Exocytotic catecholamine release is not associated with cation flux through channels in the vesicle membrane but Na⁺ influx through the fusion pore

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Release of charged neurotransmitter molecules through a narrow fusion pore requires charge compensation by other ions. It has been proposed that this may occur by ion flow from the cytosol through channels in the vesicle membrane, which would generate a net outward current. This hypothesis was tested in chromaffin cells using cell-attached patch amperometry that simultaneously measured catecholamine release from single vesicles and ionic current across the patch membrane. No detectable current was associated with catecholamine release indicating that <2% of cations, if any, enter the vesicle through its membrane. Instead, we show that flux of catecholamines through the fusion pore, measured as an amperometric foot signal, decreases when the extracellular cation concentration is reduced. The results reveal that the rate of transmitter release through the fusion pore is coupled to net Na⁺ influx through the fusion pore, as predicted by electrodiffusion theory applied to fusion-pore permeation, and suggest a prefusion rather than postfusion role for vesicular cation channels.

Neurotransmitter and hormone release occurs by exocytosis, which begins with the formation of a narrow fusion pore^{1,2}. Fusion pores in mast cells and chromaffin cells typically have an initial conductance of ~330 pS^{1,3} through which vesicular histamine or serotonin and catecholamines are released, respectively^{4,5}. The initial fusion pore of a synaptic vesicle is typically >280 pS and release of neurotransmitter should occur with a time constant <500 μ s because of the small vesicle volume⁶. Many neurotransmitters and hormones are organic ions that carry charge with them. Among these, acetylcholine, serotonin and catecholamines are mostly monovalent cations at physiological or more acidic vesicular pH. Efflux of charged molecules through a narrow fusion pore would rapidly charge the small capacitance of the vesicle and a mechanism for charge compensation is required.

Release of catecholamines from a single vesicle can be detected electrochemically⁷. The initial flux of catecholamines through the early narrow fusion pore can be measured as an amperometric foot signal preceding the amperometric spike^{3,5,8}. The amplitude of amperometric foot currents is typically ~5 pA. As most catecholamine molecules will be in monovalent cationic form, but two electrons are transferred per molecule in the oxidation giving rise to the amperometric current⁹, the catecholamines released during the foot signal carry an ionic current of ~2.5 pA with them. This would charge a typical bovine chromaffin granule with 2.5 fF capacitance by 1 V ms⁻¹. Synaptic vesicles with typical

~70 aF capacitance⁶ contain several thousand transmitter molecules per vesicle and release of only 450 monovalent molecules would charge the vesicle to >1 V. Therefore, a mechanism is necessary to compensate for the charge movement associated with the flux of charged transmitter to allow rapid and continued transmitter release through the fusion pore.

Evidence for the presence of K⁺ permeable channels in the membrane of synaptic vesicles and other neurosecretory vesicles, including chromaffin granules^{10–15}, led to the hypothesis that entry of cations may occur through cation channels in the vesicle membrane during release¹⁶. If this were the dominant mechanism mediating ion exchange, the catecholamine molecules released through the fusion pore would be replaced by cations from the cytosol, giving rise to a net outward current. To test this hypothesis, patch amperometry experiments were performed on chromaffin cells for simultaneous determination of catecholamine flux, fusion-pore conductance and net membrane current.

RESULTS

Fusion-pore conductance determines flux of catecholamines during foot signal

A cell-attached patch amperometry recording of an exocytotic event with an unusually long amperometric foot signal is shown in Fig. 1a. The catecholamine concentration of this vesicle was ~0.8 M and ~40% of the vesicle contents were released during the foot signal. The patch

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Figure 1 Flux of catecholamines during the foot signals is correlated with fusion-pore conductance. Patch amperometry recordings with small patch–CFE distance. (**a–f**) Analysis of an event with an exceptionally long foot signal. Amperometric current (**a**) indicates flux of catecholamine. The real part (**b**) and imaginary part (**c**) of patch admittance were used to calculate the time course of fusion pore conductance (**d**, continuous line). On an expanded scale, the amperometric current during the foot signal (**d**, dotted line) is observed fluctuating in parallel with the fusion-pore conductance. The ratio of catecholamine flux:fusion-pore conductance (**e**) is relatively constant for the first 800 ms of the fusion-pore opening and then decreases, suggesting depletion of free catecholamine in the vesicle. The dashed lines indicate the baselines of the respective signals. The scale bar

represents 500 ms in **a–e**. For the part indicated by the solid line in **e**, the flux is plotted versus fusion-pore conductance (**f**), showing a proportional relationship with a slope (solid line) of $\sim 3 \times 10^7$ molecules $s^{-1} nS^{-1}$, in agreement with the mean ratio indicated by the solid line in **e**. (**g, h**) A second example with shorter foot signal also shows parallel changes in fusion-pore conductance and amperometric foot current (**g**). The flux:conductance ratio (**h**) is approximately constant with, a mean value of 3.1×10^7 molecules $s^{-1} nS^{-1}$ (horizontal line). (**i, j**) A third example with unusually low catecholamine concentration (**i**) shows an initially lower flux:conductance ratio of $\sim 1.4 \times 10^7$ molecules $s^{-1} nS^{-1}$ (**j**, solid line) and decreasing foot current while fusion pore conductance remains approximately constant.

admittance measurement showed fluctuating values in the real part (Fig. 1b) and the imaginary part (Fig. 1c). From these traces, the time course of fusion-pore conductance was calculated (Fig. 1d, solid line). The fluctuations in fusion-pore conductance observed in this trace were accompanied by corresponding fluctuations in the amperometric current (Fig. 1d, dotted line) and thus, in the flux of catecholamine molecules. The correlations were well resolved in this experiment because the carbon fibre electrode (CFE) was very close to the plasma-membrane patch, thus minimizing the time for diffusion from the release site to the CFE. Eventually, the fusion pore expanded (Fig. 1d, arrow), which was accompanied by the onset of the amperometric spike indicating rapid release of the remaining catecholamine molecules from the

vesicle. The ratio of catecholamine flux to fusion-pore conductance (flux:conductance ratio) during the foot signal, gave an approximately constant value of $\sim 3 \times 10^7$ molecules per second per nS of fusion-pore conductance (molecules $s^{-1} nS^{-1}$) for the part indicated by the horizontal line (Fig. 1e), in agreement with previous estimates⁵. At later times, the flux:conductance ratio decreased somewhat, presumably because of depletion of free catecholamine in the vesicle. The data points of the part indicated by the horizontal line in Fig. 1e were plotted as flux versus fusion-pore conductance and are shown in Fig. 1f. They revealed a proportional relationship showing that the narrow fusion pore was the diffusion barrier and that its size fluctuations limited the flux of catecholamine during the amperometric foot signal.

Figure 2 Amperometric foot signals are not accompanied by net outward current. **(a)** If the positive charge carried by catecholamines (blue) is replaced by cations entering the vesicle through channels in its membrane (red), a net outward current will be generated. **(b)** Simultaneous detection of catecholamine release and patch current with cell-attached patch amperometry for an individual event shows no net outward patch current associated with the foot signal. The traces from top to bottom show the time course of: the real part of patch admittance change (Re, blue); the imaginary part of patch admittance change (Im/ω, red); fusion-pore conductance (G_p, turquoise); granule capacitance (C_v, brown); amperometric current (I_{amp}, green); and patch current (I_{patch}, black).

The fusion-pore opening and foot signal for a vesicle with similar catecholamine concentration is shown in Fig. 1g. The flux of catecholamine again showed a time course that paralleled that of the fusion-pore conductance. The flux:conductance ratio (Fig. 1h) was similar (3.1×10^7 molecules $s^{-1}nS^{-1}$) and was approximately constant for the duration of the foot signal. Because of the shorter foot duration, this vesicle released only ~25% of its contents during the foot signal. Data for a vesicle with a very low catecholamine concentration of ~0.26 M is shown in Fig. 1i. This vesicle had an average fusion pore conductance of ~270 pS, with a lifetime of 550 ms. During the first 150 ms after initial opening of the fusion pore, the flux of catecholamine again paralleled the time course of fusion-pore conductance. The flux:conductance ratio (Fig. 1j) during this time was lower (1.4×10^7 molecules $s^{-1}nS^{-1}$), consistent with the lower vesicular catecholamine concentration. From this vesicle, over 70% of the contents were released during the foot signal and a decrease in flux because of catecholamine depletion was observed after only 150 ms. The observation that such depletion was much less evident in vesicles with higher catecholamine concentration indicated that the free catecholamine concentration was buffered, presumably by binding sites on the granular matrix. The mean flux:conductance ratio for all events that were recorded with a CFE close enough to the patch membrane that the flux was accurately reflected in the amperometric foot signal was $2.2 \pm 1.2 \times 10^7$ molecules $s^{-1}nS^{-1}$ (s.d., $n = 13$).

As the ionic catecholamine current is half the amperometric current, an amperometric current of ~1 pA, as in Fig. 1d, corresponds to an ionic catecholamine current of ~0.5 pA through the fusion pore, which would

charge the 3 fF vesicle at a rate of ~170 mV ms^{-1} , and which should rapidly reduce the flux of molecules. However, the flux was maintained with an approximately constant flux:conductance ratio for hundreds of milliseconds, indicating that a mechanism for charge compensation exists.

Catecholamine release is not associated with net outward current

If a significant fraction of cations enters the vesicle through channels in the vesicle membrane (Fig. 2a), then the flux of catecholamine through the fusion pore would be associated with a net outward current. An exocytotic event measured by patch amperometry is shown in Fig. 2b. If the ionic charges of the released catecholamines were compensated by cations entering through ion channels in the vesicle membrane, the net outward current through the patch membrane would be expected to be half of the amperometric current. In contrast with this expectation, no patch outward current associated with the amperometric spike or amperometric foot signal was detected (Fig. 2b; the amperometric and patch currents were scaled such that the expected patch current would appear on the graphs with the same size as the amperometric current).

A similar recording for an expanding fusion pore is shown in Fig. 2c, but the foot current was rather small. To improve the resolution, 11 events with mean foot amplitude >1 pA and foot durations >20 ms were averaged. All events were aligned at the time of full pore expansion (onset of the spike). Even this averaged record (Fig. 2d) showed no detectable patch current associated with foot current. The patch-current trace expected if compensatory cation flux through the

Figure 3 Analysis of fusion pore properties in different extracellular solutions determined by patch amperometry, as in Fig. 2. (a, b) Fusion-pore openings were characterized by quantifying initial G_p , average G_p and fusion-pore duration as described in the Methods. (c–e) Statistical analysis of mean fusion-pore conductance (c), initial fusion-pore conductance (d) and fusion-pore lifetime (e), measured as time from fusion pore formation to rapid expansion (onset of amperometric spike). The error bars represent the s.e.m. ($n = 43$ for solution A, $n = 39$ for solution B, $n = 38$ for solution C. The single asterisk indicates $P < 0.05$ and the double asterisk indicates $P < 0.01$).

vesicular membrane occurred would be equal to the scaled amperometric current (Fig. 2d, dotted signal). However, as the amperometric foot and spike amplitudes were reduced because of diffusional broadening, the average patch current should be even larger than the dotted current signal indicates. If ion flux through the membrane only compensates for the charge while the fusion pore is small, then the patch current during the amperometric foot would be equal to or greater than the foot current and terminate with the onset of the spike (Fig. 2d, arrow and grey dotted line).

To estimate an upper limit of cation flux through the vesicle membrane during the foot signal, the mean patch current was determined during the plateau phase of the averaged amperometric foot (between the vertical dashed lines in Fig. 2c) after fitting the averaged patch current preceding the onset of the foot with a straight line and subtracting this fit as the baseline. The mean patch current during this time was 0.004 ± 0.013 pA (s.e.m.) and thus, <0.03 pA (within two s.e.m.). The mean plateau foot current was 2.9 pA, which corresponds to a cationic catecholamine current of 1.45 pA. The net outward current was thus $<2\%$ of the cationic catecholamine current, indicating that $<2\%$ of the catecholamine charge, if any, is replaced by cations entering the vesicle through channels in the vesicle membrane.

Fusion-pore conductance depends on extracellular ion concentration

Having excluded the entry of cations through the vesicle membrane, charge compensation must occur through the fusion pore — catecholamine release during the foot signal is either accompanied by co-release of anions, or by entry of cations from the extracellular space through the fusion pore. To distinguish between these two possibilities, we examined the effects of extracellular cation concentration on release from single vesicles. First, the influence of changing extracellular ions on fusion-pore properties was analysed by patch amperometry recordings with varying pipette solutions (Fig. 3). The mean capacitance step size and mean amperometrically measured charge (quantal size) were not significantly different in the different solutions.

Individual fusion-pore openings were quantified by determining initial and mean fusion-pore conductance and fusion-pore duration³ (Fig. 3a, b). The mean fusion-pore conductance during the foot signals was 740 ± 68 pS in solution A, and was reduced to 515 ± 32 pS in solution B and to 326 ± 20 pS in solution C (Fig. 3c; see Methods for compositions of solutions). A corresponding reduction was also obtained for the initial fusion-pore conductance (Fig. 3d; solution A, 383 ± 27 pS; solution B, 298 ± 29 pS; solution C, 214 ± 16 pS). The fusion-pore duration from the initial opening of the fusion pore to a rapid increase to a conductance >1 nS was ~ 26 ms, in agreement with previous results³, and was not significantly different in the different solutions (Fig. 3e).

The conductance of an aqueous fusion pore depends on the conductivity of the solution that fills the pore. The conductivities of solutions A, B and C were 18, 8.7 and 4.2 mS cm^{-1} , respectively. Although these conductivities varied with ratios of approximately 4:2:1, the initial and mean fusion-pore conductances varied approximately 4:3:2. Thus, the fusion-pore conductances varied less than the conductivities of the extracellular solutions, which is expected because the ionic composition within the pore will be a mixture of extracellular and intravesicular ions, and only the extravesicular concentrations were varied. Assuming that the fusion pore contains a 1:1 mixture of extracellular and intravesicular solution, the results are consistent with a conductivity of ~ 11 – 12 mS cm^{-1} for the intravesicular solution and unchanged fusion-pore dimensions. Therefore, the fusion pore will present a similar diffusion barrier to catecholamines and other ions in the different extracellular solutions.

Flux of catecholamines through the fusion pore depends on extracellular cations

If the charge compensation occurs by co-release of anions (such as ATP), the flux of catecholamines through the fusion pore should be similar in the different solutions. If, however, the charge compensation occurs by entry of cations from the extracellular solution through the fusion pore, the flux of catecholamines should be reduced at lower extracellular cation concentrations.

We therefore determined how the amperometric foot signals were affected by changes in extracellular cation concentration. To minimize diffusional distortion, the foot currents were measured using conventional carbon-fibre amperometry (Fig. 4a), where the membrane–CFE distance is generally very small. Four different solutions containing 140 mM, 90 mM, 40 mM and 0 mM NaCl, respectively, were used. In addition, all solutions contained 5 mM KCl, 5 mM CaCl_2 , 1 mM MgCl_2 .

Figure 4 Extracellular ion concentration modulates amperometric foot signals. **(a)** Representative amperometric events measured in three different solutions, as indicated. Each signal is shown with full amplitude (left; the scale bars represent 50 pA and 50 ms) and on expanded scale to reveal the foot signal more clearly (right; the scale bars represent 20 pA and 50 ms). The 0 mM NaCl signal has no detectable foot. **(b–f)** Statistical analysis of amperometric foot signals measured in solutions with the indicated NaCl concentrations quantifying frequency of amperometric spike with detectable foot signals **(b)**, foot charge indicating amount of catecholamine release during foot signal **(c)**, foot-signal duration **(d)**, mean of detectable foot currents **(e)**, and mean foot currents assuming an average of 0.5 pA for undetectable foot currents **(f)**. The error bars represent the s.e.m. ($n = 121$ for 140 mM NaCl, $n = 102$ for 90 mM NaCl, $n = 98$ for 40 mM NaCl, $n = 69$ for 0 mM NaCl). The single asterisk indicates $P < 0.05$ and the double asterisk indicates $P < 0.01$. In **e** and **f**, the symbols indicate foot

currents expected from constant field theory (see text and Supplementary Information, Fig. S1). The intravesicular free-catecholamine concentrations, $[CA]_{free}$, were set to reproduce the amperometric current measured in solution containing 140 mM NaCl. **(e)** Model 1, monovalent cation-selective fusion pore (filled squares, $[CA]_{free} = 205$ mM); model 2, cation-selective fusion pore (filled circles, $[CA]_{free} = 200$ mM); model 3, nonselective fusion pore $[Cl^-]_v = 0$ mM (open squares, $[CA]_{free} = 370$ mM); model 4, nonselective fusion pore $[Cl^-]_v = 50$ mM (open circles, $[CA]_{free} = 280$ mM). **(f)** Model 1, monovalent cation-selective fusion pore (filled squares, $[CA]_{free} = 135$ mM); model 2, cation-selective fusion pore (filled circles, $[CA]_{free} = 130$ mM); model 3, nonselective fusion pore $[Cl^-]_v = 0$ mM (open squares, $[CA]_{free} = 260$ mM); model 4, nonselective fusion pore $[Cl^-]_v = 50$ mM (open circles, $[CA]_{free} = 200$ mM). **(g)** Release of catecholamines through a narrow fusion pore is associated with charge compensation by cation entry through the fusion pore.

and 10 mM HEPES–NaOH. Osmolality was maintained near physiological values by adding appropriate amounts of glucose. The mean quantal size (measured as charge of integrated amperometric spikes), the mean amperometric spike amplitude and the mean half-width were not significantly affected by changing the solutions (data not shown). With 140 mM and 90 mM NaCl, foot signals were detected in ~72% of amperometric events, decreasing to 66% in 40 mM NaCl and 44% in 0 mM NaCl (Fig. 4b). The mean charge of foot signals (that is, the area under the foot), reduced progressively with lower NaCl concentrations in the bath solution (Fig. 4c). The mean foot duration was not significantly different in the different solutions (Fig. 4d), and the reduced foot charge primarily reflects a decrease in foot current amplitude (Fig. 4e). If an amperometric spike has no detectable foot signal, it is not clear, *a priori*, whether the reason is too short duration or too small amplitude. Therefore, undetected foot signals were not included in the statistical analysis of mean foot-signal properties (Fig. 4c–e). This would be correct for the mean amperometric foot current if the undetected foot signals were of too short duration. However, as the mean fusion-pore duration is more than 10 times longer than the detection limit of 3 ms, it is likely that the undetected foot signals had too small

amplitude. The detection limits for foot signal was an amplitude ≥ 1 pA and undetected foot currents should be between 0 and 1 pA. Therefore, the mean foot currents for the different NaCl concentrations were estimated assuming on average 0.5 pA for undetected foot signals (Fig. 4f). This analysis showed a further reduction at low NaCl concentrations.

As previously reported, foot-only events, also termed stand-alone foot signals¹⁷, were also observed in our recordings. The percentage of such events that did not lead to full fusion was 11.6% for 140 mM NaCl, 8.9% for 90 mM NaCl, 7% for 40 mM NaCl and 6.8% for 0 mM NaCl. These frequencies are similar to those previously reported¹⁷. The mean amperometric-current amplitudes of these events showed a similar decrease with decreasing NaCl concentrations — from 4.9 ± 0.4 pA ($n = 33$) in 140 mM NaCl solution to 2.1 ± 0.2 pA ($n = 31$) in 0 mM NaCl solution. The stand-alone foot signals had a smaller mean amplitude than the pre-spike foot signals, suggesting a smaller fusion pore. However, these events were not included in the analysis as the determination of fusion-pore conductance for such events is often uncertain because the lack of the final capacitance step leaves some uncertainty regarding the phase setting of the lock-in admittance measurement.

Efflux of catecholamines through the fusion pore occurs by electrodiffusion

The decrease in catecholamine efflux through the narrow fusion pore when the ion concentration is reduced indicated that the cation entry rate through the fusion pore limited the efflux of catecholamine release during the foot signal. For a quantitative estimate, we applied electrodiffusion theory using the Nernst-Planck equation assuming a constant field across the fusion pore, as in the Goldman-Hodgkin-Katz constant field theory (see Methods). For permeant ions, we assumed that the permeabilities of the fusion pore were proportional to the diffusion coefficients of the respective ions in aqueous solution (see Supplementary Information, Fig. S1 and Table S1). To estimate the dependence of catecholamine flux through the fusion pore on the extracellular NaCl concentration, four different models were tested: a fusion pore selective for monovalent cations; a cation-selective fusion pore allowing permeation of mono- and divalent cations; a non-selective fusion pore with a negligible intravesicular free-anion concentration; and a non-selective fusion pore with an intravesicular free-anion concentration equivalent to 50 mM Cl⁻.

The intravesicular free-catecholamine concentration was the only free parameter and was adjusted to reproduce the mean amperometric foot current amplitude measured in the presence of 140 mM NaCl (Fig. 4e and f, and see Supplementary Information, Fig. S1). Depending on the extracellular NaCl concentration, the reversal potential of the fusion pore changed, thus changing the flux of catecholamines through the fusion pore (see Supplementary Information, Fig. S1). Although an initial potential may be present across the vesicle membrane before fusion¹, the reversal potential of the fusion pore will be attained with the time constant given by chromaffin-granule capacitance and fusion-pore conductance, and was estimated to be about 3 fF/300 pS = 10 μ s.

The expected changes in foot current with changing NaCl concentration were compared with the experimental data for the four different fusion pore models in Fig. 4e and f. The most obvious result was that model 4 (allowing significant co-release of anions) showed a very weak dependence of the foot signal on the extracellular NaCl concentration and was not consistent with the measurements. Thus, it can be concluded that charge compensation occurs by entry of Na⁺ ions through the fusion pore (Fig. 4g). Cation-selective fusion-pore properties (models 1 and 2) seem to fit the data somewhat better than the non-selective pore with negligible free-anion concentration in the vesicle, but the differences are relatively small.

DISCUSSION

Release of charged transmitter molecules through exocytotic fusion pores requires a mechanism that compensates for the charge movement associated with the flux of these molecules through the fusion pore. Here, we have shown that entry of cations into the vesicle through cation channels in the vesicle membrane is not a major mechanism of ion exchange during release. Instead, catecholamine flux through the fusion pore is associated with Na⁺ influx through the fusion pore. A mechanism that involves charge compensation by co-release of anions is inconsistent with the experimental results.

In contrast with the amperometric foot current, the amperometric-spike kinetics are not significantly affected by changing the extracellular NaCl concentration, indicating that release during the amperometric spike is not limited by the fusion pore as a diffusion barrier, and is also

not limited by the rate of ion exchange. In mast cells, the fusion pore is also not rate-limiting for release during this phase¹⁸. The release kinetics of serotonin from mast-cell granules following electroporation depends on the type of cation in the solution, but not on its concentration over a wide range (1–100 mM), consistent with slow diffusion within the granular matrix and an apparent intra-matrix diffusion coefficient that depends on the cation species, but not its concentration¹⁹. It is possible that ion exchange mediating dissociation from the intravesicular matrix in chromaffin granules may involve Ca²⁺ ions — which could not be omitted in our experiments because they are essential to stimulate exocytosis. Experiments with electrode arrays revealed a reduced diffusion coefficient of catecholamines near the cell surface²⁰, suggesting that the amperometric spike kinetics may also be limited by slow diffusion near the cell membrane.

Release of catecholamines through the fusion pore is not associated with charge compensation by co-release of anions. This implies that the fusion pore is either cation selective, or that the free concentration of the major granular anion ATP is very low. If the fusion pore of chromaffin granules were cation selective, one would have to postulate that the fusion pore of glutamatergic synaptic vesicles should have very different properties, as a cation selective fusion pore would not allow effective release of anionic glutamate. As the same set of SNARE proteins mediates exocytosis in neurons and chromaffin cells²¹, a cationic fusion pore in chromaffin cells would be inconsistent with a common fusion pore lined by SNARE protein domains, as recently proposed²². However, our analysis assumes that the permeabilities for different ions through the fusion pore are proportional to their diffusion coefficients, which is a rough estimate. In particular, with complex ionic mixtures permeabilities may vary depending on ionic composition. Therefore, a precise agreement should not necessarily be expected, and a fusion pore permeable to anions and negligible free-anion concentration inside the chromaffin granule cannot be excluded. ATP in chromaffin granules has a structure similar to crystalline ATP and not to ATP in solution²³, suggesting a low free ATP concentration. The fusion-pore models indicate intravesicular free-catecholamine concentrations of 130–370 mM, much lower than the total vesicular catecholamine concentration²⁴, which is consistent with binding to the granular matrix^{25,26} or, alternatively, catecholamines and ATP forming a highly non-ideal solution²⁷. Experiments using solutions where Cl⁻ ions are substituted by large anions, with much lower diffusion coefficients or even too large to permeate the fusion pore, should allow a better estimate of the fusion-pore permeability for anions.

Catecholamine release through narrow fusion pores is significant. Amperometric recordings indicate that ~10% of catecholamine release occurs through narrow fusion pores and, under basal stimulation, catecholamine release may predominantly occur through narrow fusion pores, thus retaining the protein contents of the vesicle²⁸. Therefore, under these conditions, the rate of release from the vesicle is determined by electrodiffusion. Fast transmitter release from small synaptic vesicles is believed to occur entirely through a narrow fusion pore. It has been reported that, in Torpedo synaptic vesicles, most of the acetylcholine (ACh) or ATP content is adsorbed to an intravesicular matrix core²⁹, analogous to the situation in chromaffin cells. The hypothesis that charge compensation in these vesicles occurs through cation channels in the vesicle membrane¹⁶ has been reinforced by the recent finding that functional TRPM7 channels in cholinergic vesicles have a critical role in transmitter release^{15,30}. The results we have presented for catecholamine

release indicate that postfusion cation currents through the vesicular membrane are not detectable and are not required to sustain flux of transmitter through the fusion pore. If this is also the case for cholinergic vesicles, it would suggest that the TRPM7 modulation of quantal size may be a prefusion mechanism affecting loading of the vesicle^{15,30}, rather than a postfusion mechanism mediating ion exchange. □

METHODS

Cells and solutions. Bovine chromaffin cells were prepared and cultured as previously described³¹. Recordings were made on days 1–7 in culture. For patch amperometry³², the bath solution contained 140 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH and 10 mM glucose, the pH was adjusted to 7.3 with NaOH and the osmolality was approximately 310 mmol kg^{−1}. Pipette solution A contained 50 mM NaCl, 100 mM TEACl, 5 mM KCl, 10 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES–NaOH, the pH was adjusted to 7.3 and the osmolality was adjusted to ~290 mmol kg^{−1}. In patch amperometry experiments to study fusion pore conductance with low cation concentration in the pipette, pipette solutions contained: solution B, 0 mM NaCl, 50 mM TEACl, 5 mM KCl, 10 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH at pH 7.3; solution C, 0 mM NaCl, 0.5 mM TEACl, 5 mM KCl, 10 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH at pH 7.3. Osmolarities were adjusted to ~310 mmol kg^{−1} with glucose.

For amperometry, the bath solutions contained: 140 mM NaCl with 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH and 10 mM glucose; or 90 mM NaCl with 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH; or 40 mM NaCl with 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH; or no NaCl with 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES–NaOH. The pH was adjusted to 7.3 and the osmolalities were adjusted to ~290 mmol kg^{−1} with glucose in all bath solutions. All the experiments were performed at room temperature.

Patch amperometry. Changes in membrane capacitance and catecholamine release were recorded simultaneously by cell-attached patch amperometry³². Briefly, cell-attached patch clamp was performed with a CFE introduced into the patch pipette. The CFE was positioned 1–5 μm from the tip opening under the microscope. The CFE was continuously held at +700 mV. Amperometric currents were filtered with an 8-pole Bessel filter set at 3 kHz. Patch pipettes were pulled in four stages with a programmable puller (P-97; Sutter Instruments, Novato, CA, USA) and coated with a sticky wax (Kerr, Romulus, MI, USA). Pipettes were fire-polished and had a typical resistance in the bath of approximately 2 MΩ. Pipette resistance typically increased up to 3–4 MΩ when the CFE approached the tip opening.

For capacitance measurements, a patch clamp amplifier was used (EPC-7; HEKA-Elektronik, Lambrecht/Pfalz, Germany). Command voltage was applied to the bath. Changes of patch admittance were measured, as described previously^{33,34}, with a lock-in amplifier (SR830; Stanford Research Systems, Sunnyvale, CA, USA) using a sine wave amplitude of 50 mV (root mean square) at a frequency of 20 kHz. The output filter was set to a 1 ms time constant, 24 dB. The patch current is dominated by the capacitive currents that arise from the 20 kHz sine-wave voltage applied to measure changes in membrane capacitance. However, the 20 kHz current was filtered out by a 3 kHz low-pass filter such that patch currents below this frequency can be well resolved during the capacitance measurement. Fusion-pore openings were analysed as described previously^{3,32,34}. Pore conductance (G_p) and vesicle capacitance (C_v) were calculated from the real (Re) and imaginary (Im) parts of the admittance after baseline subtraction: $G_p = (Re^2 + Im^2)/Re$ and $C_v = ((Re^2 + Im^2)/Im)/\omega$, where $\omega = 2\pi \times 20$ kHz. The initial rise in G_p reflects the step response of the low pass filter setting of the lock-in amplifier (1 ms, 24 dB). At this setting, 90% of the final value is reached within 7 ms. The initial G_p value was thus taken as the G_p value at 7–10 ms (time of initial G_p) after the onset of the increase in the G_p trace. The fusion-pore duration was defined as the time interval between time of initial G_p and the time where G_p increased beyond 1 nS. The mean G_p was taken as the average G_p value during this time interval.

To determine vesicular catecholamine concentrations, the number of molecules released from a given vesicle was calculated from the integrated amperometric-spike charge, assuming a transfer of two electrons per molecule, and the vesicle volume was estimated from the capacitance-step size, assuming spherical geometry and a specific membrane capacitance of 9 fF cm^{−1}, as previously described⁵.

Amperometry. Conventional amperometry for catecholamine detection used 5 μm carbon fibres (ALA Scientific Instruments, Westbury, NY, USA). The tip of the electrode was positioned closely against the cell surface to minimize the diffusion distance from release sites. The amperometric current, generated by oxidation of catecholamines at the exposed tip of the CFE, was measured using a homemade amperometric amplifier, operated in the voltage-clamp mode at a holding potential of +700 mV. Amperometric signals were low-pass filtered at 1 kHz and digitized at 4 kHz. The data were collected and then analysed using IGOR software (WaveMetrics, Lake Oswego, OR). Secretion was induced by pressure ejection of 20 μM ionomycin (Sigma, St Louis, MO) diluted in the corresponding bath solution from a ~5 μm-tipped micropipette located ~30 μm away. Amperometric recordings were analysed with a program written by the authors to extract foot and spike information according to previously described criteria³⁵. The beginning of the current spike was located where the leading edge of the transient (which includes the foot signal when present) exceeded the baseline current by two times the s.d. of the baseline noise level. Foot signals and spikes were selected for analysis only when the amplitude of the spike was > 40 pA. Detectable foot signals had an amplitude ≥ 1 pA and a duration ≥ 3 ms.

Electrodiffusion calculations. The current carried by ion S through a cylindrical fusion pore of length l and cross-sectional area a is given by

$$I_s = -D_s \cdot \frac{a}{l} \cdot z_s^2 \cdot \frac{VF^2}{RT} \frac{[S_v] - [S_e] \exp(-z_s VF/RT)}{1 - \exp(-z_s VF/RT)}$$

where D_s is the diffusion coefficient for ion S , z_s its valency, $[S_v]$ its intravesicular free concentration and $[S_e]$ its extracellular free concentration. V is the voltage present across the fusion pore, F is Faraday's constant, R is the gas constant and T is the absolute temperature. For solution A, the conductivity of the solution filling the fusion pore is approximately $\sigma = 15$ mS cm^{−1}. The fusion pore conductance (G_p) can be written as

$$I_s = -D_s \cdot \frac{G_p}{\sigma} \cdot z_s^2 \cdot \frac{VF^2}{RT} \frac{[S_v] - [S_e] \exp(-z_s VF/RT)}{1 - \exp(-z_s VF/RT)}$$

and we can substitute the pore geometry in equation 1 to give

$$I_s = -D_s \cdot \frac{G_p}{\sigma} \cdot z_s^2 \cdot \frac{VF^2}{RT} \frac{[S_v] - [S_e] \exp(-z_s VF/RT)}{1 - \exp(-z_s VF/RT)}$$

which is also valid for arbitrary pore geometries³⁶.

We calculated the quantity G_p/σ from solution A to be $\sim 5 \times 10^{-8}$ cm. The assumed intravesicular concentrations of free Na⁺ and K⁺ were both ~20 mM, and free Ca²⁺ and Mg²⁺ were very low³⁷. The effective free concentration of catecholamines and soluble anions are not precisely known.

Statistical analysis. Data are expressed as mean ± s.e.m. unless otherwise indicated. Newman one-way ANOVA test was used for statistical analysis.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

Experiments were performed by L.W.G., and all authors contributed to the analysis and interpretation of the results and the writing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Supplemental Figure 1. Net current-voltage relations (blue) for fusion pore models 1-4 calculated according to Nernst-Planck electrodiffusion equation (see Methods, equation 3) for the different NaCl concentrations as indicated. The diffusion coefficients for the different ions are given in supplementary table 1. Due to the small capacitance of the vesicle (typically 3 fF) and the average initial fusion pore conductance of ~300 pS, the zero current potential will be established across the fusion pore with a time constant of ~10 μ s. Catecholamine flux through the narrow fusion pore on the millisecond time scale thus occurs at this reversal potential. Fractional catecholamine currents are indicated in red. The amperometric currents (green) are twice the fractional catecholamine currents assuming a transfer of 2 electrons per oxidized molecule⁹. The vertical arrow lines indicate the respective reversal potentials and corresponding amperometric currents. For each fusion pore model the intravesicular free catecholamine concentration was set to match the average experimental amperometric foot current in 140 mM NaCl solution (~12 pA). This was the only adjustable parameter.

Ion	Diffusion Coefficient D ($10^{-5} \text{ cm}^2 \text{ s}^{-1}$)
Na ⁺	1.33
K ⁺	1.96
CA ⁺	0.6
Ca ²⁺	0.79
Mg ²⁺	0.71
Cl ⁻	2.03

Table 1, Diffusion coefficients of ions used in the electrodiffusion calculations (from references S1, S2). CA stands for catecholamine.

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