TESTS OF AN ELECTROSTATIC SCREENING HYPOTHESIS OF THE INHIBITION OF NEUROTRANSMITTER RELEASE BY CATIONS AT THE FROG NEUROMUSCULAR JUNCTION

STANLEY MISLER AND WILLIAM P. HURLBUT, *Biophysics Department, The Rockefeller University, New York, N.Y. 10021 U.S.A.*

ABSTRACT We have investigated an electrostatic screening hypothesis of cationic inhibition of quantal release at the neuromuscular junction of the frog (*Rana pipiens*). According to this hypothesis, increasing the extracellular concentration of an inhibitory cation reduces the quantal content (m) of the end-plate potential by reducing the ability of negative surface charge to attract Ca^{2+} to the external surface of the presynaptic membrane. The inhibitory power of various cations should depend only on their net ionic charge and should increase strongly with increasing charge. We have demonstrated, in Ringer's solutions containing modified concentrations of Na^+, Ca^{2+}, and Mg^{2+}, that at fixed concentrations of Ca^{2+} and Na^+ (*a*) the dependence of m on $[Mg^{2+}]_0$ is satisfactorily accounted for by electrostatic theory and (*b*) the dependence of m on the univalent cation concentration of the modified Ringer's solution is satisfactorily predicted from the Mg^{2+} inhibition of m. (Glucosamine or arginine was used to replace a fraction of the Na^+ content of Ringer's solution in the latter experiments.) These results are consistent with electrostatic screening actions of Mg^{2+} and univalent cations in the inhibition of m. We have also re-examined the inhibition of m caused by the addition to Ringer's solution of two trace concentration divalent cations, Mn^{2+} and Sr^{2+}. Our data suggest that the inhibition of m by Sr^{2+} at high quantal contents may also be due to surface charge screening, while the potent inhibitory actions of Mn^{2+} may be due to its ability to bind negative surface charge.

INTRODUCTION

Many cations inhibit vertebrate neuromuscular transmission by reducing the average number of packets or quanta of acetylcholine, m, discharged in response to a presynaptic action potential. The search for the mechanism(s) of cationic inhibition of m has most often centered on studies of Ca^{2+}-Mg^{2+} antagonism. del Castillo and Katz (1954), Jenkinson (1957), and Dodge and Rahamimoff (1967) studied the effects on m of a wide range of extracellular Ca^{2+} and Mg^{2+} concentrations and suggested that Mg^{2+} inhibits the quantal content of the end-plate potential (e.p.p.) by competing with Ca^{2+} for binding to a set of specific, though unidentified, sites on the nerve terminal membrane which regulate quantal release. Using the Ca^{2+}-Mg^{2+} antagonism as a paradigm, other investigators have attempted similarly to account for Ca^{2+}-Na^+, Ca^{2+}-Mn^{2+}, and Ca^{2+}-Co^{2+} antagonisms on the basis of competition for specific binding sites.

More recently, Muller and Finkelstein (1974) suggested that extracellular Mg^{2+} inhibits quantal release by electrostatically screening a fixed density of negative surface charge, σ,

postulated to be on the external surface of the nerve terminal, thereby reducing the Ca^{2+} concentration nearest that membrane and hence most available for participation in a membrane process of excitation-secretion coupling. In this scheme, the principal factor in the ability of Mg^{2+} to inhibit quantal release is its net charge; Mg^{2+} antagonizes the action of Ca^{2+}, even though the two ions do not compete for occupation of specific sites. Generalizing the screening hypothesis to other cations, the inhibitory effects of other cations should be dependent on their net charges and the value of σ at a given neuromuscular junction and hence should be predictable from the inhibitory effect of Mg^{2+}, through the use of standard electrostatic theory.

We have considered electrostatic screening of surface charge as a general mechanism of cationic inhibition of quantal release. In this paper we demonstrate, at single neuromuscular junctions of the frog, that at constant $[Na^+]_o$, pH, and osmolarity, the inhibition of m by Mg^{2+} and univalent cations can be satisfactorily accounted for assuming only that these cations reduce the Ca^{2+} concentration near the presynaptic surface by screening the fixed surface charge. We also examine the ability of an electrostatic screening hypothesis to account for aspects of the inhibition of m seen on addition to Ringer's solution of two trace elements, Sr^{2+} and Mn^{2+}.

Portions of this work have previously been presented in abstract form (Misler and Hurlbut, 1975).

METHODS

Experiments were performed at 18–23° C on cutaneous pectoris muscles dissected from frogs (sp. *Rana pipiens*).

Recordings

End-plate regions of single muscle fibers were impaled with glass micropipettes filled with 3 M KCl (resistance 6–30 MΩ and tip potential ≤10 mV). End-plate potentials were displayed on and photographed from an AC or DC coupled high gain oscilloscope beam (usually 1mV/cm). A time averaging computer (Varian Associates, Inc., Instrument Div., Palo Alto, Calif.) was used in some experiments. Measurements were initiated if (*a*) the resting membrane potential was stable and > -80 mV; (*b*) the peak-to-peak baseline noise was <0.15 mV; (*c*) miniature end-plate potential (m.e.p.p.) amplitude appeared to average 0.75–1.0 mV; and (*d*) m.e.p.p. and e.p.p. rise times were ≤1.5–2.0 ms. The impalement was then maintained for the duration of the experiment, with the resting potential usually varying by <5 mV over several hours. Equilibrium in each new solution was achieved by flushing the lucite recording chamber (capacity, 3 ml) three times at 3–5-min intervals with 15 ml of the new solution. Data were usually recorded starting 12–15 min and again 20–30 min after the initial exposure to the new solution. At these times the motor nerve was stimulated at 0.4–1.0 Hz.

Most experiments were performed with the preparation bathed in a modified Ringer's solution where $[Ca^{2+}]$ was 0.25–0.6 mM and where $[Mg^{2+}]$ was 0.25–5.0 mM or where $[Ca^{2+}]$ was 1.8 mM and $[Mg^{2+}]$ was 20 mM. In these solutions where m was ≲10, m was computed on the basis of 190–210 e.p.p.s and 40–60 m.e.p.p.s. m was computed either from Eq. 1:

$$m = \frac{\text{average e.p.p. amplitude } (\overline{\text{e.p.p.}})}{\text{average m.e.p.p. amplitude } (\overline{\text{m.e.p.p.}})} \tag{1}$$

or from the average of the values of Eq. 1 and

$$m = \ln\left(\frac{\text{total stimulus trials}}{\text{number of e.p.p. failures}}\right), \tag{2}$$

when at least 10% of all stimuli resulted in e.p.p. failures. Since $\overline{\text{e.p.p.}}$ exceeded 3 mV in only two experiments, and even in these experiments did not exceed 5 mV, no correction was made for nonlinear summation of the postsynaptic effect of transmitter quanta.

Experiments were also performed in Ringer's solutions containing 1.3 mM–3.50 mM Ca^{2+} and 0.0 mM–5.0 mM Mg^{2+} in the presence of 1–2 μg/ml *d*-tubocurarine to examine the effects of Mg^{2+}, Sr^{2+}, and univalent cations on quantal release at high quantal contents. In these experiments, 50–60 e.p.p.s were recorded in each experimental solution. Data acquired in each solution are reported as average e.p.p. amplitude. Estimates of the changes in m between solutions were made from the changes in the coefficient of variation of the e.p.p. amplitude as given by Eq. 3 (Martin, 1966).

$$m = (\bar{l}/\mathrm{CV}_{\text{e.p.p.}})^2 \tag{3}$$

and/or changes in $\overline{\text{e.p.p.}}$ between solutions corrected for the changes in $\overline{\text{m.e.p.p.}}$ seen in similar solutions in the absence of *d*-tubocurarine.

Solutions

The Ringer's solution used for dissection contained NaCl, 116 mM; KCl, 2.1 mM; $CaCl_2$, 1.8 mM; and 3 mM sodium phosphate buffer adjusted to pH 6.9–7.0. Subsequent changes in the $CaCl_2$ concentrations and/or additions of $MgCl_2$ were accompanied by adjustments in NaCl concentration to maintain a constant osmolarity of 220 mosm. In several of the experiments performed in the absence of Mn^{2+} and in all of the experiments performed in the presence of Mn^{2+}, 4 mM tris (hydroxymethyl) aminomethane, buffered to pH 7.3, was substituted for the phosphate buffer.

In many experiments, large fractions of the NaCl content of Ringer's solution were nearly iso-osmotically replaced with either sucrose or chloride salts of univalent cations. Substitution of sucrose for NaCl was made by replacing each 1 mM NaCl with 1.87 mM sucrose, with periodic cryoscopic measurements revealing that the osmotic pressure (π) of a sucrose-substituted Ringer's solution was within ±3% of the corresponding full NaCl Ringer's solution. Substitutions of glucosamine · HCl (pK_a = 7.6, titrated to pH 6.5) or arginine · HCl (pK_a = 9.0, titrated to pH 6.9) were also made. π was 226 ± 3 mosm in three determinations made in 120 mM glucosamine · HCl stock solution.

THEORETICAL CONSIDERATIONS

The electrostatic screening hypothesis of cationic inhibition of quantal release is based on two assumptions: the outer surface of the presynaptic membrane bears a net negative charge which does not bind the major ions of Ringer's solution; and at fixed amplitude and shape of the nerve terminal action potential and fixed pH and osmolarity of Ringer's solution, the quantal content (m) of the e.p.p. depends only upon the surface concentration of Ca^{2+}, Ca_s; that is

$$m = f(\mathrm{Ca}_s). \tag{4}$$

The fixed charge creates a difference of electric potential, ψ_s, between the surface of the presynaptic membrane and the bulk solution and Ca^{2+} is accumulated at the surface in accordance with Boltzmann equation

$$\mathrm{Ca}_s = \mathrm{Ca}_0 e^{-2q\psi_s/kT}, \tag{5}$$

where Ca_0 is the concentration of Ca^{2+} in the bathing solution, q is the electronic charge, k is the Boltzmann constant, and T is the absolute temperature in degrees Kelvin. The magnitude of ψ_s is determined by the density of the fixed charge, σ, and by the ionic composition of the bathing solution in accordance with the Gráhame equation. When the bathing solution contains univalent and divalent cations and only univalent anions and when ψ_s is more

negative than −25mV, this equation may be written:

$$2\pi\sigma^2/\epsilon kT = D(e^{-2q\psi_s/kT} - 3) + U(e^{-q\psi_s/kT} - 2), \quad (6)$$

where D is the sum of the concentrations of divalent cations in the bathing solution, U is the sum of the concentrations of univalent cations in the bathing solution, and ϵ is the dielectric constant of the bathing solution.

This equation shows that as the ionic strength of the bulk solution increases, ψ_s decreases (i.e., becomes less negative) even though σ remains constant. As ψ_s decreases, Ca_s decreases and m declines. This is the essence of the screening hypothesis.

To apply the screening hypothesis at the neuromuscular junction one must know the functional dependence of m on Ca_s, the value of σ, and the value of ψ_s under some standard condition. The dependence of m on Ca_s cannot be determined directly, since Ca_s cannot be measured. The form of the dependence can be determined, however, if m is measured as a function of Ca_0, with ψ_s held constant, since under these conditions Ca_s is proportional to Ca_0. The curve of m vs. Ca_0 at constant ψ_s is determined empirically by varying the Ca and Mg concentrations of the bathing medium so that the sum of the concentration of these ions remains constant. The value of ψ_s appropriate for this curve can be determined from the changes in m, as outlined below.

Fig. 1 shows a hypothetical curve of m vs. Ca_0 for a constant value of $\psi_s = \psi_1$. Consider a point along this curve such that:

$$Ca_0 = Ca_1,\ Mg_0 = Mg_1, \text{ and } m = m_1 = f(Ca_{s,1}) = f(Ca_1 e^{-2q\psi_1/kT}). \quad (7a)$$

If Mg_0 is changed to Mg_2 (with Ca_0 held constant), m is observed to change to value m_2.

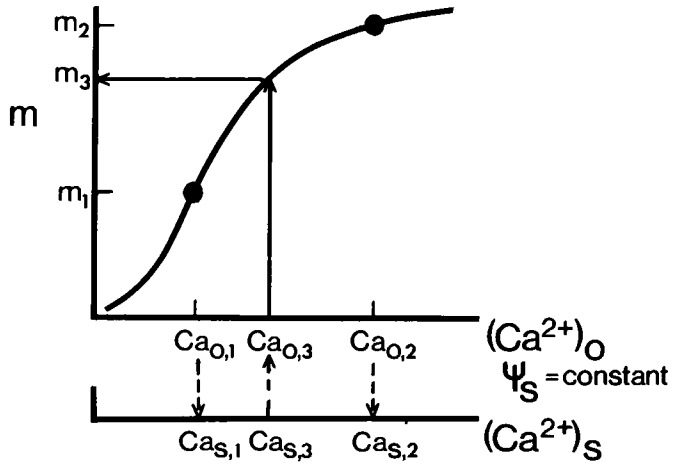

FIGURE 1 Hypothetical graph of m vs. $[Ca^{2+}]_0$ obtained at a constant value of surface potential, ψ_s. Assuming that presynaptic action potential waveform, and the osmolarity and pH of the Ringer's solution remain constant throughout the various manipulations, then according to the electrostatic screening hypothesis m is uniquely determined by $[Ca^{2+}]_s$. However, at constant ψ_s, $[Ca^{2+}]_s$ is directly proportional to $[Ca^{2+}]_0$. Hence, given the measured values m_1 and m_2, the ratio $[Ca^{2+}]_{s,1}$:$[Ca^{2+}]_{s,2}$ can be found by projecting m_1 and m_2 onto the abscissa (see arrows pointing downward) and computing the ratio $[Ca^{2+}]_{0,1}$:$[Ca^{2+}]_{0,2}$. Similarly having computed the ratio $[Ca^{2+}]_{s,3}$:$[Ca^{2+}]_{s,1}$, which is equal to the ratio $[Ca^{2+}]_{0,3}$:$[Ca^{2+}]_{0,1}$, the ratio m_3:m_1 can be found by projecting $[Ca^{2+}]_{0,3}$ and $[Ca^{2+}]_{0,1}$ onto the ordinate (see arrows pointing upward and to the left).

The screening hypothesis assumes that this change in m is due entirely to a change in ψ_s so that

$$m_2 = f(\mathrm{Ca}_{s,2}) = f(\mathrm{Ca}_1 \mathrm{e}^{-2q\psi_2/kT}). \tag{7b}$$

This same value of m could have been achieved at the original surface potential if Ca_0 had been changed to the value Ca_2 (Fig. 1). Hence

$$m_2 = f(\mathrm{Ca}_2 \mathrm{e}^{-2q\psi_1/kT}). \tag{7c}$$

Since the arguments of the two functions 7b and c must be equal for the m's to be equal:

$$\mathrm{Ca}_1 \mathrm{e}^{-2q\psi_2/kT} = \mathrm{Ca}_2 \mathrm{e}^{-2q\psi_1/kT}$$

and

$$\mathrm{e}^{-2q\Delta\psi/kT} = (\mathrm{Ca}_2/\mathrm{Ca}_1)_{\psi_1} = (\mathrm{Ca}_{s,2}/\mathrm{Ca}_{s,1}).$$

Thus the empirical relation between m and Ca_0 at constant ψ_s can be used to determine the change in ψ_s caused by changing Mg_0 at fixed Ca_0.

The magnitude of ψ_1 can be determined by equating the simplified Grahame equations for the two test solutions:

$$U_1(\mathrm{e}^{-q\psi_1/kT} - 2) + D_1(\mathrm{e}^{-2q\psi_1/kT} - 3) = U_2(\mathrm{e}^{-q\psi_2/kT} - 2) + D_2(\mathrm{e}^{-2q\psi_2/kT} - 3). \tag{8}$$

If:

$$\Delta\psi = \psi_2 - \psi_1, \tag{8a}$$

$$\alpha = \mathrm{e}^{-q\Delta\psi/kT} = (\mathrm{Ca}_2/\mathrm{Ca}_1)_{\psi_1} \tag{8b}$$

$$\xi = \mathrm{e}^{-q\psi_1/kT} \tag{8c}$$

this equation can be rewritten:

$$\xi^2(D_1 - \alpha^2 D_2) + \xi(U_1 - \alpha U_2) + 2(U_2 - U_1) + 3(D_2 - D_1) = 0. \tag{9}$$

Eq. 9 can be solved for ξ since all the other parameters are known.

If a third bathing solution is applied (with Ca_0 held constant), the expected value of m, m_3, is determined as follows. The change in surface potential produced by the third solution is obtained by equating the simplified Grahame equations for solutions (1) and (3) to give:

$$\beta^2\xi^2 D_3 + \beta\xi U_3 - 2(U_3 - U_1) - \xi U_1 + 3(D_3 - D_1) - \xi^2 D_1 = 0 \tag{10}$$

where:

$$\beta = \mathrm{e}^{-q\Delta\psi'/kT} = (\mathrm{Ca}_3/\mathrm{Ca}_1)_{\psi_1} \tag{10a}$$

$$\Delta\psi' = \psi_3 - \psi_1. \tag{10b}$$

Eq. 10 can be solved for β since ξ and the other parameters are known. Eq. 10a can be solved to give Ca_3, the value of Ca_0 which would give m_3 at the original surface potential. The expected value of m_3 is then read off the empirical curve of m vs. Ca_0 for $\psi_s = \psi_1$.

Most of our experiments were done at low quantal contents where m is exponentially dependent upon Ca_0:

$$m = k\,\mathrm{Ca}_0^n, \tag{11}$$

where n is independent of Mg_0 and, therefore, of ψ_s (Dodge and Rahamimoff, 1967; Misler,

1977). In these experiments the following procedure was used to determine ψ_s: (*i*) n was determined as the slope of the log-log plot of m vs. Ca_0, when Ca_0 was varied from 0.25–0.6 mM in the presence of 4–5 mM Mg_0 (the change in ψ_s caused by this small increase in D was negligible); (*ii*) Mg_0 was changed from 4 mM to 1 mM and the change in ψ_s computed from the equation:

$$e^{-2q\Delta\psi/kT} = (m_2/m_1)^{1/n}, \tag{12}$$

where m_2 and m_1 are the quantal contents at the two concentrations of Mg_0; (*iii*) ψ_1 was computed from Eqs. 9 and 8c; (*iv*) the change in ψ_s produced by a second change in Mg_0, or by a change in the concentration of a monovalent ion, was computed from Eqs. 10 and 10a; and (*v*) the expected value of m_3 in the third solution was computed from the equation:

$$(m_3/m_1) = \beta^{2n}. \tag{13}$$

We also performed several experiments at high quantal contents where m is not related exponentially to Ca_0. In these experiments we used the graphical procedure outlined above and varied Ca_0 between 1.3 and 3 mM in the presence of 5 mM Mg_0. The total concentration of divalent ions varied from 6.3 to 8.0 mM in these experiments and caused ψ_s to vary by 0.8 mV. This variation in ψ_s was not a serious source of error, however, since the slope of the curve of $\overline{\text{e.p.p.}}$ amplitude vs. Ca_0 at constant Mg_0 was only 5% less than the slope of this curve at constant ψ_s (e.g., see Fig. 3 *a*).

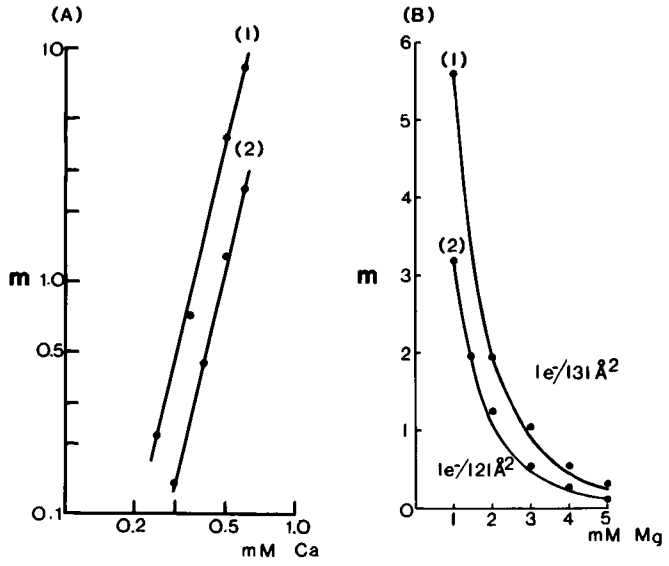

FIGURE 2 The Ca^{2+} and Mg^{2+} dependencies of m at low quantal contents. In *A*, a double logarithmic plot of m vs. $[Ca^{2+}]_0$ was fitted by eye to lines of slope 4.26 (exp. 1) and 4.4 (exp. 2). In *B*, the solid curves represent the predicted values of m as a function of $[Mg^{2+}]_0$ based on the Ca^{2+} dependencies of m determined in *A* and the effect on m of increasing $[Mg^{2+}]_0$ from 1 to 2 mM (exp. 1) or from 1 to 1.45 mM (exp. 2).

In exp. 1, m was 5.6 in 0.35 mM Ca^{2+} and 1 mM Mg^{2+} and 1.95 in 0.35 mM Ca^{2+} and 2 mM Mg^{2+}. Using Eq. 12, the increase in $[Mg^{2+}]_0$ is calculated to decrease ψ_s by 3.1 mV. With this we solved Eq. 8 for ψ_s and found that $\psi_s = -86.1$ mV in 0.35 mM Ca^{2+} and 1 mM Mg^{2+}. We computed σ as 7.63×10^{-13} negative charges per cm^2 or 1 $e^-/131$ Å^2. This information is sufficient to generate the remainder of the m vs. $[Mg^{2+}]_0$ curve, using Eqs. 8–13.

RESULTS

Magnesium Inhibition of Quantal Release

The electrostatic screening hypothesis predicts that increasing the extracellular concentration of Mg^{2+} should decrease m. Fig. 2 presents the Ca^{2+} dependence and Mg^{2+} inhibition of m observed at low quantal contents (i.e., $m \leq 10$) in two separate experiments performed at pH 7.3. Note that under these conditions m shows nearly a fourth power dependence on $[Ca^{2+}]_0$. In both cases the Mg^{2+} inhibition of m was satisfactorily predicted, for a range of Mg^{2+} concentrations, using the value of σ computed from the effect on m of a small decrease in $[Mg^{2+}]_0$. This confirms, at single neuromuscular junctions, the predictions which Muller and Finkelstein (1974) made using average data of Dodge and Rahamimoff (1967).

Fig. 3 presents the Ca^{2+} dependence and Mg^{2+} inhibition of e.p.p. amplitude seen at more nearly physiological quantal contents (i.e., $m \geq 50$) at pH 6.6, in the presence of a small concentration of *d*-tubocurarine. Under these conditions, $\overline{\text{e.p.p.}}$ varies linearly with $[Ca^{2+}]_0$. We felt confident in assuming that $\overline{\text{e.p.p.}}$ was proportional to m because several authors (e.g., del Castillo and Stark, 1952; Jenkinson, 1960) demonstrated that postsynaptic sensitivity of the curarized end-plate to transmitter agonist varied by 10% or less when $[Ca^{2+}]_0$ was varied between 1.8 and 7.2 mM and $[Mg^{2+}]_0$ was varied between 0.0 and 8.0 mM. Using the graphical computational methods outlined above, we were able to predict Mg^{2+} inhibition of e.p.p. at the higher quantal contents.

In the experiment shown in Fig. 3, it was possible to demonstrate that when $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$ are varied but their sum remained constant, a single value of σ fitted data obtained over a wide range of quantal contents. In this experiment, before the addition of *d*-tubocurarine to the modified Ringer's solution, $[Ca^{2+}]_0$ was increased from 0.40 to 0.55 mM in the presence of 6 mM Mg^{2+}, and then $[Mg^{2+}]_0$ was reduced from 6 to 3 mM in the presence of 0.55 mM Ca^{2+}. At the low values of m, m showed a 3.7 power dependence on $[Ca^{2+}]_0$ and in the presence of 0.55 mM Ca^{2+}, the ratio of m in 3 mM Mg^{2+} to m in 6 mM Mg^{2+} was 3.05. Hence ψ_s and σ were calculated as -71.0 mV and 1 $e^-/164$ Å^2, respectively, in 0.55 mM Ca^{2+} and 3.0 mM Mg^{2+}, as compared with $\psi_s = -72.0$ mV and $\sigma = 1$ $e^-/162$ Å^2 in 1.3 mM Ca^{2+} and 2 mM Mg^{2+}. These results are consistent with the possibility that in the range of concentrations investigated, Ca^{2+}, like Mg^{2+}, chiefly screens rather than binds presynaptic surface charge.

The Effects of Univalent Cations on Quantal Release. The Effect on m of Substituting Sucrose for Na^+ in Modified Ringer's Solutions.

The electrostatic screening hypothesis predicts that decreasing the univalent cation concentration of Ringer's solution should increase m.

When m is small (i.e., ≤ 10) in full Na^+ Ringer's solution, iso-osmotic substitution of a portion of the Na^+ content of Ringer's solution with sucrose results in an increase in m (Kelly, 1968; Colomo and Rahamimoff, 1968). Fig. 4 *a* and *b* depict a 3.5-fold increase in m seen on replacing 51 mM NaCl with 95 mM sucrose in the presence of 0.25 mM Ca^{2+} and 1 mM Mg^{2+} at pH 6.9. Using the average values of 1 $e^-/146$ Å^2 for σ at pH 6.9 (see below) and 3.8 for the power dependence of m on $[Ca^{2+}]_0$, the electrostatic screening hypothesis predicts that this reduction in univalent cation concentration should result in an 8.0-fold increase in m.

Fig. 4 *c* and *d* demonstrate a more complex facet of the effect on m of reducing the Na^+

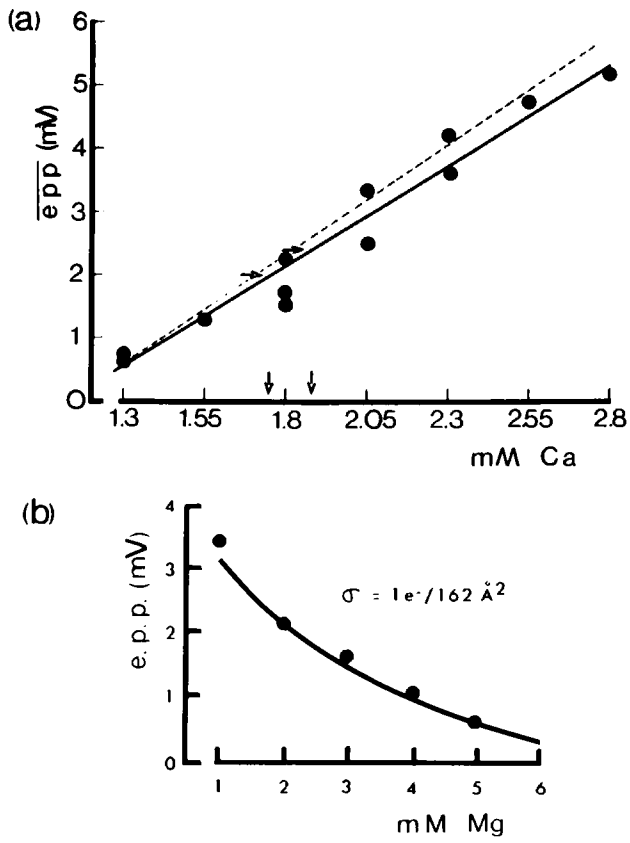

FIGURE 3 The Ca^{2+} and Mg^{2+} dependencies of e.p.p. amplitude at high quantal contents. (*a*) The Ca^{2+} dependence of the average e.p.p. amplitude ($\overline{e.p.p.}$) was determined over the range of $[Ca^{2+}]_0$'s between 1.3 and 2.8 mM, in the presence of 5.0 mM Mg^{2+}, and was fit by least squares analysis to a straight line $\overline{e.p.p.}$ (millivolts) = 3.3 $[Ca^{2+}]_0$ − 3.9 (solid line). The effect of $[Mg^{2+}]_0$ on $\overline{e.p.p.}$ was determined by recording $\overline{e.p.p.}$ twice in 1.3 mM Ca^{2+} and 5.0 mM Mg^{2+} and then twice in 1.3 mM Ca^{2+} and 2.0 mM Mg^{2+}. The values observed in 2.0 mM Mg^{2+} were mapped onto the graph of $\overline{e.p.p.}$ vs. $[Ca^{2+}]_0$, obtained in 5.0 mM Mg^{2+} (horizontal arrows). The corresponding values of $[Ca^{2+}]_0$, which in the presence of 5.0 mM Mg^{2+} should yield identical levels of quantal release, were 1.75 and 1.88 mM, respectively (vertical arrows). Averaging the latter values and using Eqs. 11 and 18, ψ_s was calculated as −72.7 mV in 1.30 mM Ca^{2+} and 2.0 mM Mg^{2+} and σ was computed as 1 $e^-/162$ Å^2. In this experiment ψ_s varied by 0.78 mV over the range of Ca^{2+} concentrations. The dashed line shows the corrected curve of $\overline{e.p.p.}$ vs. $[Ca^{2+}]_0$ at constant ψ_s. Note that in the range of values of interest in this experiment, the error contributed by the inconstancy of ψ_s is less than the variation in measured $\overline{e.p.p.}$'s.

(*b*) Comparison of the observed dependence of $\overline{e.p.p.}$ on $[Mg^{2+}]_0$ in 1.3 mM Ca^{2+} (single points) and the predicted dependence of e.p.p. on $[Mg^{2+}]_0$ (solid line) computed from σ = 1 $e^-/162$ Å^2, Eqs. 8–10, and the results in (*a*).

concentration of Ringer's solution. Note that when 95 mM sucrose is substituted for 51 mM NaCl in the presence of 1.35 mM Ca^{2+}, where m approaches more physiological levels (i.e., $m \geq 50$), both the amplitude of the curarized e.p.p. and the estimated value of m decrease. Similar data were obtained by Fatt and Katz (1952), Kelly (1968), and Colomo and Rahamimoff (1968).

A single deficiency of the experimental design might account for both the quantitative and qualitative differences between the predicted and observed effects on m of these reductions in

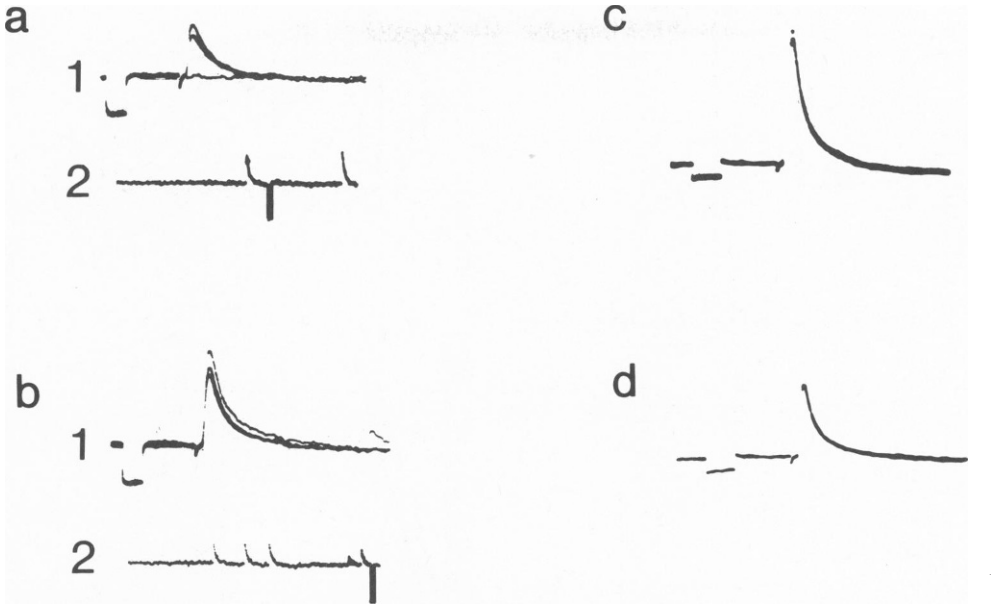

FIGURE 4 The effects on quantal release of substituting 51 mM sodium chloride with 95 mM sucrose at low quantal contents (in 0.35 mM Ca^{2+} and 1 mM Mg^{2+}) (*a*, *b*) and high quantal contents (in 1.35 mM Ca^{2+} and no added Mg^{2+}) (*c*, *d*). At the low quantal contents ($m < 5$) Na^+ replacement resulted in a 2.4-fold increase in $\overline{\text{e.p.p.}}$ (compare a_1 and b_1), a 25% reduction in $\overline{\text{m.e.p.p.}}$ (compare a_2 and b_2), and hence a 3.5-fold increase in *m*. In the same total divalent cation concentration but significantly higher quantal contents ($m > 50$), Na^+ replacement led to a 38% decrease in $\overline{\text{e.p.p.}}$ recorded in 1 μg/ml *d*-tubocurare and an estimated 12% decrease in *m*. In a_1, b_1, *c*, and *d* there are three e.p.p.'s per trace.

$[Na^+]_0$. Since transmitter release at synapses is directly related to the peak level of presynaptic depolarization (Liley, 1956; Katz and Miledi, 1967), reducing $[Na^+]_0$ might significantly reduce *m* by decreasing the overshoot of the presynaptic action potential. Hence, in the experiment illustrated above, the increment in *m* resulting from a reduction in ionic strength might be opposed by the decrement in *m* resulting from a reduction in action potential overshoot. At low levels of *m*, where *m* displays approximately a fourth power dependence on $[Ca^{2+}]_0$, the increment in *m* reflecting the increase in ψ_s might predominate. At higher levels of *m*, where *m* displays a nearly linear dependence on $[Ca^{2+}]_0$, the decrement in *m* reflecting the reduced overshoot of the action potential might predominate and result in a net reduction in *m* on reducing $[Na^+]_0$.

Quantitative data on the effect of *m* of reducing presynaptic action potential overshoot is not available for the vertebrate neuromuscular junction. To provide an internal correction for the latter effect, all further experiments involving reduction of univalent cation concentration were performed, at constant $[Na^+]_0$, by iso-osmotically substituting sucrose for a univalent cation which would be impermeant to the nerve terminal during the action potential.

The Use of Glucosamine and Arginine as Na^+ Substitutes

Glucosamine and arginine were chosen for use as Na^+ substitutes in the experiments performed to test the effects on *m* of reducing the univalent cation concentration of Ringer's solution at a constant $[Na^+]$. Each cation has larger molecular dimensions than the equivalent

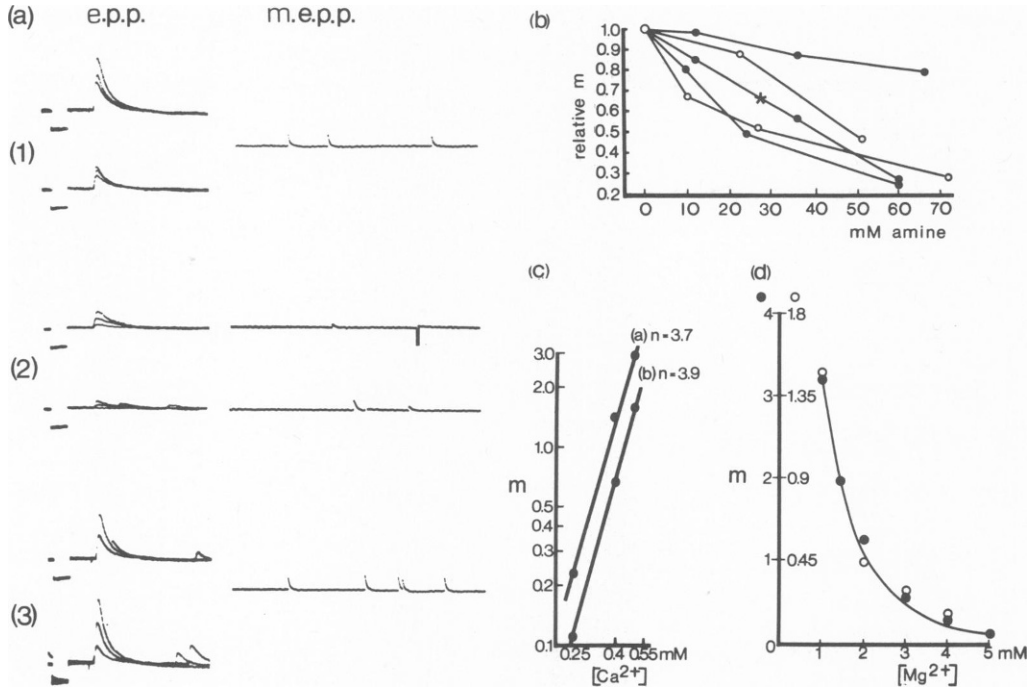

FIGURE 5 The effects on quantal release of substituting glucosamine hydrochloride or arginine hydrochloride for sodium chloride. (*a*) A preparation bathed in 0.25 mM Ca^{2+} + 1.0 mM Mg^{2+} Ringer's solution (pH 6.6) was exposed to a 0.25 mM Ca^{2+} + 1.0 mM Mg^{2+} 51 mM glucosamine Ringer's solution (pH 6.5). e.p.p.'s (left column) and m.e.p.p.'s (right column) were recorded immediately before (row 1) and 18 min after (row 2) exposure to the glucosamine Ringer's solution, and again 15 min after the return to the original full Na^+ Ringer's solution (row 3). The resting membrane potential was −85 mV throughout. Calibrations in all traces were 1 mV × 10 ms. (*b*) A plot of the ratio of *m* in *X* mM amine substituted Ringer's solution to *m* in full Na^+ Ringer's solution as a function of the glucosamine (filled circles) or arginine (open circles) concentration of the amine substituted Ringer's solution. Straight lines connect the data points determined at a single end plate. The concentration of the substituting amine was varied at random. (*c*) Log-log plots of quantal content vs. $(Ca^{2+})_0$ in full Na^+ Ringer's solution and 51 mM glucosamine substituted Ringer's solution obtained at a single end plate. (*d*) Mg^{2+} inhibition of *m* in full Na^+ Ringer's solution (filled circles) and 51mM arginine substituted Ringer's solution (open circles). The solid line is a predicted curve of *m* vs. $(Mg^{2+})_0$ using σ = 1 e^-/121 Å^2. This experiment was performed at the end plate introduced in curves a_2 and b_2 of Fig. 2.

pore radii calculated for the Na^+ and K^+ channels of the node of Ranvier on the frog (Hille, 1971 and 1975) and neither supports a nodal action potential when used as a total cation replacement for Na^+ (Deck, 1958).

Fig. 5 illustrates some basic effects of these two cations on the frog end plate. Substituting increasing fractions of the Na^+ content of Ringer's solution (up to 60%) with either glucosamine or arginine resulted in increasing reductions in *m*. For example, replacing 51 mM Na^+ with glucosamine led to a twofold reduction in *m* (Fig. 5 *a* and *b*). Assuming that a 51-mM reduction in $[Na^+]_0$ leads to a 15-mV reduction in presynaptic action potential overshoot, as it does at the isolated mode of Ranvier (Huxley and Stämpfli, 1951), this data is quantitatively similar to the functions of "synaptic transfer of voltage" found at the squid and lamprey giant synapses. At the latter synapses, 15-mV reductions in presynaptic action

potential overshoots achieved by treatment of the synapse with tetrodotoxin result in a 1.25–1.50-fold reduction in average postsynaptic potential amplitude (Katz and Miledi, 1967; Martin and Ringham, 1975). A 51-mM substitution of glucosamine or arginine for Na^+ did not, however, alter the exponential dependence of m on $[Ca^{2+}]$ seen at low quantal contents (Fig. 5 *c*) or alter that ability of an electrostatic screening hypothesis to account for Mg^{2+} inhibition of m (Fig. 5 *d*). This suggests that these substituting cations do not alter a basic intracellular process of excitation-secretion coupling or bind to membrane surface change.

Data presented in the Appendix demonstrate that substituting either glucosamine or arginine for Na^+ has little effect on action potential electrogenesis of the directly stimulated frog sciatic nerve trunk or frog skeletal muscle fiber beyond reducing the action potential overshoot and maximum rate of rise. The total experimental data then suggest that substituting either glucosamine or arginine for Na^+ may not alter the quantity of transmitter released beyond that expected from altering the rising phase of the nerve terminal action potential.

The Effect on m *of* $[U^+]_0$ *Reduction using Glucosamine or Arginine as the* Na^+ *Replacement*

Fig. 6 illustrates a typical experiment, performed at a low level of m, to compare quantitatively the observed and theoretically predicted effect of iso-osmotic substitution of 95 mM sucrose Ringer's solution for 51 mM arginine Ringer's solution (at pH 6.9) in the presence of 0.25 mM Ca^{2+} and 1.0 mM Mg^{2+}. The observed ratio of m in 95 mM sucrose Ringer's solution to m in 51 mM arginine Ringer's solution was 9.0. The experiment began and ended with tests of both Ca^{2+} dependence and Mg^{2+} inhibition of m, hence permitting two separate predictions of the latter ratio. From the reductions in m seen on reducing $[Ca^{2+}]_0$ from 0.5 to 0.25 mM (in the presence of 4.0 mM Mg^{2+}), n, the exponential dependence of m on $[Ca^{2+}]_0$, was 4.02 and later 3.8. Reducing Mg^{2+} from 4 to 1 mM resulted first in a 8.3-fold

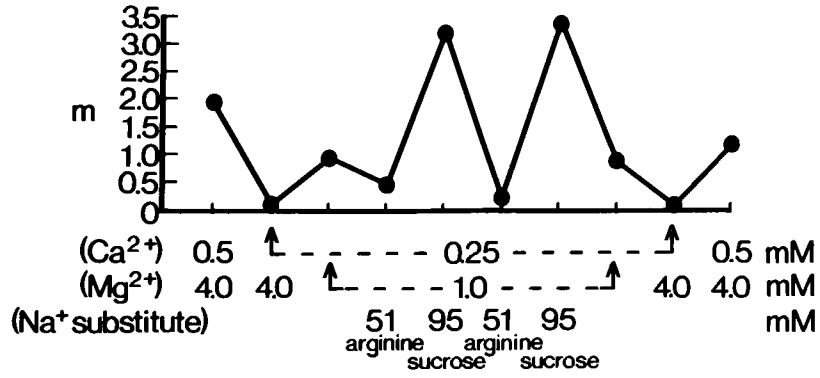

FIGURE 6 Observation and prediction of the effect of univalent cation concentration reduction on e.p.p. quantal content at a single end plate at low quantal contents. In this experiment the effect of U^+ reduction on m was tested by iso-osmotically substituting 95 mM sucrose for 51 mM arginine, at pH 6.9, in the presence of 0.25 mM Ca^{2+} and 1 mM Mg^{2+}. The resultant ratio of m in 95 mM sucrose Ringer's solution to m in 51 mM arginine Ringer's solution was 9.0 as compared with predicted ratios of 10.1 and 6.9, calculated from two separate determinations of the Ca^{2+} dependence and Mg^{2+} inhibition of m (see text). Note that in this preparation a 51-mM substitution of arginine chloride for NaCl resulted in a threefold depression of m.

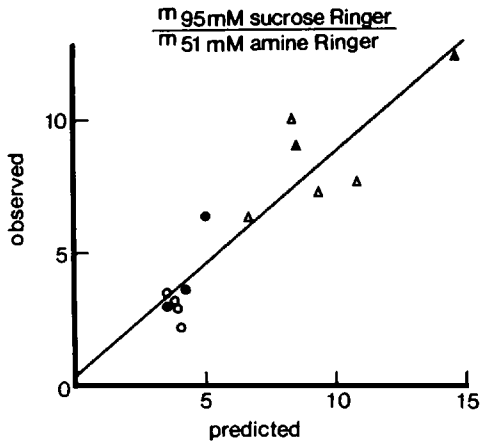

FIGURE 7 Correlation of the observed and predicted changes in quantal content resulting from the iso-osmotic substitution of either glucosamine (open symbols) or arginine (filled symbols) for sucrose at low quantal contents. Experiments were performed in either 0.50 mM Ca^{2+} and 4 mM Mg^{2+} (circles) or 0.25 mM Ca^{2+} + 1 mM Mg^{2+} (triangles). The values of the ratio ($m_{95\text{ mM sucrose Ringer's solution}}/m_{51\text{ mM amine Ringer's solution}}$) obtained in the experiments are plotted on the ordinate; the values of the ratio predicted from the Ca^{2+} and Mg^{2+} dependences of m obtained at the same end plate are plotted on the abscissa. The slope of the regression line is 0.89. $r = 0.92$.

and later a 10.45-fold increase in m. Hence, ψ_s was estimated as -75.7 mV and -82.5 mV in the presence of 0.5 mM Ca^{2+} and 4 mM Mg^{2+}, and σ was computed as 1 $e^-/142$ Å^2 or 1 $e^-/116$ Å^2, at pH 6.9. Hence the ratio of m in 95 mM sucrose Ringer's solution to m in 51 mM arginine Ringer's solution was predicted to be 10.1 or 6.9.

Fig. 7 compares the observed and predicted changes in quantal content on substituting either 51 mM Glucosamine (at pH 6.6) or arginine (at pH 6.9) for 95 mM sucrose in the presence of either 0.25 mM Ca^{2+} + 1 mM Mg^{2+} or 0.50 mM Ca^{2+} + 4 mM Mg^{2+} for an entire series of experiments. Using glucosamine as a Na^+ substitute, the ratio of the observed to the predicted effect of reducing $[U^+]_0$ averaged 0.85 ± 0.20 in eight experiments. Using arginine, the ratio of the observed to the predicted effect averaged 0.98 ± 0.19 in five experiments. From the experiments using glucosamine, σ was estimated to average 1 $e^-/164$ Å^2 (range 1 $e^-/136$ Å^2 to 1 $e^-/180$ Å^2) at pH 6.6. From the experiments using arginine, σ was estimated to average 1 $e^-/142$ Å^2 (range 1 $e^-/110$ Å^2 to 1 $e^-/170$ Å^2) at pH 6.9.

The restricted range of total divalent cation concentration, $[D^{2+}]_0$, in these experiments performed at low quantal contents, reflects the difficulty in controlling nerve terminal excitability when $[D^{2+}]_0$ is >12.5 mM or < 0.5 mM, in the presence of reduced $[Na^+]_0$. Repetitive firing (i.e., a doublet or triplet e.p.p. response) was encountered at the lower end of divalent cation concentrations and conduction block at the higher end. The restricted range of $[U^+]_0$ reduction reflects on the one hand the inability to measure $\overline{\text{m.e.p.p.}}$ reliably, on a routine basis, in a solution where more than 65 mM Na^+ was replaced by either amine, and on the other hand, the desire to work with a reduction in $[U^+]_0$ large enough to produce readily observable effects on m. In three out of six endplates impaled in the presence of 0.30 mM Ca^{2+} + no added Mg^{2+}, however, repetitive e.p.p. firing occurred only intermittently. In these experiments the ratio of m in 95 mM sucrose Ringer's solution to m in 51 mM glucosamine

Ringer's solution was 20.2, 22.3, and 25, as compared with a predicted value of 27.3 using σ = 1 $e^-/164$ Å^2 as calculated for pH 6.5 (see above). In another three experiments performed in the presence of a hypertonic Ringer's solution containing 116 mM NaCl, 1.8 mM $CaCl_2$, and 20 mM Mg^{2+}, the ratio of m in 47 mM sucrose Ringer's solution to m in 25 mM glucosamine Ringer's solution was 1.53 ± 0.08 as compared with a predicted value of 1.7, again using σ = 1 $e^-/164$ Å^2.

Fig. 8 shows one of four experiments performed to examine the effect on m of reducing $[U^+]_0$ at high quantal contents. In these experiments the bathing medium contained *d*-tubocurarine (1–2 μg ml), 1.3 mM Ca^{2+}, and 2 mM Mg^{2+}. The ratio of $\overline{e.p.p.}$ in full Na^+ Ringer's solution to $\overline{e.p.p.}$ in 51 mM glucosamine was 2.84 (range 1.85–3.47). The ratio of m in 95 mM sucrose to m in 51 mM glucosamine was 2.29 (range 1.69–2.77), when m was estimated from the coefficients of variation of the e.p.p. amplitudes, and 1.64 (range 1.07–2.01), when m was estimated from the ratio of the $\overline{e.p.p.}$'s corrected for changes in $\overline{m.e.p.p.}$ in the absence of curare. These estimates of m suggested that reductions in $[U^+]_0$ of modified Ringer's solution, at constant $[Na^+]_0$, increase quantal release even at high quantal contents.

Our measured ratio of $\overline{m.e.p.p.}$ in 95 mM sucrose Ringer's solution to $\overline{m.e.p.p.}$ in 51 mM glucosamine Ringer's solution was 0.74/0.44, where 1.0 is taken as the normalized $\overline{m.e.p.p.}$ in full Ringer's solution. We have assumed that the coefficient of variation method tends to overestimate the actual ratio of m's, while the corrected $\overline{e.p.p.}$ method tends to underestimate this ratio. (In the latter case there is some evidence that reducing $[U^+]_0$ of Ringer's solution may decrease the postsynaptic sensitivity of the curarized end plate to transmitter agonist [Jenkinson, 1960].) One of these experiments was performed on the neuromuscular junction studied in Fig. 3. Using the computed value σ = 1 $e^-/162$ Å^2, replacing 51 mM glucosamine with 95 mM sucrose in 1.3 mM Ca^{2+} + 2 mM Mg^{2+} should have the equivalent effect on $[Ca^{2+}]_s$ as increasing $[Ca^{2+}]_0$ from 1.82 to 2.73 mM in the presence of 5 mM Mg^{2+}, assuming the slopes of the curves of $\overline{e.p.p.}$ vs. $[Ca^{2+}]_0$ are very similar in full Na^+ and 66 mM Na^+ Ringer's solution. The reduction in $[U^+]_0$ should result in a 2.25-fold increase in m as compared with a 2.77-fold increase in m estimated from $(1/CV)^2$ and a 1.74-fold increase in m estimated from the corrected $\overline{e.p.p.}$

Sr^{2+} Inhibition of the e.p.p.

Sr^{2+} is the only trace concentration divalent cation which can routinely support the e.p.p. in the absence of Ca^{2+}, albeit ten times less effectively than Ca^{2+}. Sr^{2+} also enhances m when added to modified Ringer's solutions in which m is low (Dodge et al., 1969). When added to Ringer's solution in which m is high (i.e., ≥ 50), Sr^{2+} however reduces m. Meiri and Rahamimoff (1971) have suggested that Sr^{2+} may serve both as partial agonist and antagonist of quantal release by binding to release activating sites at which it is only partly as effective as Ca^{2+}.

We have reexamined Sr^{2+} inhibition of quantal release in normal Ca^{2+} Ringer's solution. Fig. 9 shows one example out of three experiments performed to examine the effect on the average amplitude of the curarized e.p.p. of adding up to 7 mM Sr^{2+} or Mg^{2+} to 1.8 mM Ca^{2+}, Mg^{2+}-free Ringer's solution. At concentrations up to 1.0–1.5 mM, where Sr^{2+} by itself is unable to sustain significant quantal release, equal concentrations of Sr^{2+} and Mg^{2+}

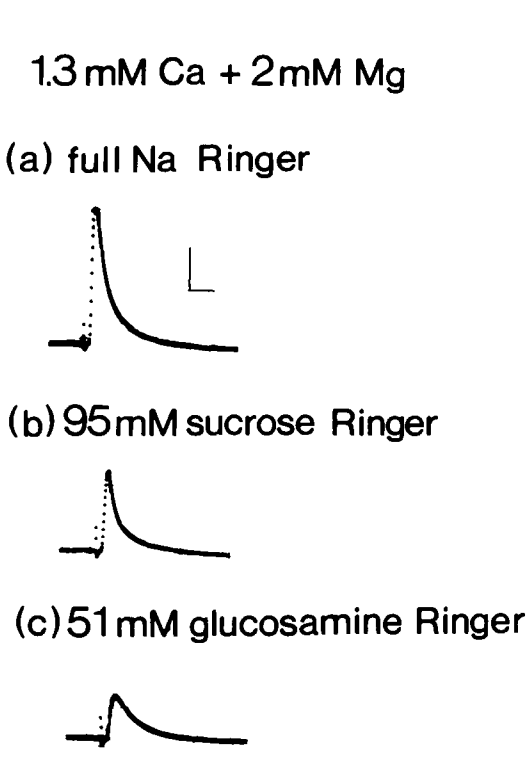

FIGURE 8

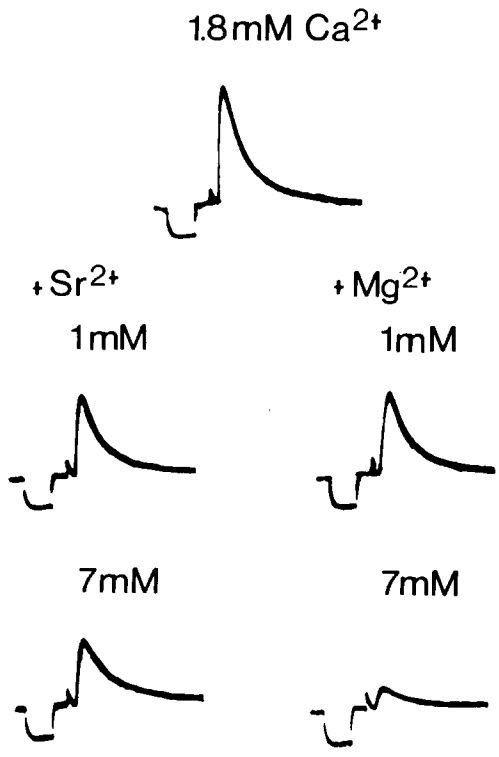

FIGURE 9

FIGURE 8 A comparison of the effect on average e.p.p. amplitude ($\overline{e.p.p.}$) of replacing 95 mM sucrose for 51 mM NaCl and then replacing 95 mM sucrose with 51 mM glucosamine in the presence of 1.55 mM Ca^{2+}, 2 mM Mg^{2+}, and 1.5 μg/ml *d*-tubocurarine. Each trace is the computer sum of 50 e.p.p.'s. Calibration: 1 mV × 10 ms.

FIGURE 9 A comparison of the effect on $\overline{e.p.p.}$ of adding 1 or 7 mM Sr^{2+} or 1 or 7 mM Mg^{2+} in the presence 1.8 mM Ca^{2+} and 1.2 μg/ml *d*-tubocurarine. Each trace is the computer sum of 50 e.p.p.'s. Calibrations 1 mV × 10 ms. In three experiments the ratio of $\overline{e.p.p.}$ in 1 mM Sr^{2+} to that in 1 mM Mg^{2+} averaged 1.05, while the ratio of $\overline{e.p.p.}$ in 7 mM Sr^{2+} to that in 7 mM Mg^{2+} averaged 2.5. When *m* is estimated from the coefficient of variation of e.p.p. amplitude, the corresponding ratios of *m* averaged 1.10 and 2.0.

produce nearly comparable reductions in $\overline{e.p.p.}$ With further increases in the concentration of added divalent cation up to 7 mM, where Sr^{2+} can support significant quantal release, $\overline{e.p.p.}$ in Sr^{2+}-enriched solutions greatly exceeds $\overline{e.p.p.}$ in Mg^{2+}-enriched solutions. These changes in $\overline{e.p.p.}$ probably reflect changes in *m*, because estimates of *m* made from the coefficients of variations of e.p.p. amplitudes have consistently shown parallel changes and $\overline{m.e.p.p.}$'s differed by $\leq 10\%$ in Sr^{2+}- and Mg^{2+}-enriched solutions in the absence of curare. These data suggest that at concentrations where it does not significantly support release, Sr^{2+}, like Mg^{2+}, may inhibit *m* by screening surface charge.

Mg^{2+} Inhibition of m in the Presence of Mn^{2+}

Manganese is one of several nonalkali earth divalent cations (including beryllium, cobalt, nickel, and zinc) whose inhibitory actions on e.p.p. quantal content are too potent to be

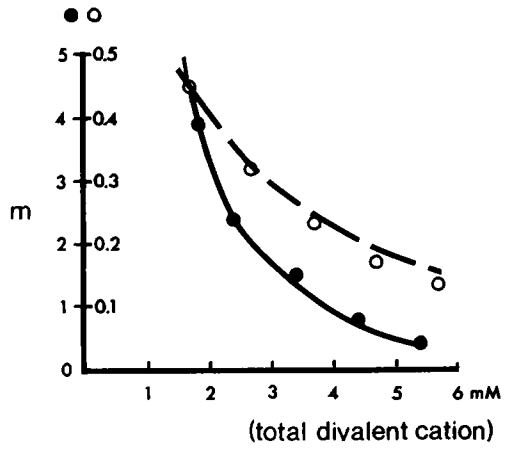

FIGURE 10 Mg^{2+} inhibition of m in the absence (filled circles) and presence (open circles) of Mn^{2+}. At constant $[Ca^{2+}]_0$ (= 0.35 mM), Mg^{2+} was varied between 1.45 and 5.0 mM in the absence of Mn^{2+} and between 1.0 and 5.0 mM in the presence of 0.20 mM Mn^{2+}. The solid line shows the predicted curve of Mg^{2+} inhibition of m in the absence of Mn^{2+} based on the computed value of σ = 1 $e^-/130$ Å^2 (see text). The broken line shows the predicted curve of Mg^{2+} inhibition of m in the presence of 0.20 mM Mn^{2+}, as explained in the text.

accounted for by surface charge screening, in that they inhibit m up to twenty times as effectively as an equimolar quantity of magnesium (e.g., Benoit and Mambrini, 1970; Meiri and Rahamimoff, 1972). If Mn^{2+} affects m by a mechanism other than surface charge screening, such as by binding surface charge, then m should be dependent on the surface concentrations of both Ca^{2+} and Mn^{2+}. Increasing $[Mg^{2+}]_0$ should not only decrease $[Ca^{2+}]_s$ but also $[Mn^{2+}]_s$, hence in part relieving the latter's inhibitory effect. The Mg^{2+} inhibition of m should be smaller in the presence than in the absence of Mn^{2+}. This prediction is confirmed by Fig. 10, which shows that at low quantal contents, Mg^{2+} inhibition of m in the presence of 0.20 mM Mn^{2+} is substantially smaller than in the absence of Mn^{2+}. In addition, no one value of σ fits the entire curve obtained in the presence of Mn^{2+}. This experiment suggests that our overall experimental approach was sensitive enough to detect a major nonscreening action of a cation and increases our confidence that the major cationic components of Ringer's solution have at best only minimal surface charge binding activity.

Several factors suggest that Mn^{2+} might inhibit quantal release by binding to presynaptic surface charge. (*a*) Addition of small quantities of Mn^{2+} to Ringer's solution does not alter the magnitude or duration of the action current recorded at the isolated frog node of Ranvier (Takahashi et. al., 1960) or the exponential dependence of m on $[Ca^{2+}]_0$ seen at low quantal contents (Balnave and Gage, 1973). (*b*) Mn^{2+} shifts the conductance vs. voltage characteristic of the Na^+ channel of the frog node of Ranvier more strongly than does Mg^{2+} (Hille et al., 1975) and inhibits the Ca^{2+} current of barnacle muscle fibers more strongly then does Mg^{2+} (Hagiwara and Takahashi, 1967).

To examine a surface charge binding action of Mn^{2+}, we assumed that single manganese ions could bind to and neutralize divalent anionic sites, S^{2-}, thus decreasing σ. That is,

$$S^{2-} + Mn^{2+} \rightleftharpoons Mn\,S, \tag{14}$$

where the dissociation constant of the reaction, $K_{Mn^{2+}}$, is given by

$$\frac{\sigma_{\text{free (in the presence of } Mn^{2+})}}{\sigma_{\text{total (in the absence of } Mn^{2+})}} = \frac{1}{1 + ([Mn^{2+}]_s/K_{Mn^{2+}})} \tag{15}$$

where

$$[Mn^{2+}]_s = [Mn^{2+}]_0 \, e^{-2q\psi_s/kT}. \tag{16}$$

We were able to predict satisfactorily the Mg^{2+} inhibition of m in the presence of a small concentration of Mn^{2+} from both the Mg^{2+} inhibition of m in the absence of Mn^{2+} and the effect on m of adding a small quantity of Mn^{2+} to modified Ringer's solution. Fig. 10 demonstrates one such successful prediction.

From the value of $n = 4.03$ and the sixfold decrease in m seen on increasing $[Mg^{2+}]_0$ from 2 to 5 mM, we computed that $\psi_s = -82$ mV and $\sigma_{total} = 1\ e^-/130\ Å^2$ in 0.35 mM Ca^{2+} and 2.0 mM Mg^{2+}. From the 7.06-fold decrease in m seen on the addition of 0.20 mM Mn^{2+}, we computed a 6.2-mV reduction in ψ_s. Using the new value of $\psi_s = -75.8$ mV, we calculated that $\sigma_{free} = 1\ e^-/152\ Å^2$ (via Eq. 6). From the ratio $\sigma_{free}/\sigma_{total}$, we computed $K_{Mn^{2+}} = 0.92$ M (or 1.5 mM when referred to the bulk solution).

Increasing $[Mg^{2+}]_0$ in the presence of Mn^{2+} reduces ψ_s by screening surface charge, but in doing so also decreases $[Mn^{2+}]_s$, hence reexposing some previously bound surface charge. We were able to predict the effects on m of changing $[Mg^{2+}]_0$ in the presence of Mn^{2+} by substituting Eq. 15 into Eq. 6 to give Eq. 17:

$$2\pi(\sigma_{free})^2/\epsilon\, kT\left(1 + \frac{\gamma^2[Mn^{2+}]_0\, e^{-2q\psi_s/kT}}{K_{Mn^{2+}}}\right)^2 = [D^{2+}]_0(\gamma^2\, e^{-2q\psi/kT} - 3) + [U^+]_0(\gamma e^{-q\psi_s/kT} - 2), \tag{17}$$

where

$$\gamma = e^{-2q\Delta\psi_s/kT}; \tag{18}$$

solving Eq. 18 for $\Delta\psi_s$; and then solving Eq. 12 for the ratio of m's. Using this computational scheme, we calculated that increasing $[Mg^{2+}]_0$ from 2 to 5 mM in the presence of 0.35 mM Ca^{2+} and 0.20 mM Mg^{2+} should reduce ψ_s to -73.1 mV, increase σ_{free} to $1\ e^-/150\ Å^2$ and decrease m by 2.14-fold. This compares favorably with the 2.37-fold reduction in m actually observed. A comparison of the predicted curve (dashed line) with the experimental points (open circles) demonstrates the satisfactory agreement between observed and predicted effects at other values of m.

DISCUSSION

We have demonstrated that the inhibition of m caused by Mg^{2+} and some univalent cations can be accounted for quantitatively by a model which assumes that these cations act by reducing the concentration of Ca^{2+} near the presynaptic membrane surface by screening negative surface charges. Sr^{2+} inhibition of m can be at least qualitatively accounted for by this model. Mn^{2+} inhibition of m cannot be attributed chiefly to surface charge screening, but can be better accounted for assuming that Mn^{2+} tightly binds surface charge. A similar

charge binding scheme was proposed by Landau and Nachshen (1975) to account for the effects of H^+ on m. The ability of the electrostatic screening hypothesis to account for several interactions between inhibitory cations and Ca^{2+}, without the addition of new parameters, makes this model a reasonable alternative to the model of cationic inhibition of quantal release which postulates competitive binding to unspecified Ca^{2+}-dependent release activating sites.

The electrostatic screening hypothesis does not and need not specify the precise mechanism by which extracellular Ca^{2+} affects transmitter release, for as long as transmitter release is both a presynaptic membrane-related function and dependent on $[Ca^{2+}]_0$, changes in the concentration of Ca^{2+} nearest the presynaptic membrane surface should affect m. When considered in the light of a currently accepted, more detailed mechanism of Ca^{2+} action, which includes Ca^{2+} influx into the nerve terminal via voltage and time-dependent conductance channels (Katz and Miledi, 1969; Llinás et al., 1976), the electrostatic screening hypothesis suggests two interesting possibilities. (*a*) The surface charge density σ is the surface charge surrounding the entrances to presynaptic Ca^{2+} channels. (*b*) Cations which inhibit m by screening surface charge reduce Ca^{2+} influx through Ca^{2+} channels during membrane depolarization by reducing the Ca^{2+} concentration nearest the outer surfaces of the channels. These suggestions deserve some comment.

(*a*) The range of values of surface charge density reported here (i.e., 1 $e^-/164$ Å^2 at pH 6.6 to 1 $e^-/126$ Å^2 at pH 7.3) is similar to those calculated to be associated with the external surfaces of Na^+ and K^+ channels of various axons (Schauf, 1975). In the case of each of the latter channels, the negative surface charges are postulated to generate a negative surface potential which is thought to contribute to the electric field sensed by gating particles within the membrane. In these cases, when the effects of inactivation are removed, the major effect of changing ψ_s is considered to be the translation of the conductance vs. voltage characteristic of the channel along the voltage axis by an amount equivalent to the calculated change in ψ_s. The effect of a change in $[Na^+]_s$ or $[K^+]_s$ on channel conductance at a given voltage is, by this analysis, quite small. In contrast, we have implicitly assumed that the effects of changes of ψ_s on m is due to changes in the Ca^{2+} concentration at the external surfaces of the Ca^{2+} channels rather than changes in the electric field sensed by gating particles in these channels. This may be an oversimplification, but we feel that the following argument suggests that it is a reasonable first approximation.[1]

Let us assume that in the range of conditions pertinent to these experiments the presynaptic Ca^{2+} channel shows little or no inactivation and that shifts in the conductance vs. voltage characteristics of the Na^+ and K^+ channels due to changes in the divalent cation concentration are not reflected in change in action potential amplitude or duration.[1] Typically, increasing $[Mg^{2+}]_0$ from 1 to 4 mM results in a 5-mV reduction in ψ_s at constant membrane potential. This should increase the total potential difference seen by gating elements of the

[1](*a*) While no evidence is available concerning long-term inactivation of the presynaptic Ca^{2+} conductance, short-term inactivation seems unlikely in that the Ca^{2+} current at the squid stellate ganglion does not inactivate and the synaptic transfer function of the lamprey eel giant synapses is not altered by prior hyperpolarization of the nerve terminal (Martin and Ringham, 1975).

(*b*) Altering the total concentration of Ca^{2+} and Mg^{2+} from 0.5 to 5 mM does not change the amplitude or duration of the action potential of the node of Ranvier (Frankenhäuser, 1957; Frankenhäuser and Meves, 1958).

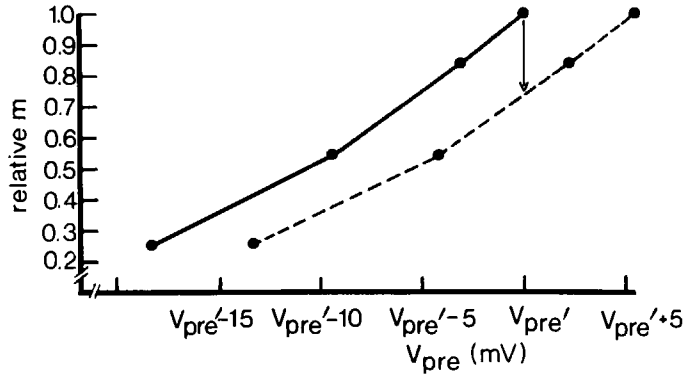

FIGURE 11 Solid line: m as a function of V_{pre} in 0.25 mM Ca^{2+} and 1 mM Mg^{2+}. This curve was computed from Fig. 5*b* (starred curve) assuming that the change in V_{pre} on reducing $[Na^+]_0$ is given by

$$\frac{RT}{F} \log \frac{[Na^+]_0 \text{ in substituted Ringer's solution}}{[Na^+]_0 \text{ in full } Na^+ \text{ Ringer's solution}}$$

and assigning a value of V'_{pre} to V_{pre} in full Na^+ Ringer's solution. m in full Na^+ Ringer's solution is taken as unity. Dashed line: solid line displaced to the right by 5 mV. Vertical arrow indicates the reduction in m, at constant $[Ca^{2+}]_s$ and V'_{pre}, predicted to result from the 5 mV reduction in the total potential difference across the Ca^{2+} channel. Note that in this analysis a 5-mV reduction in ψ_s is equivalent to shifting the curve of m vs. V_{pre} to the right by 5 mV.

Ca^{2+} channels by 5 mV, much like a 5-mV hyperpolarization of the membrane potential would. Fig. 11 presents a curve of m vs. presynaptic action potential amplitude (V_{pre}) at constant $[Ca^{2+}]_s$. (It is derived from a curve of m vs. glucosamine concentration in modified Ringer's solution containing 0.25 mM Ca^{2+} and 1 mM Mg^{2+}—the starred curve of Fig. 5 *b*—assuming that the reduction in m seen on replacing Na^+ with glucosamine is due to a reduction in action potential overshoot and that this reduction in overshoot is given by the Nernst potential for Na^+). Shifting the curve of m vs. V_{pre} to the right by 5 mV should reduce m seen in full Na^+ Ringer's solution by 1.28-fold. This is small compared with the nearly tenfold reduction in m observed on raising $[Mg^{2+}]_0$ from 1 to 4 mM and likewise predicted from our analysis.

This argument is at variance with Mathews and Winkelgren (1977) and Madden and van der Kloot (1978), who have suggested that the abilities of small concentrations of Mg^{2+} and large concentrations of Ca^{2+} to reduce the Ca^{2+} dependent rise in m.e.p.p. frequency seen on rising $[K^+]_0$ is qualitatively consistent with surface charge screening by divalent cations, where the changes in ψ_s strongly affect the total potential difference seen by a voltage dependent channel.

(*b*) The success of electrostatic screening in accounting for the action of Mg^{2+} and univalent cations in inhibiting m suggests that these cations do little to occlude Ca^{2+} channels, slow the passage of individual Ca^{2+} ions through them, or compete for binding to release activating sites on the inside of the presynaptic membrane. In the case of Mg^{2+}, two lines of evidence available to address these points appear to support this argument. First, the most direct evidence for a presynaptic Ca^{2+} conductance channel is the voltage dependent delayed inward current recordable in the presence of extracellular Ca^{2+} across the presynaptic

terminal of the squid stellate ganglion pretreated with tetrodotoxin and 4-aminopyridine (Llinás et al., 1976). The amplitude of the postsynaptic potential correlates well with the amplitude of the Ca^{2+} current. No inward current is recorded under similar conditions in the absence of Ca^{2+} but in the presence of 20 mM Mg^{2+}. Second, Miledi (1973) showed that co-injection of Mg^{2+} and Ca^{2+} into the presynaptic terminal of the squid stellate ganglion at best "only slightly reduces the amount of transmitter release" below that seen with Ca^{2+} injection alone, suggesting that even if Mg^{2+} were to enter the nerve terminal by a route other than the Ca^{2+} channel, it still might not significantly inhibit quantal release. Interestingly, we have found that either tetanic stimulation of the motor nerve or application of black widow spider venom, in Ca^{2+}-free, Mg^{2+}-enriched solutions, actually increases asynchronous transmitter release (Misler and Hurlbut, 1979). These are two conditions where intraterminal $[Mg^{2+}]$ might be expected to rise significantly.

It is clear that neither the electrostatic screening nor the competitive binding hypotheses of cationic inhibition of quantal release have been independently verified, since neither the surface charge nor the binding sites have been clearly identified. While both analyses are based on curve fitting, the electrostatic screening hypothesis has clear value in predicting the inhibitory effects of several cations when the effect of one cation is known. Using a preparation which permits presynaptic intracellular recording it may be possible however to infer more closely the existence of σ and its role in modulating quantal release. This may be possible by examining the ability of various inhibitory cations (*a*) to reduce the Ca^{2+} current measured at a given clamping potential (by shifting the conductance vs. voltage curve of the channel or by reducing peak Ca^{2+} current) and (*b*) to change the magnitude or power spectrum of the Ca^{2+} current noise or alter single Ca^{2+} channel conductance.

APPENDIX

Effects of Glucosamine and Arginine on Action Potential Electrogenesis

Fig. A-1 compares the effects of glucosamine, arginine, and tetramethylamine (TMA), a known impermeant and nearly inert Na^+ substitute, on the monophasic compound action potential (MCAP) of the desheathed frog sciatic nerve trunk. Note that the MCAP waveforms are nearly identical in 51 mM glucosamine and 51 mM arginine substituted Ringer's solution, and that MCAP amplitudes in these solutions differed little from MCAP amplitude in 51 mM TMA substituted Ringer's solution. (Note,

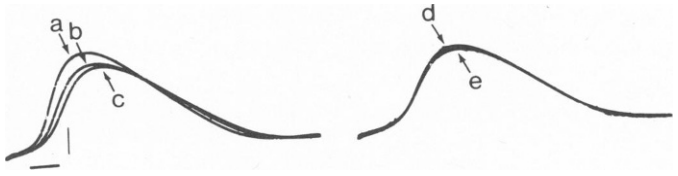

FIGURE A-1 The effects of univalent cations used as Na^+ substitutes on the monophasic compound action potential of the desheathed frog sciatic nerve trunk. All test solutions contained 1.8 mM Ca^{2+} and 3 mM Mg^{2+} and were buffered to pH 6.5–6.6 Stimuli were delivered to the proximal end of the nerve trunk across a petroleum jelly seal separating two pools of full Na^+ Ringer's solution. Stimulus intensity was adjusted to recruit only the largest diameter (Aα) fibers. The action potential was recorded across a seal separating a pool containing the test solution and a pool containing full (116 mM) TMA Ringer's solution. (*a*) Full Na^+ Ringer's solution; (*b*) 51 mM TMA Ringer's solution; (*c, d*) 51 mM glucosamine Ringer's solution; and (*e*) 51 mM arginine Ringer's solution. Calibration 0.1 ms × 5 mV.

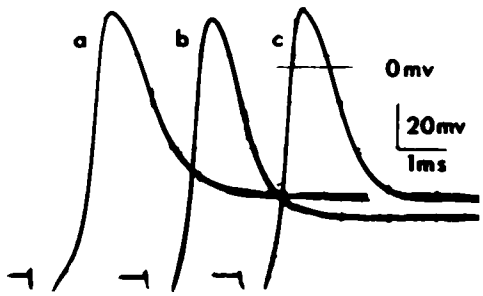

FIGURE A-2 Action potentials recorded from a single fiber of frog cutaneous pectoris muscle (resting potential = −88 mV) exposed to (*a*) 51 mM glucosamine Ringer's solution, (*b*) 51 mM TMA Ringer's solution, and (*c*) 95 mM sucrose Ringer's solution, all containing 1.8 mM Ca^{2+}, 3.0 mM Mg^{2+}, and *d*-tubocurarine (5 μg/ml) and all buffered to pH 6.5–6.6. Traces were recorded during the continuous impalement of a nonjunctional region of a fiber selected from a fiber bundle stretched to one and a half times the resting length and stimulated by an extracellular current source. The overshoot potentials were +25, +23, and +25 mV, respectively. The maximum upstroke velocity was nearly 350 mV/ms in all three solutions. Similar results were obtained with 51 mM arginine Ringer's solution (not shown). In this fiber the overshoot potential and maximum upstroke velocity were +38 mV and 525 mV/ms, respectively in full Na^+ Ringer's solution.

however, that when compared to TMA, glucosamine and arginine produced small reductions in conduction velocity and rate of rise of the MCAP, as well as increased MCAP dispersion.)

Fig. A-2 compares the intracellularly recorded action potential of a frog cutaneous pectoris muscle fiber in a variety of Na^+ substituted Ringer's solutions. The skeletal muscle fiber was chosen as a test system for the intracellular recording of action potentials because the organic cation selectivity of the Na^+ channel of this tissue closely resembles that of the node of Ranvier (Campbell, 1976). Note that the amplitudes of the muscle action potentials were very similar when 51 mM Na^+ was iso-osmotically replaced by sucrose, glucosamine, arginine, or TMA. Similar results were obtained with 51 mM arginine in other experiments. In addition, the overall waveform of the action potential was only minimally altered when the univalent cation concentration of the Ringer's solution was reduced by replacing either TMA or glucosamine with sucrose.

These data suggest that glucosamine and arginine may have only minimal effects on the Na^+ channel of the motor nerve terminal beyond being nearly impermeant to it, and that in the range of conditions tested in these experiments, possible changes in the conductance-voltage relationships of Na^+ and K^+ conductance channels resulting from surface potential changes, may not be reflected in action potential waveform.

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