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

**Liang-Wei Gong**[1,3], **Guillermo Alvarez de Toledo**[2], and **Manfred Lindau**[1,4]

[1]School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14850

[2]Department of Physiology and Biophysics, Faculty of Medicine, University of Seville, E-41009 Seville, Spain

## Abstract

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. We tested this hypothesis in chromaffin cells using cell-attached patch amperometry measuring simultaneously catecholamine release from single vesicles and ionic current across the patch membrane. No detectable current was associated with catecholamine release indicating that <2% (if any) of cations 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 have typically an initial conductance of ~330 pS[1,3] through which vesicular 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 μs due to the small vesicle volume[6]. 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 of charge compensation is required.

Release of catecholamines from a single vesicle can be detected electrochemically[7]. 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. Since most catecholamine molecules will be in monovalent cationic form but two electrons are transferred per molecule in the oxidation giving rise to the amperometric current[9], 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

4correspondence should be addressed to M.L. (ml95@cornell.edu).
[3]Present address: Department of Cell Biology, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510

with 2.5 fF capacitance by 1 V/ms. Synaptic vesicles with typical ~70 aF capacitance[6] 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 the charge movement associated with the flux of charged transmitter to allow for 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 during release entry of cations may occur through cation channels in the vesicle membrane[16]. 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 we performed patch amperometry experiments 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

Fig. 1 shows a cell attached patch amperometry recording of an exocytotic event with an unusually long amperometric foot signal (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 admittance measurement shows 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 are accompanied by corresponding fluctuations in the amperometric current (Fig. 1d, dotted line) and thus in the flux of catecholamine molecules. The correlations are well resolved because in this experiment the carbon fibre electrode (CFE) was very close to the plasma membrane patch minimizing the time for diffusion from the release site to the CFE. Eventually the fusion pore expanded (arrow), which was accompanied by the onset of the amperometric spike indicating rapid release of the remaining catecholamine molecules from the vesicle. Fig. 1e shows the ratio of catecholamine flux/fusion pore conductance (flux/conductance ratio) during the foot signal, giving a rather constant value of $\sim 3\times10^7$ molecules per second per nS of fusion pore conductance (molecules $s^{-1}nS^{-1}$) for the part indicated by the horizontal line, in agreement with previous estimates[5]. At later times the flux/conductance ratio decreases somewhat, presumably due to depletion of free catecholamine in the vesicle. Fig. 1f shows the data points of the part indicated by the horizontal line in Fig. 1e plotted as flux vs. fusion pore conductance and reveals a proportional relationship. It shows that the narrow fusion pore is the diffusion barrier and that its size fluctuations limit the flux of catecholamine during the amperometric foot signal.

Fig. 1g shows the fusion pore opening and foot signal for a vesicle with similar catecholamine concentration. The flux of catecholamine again shows a time course that parallels that of the fusion pore conductance. The flux/conductance ratio (Fig. 1h) is similar ($3.1\times10^7$ molecules $s^{-1}nS^{-1}$) and is rather constant for the duration of the foot signal. Due to the shorter foot duration this vesicle releases only ~25% of its contents during the foot signal. Fig. 1i shows the data for a vesicle with a very low catecholamine concentration of ~0.26 M. 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 parallels the time course of fusion pore conductance. The flux/conductance ratio (Fig. 1j) during this time was lower ($1.4\times10^7$ 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 it can be seen that a decrease in flux due to catecholamine depletion already sets in after 150 ms. The fact that such depletion is much less evident in vesicles with higher catecholamine

concentration indicates that the free catecholamine concentration is 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 $2.2\pm1.2\times10^7$ molecules $s^{-1}nS^{-1}$ (SD, n=13).

Since 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, and which should rapidly reduce the flux of molecules. However, the flux is maintained with a rather constant flux/conductance ratio for hundreds of milliseconds indicating that a mechanism of charge compensation is present.

## Catecholamine release is not associated with net outward current

If a significant fraction of cations enters the vesicle via channels in the vesicle membrane (Fig. 2a), then the flux of catecholamine through the fusion pore would be associated with a net outward current. Fig. 2b shows an exocytotic event measured by patch amperometry. If the ionic charges of the released catecholamines were to be 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 to this expectation, no patch outward current associated with the amperometric spike or amperometric foot signal is detectable in Fig. 2b (bottom trace). Note that the amperometric and patch currents are scaled such that the expected patch current would appear on the graphs with the same size as the amperometric current.

The event of Fig. 2c shows a similar recording for an expanding fusion pore but the foot current was rather small. To improve the resolution we averaged 11 events with mean foot amplitude >1 pA and foot durations >20 ms. 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 that would be expected if compensatory cation flux through the vesicular membrane occurred, would actually be equal to the scaled amperometric current (Fig.2d, dotted signal). However, since the amperometric foot and spike amplitudes are reduced due to diffusional broadening, the average patch current should be even larger than the dotted current signal indicates. If ion flux through the membrane would only compensate the charge while the fusion pore is small, then the patch current during the amperometric foot would be equal or greater than the foot current and terminate with the onset of the spike (arrow, grey dotted line).

To estimate an upper limit of cation flux through the vesicle membrane during the foot signal we determined the mean patch current during the plateau phase of the averaged amperometric foot (between 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 $0.004\pm0.013$ pA (s.e.m.) and thus <0.03 pA (within 2 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 is 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 via the fusion pore. Either catecholamine release during the foot signal is 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 we analyzed the influence of changing extracellular ions on fusion pore properties by patch amperometry recordings varying the pipette solution (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 duration[3] (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, B, C). A corresponding reduction was also obtained for the initial fusion pore conductance (solution A: 383±27 pS, solution B: 298±29 pS, solution C: 214±16 pS) (Fig. 3d). The fusion pore duration from the initial opening of the fusion pore to a rapid increase to a conductance >1 nS was ~26ms, in agreement with previous results[3], and was not significantly different in the different solutions (Fig. 3e).

For an aqueous fusion pore, its conductance depends on the conductivity of the solution that fills the pore. The conductivities of solutions A, B, and C were measured to be 18, 8.7, and 4.2 $mScm^{-1}$, respectively. While these conductivities vary approximately as 4:2:1, the initial and mean fusion pore conductances vary approximately as 4:3:2. The fusion pore conductances thus vary less than the conductivities of the extracellular solutions, which is expected because the ionic composition in 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 result would be consistent with a conductivity of ~11-12 $mScm^{-1}$ for the intravesicular solution and unchanged fusion pore dimensions. The fusion pore will thus 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 are affected by changes in extracellular cation concentration. To minimize diffusional distortion we measured the foot currents using conventional carbon fibre amperometry (Fig. 4a) where the membrane-CFE distance is generally very small. We used four different solutions containing (in mM) 140, 90, 40, and 0 NaCl. All solutions contained in addition 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$ and 10 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 and 90 mM NaCl foot signals were detectable in ~72% of amperometric events, decreasing to 66% in 40 mM NaCl and 44% in 0 NaCl (Fig. 4b). The mean charge of foot signals, i.e. the area under the foot, was reduced progressively with reducing the NaCl concentration ([NaCl]) 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 reflects primarily a decrease in foot current amplitude (Fig. 4e). If an amperometric spike has no detectable foot signal, it is not *a priori* clear if 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). For the mean amperometric foot current, this would be correct if the undetected foot signals were of too short duration. Since the mean fusion pore duration is more than 10 times longer than our detection limit of 3

ms, it is, however, 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. We thus estimated the mean foot currents for the different [NaCl] assuming on average 0.5 pA for undetected foot signals (Fig. 4f). This analysis shows a further reduction at low [NaCl].

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 NaCl, 8.9% for 90 NaCl, 7% for 40 NaCl and 6.8% for 0 NaCl. These frequencies are similar to those previously reported[17]. The mean amperometric current amplitudes of these events showed a similar decrease with decreasing [NaCl] from 4.9 +/− 0.4 pA (n=33) in 140 NaCl solution to 2.1+/−0.2 pA (n=31) in 0 NaCl solution. The stand-alone foot signals had a smaller mean amplitude than the pre-spike foot signals suggesting a smaller fusion pore. However, we did not include these events in the analysis because 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 indicates that the cation entry rate via the fusion pore limits 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). We assumed that for permeant ions, the permeabilities of the fusion pore are proportional to the diffusion coefficients of the respective ions in aqueous solution (see supplementary fig.1 and supplementary table 1). To estimate the dependence of catecholamine flux through the fusion pore on the extracellular [NaCl], we tested four different models:

1. Fusion pore selective for monovalent cations
2. Cation selective fusion pore (allowing permeation of mono- and divalent cations)
3. Non-selective fusion pore, negligible intravesicular free anion concentration
4. Non-selective fusion pore, 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 (see legend of Fig. 4 e,f and supplementary fig. 1). Depending on the extracellular [NaCl] the reversal potential of the fusion pore changes, thus changing the flux of catecholamines through the fusion pore (see supplementary fig. 1). Although an initial potential may be present across the vesicle membrane before fusion[1], the reversal potential of the fusion pore will be attained with the time constant given by chromaffin granule capacitance and fusion pore conductance and is estimated to be about 3 fF/300 pS=10 μs.

The expected changes in foot current with changing [NaCl] are compared with the experimental data for the four different fusion pore models in Figs. 4 e,f. The most obvious result is that model 4, allowing significant co-release of anions, shows a very weak dependence of the foot signal on the extracellular [NaCl] and is not consistent with the measurements. It can thus 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) appear 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. We have shown here that entry of cations into the vesicle via 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 to the amperometric foot current, the amperometric spike kinetics are not significantly affected by changing the extracellular [NaCl], indicating that release during the amperometric spike is not limited by the fusion pore as a diffusion barrier and not limited by the rate of ion exchange. In mast cells the fusion pore is also not limiting the rate of release during this phase[18]. 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 $Ca^{2+}$ 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 surface[20] 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 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 since a cation selective fusion pore would not allow effective release of anionic glutamate. Since the same set of SNARE proteins mediates exocytosis in neurons and chromaffin cells[21], a cationic fusion pore in chromaffin cells would be inconsistent with a common fusion pore lined by SNARE protein domains as recently proposed[22]. 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, not to ATP in solution[23], 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 concentration[24], which is consistent with binding to the granular matrix[25,26] or alternatively catecholamines and ATP forming a highly non-ideal solution[27]. Experiments employing 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 retaining the protein contents of the vesicle[28]. Under these conditions the rate of release from the vesicle is therefore 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 core[29] analogous to the situation in chromaffin cells. The hypothesis that charge compensation in these vesicles occurs via cation channels in the vesicle membrane16 has been reinforced by the recent finding that functional TRPM7 channels in cholinergic vesicles play a critical role in transmitter release15,30. The results presented here 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 role in mediating ion exchange.

## METHODS

### Cells and Solutions

Bovine chromaffin cells were prepared and cultured as described31. Recordings were made on days 1–7 in culture. For patch amperometry[32], the bath solution contained (in mM) 140 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH, 10 glucose, and pH was adjusted to 7.3 with NaOH and the osmolality was around 310 mmol/kg. Pipette solution A contained (in mM) 50 NaCl, 100 TEACl, 5 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH, the pH was adjusted to 7.3 and the osmolality was adjusted to ~290 mmol/kg. In patch amperometry experiments to study fusion pore conductance with low cation concentration in the pipette, pipette solutions contained (in mM): (solution B) 0 NaCl, 50 TEACl, 5 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH pH 7.3, and (solution C) 0 NaCl, 0.5 TEACl, 5 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH pH 7.3, osmolarities were adjusted to ~310 mmol/kg with glucose.

For amperometry, the bath solutions contained (in mM): (140 NaCl solution) 140 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH, 10 glucose; (90 NaCl solution) 90 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH; (40 NaCl solution) 40 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH; or (0 NaCl solution) 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH; the pH was adjusted to 7.3 and the osmolarities were adjusted to ~290 mmol/kg with glucose. All the experiments were performed at room temperature.

### Patch amperometry

Changes in membrane capacitance and catecholamine release were recorded simultaneously by the cell-attached patch amperometry[32]. Briefly, cell-attached patch clamp was performed with a CFE introduced into the patch pipette. The CFE was positioned at a distance 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) and coated with a sticky wax (Kerr). Pipettes were fire-polished and had a typical resistance in the bath of around 2 MΩ. Pipette resistance typically increased up to 3-4 MΩ when the CFE approached the tip opening.

For capacitance measurements, we used a patch clamp amplifier (EPC-7; HEKA-Elektronik). Command voltage was applied to the bath. Changes of patch admittance were measured as described previously33,34 with a lock-in amplifier (SR830; Stanford Research Systems) using a sine wave amplitude of 50 mV (root mean square) at a frequency of 20 kHz. The output filter was set to a 1ms 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 analyzed as described previously3,32,34. Pore conductance ($G_P$)

and vesicle capacitance were calculated from the real (Re) and imaginary (Im) parts of the admittance after baseline subtraction: $G_P = (Re^2 + Im^2)/Re$ and $C_V = [(Re^2 + Im^2)/Im]/\omega$. The initial rise of $G_P$ 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 2 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 described[5].

## Amperometry

Conventional amperometry for catecholamine detection used 5 μm carbon fibres (ALA Scientific Instruments). 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 analyzed by computer using IGOR software (WaveMetrics, Lake Oswego, OR). Secretion was induced by pressure ejection of 20 μM ionomycin (Sigma, St. Louis) diluted in the corresponding bath solution from a ~ 5 μm tipped micropipette located ~ 30 μm away. Amperometric recordings were analyzed with a program written in this laboratory to extract foot and spike information according to the criteria of Chow and Von Ruden[35]. 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 SD of the baseline noise level. Foot and spikes were selected for analysis only when the amplitude of the spike was > 40 pA. Detectable foot signals had an amplitude ≥1pA and a duration ≥3ms.

## Electrodiffusion Calculations

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

$$I_S = -D_S \cdot \frac{a}{l} \cdot z_S^2 \cdot \frac{VF^2}{RT} \frac{[S_V] - [S_E]\exp(-z_S VF/RT)}{1 - \exp(-z_S VF/RT)} \quad (1)$$

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 and F,R,T have their usual meaning. For solution A the conductivity of the solution filling the fusion pore is approximately σ=15 $mScm^{-1}$. The fusion pore conductance $G_P$ can be written as

$$G_P = \sigma \cdot a/l \quad (2)$$

and we can substitute the pore geometry in eq. 1 giving

$$I_s = -D_s \cdot \frac{G_P}{\sigma} \cdot z_s^2 \cdot \frac{VF^2}{RT} \frac{[S_V] - [S_E]\exp(-z_s VF/RT)}{1 - \exp(-z_s VF/RT)} \quad (3),$$

which is valid also for arbitrary pore geometries[36].

We calculate the quantity $G_P/\sigma$ from solution A to be ~$5\times10^{-8}$ cm. The intravesicular concentrations of free $Na^+$ and $K^+$ are both ~20 mM and free $Ca^{2+}$ and $Mg^{2+}$ are very low[37]. The effective free concentration of catecholamines and soluble anions are not precisely known.

### Statistical Analysis

Data is expressed as Mean±SEM if not otherwise indicated. Newman of one-way ANOVA tests was used for statistical analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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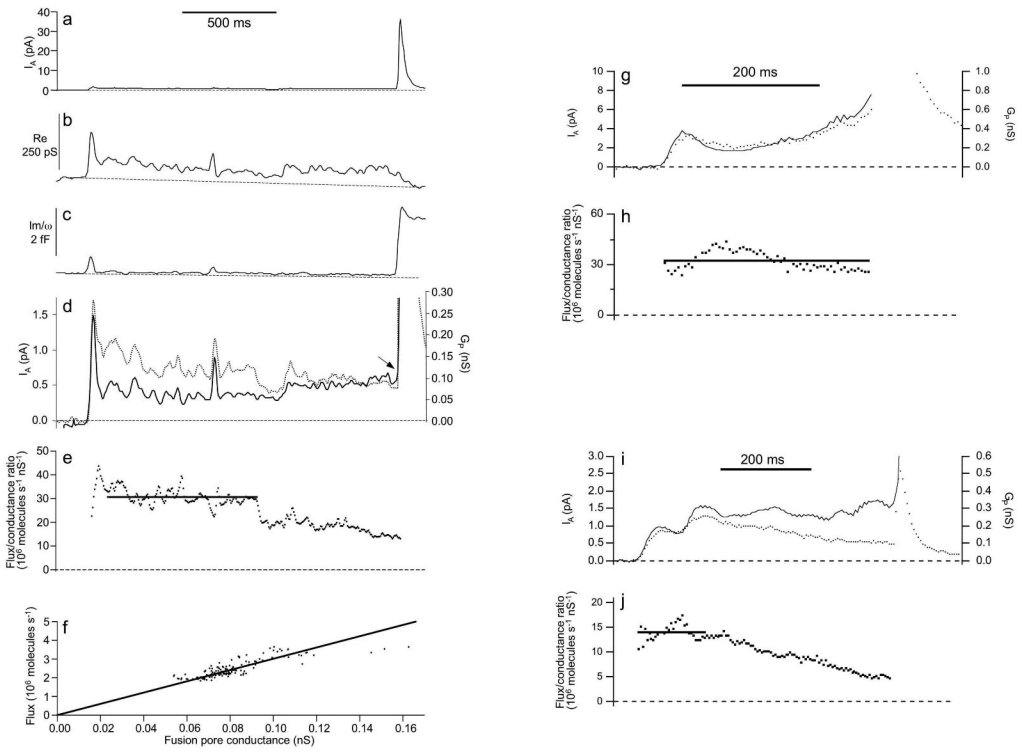


**Figure 1.**

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 exceptionally long foot signal. Amperometric current (a) indicates flux of catecholamine. 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 seen to fluctuate 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 baseline of the respective signals. The 500 ms scale bar at the top is for panels a-e. For the part indicated by the solid line in (e) the flux is plotted vs. fusion pore conductance (f) showing a proportional relationship with a slope (solid line) of ~$3\times10^7$ molecules $s^{-1}$ $nS^{-1}$ in agreement with the mean ratio indicated by the solid line in (e). A second example with shorter foot signal also shows parallel changes in fusion pore conductance and amperometric foot current (g). The flux/conductance (h) is rather constant with a mean value of $3.1\times10^7$ molecules $s^{-1}$ $nS^{-1}$ (horizontal line). A third example with unusually low catecholamine concentration (i) shows initially lower flux/conductance of ~$1.4\times10^7$ molecules $s^{-1}$ $nS^{-1}$ (j, solid line) and decreasing foot current while fusion pore conductance remains rather constant.

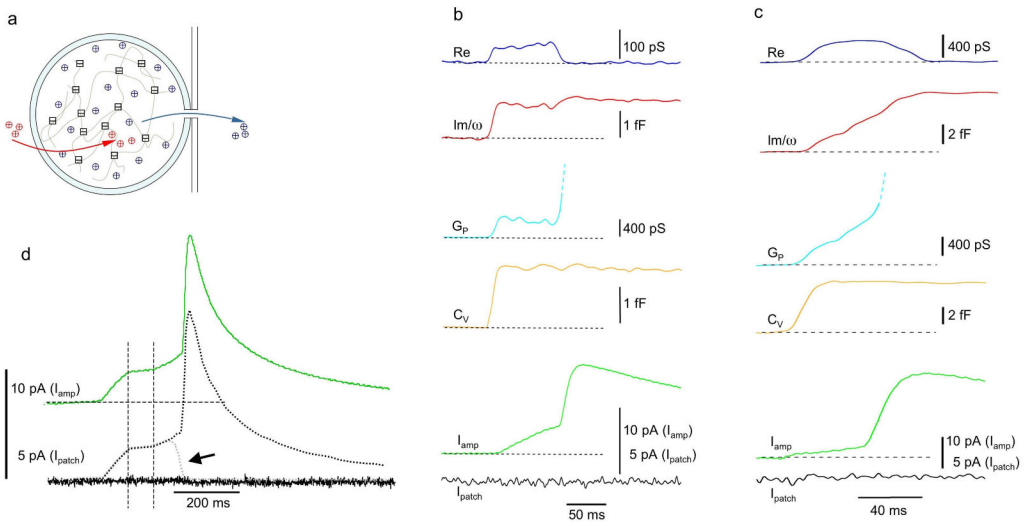


**Figure 2.**

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: Real part of patch admittance change (Re, blue), 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 horizontal dashed lines indicate the baselines of the respective signals. (c) Similar analysis of an event with an expanding fusion pore conductance ($G_P$). (d) Averaged amperometric current (green) and patch current (black) from 11 events with foot amplitude > 1 pA and foot duration >20 ms. The events were aligned at the time of rapid fusion pore expansion (end of foot = onset of the spike). The black and grey dotted lines indicate respectively the lower limits for expected patch currents if charge compensation occurs through channels in the vesicle membrane for total release or foot only (arrow). The vertical dashed lines indicate the plateau of the averaged foot current during which the patch current was averaged (see text).

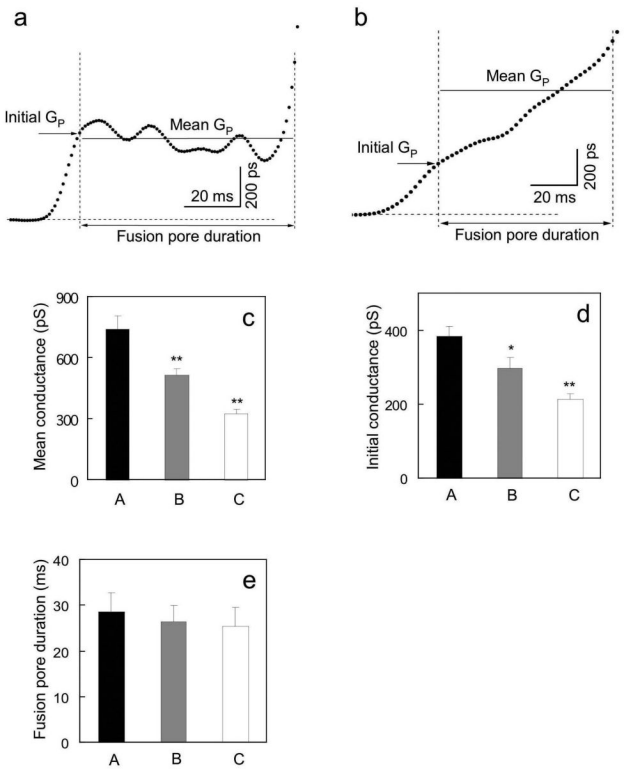


**Figure 3.**

Analysis of fusion pore properties in different extracellular solutions determined by patch amperometry as in Fig. 2. See Methods for compositions of solutions. (a,b) Fusion pore openings were characterized by quantifying initial $G_P$, average $G_P$ and fusion pore duration as described in Patch Amperometry section of Methods. Panels c-e show 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). Error bars are s.e.m, n=43 for solution A, n=39 for solution B, n=38 for solution C (* $p<0.05$, ** $p<0.01$).

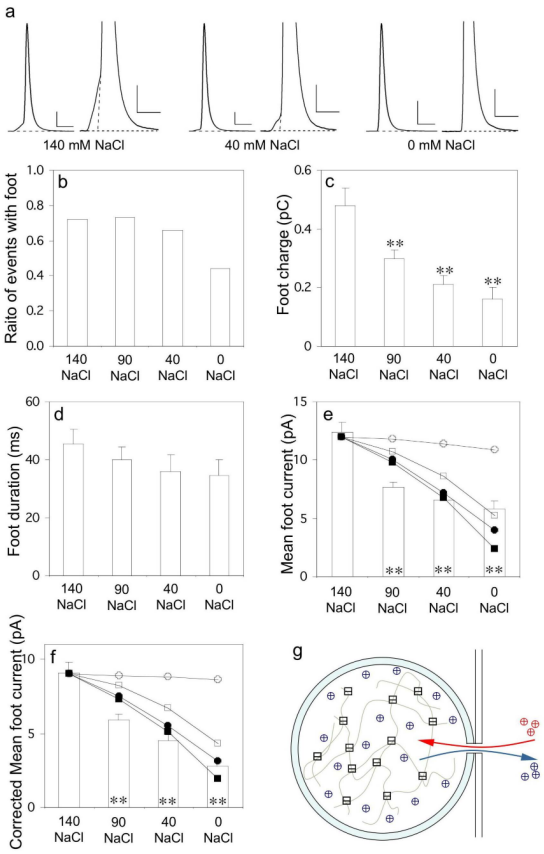


**Figure 4.**

Extracellular ion concentration modulates amperometric foot signals. (a) Representative amperometric events measured in 3 different solutions as indicated. Each signal is shown with full amplitude (left, scale bars 50 pA, 50 ms) and on expanded scale to reveal the foot signal more clearly (right, scale bars 20 pA, 50 ms). The 0 NaCl signal has no detectable foot. (b-f) Statistical analysis of amperometric foot signals measured in solutions with the indicated [NaCl] quantifying frequency of amperometric spike with detectable foot signals (b), foot charge indicating amount of catecholamine release during foot (c), foot duration (d) and mean of detectable foot currents (e), and mean foot currents assuming an average of 0.5 pA for undetected foot currents (f). Error bars are s.e.m., n=121 for 140 NaCl, n=102 for 90 NaCl, n=98 for 40 NaCl, n=69 for 0 NaCl (* $p<0.05$, ** $p<0.01$). In panels e and f the symbols indicate foot currents expected from constant field theory (see text and supplementary fig. 1). 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 (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 (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.