Cations Residing at the Selectivity Filter of the Voltage-Gated Ca\textsuperscript{2+} -Channel Modify Fusion-Pore Kinetics

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ABSTRACT

Strontium (Sr\textsuperscript{2+}), Barium (Ba\textsuperscript{2+}) and Lanthanum (La\textsuperscript{3+}) can substitute for Ca\textsuperscript{2+} in driving synaptic transmission during membrane depolarization. Ion recognition at the polyglutamate motif (EEE), comprising the channel selectivity-filter, during voltage-driven transitions, controls the kinetics of the voltage-gated calcium channel (VGCC) and its interactions with the synaptic proteins. We tested the effect of different charge carriers on evoked-release, as a means of exploring the involvement of VGCC in the fusion pore configuration. Employing amperometry recordings in single bovine chromaffin cells we show that the size of the fusion pore, designated by the ‘foot’-amplitude, was increased when Ba\textsuperscript{2+} substituted for Ca\textsuperscript{2+} and decreased, with La\textsuperscript{3+}. The fusion pore stability, indicated by ‘foot’-width, was decreased in La\textsuperscript{3+}. Also, the mean open time of the fusion pore (\(t_{\text{open}}\)) was significantly lower in Sr\textsuperscript{2+} and La\textsuperscript{3+} compared to Ba\textsuperscript{2+} and Ca\textsuperscript{2+}. These cations when occupying the selectivity filter reduced the spike frequency in the order of Ca\textsuperscript{2+} > Sr\textsuperscript{2+} > Ba\textsuperscript{2+} > La\textsuperscript{3+}, which is parallel to the reduction in total catecholamine release. The correlation between ion binding at the selectivity filter and fusion pore properties supports a model in which the Ca\textsuperscript{2+} channel regulates secretion through a site at the selectivity filter, upstream to cation entry into the cell.

INTRODUCTION

Voltage-gated ion channels propagate electrical signaling by responding to changes in membrane potential. Analogous to the Kv channel,\textsuperscript{1} the functional channel complex of VGCC is believed to be comprised of a single pore domain formed by the S5–S6 regions from four subunits, and four surrounding voltage-sensing domains, each one formed by the S1–S4 segments from a single subunit. The presence of four consecutive negatively charged amino acids, the EEEE locus, at the VGCC cavity is highly conserved throughout the VGCC family, and is considered to underline the basis of VGCC selectivity.\textsuperscript{2} The pore domain containing the conduction pathway for calcium ions is believed to have two Ca\textsuperscript{2+} binding sites, a high and low affinity site. The high affinity site blocks entry of Na\textsuperscript{+} and the flow of ions in the closed state\textsuperscript{2} and the low affinity site generated by voltage-dependent pore transitions is responsible for the Ca\textsuperscript{2+} current.

A depolarizing signal transforms the channel to an ion conducting molecule as atoms of the EEEE site make a direct contact with bound cations, recently shown for the K channel.\textsuperscript{3} Ca\textsuperscript{2+}, Sr\textsuperscript{2+} and Ba\textsuperscript{2+} are conducted through the pore and the differences in ion-sizes affect the binding and conductivity of the filter.

Heterologous expression studies have shown that the kinetics of VGCC,\textsuperscript{4–9} Ca\textsubscript{v}1.2 (L-type), Ca\textsubscript{v}2.2 (N-type),\textsuperscript{7} Ca\textsubscript{v}2.3 (R-type)\textsuperscript{8} and Ca\textsubscript{v}2.1 (P/Q)\textsuperscript{5,9} are altered by various synaptic proteins such as syntaxin 1A, SNAP-25, and synaptotagmin. The VGCC interaction with syntaxin 1A is further modified when Sr\textsuperscript{2+} or Ba\textsuperscript{2+} is substituted for Ca\textsuperscript{2+} (refs. 9 and 10) indicating a linkage between the exocytotic machinery and the selectivity filter of the channel. Further support for this linkage was demonstrated by conferring a prolonged ‘releasing state’ upon the channel when the non-permeable La\textsuperscript{3+} was substituted for Ca\textsuperscript{2+}, producing amperometric currents (spikes) and pre-spike currents (‘foot’).\textsuperscript{11} The differences observed in the ‘foot’ parameters with La\textsuperscript{3+} compared to Ca\textsuperscript{2+}, indicate an intimate affiliation between the interaction of the resident ion at the VGCC cavity and the fusion pore configuration. The VGCC was therefore, proposed a likely candidate as a structural constituent of the fusion pore.\textsuperscript{11}

Based on modulation of ‘foot’ parameters, both synaptotagmin and syntaxin 1A have been suggested to be associated with the fusion pore configuration.\textsuperscript{12–15} To address the
The possibility that the calcium channel could be a constituent of the fusion pore, we examined the relationship between the kinetic properties of the fusion pore and the voltage-driven transitions at the channel cavity. Spikes and pre-spikes were triggered by 60 mM KCl and were measured by substituting Sr²⁺, Ba²⁺ or La³⁺ for Ca²⁺ in single chromaffin cells.

Differences were observed primarily in 'foot' amplitude, indicating different pore size, and in 'foot'-width, designating changes in 'foot' stability. Our results suggest a direct link between fusion pore openings and resident ion-interaction at the VGCC cavity. We propose that distinct structural transitions at the cavity control exocytosis dynamics and trigger the early stages of the fusion pore opening. Voltage-driven changes at the channel cavity during cation occupancy are directly transmitted to the exocytotic protein complex to mediate exocytosis. These results support the view that the channel is intimately associated with the fusion pore structure. The results favor a model of a proteinacious structure for the fusion pore, and offer a unique opportunity for gaining a deeper understanding of the basis for VGCC coupling to exocytosis.

**MATERIALS AND METHODS**

Chromaffin cell preparation and culture. Bovine adrenal glands were obtained at a local slaughterhouse. The adrenal medulla cells were isolated (previously described in ref. 11), cultured and plated at a density of 5 x 10⁴ cells/cm² on glass cover slips placed in 35 mm plates, in DMEM (Gibco) supplemented with ITS-X (Sigma, Jerusalem, Israel). Cells were incubated at 37°C in 5% CO₂ and used for 5–14 days.

Amperometric recordings of catecholamine release from chromaffin cell. Amperometry recordings were carried out using 5μm thin carbon fiber electrodes (CFS A La Incorp, Westbury, NY, USA) and a VA-10 amplifier (NPI-electronic, Tamm, Germany) held at 800 mV (previously described in ref. 16). Cells were rinsed 3–4 times prior to the experiment and bathed during the recordings at 23°C in iso-osmotic physiological solution (149 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Glucose, 10 mM HEPES, pH 7.3 (adjusted with NaOH). Individual cells were stimulated to release by a 30 sec application of iso-osmotic 60 mM KCl buffer from a 3-μm tipped micropipette placed 30 μm from the cell in the bath. Amperometric currents were sampled at 10 kHz, using clampex 9.0 (Axon Instruments) and low pass filtered at 0.2kHz.

Amperometric data acquisition and analysis. Amperometry records were analyzed with IGOR PRO (WaveMetrics, Lake Oswego, OR, USA) to extract 'foot' information according to the criteria of Chow et al.16 “Feet” were analyzed for spikes > 10 pA (peak-to-peak noise was ≤2 pA). The beginning of the ‘foot’ was defined as the time when the signal exceeds the noise. The end of the ‘foot’ was defined as the inflection point between the ‘foot’ and the fast rise of the spike. ‘Foot’ width corresponds to the lifetime of an open state (O) of a dynamic fusion pore,17 governed by the rate constants for dilation (D) and closure (C), (kd and kc; ref. 18):

\[ k_c \rightarrow C \rightarrow k_e \rightarrow O \rightarrow k_d \rightarrow D \]

Mean fusion pore open time (τ_Fp) was obtained by fitting a single exponential to the ‘foot’ widths’ distribution of each group computed (as described by Wang15).

Rates of secretion were determined for individual cells and averaged. The initial (10–40 sec) and sustained (60–180 sec) rates of secretion were determined from the initial and sustained slopes of the corresponding cumulative spike plots.

Data was analyzed as described in the text and figure legends. Error bars give standard errors. Spikes exceeding three times the background noise (>10 pA) were analyzed. All peaks identified by the program were inspected visually and bad signals were excluded manually.

Ca²⁺ measurements. Ca²⁺ imaging employed the fluorescent dye, fura-2 (fura-2 AM), and an imaging system, a Zeiss Axiovert 35 microscope coupled to a Till photonics system. Cells were incubated with 3 μM of acetoxymethyl ester form of fura-2 AM for 30 min at 37°C and then washed X3 with iso-osmotic solution. Intracellular fura-2 bound to Ca²⁺ was excited at 350 nm while free fura-2 was excited at 380 nm. Emission was monitored at 510 nm using a digital camera PCD Sensicam CCD; oil lens FLUAR 40X NA 1.3 and analyzed by the Image J software. The ratio of the emission with 350 nm and 380 nm excitation was determined. On each cover slip, 1–4 chromaffin cells were selected and individually imaged. Image pairs (one at 350 nm and 380 nm) were obtained every sec for 300 sec. KCl (60mM) was added 30 sec after fluorescence monitoring began. Backgrounds were subtracted from the individual wavelengths, and the 350 nm image was divided by the 380 nm image to provide a ratiometric image.

**RESULTS**

Amperometric spikes elicited during membrane depolarization in Ca²⁺, Sr²⁺ and La³⁺. To explore cations effects on secretion on catecholamine secretion, chromaffin cells were depolarized and release

| Cation | Cells (n) | Spikes (n) | Amplitude (pA) | Half Width (ms) | Rise Time (ms) | Charge (pC) |
|--------|----------|-----------|---------------|----------------|---------------|------------|
| Ca²⁺   | 37       | 916       | 35.8 ± 2.3    | 24.4 ± 1.3     | 3.9 ± 0.2     | 1.0 ± 0.1  |
| Sr²⁺   | 32       | 515       | 32.6 ± 2.3    | 22.4 ± 1.3     | 3.6 ± 0.2     | 0.9 ± 0.1  |
| Ba²⁺   | 30       | 573       | 86.6 ± 8.5 ***| 13.6 ± 1.0 ***| 2.4 ± 0.2 ***| 1.2 ± 0.1  |
| La³⁺   | 30       | 524       | 31.5 ± 3      | 17.7 ± 0.9 ***| 3.2 ± 0.1 **  | 0.6 ± 0.1 ***|

**p < 0.01; ***p < 0.001.
The Ca\textsuperscript{2+} Channel as a Constituent of the Fusion Pore

When an amperometry electrode was positioned at the surface of a chromaffin cell, catecholamine secretion of a single vesicle registered as a spike of current caused by the oxidation of catecholamine at the electrode surface. Cells were stimulated by 60 mM KCl (n = 30–37) applied in a physiological solution containing 2 mM Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, or 0.2 mM La\textsuperscript{3+} for 30 sec and produced amperometric currents (spikes), within 5–40 sec from stimuli application shown by representative traces (Table 1; Fig. 1A). 10-sec control recording of spontaneous release was performed for each cell prior to stimulation (Fig. 1). A physiological solution lacking 60 mM KCl elicited no amperometric currents. The peak amplitude, half-width, 50–90% rise-time, and mean charge of single amperometric events elicited by 60 mM K\textsuperscript{+} were quantified. For every parameter the values from each cell were averaged and presented as the mean of cell averages ± SEM for each group, assigning the same weight to each cell, regardless of the total number of spikes (Table 1; ref. 20). Spike parameters observed with Ca\textsuperscript{2+} or Sr\textsuperscript{2+} displayed similar characteristics (Fig. 1; Table 1). Substitution of Ba\textsuperscript{2+} for Ca\textsuperscript{2+} led to an increase in peak amplitude (>2 fold), accompanied by a decrease in spike half-width (13.6 ± 1 ms p < 0.001). This value was significantly shorter than in Ca\textsuperscript{2+} (24.4 ± 1.3 ms) or Sr\textsuperscript{2+} (22.4 ± 1.3 ms) or even in La\textsuperscript{3+} (17.7 ± 0.9 ms; p < 0.001). Rise time (50–90%) was also significantly faster (Table 1; Fig. 1A). The amperometric spikes in La\textsuperscript{3+}, a non-permeable trivalent cation, displayed peak amplitudes similar to Ca\textsuperscript{2+} or Sr\textsuperscript{2+}, exhibiting a slightly smaller half-width (Fig. 1C). Rise-time 50–90% was faster (3.2 ± 0.1 ms) compared to Ca\textsuperscript{2+} (3.9 ± 0.2 ms; p < 0.01), indicating a more rapid development of the amperometric current (Fig. 1D) and mean charge value was smaller (Fig. 1E). In previous studies from this laboratory\textsuperscript{11} the peak amplitude in La\textsuperscript{3+} was lower as a result of analyzing a significantly smaller population of cells and amperometric events (Table 1). A large number of metal ions including Cd\textsuperscript{2+}, Mn\textsuperscript{2+}, Co\textsuperscript{2+} but not Mg\textsuperscript{2+} elicited catecholamine release in PC12 cells when introduced intracellularly in a caged form.\textsuperscript{21} It should be mentioned that Mg\textsuperscript{2+} has a rather low affinity for the selectivity filter of the channel, was present in all the experiments.

Cation permeation through Ca\textsubscript{V1.2} detected by Fura-2 imaging. Permeability of the cations was established in chromaffin cells using
Figure 2. Increase in fura 2 imaging during membrane depolarization in Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, and La\textsuperscript{3+}. Cells had been loaded for 30 min with 3 μM fura-2AM washed extensively and stimulated with 60 mM KCl (arrow) in 2 mM Ca\textsuperscript{2+}, 2 mM Sr\textsuperscript{2+}, 2 mM Ba\textsuperscript{2+}, and 0.2 mM La\textsuperscript{3+}, solutions as indicated. Inset, cells stimulated with 60mM KCl in cation-free solution. Results are expressed as the ratio of fluorescence at 350/380nm. Traces are representative of 2–4 single cells present in the same microscope field. In La\textsuperscript{3+} there was no detectable change in 350/380 ratio, in spite high sensitivity towards fura 2 (Kd= 27pM).

fura 2-fluorescence. Cells loaded with fura 2 were depolarized with 60mM KCl and changes in intracellular Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, and La\textsuperscript{3+} were determined. Fluorescence ratio (350/380 nm) showed a rise in intracellular Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, and Ba\textsuperscript{2+} (Fig. 2). Fluorescence was measured in a control solution in the absence of divalent cations (inset). In spite of the high affinity of fura 2 to free La\textsuperscript{3+} (Kd = 27pM), that allows detection of concentrations 10,000 lower than Ca\textsuperscript{2+} (Kd = 0.2 μM), no change in fluorescence ratio was observed during membrane depolarization in La\textsuperscript{3+} solution. These results further confirm La\textsuperscript{3+} exclusion from entry into chromaffin cell, during depolarization consistent with previous studies.\textsuperscript{11,22}

‘Foot’ parameters are affected by the charge carrier cations. In chromaffin cells, amperometric spikes recorded during membrane depolarization are often preceded by a pre-spike current event (‘foot’) corresponding to the open state of the fusion pore.\textsuperscript{16} Feet were seen in association with most of the spikes elicited by 60 mM KCl. The percentage of spikes without ‘foot’ in Ca\textsuperscript{2+} was 17.8%, 14.4% in Sr\textsuperscript{2+}, 13.7% in Ba\textsuperscript{2+} and 20% La\textsuperscript{3+}. Interestingly, spikes elicited by means other than VGCC activation, displayed a significantly larger number of “footless” spikes.\textsuperscript{23,24}

‘Foot’ amplitude relates directly to the size of the fusion pore while ‘foot’-width indicates its stability (Fig. 3A–C). These kinetic parameters were quantified and analyzed in a complementary approach, revealing significant differences caused by substituting Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, or La\textsuperscript{3+} for Ca\textsuperscript{2+} (Fig. 3; Table 2).

‘Foot’ amplitudes were similar with Ca\textsuperscript{2+} or Sr\textsuperscript{2+}, 5.4 ± 0.5 and 5.5 ± 0.6 pA, respectively, but were > 2-fold higher in Ba\textsuperscript{2+} (12.6 ± 1.3 pA; p < 0.001), and smaller in La\textsuperscript{3+} (3.8 ± 0.4 pA p < 0.05; Fig. 3A). These differences provide a quantitative index of the fusion pore size, which is correlated with the ionic radius that affects energy of hydration and the charge of the cations residing at the selectivity filter of the channel.

The mean ‘foot’ width or ‘foot’ duration were similar in Ca\textsuperscript{2+}, Sr\textsuperscript{2+} and Ba\textsuperscript{2+} (11.2 ± 0.9, 10.2 ± 0.7 and 12.4 ± 1.1 ms, respectively), and significantly smaller in 0.2 mM La\textsuperscript{3+} (6.6 ± 0.3 ms; p < 0.001; Fig. 3B; Table 2).

Mean ‘foot’ charge was decreased in the order of Ba\textsuperscript{2+} > Ca\textsuperscript{2+} > Sr\textsuperscript{2+} > La\textsuperscript{3+} (67 ± 9.5; 52.8 ± 8.3; 29.2 ± 4.2; and 15 ± 1.3 fC; Fig. 3C; Table 2).

‘Foot’ width corresponds to the lifetime of an open state (O) of a dynamic fusion pore (ref. 20; see Experimental Procedures) and the lifetime distribution can be described by a single exponent. The mean open-time of the fusion pore (t\textsubscript{O}) obtained by fitting a single exponential to the ‘foot’ widths’ distribution of each group, was computed (previously described in ref. 15).

As shown in Table 2, t\textsubscript{O} values of Ca\textsuperscript{2+} and Ba\textsuperscript{2+} were similar, while those observed for Sr\textsuperscript{2+} and La\textsuperscript{3+}, were significantly smaller (Fig. 3C; Table 2). Smaller t\textsubscript{O} value indicate a faster rate of dilation, appeared to be affected by the cation residing at the selectivity filter.

The cation bound at the channel cavity and the ensuing channel conformation seem to be directly responsible for modifying the kinetic properties of the fusion pore, strongly supporting the likelihood of the channel being part of the fusion pore configuration.

An alternative possibility that kinetics of the fusion pore were affected by the cation bound to a putative cytosolic Ca\textsuperscript{2+} sensor protein was explored using Ca\textsuperscript{2+} ionophore. The cells were stimulated by 10 μM ionomycin applied to the cell for 30 sec in 2 mM Ca\textsuperscript{2+} (23 cells; 309 spikes) or 2 mM Sr\textsuperscript{2+} (55 cells; 301 spikes) in the presence of 5 μM nifedipine. The kinetic parameters were virtually identical. Foot amplitude in Ca\textsuperscript{2+} was 3.8 ± 0.4 and 4.16 ± 0.3 pA in Sr\textsuperscript{2+}; foot width, 9.9 ± 0.7 and 9.8 ± 0.7 ms, and foot area 26.4 ± 4.1 and 27.6 ± 4.2 pC. The mean open time of the fusion pore (t\textsubscript{fp})
The Ca$_2^+$ Channel as a Constituent of the Fusion Pore

was 8.0 ± 0.3 and 7.9 ± 0.2 ms, in Ca$_2^+$ and Sr$_2^+$, respectively. The lack of sensitivity to the type of intracellular ion stands in marked contrast to the effect of ion exchange within the selectivity filter of the channel.

The frequency of catecholamine secretion in chromaffin cells. The overall time course of secretion was determined from normalized waiting time distributions constructed by spike counting, in Ca$_2^+$ (number of cells; n = 37); Sr$_2^+$ (n = 32), Ba$_2^+$ (n = 30), and La$_3^+$ (n = 30). Two-phase slopes were observed, an initial slope during the 30 sec stimulation representing the initial secretion, followed by an apparent sustained slope, ranging from 60 to 180 sec. Rates of secretion were determined for individual cells and then averaging over cells. The slopes of the corresponding cumulative spike plots represent the initial and sustained rates of secretion (Fig. 4). The initial rate was highest in Ca$_2^+$ (0.21 ± 0.002 spike/sec), slightly higher in Sr$_2^+$ (0.16 ± 0.003 spike/sec) and slower in Ba$_2^+$ (0.11 ± 0.002 spike/sec). The initial rate of secretion in La$_3^+$ was significantly lower >1/10 of Ca$_2^+$ (0.02 ± 0.001 spike/sec) (Fig. 4 A and B).

After the initial rate, spike frequency was lowered and the cumulative slopes provided an estimate for the apparent sustained rate of secretion. While the sustained rates for Ca$_2^+$, Sr$_2^+$ and Ba$_2^+$ (Fig. 4C–F) were lower compared to initial rate, in La$_3^+$, the sustained rate of secretion was increased >3-fold (0.06 ± 0.001 spike/sec; Table 3).

Efficacy of catecholamine secretion. The observation of two distinct phases of spike frequency, led us to evaluate the efficacy of 60 K$^+$-induced release at different time points following a 30 sec stimulation period. Two different parameters were calculated: the sum of total mean charge (TMC) of the spikes over time at three time intervals 10–40 sec, 40–100 sec, and 100–180 sec, and the total mean charge of 'feet'. When combined these values designate total amount of catecholamine released. As shown in Figure 5, (see also Table 4) during the first 30 sec time period (between 10–40sec), TMC was highest with Ca$_2^+$ (10.0 ± 1.5 pC), followed by Sr$_2^+$ (7.9 ± 0.9 pC), Ba$_2^+$ (5.9 ± 1.1 pC), and more than 30 fold lower in La$_3^+$ (0.3 ± 0.09 pC). Over time, at the 40-100 sec and 100-180 sec time periods, total secretion remained constant in Ca$_2^+$, with slight changes in Sr$_2^+$. In contrast, total mean charge was >2-fold larger in Ba$_2^+$ during the following 40–100 sec and 100-180 sec time periods, showing a certain tendency towards a steady rate of secretion.
The Ca\(^{2+}\) Channel as a Constituent of the Fusion Pore

Table 3: Effects of cations bound at the selectivity filter of the VGCC on spike frequency elicited by 60 mM KCl in bovine chromaffin cells

| Cation | Cells (n) | Initial Rate (10–40 sec) | Sustained Rate (60–180 sec) |
|--------|-----------|--------------------------|-----------------------------|
| Ca\(^{2+}\) | 37        | 0.21 ± 0.002             | 0.11 ± 0.001                |
| Sr\(^{2+}\) | 32        | 0.16 ± 0.003             | 0.06 ± 0.001                |
| Ba\(^{2+}\) | 30        | 0.11 ± 0.002             | 0.09 ± 0.001                |
| La\(^{3+}\) | 30        | 0.02 ± 0.001             | 0.06 ± 0.0004               |

DISCUSSION

VGCC are physically and functionally coupled to synaptic proteins, leading to a bidirectional crosstalk between VGCC and the exocytotic process. The present results which are aimed at understanding the role of the Ca\(^{2+}\) channel in secretion, show significant alterations in ‘foot’ current kinetics upon substituting Sr\(^{2+}\), Ba\(^{2+}\), or La\(^{3+}\) for Ca\(^{2+}\). They establish a linkage between the cations that are in contact with atoms of the selectivity filter, and the kinetics of secretion, raising the possibility that the VGCC is a constituent of the fusion pore.

Once vesicle fusion occurs with the pre-synaptic membrane, the continuation of the fusion process is mainly regulated by factors unrelated to its initiation. Since amperometric spikes represent the collapse of single vesicles, substituting the charge carrier would not be expected to lead to large changes in spikes kinetics. Indeed, spike parameters, peak-amplitude, half-width, rise-time (50–90%) and the mean charge were not affected when Sr\(^{2+}\) was substituted for Ca\(^{2+}\) and only small differences were observed when La\(^{3+}\) was substituted for Ca\(^{2+}\). A larger mean peak current, a faster rise-time, and a smaller half-width were observed when Ba\(^{2+}\) was substituted for Ca\(^{2+}\). Further studies are needed to explain these effects, which could be related to the ionic radius, matrix dissolution, or Ba\(^{2+}\) binding to other channels expressed at the cell membrane.
The type of cation bound at the selectivity filter affects the fusion pore stability and size. More relevant to exocytic mechanism were the pre-spike (‘foot’) parameters, portraying fusion pore kinetics. The kinetics of the pore stability is controlled by two opposing steps $k_{off}$ that closes the pore and a mechanism that opens the pore $K_{15}$ (see Experimental Procedures).

‘Foot’ analysis showed distinct changes in the size and stability of the fusion pore when either La$^{3+}$ or Ba$^{2+}$ was substituted for Ca$^{2+}$. These changes are closely correlated to the ionic radius and charge of the cation. ‘Foot’ stability and ‘foot’ size in Ca$^{2+}$ (ionic radius of 0.99 Å) and Sr$^{2+}$ (ionic radius of 1.24 Å) were similar, while the mean ‘foot’-charge was significantly lower with Sr$^{2+}$. Ba$^{2+}$ had a more pronounced effect. It is well established that although Ba$^{2+}$ and Ca$^{2+}$ carry the same charge, the ionic radius of Ba$^{2+}$ (1.43 Å) is 36% larger, lowering its affinity for the EEEE locus, thereby increasing its conductance.30 This leads to significant alterations within the selectivity filter, and in our studies, to more than a 2-fold increase in the fusion-pore size (see schematic model in Fig. 7).

Extracellular effects of ions on the fusion pore kinetics. Differences in fusion pore kinetics could result from the extracellular ion binding at the selectivity filter of the channel, from intracellular binding of ions to Ca$^{2+}$-binding proteins, or from a direct interaction with the fusion pore.

Experiments with La$^{3+}$, with an almost identical ionic radius (1.061 Å) to Ca$^{2+}$ and a considerable higher affinity for the negatively charged EEEE locus,31-33 enabled us to distinguish between extracellular and intracellular cation effects.

We showed that occupancy of the selectivity filter, without permeation, altered the fusion pore kinetics. A shorter ‘foot’-width and a smaller ‘foot’-amplitude in La$^{3+}$, revealed an unstable, and a small-size fusion pore. The overall reduction in ‘foot’ charge was > 50%.

These changes can be explained by assuming that the calcium channel is an integral part of the transmitter release site, either proximal to it or linked directly to it, perhaps via interaction with syntaxin 1A.12,34 In addition, these results imply that for the formation of a ‘releasing’ structure, ion binding at the selectivity filter is sufficient. It allows the channel to control vesicle fusion by adjusting the size and stability of the ‘foot’.

These results highlight that the important link between occupancy at the channel cavity and the onset of secretion is independent of rise in intracellular cation.

Intracellular effects of ions on the fusion pore kinetics. In the present study we emphasize mainly the role of the Ca$^{2+}$ channel as a potential constituent of the fusion pore.

An alternative site of ion action would be near the site of entry, where ions accumulate and control the fusion pore from within. A commonly proposed locus for the initial site of Ca$^{2+}$ binding and action is at the C2 domains of synaptotagmin.15 Previously, the rate of fusion pore dilation was reported to be Ca$^{2+}$-dependent. A shortening of the latency period between fusion and release

Table 4  Effects of cations bound at the selectivity filter of the VGCC on total mean charge of spikes and ‘foot’ currents elicited by 60 mM KCl in bovine chromaffin cells

| Cation  | 10–40 sec | 40–100 sec | 100–180 sec | 30 sec | ‘Foot’ 90 sec | 180 sec |
|---------|-----------|-----------|-----------|-------|-------------|--------|
| Ca$^{2+}$ | 10.0 ± 1.5 | 12.0 ± 1.6 | 11.0 ± 2.8 | 0.51 ± 0.2 | 0.95 ± 0.2 | 1.2 ± 0.2 |
| Sr$^{2+}$ | 7.9 ± 0.9 | 11.3 ± 2.0 | 7.4 ± 1.4 | 0.12 ± 0.02 * | 0.23 ± 0.03 ** | 0.3 ± 0.04 *** |
| Ba$^{2+}$ | 5.9 ± 1.1 * | 13.5 ± 3.2 | 13.9 ± 2.6 | 0.14 ± 0.04 | 0.57 ± 0.1 | 0.86 ± 0.2 |
| La$^{2+}$ | 0.3 ± 0.09 *** | 2.7 ± 0.6 *** | 4.8 ± 0.9 *** | 0.005 ± 0.002 ** | 0.05 ± 0.01 *** | 0.14 ± 0.03 *** |

*p < 0.05; **p < 0.01; ***p < 0.001. The number of cells used for the analysis is as indicated in Table 3.
The Ca\textsuperscript{2+} Channel as a Constituent of the Fusion Pore

Figure 6. Sum of total mean charge of ‘feet’ (TMC) during 30, 90, and 180 sec. The sum of total mean 'foot' charge triggered by 60 mM KCl was calculated by the summation of 'foot' areas for 30 sec (left) 90 sec (middle) and 180 sec (right) in Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+} and La\textsuperscript{3+}. The gradual increase in total release from the fusion pore prior to dilation for the different cations is shown (inset). *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 7. Schematic model correlating fusion pore stability and size to ionic radius of cations residing at the pore of the channel. Averaged 'foot' parameters, 'foot'-amplitude and 'foot'-width, observed during single-vesicle release triggered by depolarization with high KCl (Top). 'Foot'-width that reflects the stability of the fusion pore was similar for Ca\textsuperscript{2+} and Sr\textsuperscript{2+} was longer in Ba\textsuperscript{2+} and shorter in La\textsuperscript{3+}. 'Foot'-amplitude that reflects the size of the fusion pore was the largest with Ba\textsuperscript{2+} and was correlated with its largest ionic radius (bottom). The smallest pore size was observed with La\textsuperscript{3+}, the impermeable trivalent cation. The law of mass action linking the protein and the stimulus determines the extent of the biological response. Hence, the binding of Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+} or La\textsuperscript{3+} at the selectivity filter is understood to induce a change in channel conformation, such that it communicates with its affiliated synaptic proteins, affecting the kinetics of secretion.

was seen with high [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{35} These results were explained by a synaptotagmin/Ca\textsuperscript{2+} interaction. Because Ba\textsuperscript{2+} is less effective at binding to synaptotagmin, latency should be longer. Indeed, our present findings showed a larger latency when Ba\textsuperscript{2+} substituted for Ca\textsuperscript{2+}, consistent with previous findings by Wang et al.\textsuperscript{35} However, a significant shorter delay was observed also in La\textsuperscript{3+}, despite its impermeability and having no effect on either [La\textsuperscript{3+}]\textsubscript{i} or [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{11}

Furthermore, when either intracellular Ca\textsuperscript{2+} or Sr\textsuperscript{2+} was elevated by ionophore the kinetics of the fusion pore was virtually identical. The insensitivity of the fusion pore to the type of the cytosolic cation stands in marked contrast to changes in fusion pore kinetics observed by the extracellular ion binding at the channel. These results suggest that initially, the channel conformation regulates fusion pore kinetics, independent of intracellular cations or ion transport.

Direct effect on the fusion pore. A third possibility would be a direct action of the ions on the fusion pore itself (neither via the Ca\textsuperscript{2+} channel nor synaptotagmin). This requires that one of the constituents of the fusion pore should be a cation-binding protein. While such a protein has still to be identified, the VGCC could qualify provided it is an integral constituent of the fusion pore.

We therefore suggest that the VGCC could be the site through which the ions directly affect the fusion pore. Our model proposes that the VGCC is a constituent of the fusion pore configuration.
The relation between release frequency and total catecholamine secretion. Permeable ions differentially affect gating kinetics and the unitary conductance of Ca$_{\text{L}}$1.2, the L-type calcium channel. The channel kinetics and its interaction with the synaptic proteins are controlled by the ions bound at the channel cavity. This leads to the prediction that the type of cation bound at the channel cavity could determine the overall frequency of release and thereby, total secretion. Analogous to receptor-ligand interactions, where the better agonist has the highest probability of generating a strong response, Ca$^{2+}$ is expected to confer the most favorable form of the channel yielding the foremost efficient stimulus-secretion coupling. La$^{3+}$ on the other hand, would be the least effective. Consistent with this view, secretion rate during depolarization decreased in the order of: Ca$^{2+}$ > Sr$^{2+}$ > Ba$^{2+}$ > La$^{3+}$.

Distribution of spike waiting times showed a two-phase profile that fitted an initial rate of release followed by a sustained rate. The highest spike frequency seen with Ca$^{2+}$ was correlated with the highest total catecholamine secretion. The lower spike frequency in La$^{3+}$ correlated with the lower efficacy (<6) of catecholamine release. A significant increase in the sustained rate of secretion in La$^{3+}$, which led to an increase in secretion over time, could be accounted for by the impermeability and continuous binding of La$^{3+}$ at the EEEE site, preventing channel inactivation.

The channel as part of the fusion pore. As previously stated, the proteins that constitute the fusion pore assembly determine its initial static state and its configuration. If the channel were a constituent of the fusion pore, conformational changes induced by cation rearrangements at the channel selectivity filter, should affect the fusion-pore dynamics.

It is well established that depolarization in the absence of an extracellular divalent cation does not elicit secretion. However, the role of the calcium channel as a signaling molecule in stimulus secretion-coupling, cannot be ignored. Secrecion triggered by an impermeable cation bound at the cavity, would seem to suggest that the permeating ions Ca$^{2+}$, Sr$^{2+}$ or Ba$^{2+}$ actually trigger secretion upstream to cell entry, primarily by recognition and specific binding to the VGCC cavity. The signaling role of the Ca$^{2+}$ channel is strengthened by the ability of La$^{3+}$ to support, in nominally Ca$^{2+}$-free solution, KCl induced catecholamine release in chromaffin cells and glucose induced-insulin release in pancreatic cells. The functional coupling of VGCC to the synaptic proteins enables the priming, spatial, and rapid temporal requirements of fast release.

In summary, we have shown a linkage between fusion pore kinetics and permeable and non-permeable cations bound at the channel selectivity-filter. The possibility of having an additional site of regulation within the selectivity filter strengthens the view that re-arrangements of the cation residing at the cavity trigger release. Inter-conversion from a ‘non-releasing’ to a ‘releasing’-competent conformation of the channel could be responsible for the initiation of depolarization-evoked-transmitter release prior to Ca$^{2+}$ entry. A model, in which the Ca$^{2+}$ channel simultaneously senses voltage-transitions, selectively binds ions, and is a structural constituent of the fusion pore, is attractive, because it provides a direct and a temporally tight control of neurosecretion.

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