Temperature-dependent differences between readily releasable and reserve pool vesicles in chromaffin cells

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Abstract

Statistical differences between amperometric traces recorded from chromaffin cells using K⁺ and Ba²⁺ secretagogues support the assertion that readily releasable pool (RRP) and reserve pool (RP) vesicles can be probed with pool-specific secretagogues. Release from the RRP was evoked by K⁺ while release from the RP was evoked by Ba²⁺. Similar temperature-dependent changes in spike area and half-width for both pools suggest that the content of RRP and RP vesicles is similar and packaged in the same way. Differences between the vesicle pools were revealed in the temperature dependence of spike frequency. While the burst spike frequency of the RRP, which is comprised of pre-docked and primed vesicles, increased 2.8% per °C, the RP spike frequency increased 12% per °C. This difference is attributed to a temperature-dependent mobilization of the RP. Furthermore, the RP exhibited more foot events at room temperature than the RRP but this difference was not apparent at 37 °C. This trend suggests that RP vesicle membranes have a compromised surface tension compared to RRP vesicles. Collectively, the changes of release characteristics with temperature reveal distinctions between the RRP and the RP.

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1. Introduction

A common property of neurons and neuroendocrine cells that secrete via exocytosis is that their vesicles appear to be sequestered into different compartments [1–3]. One population of vesicles, the readily releasable pool (RRP) is ready for immediate release. This set of vesicles is near the cell membrane and has undergone an ATP-dependent priming step [4] that causes a conformation change of the SNARE and/or SM protein complex. This makes the docked vesicle fusion competent upon elevation of intracellular Ca²⁺ that causes a SNARE complex conformation change [5]. In chromaffin cells, 1–2% of the 22,000 large dense-core vesicles comprise the RRP and these can be mobilized for release within seconds upon stimulation [6]. Another major population is termed the reserve pool (RP). This population is not available for immediate release and requires mobilization to the release zone via the actin–myosin cytoskeletal system [7], followed by docking in the release zone either through the formation of the SNARE complex [8] or interaction with Sec1/Munc18 (SM) proteins [9]. Thus, release from this pool occurs tens of seconds after prolonged stimulation.

In a recent report, Wiegand et al. suggest that the RRP and RP can be selectively activated in chromaffin cells based on secretagogue choice [10]. They used a fluorescent timer protein to label the secretory vesicles [10,11]. Fluorescence images of chromaffin cells containing the timer protein show that the youngest vesicles, immobilized at the periphery of the cell [12], are selectively released with nicotine stimulation. Nicotine evokes immediate entry of Ca²⁺ through voltage sensitive channels and this evokes a high frequency burst of vesicular release. In contrast, stimulation with Ba²⁺ caused release from older vesicles, and these are vesicles that are loosely tethered in the center of the cell. Cells exposed to Ba²⁺ show sustained low frequency secretion. The mechanism whereby Ba²⁺ evokes exocytosis is not well understood, but it is thought to enter through Ca²⁺ channels and stimulate the exocytotic cycle without interacting with the Ca²⁺ pump or buffering system.
Thus, this work by Wiegand et al. suggests that the RRP and RP can be distinguished by the judicious selection of secretagogues.

This work compares the characteristics of exocytosis from vesicles evoked by K$^+$ and Ba$^{2+}$ from chromaffin cells by monitoring catecholamine release with amperometry at different temperatures [14,15]. Prior work has shown that temperature is an important parameter that reveals facets of the refilling of the RRP as studied by simultaneous Ca$^{2+}$-imaging and membrane capacitance Dinkelacker et al. [16]. This study demonstrated that the size of RRP adapts rapidly based on intracellular Ca$^{2+}$ concentration and that RRP refilling is more efficient at physiological temperature than at lower temperatures. The data presented herein confirm that K$^+$ and Ba$^{2+}$ selectively stimulate different vesicle pools. Further, the amperometric characteristics of these pools lend insight into their biochemical distinctions.

2. Experimental procedures

2.1. Chromaffin cell culture

Acutely dissociated bovine adrenal medullary chromaffin cells were maintained in culture as previously described [17]. Briefly, adrenal glands were collected from a local abattoir, rinsed, and digested using collagenase (Worthington Biochemical Corp, Lakewood, NJ). The epinephrine-containing chromaffin cells were isolated using a Renografin (Bracco Diagnostics, Inc., Mt. Vernon, IN) density gradient and then plated in DMEM/F-12 nutrient medium (Invitrogen Corp., Carlsbad, CA). Cells were used on days 3 through 7 of culture. Experimental buffers were prepared weekly with the following composition: 150 mM NaCl, 5 mM KCl, 10 mM Tris–HCl, 2 mM CaCl$_2$·2H$_2$O, 1.2 mM MgCl$_2$·6H$_2$O, and 5 mM glucose at pH = 7.4 for experiments with K$^+$ stimulation and 150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.2 mM MgCl$_2$·6H$_2$O, and 5 mM glucose at pH = 7.4 for experiments with Ba$^{2+}$ stimulation. There was no Ca$^{2+}$ present in the experiments using the Ba$^{2+}$ secretagogue to avoid competition between Ca$^{2+}$ and Ba$^{2+}$-influx triggering the release of vesicles. The osmolarity of secretagogue solutions was maintained by adjusting the concentration of NaCl. During secretion measurements, the cell culture plate temperature was controlled and measured using a heating stage (TC-324B, Warner Instrument Corp., Hamden, CT) placed on an inverted microscope with differential interference contrast optics (Eclipse TE2000-S, Nikon, Melville, NY).

2.2. Microelectrode preparation and operation

Carbon-fiber disk microelectrodes were fabricated as previously described [15]. Briefly, single 5-μm-diameter T-650 carbon fibers (Thornel, Amoco Co., Greenville, SC) were aspirated into glass capillaries (A-M Systems, Inc, Carlsborg, WA). The capillaries were heated and pulled to create a glass seal around the fiber. The protruding fiber was cut flush with the glass capillary and sealed with epoxy (EPON 828 resin from Miller-Stephenson, Danbury, CT and m-phenylenediamine hardener from DuPont Specialty Chemicals, Wilmington, DE). After the epoxy was cured, the microelectrode tips were beveled at a 45° angle on a micropipette polishing wheel and soaked for at least 10 min in 2-propanol purified with Norit A activated carbon [18].

The carbon-fiber working electrode was positioned with a piezoelectric driver (PCS-5000, Burleigh Instruments, Fishers, NY) to rest firmly on the cell membrane, covering ~10% of the cell surface area. Glass stimulating pipettes, tapered to a 10-μm-diameter and fire-polished, were filled with a secretagogue solution (60 mM K$^+$ or 5 mM Ba$^{2+}$) and positioned ~20 μm from the cell. Using forced air delivery (Picospritzer II, Parker Hannifin Corp, Fairfield, NJ), the solution was applied to the cell membrane for 1 min. The carbon-fiber microelectrode was held at a potential of +700 mV versus a Ag/AgCl reference electrode to oxidize released catecholamines. The potential was applied and the current was measured using an Axopatch 200B (Axon Instruments, Union City, CA), and a data acquisition board with locally written LabVIEW software (National Instruments, Austin, TX). The signal was filtered at 5 kHz with a low-pass Bessel filter and collected at 20 kHz. Amperometric traces were recorded for a minimum of 60 s and until 15 s passed without a vesicular release event. Before statistical analysis, each trace was digitally low-pass filtered at 400 Hz. During...
preliminary data analysis, amperometry traces were filtered at 25, 400, and 1000 Hz, and the same spikes were identified in all cases (data not shown).

2.3. Cell stimulation procedure

With both secretagogues, amperometric traces were collected both from individual cells at all four temperatures (>2 min between each stimulation) and from individual cells at each of four temperatures (stimulated only once). In the case of cells that were stimulated at all four temperatures, the order of the applied temperatures was randomized. In both K⁺ and Ba²⁺ experiments, there was no significant difference in the trace-averaged spike characteristics when one cell was sampled at all four temperatures versus when cells were only sampled once. In all cases, cells were allowed to equilibrate at a given temperature for more than 2 min before stimulation, and there was always at least a 2-min interval between stimulations to allow cell recovery.

2.4. Amperometric spike analysis

Spike analysis employed MiniAnalysis software (Synaptosoft, Decatur, GA). The spike detection threshold was set to five times the root-mean-square of the current noise. The spike area threshold was set at 60 fC, and spikes were only included in the trace average if the analysis routine could identify the three key spike characteristics: baseline, apex, and at least 80% decay back to baseline. After automated evaluation of the amperometric trace, the chosen spikes were visually examined so that spikes in close proximity to one another were not excluded. In cases where it appears that spikes are superimposed on a broader background current, the baseline for the spike was assumed to occur on top of the background current. The most likely cause of any background current is the nearly simultaneous exocytosis, as is common during the burst of release after K⁺ stimulation, of more vesicles than can be detected at the carbon-fiber microelectrode surface. The background current appears as an envelope because an amperometric spike is broader and the amplitude is diminished if release occurs at a site not directly beneath the electrode [19]. When less intense stimulation conditions are used, the background current is no longer measured; however, the stimulation conditions for this work were chosen to allow comparison to similar literature precedents.

For each amperometric trace, the average spike area ($Q$), width at half height ($t_{1/2}$), and frequency of spikes ($f$) were determined [20]. The $Q$ value reveals the number of molecules released from a vesicle and the $t_{1/2}$ value is a measure of the rate of extrusion of chemical messengers following cell-vesicle fusion (Fig. 1). Spike frequency was determined simply by dividing the total number of spikes by the time elapsed from the occurrence of the first spike to the occurrence of the last spike or a fixed time, as specified in the text. This procedure provides a measure of the number of spikes elicited by a particular stimulus, but masks burst-like behavior. In cases where the amperometric spikes occur on top of a broad background current, errors may be introduced in calculations of $Q$ or $t_{1/2}$ but frequency values will not be influenced.

A minority of amperometric events are preceded by a pre-spike feature, also known as a foot. The foot reveals an arrested state of vesicle-cell membrane fusion where vesicle contents leak through a small fusion pore. To verify that feet are not the result of two overlapping full fusion spikes, extensive analysis was performed considering the frequency of time intervals between spikes elicited with both K⁺ and Ba²⁺ stimulation (vide infra, Fig. 5C). Feet were identified manually during trace analysis. The relative area of feet to their associated spikes was determined with a MiniAnalysis sub-routine. The apex of the spike, the baseline of the spike, and the inflection point between the foot and the full fusion spike were marked. The integrated area was split into foot area
and full fusion spike area based on the inflection point and their ratio was taken. The mean values of $Q$, $t_{1/2}$, $f$, and foot spike ratios were determined at each cell and condition tested, and then the data from multiple cells were combined to give the mean values presented.

2.5. Statistics

Linear regression (data series) or $t$-test (data pairs) analysis to evaluate statistical significance ($p<0.05$) was accomplished in Excel. All data are reported as the mean±SEM. Cells were pooled based on stimulation conditions across three separate preparations and different days within each preparation; in each case, data collected with different electrodes or on different days were statistically indistinguishable from other data collected with the same stimulation conditions. Further, the slopes and intercepts from the linear regression analyses were compared using GraphPad Prism. Calculation of the two-tailed $p$ value for each pair of linear regression slopes revealed whether or not the slopes were identical (parallel lines). If the $p$ value was greater than 0.05, a pooled slope was calculated. A second $p$ value could then be calculated to compare the intercepts. If this $p$ value was less than 0.05, then the regression lines were reported parallel but not identical.

2.6. Materials

NORIT A decolorizing activated carbon was obtained from ICN biomedicals, Inc. (Aurora, OH). NaCl and KCl were purchased from Fisher Scientific (Fair Lawn, NJ). Anhydrous alpha-D(+)-glucose was obtained from Acros Organics (Morris Plains, NJ). All other compounds were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were used without further purification.

3. Results

3.1. Temperature-dependent exocytosis using the $K^+$ secretagogue

A 3-s bolus of 60 mM $K^+$ was delivered to individual chromaffin cells at four different temperatures between 25 °C and 37 °C (example trace in Fig. 2A). At all temperatures, $K^+$ elicits short, high frequency bursts of secretion. Each spike represents the exocytosis of catecholamine from an individual vesicle [21]. Fig. 3 presents the trace-averaged spike characteristics as a function of four temperatures ($n>12$ cells at all temperatures). Linear regression analysis reveals that spike area increases significantly ($y=16x+35$, $p<0.004$) with increased buffer temperature. The area under a spike can be directly related to the number of molecules released by using Faraday’s Law. In the data shown in Fig. 3A, the average quantal size as a function of increasing temperature is: 2.2, 2.6, 2.6, and 3.4 attomoles. Linear regression analysis in Fig. 3B reveals that $t_{1/2}$ decreased significantly ($y=-0.82x+51$, $p<0.00003$) with increased buffer temperature. Finally, the spike frequency was analyzed for the first 10 s of release (Fig. 3C). Linear regression analysis in Fig. 3C reveals that the burst frequency increases significantly ($y=0.07x+0.50$, $p<0.005$) with increased buffer temperature. These trends of increased spike area, decreased spike $t_{1/2}$, and increased spike frequency with increased buffer temperature are in agreement with an abbreviated study previously performed by Pihel et al. where only 22 °C and 37 °C temperatures were used on a smaller population of cells [22]. Quantitative differences between the previous study and this work are likely due to variations in cell culture procedure, carbon-fiber microelectrode fabrication, or analysis software.

3.2. Temperature-dependent exocytosis using the $Ba^{2+}$ secretagogue

A 5-s bolus of 5 mM $Ba^{2+}$ was delivered to individual chromaffin cells at four different temperatures from 25 °C to 37 °C (example trace in Fig. 2B). Note the prolonged time of spike occurrence when stimulating with $Ba^{2+}$ in comparison to the short bursts measured when exocytosis is induced using $K^+$ stimulation (Fig. 2A). Fig. 4 presents the trace-averaged spike characteristics as a function of extracellular buffer temperature ($n>12$ cells for all temperatures). Linear regression analysis reveals that spike area increased significantly ($y=25x+130$, ...
p<0.03) with increased buffer temperature. In the data shown in Fig. 4A, the average quantal size as a function of increasing extracellular buffer temperature is: 3.6, 4.7, 5.1, and 5.2 attomoles. Linear regression analysis in Fig. 4B reveals that \(t_{1/2}\) decreases significantly \((p<0.001)\) as would be expected based on the aforementioned point-by-point t-test results. The spike \(t_{1/2}\) pooled slope indicates a 0.72 ms decrease per °C but, again, the line intercepts are significantly different \((p<0.0003)\). However, the change in average spike frequency per °C was statistically distinct \((p<0.03)\); while the spike frequency increased 3.1% per °C with K⁺ stimulation, it increased 12% per °C with Ba²⁺ stimulation.

3.3. Foot events occurring with K⁺ or Ba²⁺ secretagogues

A representative example of a foot event during K⁺-stimulated exocytosis is shown in Fig. 5A. On average, 5% of the full fusion events caused by K⁺ stimulation had a foot, and the foot area was 20% of the full fusion spike area. Linear regression analyses of both relative foot event occurrence (Fig. 5B, triangles) and relative foot area (5D, triangles) for events associated with K⁺ stimulation revealed no significant trend as a function of buffer temperature. For Ba²⁺-stimulated events at 37 °C, 5% of the full fusion events had a foot, and the foot area comprised 15% of the full fusion spike area. However, unlike K⁺-stimulated secretion, the occurrence of feet for Ba²⁺-stimulated secretion was temperature dependent; linear regression analysis of relative foot occurrence revealed an increased occurrence of feet (Fig. 5B, diamonds) with decreased buffer temperature \((p<0.001)\). However, like K⁺-stimulated exocytosis, the relative area of feet from Ba²⁺-stimulation was not significantly influenced by buffer temperature (Fig. 5D, diamonds).

To assess the possibility that foot events are not revealing fusion pore formation but instead nearly coincident full fusion events, histograms were constructed for the interspike intervals in both K⁺ \((n=15 \text{ cells})\) and Ba²⁺-stimulated \((n=14 \text{ cells})\) secretion at 37 °C. Based on the average spike \(t_{1/2}\) of approximately 20 ms at 37 °C in both cases (Figs. 3B and 4B), an interspike interval of 25 ms or less may result in the misidentification of coincident spikes as a foot-spike combination. In K⁺-stimulated release, the average interspike interval in the first 10 s of secretion (measured from apex to apex) is 275 ms and only 0.72% of the measured spike intervals are 25 ms or less (Fig. 5C, squares). In Ba²⁺-stimulated release, the average...
interspike interval is 453 ms and only 0.42% of the measured spike intervals are 25 ms or less (Fig. 5C, diamonds). In both cases, there is approximately a 10-fold difference between the number of spikes that could be misidentified as a foot-spike combination and the actual number of feet detected.

4. Discussion

This work explores the possibility of difference in vesicle pools in chromaffin cells released by two secretagogues using amperometry and temperature variation. Prior work has suggested that high K⁺ elicits transient exocytosis from the docked and primed vesicles of the RRP and that Ba²⁺ predominantly releases vesicles from the RP [11]. Direct comparison of secretion characteristics reveals that K⁺ and Ba²⁺ do recruit pools of vesicles with different characteristics. In the simplest comparison, the average number of spikes per trace (representative of the number of vesicles undergoing fusion) was calculated using each secretagogue at 37 °C. Nearly two times more vesicles are secreted when Ba²⁺ is used as the secretagogue instead of K⁺. This leads to the hypothesis that Ba²⁺ and K⁺ stimulate release of the same vesicles but that Ba²⁺ acts for an extended period of time. In order to prove or disprove this hypothesis, it is necessary to compare the temperature-dependent Q, 1/2, frequency, and foot characteristics using both secretagogues.

The efficiency of the last step in the vesicular secretion sequence, vesicle extrusion, is apparent in the spike area that reveals the number of catecholamine molecules extruded from each vesicle. While this quantity increases with temperature for both the K⁺- and Ba²⁺-stimulated secretion, significantly more molecules are secreted from Ba²⁺-stimulated vesicles at all four temperatures investigated. Similarly, for both secretagogues, the rate of vesicle extrusion, as revealed by the mean 1/2 values, increases as the temperature is raised to 37 °C; however, the extrusion times are shorter for vesicles secreted following Ba²⁺ stimulation. The similarities in the temperature dependence of the extrusion amount and time course suggests that the underlying extrusion processes for both K⁺- and Ba²⁺-stimulated secretion are controlled by the same factors. The frequency of release, governed by mobilization, docking, priming, and fusion, increases with increasing buffer temperature when using either secretagogue. However, statistical comparison of the linear regression slopes reveals a substantial difference in vesicle fusion competency which could be attributed either to differences in membrane characteristics or the actions of Ca²⁺ versus Ba²⁺. Similarly, foot occurrence is temperature independent with K⁺ stimulation but is temperature dependent with Ba²⁺ stimulation. Overall, the amperometric characteristics reveal that K⁺ and Ba²⁺-stimulated vesicles have some common characteristics but likely represent distinct populations of vesicles.

The results show that the last step of the exocytotic sequence, vesicle extrusion, becomes more efficient as the bath
temperature is raised to physiological temperature for both the K\(^{+}\)- and Ba\(^{2+}\)-stimulated vesicles. Indeed, linear regression reveals that \(\sim 6 \times 10^4\) more molecules are released for each additional degree from both pools. The most likely explanation for the temperature dependence of the spike area and \(t_{1/2}\) values is the thermodynamic behavior of the chromogranin A intravesicular matrix. Yoo [23] characterized the pH- and temperature-dependent expansion of the chromogranin A polymer. Under vesicular conditions (pH=5.5, \([\text{Ca}^{2+}] = 35–40\) mM, osmolarity>750 mOsM), the tetrameric chromogranin A is composed of 60–65% random coil with 25–40% \(\alpha\)-helicity. As the vesicle fuses with the cell membrane, exposing the vesicular contents to the extracellular environment (pH=7.4, \([\text{Ca}^{2+}] = 2\) mM, osmolarity=310 mOsM), pH, \([\text{Ca}^{2+}]\), and osmolarity gradients drive the conformational change of chromogranin A to a dimer with increased random coil character. In a thermodynamic study, Yoo et al. demonstrated that the interaction between chromogranin A and the intravesicular membrane, once exposed to extracellular conditions, becomes less stable and the polymer matrix becomes more disordered as temperature increases towards physiological temperature [24]. As chromogranin A undergoes this conformational change, catecholamines associated with ATP in the intravesicular matrix can be released into the extracellular space as cation exchange occurs with the monovalent ions in the extracellular buffer. This similar temperature dependence indicates that the contents of the two distinct vesicles pools are packaged in the same way. Alternate explanations, such as diminished vesicular catecholamine stores at lower temperature, would lead to shorter \(t_{1/2}\) values because extrusion time would decrease due to the weakened intravesicular associations following the loss of stored catecholamine. Also, control experiments with direct delivery of catecholamine to the electrode were done at various temperatures to rule out a temperature-dependent change in detection efficiency.

While the temperature-dependent matrix expansion is the same for the both vesicle pools, statistical analysis of other spike characteristics reveals significant distinctions. For example, the average temperature-dependent spike frequency of the K\(^{+}\)-stimulated secretion increased 3.1% per °C. This result is consistent with the pre-docked and primed nature of RRP vesicles and indicates that vesicle–cell membrane fusion is a temperature independent process. In contrast, the Ba\(^{2+}\)-induced spike frequency increases 12% per °C. During release from the RP, the vesicles (stored remote from the release zone, unlike RRP vesicles) must be mobilized, docked, and primed before vesicle–cell membrane fusion can occur. Therefore, the data indicate that one of these steps must be highly temperature dependent. Consistent with these results, capacitance and amperometric measurements by Neher and coworkers demonstrated that mobilization is a slow step with the transition from the reserve pool to a docked state requiring 10 or more seconds [25]. These data support the conclusion that K\(^{+}\) and Ba\(^{2+}\) are selective secretagogues for the RRP and RP, respectively. It is impossible, using only the presented data, to exclude the alternative that Ba\(^{2+}\) is stimulating release of both the RRP and RP vesicles, though this possibility seems unlikely considering that the amperometric traces recorded with Ba\(^{2+}\)-incited secretion do not include the burst of RRP spikes and that the vesicle contents are distinct. If the Ba\(^{2+}\) secretagogue does solicit exocytosis of both the RRP and RP, the differences between the RRP and RP vesicles are even larger than those represented by the average data presented in this work.

As diagrammed in the inset of Fig. 1, the three steps by which a docked and primed vesicle progresses from a fusion-competent state to a fully fused state can be viewed as a transition from a primed state (before a secretion event) to a state where vesicle–cell membrane fusion has occurred (foot current) and to the final state that culminates when the vesicle is fully fused, maximizing the contact between the intravesicular contents and the extracellular space (spike). The factors influencing the transition from the primed to initial fusion state are the arrangement of SNARE and SM proteins and the Ca\(^{2+}\)-triggered vesicle–cell membrane fusion [9]. The transition from the foot to the full fusion state is promoted by the chromogranin A matrix expansion [26] and restrained by the energy required to separate the intracellular plasma membrane from the cytoskeleton [27]. Thus, feet are only observed when matrix expansion is counterbalanced by the resistance of the fusion pore to open further for greater than one ms [28], allowing for a finite duration of the foot current. The proteinaceous or lipidic nature of the fusion pore is under debate [29,30]. However, because matrix expansion is facilitated by higher temperatures, maintenance of constant foot probability, as observed in K\(^{+}\)-stimulated release events, reveals that the energy barrier for fusion pore enlargement also increases proportionally to temperature. Theoretical models have demonstrated that this energy barrier depends on the sum of the vesicle and plasma membrane surface tensions [27]; while the membrane surface tensions are known to be largely invariable at temperatures above 37 °C [31], the surface tension characteristics at subphysiological temperatures are unknown.

Release from the RP differs from the RRP in that more feet are observed at lower temperatures. As the extracellular buffer temperature is increased, the percentage of spikes with feet decreases, approaching the number of temperature-independent fusion pore events measured from the RRP at physiological temperature. Thus, at sub-physiological temperatures, the transition from primed vesicle to fully fused vesicle occurs in a suboptimal fashion for some RP vesicles, leading to more frequent feet. This could be due to either inferior priming of RP vesicles (non-optimal protein conformation) or compromised vesicle–cell membrane interaction during RP secretion. Based on the combined facts that increased temperature leads to increased fusion efficiency (fewer feet and more full fusion events) when RP vesicles are stimulated and that the temperature-dependent chromogranin A matrix expansion is indistinguishable in RRP and RP vesicles, it is clear that RP vesicles experience a lower energy barrier to expand the fusion pore. This trend suggests that RP vesicle membranes have a compromised surface tension compared to RRP vesicles.

In summary, this work has exploited controlled temperature and single cell amperometry to verify that K\(^{+}\) and Ba\(^{2+}\) secretagogues can be exploited to explore differences between
the RRP and the RP vesicles. Comparison of RRP vesicle spike characteristics with varied extracellular buffer temperature conditions reveals that chromogranin A matrix expansion is the rate-determining step in secretion from this small pool of vesicles that responds immediately to physiological stimulation; this conclusion is in agreement with work done by Trifaró and coworkers [32]. The extent and rate of the RP vesicle chromogranin A matrix expansion is distinct from that of RRP vesicles, with more catecholamine being released faster from RP vesicles. However, temperature also influences RP vesicle mobilization and protein machinery in the transition from the primed vesicle to a fully fused vesicle. Upon comparing the spike frequency and foot characteristics from RRP and RP vesicles, it becomes clear that there is a functional distinction between the newly synthesized vesicles that are used immediately upon stimulation and the older vesicles that are only exocytosed with prolonged and intense stimulation in spite of the fact that these vesicles are likely to dock, prime, and fuse in the same release zone.

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