a single Lorentzian could be detected up to 10 times the cut off frequency.) The effects of replacement of Na by glucosamine on the mean channel open time calculated from the noise power spectra are shown in Fig. 3 *B*. They are very similar to those observed at 12 °C in relaxation experiments (*compare* Figs. 2 and 3 *B*).

Other experiments gave results consistent with those illustrated in Fig. 3. Table 1 summarizes results obtained at −60 and −80 mV in 6 independent experiments (including those of Figs. 3 and 4).

Steady-State Current

Partial replacement of Na with glucosamine results in a reduction of the steady-state ACh-induced current. At 12 °C, the reduction in 1/4 glucosamine seawater was to 82±12 % of the control (mean±SE, 5 experi-

Table 1. Averaged ratios of τ and i_{el} values in 1/4 glucosamine and control seawater

V (mV)	$\frac{i_{el}(1/4\ \text{glucosamine})}{i_{el}(\text{control})}$	$\frac{\tau(1/4\ \text{glucosamine})}{\tau(\text{control})}$
−60	0.58 ± 0.01 (6)	1.2 ± 0.1 (6)
−80	0.47 ± 0.03 (5)	1.51 ± 0.08 (5)

Noise experiments were performed on a total of 6 cells at 20–23 °C, in control and in 1/4 glucosamine seawater. Means (and SEM) of the i_{el} and ratios are indicated.

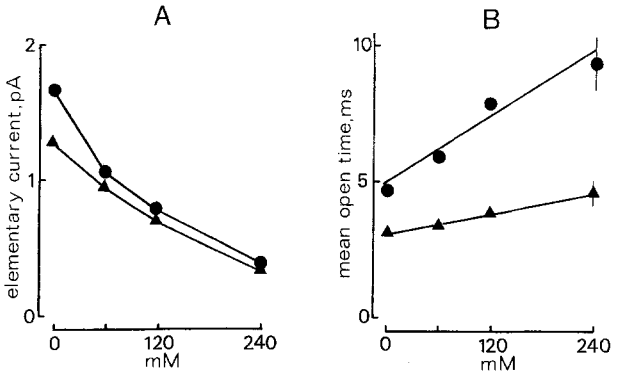

Fig. 4. Effects on i_{el} and τ of progressive replacement of Na by glucosamine. Acetylcholine current fluctuations were recorded in control seawater and after replacement of 1/8, 1/4 and 1/2 extracellular Na ions by glucosamine. Elementary current (*A*) and mean channel open time (*B*) are drawn as a function of glucosamine concentration at −60 mV (▲) and −80 mV (●). As already observed in Figs. 1–3, the effects of glucosamine are enhanced by membrane hyperpolarization. After substitution of half of the Na ions by glucosamine, the estimate of τ was not very accurate (vertical bars indicate the uncertainty of this determination). Temperature, 26 °C.

ments) at −60 mV and 67 ± 9 % at −100 mV. This reduction corresponds roughly to what is expected from the variation of the product $i_{el}\ \tau$ calculated from the results of noise experiments (*see* Fig. 3). Therefore the data suggest that glucosamine has little or no effect on the opening rate p.

Influence of Glucosamine Concentration

All the experiments reported above deal with replacement of 1/4 of the Na ions by glucosamine. In the experiment illustrated in Fig. 4, ACh current fluctuations at −60 and −80 mV were analyzed after replacement of 1/8, 1/4 and 1/2 of the extracellular Na ions by glucosamine. The prolongation of τ and the reduction of i_{el} by glucosamine are clearly dependent on the amount of glucosamine present in the extracellular medium. The estimates of τ in 1/2 glucosamine seawater are not very accurate, but the results are consistent with a linear dependence of τ on glucosamine concentration (Fig. 4*B*). The reduction of i_{el} by glucosamine is also strongly dose dependent, with a 4.5-fold reduction at −80 mV in 1/2 glucosamine seawater (Fig. 4*A*).

Table 2. Replacement of Na by glucosamine at pH 9.6

Cell #	V (mV)	i_{el} (pA) no glucosamine	i_{el} (pA) 1/4 glucosamine	τ (ms) no glucosamine	τ (msec) 1/4 glucosamine
1	−60	1.0	0.81	23	26
	−80	1.2	0.85	28	34
2	−60	0.93	0.68	26	20
	−80	1.0	0.90	31	25

In two noise experiments, i_{el} and τ were measured on the same cell at −60 and −80 mV in control seawater (data not shown), in pH 9.6 seawater, and in 1/4 glucosamine, pH 9.6 seawater. At this pH, substituting glucosamine for Na does not alter i_{el} or τ markely. Temperature, 12 °C

The Active Form of Glucosamine is Positively Charged

Glucosamine has a titrable amine group with a pK which we estimated at 7.8. Since this is the pH of normal seawater, 50 % of the glucosamine molecules were neutral and 50 % were positively charged in the experiments of Figs. 1–4. In order to know which form is active, we performed a few experiments at high pH, where most glucosamine molecules are neutral. If the active form of glucosamine is positively charged, it is expected that under these conditions τ and i_{el} should not be much affected by the replacement of 25 % Na ions by glucosamine. (According to results previously obtained after replacement of 75 % of the Na ions by sucrose (Marchais & Marty, 1979), a 25 % reduction of the extracellular Na concentration is not expected to induce by itself a strong modification of τ.)

In these experiments, i_{el} was slightly larger at pH 9.6 than at pH 7.8 (by 10 to 40 %), while the values of τ were similar at the two pH values. These results differ from those at the frog endplate, where the time course of decay of miniature endplate currents, but not their peak amplitude, is affected by pH (Mallart & Molgo, 1978). However, the present results are far too limited to ascertain a genuine difference with the data at the neuromuscular junction.

Table 2 shows the results of experiments investigating the effects of glucosamine at pH 9.6. Only small differences were found between τ values obtained in (1/4 glucosamine, pH 9.6) seawater, and in pH 9.6 seawater. The ratio between these values (1.1 and 0.77 at −60 mV, and 1.2 and 0.81 at −80 mV) is clearly lower than at pH 7.8 (1.29 ± 0.07 (3) at −60 mV, and 1.9 ± 0.1 (3) at −80 mV (data from Fig. 2)). The elementary current measured in the same experiments is slightly reduced in the presence of glucosamine (Table 2). But this reduction is small compared to the twofold change observed in the

same potential range at pH 7.8 (Table 1). (Note that in Table 1 i_{el} was measured at 20–23 °C at pH 7.8, whereas the temperature was 12 °C for the experiments at pH 9.6. However, comparison of noise and relaxation results at 12 °C and at room temperature does not reveal obvious effects of temperature on the action of glucosamine).

The results of Table 2 demonstrate that, at hyperpolarized potentials, the positively charged form of glucosamine is much more efficient than the neutral form to prolong τ and reduce i_{el}. Furthermore, these experiments confirm that the effects seen in Figs. 1–4 are due to a specific action of glucosamine and not to the reduction in Na concentration concomittant with the addition of glucosamine.

Discussion

The results indicate that glucosamine reduces the elementary current amplitude and increases the mean channel open time. Thus, the assumption that glucosamine does not interfere with the ACh receptor-channel complex is unwarranted in *Aplysia* neurones.

The data are reminiscent of the results previously obtained with Sr on the same preparation (Marchais & Marty, 1979). Like glucosamine, Sr decreases i_{el} and increases τ in a voltage-dependent way. We suggest that glucosamine, like Sr, has a weak binding site in the channel itself. While a glucosamine ion is bound to this site, other ions such as Na cannot cross the channel (so that i_{el} is reduced), and the channel cannot close (so that τ is lengthened). A specific version of this model, which assumes competition between glucosamine and permeant ions for a single binding site, is discussed in the Appendix.

In many ways this model is similar to the channel block hypothesis, which has recently proved very successful in explaining the effects of local anesthetics (Adams, 1975, 1977; Ruff, 1977; Marty, 1978; Neher & Steinbach, 1978) and of other blockers of the cholinergic response. However, procaine is active in the range 10^{-5} to 10^{-4} M (Marty, 1978), whereas glucosamine acts at about 10^{-1} M. Because all channel blockers present similar association rate constants (*see* Ascher et al., 1978*b*), it follows that glucosamine presumably stays during a 10^3–10^4 times shorter period than procaine at its binding site (i.e., during 10 to 100 μsec). Such short intervals are beyond the time resolution of our voltage clamp. Thus, although the channel block model implies that two components should be obtained in noise and relaxation experiments, it is expected that in our experimental conditions the fast component induced by glucosamine is too rapid to be observed. In fact, one could envisage the anomalous instantaneous current change seen in Fig. 1 as being due to a very fast "inverse" relaxation similar to that observed in the presence of procaine (Adams, 1977; *see* Fig. 4 in Marty, 1978), gallamine (Colquhoun & Sheridan, 1979), or decamethonium (Adams & Sakmann, 1978; Ascher, Large & Rang, 1979), which presumably act, at least in part, as fast dissociating channel blockers.

Many putative channel blockers are thought to act not only on the open channel but also on the nonactivated receptor-channel complex (*see*, e.g., Adams, 1977). This type of mixed action was proposed to account for the effects of Tris (Ascher et al., 1978*a*), which blocks ACh-sensitive channels at large concentrations (10 mM or more). Tris reduces i_{el} in a voltage-dependent manner but does not modify τ appreciably. It was proposed that Tris has similar affinities for the closed and open configurations of the receptor-channel complex, and that bound complexes can undergo the open $\rightleftharpoons$ closed conformation change (cyclic model).

Since i_{el} and τ are simultaneously affected by glucosamine, the cyclic model is not attractive here. Another possibility would be that glucosamine would act independently on the closed and open forms of the complex, but that the closed-bound $\rightleftharpoons$ open-bound transition would be forbidden. This is unlikely because the opening rate of the channels, p, is not significantly affected by glucosamine. Thus, the main action of glucosamine is on the open form of the receptor-channel complex.

Appendix

Following the theory outlined before (Marchais & Marty, 1979), we suppose in this section that permeant ions, I, and glucosamine, G, bind to a common site of the open channel-receptor complex R:

$$\begin{array}{ccc} & R^*I & \\ & \updownarrow K_I & \\ R \underset{q_0}{\overset{p}{\rightleftharpoons}} & R^* & \\ & \updownarrow K_G & \\ & R^*G & \end{array} \qquad \text{(A1)}$$

The binding of I and G is supposed to be much faster than the $R \rightleftharpoons R^*$ conformation change and to effectively slow down the closing rate of open channels. With these assumptions, the value of τ in the presence of glucosamine exceeds its control value by

$$\tau - \tau_{cont} = \frac{1}{q_0}[G]\left(\frac{1}{2K_G} - \frac{1}{K_{Na}}\right). \qquad \text{(A2)}$$

In this equation, q_0 is the closing rate in the absence of permeant ions; K_{Na} and K_G are the apparent dissociation constants of Na and of the ionized from of glucosamine for the channel site, respectively. The influence of intracellular ions on τ is not taken into account to derive Eq. (A2).

Equation (A2) predicts a linear dependence of τ on glucosamine concentration, as is experimentally found (Fig. 4). Using the results of Fig. 4, one obtains at -80 mV

$$K_G \simeq \tfrac{1}{10} K_{Na}.$$

Using the same model, if the role of divalent ions is neglected, and assuming that glucosamine is impermeant, one finds that

$$i_{el} \cdot \tau = i_{el,\,cont} \cdot \tau_{cont} \frac{[Na_{cont}] - [G]}{[Na_{cont}]}. \quad (A3)$$

Equation (A3) predicts that, in 1/4 glucosamine seawater, the product $i_{el} \cdot \tau$ should be about 3/4 of its control value. This is in agreement with the corresponding products calculated from Table 1. Equation (A3) also gives a theoretical basis for the observation that the ratio $i_{el}/i_{el,\,cont}$ is smaller than τ/τ_{cont} (*see* Fig. 3 and Table 1).

Dropping the assumptions used to derive Eqs. (A2) and (A3) (i.e., that the role of intracellular K and of extracellular divalent ions can be neglected), a simulation of the $i_{el}(V)$ and $\tau(V)$ curves in the presence of glucosamine was performed using the same methods as previously (Marchais & Marty, 1979). Although the results could be accounted for on a semi-quantitative level by model (A_1), some quantitative discrepancies arose. The action of glucosamine is more enhanced by hyperpolarization than what is expected by assuming in model (A_1) that glucosamine, like Na and Mg, binds to a channel site feeling about 7/10 of the total electric field. It is not likely that the explanation of this effect could simply be that glucosamine goes further than Na or Mg ions in the channel, since this would lead to assume that glucosamine binds almost at the interface with the intracellular solution. Two more probable interpretations are: (i) Glucosamine ions may displace Na ions from the binding site by a knock-on effect (Armstrong, 1975), and (ii) once glucosamine ions are bound, extracellular Na ions can enter the mouth of the channel and thus hinder the return of glucosamine to the extracellular solution.

This work was supported by grants from INSERM (ATP 78-90) and DGRST (79·7·0785).

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Received 12 December 1979; revised 20 March 1980