Single channel measurements demonstrate the voltage dependence of permeation through N-type and L-type CaV channels

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The delivery of Ca2+ into cells by CaV channels provides the trigger for many cellular actions, such as cardiac muscle contraction and neurotransmitter release. Thus, a full understanding of Ca2+ permeation through these channels is critical. Using whole-cell voltage-clamp recordings, we recently demonstrated that voltage modulates the apparent affinity of N-type (CaV2.2) channels for permeating Ca2+ and Ba2+ ions. While we took many steps to ensure the high fidelity of our recordings, problems can occur when CaV currents become large and fast, or when currents run down. Thus, we use here single channel recordings to further test the hypothesis that permeating ions interact with N-type channels in a voltage-dependent manner. We also examined L-type (CaV1.2) channels to determine if these channels also exhibit voltage-dependent permeation. Like our whole-cell data, we find that voltage modulates N-channel affinity for Ba2+ at voltages > 0 mV, but has little or no effect at voltages < 0 mV. Furthermore, we demonstrate that permeation through L-channel is also modulated by voltage. Thus, voltage-dependence may be a common feature of divalent cation permeation through CaV1 and CaV2 channels (i.e., high-voltage activated CaV channels). The voltage dependence of CaV1 channel permeation is likely a mechanism mediating sustained Ca2+ influx during the plateau phase of the cardiac action potential.

Introduction

The permeation of CaV channels has been an important area of study for the past 30 years. The movement of ions through CaV channels is driven by the ions' electrochemical gradient, or the driving force, which depends on the ion concentration and the transmembrane voltage. Permeation, however, is a less predictable phenomenon that describes interactions that occur inside the CaV channel’s pore, between the permeating ions and channel itself.1,2 These interactions occur at apparent ion binding sites and endow CaV channels with their specificity (e.g., for Ca2+) and shape the magnitude of ionic flux.1,2 CaV channels are thought to have one high affinity Ca2+ binding site, to which Ca2+ and other divalent cations bind at μM concentrations, and which endows the channel with its specificity for Ca2+ over Na+ and K+.3 As the exit rate from a high affinity site is small, it has been proposed that CaV channels have adjacent low affinity sites within the pore.4 These low affinity sites effectively increase the Ca2+ off-rate from the channel by providing “steps” (i.e., low energy transition sites) for ions to move out of the high affinity site.1,2,4 An important question is whether transmembrane voltage can influence the affinity of these sites and, thus, ion permeation through CaV channels.

One way to measure the affinity of Ca2+ for these sites is to study ionic currents in different concentrations of Ca2+. A few studies examined CaV current permeation at voltages < 0 mV and concluded that permeation is not voltage dependent.6,7 However, there were clues within the literature that voltage could affect permeation at more depolarized voltages.8,9 To reconcile these findings, we
examined permeation through N-type CaV channels (CaV2.2) over a wide range of Ca$^{2+}$ concentrations and voltages.\textsuperscript{10} Using whole-cell patch clamp recordings and computer modeling, we showed that the apparent affinity of the channel for Ca$^{2+}$ decreased with increased depolarization, but only for voltages > 0 mV. Thus, we concluded that N-type calcium channel permeation is voltage-dependent,\textsuperscript{10} unlike that of T-type channels.\textsuperscript{11} Our study also revealed a physiological consequence of voltage-dependent permeation, which is that at depolarized voltages, where driving force is small, the Ca$^{2+}$ ions flow into the cell more easily than they would if the channels’ affinity for Ca$^{2+}$ was unchanged by voltage.\textsuperscript{10}

The use of the whole-cell recording method in our previous study presented some limitations. One difficulty was in isolating one CaV current type from another. We utilized bullfrog sympathetic neurons in which 90–95% of the CaV current (2–3 mM Ba$^{2+}$) was generated by N-type channels.\textsuperscript{12,13} However, in the presence of Ca$^{2+}$ or high divalent cation concentrations, another CaV channel, called E$\nu$-channel, can contribute up to 25% of the total current.\textsuperscript{14,15} Thus, the fraction of current contributed by N-type channels changes as the external divalent cation concentration increases. We believe that this did not adversely affect our results, but this assumption must be tested.

Importantly, single channel recordings allow the isolation of channel types in cells with mixed channel populations. Thus, we utilized this technique to further test the hypothesis of voltage control of permeation through N-type channels. In addition, we examined L-type (CaV1.2) channels, which can easily be isolated in this configuration\textsuperscript{16} in spite of the fact they comprise a minor component of the whole-cell CaV current in Bullfrog sympathetic neurons.\textsuperscript{12,13} We find that the 2 channel types exhibit remarkably similar permeation characteristics. Thus, it is likely that voltage control of permeation at voltages > 0 mV (i.e., the reduced channel affinity for ions) is a common feature of CaV1 and CaV2 (i.e., high-voltage activated) channels. In addition, our results support a mechanism by which Ca$^{2+}$ influx could be maintained at a higher-than-expected level during the long depolarization phase of the cardiac action potential.

### Results

**Permeation of single N-channels**

We examined voltage control of permeation using single channel recordings, where CaV channels can be unambiguously isolated. Bullfrog sympathetic neurons express 3 CaV channel types, N-type (CaV2.2), L-type (CaV1.2) and E$\nu$-type (likely CaV2.3).\textsuperscript{14,16} N-type and L-type channels have very similar conductances,\textsuperscript{16} which can make them difficult to separate. However, all of our recordings were done in the presence of 1 μM BayK-6844, which selectively increases L-channel open times at negative voltages (e.g., −40 mV) to facilitate identification of N-type from L-type channels.\textsuperscript{16,17} E$\nu$-channels were easily distinguished from N- and L-type channels by their smaller unitary conductance.\textsuperscript{16} Since we were only interested in permeation (not gating), patches with multiple CaV channels were used as long as contaminating channels (i.e. E$\nu$-channels) could easily be excluded from the analysis.\textsuperscript{16,18,19} Thus, patches containing both N-type and L-type channels were not utilized. Ba$^{2+}$ was used as the charge carrier instead of Ca$^{2+}$ to increase unitary current amplitude and block potassium channels in the patches.

**Figure 1** shows N-channel activity under different conditions along with the resulting Ba$^{2+}$ dose-response curves at different voltages. As expected, the unitary current amplitude increased with Ba$^{2+}$ concentration and with hyperpolarization. The unitary current amplitude plotted vs. Ba$^{2+}$ concentration shows a monotonic increase that is well fitted by the mass-action equation (Fig. 1B). The resulting EC$_{50}$ values increased with voltage from 0 to 50 mV (Table 1). The voltage dependence of permeation is better visualized from the normalized curves in Fig. 1C, which show that N-channel permeation is voltage-dependent at voltages > 0 mV. Since unitary current amplitude does not depend on gating,\textsuperscript{20} our data confirm the voltage dependence of permeation measured from whole-cell recordings. Interestingly, the difference in EC$_{50}$ between 0 and −10 mV was relatively small, which is also consistent with both our whole-cell data and our permeation model.\textsuperscript{10}

One limitation was the low number of patches (N) measured for some Ba$^{2+}$ concentrations. For example, the N was only 1 or 2 for 3 mM and 300 mM Ba$^{2+}$. In addition, some voltages also had measurements from only a single patch (see Fig. 1 legend for details). Since the EC$_{50}$ values could have been negatively impacted by including points with only 1 or 2 observations, we re-fit the data using only points with N ≥ 3. The resulting EC$_{50}$ were almost identical to those from fits that included all points (compare with Table 1). The values were EC$_{50}$ = 13 mM for 0 mV, 17 mM for +10 mV, 33 mM for +20 mV, 52 mM for +30 mV and 73 mM for +40 mV. Thus, the voltage-dependence of N-channel permeation at voltages > 0 mV is strongly supported.

**Permeation of single L-channels**

We wanted to confirm previous studies from L-type channel recordings that showed little or no voltage dependence of permeation at voltages < 0 mV,\textsuperscript{2,6,21} but also to investigate permeation at voltages > 0 mV. We recorded unitary calcium channel currents in BayK-8644 (1 μM), which increases L-channel open times so that tail openings could be observed at hyperpolarized voltages (Fig. 2A), without impacting permeation.\textsuperscript{22} **Figure 2B** shows dose-response curves for L-channels at different voltages. These current-concentration relationships could only be fit by a double mass-action equation, while the N-channel data was consistently well fit by a single mass-action equation for both whole-cell\textsuperscript{10} and single channel data (Fig. 1). Such behavior was previously observed in single L-channel recordings by Yue and Marban,\textsuperscript{23} which led them to conclude that the channel had at least 3 Ca$^{2+}$-binding sites within the pore: 2 low affinity sites saturated by millimolar concentrations of Ca$^{2+}$ or Ba$^{2+}$, and one high affinity site being saturated by micromolar concentrations. We found that the smaller of the 2 EC$_{50}$ values (generated from double mass-action equation fitting,
Fig. 2B) at each voltage was close to values obtained for N-channels and those values were used for comparison between voltages. We found little or no change at voltages < 0 mV but a notable increase in EC50 with voltages > 0 mV (Fig. 2B, Table 1). This evaluation lends support to the hypothesis that L-channel permeation is voltage-dependent, but the effect of voltage is only detectable at voltages > 0 mV. The effect of voltage on the second component of the current-concentration relationship was difficult to estimate because the EC50s were large (68–187 mM) and near the maximum Ba2+ concentration used in this study, but no voltage-dependence was noted (see Fig. 2 legend).

**Discussion**

We previously utilized whole-cell recording and computer modeling to
investigate permeation of N-type CaV channels. These recordings determined the Ca2⁺ dose-response relationship for permeation at different voltages, and investigated the Ca2⁺-Ba2⁺ anomalous mole fraction effect (AMFE; smaller currents in Ca2⁺-Ba2⁺ mixtures) to show a reduced affinity of N-channels for both Ca2⁺ and Ba2⁺ as the voltage was depolarized from 0 mV. However, this voltage dependence of permeation was not observed at voltages < 0 mV. Here we extended our results by using single channel recordings to further support the voltage-dependence of N-channel permeation at voltages > 0 mV. We were also able to observe a similar voltage-dependence in L-type channels. Thus, voltage-dependent permeation appears to be an attribute of both CaV1 and CaV2 channels. Interestingly, voltage does not appear to affect permeation through CaV3 (T-type) channels.

Voltage dependence of N-channel permeation

One problem with our whole-cell data that we noted previously was current isolation. When recorded in 2–3 mM Ba2⁺, the CaV current in bullfrog sympathetic neurons is comprised of >90% N-type and 10% L-type current, but the N-current contribution drops to >75% in 3 mM Ca2⁺. This reduction results from a CaV current that we call Ef-current, which becomes a measurable component of the whole-cell current in Ca2⁺ and at high divalent cation concentrations. Thus, the fraction of N-type current will decrease as Ca2⁺ and Ba2⁺ concentrations increase. We previously estimated that the largest impact of Ef-current would be 25% of the total CaV current, so that N-type current would dominate at all Ca2⁺ and Ba2⁺ concentrations. Thus, our observations reflected N-channel activity.

With our single channel data, we were able to test this assumption and we demonstrated the same voltage-dependence of permeation. One unexpected finding was that the EC50 values for the single channel and whole-cell data were nearly identical (e.g. 13 mM at 0 mV, Table 1). This was surprising because the whole-cell data were recorded using Ca2⁺, while the single channel data utilized Ba2⁺, and many studies have established that Ba2⁺ has a lower affinity for the channel relative to Ca2⁺ (25 mM vs. 13 mM). Furthermore, the EC50 value at each voltage was almost identical to that measured from our whole-cell recordings in Ca2⁺ (Table 1). We have not determined the reason for this unexpected similarity, but there are several possibilities. First, the Ef-current could have impacted the whole-cell recordings more than we had assumed, which may have increased the EC50 values for Ca2⁺. Second,
Voltage control can become difficult for large whole-cell currents, and it is possible that membrane voltage control was compromised for the large CaV currents at high divalent cation concentrations, even though precautions were taken to prevent such a problem.\(^{10,25}\) Regardless of the reason for the similar EC\(_{50}\) values, all of the techniques that we have employed, including whole-cell dose-response, whole-cell AMFE and single channel dose-response measurements, converge to support strong voltage control of N-channel permeation at voltages \(> 0 \text{ mV}\), but not at more hyperpolarized voltages.

**Voltage Dependence of L-Channel Permeation**

The voltage dependence of L-type channel permeation was previously examined at voltages \(< 0 \text{ mV}\), where little or no effect was measured.\(^6\) However, Friel and Tsien showed an effect of voltage on L-channel AMFE, which supported an effect of voltage on permeation at depolarized voltages.\(^9\) Our single L-channel results reconcile these 2 observations. We found no difference in the permeation EC\(_{50}\) from \(-40 \text{ mV}\) (8 mM) to 0 mV (10 mM), but the EC\(_{50}\) increased to 25 mM at +20 mV. Indeed, the EC\(_{50}\) values were remarkably similar to those from N-channels for the same voltages (Table 1). Thus, permeation through both L-type and N-type channels is similar, which is expected based on high amino acid sequence homology in the pore-forming region (S5, S6 and P-loop) of these 2 channels.\(^{26}\)

However, there was one difference between N-type and L-type channels, which was the 2 components required to fit L-channel current-concentration curves. The higher affinity component matched that of N-type channels as discussed above, but the lower-affinity component apparently lacked voltage dependence. However, the apparent affinities of the lower affinity component (68–187 mM) were at the edge of the concentration range that could be tested, so they were poorly described by our data. A second component to the dose-response relationship was also observed for L-channels by Yue and Marban.\(^{23}\)

Thus, we cautiously conclude that this represents a low-affinity site that lies outside the transmembrane electric field. One clue that supports this idea is that the second component of the L-channel dose-response relationship appears to be reduced at depolarized voltages. One explanation is that the affinity of the voltage-dependent site(s) becomes close to that of the putative voltage-independent site, so that a single binding site equation can reproduce the data.

### The Physiological Impact of Voltage-Dependent Permeation

Ca\(^{2+}\) is critical for many action potential-triggered cellular functions, such as neurotransmitter release\(^{27}\) and muscle contraction,\(^{26}\) that require Ca\(^{2+}\) delivery over a wide range of voltages. We previously discussed how the impact of voltage-dependent Ca\(^{2+}\) permeation helps to maximize Ca\(^{2+}\) influx through N-type channels\(^{10}\) when the electrochemical gradient (driving force) is unfavorable. The reduced Ca\(^{2+}\) affinity at depolarized voltages decreases the dwell time of Ca\(^{2+}\) within the channel to increase permeation relative to other ions. Whereas the absence of increased affinities with voltages \(< 0 \text{ mV}\) prevents Ca\(^{2+}\) from becoming a channel blocker at hyperpolarized voltages.\(^{10}\)

We also previously speculated that if L-channel permeation was similarly voltage dependent, this mechanism could be important for providing Ca\(^{2+}\) for cardiac contractions.\(^{10}\) Our finding that single L-channel permeation is voltage-dependent at depolarized voltages supports such a mechanism. It is likely that voltage-dependent permeation, along with nanodomain-restricted Ca\(^{2+}\) diffusion,\(^{28}\) helps maintain intracellular Ca\(^{2+}\) levels during the plateau phase of the cardiac action potential to sustain the cardiac contraction.

### Methods

#### Single channel recording

These methods have been previously published.\(^{16,18,19}\) The pipette solutions are listed in Table 2. The pH (7.0–7.2) was adjusted using NMG-base, and the osmolarity was \(\sim 280 \text{ mOsm}\) (adjusted using TEA-Cl). The exception was 300 mM Ba\(^{2+}\), which had osmolarity \(= 670 \text{ mOsm}\). Voltages were not corrected for possible junction potential effects. The exclusion of Na\(^+\) prevented currents through voltage-dependent sodium channels, while the TEA and Ba\(^{2+}\) effectively blocked outward potassium currents.

The bath solution contained (in mM) 100 KCl, 10 K-HEPES and 5 NMG-EGTA, and was adjusted to pH 7.2 with NMG base. This isotonic K\(^+\) solution zeroed the membrane potential, allowing precise voltage control of the isolated patch.\(^{18}\)

To overcome the problem of high calcium channel density we used high resistance electrodes. Since we were only interested in current amplitude, multiple channels in a patch were not a problem, as long as the patch contained either N- or L-channels. To facilitate the identification of L-channels, 1 \(\mu\text{M} \pm \text{BayK-8644}\) was added to the pipette solution.\(^{16,17}\) The holding potential was typically –40 mV to inactivate E\(_p\)-channels.\(^{16}\) Occasionally, N- or L-channel patches were held at –80 mV, but active E\(_p\)-channels could easily be distinguished by their smaller unitary current amplitude, lower activation voltage, and inactivation at HP –40 mV.

Channel identification was carried out during the first 5 min of recording using 100 ms voltage steps to 0 and +40 mV in blocks of 100 sweeps (2 sec interval). The number of channels in a patch was determined from superimposed openings at +40 mV where the open probability is high (0.8–0.9 for N-channel \(^{18}\)). Patches having both N- and L-channel types were not used, since their current amplitude cannot be easily distinguished.\(^{16}\) Currents were filtered at 2 kHz before digitization at 10 kHz.

| \([\text{Ba}^{2+}]_0 \text{(mM)}\) | 3 | 10 | 30 | 100 | 300 |
|-----------------|---|----|----|-----|-----|
| NMG-HEPES (mM) | 10 | 10 | 10 | 10  | 10  |
| TEA-Cl (mM)    | 128| 107.5 | 77.5 | 10  | —   |
Single channel analysis

All single channel data were analyzed using routines written in IgorPro (WaveMetrics, Lake Oswego, OR). Capacitative and leak currents were removed from records by subtracting the average of sweeps without channel activity (null sweeps). If null sweeps were rare or absent, one was made by averaging sweeps after active regions had been blanked.18,19

The amplitude of unitary currents was determined using mean-low variance analysis16,29,30 as follows. The mean current was calculated using mean-low variance levels.18,19

Each component from the amplitude histogram was fit with a Gaussian equation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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