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Effects of Divalent Cations on Toad End-Plate Channels

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Summary. Miniature end-plate currents (MEPCs) and acetylcholine-induced current fluctuations were recorded in voltage-clamped, glycerol-treated toad sartorius muscle fibers in control solution and in solutions with added divalent cations. In isosmotic solutions containing 20 mM Ca or Mg, MEPCs had time constants of decay (τ_D) which were about 30% slower than normal. In isotonic Ca solutions (Na-free), greater increases in both τ_D and channel lifetime were seen; the null potential was −34 mV, and single-channel conductance decreased to approximately 5 pS. Zn or Ni, at concentrations of 0.1–5 mM, were much more effective in increasing τ_D than Ca or Mg, although they did not greatly affect channel conductance. The normal temperature and voltage sensitivity of τ was not significantly altered by any of the added divalent cations. Surface potential shifts arising from screening of membrane fixed charge by divalent cations cannot entirely explain the observed increases in τ, especially when taken together with changes in channel conductance.

Key words endplate channel · divalent cations · ACh-noise · calcium

Introduction

It has been known for some time that many cations, both monovalent and divalent, can pass through the ion channels activated by acetylcholine at the motor end-plate (Takeuchi & Takeuchi, 1960; for review *see* Rang, 1975). Furthermore, because end-plate channels are apparently impermeable to anions (Takeuchi & Takeuchi, 1959) and because single-channel conductance depends on the nature of the permeating cation (Van Helden, Hamill & Gage, 1977; Gage & Van Helden, 1979), it has been proposed that the channel contains negatively charged sites with which permeant cations interact (Barry, Gage & Van Helden, 1979*a*, *b*; Lewis & Stevens, 1979; Takeda, Barry & Gage, 1980*a*). The observation of ion-dependent, reciprocal changes in open channel lifetime and conductance at both the end-plate (Van Helden et al., 1977; Gage & Van Helden, 1979) and in *Aplysia* neurones (Ascher, Marty & Neild, 1978) has led to the suggestion that ion binding to critical intrachannel sites influences channel lifetime. However, more recent reports (Nonner, Adams, Dwyer & Hille, 1980; Takeda et al., 1980*a*) indicate that ion-dependent changes in channel lifetime need not be reciprocally related to conductance changes, although the ion binding hypothesis would explain the increases in voltage sensitivity of channel lifetime seen in the presence of some divalent cations in *Aplysia* neurones (Marchais & Marty, 1979).

When calcium ions are substituted for sodium ions in the external medium, single-channel conductance is clearly reduced (Bregestovski, Miledi & Parker, 1979; Lewis, 1979). However, there have been varying reports on the effects of high calcium ion concentrations on channel lifetime: in some experiments it was increased (Cohen & Van der Kloot, 1978) whereas in others, it was unchanged or decreased (Bregestovski et al., 1979; Magleby & Weinstock, 1980). Effects of calcium ions on channel characteristics could be related to their affinity for intrachannel sites or to other effects on the channel or its close environment. Alternatively, it has been suggested that the increase in channel lifetime caused by elevated levels of calcium ions (and also hydrogen ions; Scuka, 1975; Mallart & Molgó, 1978) might be due to screening of membrane surface charge (Van der Kloot & Cohen, 1979). Our aim here was to investigate the effect of several divalent cations, particularly calcium ions, on the voltage- and temperature-dependent characteristics of end-plate channels in an attempt to obtain more information about what controls open channel lifetime and conductance.

Brief accounts of some of this work have been presented to the Australian Physiological and Pharmacological Society (Takeda, Datyner, Barry & Gage, 1978) and to the Australian Society for Biophysics (Takeda, Barry & Gage, 1980*b*).

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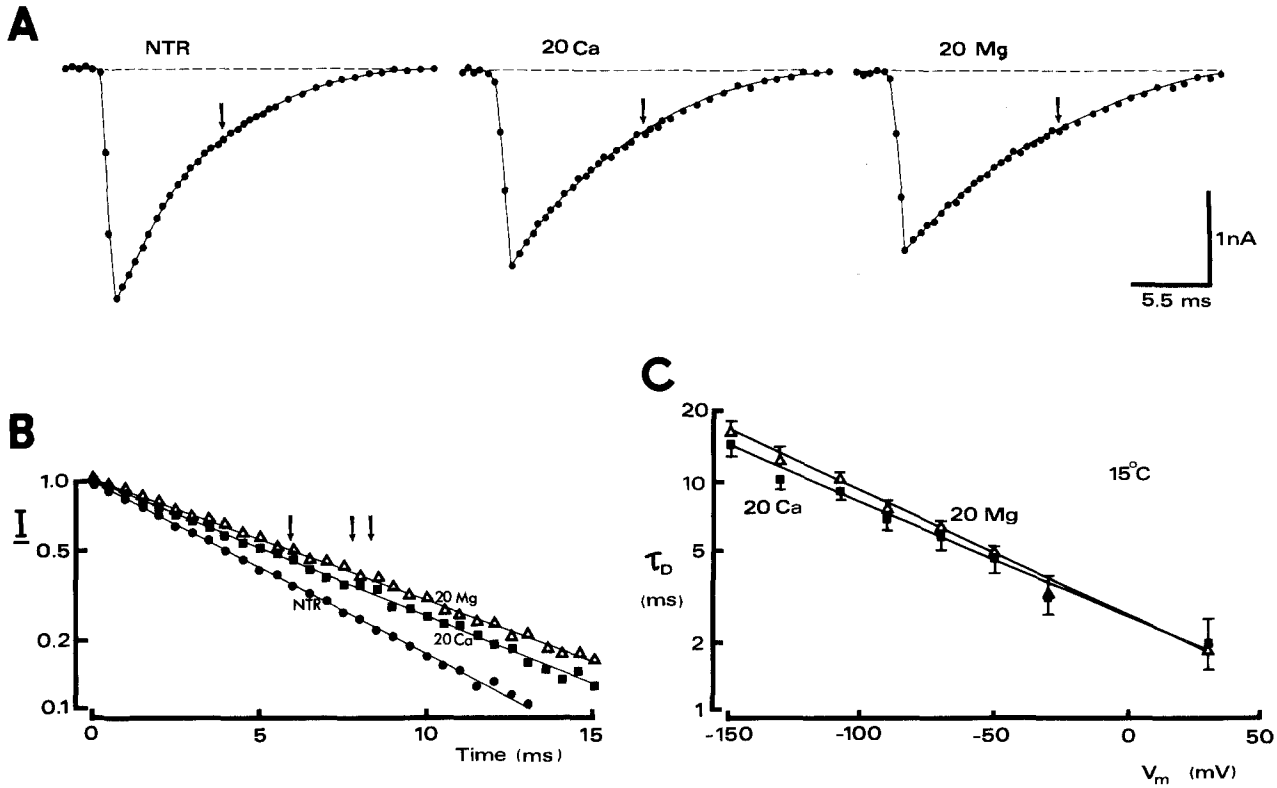

Fig. 1. Addition of 20 mM Ca or Mg lengthens MEPC decay. *A*. Averaged MEPCs ($n=20$) recorded under voltage-clamp at -90 mV in NTR, 20 Ca and 20 Mg solutions. MEPC time constants of decay (τ_D, arrows) were 6.0 (NTR), 7.13 (20 Ca) and 8.33 msec (20 Mg). Temperature, 15 °C. *B*. The decay of the normalized MEPCs shown in *A* is well-fitted by a single exponential. *C*. Voltage-dependence of τ_D. Averaged data at each potential from 5 cells in each solution were well described by the equation $\tau_D(V)=\tau_D(0)\exp(-V_m/H)$. H values were 94.2 and 86.7 mV, $\tau_D(0)$ values were 2.66 and 2.50 msec and correlation co-efficients were 0.980 and 0.991 in 20 Ca (■) and 20 Mg (△) solutions, respectively, for each parameter. In this set of control experiments (NTR), H was 98.4 mV and $\tau_D(0)$ was 2.01 msec

Materials and Methods

The methods used here were essentially the same as those recently described in detail by Takeda et al. (1980*a*). Briefly, toad (*Bufo marinus*) sartorius muscles were glycerol-treated to prevent contraction, and MEPCs were recorded extracellularly or in voltage-clamped fibers using conventional electrophysiological techniques. Extracellular electrodes were filled with normal toad Ringer's (NTR: NaCl, 115 mM; KCl, 2.5 mM; $CaCl_2$, 1.8 mM; Na-Hepes buffer, 2 mM; pH 7.2) and had resistances of 2–4 MΩ, while filament-containing microelectrodes (Clark Electromedical) filled with 2 M KCl for voltage recording or a 2 M KCl-0.8 M K citrate mixture for current passing (resistance 4–8 MΩ) were used in voltage-clamp experiments. Analysis of end-plate current fluctuations (noise) produced by iontophoresis of ACh was based on the model and theory presented by Anderson and Stevens (1973). Experimental data were recorded on FM tape for off-line computer analysis (*see* Van Helden et al., 1977; Gage, McBurney & Van Helden, 1978; Takeda et al., 1980*a*, for details). A minor change was the use of 16,384 data points for fluctuation analysis: i.e. 8 sec of noise sampled at 2 kHz in 32 blocks. Baseline noise in the absence of agonist was always subtracted. Usually, control data from several cells in each muscle were obtained in NTR at 15 °C at a series of potentials before the temperature was changed or test solutions introduced. Solution changes were made by superfusing 3 to 5× bath volume (2–3 ml) of the test solution over a ~5-min period – the bath changeover time was deliberately kept slow to facilitate holding cells through the solution change. Data then collected from cells in the test solutions always showed the same trends as data from the paired cells. Divalent cations (up to 1 mM) were added directly to NTR; NaCl was reduced appropriately to maintain osmotic strength when greater divalent cation concentrations were used (20 mM divalent cation solutions also contained 90 mM NaCl, 2.5 mM KCl and 2 mM Na-Hepes buffer; pH 7.2). Isotonic calcium solutions (80 Ca solution) contained 80 mM $CaCl_2$, 2.5 mM KCl and 2 mM J-Hepes buffer; pH 7.2. Results are presented as means ±1 SEM unless otherwise noted.

Results

20 mM Ca and Mg

Isosmotic solutions containing 20 mM $CaCl_2$ (20 Ca solution) produced an increase in the time constant of decay (τ_D) of miniature end-plate currents (MEPCs), as illustrated in Fig. 1. This increase in τ_D, though small, was regularly seen. In five paired cells, there was, on average, a 27.6±4.3% (mean ±1 SEM) increase in τ_D in the 20 Ca solution (Table 1). This effect was not specific for calcium ions: an increase in τ_D of 36.6±3.8% was also produced by solutions containing 20 mM $MgCl_2$ (20 Mg solution). In both 20 Ca and 20 Mg solutions, the

Table 1. Addition of 20 mM Ca or 20 mM Mg increases MEPC time constant of decay (τ_D)[a]

Cell	τ_D (msec)		Cell	τ_D (msec)	
	NTR	20 Ca		NTR	20 Mg
A	5.01	6.83	F	5.62	7.62
B	5.46	6.87	G	4.98	6.12
C	6.10	7.13	H	5.91	8.33
D	5.59	7.77	I	4.90	6.86
E	5.37	6.45	J	5.21	7.49

[a] Data were obtained from paired cells under voltage clamp at −90 mV; temperature, 15° C.

Table 2. Voltage sensitivity of τ_D in normal toad Ringer's (NTR) and in solutions containing added divalent cations[a]

Solution	Temperature (°C)	H (mV)	$\tau_D(0)$ (msec)	$\bar{r}$	n
NTR	15	105.1 ± 4.2	2.02 ± 0.14	0.994 ± 0.004	18
NTR	25	101.5 ± 10.8	0.98 ± 0.25	0.992 ± 0.007	9
20 Ca	15	89.8 ± 9.0	2.47 ± 0.30	0.988 ± 0.005	5
20 Mg	15	83.5 ± 11.7	2.40 ± 0.20	0.989 ± 0.006	5
80 Ca	15	104.6 ± 8.3	3.46 ± 0.40	0.979 ± 0.018	6
80 Ca	25	94.9 ± 7.9	1.68 ± 0.16	0.982 ± 0.014	11
1 Zn	15	92.7 ± 8.8	4.16 ± 0.36	0.987 ± 0.009	7
1 Ni	15	95.3 ± 6.6	3.61 ± 0.21	0.981 ± 0.008	12

[a] Data from single cells voltage clamped over wide ranges of potential were fitted (using least-squares) by the equation: $\tau_D(V_m) = \tau_D(0) \exp(-V_m/H)$. Values of $\tau_D(0)$, H and $\bar{r}$ (correlation coefficient) are shown as mean ± 1 SEM (n, number of fibers)

decay of MEPCs remained exponential (Fig. 1*B*), and the increases in τ_D were fully reversible on return to control solution.

The voltage sensitivity of τ_D (Magleby & Stevens, 1972) recorded in these solutions containing raised divalent ion concentrations was not significantly different from normal. Mean values of τ_D obtained from five cells in 20 Ca (squares) and 20 Mg solutions (open triangles) are plotted semilogarithmically against membrane potential (V_m) in Fig. 1*C*. The straight lines are least-squares fits to the equation $\tau_D(V_m) = \tau_D(0) \exp(-V_m/H)$, where $\tau_D(0)$ is the time constant of decay at zero membrane potential and H is the change in membrane potential for an e-fold change in τ_D (Magleby & Stevens, 1972; Gage & McBurney, 1975). The H value (volt constant) obtained in this way in control solution was 98.4 mV: H values in 20 Ca and 20 Mg solution were 94.2 and 86.7 mV, respectively. Values of H and $\tau_D(0)$ obtained in individual cells were also averaged and are given in Table 2.

80 mM Ca

Higher concentrations of calcium produced larger increases in τ_D. Changes in MEPCs recorded in an isotonic solution containing 80 mM $CaCl_2$ and zero Na (80 Ca solution) are illustrated in Fig. 2. MEPCs had a slower decay, and were smaller in amplitude in 80 Ca than in control solution. A large decrease in resting membrane conductance occurred in the 80 Ca solution and this facilitated the recording of MEPCs over large potential ranges, as less holding current was required to displace membrane potential. The voltage sensitivity of τ_D obtained using average values of τ_D from several cells showed little change in 80 Ca solution (Fig. 3*B*; *see* Table 2 for averaged H values obtained from individual cells), and was not detectably different at 15° and 25 °C.

The reduction in peak MEPC amplitude in 80 Ca (Figs. 2*B* and 3*A*) was more pronounced at negative potentials, as is illustrated in Fig. 4*A* in which mean MEPC amplitude is plotted against membrane potential (averaged data from six cells). In fact, at positive potentials, there was no significant reduction in MEPC amplitude. A contributory factor to the decrease in MEPC amplitude was a negative shift in the null (zero-current) potential in 80 Ca. In normal toad Ringer's, the null potential (ε_o) was −3.1 ± 1.2 mV (n=11) and in 80 Ca, ε_o was −34.3 ± 2.9 mV (n=4). The shift in ε_o in 80 Ca was not different at 15° and 25 °C.

The shift in null potential in 80 Ca solutions was not sufficient to account for the whole of the reduction in MEPC amplitude. The magnitude of the conductance change produced by ACh was also reduced. This is illustrated in Fig. 4*B* in which the conductance at the peak of a MEPC (G_p, calculated by dividing peak MEPC amplitude by the driving force, $V_m - \varepsilon_o$) is plotted against membrane potential. In 80 Ca, G_p was reduced at all potentials, but it is clear that the reduction at +50 mV was less than at −150 mV.

In order to test whether the increase in τ_D was caused by inhibition of acetylcholinesterase in 80 Ca solution, the effectiveness of an anticholinesterase, prostigmine, was compared in normal toad Ringer's and in 80 Ca. There should be little effect of prostigmine in 80 Ca if the latter had already significantly inhibited acetylcholinesterase. In Fig. 5, it can be seen that addition of 3 μM prostigmine to normal toad Ringer's produced an increase in τ_D from 7.0 to 11.2 msec, while in 80 Ca, 3 μM prostigmine resulted in τ_D increasing from 13.6 to 17.0 msec. Thus, prostigmine clearly caused an increase in τ_D in 80 Ca. However, the observation does not exclude the possibility that acetylcholinesterase was partially depressed, especially as the increase in τ_D caused by prostigmine

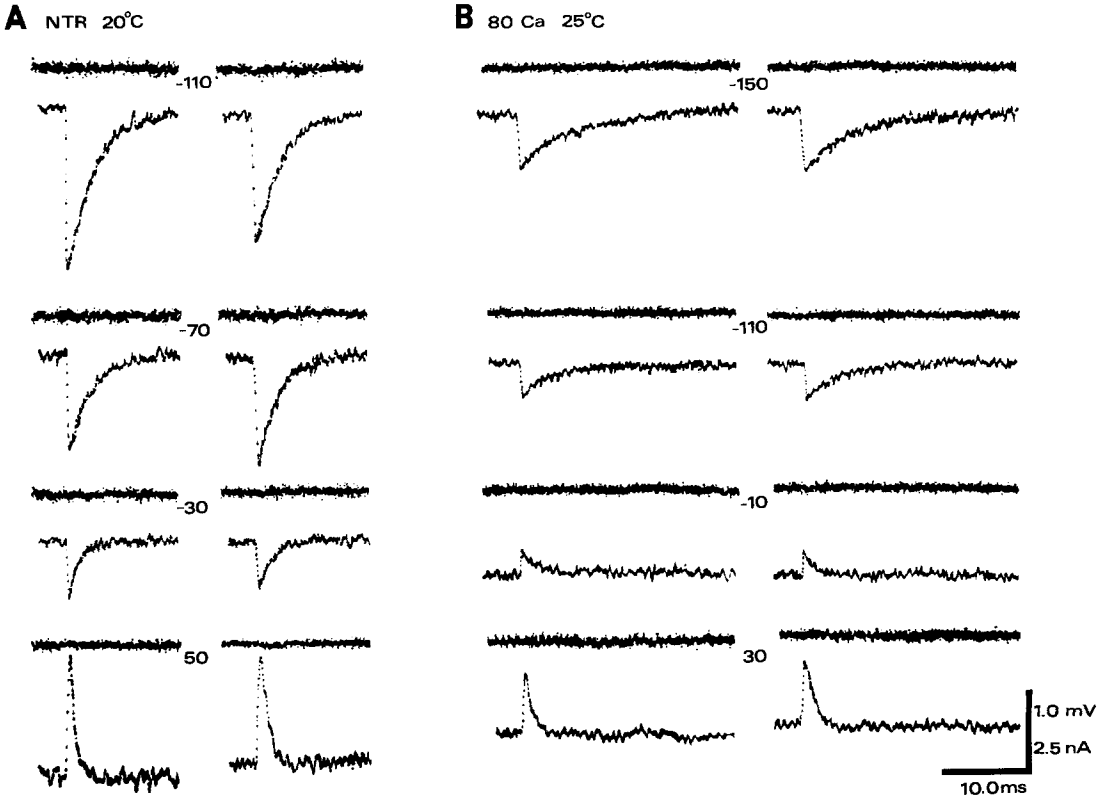

Fig. 2. MEPCs recorded in a voltage-clamped fiber in NTR (*A*) and 15 min later in 80 Ca solution (*B*). MEPCs were smaller, and decayed more slowly in 80 Ca solution at all potentials. Note the shift in null potential to a more negative value in the 80 Ca solution. The voltage trace is shown above each current trace

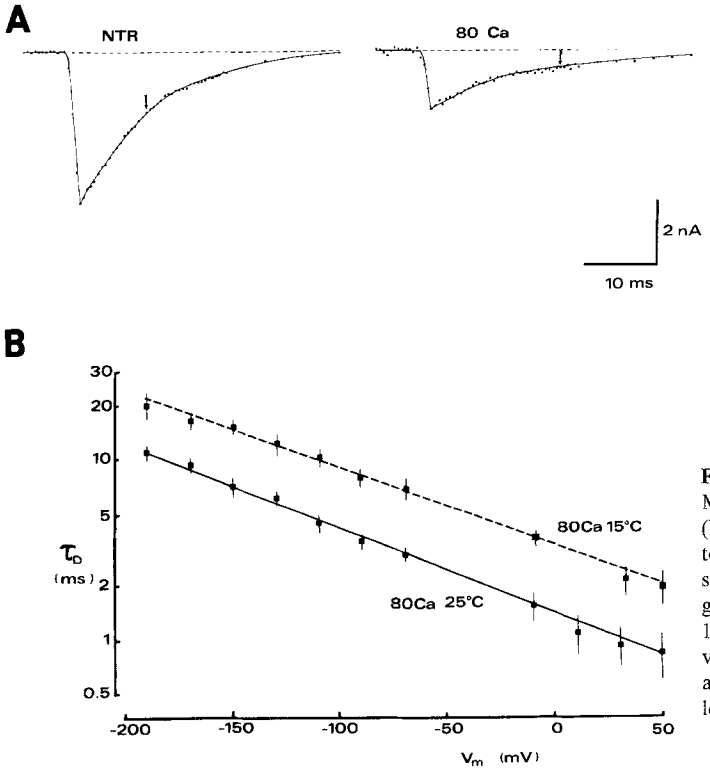

Fig. 3. *A*. Averaged MEPCs recorded in NTR and 80 Ca. MEPC time constant of decay (τ_D, arrows) was 9.5 msec (NTR) and 17.6 msec (80 Ca). Holding potential, −150 mV; temperature, 14.5 °C. *B*. Voltage-dependence of τ_D in 80 Ca solutions. Correlation coefficients for least-squares fits to single exponentials were 0.980 (80 Ca, 25 °C) and 0.932 (80 Ca, 15 °C). *H* values calculated were 101.4 and 103.9 mV; $\tau_D(0)$ values were 1.49 and 3.74 msec, respectively, for 80 Ca (25 °C) and 80 Ca (15 °C). At each potential, the average τ_D from at least 6 cells is shown. *See* Table 2 for control (NTR) data

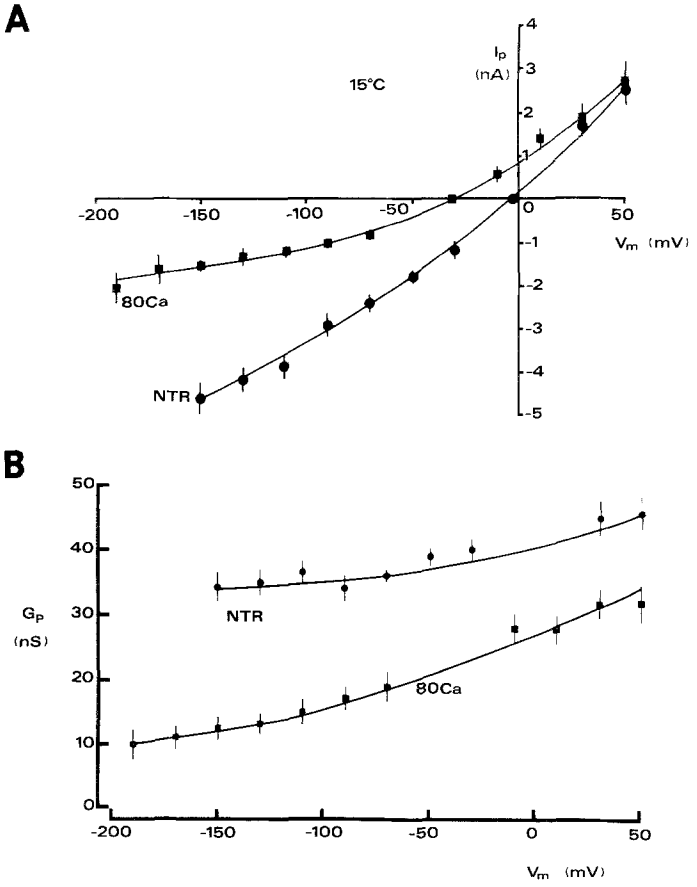

Fig. 4. The effect of membrane potential on mean peak MEPC amplitude and conductance in NTR and 80 Ca solutions. *A.* MEPCs had reduced amplitudes at negative potentials in 80 Ca, but were near normal-sized at positive potentials. The null potential shifted from −3 mV in NTR to −34 mV in 80 Ca, and was not affected by temperature. Data are averaged MEPC amplitudes ($n \geqq 20$) from at least 8 cells. *B.* Mean peak MEPC conductance was obtained by dividing the peak MEPC amplitude by the driving force and the lines were fitted by eye. Note that G_p was always smaller in 80 Ca and that as the holding potential was made more positive, G_p increased slightly in both solutions. Temperature, 15 °C

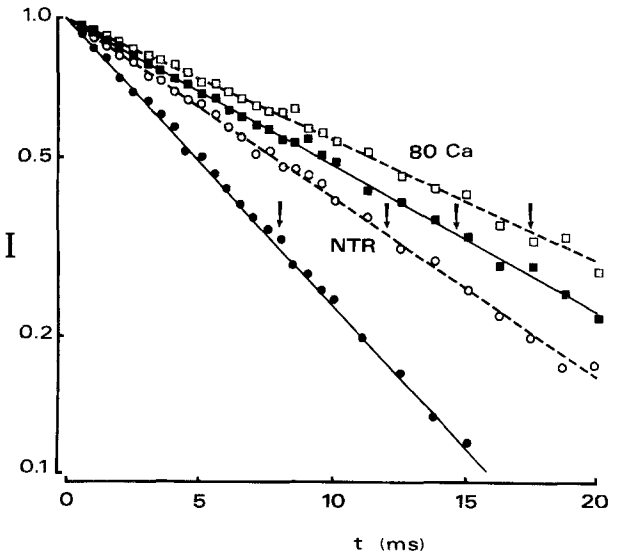

Fig. 5. Addition of 3 μM prostigmine (broken lines) lengthens MEPCs in normal toad Ringer's (NTR) and the 80 Ca solution. The decay of averaged ($n=25$), normalized MEPCs recorded under voltage clamp is shown plotted semilogarithmically. In NTR (●), τ_D (arrow) was 7.0 msec, and after addition of prostigmine (○), τ_D was increased to 11.2 msec. In 80 Ca (■), τ_D was 13.6 msec, and after addition of prostigmine (□), τ_D was increased to 17.0 msec. Holding potential, −130 mV; temperature, 15 °C

was less than in the control solution. On the other hand, the increase in τ_D cannot be due wholly to inhibition of acetylcholinesterase as 3 μM prostigmine had less effect than 80 Ca solution on τ_D (Fig. 5).

Further evidence against the idea that the increase in τ_D caused by 80 mM Ca might be due to inhibition of acetylcholinesterase came from comparison of channel open time in normal toad Ringer's and in 80 Ca. Power density spectra of fluctuations in end-plate current generated by iontophoresis of ACh are presented in Fig. 6. Spectra recorded in normal toad Ringer's and 10 min later in 80 Ca clearly show a shift to lower frequencies in 80 Ca, an effect not seen with anticholinesterases (Katz & Miledi, 1973). Average channel lifetime (τ_N) calculated from the half-power frequency was 6.9 msec in normal toad Ringer's and 13.7 msec in 80 Ca, an increase similar to the increase in τ_D caused by 80 Ca. Furthermore, MEPCs recorded in 80 Ca (Figs. 2*B* and 3*A*) showed no pronounced increase in rise-time or rounding of the peak, features observed in prostigmine-containing solutions (Katz & Miledi, 1973).

Another effect of the 80 Ca solution was to decrease single-channel conductance (γ), as has been previously reported (Bregestovski et al., 1979; Lewis, 1979). Values of τ_N and γ obtained from noise analysis in NTR and 80 Ca are given in Table 3.

Zinc and Nickel

Other divalent ions affected MEPCs in a similar way. Both zinc and nickel slowed the decay of MEPCs when added to normal toad Ringer's at relatively low concentrations (0.1–5 mM). The effect of these ions on τ_D increased with concentration in this range, as can be seen in Fig. 7, and were rapid in onset. Zinc was somewhat more potent than nickel and while the effects of nickel were largely reversible, those of zinc were less so.[1] In the experiments illustrated in Fig. 7, τ_D was doubled by a solution containing

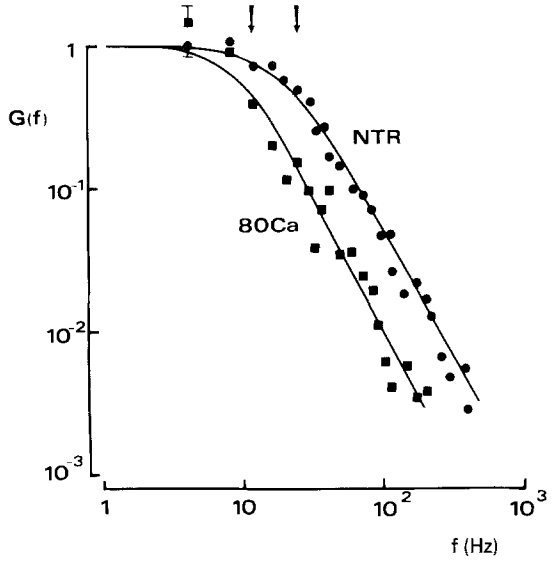

Fig. 6. Power spectral density of ACh-induced fluctuations in NTR, and 10 min later in 80 Ca solution. Channel lifetime increased from 6.9 msec in NTR to 13.7 msec in 80 Ca, corresponding to half-power frequencies (arrows) of 23.1 and 11.6 Hz, respectively. Data were normalized and were well fitted (by least-squares) to single Lorentzian curves. Channel conductances calculated from the spectra were 22.5 pS in NTR, and 4.7 pS in 80 Ca. Asymptotic spectral densities were 2.92×10^{-21} and 1.48×10^{-22} A^2 sec for steady-state currents of 37 and 6 nA, in NTR and 80 Ca, respectively. Membrane potential, −130 mV; temperature, 15 °C

[1] Since the preparation of this manuscript, Miledi and Parker (1980) have reported that strontium ions have effects similar to those of zinc and nickel.

Table 3. Single-channel characteristics obtained from noise analysis[a]

Solution	V_m (mV)	τ_N (msec)	γ (pS)	n
NTR	−130	7.1±0.3	23.7±2.0	5
80 Ca	−130	14.3±0.5	5.2±1.7	3
NTR	−90	5.9±0.3	25.2±0.9	7
1 mM Zn	−90	11.4±0.9	20.4±1.1	4
1 mM Ni	−90	9.0±0.6	19.8±1.0	6

[a] Mean channel lifetime (τ_N) was calculated using the equation $\tau_N = \frac{1}{2\pi f_{\frac{1}{2}}}$ where $f_{\frac{1}{2}}$ is the half-power frequency. Single-channel conductance was calculated using the relationship $\gamma = \frac{G(0)}{4\mu_i(V_m-\varepsilon_o)\tau_N}$, where $G(0)$ is the zero frequency asymptote and μ_i is the mean ACh-induced end-plate current. Estimates of γ obtained in this way were not different from values calculated using the equation $\gamma = \frac{\sigma_i^2}{\mu_i(V_m-\varepsilon_o)}$, where σ_i^2 is the variance of the current fluctuations. Temperature, 15 °C; n, number of cells.

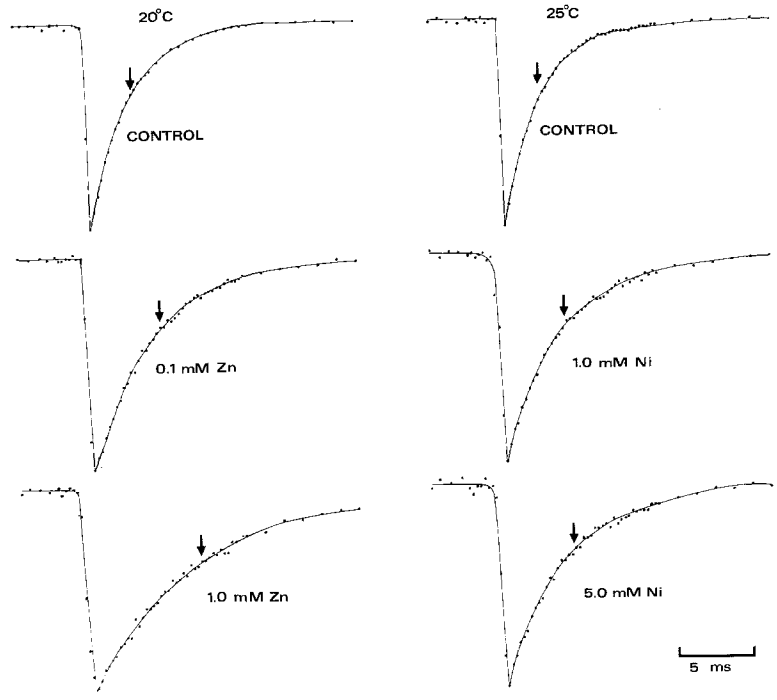

Fig. 7. The increase in τ_D caused by zinc and nickel. Left panel shows normalized averages ($n=20$) of MEPCs recorded extracellularly from the same cell in control solution, 0.1 mM and 1.0 mM Zn solutions (20 °C). Arrows indicate time constants of decay which were 2.29 (control), 4.58 (0.1 mM Zn) and 7.29 msec (1.0 mM Zn). Right-hand panel shows normalized averages of MEPCs recorded extracellularly from 1 cell in control and in nickel-containing solutions (25 °C). τ_D's were 1.87 (control), 3.96 (1.0 mM Ni) and 4.79 msec (5.0 mM Ni)

0.1 mM Zn, whereas 1 mM Ni was required to produce a similar effect. There was no significant change in the growth phase of MEPCs in the presence of these ions and the decay phase remained exponential. No increase in τ_D was observed when 1 mM Mg was added to the control solution. The observations in Ni-containing solutions confirm those reported recently by Magleby and Weinstock (1980).

Although low concentrations of zinc and nickel had a greater effect than 80 Ca on τ_D, they caused much less depression of MEPC amplitude. The mean peak amplitude of MEPCs recorded from several cells under voltage clamp in normal toad Ringer's, 1 mM Zn and 1 mM Ni is shown plotted against membrane potential in Fig. 8*A*. It can be seen that there was only a slight decrease in MEPC amplitude in the presence of Zn or Ni. Furthermore, in contrast to the effects in 80 Ca, there was no appreciable shift in the null potential (Fig. 8*A*). Although Zn and Ni increased τ_D, they had no significant effect on the voltage dependence of τ_D, as illustrated in Fig. 8*B* (*see also* Table 2).

Analysis of power density spectra of end-plate current fluctuations showed that both Zn and Ni increased average channel lifetime. This is illustrated for a 1 mM Zn solution in Fig. 9. The half-power frequency of 38.2 Hz in normal toad Ringer's gave an average channel open time of 4.2 msec and the shift in half-power frequency to 22.6 Hz produced by 1 mM Zn gave a channel open time of 7.0 msec. In the same cell, average channel open time was 6.2 msec in a solution containing 1 mM Ni. Both Zn and Ni reduced single-channel conductance, but to a lesser extent than 80 Ca. The effects of Zn and Ni on end-plate channels in several preparations can be seen in Table 3.

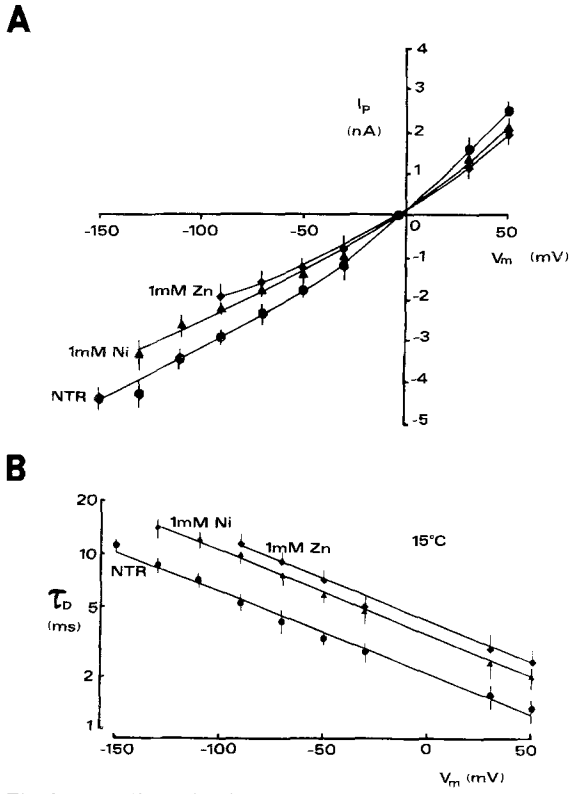

Fig. 8. The effect of voltage on MEPC peak amplitude (I_p) and time constant of decay (τ_D). *A*. Current-voltage relationships for averaged MEPCs ($n \geqq 15$) recorded from at least 7 cells in NTR (●), 1 mM Ni (▲) and 1 mM Zn (◆) solutions (15 °C). Peak MEPC amplitude was reduced slightly in the presence of the divalent cation, but no change in the null potential or in the overall shape of the *I–V* curve was apparent. *B*. Voltage sensitivity of τ_D. Averaged values of τ_D at each potential (at least 7 cells) were well fitted (least-squares) by: $\tau_D(V_m) = \tau_D(0) \exp(-V_m/H)$. For normal toad Ringer's, 1 mM Ni and 1 mM Zn solutions, H values were 99.8, 95.3 and 93.5 mV, $\tau_D(0)$ values were 2.06, 3.63 and 4.06 msec and correlation coefficients were 0.994, 0.989 and 0.992

Effect of Temperature

If the divalent cations affect channel lifetime (τ) by changing the nature of the reaction that is normally rate-limiting, then a change in the temperature sensitivity of τ would be expected. Changes in τ_D with temperature in normal toad Ringer's (circles), 1 mM Ni (triangles), 1 mM Zn (diamonds) and 80 Ca solution (squares) are shown in Fig. 10. From the regression lines fitted to the points, Q_{10}'s of 2.60 in normal toad Ringer's, 2.55 in 1 mM Ni, 2.47 in 1 mM Zn

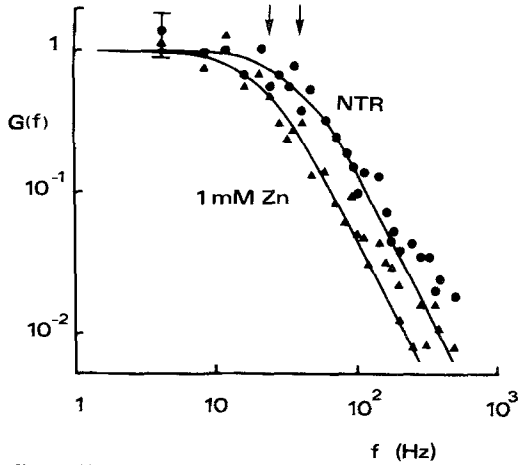

Fig. 9. Channel lifetime (calculated from the half power frequency, arrow) increased from 4.17 msec in normal toad Ringer's (NTR) to 7.03 msec in 1.0 mM Zn solution. Current fluctuations were recorded from the same cell in NTR, and after 5 min in 1.0 mM Zn. Holding potential, −70 mV, temperature 15 °C. Spectra are shown normalized, and were well-fitted (by least-squares using log $G(f)$ values) to single Lorentzian curves. Asymptotic spectral densities were 9.1×10^{-22} and 1.1×10^{-21} A^2 sec, for mean end-plate currents of 32 and 28 nA in NTR and 1.0 mM Zn, respectively. Single-channel conductance values were 28.2 pS in control solution and 19.2 pS in 1.0 mM Zn. In 1.0 mM Ni (not shown), noise spectra gave channel lifetimes of 6.23 msec and conductances of 19.4 pS, under the same conditions

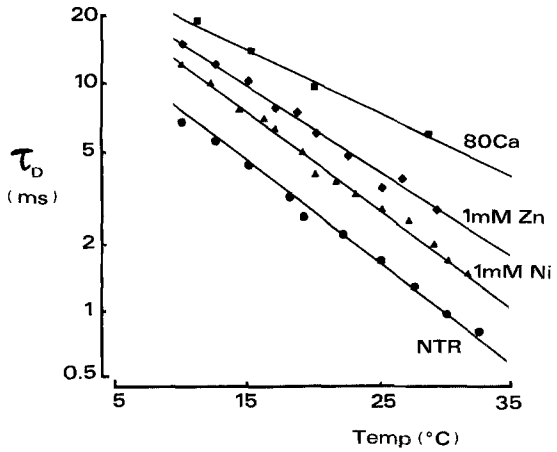

Fig. 10. Temperature dependence of τ_D. MEPCs from single cells were recorded extracellularly in NTR, 1 mM Ni and 1 mM Zn solutions, and under voltage clamp in 80 Ca (holding potential, −130 mV). Data were also least-squares fit to Arrhenius plots (*not shown*) and activation energies of 65.9, 64.5, 62.4 and 54.4 kJ/mol were calculated for NTR, 1 mM Ni, 1 mM Zn and 80 Ca solutions, respectively, corresponding to Q_{10}'s of 2.60, 2.55, 2.47 and 2.20

Table 4. Effect of temperature on τ_D[a]

Solution	Activation energy (kJ/mol)	Q_{10}	n
NTR	68.3±3.0	2.66±0.1	5
80 Ca	57.9±4.9	2.33±0.3	3
1 mM Zn	65.7±3.8	2.60±0.1	3
1 mM Ni	63.1±3.7	2.50±0.1	4

[a] MEPCs were recorded extracellularly (except in 80 Ca, where cells were voltage-clamped at −130 mV). Values of Q_{10} and activation energy for individual cells were obtained from least-squares fits to Arrhenius plots, and are shown as mean ±1 SEM. MEPCs were recorded at a minimum of 3 temperatures over at least a 15 °C range. n, number of cells.

and 2.20 in 80 Ca solution were obtained. Mean values of Q_{10} and activation energy obtained in several experiments are presented in Table 4.

Charge Movement

It has been reported previously (Van Helden et al., 1977; Gage & Van Helden, 1979) that some permeant monovalent cations have opposite effects on the amplitude and time constant of decay of MEPCs so that total charge movement during a MEPC is changed little when the cations are substituted for Na in the extracellular solution. The divalent cations, Zn and Ni, slowed the decay of MEPCs but caused only a small reduction in peak amplitude so that charge movement was greater than in normal toad Ringer's. In contrast, the decrease in MEPC amplitude in 80 Ca caused a decrease in charge movement compared to normal toad Ringer's, in spite of the increase in τ_D. For example, charge movement (calculated using MEPC peak amplitude $\times \tau_D$) at −110 mV was 36.8±1.5, 30.7±0.9, 25.9±0.7 and 12.2±1.4 pC for 1 mM Zn, 1 mM Ni, normal toad Ringer's and 80 Ca solutions, respectively (15 °C).

Discussion

Channel lifetime, whether estimated from the time constant of decay of MEPCs or from fluctuation analysis, was clearly increased in the presence of the divalent cations and this is in agreement with some previous observations. Tenfold increases in Ca and Mg concentrations (from 1 to 10 mM) have been reported to cause increases in τ_D in frogs that would occur with hyperpolarizations of about 20 and 12 mV, respectively (Cohen & Van der Kloot, 1978). It has also been found that end-plate currents recorded in solutions containing 8–10 mM Mg have a 50% greater time constant of decay than EPCs recorded at curare-blocked (3 μM) end-plates (Mallart & Molgó, 1978). In both cases, the voltage sensitivity of τ_D was unchanged. In earlier experiments, it had been found that miniature end-plate potentials and end-plate potentials recorded in isotonic calcium (Na-free) solutions have very prolonged decay phases (Katz & Miledi, 1969). An increase in membrane resistance may partly account for this, but the decay of end-plate currents was probably prolonged also. In contrast, some workers have found that an increase in calcium concentration decreases or has little effect on the time constant of decay of end-plate currents. For example, Bregestovski et al. (1979) found that 10 mM Ca caused little change in τ_D, but that in isotonic Ca solution (82 mM $CaCl_2$, 0 NaCl) τ_N approximately halved, with no change in voltage sensitivity. Magleby and Weinstock (1980) reported a 17% decrease in τ_D and an 8% decrease in τ_N in 10 mM Ca solution but they did find that τ_D was increased by 80% and τ_N by 50% in the presence of 10 mM Ni. The voltage sensitivity of τ_D was unchanged either by Ca or Ni. We are unable to explain these divergent findings. A remote possibility is that there may be species differences: we used toads whereas most of the other work was done with frogs.

The increase in τ_D we have seen seems unlikely to have resulted from an anticholinesterase action of the divalent cations, as similar increases in τ_N were observed (Table 3, Figs. 6 and 9), and 3 μM prostigmine caused a further increase in τ_D in 80 Ca solution (Fig. 5).

It has been suggested (Cohen & Van der Kloot, 1978; Van der Kloot & Cohen, 1979) that calcium

ions may increase τ_D by reducing the surface charge potential (Hille, Woodhull & Shapiro, 1975). Our results are not completely compatible with this hypothesis. Raising Ca concentration from 1.8 to 20 mM increased $\tau_D(0)$ from 2.02 ± 0.14 to 2.47 ± 0.30 msec. Using an external surface charge density of 10^{-5} C/cm^2, similar to the value of 0.8×10^{-5} C/cm^2 used by Lewis (1979), the above change in ionic strength would be expected (Grahame, 1947)[2] to screen surface charge and result in a hyperpolarization, $\Delta\psi$, of 17 mV. Using the same fixed-charge density, the higher ionic strength in the 80 Ca solution would result in a further hyperpolarization (relative to NTR) of 30 mV. Using the H value for NTR at 15 °C (Table 2) and assuming that the change in τ_D as calcium concentration was increased could be explained solely in terms of surface charge screening, it can be calculated that $\tau_D(0)$ in the 20 Ca solution would be 2.38 ± 0.18 msec (experimental value, 2.47 ± 0.30 msec) and $\tau_D(0)$ in the 80 Ca solution would be 2.69 ± 0.22 msec (experimental value 3.46 ± 0.40 msec). It can be seen that the increase in $\tau_D(0)$ in the 20 Ca solution is consistent with simple screening of surface charge by the increased ionic strength. However, the increase in $\tau_D(0)$ in the 80 Ca solution is somewhat greater than predicted. This higher value in the 80 Ca solution may possibly be due to some specific binding of calcium to surface charge but it seems more likely that calcium ions have some other action that increases channel lifetime.

An alternative possibility is that the value of surface charge density assumed is too low. If a higher value of 1.6×10^{-5} C/cm^2 is used for surface charge density, as seems appropriate for the ion gating system of Na channels (Hille et al., 1975), then $\Delta\psi$ in 80 Ca solution (relative to NTR) becomes 35.7 mV and the predicted value of $\tau_D(0)$ is 2.84 ± 0.25 msec. However, this is still somewhat lower than the experimental value of 3.46 ± 0.40 msec. It should be noted that if a lower value of 0.2×10^{-5} C/cm^2 is chosen for surface charge density (Adams, Dwyer & Hille, 1980) then $\Delta\psi$ in 20 Ca solution would be 3.8 mV and the predicted value of $\tau_D(0)$ becomes 2.09 ± 0.15 msec, which is barely different from the value in NTR and is somewhat lower than the experimental value of 2.47 ± 0.30 msec. The deviation between observation and prediction is even greater with 80 Ca solution. The increases in $\tau_D(0)$ produced by Zn and Ni are even more difficult to explain in terms of screening of negative surface charges. For example, it can be calculated (assuming a charge density of 10^{-5} C/cm^2), that the addition of 1 mM Zn would increase the actual transmembrane voltage by only 2 mV due to screening of negative fixed surface charge, yet Zn (and Ni) cause much greater lengthening than 20 Mg, 20 Ca or 80 Ca solutions. It may be that Zn and Ni bind specifically to membrane fixed-charge and exert a much greater effect on surface potential than would be expected if they simply screened surface charge. Such an explanation was originally proposed by Huxley (in Frankenhaeuser & Hodgkin, 1957) to account for the shifts in voltage-dependent membrane characteristics produced by increases in external Ca concentration. Consistent with this hypothesis is the observation that both Zn and Ni are among the most potent divalent cations in shifting the voltage-dependence of Na activation curves in nerve membranes, and that Ca and Mg have much smaller effects (e.g. Blaustein & Goldman, 1968; Hille et al., 1975). However, for lipid bilayers, McLaughlin, Szabo and Eisenman (1971) showed that the major effect of increasing divalent cation concentration (in the range ~ 100 μM to 100 mM) on surface potential arises from a simple screening mechanism, irrespective of whether the ion exhibits pure screening or whether it both binds and screens. Cations that exhibit pure binding, like UO_2 (divalent) and Th (tetravalent) were effective at concentrations of 1–10 μM. In preliminary experiments, no lengthening of τ_D was seen when Th was added to normal toad Ringer's to give final concentrations of 1 or 20 μM (Takeda, *unpublished observations*). On the other hand, surface potential calculations assume a uniform, "smeared" fixed-charge density. If the end-plate region has abnormally high fixed-charge density, local potential changes may be much greater for cations like Zn and Ni, which have apparently higher association constants.

The effects of the divalent cations on MEPC peak conductance and on single-channel conductance are not easily explained by surface potential changes. Certainly, negative surface charges may give membrane cation concentrations which are significantly greater than in the bulk solution. Assuming a fixed-charge concentration of 10^{-5} C/cm^2, the surface concentration of monovalent cations in normal toad Ringer's would be 20 times greater than in the bulk solution. In contrast, monovalent anions would be decreased to about one-twentieth of their bulk solution values. A tenfold decrease in the surface charge would reduce this surface concentration ratio from 20 to 1.6. In

[2] These values were obtained by solving numerically for ψ in the surface charge equation (Grahame, 1947), which may be re-expressed as

$$\sigma = [2\varepsilon\varepsilon_0 RT \sum_i m_i (e^{-ZF\psi/RT} - 1)]^{\frac{1}{2}}$$

where σ is the surface charge density in C·m^{-2}, ε is the relative dielectric constant ε_0 is the permittivity of free space ($=8.854 \times 10^{-12}$ F·m^{-1}); R and T are the gas constant and temperature in °K so that $(2\varepsilon\varepsilon_0 RT)^{\frac{1}{2}} = 1.86 \times 10^{-3}$ C·m$^{-\frac{1}{2}}$. mole$^{-\frac{1}{2}}$ and m_i is in moles m^{-3}.

these terms, cations causing the largest effective hyperpolarizations (and the greatest increases in τ_D) might be expected to reduce conductance the most. However, Zn and Ni, the most potent cations in prolonging τ, reduced both G_p and γ to a much smaller extent than 80 Ca solution. Of course, the conductance of end-plate channels may not be set solely by surface cation concentrations (although, the bulk concentration does affect γ; Barry et al., 1979*a*; Lewis, 1979). In the neutral site end-plate channel model (Barry et al., 1979*a*), absolute values of conductance tend to be independent of surface potential because the conductance is influenced by both cation and anion concentrations, even though the anion mobility is considered to be small. Thus the reciprocal changes in surface concentrations of anions and cations (of the same absolute valency) caused by surface potential shifts would tend to balance each other with respect to the channel conductance. The possibility that the low anion permeability of end-plate channels is due to a very low surface anion concentration caused by negative surface charge seems unlikely because other ACh-activated channels can have a high chloride conductance (e.g. Kehoe, 1972), although presumably the surface membrane bears negative charges also. Finally, it should be noted that the normal voltage sensitivity of τ_D in the presence of the divalent cations is consistent with surface potential shifts.

A simple explanation for ion-dependent changes in τ is that permeant ions bind to intrachannel sites and create a favorable energy conformation for the open channel: thus, less mobile ions (producing a lower channel conductance) would give a longer channel open time (Van Helden et al., 1977; Ascher et al., 1978; Gage & Van Helden, 1979; Marchais & Marty, 1979). Similarly, Kolb and Bamberg (1977) originally proposed that, in lipid bilayers, the longer Gramicidin A channel lifetimes observed with increasing cation concentrations resulted from an "electrostatic stabilization" of (open) channels containing permeating ions. For ACh-activated channels, the evidence rests on the reciprocal changes in τ and γ seen with monovalent alkali cations at the end-plate giving rise to constant charge transfer (Van Helden et al., 1977; Gage & Van Helden, 1979) and on the increased voltage sensitivity of τ observed in the presence of divalent cations in aplysia (Ascher et al., 1978; Marchais & Marty, 1979). However, ion-dependent increases in both τ and γ occur at the end-plate (Nonner et al., 1980; Takeda et al., 1980*a*) and in *Aplysia* (Ascher et al., 1978). Clearly, the divalent cations result in a total charge transfer different from normal. Marchais and Marty (1979) showed that, for divalent cations, surface potential shifts cannot account for both the prolonged τ, and for the increased voltage sensitivity of τ. Rather, they proposed that the voltage-dependence of τ arises from the ions binding to intrachannel sites and encountering asymmetric energy barriers. A corollary would be that low temperatures should favor divalent cation binding and result in an increased voltage sensitivity of τ. The normal voltage and temperature sensitivity of τ observed here in the presence of divalent cations is difficult to reconcile with the Marchais and Marty (1979) model. Other workers (Bregestovski et al., 1979; *see also* references in Marchais & Marty, 1979) have also observed normal voltage sensitivities of τ in isotonic Ca solutions.

Permeability ratios calculated from null (zero current) potentials have been compiled for an exhaustive list of cations, both organic and metal, monovalent and divalent (Van Helden et al., 1977; Linder & Quastel, 1978; Gage & Van Helden, 1979; Lewis, 1979; Watanabe & Narahashi, 1979; Adams et al., 1980; Dwyer, Adams & Hille, 1980). Some anomalies appear when these ratios are compared to conductance values obtained from noise analysis (Barry et al., 1979*a*; Nonner et al., 1980; Takeda et al., 1980*a*). As permeability ratios obtained from null potential measurements using the Goldman-Hodgkin-Katz Constant Field Equation give good estimates of conductance ratios only in systems where the independence principle applies, it seems necessary to conclude that ion interaction occurs, which could give rise to competition, blocking or saturation effects. Values of P_{Ca}/P_K obtained from null potential measurements using the Constant Field Equation are dependent on the surface charge density assumed (Lewis, 1979; Adams et al., 1980). In solutions containing only permeant monovalent cations, permeability ratios are independent of surface charge density, as the effects of surface potentials are exactly balanced by the changes in surface concentration of cations. If surface charge effects are ignored, $P_{Ca}/P_K = 0.14$. On the other hand, with a surface charge density of 10^{-5} C/cm^2, $P_{Ca}/P_K \simeq 0.04$.

The increase in MEPC peak conductance in 80 Ca solution with increasing membrane depolarization (Fig. 4*B*) can be explained if Ca, like Li (Barry et al., 1979) and NH_4 (Takeda et al., 1980*a*), has a very much lower mobility than K. The voltage-dependence of conductance then results from the asymmetrical nature of the solution composition. At depolarizing potentials the channel tends to be occupied by K ions with a resonably high mobility and so the conductance is high, whereas at hyperpolarizing potentials the channel tends to be occupied by Ca ions with a much lower mobility and hence a resultant lower conductance. The actual slope of the conductance-voltage curve and the absolute value of conductance is dependent on the value of the Ca equilibrium

constant. Quantitative fitting of the data to either neutral or charged site models was not undertaken because of the considerable theoretical complications arising from divalent and monovalent ions competing for sites in models where the independence principle is violated.

The blocking actions of some anaesthetics and partial agonists have been interpreted in terms of the blocking molecule entering open end-plate channels and binding to some critical intrachannel site, thereby presumably decreasing the flow of permeant ions (Adams, 1976, 1977; Ruff, 1977; Adams & Sakmann, 1978; Neher & Steinbach, 1978). For Gramicidin A channels, Bamberg and Läuger (1977) have proposed that the blocking effects of Ca and Mg (which are impermeable) result not from binding to an intrachannel site, but rather to a site distinct from the main pathway for permeant ions, thus impeding the access of permeable ions to the channel. Also by analogy with the effects of quaternary ammonium ions on K channels (Armstrong, 1975), blocking molecules could exert their actions from sites just outside the channel proper. However, the fact that Ca is permeant suggests that in 80 Ca solution, the increased τ and smaller γ arises from interaction of Ca with intrachannel sites. On the other hand, although no experiments have been reported for isotonic Zn solutions, the high potency of 1 mM Zn in prolonging τ (without a corresponding decrease in γ) indicates that the mechanism of action for Zn might be different. It may be that blocking and permeant ions compete for the same site(s). A more likely explanation would be that Zn directly affects the "gate" that controls channel lifetime (Begenisich & Lynch, 1974; Armstrong & Gilly, 1979).

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