MONOVALENT ION CURRENT THROUGH SINGLE CALCIUM CHANNELS OF SKELETAL MUSCLE TRANSVERSE TUBULES

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ABSTRACT Akali monovalents, Li, Na, K, Cs, and organic monovalents of molecular cross section <20 Å^2, ammonium, methylammonium, hydrazinium, guanidinium, are shown to have a measurable conductance through Ca channels of muscle transverse tubules reconstituted into planar bilayers. For the alkali series, single channel conductances follow the sequence Cs ≈ K > Na > Li with a conductance ratio $[g(Cs)/g(Li)] = 1.7$. For permeability ratios, the sequence is Li > Na > K ≈ Cs with $[P(Li)/P(Cs)] = 1.5$. Monovalent current is only unmasked when Ba ions are not present. In mixtures of Cs and Ba, single channel current reverses close to the Ba equilibrium potential and more than 100 mV away from the Cs equilibrium potential. A cutoff in conduction is found for organic cations larger than trimethylammonium; this suggests an apparent pore aperture of about 5 × 5 Å. Even in such a large pore, the fact that the alkali cation permeability sequence and conductance sequence are inverted rules out molecular sieving as the mechanism of selection among monovalents.

INTRODUCTION

Studies in vivo have shown that when Ca channels are bathed in solutions containing a low concentration of free divalent ions (< 1 μM), they lose their divalent ion selectivity given that under this condition, alkali ions and small organic cations show a measurable permeability (Kostyuk and Krishtal, 1977; Yamamoto and Washio, 1979; Yoshida, 1983; Kostyuk et al., 1983; Almers et al., 1984; McCleskey and Almers, 1985; Fukushima and Hagiwara, 1985). In the molluscan neuron, Kostyuk et al. (1983) proposed that divalent ion selectivity is conferred by a single high affinity divalent-only binding site, which in their analysis is located somewhere in the outer surface of the Ca channel protein, i.e., an "external ion-selecting filter," which in the absence of divalents loses its specificity. In skeletal and cardiac muscle and in snail neurons, the evidence has been lately in favor of two divalent binding sites within the pore because in mixed divalent electrolyte conditions, currents through Ca channels display a characteristic anomalous mole fraction behavior (Hess and Tsien, 1984; Almers and McCleskey, 1984; Byerly et al., 1985). In this report, we describe similar conductive features in muscle Ca channels in planar bilayers (Affolter and Coronado, 1985). We find that in the absence of free divalent ions, transverse-tubule (t-tubule) Ca channels discriminate very little among alkali monovalents. Under the same conditions organic monovalent cations of the size of ammonium, hydrazinium, and methylammonium also show significant conductance. A cutoff in conduction for molecules larger than trimethylammonium yields an approximate pore cross-section of 25 Å^2. This estimate is in good agreement with measurements in frog skeletal muscle (McCleskey and Almers, 1985).

MATERIALS AND METHODS

T-tubules from rat back and leg muscles were purified by the procedure of Rosemblatt et al. (1981) with minor modifications described elsewhere (Affolter and Coronado, 1985). Bilayers were cast from 50 mg/ml phospholipid solutions of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids, Birmingham, AL) at a 1:1 molar ratio in decane. Insertion of channels, low pass filtering (0.1–0.5 kHz corner frequency on an 8-pole Bessel filter) and handling of single channel data has been described in detail elsewhere (Coronado and Affolter, 1986*a*). Activity coefficients were obtained from Parsons (1959) and they correspond to the 25°C mean activity coefficient of the chloride salt of alkali cations. Activity coefficients were as follows: LiCl (0.921, 0.750); NaCl (0.928, 0.704); CsCl (0.809, 0.675); KCl (0.816, 0.703); where the two values given in parenthesis correspond to the 0.05 and 0.25 M activity coefficient, respectively. Channel conductance in single salts of alkali and organic cations corresponds to the slope conductance at a holding potential (HP) of 0 mV. Permeability ratios for cation pairs X, Y with X on the external, and Y on the internal sides were calculated according to

$$P(Y)/P(X) = A(X)/A(Y) \exp(-V_0/25.3), \quad (1)$$

where $A(X)$ and $A(Y)$ denote activities, V_0 is the zero-current potential in millivolts, and 25.3 is the value in mV calculated from the appropriate thermodynamic constants at room temperature. Organic cations purchased as hydrochloride salt from Sigma Chemical Co. (St. Louis, MO) were titrated to pH 7.0 with Tris (trishydroxymethylaminomethane). Alkali cations (chloride salt) were American Chemical Society grade from Fisher Scientific Co. (Fair Lawn, NJ). Solutions were adjusted to pH 7.0 with 10 mM HEPES-Tris, 0.5 mM EDTA. Bay K8644 (1–5 μM) was added to all solutions. Cross-sectional areas of organic cations were calculated from the atomic dimensions reported by Moreno and Diamond (Table 5, 1975) obtained from unmodified Corey Pauling Koltun (CPK) molecular models. The reported cross section corresponds to the area of the smallest rectangle through which a given cation could physically fit and pass through.

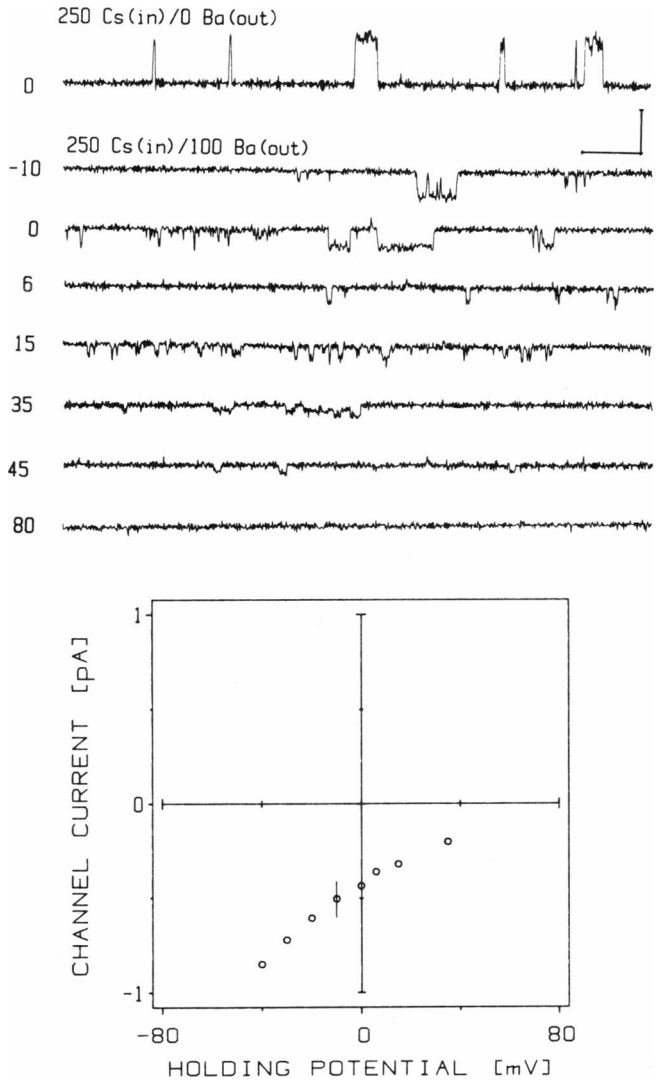

FIGURE 1. Ca channel reversal potential in internal Cs and external Ba. Records at the holding potential indicated at the left of each trace were taken in 250 mM internal/50 mM external CsCl (labeled 250 Cs (in)/0 Ba (out)) and after addition of 100 mM $BaCl_2$ to the external side without removing the CsCl gradient (labeled 250 Cs (in)/100 Ba (out)). When the current is in the outward direction, channel openings deflect the trace upwards. Inward currents deflect the trace downwards. Time and current marks correspond to 400 ms, 1 pA. Mean amplitudes of openings collected in the range of -40 to $+35$ mV are shown in the bottom panel for channels in 250 mM Cs, 0 Ba internal/50 mM Cs, 100 mM Ba external. Bars, two SD, the data point with the largest error.

RESULTS

Internal and external sides of the channel have been previously shown to be located in the *cis* (voltage command) and *trans* (ground) bilayer chambers, respectively (Affolter and Coronado, 1986). As such, all holding potentials, equilibrium potentials, and designations of internal and external solutions given here have the same meaning as in cellular recordings. Fig. 1 (*top record*) shows unitary outward Cs currents through Ca channels in the absence of Ba at HP 0 mV in 250 mM internal/50 mM external CsCl. Under these conditions, channel reversal is at ~ -40 mV, which is the Cs equilibrium potential for such a salt gradient (see Fig. 3 *C* for a reversal experiment for the same gradient of NaCl). That the Cs current is transported via Ca channels is directly shown in the records immediately below the top record in which 100 mM $BaCl_2$ is added to the external side without removal of internal or external CsCl. Under these conditions, unitary currents turn inward and a scan of opening events at different holding potentials (indicated at the left of each trace) show that channel reversal in Cs plus Ba is more positive than +45 mV. Actually, at holding potentials of up to +80 mV (Fig. 1, *bottom trace*) there is no sign of outward Cs current. Mean amplitudes in the range of -40 to +35 mV are given in the current-voltage curve of Fig. 1. This curve, which is obtained in the mixed Cs-Ba conditions, shows a unitary slope conductance of ~8 pS at HP 0 mV and a reversal potential beyond +40 mV, probably close to +80 mV as judged by the single channel records. Thus, channels are divalently-selective by a wide margin when divalents are present given that reversal is closer to the Ba equilibrium potential ($E(\text{Ba}) \gg +100$ mV) and about 100 mV away from the Cs equilibrium potential ($E(\text{Cs}) = -40$ mV). The impermeability of Cl is shown here by the reversal of channels at $E(\text{Na})$ in a NaCl gradient (Fig. 3 *B*). The same result has been shown elsewhere at a different concentration of NaCl (Coronado and Affolter, 1986*a*).

Using the reversal potential in 100 mM external Ba as a criterion, we were able to further identify monovalent current through Ca channels for the rest of the alkali series. This is shown in Fig. 2 for Na and Li in addition to Cs. Histograms of single openings collected at HP 0 mV for the same gradient of alkali ion (250 mM internal, 50 mM external) shows that currents in Cs exceed 1 pA, a value that is approximately two-fold larger than the saturating current in 100 mM Ba at the same potential (Coronado and Affolter, 1986*a*). Currents in Li are about half of that in Cs while determinations in Na and K (not shown) are contained within the conductance limits given by the less mobile Li and most mobile Cs ions. Current-voltage curves for channels in a gradient of a single salt and separately in gradients of two salts where used to construct a sequence of single channel conductances and a sequence of permeability coefficient ratios, respectively. These results are shown in Fig. 3. Relative permeabilities were

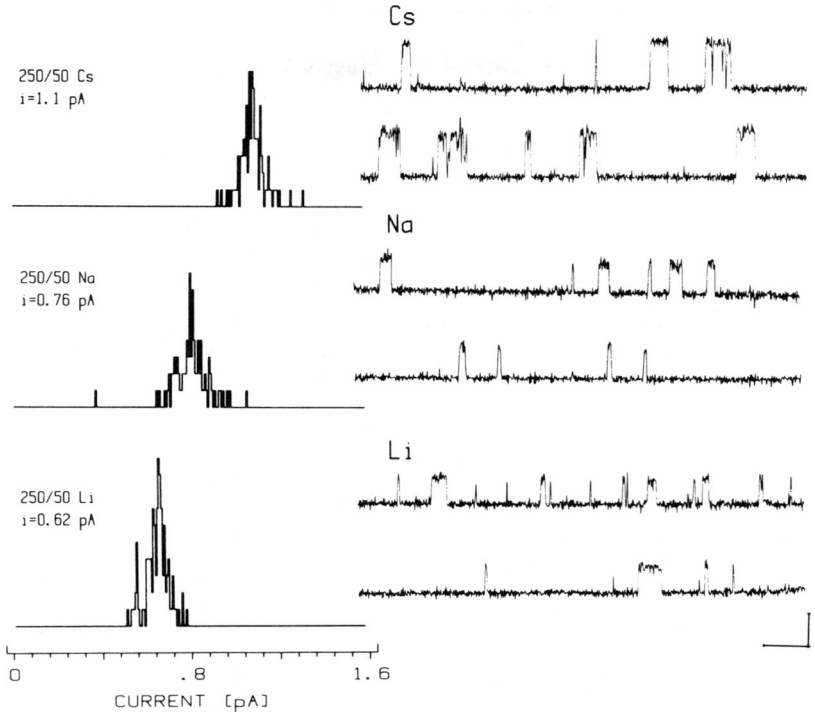

FIGURE 2. Alkali monovalent current through t-tubule Ca channels. Records are shown at HP 0 mV in 250 mM internal/50 mM external CsCl, NaCl, and LiCl, each salt tested separately. Time marks and current marks correspond to 400 ms, 1 pA. Left panel, histograms of opening events with the *y*-axis (not labeled) corresponding to occurrences and the *x*-axis corresponding to pA of unitary current. The mean current and salt condition is indicated in each histogram. Total number of events in the histograms are Cs (n = 125; mean = 1.1 pA, 0.17 SD), Na (n = 135; mean = 0.76 pA, 0.07 SD), Li (n = 105; mean = 0.62 pA, 0.082 SD).

obtained from pairs of test cations (X, Y), one on each side of the bilayer, at two concentrations: 250 mM internal X/50 mM external Y, and a strictly bi-ionic condition with 50 mM internal X/50 mM external Y (X, Y = Li, Cs; Na, K; and Na, Cs). Fig. 3 *A* shows determinations of reversal potentials for Cs and Li, the pair that gives the largest difference in permeability ratios. In the bi-ionic case (50 mM internal LiCl/50 mM external CsCl) there is a net outward Li current at HP 0 mV and the extrapolated zero-current potential is −10 mV. In the five-fold gradient situation (250 mM internal LiCl/50 mM external CsCl) there is an outward Li current that extrapolates to zero current at −50 mV. In both conditions the reversal for the Li (internal)–Cs (external) cation pair is at a voltage more negative than the cationic equilibrium potential that we calculate to be −0.3 mV in symmetrical 50 mM Li/50 mM Cs, and −38.8 mV in 250 mM Li/50 mM Cs. The difference between the actual reversal and the calculated cationic reversal is clearly reflected in the permeability ratio $P(\text{Li})/P(\text{Cs})$, which from Eq. 1 gives a value of 1.46 at 50 Li/50 Cs and 1.55 at 250 Li/50 Cs. Averaged over several experiments, these values were found to be statistically similar. In separate experiments, single channel conductance was measured for each salt separately in 250 mM internal/50 mM external salt using current-voltage curves as in Fig. 3 *B* shown for NaCl. For all salts the extrapolated zero-current potential was within −35 to −40 mV, which is well accounted for by cationic equilibrium potential with $P(\text{Cl}) = 0$. Permeability coefficients for the four test cations are shown in Fig. 3 *C* using the Na coefficient as unity. Single channel slope conductance at HP 0 mV is shown in Fig. 3 *D* for the same cations. In Fig. 3, *C* and *D*, cations have been arbitrarily ranked according to atomic radii only for the ease of comparison. We find small but significant differences in permeability coefficients as well as in channel conductance for the cation pairs of most extreme size such as in Li vs K or Li vs Cs. The K and Cs data in Fig. 3, *C* and *D*, however may not be significantly different given the clear overlap in standard deviations. Notwithstanding the latter uncertainty, the important observation here is that the permeation sequence and the conductance sequence are inverted, in an almost symmetrical way with $P(\text{Li})/P(\text{Cs}) = 1.5$ and $g(\text{Cs})/g(\text{Li}) = 1.7$.

The sieving characteristics of cations where further investigated in Fig. 4 for a series of amino and methyl derivatives of quaternary ammonium. The size of cations is given in terms of cross-sectional areas, that is, the smallest conceivable rectangular hole that would have to be poked into a bilayer for a cation to fit and pass through. Test cations in this series, ammonium (1), hydrazinium (2), methylammonium (6) were chosen (*a*) for having a *pKa* such that 95% or more of the compound in solution would be protonated at pH 7.0, (*b*) for having a roughly spherical shape so that collision factors between the test cation and the channel along the different axes as the cation could be

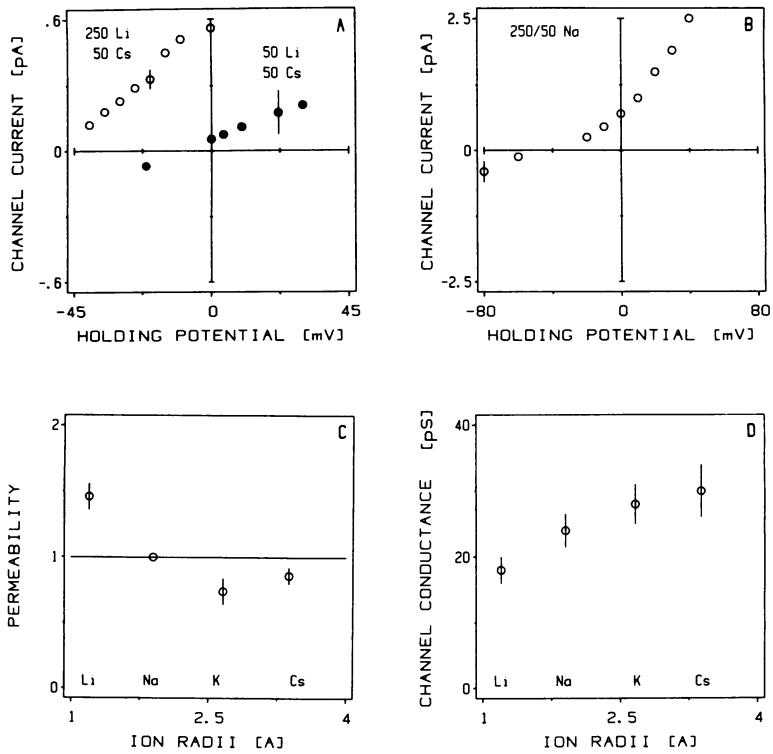

FIGURE 3. Permeability and conductance sequences for alkali cations. *A* and *B* correspond to current-voltage curves of single channels in two salts (LiCl-internal, CsCl-external) or single salt (NaCl), respectively. In *A* data are shown for two separate gradients corresponding to 250 mM Li/50 mM Cs (data points in the upper left quadrant of the I-V curve with a reversal more negative than −40 mV, *open circles*) and 50 mM Li/50 mM Cs (data points in the upper right quadrant of the I-V curve with a reversal more negative than 0 mV, *filled circles*). In *B* the ionic condition was 250 mM Na internal/50 mM Na external. Bars (2 SD) are given for data with largest error. *C* and *D* correspond to ratios of permeability coefficients and single channel slope conductances (taken at HP 0 mV), respectively. Permeability ratios in *C* were calculated from Eq. 1 of text and normalized by assuming $P(\mathrm{Na}) = 1.0$. Bars, SD of four separate experiments, two determinations in 250 *X*/50 *Y* and two determinations in 50 *X*/50 *Y* (*X*, *Y* test salts) as described in (*A*) and text. The extrapolated zero current potential and the closest data point in the I-V curve were never separated by more than 10 mV. Slope conductance at HP 0 mV in (*D*) were determined for each cation separately in 250 mM internal/50 mM external test cation.

neglected, at least to a first approximation, and (*c*) for being sufficiently conductive through the Ca channel as to make the single channel conductance a reliable measurement. As shown in top records Fig. 4 (*top records*), when cations are tested in a concentration gradient of 250 mM internal/50 mM external, the two extremes of unitary current amplitudes are given by ammonium and methylammonium. Ammonium (1) is the most permeant with a slope conductance of 60 pS at HP 0 mV while substitution of a single hydrogen by a bulkier amino or methyl group, as in hydrazinium (2) or methylammonium (3), produces a drop in conductance to 15 and 6 pS, respectively. Further substitutions by two or more amino and methyl groups as in guanidinium (4) and trimethylammonium (5) reduce conductance only slighly further to 5 and 3 pS, respectively. A true cutoff in conductance seems to be for molecules such as tetramethylammonium with a conductance < 2 pS, which is at the edge of our current resolution. Thus, by this analysis we conclude that organic molecules larger than 5 × 5 Å are excluded from the channel primarily on the basis of size.

DISCUSSION

The data presented here, in Ca channels in an in vitro recording system, indicate that in monovalent solutions containing 0.5 mM EDTA, which is sufficient to chelate the contaminant levels of divalents found in monovalent salts, there is a large alkali and organic cation current through the same channel, which in the presence of Ba and monovalent salt is highly divalent selective. This was shown in Fig. 1 for Cs and Ba and the same has been shown previously for Na and Ba (Coronado and Affolter, 1986*a*). Additional pharmacological evidence in this system showing that monovalent current is carried in fact through Ca channels has been reported recently (Coronado and Affolter, 1986*b*). The cutoff in conductance for organic cations larger than trimethylammonium (5 × 5 Å) is similar to that found by McCleskey and Almers (1985) in the slow Ca channel of frog muscle. This result and the mole fraction behavior in mixed Na/Ba solutions (Coronado and Affolter, 1986*a*) suggests that the slow Ca channel is the physiological correlate of the channel studied here in

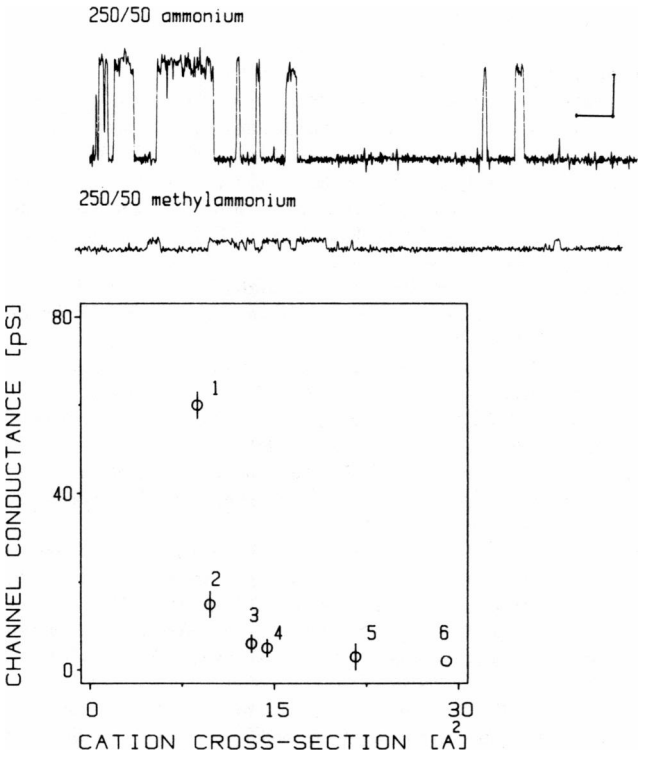

FIGURE 4. Conduction of organic cations through t-tubule Ca channels. Single channel recordings of outward current are shown at HP 0 mV for 250 mM internal/50 mM external ammonium and methylammonium. Time and current marks correspond to 400 ms, 1 pA. Bottom panel, slope conductance at HP 0 mV plotted as a function of cation cross section. (1) Ammonium (60 pS, 3 SD); (2) hydrazinium (15 pS, 3 SD); (3) methylammonium (6 pS, 2 SD); (4) guanidinium (5 pS, 2 SD); (5) trimethylammonium (3 pS, 3 SD); (6) tetramethylammonium (<2 pS).

bilayers. Also, the slow Ca channel is present in rat skeletal muscle, the same tissue used here to purify t-tubules (Donaldson and Beam, 1983). Some heterogeneity of current amplitudes seen in Ba solutions (Fig. 1) but not in monovalent ion solutions (Fig. 2) most probably arises from substate conductances of the slow Ca channel, which we have recently described (Ma and Coronado, 1987). Evidence for slow and fast types of Ca channels in frog skeletal muscle has been presented (Cota and Stefani, 1986), but to date, all the data in planar bilayers seem to indicate that in this system only one type is present, the slow type.

The finding that the ammonium conductance is outstandingly large (60 pS) compared with that of alkali ions such as Li (18 pS) and Cs (30 pS) is significant given that it may reflect important energy transactions involving hydrogen bonds that take place within the Ca channel. Unfortunately, there seems to be only few cations besides ammonium that could be of further use as probes of structural features, given that single substitutions in the ammonium molecule such as in methylammonium generate a 10-fold reduction in conductance, from 60 to 6 pS, respectively. Obviously the amplitude of the channel currents limits in this case the type and design of experiments. It has been suggested in Na and K channels that the drop in conductance or permeability when hydrogens are replaced by methyl groups may not be accounted for by a steric factor alone, but it may also involve the loss of hydrogen bonding capacity of the cation to acceptor groups in the channel (Hille, 1975). However, this rule cannot be applied in any sample way to larger molecules such as guanidinium, which has six hydrogen donors, or trimethylammonium, which has only one hydrogen donor because both cations here have essentially the same low conductance. Size limitation seems to be the most direct explanation in this case.

Even if large organic cations are excluded on the basis of size, for alkali cations permeation is not governed by molecular sieving. The data on alkali cations show that the sequence of single channel conductance and that of permeability obtained from bi-ionic potentials are quite the opposite of each other. The extremes of these sequences are given by Li and Cs with $g(\mathrm{Cs})/g(\mathrm{Li}) = 1.7$ and $P(\mathrm{Li})/P(\mathrm{Cs}) = 1.5$. This discrepancy can only be understood when ions crossing the channel encounter at least two energetically unfavorable energy barriers or peaks and one energetically favorable binding site or well (Lauger, 1973). This immediately raises the possibility of having selectivity sequences entirely different for peaks and wells, which depending on experimental protocol, ion concentration, and given barrier profile, will reflect themselves differently in the permeability ratio or the conductance ratio (see Eisenman and Horn, 1983). In the case when a single site is considered and no more than one ion can occupy the site at any given time, it can be shown experimentally that permeability ratios and conductance ratios only agree when measurements are done at low salt, i.e., under conditions where occupancy of the channel by ions is sufficiently low so that passage of one ion does not depend on the presence of other ions in the pore (Coronado et al., 1980). Additional sources of discrepancy for conductances and permeabilities have been mentioned extensively when multiple sites and multiple ions are considered, (Hille and Schwartz, 1978; Finkelstein and Andersen, 1981; Eisenman and Horn, 1983).

The sequence of permeability coefficients and the sequence of single channel conductances (Fig. 3, *C*, and *D*) raises several questions about the forces governing selectivity for monovalent cations in the Ca channel. The permeability sequence $P(\mathrm{Li})1.4 > P(\mathrm{Na})1.0 > P(\mathrm{K})0.7 \approx P(\mathrm{Cs})0.8$ is analogous to an Eisenman sequence of binding to a site of high field strength, i.e., a site that favors the binding of Li, the smallest cation (Eisenman, 1962). A similar series has been previously shown to hold for Ca channels from heart (Hess and Tsien, 1983) and probably also for Ca channels in lymphocytes (Fukushima and Hagiwara, 1985). However, the conductance sequence

g(Li)18 pS < g(Na)24 pS < g(K)28 pS ≈ g(Cs)30 pS was previously unknown and because it is the opposite of the permeability series, it is analogous to an Eisenman sequence of binding to a site of low field strength, i.e., a site that favors binding of Cs, the less hydrated cation. This apparent contradiction (high vs. low field strength binding) is not unexpected because, as mentioned previously, at the concentrations used here (50 mM external/250 mM external cation) channel conductances in single salt and zero-current potentials in mixed salts may have entirely different rate-limiting steps. Nevertheless, the fact that the less hydrated Cs also has the highest conductance prompts the question of whether this is related to (*a*) binding affinity, or (*b*) high mobility inside the channel, or (*c*) higher rate of access of this cation to the pore entry; (*b*) or (*c*) may be suggested by the fact that our conductance data also correlate well with the mobilities of alkali cations in dilute solutions,

	Li	Na	K	Cs	Cs/Li
g =	.75	< 1.0	< 1.16	< 1.25	1.66
u =	.76	< 1.0	< 1.46	< 1.54	2.02 ,

where g denotes conductance, u denotes mobility (Parsons, 1959), and Na is taken as unity. At least for Na, the half saturation conductance is at ~0.3 M (Coronado and Affolter, 1986*a*). Thus, conductances reported here probably reflect values in the vicinity or immediately below the half saturation. If this sequence also holds at low concentrations, i.e., when conductance ratios and permeability ratios are expected to be the same and to be proportional to the mobility of ions inside the pore (Lauger, 1973), it would be strong evidence in favor of the Ca-free Ca channel as being a large water-filled pore.

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