Protons Resolve Dual Effects of Calcium on Miniature End-Plate Potential Frequency at Frog Neuromuscular Junctions

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ABSTRACT   Inhibition of transmitter release by protons (H^+) was studied at the frog neuromuscular junction at various extracellular concentrations of calcium ($[Ca^{++}]_o$) and potassium ($[K^+]_o$) by recording miniature end-plate potential (MEPP) frequency with the intracellular microelectrode. H^+ decreased K^+-stimulated MEPP frequency. A double logarithmic graph of MEPP frequency at 7.5 mM K^+ vs. $[H^+]_o$ yielded a straight line with negative slope. At 10 mM K^+, there was a parallel shift to the right of the graph. According to the surface charge model, K^+ acts solely to depolarize the prejunctional membrane in accordance with the Nernst equation. By decreasing the prejunctional negative surface charge, H^+ decreases K^+-stimulated MEPP frequency by decreasing $[Ca^{++}]_o$ at the Ca^{++} channel. An estimated pK_a of 4.20 may represent an acidic site at the Ca^{++} channel associated with Ca^{++} influx.

As $[Ca^{++}]_o$ increased above 1 mM for pH 7.40 and 10 mM K^+, MEPP frequency decreased, i.e., the inhibitory component of dual effects of Ca^{++} occurred. At pH 6.40, the inhibitory component was abolished, unmasking the stimulatory effect of Ca^{++} on MEPP frequency. Reversal of Ca^{++} action by H^+ could not be explained by surface charge theory alone.

A double logarithmic graph of MEPP frequency vs. $[K^+]_o$ at 8.5–10.5 mM was linear with a slope of 4. There were parallel shifts to the right of this graph for changes in pH from 7.40 to 6.90 and in $[Ca^{++}]_o$ from 1 to 2.5 mM. These results are explained on the hypothesis that K^+ also acts at an acidic prejunctional site to increase Ca^{++}-dependent quantal transmitter release. This action of K^+ was inhibited by H^+ and raised Ca^{++}. Based on kinetic theory, the estimated pK_a of the acidic prejunctional K^+ site was 6.31. Based on free energy calculations, its cation preference was $H^+ > K^+ > Ca^{++}$.

INTRODUCTION

The effect of extracellular Ca^{++} on transmitter release is presumably due to an alteration of intracellular Ca^{++} (Hubbard, 1973; Miledi, 1973). In the presence of

raised $[K^+]_o$, Ca^{++} has dual or nonmonotonic effects (i.e., inhibitory as well as stimulatory) on transmitter release. The nonmonotonic effect of Ca^{++} has been observed at pH 6.7–7.4 at neuromuscular junctions of frog (Birks et al., 1968) and rat (Cooke and Quastel, 1973).

Attempts have been made to explain the nonmonotonic effect of Ca^{++}. One explanation is that Ca^{++} inhibits a fast stimulatory component of K^+ action on the transmitter release process, which is different from K^+-induced depolarization of the prejunctional membrane (Cooke and Quastel, 1973). The possibility that K^+ has two separate effects on transmitter release was challenged on the basis that surface charge theory alone adequately explains Ca^{++} inhibition (Matthews and Wickelgren, 1977; Madden and Van Der Kloot, 1978; Kim and Sanders, 1979). Alternatively, when Ca^{++} enters the nerve terminal at high concentrations, the cation may stimulate either its own efflux or perhaps uptake by intraneuronal organelles (Otha and Kuba, 1980).

In the present study, kinetic and surface charge theories were used to analyze the action of H^+ on the nonmonotonic effect of Ca^{++}. Reversal of the Ca^{++} effect, i.e., the change from Ca^{++} inhibition of MEPP frequency to Ca^{++} stimulation, by H^+ could not be explained by surface charge theory alone. Based on kinetic theory, it was shown that K^+ increased Ca^{++}-dependent (mediated) quantal transmitter release at depolarized nerve terminals by binding to an acidic prejunctional site with pK_a 6.31. The analysis showed that H^+ was more effective than Ca^{++} in competing with K^+ for this site.

METHODS

Preparations, Solutions, and MEPP Frequency Recordings

Intracellular microelectrode recordings were made at end-plates of sartorius preparations in vitro from 1.5–2-in northern grass frogs (*Rana pipiens pipiens*). Glass micropipettes filled with 3 M KCl and with tip resistances of 10–30 MΩ were used. After dissection, the preparation was mounted in a 1.5–ml Lucite chamber, submerged in Ringer's (saline) and MEPPs were recorded. Experiments were done at room temperature (20°C).

Preparations were continuously superfused with saline by using a model 375 Sage tubing pump (Orion, Cambridge, MA); saline flux was 5 ml/min. Within 5 min, equilibration of the bathing solution in the chamber was virtually complete according to a first order kinetic process with the time constant equal to chamber volume/flux of 0.3 min. To reduce the time required for equilibration of the tissue with test saline, only surface fibers were used. Recordings were begun after steady state MEPP frequencies were achieved at the end-plate. Steady state was achieved within 2–5 min from the time saline reached the chamber. MEPPs were recorded via a model 750 preamplifier (World Precision Instruments, Inc., New Haven, CT) through a 5-kHz filter to a model 5A48 amplifier (Tektronix, Inc., Beaverton, OR) and a model 5223 oscilloscope (Tektronix). The resting membrane potential (RMP) was recorded via a model 360-2 digital millivoltmeter (Simpson Electric Co., Elgin, IL). Permanent records of MEPPs were achieved by using a model C-4 kymograph camera (Grass Instrument Co., Quincy, MA). At least 300 MEPPs were counted and averaged per recording.

The mean MEPP frequency recorded in saline A (Table I) was used as the standard of reference (F_o) in most experiments. Exceptions were the use of (a) saline B to determine if Tris buffer can be substituted for phosphate buffer, and (b) saline F in some experiments in which increases in MEPP frequency by test salines relative to the control were anticipated.

Each F_0 was recorded at pH 7.40. At each end-plate, the average MEPP frequency per recording in a test saline (F) was expressed relative to F_0 as a ratio, F/F_0. This method allowed results of the same test salines at different end-plates to be compared. Only one experiment was performed per end-plate.

Osmolality, in milliosmoles per kilogram, was measured by using an Osmette A osmometer (Precision Systems, Inc., Natick, MA). A standard pH meter and Ross pH electrode (Orion) were used to measure pH; pH was recorded up to 1/100 pH unit. Ca^{++} ionic activity was measured with a Ca^{++}-selective electrode (World Precision Instruments). All salines contained 10^{-7} M tetrodotoxin and 10^{-6} M neostigmine bromide. Tris, tetrodotoxin, and neostigmine were obtained from Sigma Chemical Co., St Louis, MO.

Theoretical Considerations

In these experiments, surface charge and kinetic models were used to make predictions, and to analyze data.

Surface charge theory. H^+ decreases the negative surface potential at the neuronal membrane by decreasing the total negative surface charge (Gilbert, 1971). The change in surface

TABLE I

Composition of Salines in Millimolar

Saline*	NaCl	KCl	CaCl$_2$	Dextrose	NaH$_2$PO$_4$	Na$_2$HPO$_4$	Tris maleate	Milliosmoles/ kilogram
A	104	10.0	1	—	0.6	2.25	—	218
B (B')[B"]	104	10.0	1 (5)[10]	—	—	—	1.2	218 (225)[240]
C (C')	105 (96)	10.0	0.2 (5)	—	—	—	1.2	218 (218)
D	107	7.5	1	—	0.6	2.25	—	218
E (E')	106 (99)	2.5	5 (10)	—	—	—	1.2	218 (218)
F	112	2.5	1	—	0.6	2.25	—	218
G (G')[G"]	112	2.5	1 (5)[10]	—	—	—	1.2	218 (225)[240]
H (H')	112	2.5	1 (5)	20 (9)	—	—	1.2	240 (240)

*Parentheses show variations in ionic compositions of salines at a given $[K^+]_0$. For instance, 1, 5, and 10 mM CaCl$_2$ yielded salines B, B', and B" with osmolalities of 218, 225, and 240 mosmol, respectively. Other alternatives of saline B used in this study (not shown) were 1–2.5 mM Ca and 8.5–10.5 mM K. Concentrations of Na phosphates shown above yielded pH 7.40. Concentrations of Na phosphates were adjusted, while maintaining osmolality and ionic strength, to achieve pH values below pH 7.40. To achieve the desired pH with Tris maleate, HCl was added.

potential, $\Delta\psi(0)$, was estimated by using the procedure of Madden and Van Der Kloot (1978). Because H^+ decreased MEPP frequency at raised K^+, equivalent MEPP frequencies were obtained at pH 7.40 and 6.40 by elevating $[K^+]_0$ at pH 6.40. The hypothesis is that the incremental increase in prejunctional depolarization, ΔV_m (in millivolts), induced by elevating $[K^+]_0$, is equal to $\Delta\psi(0)$, i.e.,

$$\Delta V_m = RT/F \ln [K(\infty)]'_0/[K(\infty)]_0 \qquad (1A)$$

$$\Delta\psi(0) = \Delta V_m \qquad (1B)$$

Where $[K^+]_0$ in bulk superfusing salines (in the chamber) are $[K(\infty)]'_0$ and $[K(\infty)]_0$ at pH 7.40 and 6.40, respectively, and RT/F is 25.3 mv at room temperature. The prejunctional ΔV_m predicted by Eq. 1A was compared with the resting membrane potential change at the end-plate of the muscle when $[K^+]_0$ was changed.

The pK_a of the acidic nerve terminal site associated with the decrease in negative surface potential by H^+ was estimated. The hypothesis is that this pK_a represents an acidic site at the

Ca^{++} channel associated with Ca^{++} influx (Landau and Nachshen, 1975). The relation between the dissociation constant for H^+ (K_H), the negative surface potential [$\psi(0)$]; and the negative surface charge (σ) of the prejunctional membrane is

$$[H(\infty)]_o/K_H \times \exp -F\psi(0)/RT = \sigma_T/\sigma - 1 \tag{2}$$

where $[H(\infty)]_o$ represents $[H^+]_o$ in bulk saline, and σ_T represents the total negative surface charge of the prejunctional membrane (Gilbert, 1971). Eq. 2 may be rearranged to solve for K_H as follows,

$$K_H = \frac{\sigma[H(\infty)]_o \exp[-F\psi(0)/RT] - \sigma'[H(\infty)]_o' \exp[-F\psi'(0)/RT]}{\sigma' - \sigma} \tag{3}$$

for σ' and σ at pH 7.40 and 6.40, respectively. σ' and σ (in electronic charges/$Å^2$) will be obtained by using the Gouy-Chapman equation, i.e.,

$$\sigma = 1/270 \sum_{i=1}^{n} (c_{i\infty} \{\exp[-z_iF\psi(0)/RT] - 1\})^{1/2} \tag{4}$$

where $c_{i\infty}$ (in moles/liter) is the concentration of the i-th ion in bulk saline, and z_i is its valence. At pH 7.40, $\psi'(0)$ equals -100 mv (Madden and Van Der Kloot, 1978), and $\psi(0)$ equals $\psi'(0) - \Delta\psi(0)$. The pK_a is $-\log K_H$.

Based on the surface charge model, K^+ acts solely to depolarize the nerve terminal in accordance with the Nernst equation (Madden and Van Der Kloot, 1978; Kim and Sanders, 1979). In the absence of a direct interaction between K^+ and the prejunctional membrane, H^+ may decrease K^+-stimulated MEPP frequency by decreasing $[Ca^{++}]_o$ at the Ca^{++} channel. For instance, using $\Delta\psi(0)$ (Eq. 1B) and the Boltzmann distribution, a pH change from 7.40 to 6.40 yields
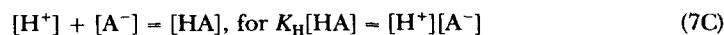
$$\frac{[Ca(0)]_o'}{[Ca(0)]_o} = \exp -2F\Delta\psi(0)/RT \tag{5}$$

where $[Ca(0)]_o'$ and $[Ca(0)]_o$ represent $[Ca^{++}]_o$ at the prejunctional membrane, i.e., at the Ca^{++} channel, at pH 7.40 and 6.40, respectively.

Kinetic theory. The hypothesis is that K^+ has a specific action at the nerve terminal that is blocked by raised Ca^{++} (Cooke and Quastel, 1973). The hypothesis requires that K^+ binds to an acidic nerve terminal site to increase MEPP frequency (F) in the presence of raised $[K^+]_o$. Thus, at raised $[K^+]_o$,

$$F = [C \times V_K(K)]^u + f_i \tag{6}$$

where C and u are proportionality factors, f_i represents MEPP frequency at 2.5 mM K^+, and $V_K(K)$ represents the fractional occupancy of K^+ at a negatively charged site, A^-, at the prejunctional membrane. According to this hypothesis, K^+ acts at site A^- to increase quantal transmitter output by Ca^{++} at depolarized nerve terminals. The assumption is that this specific effect of K^+ is linearly related to the one reported for Ca^{++} (Dodge and Rahamimoff, 1967). The validity of this assumption was evaluated by determining if the term u is equal to the exponential factor, n, in Dodge and Rahamimoff's (1967) model (i.e., $n = \log$ quantum content/$\log [Ca^{++}]_o$). Binding of K^+ to A^- may be competitively inhibited by H^+ and Ca^{++} as follows,

$$[K^+] + [A^-] = [KA], \text{ for } K_K[KA] = [K^+][A^-] \tag{7A}$$

$$[Ca^{++}] + [A^-] = [CaA]^+, \text{ for } K_{Ca}[CaA]^+ = [Ca^{++}][A^-] \tag{7B}$$

$$[H^+] + [A^-] = [HA], \text{ for } K_H[HA] = [H^+][A^-] \tag{7C}$$

where K_K and K_H are dissociation constants (K_d) of K^+ and H^+, respectively, and K_{Ca} is the intrinsic (microscopic) K_d of the divalent cation Ca^{++} (Metzler, 1977). The total number of binding sites is

$$[A^-]_T = [A^-] + [CaA]^+ + [HA] + [KA] \tag{8}$$

The fractional occupancy of K^+ at A^-, i.e., $[KA]/[A^-]_T$ (or $V_K[K]$), is

$$V_K = \frac{[K^+]/K_K}{1 + [Ca^{++}]/K_{Ca} + [H^+]/K_H + [K^+]/K_K} . \tag{9}$$

For $F \gg f_i$ in Eq. 6, i.e., at raised $[K^+]_o$,

$$\log F = u \log \frac{C\,[K^+]/K_K}{1 + [Ca^{++}]/K_{Ca} + [H^+]/K_H + [K^+]/K_K} \tag{10}$$

where u represents the slope of a graph of $\log F$ vs. $\log [K^+]_o$. The Lineweaver-Burke expression may be written as follows,

$$F^{-1/u} = m \times 1/[K^+] + 1/C \tag{11}$$

$$m = (1 + [Ca^{++}]/K_{Ca} + [H^+]/K_H) \times K_K/C \tag{12}$$

$[K^+]_o$ was used at 8.5–10.5 mM for (a) control condition: 1 mM Ca^{++} $([Ca^{++}]_1)$ and pH 7.40 $(-\log [H^+]_1)$; (b) test condition I: test Ca^{++} $([Ca^{++}]_2)$ and pH 7.40; and (c) test condition II: $[Ca^{++}]_1$ and test pH $(-\log [H^+]_2)$. In each experiment, u, u', and u'' were estimated from graphs of $\log F$ vs. $\log [K^+]_o$ for control condition, test condition I, and test condition II, respectively. For u, u', and u'', estimates of m, m', and m'' were obtained, respectively, (using Eq. 11). Thus, K_{Ca} and K_H were obtained in each experiment as follows,

$$m/(1 + [Ca^{++}]_1/K_{Ca} + [H^+]_1/K_H) = m'/(1 + [Ca^{++}]_2/K_{Ca} + [H^+]_1/K_H) \tag{13A}$$

$$m/(1 + [Ca^{++}]_1/K_{Ca} + [H^+]_1/K_H) = m''/(1 + [Ca^{++}]_1/K_{Ca} + [H^+]_2/K_H) \tag{13B}$$

where $[Ca^{++}]_1$, $[Ca^{++}]_2$, $[H^+]_1$, and $[H^+]_2$ were known.

Since the constant C is unknown, K_K cannot be determined from Eq. 12. For constant values of K^+ and Ca^{++}, i.e., $[K^+]_1$ and $[Ca^{++}]_1$, $\log C\,V_K$ may be a function of pH $(-\log [H^+])$, i.e.,

$$\log C\,V_K = -\log (1 + [K^+]_1/K_K + [Ca^{++}]_1/K_{Ca} + [H^+]/K_H) + \log C\,[K^+]_1/K_K \tag{14}$$

if the term $[H^+]/K_H$ is not negligible. Based on Eq. 6,

$$\frac{d\log F}{d\mathrm{pH}} = (u \times S_e) \tag{15}$$

for $du/d\mathrm{pH}$ equal to zero, and S_e equal to $d \log C\ V_K/d\mathrm{pH}$. $du/d\mathrm{pH}$ may be zero based on the hypothesis that u, defined only for $F \gg f_i$, is equal to n in Dodge and Rahamimoff's model (1967); n is independent of pH (Landau and Nachshen, 1975). If it is assumed that a graph of $\log F$ vs. pH can be approximated by a straight line with slope magnitude $(u \times S_e)$, then based on this criterion,

$$S_e = 1/u \times \frac{\log \dfrac{F\,(\mathrm{pH}_1)}{F\,(\mathrm{pH}_2)}}{\mathrm{pH}_1 - \mathrm{pH}_2} \tag{16}$$

at pH_1 and pH_2. The validity of this criterion was tested experimentally. If (a) the term $[H^+]/$

K_H in Eq. 14 is not negligible, and (b) the pH interval at pH_1 and pH_2 is small such that the pH dependence of S_e is negligible, then for S_e equal to $d \log C \ V_K/d$pH,

$$S_e = \frac{\log\left[1 + \dfrac{([H^+]_2 - [H^+]_1)/K_H}{1 + [K^+]_1/K_K + [Ca^{++}]_1/K_{Ca} + [H^+]_1/K_H}\right]}{pH_1 - pH_2} \tag{17}$$

at pH_1 and pH_2. Rearranging Eq. 17 yields an estimate of K_K as follows,

$$K_K = \frac{([H^+]_2/[H^+]_1)^{S_e} - 1) \times [K^+]_1}{\dfrac{[H^+]_2 - [H^+]_1([H^+]_2/[H^+]_1)^{S_e}}{K_H} - [([H^+]_2/[H^+]_1)^{S_e} - 1] \times (1 + [Ca^{++}]_1/K_{Ca})} \tag{18}$$

K_K was determined (Eq. 18) by using estimates of K_H, K_{Ca}, and S_e.

The strength of the electrostatic interaction between cation and site A^- is expressed as the free energy change associated with bond formation, i.e.,

$$\Delta G_f = -1.36 \, pK_d \tag{19}$$

in kilocalories per mole at room temperature, where pK_d is $-\log K_d$. Mean values ± SEM were obtained for pK_a ($-\log K_H$), pCa ($-\log K_{Ca}$), and pK ($-\log K_K$), and respective estimates of ΔG_f were determined.

Statistical Analysis

Data were reported as means ± SEM. When two means were compared, an unpaired t test was used; for paired data, a paired t test was used. When comparing more than two means, an analysis of variance was used. A chi square test was used to determine the goodness of fit of data to a straight line. The critical level of significance (P) was <5%.

RESULTS

Effect of H^+ on MEPP Frequency in the Presence of Raised $[K^+]_o$

When pH was decreased below 7.40, MEPP frequency decreased at 10 mM K^+ (Fig. 1, A and B). Maximum changes in MEPP frequency occurred at 2–5 min after inflow of low pH test saline into the chamber. It was independent of the order in which test salines were used. Similar results were obtained whether pH 6.40 saline was used first or if it were last in the experimental sequence shown in Fig. 1 A.

MEPP frequencies recorded during return to pH 7.40 did not significantly differ from the initial recording. H^+ decreased MEPP frequency at various $[Ca^{++}]_o$. This effect occurred for inhibitory $[Ca^{++}]_o$ (5 mM) as well as stimulatory $[Ca^{++}]_o$ (0.2 mM) (Fig. 2). In this and all subsequent experiments in which F_o (always recorded at pH 7.40 and 1 mM Ca^{++}) was obtained at 10 mM K^+, the mean ± SEM was 13.64 MEPPs s^{-1} ± 1.83 at 41 end-plates; the range was 2.16–54.12 MEPPs s^{-1}.

Effect of H^+ on MEPP Frequency in the Absence of Raised $[K^+]_o$

A constant $[Na^+]_o$ was used in test salines at various $[Ca^{++}]_o$. The reason was because, at or above 1 mM Ca^{++}, reductions in $[Na^+]_o$ were associated with decreases in MEPP frequency (Fig. 3 A); the mechanism is unknown (see Fig. 4 in
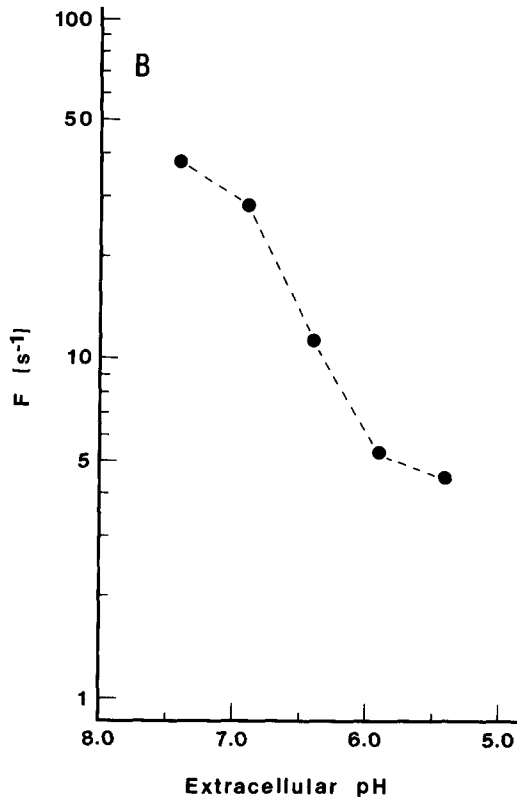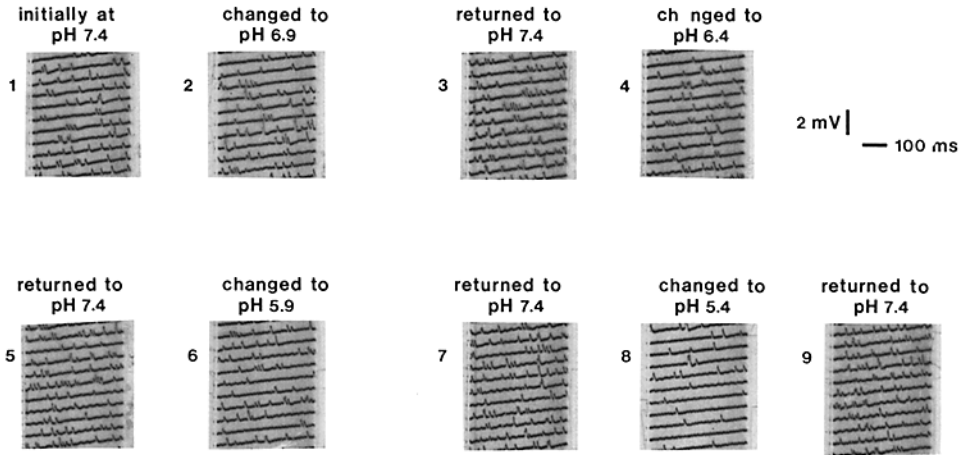
A

FIGURE 1. (A) The experimental sequence is shown for effects of H^+ on MEPP frequency in a representative experiment at a single end-plate. Superfusing Ringer's contained 10 mM K^+; saline A of Table I was used. (B) The experiment shown in A is presented graphically. Each point represents MEPP frequency (F) recorded during superfusion of the preparation with test salines below pH 7.40. At pH 7.40, the initial MEPP frequency was 39 MEPPs s^{-1}. These data are presented in the first row of Table II.

Birks et al., 1968). When $[Ca^{++}]_o$ was changed from 1 to 10 mM, the osmolality of the test saline changed from 218 to 240 mosmol. The 22 mosmol difference did not account for changes in MEPP frequency observed at various $[Ca^{++}]_o$ because similar changes in MEPP frequency occurred when dextrose was used to maintain isosmolality (Fig. 3 B).

At pH 7.40 and 2.5 mM K^+, the mean slope ± SEM of the double logarithmic graph of MEPP frequency vs. $[Ca^{++}]_o$ was 0.90 ± 0.10 at three end-plates. At the same end-plates, the slope at pH 6.40 (low pH) was expressed as a percentage of the value at neutral pH. When pH was decreased from 7.40 to 6.40, the slope decreased by 42%: expressed as a mean percentage ± SEM, it was 57.8 ± 2.3% at
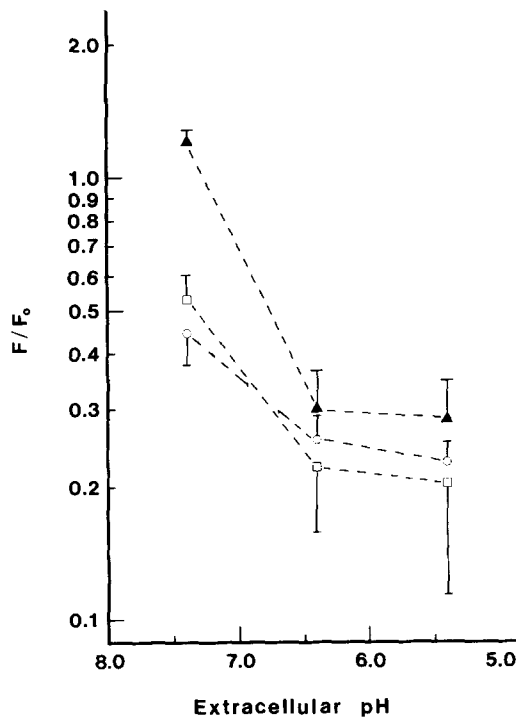

FIGURE 2. Effects of H^+ on MEPP frequency at 10 mM K^+ and various $[Ca^{++}]_o$ are shown. The ordinate shows results expressed relative to F_o (recorded in saline A) as a ratio, F/F_o. The abscissa shows pH of test salines. $[Ca^{++}]_o$ at different end-plates were: □, 0.2 mM (saline C); ▲, 1 mM (saline B); and O, 5 mM (saline C'). Each point, F/F_o, is a mean of four experiments; vertical bars show SEM.

the three end-plates used. At low pH, a significant linear increase in MEPP frequency ($P < 0.5\%$) still occurred when $[Ca^{++}]_o$ was increased (Fig. 3 C).

Use of the Surface Charge Model to Estimate $\Delta\Psi(0)$

An estimate of $\Delta\psi(0)$ was obtained for a change in pH from 7.40 to 6.40 by using Eqs. 1, A and B. Fig. 4 shows equivalent MEPP frequencies at pH 6.40 for 10 mM K^+, and at pH 7.40 for 7.5 mM K^+. Mean absolute MEPP frequencies ± SEM were: 6.74 MEPPs s^{-1} ± 1.36 at pH 6.40 for 10 mM K^+ (Tris buffer); and 5.97 MEPPs s^{-1} ± 0.38 at pH 7.40 for 7.5 mM K^+ (phosphate buffer). The use of Tris buffer had no effect on these results because, in Table II, the mean absolute MEPP frequency ± SEM at pH 6.40 for 10 mM K^+ (phosphate buffer) was 5.26 MEPPs s^{-1} ± 2.32. No
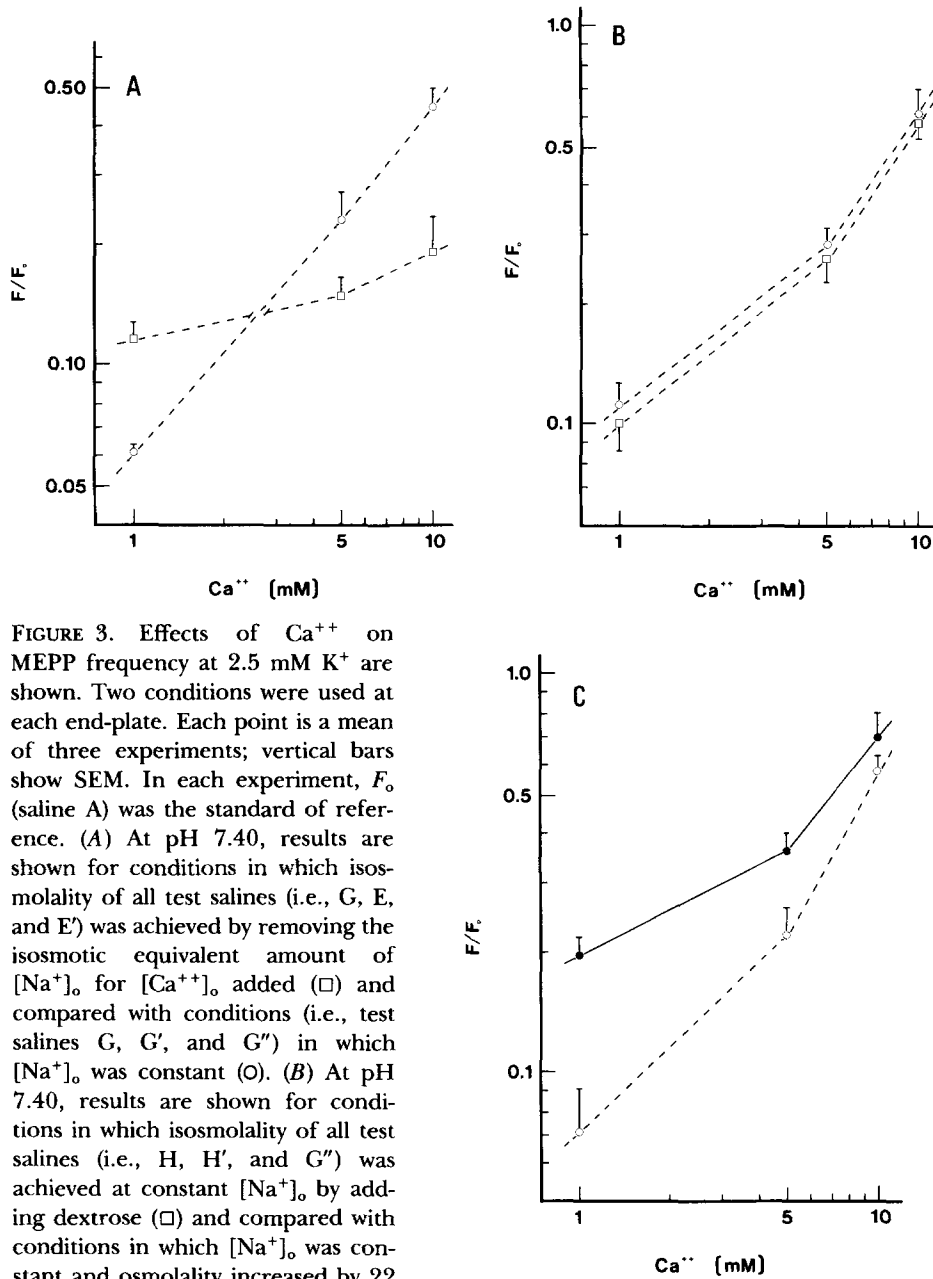
FIGURE 3. Effects of Ca^{++} on MEPP frequency at 2.5 mM K^+ are shown. Two conditions were used at each end-plate. Each point is a mean of three experiments; vertical bars show SEM. In each experiment, F_o (saline A) was the standard of reference. (A) At pH 7.40, results are shown for conditions in which isosmolality of all test salines (i.e., G, E, and E') was achieved by removing the isosmotic equivalent amount of $[Na^+]_o$ for $[Ca^{++}]_o$ added ($\square$) and compared with conditions (i.e., test salines G, G', and G") in which $[Na^+]_o$ was constant (O). (B) At pH 7.40, results are shown for conditions in which isosmolality of all test salines (i.e., H, H', and G") was achieved at constant $[Na^+]_o$ by adding dextrose ($\square$) and compared with conditions in which $[Na^+]_o$ was constant and osmolality increased by 22 mosmol for a change in $[Ca^{++}]_o$ from 1 mM to 10 mM (i.e., salines G, G', and G") (O). (C) The action of H^+ on the stimulatory effect of Ca^{++} is shown at pH 7.40 (O) and pH 6.40 ($\bullet$). Test salines G, G', and G" were used.

significant difference existed between these three mean MEPP frequency values. Thus, the decrease in MEPP frequency resulting from a change in pH from 7.40 to 6.40 at 7.5 mM K^+ was counteracted by raising $[K^+]_o$ to 10 mM (at pH 6.40). The estimate of ΔV_m and, therefore $\Delta\psi(0)$, was -7.3 mv; it was obtained by substituting 10 mM K^+ and 7.5 mM K^+ for $[K(\infty)]_o$ and $[K(\infty)]'_o$, respectively, in Eq. 1A.

At the end-plate region of a representative muscle fiber, the observed RMP change associated with a change in $[K^+]_o$ from 7.5 to 10 mM was -5.7 mv: -58.7 mv recorded at pH 7.40 for 7.5 mM K^+ minus -53.0 mv recorded at pH 6.40 for 10 mM K^+. No significant change in the muscle RMP occurred for a change in pH from 7.40 to 6.40. The RMP change (-5.7 mv) of the muscle fiber was of the same order of magnitude as the prejunctional ΔV_m (-7.3 mv) predicted by Eq. 1A. Using Eqs. 2–4, the pK_a estimate was 4.20. This individual estimate of the pK_a obtained by
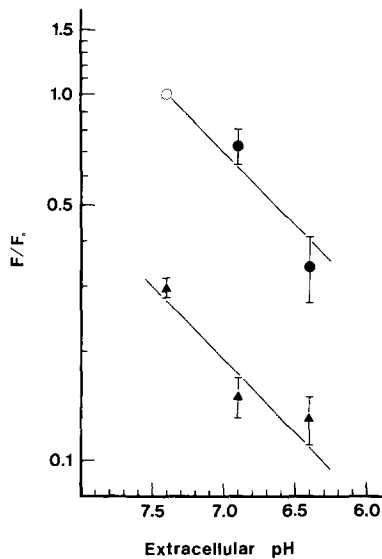

FIGURE 4. Effects of H^+ on MEPP frequency at various raised $[K^+]_o$ are shown. F_o (O) as well as results obtained in test salines below neutral pH at 10 mM K^+ (●) were recorded in saline B (Table I). Each point is a mean of three experiments; vertical bars show SEM. Table II shows these results at muscle numbers 5, 6, and 7. At 7.5 mM K^+, results obtained in test salines at pH 6.40–7.40 (▲) were recorded in saline D; F_o, in this instance, was saline A. Each point is a mean of four experiments; vertical bars show SEM. Straight line fits to the data were obtained. A parallel shift to the right was observed when $[K^+]_o$ was changed from 7.5 to 10 mM. Slopes were not significantly different: mean slopes ± SEM were 0.49 ± 0.09 at 10 mM K^+, and 0.37 ± 0.09 at 7.5 mM K^+.

using surface charge theory was consistent with reported pK_a values of 3.6 and 5.7 (Landau and Nachshen, 1975).

Effect of H^+ on the Nonmonotonic Effect of Ca^{++}, Predictions Based on the Surface Charge Model

For $\Delta\psi(0)$ equal to -7.3 mv, the ratio of $[Ca(0)]_o$ at pH 7.40 to $[Ca(0)]_o$ at pH 6.40 was determined (see Eq. 5 of the Methods). The ratio was 2. Thus, according to surface charge theory, $[Ca^{++}]_o$ at the Ca^{++} channel at pH 7.40 was twice as large as that at pH 6.40. The theory assumed that if $[Ca^{++}]_o$ in bulk saline at pH 6.40 was doubled, then $[Ca^{++}]_o$ at the Ca^{++} channel before and after the pH change were equivalent. For instance, the surface charge model predicted that a K^+-stimulated

MEPP frequency due to $[Ca^{++}]_o$ at the Ca^{++} channel would be equivalent at pH 7.40 for 5 mM Ca^{++} in bulk saline and pH 6.40 for 10 mM Ca^{++} in bulk saline. This prediction was consistent with observed mean MEPP frequencies at pH 7.40 for 5 mM Ca^{++}, and pH 6.40 for 10 mM Ca^{++} (Fig. 5, legend). Ca^{++} stimulation of MEPP frequency was observed at pH 6.40 for 10 mM Ca^{++} and 10 mM K^+, whereas Ca^{++} inhibition was observed at pH 7.40 for 5 mM Ca^{++} (Fig. 5). At low pH, the mean slope ± SEM of the double logarithmic graph of MEPP frequency vs. $[Ca^{++}]_o$ was 0.46 ± 0.06 (three end-plates). At pH 6.40, there was no significant difference between the slope at 10 mM K^+ (Fig. 5) and 2.5 mM K^+ (Fig. 3 *C*). Reversal of the Ca^{++} effect at 10 mM K^+, i.e., a change from Ca^{++} inhibition of MEPP frequency to

TABLE II

Effect of H^+ on MEPP Frequency (F) at 2.5 and 10 mM K^+

Muscle number	$[K^+]_0$	pH				
		7.40	6.90	6.40	5.90	5.40
	mM					
1	10.0	1.00 (38.87)	0.75	0.30	0.14	0.12
2	10.0	1.00 (12.34)	—	—	0.40	0.31
2	10.0	1.00 (10.66)	0.66	0.21	—	—
3	10.0	1.00 (7.97)	0.81	0.18	0.10	0.07
3	10.0	1.00 (12.69)	1.00	0.45	—	—
4	10.0	1.00 (5.97)	—	—	0.17	0.16
4	10.0	1.00 (2.39)	1.02	0.38	0.19	0.13
5	10.0	1.00 (20.14)	0.89	0.31	0.16	0.15
6	10.0	1.00 (20.34)	0.69	0.46	0.12	—
7	10.0	1.00 (13.73)	0.61	0.23	—	—
8	2.5	1.00 (0.45)	0.79	0.88	1.37	1.21
9	2.5	1.00 (0.50)	1.16	1.06	1.98	1.19
10	2.5	1.00 (1.61)	1.13	1.32	2.50	2.75
11	2.5	1.00 (2.11)	1.43	2.28	13.29	22.78
12	2.5	1.00 (2.22)	—	—	—	1.40

In parenthesis is the initial MEPP frequency (in MEPPS s^{-1}) at pH 7.40 (F_0) at each end-plate. Each number at a particular pH represents normalized MEPP frequency, i.e., F/F_0. Rows show the effect of H^+ at each end-plate. Saline A (in Table I) was used for muscles 1–4, saline B was used for muscles 5–7, and saline F was used for muscles 8–12. The experiment in Fig. 1, *A* and *B* is shown at muscle 1. Depression of MEPP frequency by H^+ at 10 mM K^+ was independent of F_0 and the type of buffer used.

Ca^{++} stimulation, by H^+ at low pH cannot be explained by surface charge theory alone.

Use of the Kinetic Model to Estimate ΔG_f

To estimate ΔG_f for interactions of H^+, Ca^{++}, and K^+ with a K^+ site (see the Methods), estimates of K_d for these cations were obtained at each end-plate for test $[Ca^{++}]_o$ of 1.5, 2.0, and 2.5 mM, and for test pH 6.90. Parallel shifts to the right of the double logarithmic graph of MEPP frequency vs. $[K^+]_o$ were observed for decreases in pH from 7.40 to 6.90, and increases in $[Ca^{++}]_o$ from 1 to 2.5 mM (Fig. 6 *A*). At 16 end-plates, mean estimates of u ± SEM were 3.90 ± 0.23 (range, 2.63–5.28) for control; 3.78 ± 0.37 (range, 2.06–7.04) for test pH (pH 6.90); and 4.11 ±

0.20 (range, 2.27–5.37) for test Ca^{++} (1.5–2.5 mM Ca^{++}). To determine whether H^+ and Ca^{++} competitively inhibited the actions of raised K^+ on MEPP frequency, Lineweaver-Burke graphs of the data were obtained (as described in the Methods). Test salines increased the slope without changing the y-intercept (Fig. 6 B).

The pK_a range was 5.84–7.03 with a mean ± SEM of 6.31 ± 0.08 at 16 end-plates; K_H was 0.49 μM. ΔG_f associated with protonation of the acidic site was −8.58 kcal/mol. The pCa range was 1.49–2.66 with a mean ± SEM of 1.97 ± 0.10 at the same 16 end-plates; K_{Ca} was 10.62 mM. ΔG_f associated with the interaction between Ca^{++} and the acidic site was −2.68 kcal/mol.

To estimate K_K, it was important to know if a graph of log MEPP frequency vs. pH can be approximated by a straight line with slope magnitude $(u \times S_e)$ (see the Methods). Fig. 4 showed that graphs of MEPP frequency vs. pH yielded straight lines at the pH range 6.40–7.40. S_e was obtained at control pH 7.40 and test pH 6.90 by using Eq. 16; the integer value of estimates of u (a value of 4) was used. The
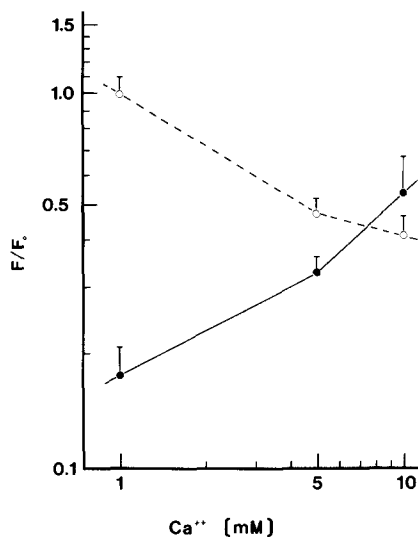

FIGURE 5. The discriminatory action of H^+ on the nonmonotonic (or dual) effect of Ca^{++} on MEPP frequency is shown at 10 mM K^+. In each experiment, F_o (saline A) was the standard of reference; test salines B, B', and B" were used. Results in each experiment were recorded at pH 7.40 (O), and at 6.40 (●). Each point is a mean of three experiments; vertical bars show SEM. The mean MEPP frequency ± SEM (i.e., F/F_o recorded as a percentage) at pH 7.40 for 5 mM Ca^{++} was 47.31 ± 5.11%. It was not significantly different from the mean MEPP frequency at pH 6.40 for 10 mM Ca^{++}, i.e., 53.62 ± 14.57.

pK range was 2.28–2.69 with a mean ± SEM of 2.51 ± 0.12 at 3 of the 16 end-plates: K_K was 3.09 mM. It was only possible to estimate K_K at three end-plates, at test pH 6.90, in which the pK_a range was 6.58–7.03; K_K could not be determined at the remaining 13 end-plates in which pK_a estimates were less than this range. This observation was consistent with the requirement that Eq. 17 holds (see Methods) provided that (a) the term $[H^+]/K_H$ in Eq. 14 is not negligible, and (b) the interval between pH_1 and pH_2 is small such that the pH dependence of S_e is negligible. These conditions were apparently met at three end-plates in which (a) control pH 7.40 and test pH 6.90 were near the pK_a (range of 6.58–7.03) such that $[H^+]/K_H$ was not negligible, and (b) the difference between control and test pH was small, i.e., 0.5 pH units. ΔG_f associated with the interaction between K^+ and the acidic site was −3.41 kcal/mol. Based on ΔG_f, the order of cation preference with the acidic site was $H^+ > K^+ > Ca^{++}$.

DISCUSSION

In the present study, membrane voltages of the muscle fiber and apparently also the nerve terminal were not affected by H^+. The surface charge model appeared to be consistent with the decrease in K^+ stimulated MEPP frequency by H^+. In this model, the effect of extracellular K^+ is independent of a direct interaction between K^+ and the prejunctional membrane. K^+-induced membrane depolarization follows from the Nernst equation when $[K^+]_o$ is increased. It results in an increase in voltage-dependent Ca^{++}-mediated MEPP frequency (Liley, 1956). In accordance with
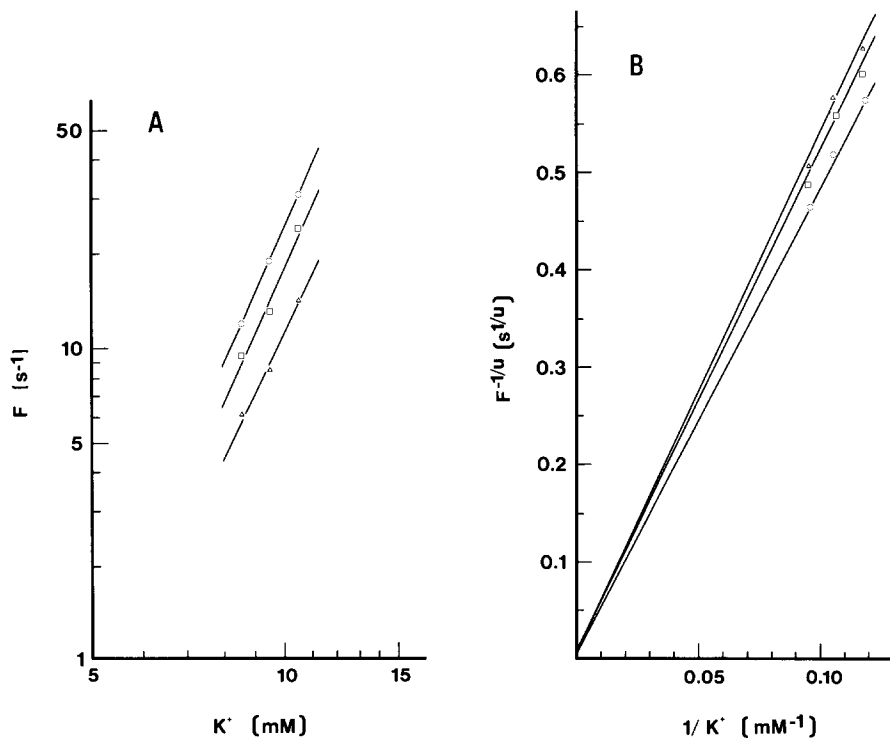

FIGURE 6. The nature of inhibition of MEPP frequency by H^+ and Ca^{++} is shown for an individual experiment at a single end-plate. Results were recorded at 1 mM Ca^{++}, pH 7.40 (O); 2.5 mM Ca^{++}, pH 7.40 (□); and 1 mM Ca^{++}, pH 6.90 (△). Test salines were similar to saline B (see Table I, legend). (A) Double logarithmic graphs are shown for MEPP frequency (F) vs. $[K^+]_o$, in which slopes represent estimates of u. (B) Lineweaver-Burke graphs of the same data in A are shown in which slopes represent estimates of m (Eq. 11).

surface charge theory, H^+ (at low pH, i.e., pH 6.40) reduced the total negative surface charge of the membrane and, therefore, reduced the negative surface potential, $\psi(0)$ (Gilbert, 1971). In accordance with the Boltzmann distribution, $[Ca^{++}]_o$ at the Ca^{++} channel may have decreased at low pH. Consequently, Ca^{++}-mediated transmitter release at raised $[K^+]_o$ decreased. In comparison, divalent cations may reduce $\psi(0)$ without affecting the total negative surface charge of the membrane

(Muller and Finkelstein, 1974). The surface charge model also appeared to be consistent with the decrease in Ca^{++}-mediated transmitter release at raised $[K^+]_o$ caused by divalent cations (Madden and Van Der Kloot, 1978).

In the present study, reversal of the effect of Ca^{++}, i.e., the change from Ca^{++} inhibition of MEPP frequency to Ca^{++} stimulation, by H^+ could not be explained by the surface charge model. Based on this model, $[Ca^{++}]_o$ in bulk saline (superfusing the nerve terminal) may be increased such that predicted $[Ca^{++}]_o$ near the Ca^{++} channel are equivalent before and after a change from neutral to low pH. Ca^{++} inhibition was predicted before and after the pH change. Based on experimental findings, however, Ca^{++} inhibition occurred before the change to low pH, whereas Ca^{++} stimulation occurred after the pH change. Based on these results, the hypothesis is that (a) K^+ ions normally act at an acidic prejunctional site, i.e., a K^+ site, to maintain voltage-dependent Ca^{++}-mediated transmitter release, and (b) Ca^{++} and H^+ may compete with K^+ for this site. This mechanism requires direct interaction between K^+ and the prejunctional membrane. It offers an explanation for (a) the increase in quantum content by raised K^+ (Takeuchi and Takeuchi, 1961), and (b) the fast action of raised K^+ that increases MEPP frequency by a mechanism which is inhibited by raised Ca^{++}, and is different from K^+ induced membrane depolarization (Cooke and Quastel, 1973).

Using the kinetic model in the present study, the dissociation constant for K^+ was 3.09 mM. The K^+ site may be distinguished from other acidic sites at the Ca^{++} channel by differences in pK_a and K_d for Ca^{++} (K_{Ca}). A K_{Ca} of 1.1 mM (Dodge and Rahamimoff, 1967), and pK_a of 3.6–5.7 (Landau and Nachshen, 1975) or of 4.20 (the present study) were reported for an acidic site at the Ca^{++} channel associated with Ca^{++} influx. In contrast, the K^+ site was shown to have a K_{Ca} of 10.62 mM, and pK_a of 6.31. Based on free energy (ΔG_f) calculations, the analysis showed that H^+ was much more effective than Ca^{++} in competing with K^+ for this site. Thus, at pH 6.40 and raised $[K^+]_o$, Ca^{++} inhibition of MEPP frequency was abolished by H^+ unmasking Ca^{++} stimulation. Ca^{++} stimulation of MEPP frequency at pH 6.40 occurred perhaps because some Ca^{++} channels remained functional at this pH. The reported Ca^{++} site is presumably located at the external entrance of the Ca^{++} channel (Dodge and Rahamimoff, 1967; Landau and Nachshen, 1975). In contrast, the K^+ site may be located either within the Ca^{++} channel near its external entrance, making the site accessible to extracellular K^+ ions, or at the Na^+-K^+ pump.

The first possible location for the K^+ site is consistent with one originally proposed by Cooke and Quastel (1973). These investigators proposed that K^+ acts at a prejunctional site to cause rapid activation of an inward presynaptic Ca^{++} current, i.e., to increase Ca^{++} ion availability at transmitter release sites. This effect of K^+ was inhibited by raised Ca^{++}. The proposed action of K^+ is not unique. Ca^{++} channels may be activated by a wide variety of substances (Brown, 1984). The second possibility is consistent with evidence that the Na^+-K^+ pump rate is linked to high affinity choline uptake, i.e., high affinity choline uptake may be coupled to the inward movement of K^+ at the Na^+-K^+ pump (Birks, 1985). Choline uptake is associated with transmitter acetylcholine synthesis, and it is newly synthesized transmitter which is preferentially released by the nerve impulse (MacIntosh and Collier, 1976). Consequently, it may be inferred that raised K^+, at concentrations that do

not block action potential conduction, may increase the availability of newly synthesized transmitter for Ca^{++}-mediated transmitter release. Thus, K^+ binding may regulate (a) Ca^{++} ion availability at transmitter release sites and/or (b) the availability of newly synthesized transmitter for Ca^{++}-mediated release.

A fourth power relationship was observed between Ca^{++}-mediated transmitter release (recorded as MEPP frequency) and K^+ (see Fig. 6 A and Results). Estimates of u in the present study (i.e., $u = \log$ MEPP frequency/log $[K^+]_o$) were equivalent to the reported value of n (i.e., $n = \log$ quantum content/log $[Ca^{++}]_o$) (Dodge and Rahamimoff, 1967): u and n equal 4. The equivalence of factors u and n may be a reflection of a linear relationship between extracellular K^+ and Ca^{++} ions in the sequence of events leading to voltage-dependent release of a quantum of transmitter. Binding of K^+ to the K^+ site may not be the final step in this sequence. Thus, the fourth power relationship observed with K^+ does not necessarily imply that four K^+ ions are required to release a quantum of transmitter. It may, however, be a reflection of the reported fourth power relationship between quantal transmitter release and Ca^{++} (Dodge and Rahamimoff, 1967). For instance, binding of K^+ to a prejunctional K^+ site may be linearly related to Ca^{++} ion availability at transmitter release sites and/or the availability of newly synthesized transmitter for Ca^{++}-mediated quantal release.

In summary, inhibition of K^+-stimulated MEPP frequency by H^+ was consistent with the surface charge model. A pK_a of 4.20 was estimated for an acidic prejunctional site, which is presumably located at the external entrance of the Ca^{++} channel. The surface charge model could not account for reversal of the Ca^{++} effect, i.e., the change from Ca^{++} inhibition of transmitter release to Ca^{++} stimulation, by H^+ at K^+-stimulated nerve terminals. Based on kinetic theory, the estimated pK_a of a prejunctional K^+ site was 6.31. The dissociation constant for K^+ (K_K) was 3.09 mM. Based on ΔG_f values, its cation preference was $H^+ > K^+ > Ca^{++}$.

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