Hypotonicity and peptide discharge from a single vesicle

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AMONG ITS MANY FUNCTIONS, peptidergic hormone prolactin plays also a role in osmoregulation (14). Previous studies demonstrated prolactin release from isolated tissue (18, 34, 41, 42) or cells in response to hyposmotic stimulation (41, 50), which required extracellular calcium (23, 40, 41). While these findings suggest that prolactin is likely released by exocytosis in response to hyposmotic stress, secretory activity at the single vesicle level in response to hypotonicity was not monitored directly yet. In particular, it is unclear whether the hormone is released rapidly after complete collapse of vesicles into the plasma membrane (24) by full fusion exocytosis (21) or by kiss-and-run exocytosis (1, 13), whereby vesicles transiently fuse with the plasma membrane, forming a channel with the extracellular space, termed the fusion pore.

To address these questions, we studied rat pituitary lactotrophs, which release prolactin stored in a highly aggregated form in large dense-core secretory vesicles (10). To monitor secretory activity, we used perfusion experiments and radioimmunoassays and recorded single exocytotic and vesicle release events in real time, using confocal microscopy, steryl dyes, and fluorescently labeled peptides (2, 45). To directly study fusion pore properties, we used electrophysiological methods to monitor membrane capacitance (Cm), which is linearly related to changes in membrane area (30, 35, 48, 53).

Experimental evidence indicates that fusion of secretory vesicles in lactotrophs occurs spontaneously and is facilitated by hypotonicity. Moreover, the results show that hypotonicity-induced hormone discharge from a single vesicle involves transient fusion pore openings.

MATERIALS AND METHODS

Measuring prolactin release. For perfusion experiments, 1.2 × 10⁷ lactotroph cells were incubated with preswollen Cytodex-1 beads in 60-mm petri dishes for 24 h. The beads were transferred to 0.5-ml chambers and perfused with Hanks’ medium 199 for 90 min at 0.8 ml/min, at room temperature. During perfusion, cells were stimulated with hyposmolar solution or with KCl depolarization. Fractions were collected at 1-min intervals, stored at −20°C, and assayed for prolactin by radioimmunoassay. The primary antibody and standard for the prolactin assay were from the National Pituitary Unitary Agency (Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA), the secondary antibody was from Sigma, and 125I-labeled prolactin tracer was from PerkinElmer Life Sciences (Boston, MA).

Cell culture and microscopy. Pituitaries were obtained after decapitation from male Wistar rats and enriched for lactotrophs (5). The care of experimental animals was in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and the Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia 40/85 and 22/87). The animal use procedures were approved by the Veterinary administration of the Republic of Slovenia (approval no. 3440-29/2006). Briefly, lactotrophs were obtained by enzymatic dispersion, enriched with Percoll gradient, plated on glass coverslips coated with poly-L-lysine, and maintained in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% newborn calf serum and l-glutamine in an atmosphere of humidified air (92%) and CO2 (8%). The level of lactotroph purification exceeded 80% (data not shown). The lactotroph purification exceeded 80% (data not shown). A plasmid encoding atrial natriuretic peptide-enhanced green fluorescent protein (ANP-EGFP) (a gift from Dr. Ed Levitan) was introduced into the lactotrophs by lipofection (Invitrogen). The level of lactotroph purification exceeded 80% (data not shown). Cell-loaded coverslips were placed into a recording chamber on an inverted confocal microscope (LSM 510; Zeiss, Jena, Germany), and extracellular solution (400 μl) containing 4 μM FM 4-64 (Molecular Probes, Leiden, Netherlands) was added. Fluorescence images were acquired

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with a plan-apochromatic oil-immersion objective (×63, 1.4 numerical aperture) using 488-nm Ar-ion laser excitation and filtered with LP 580-nm emission filter.

Hypotonicity-induced cell swelling was monitored in the equatorial plane of the cells as changes in cell diameter. Initially, cells were kept in normal extracellular solution containing 4 mM FM 4-64. The osmolarity of the solution was then reduced to ~200 mosM by bath application of hypotonic extracellular solution containing 4 mM FM 4-64. Cell swelling was measured as a change in the cell diameter, determined from fluorescence intensity line profiles drawn perpendicularly over the stained cells with LSM 510 software (Zeiss). The fluorescence peaks marked the cell boundary. The distance between the peaks was determined before and 2 min after hypotonic stimulation. Hypotonicity-induced hormone secretion from a single granule was studied by using FM 4-64 and ANP-EGFP; emission fluorescence was filtered with a 505- to 530-nm band-pass filter. In KCl depolarization experiments, cells were superfused with 100 mM KCl solution. Images were acquired every 493 ms (~2 Hz) and analyzed with an LSM 510 laser-scanning confocal microscope (Zeiss); custom software was written in MATLAB (Math Works, Natick, MA). The average fluorescence intensity changes were monitored within a circular region (diameter 14 pixels) at the locus of a single vesicle by measuring the time required for a 20% to 80% intensity change in the fluorescent signal.

Electrophysiology. Capacitance in attached cells was measured as described previously (26, 53, 45). Briefly, a sine wave voltage (1,591 Hz, 111 mV root mean square; SWAM IIC, Celica, Ljubljana, Slovenia) was applied to the pipette. The pipette potential was held at 0 mV. The resistance of thick-walled pipettes, which were fire polished and coated with Sylgard, was 2–6 MΩ. Responses were recorded under basal conditions (bath and pipette contained extracellular solution) in the first set of experiments and under both basal and stimulated conditions (hypotonic stimulation or KCl depolarization) in two other sets, typically for 7 min. During stimulation with hypotonic solution, a bolus of saline was added to reduce osmolarity to ~200 mosM. Stimulation with depolarization was achieved by adding a bolus solution to reach a final KCl concentration of 100 mM. The phase of the lock-in amplifier was adjusted to nullify the changes in the real (Re) portion of the admittance signal by manually generating 10-Hz calibration pulses with an in-built calibration in patch-clamp instrument.

Transient events in the Im portion of the admittance signal that exhibited measurable projections to the Re portion (“cross talk”) were used to calculate fusion pore conductance \[ G_p = (R_e + Im^2)/Re \] (32) and vesicle capacitance \[ C_v = (Re^2 + Im^2)/Im/\omega, \text{ where } \omega \text{ denotes angular frequency}. \] Fusion pore diameter was determined from the equation \[ G_p = (\pi r^2)/d(\lambda), \] where \( r \) denotes fusion pore radius, \( \rho \) is the resistivity of the saline (100 Ω·cm), and \( \lambda \) is the estimated length of a gap junction channel (15 nm) (44). Vesicle diameter was determined from specific membrane capacitance \( C_m = 8 fF/\mu m \) and vesicle capacitance \( C_v \). All recordings were performed at room temperature.

Events were analyzed by using the cursor option in the software subroutine (CellAn, Celica, Slovenia) written for MATLAB. We considered an event to be reversible if an on-step in Im was followed by an off-step within 2 s. The appearance of events was ascertained by progressive filtering. We considered an event to be detectable if the signal-to-noise ratio was at list 3:1 and the event did not exhibit a projection in the current trace. Sigma Plot was used for statistical analysis. Values are presented as means ± SE. Differences between samples were analyzed with t-tests unless stated otherwise.

Solutions. Extracellular solution contained in (mM) 130 NaCl, 5 KCl, 8 CaCl2, 1 MgCl2, 10 d-glucose, 10 HEPS (N-2-hydroxylethylpiperazine-N’-2-ethanesulfonic acid), and pH 7.2/NaOH. Hypotonic extracellular solution used for cell perfusion contained (in mM) 90 NaCl, 5 KCl, 8 CaCl2, 1 MgCl2, 10 d-glucose, 10 HEPS, and pH 7.2/NaOH. Hypotonic extracellular solution added as bolus to reduced osmolarity of the extracellular solution contained (in mM) 5 KCl, 8 CaCl2, 1 MgCl2, 10 d-glucose, 10 HEPS, and pH 7.2/NaOH. KCl solution added as a bolus contained (in mM) 5 NaCl, 130 KCl, 8 CaCl2, 1 MgCl2, 10 d-glucose, 10 HEPS, and pH 7.2/NaOH. A 100 mM KCl solution was prepared by replacing 95 mM NaCl in the standard saline solution with 95 mM KCl. Osmolarity was measured with a freezing-point osmometer (Osmomat030; Gonotech, Berlin, Germany). Chemicals were from Sigma (Darmstadt, Germany) and were of highest grade of purity available.

RESULTS

Hypotonicity, cell volume increase, and prolactin release. Pituitary lactotrophs of lower vertebrates respond to reduced extracellular osmolarity by increasing prolactin release (18, 34, 41, 42), which reduces ion and water permeability and increases solute retention (7, 22, 33). However, whether hypotonicity affects prolactin release from mammalian lactotrophs is unclear. Here we exposed rat lactotrophs to hypotonicity of ~200 mosM, which is encountered clinically (3, 9).

Under hypotonic conditions, cells generally swell and undergo a regulatory volume decrease (27). Consistent with previous reports (41), hypotonicity increased the volume of rat lactotrophs, measured by fluorescence intensity line profiles (Fig. 1, A–C) in the presence of FM 4-64, a membrane area marker (39). Hypotonicity increased the interpeak distance, an estimate of cell diameter, by 0.58 ± 0.13 μm (Fig. 1D), significantly different from zero (\( P < 0.001 \)) (from 10.65 ± 0.16 μm to 11.23 ± 0.22 μm, \( n = 28 \)), and FM 4-64 peak intensity; regular extracellular solution elicited no such changes (Fig. 1C).

Perfused cells spontaneously released prolactin at room temperature, and hypotonicity evoked a transient increase in prolactin release, followed by a sustained depression (Fig. 2), likely reflecting reduced basal spontaneous secretion, which is controlled by spontaneous voltage-gated calcium influx (47). The transient increase did not reflect depletion of the pool of readily releasable vesicles, since KCl depolarization of cells yielded a sustained increase in prolactin release that continued after the stimulus was discontinued (Fig. 2).

Hypotonicity and hormone discharge from a single vesicle. To determine whether hypotonicity induces hormone discharge from a single vesicle, we transfected cells with a construct that encodes ANP-EGFP and is targeted to secretory vesicles (19). In transfected lactotrophs, ANP-EGFP fluorescence (Fig. 3A) had a punctate appearance and colocalized with prolactin-containing vesicles, as reported previously (45).

To analyze the properties of stationary vesicles at the plasma membrane, we monitored changes in the fluorescence intensity of labeled vesicles (Fig. 3A, insets). In lactotrophs exposed to hypotonic solution, ANP-EGFP fluorescence displayed complex time-dependent changes. Initially, fluorescence intensity increased. Since EGFP is pH sensitive (38), the increase likely indicates alkalinization of the vesicle lumen after the exposure to the extracellular space through the fusion pore. Thereafter, fluorescence intensity decreased, likely reflecting ANP-EGFP discharge into the extracellular space (Fig. 3B, bottom), since FM 4-64 fluorescence increased simultaneously (45; Fig. 3B, top). FM-dyes are widely used to study exo-/endocytosis, given that these amphiphilic styryl fluorescent dyes are virtually nonfluorescent in water but show a dramatic increase in the quantum yield upon incorporation into the cell membranes or any other hydrophobic environment (6).
In 4 of 11 image planes at cell base, 7 of 448 vesicles (2 ± 1 vesicles/cell image) that were initially labeled by ANP-EGFP and exhibited green fluorescence dimming were loaded with FM 4-64. This corresponds to hypotonicity-induced release from 2.4 ± 1.6% (n = 4) of ANP-EGFP-labeled vesicles determined as a fraction (%) of responding vesicles per cell image. Measurements of the time required for a 20–80% change of the fluorescent signal revealed that ANP-EGFP fluorescence decayed in 5.5 ± 1.4 s, which appeared slower than vesicle loading with FM 4-64 (2.8 ± 0.6 s; Fig. 3C), however, not significantly different (P = 0.23, n = 7). Upon hypotonic stimulation in four cells, eight vesicles loaded FM 4-64 with a 20–80% of 2.6 ± 0.5 s (not shown) but did not fluoresce green before stimulation, consistent with the view that not all exocytotic vesicles are preloaded with ANP-EGFP (45).

KCl depolarization generated a much more robust response (Fig. 3D). In 9 of 10 image planes at cell base, 66 of 585 ANP-EGFP-labeled vesicles (13 ± 7 vesicles/cell image) discharged their cargo and were loaded with FM 4-64 upon KCl depolarization (Fig. 3E); this corresponds to fluorescent cargo release from 9.8 ± 3.2% of ANP-EGFP-labeled vesicles. Thus, four- to fivefold more vesicles discharged ANP-EGFP and were loaded with FM 4-64 in response to KCl depolarization than in response to the hypotonic solution. The ANP-EGFP fluorescence decay (5.0 ± 0.5 s) differed significantly from the increase in FM 4-64 fluorescence (3.1 ± 0.3 s) in KCl-depolarized vesicles (P < 0.01, n = 66; Fig. 3F), consistent with our previous report (45). These results show that hypotonicity induces cargo release from vesicles associated with the plasma membrane. However, a significantly smaller fraction of vesicles responded to hypotonicity than to KCl depolarization.

Transient fusion pore events and hypotonicity. Next we studied fusion pore properties. For this, high-resolution changes in \( C_m \), which are related to surface area changes, were recorded. Discrete steps in \( C_m \) correspond to elementary membrane fusion and fission events (35, 48). In 11 of 34 cells exposed to hypotonicity (total recording time, 5.06 h; average, 536 s per patch), we observed spontaneous discrete steps in \( C_m \) of around 1 fF (range, 0.4 to 6 fF). In 10 of the cells, discrete steps were seen under resting conditions and after stimulation. In one cell, hypotonicity induced discrete \( C_m \) steps in a previously silent patch. In addition, the spontaneous discrete \( C_m \) steps were silenced after stimulation in one cell. Thus, rather than generating new discrete steps,
hypotonicity modulates the activity of discrete \( C_m \) steps present under nonstimulated conditions.

Irreversible \( C_m \) steps, representing full fusion/fission (Fig. 4A, left), and reversible \( C_m \) steps (Fig. 4A, right), likely representing transient kiss-and-run events, were observed in nonstimulated and hypotonic-stimulated cells (Fig. 4B). Reversible \( C_m \) steps in the imaginary part (Im) of the admittance signal were often accompanied by projections to the real part (Re) of the admittance signal (Fig. 4B). The amplitude distribution of the upward steps and of the downward steps that followed within 2 s was best fitted with a regression line \( (n = 619; \text{correlation coefficient } r = 0.93; \text{Fig. } 4C) \), with a slope near 1. This finding supports the idea that reversible \( C_m \) steps reflect the fusion and subsequent fission of a single vesicle. The amplitude of upward \( C_m \) steps, corresponding to \( C_v \) (Fig. 4D), were similar before and after hypotonic stimulation (Kolomogorov-Smirnov test, \( P > 0.05; n = 179 \) and \( n = 440 \), respectively). Thus, hypotonicity had little effect on the amplitude of these events. The calculated diameter of vesicles was \( 150–450 \) nm (Fig. 4D), consistent with the size of prolactin-containing vesicles in lactotrophs (2, 43, 53).

To study whether transient fusion proceeds into full fusion (12, 15), we analyzed fusion events and the mode of secretory activity after hypotonic stimulation and after KCl depolarization. The frequency of transient events, measured in 10-s epochs, increased from \( 0.07 \pm 0.01 \) (\( n = 761 \) events in 895 epochs) to \( 0.10 \pm 0.01 \) events/s (\( P < 0.05; n = 440 \) events in 428 epochs) after hypotonic stimulation but was sixfold higher after KCl depolarization \( (0.66 \pm 0.06 \text{ events/s; } P < 0.01; n = 2,372 \text{ events in } 366 \text{ epochs}) \) (Fig. 5A). The frequency of irreversible \( C_m \) steps increased only slightly, however, not significantly different \( (P > 0.05) \), and was considerably lower than the frequency of transient events occurring spontaneously \( (0.006 \pm 0.001 \text{ events/s; } n = 35 \text{ events}) \) or in response to hypotonicity \( (0.007 \pm 0.001; n = 29 \text{ events}) \) or KCl depolarization \( (0.009 \pm 0.001; n = 32 \text{ events}) \) (Fig. 5A). Therefore, transient, reversible fusion events were the predominant mode of exocytosis before and after stimulation, consistent with a previous report (48).

To further test whether the repetitive transient fusion events can be attributed to the same vesicles in particular patches, we analyzed the amplitude of \( C_m \) steps in each recording separately. Plots of average \( C_v \) amplitude before and after stimulation show a high correlation coefficient \( (r = 0.85 \text{ for hypotonic-stimulated cells}) \).

**Fig. 3.** Induced hormone release from single vesicles. A: confocal image of hypotonicity triggered peptide release from a lactotroph granule at time of fusion \((0 \text{ s; left})\) and 40 s later \((40 \text{ s; right})\). Scale bar: 5 \( \mu \text{m. Bottom: atrial natriuretic peptide-enhanced green fluorescent protein (ANP-EGFP; green) and FM 4-64 (red) fluorescence from a selected vesicle (squares at top). Scale bar: 0.5 \( \mu \text{m. Bottom: increase in FM 4-64 fluorescence (top) normalized to minimal and maximal fluorescence (thin solid lines) and ANP-EGFP fluorescence decrease (black trace, bottom) in response to hypotonic stimulation. Gray trace indicates baseline fluorescence before opening of the vesicle fusion pore and increase in fluorescence due to pH neutralization after fusion pore opening. Dotted lines represent 20% and 80% fluorescence intensity levels. C: the rise times (\( \tau_{20-80\%} \) means \( \pm \text{SE}) \text{ of FM 4-64 and ANP-EGFP fluorescence. Numbers adjacent to bars indicate the number of vesicles analyzed. D: high potassium triggered fluorescent peptide release from lactotroph vesicles displayed at stimulation onset (0 s; left) and 40 s after (right). Scale bar: 5 \( \mu \text{m. Note substantial increase in number of labeled vesicles that discharged fluorescent peptides and were loaded with FM 4-64 (circles at right) in comparison to vesicle cargo discharge in A. White arrowheads indicate selected initially nonfluorescing vesicles that fused with the plasma membrane upon stimulation and were loaded with FM 4-64. Bottom: ANP-EGFP (green) and FM 4-64 (red) fluorescence in a selected vesicle (squares at top). Scale bar: 0.5 \( \mu \text{m. E: FM 4-64 fluorescence increase (top) normalized to minimal and maximal fluorescence (thin solid lines) and ANP-EGFP fluorescence decrease (black trace, bottom) in response to 100 mM K+. F: the rise times (\( \tau_{20-80\%} \) means \( \pm \text{SE}) \text{ of FM 4-64 and ANP-EGFP signals. Numbers adjacent to bars indicate the number of vesicles analyzed. *P < 0.01. \)
tonicity and $r = 0.93$ for depolarization; $n = 10$ and 8 patches, respectively) and regression line slopes near 1 ($1.14 \pm 0.25$ and $1.05 \pm 0.17$, respectively; Fig. 5B). Therefore, the average $C_v$ amplitudes before and after stimulation were similar.

Hypotonicity increases fusion pore dwell-time and conductance. The secretory output from a cell can be increased in several ways. It can increase by an increase in the frequency of fusion events (Fig. 5A), but also by a prolonged fusion pore dwell-time and a wider fusion pore diameter. Hypotonicity prolonged the fusion pore dwell-time ($0.16 \pm 0.01$ vs. $0.20 \pm 0.01$ s; $P < 0.05$; $n = 716$ events and $n = 440$ events, respectively; Fig. 6A) to approximately the same extent as depolarization ($0.20 \pm 0.01$ s; $n = 2,372$ events), consistent with a previous report (48). The probability of observing an open fusion pore (calculated as the sum of all pore dwell-times divided by the duration of each recording) increased from $0.012 \pm 0.002$ under resting conditions ($n = 37$ recordings) to $0.019 \pm 0.005$ ($P < 0.05$; $n = 11$ recordings) after hypotonic
Fig. 6. Hyposmotic stress increases the average fusion pore conductance, pore dwell-time, and the probability of open fusion pore state. A: the average pore dwell-time increased significantly (0.16 ± 0.01 s at spontaneous conditions; n = 716 events) after hyposmotic stimulation (0.20 ± 0.01 s; n = 440 events) and KCl depolarization (0.20 ± 0.01 s; n = 2,372 events). Numbers above bars indicate the number of fusion events. B: hyposmotic stress increased the probability of observing an open fusion pore from 0.012 ± 0.002 (n = 37 patches) to 0.019 ± 0.005 (n = 11 patches), and depolarization increased the probability to 0.110 ± 0.04 (n = 8 patches). Numbers above bars indicate the number of patches. C: the mean Gp increased from 47 ± 4 pS (n = 261) to 73 ± 5 pS (n = 142) after hyposmotic stimulation and to 68 ± 4 (n = 200) pS after KCl depolarization. Numbers above bars indicate the number of fusion events with measurable Gp. *P < 0.05 and **P < 0.01.

stimulation. An even greater increase was observed after KCl depolarization (0.110 ± 0.04; P < 0.01; n = 8 recordings) (Fig. 6B).

Transient exocytosis could constrain peptide discharge from large dense-core vesicles owing to a narrow fusion pore (4, 8, 48, 49). Narrow fusion pore can act as a resistor in series and thus can reduce the signal in Im part of admittance by producing a correlated increase in Re part (cross talk) (32). Therefore, we analyzed transient fusion events with detectable cross talk to calculate the fusion pore conductance [Gp = (Re² + Im²)/Re] and vesicle capacitance [Cv = ([Re² + Im²]/Im)/φ] (32; Fig. 4C). The percentage of reversible events that exhibited cross talk was similar under isotonic, spontaneous (34%), and hypotonic conditions (32%). However, after KCl depolarization, only 8% of events exhibited cross talk. The mean fusion pore conductance (Gp) of spontaneous events with measurable cross talk was 47 ± 4 pS (range, 8–251 pS; n = 261 events). Gp increased to 73 ± 5 pS (range, 13–288 pS; P < 0.01; n = 142 events) after hyposmotic stimulation and to 68 ± 4 pS (range 9–537 pS; P < 0.01; n = 200 events) after KCl depolarization (Fig. 6C). Therefore, the mean pore diameter for events with measurable Gp increased from 0.94 ± 0.04 nm under spontaneous conditions to 1.18 ± 0.04 nm after hyposmotic stimulation and to 1.14 ± 0.04 nm after KCl depolarization.

DISCUSSION

The present study shows that mammalian lactotrophs release prolactin in a hypotonic environment and that this involves transient vesicle fusion pore openings.

Cell swelling and surface area changes in hypotonic conditions. Cells generally swell in response to hyposmotic conditions (27). Hypotonic stimulation of lactotrophs induced sustained cell swelling for more than 120 s. (Fig. 1). Using data on the cell diameter increase and assuming spherical geometry of lactotrophs, we estimate that the cell surface area increased by ~11% under our experimental conditions. Simultaneously, the peak fluorescence intensity profile also increased, indicating enhanced labeling of plasma membrane with FM 4-64. This may reflect exocytic addition of vesicles into the membrane. After full fusion with the plasma membrane, a single spherical dense-core vesicle 200 nm in diameter (43) may increase the membrane surface by 0.126 μm². Thus, an 11% increase in the surface area would correspond to ~78 vesicles fusing with the plasma membrane throughout the cell. Alternatively, increased staining of plasma membrane by FM 4-64 may be attributed to membrane expansion that changes local membrane curvature in response to hypotonic cell stimulation. Measurements with preloaded fluorescent peptides showed that one to five vesicles fused with the plasma membrane per 2-μm-thick optical slice (Fig. 3). In a cell 10–12 μm in diameter, this corresponds to a maximum of 25–30 fusing vesicles. These considerations support the notion that hypotonicity-induced cell swelling is associated with an increased surface membrane area. However, it is not clear why the actual number of vesicles that fused with the plasma membrane differed from the estimate. One possibility is that there is no simple relationship between cell swelling, surface membrane increase, and FM 4-64 staining, which is thought to reflect the membrane surface area (25). Another likely possibility is that hyposmotic challenge of lactotrophs resulted in expansion of the existing plasma membrane reservoirs-membrane folds, as described previously in astrocytes (36, 37) and in hepatocytes (17). Thus, both the exocytotic vesicle membrane additions and unfolding of the lactotrophs plasma membrane likely contributed to the increased plasma membrane staining by FM 4-64.

Cell volume increased by ~17% after hypotonic stimulation, as estimated from the change in cell diameter. When the osmolarity of the extracellular solution was reduced by ~30%, regulatory volume decrease was absent over the 2 min after the stimulation. Consistent with this finding, regulatory decreases in cell volume occur over 1–2 h (41), and cell volume and prolactin release are closely associated as extracellular osmolarity decreased (51).

Prolactin release from cell populations and from single vesicles in hypotonicity. To study hypotonicity-induced hormone discharge, we measured prolactin release from perfused rat lactotrophs (47) and monitored the dynamics of single large dense-core vesicles by fluorescence and electrophysiology techniques (4, 45, 53).

The amount of stimulus-induced prolactin release, estimated by integrating the signal over the basal level (Fig. 2), was ~30 ng during hypotonic stimulation and ~360 ng during KCl
depolarization. Since the density of cells was similar under both conditions, the reduced amount of hormone release after hypotonic stimulation may reflect reduced release of prolactin per cell. However, it cannot be entirely excluded that lactotrophs are functionally heterogeneous cells (29) and may thus exhibit distinct sensitivity to the two stimuli.

To examine this question further, we transfected cells with the ANP-EGFP construct that targets secretory vesicles (19). Prolactin (23 kDa; 14) and ANP.emd (32 kDa) appear to colocalize in the same vesicles (45) and have similar molecular weights; hence measurements of ANP-EGFP discharge likely reflect the rate of prolactin release from a single vesicle. We also monitored single vesicle exocytosis with FM 4-64, which stains the membrane and the matrix of individual vesicles (2) when a fusion pore forms between the docked vesicle and the plasma membrane. Since fewer vesicles discharged their cargos after hypotonic stimulation than after KCl depolarization (Fig. 3), the reduced prolactin release elicited by hypotonic stimulation vs. KCl depolarization (Fig. 2) is likely due to the reduced discharge of hormone per cell rather than due to response of a smaller, distinct fraction of cells in a heterogeneous population of lactotrophs (29).

Sustained prolactin release from KCl-stimulated cell population of lactotrophs (Fig. 2) likely reflects the discharge from readily docked/primed (45) vesicles as well as the newly recruited vesicles. In contrast, hypotonicity-evoked transient increase followed by the subsequent depression appears to preferentially involve hormone discharge from vesicles in the postfusion state only. As discussed below, in the majority of patches, hypotonicity failed to evoke new fusion events in previously silent patches and thus affected vesicle discharge properties at the postfusion state.

Transient fusion events are the predominant form of exocytosis in hypotonicity. In neurons, kiss-and-run exocytosis is unexpectedly common (16), although the appearance of this mode of exocytosis is debated (20). Transient fusion pore openings, although uncommon, were identified in neuroendocrine cells by electrophysiological measurements (28). Using cell-attached patch-clamp recordings, we found that reversible and irreversible steps occur spontaneously in lactotrophs. Irreversible steps (Fig. 3A, left) most likely represent full fusion exocytosis (35), whereas reversible steps (Fig. 4A, right, and Fig. 4B) probably represent kiss-and-run exocytosis (1, 13). Hypotonic stimulation increased the occurrence of transient fusion events, whereas it did not affect the occurrence of full fusion events (Fig. 5A).

The main obstacle for hormone release from a single vesicle is the diffusion through fusion pore, which opens transiently in our experiments (Fig. 4B). Increased hormone release from a single vesicle may be attained if stimulation affected the fusion pore dwell-time, prolonging the connection between vesicle lumen and the extracellular space (46, 48). Indeed, in our experiments, stimulation prolonged dwell-time and increased fusion pore open probability (Fig. 6, A and B). On the other hand, the pore diameter should be sufficiently wide to permeate the relatively large peptidergic hormones. The results revealed that stimulation affected fusion pore properties by also increasing the average $G_p$, which reflects widening of fusion pore diameter (Fig. 6C). Moreover, the incidence of events with measureable $G_p$ was fourfold smaller in KCl-stimulated cells, which shows that KCl is a stronger modulator of fusion pore diameter in comparison to hypotonicity. This is consistent with the difference in prolactin release responses of cell populations to the two respective stimuli (Fig. 2).

Hypotonicity modulates postfusion vesicle hormone discharge. Reversible exocytosis has been measured in various cell types (11, 16, 28, 31, 45, 48, 52) and appears to be the dominant mode of exocytosis in both nonstimulated and stimulated lactotrophs (48). Of 11 patches in which we observed discrete steps in $C_m$, in only one did hypotonic stimulation induce steps in a previously silent patch of membrane. This indicates that hypotonicity mainly affects the postfusion events, of which the molecular nature and Ca$^{2+}$ dependence awaits further studies.

In summary, the exposure of perfused rat lactotrophs to hypotonic stimulation caused a transient increase in prolactin release, followed by a sustained decrease below the basal level, which contrasts the sustained KCl-evoked release. This is likely due to the reduced fraction of release competent vesicles per cell in hypotonic vs. KCl depolarization. Vesicle release competence appears to be affected by postfusion pore properties, whereby transient vesicle fusion mediates hormone release.

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DISCLOSURE

The authors declare that they have no competing financial interests.

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