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EXTRACELLULAR IONIC COMPOSITION ALTERS KINETICS OF VESICULAR RELEASE OF CATECHOLAMINES AND QUANTAL SIZE DURING EXOCYTOSIS AT ADRENAL MEDULLARY CELLS

by

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Exocytosis caused by Ba^{2+} in Ca^{2+}-free medium had a larger mean spike area at pH 8.2 than at pH 7.4. When Ba^{2+}-induced spikes measured at pH 7.4 were compared, the spikes in Ca^{2+}-free medium were found to be broader and shorter, but had the same area. Release induced by K^+ after exposure to Ba^{2+} was comprised of larger quantal events when compared to preceding K^+ stimulations. Finally, spikes obtained with Ba^{2+} exposure at an extracellular pH of 5.5 had a different shape than those obtained in more basic solutions. These changes in spike size and shape are consistent with the interactions between catecholamines and other intravesicular components.

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EXTRACELLULAR IONIC COMPOSITION ALTERS KINETICS OF VESICULAR RELEASE OF CATECHOLAMINES AND QUANTAL SIZE DURING EXOCYTOSIS AT ADRENAL MEDULLARY CELLS

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ABSTRACT

The temporal resolution of carbon-fiber microelectrodes has been exploited to examine the plasticity of quantal secretory events at individual adrenal medullary cells. The size of individual quantal events, monitored by amperometric oxidation of released catecholamines, was found to be dependent on the extracellular ionic composition, the secretagogue, and the order of depolarization delivery. Release was observed with either exposure to 60 mM K^+ in the presence of Ca^{2+} or exposure to 3 mM Ba^{2+} in solutions of different pH, with and without external Ca^{2+}. Ba^{2+} was demonstrated to induce Ca^{2+}-independent exocytotic release for an extended period of time (>4 min) relative to release induced by K^+ (~30 s), which is Ca^{2+}-dependent. In all cases, simultaneous changes of intracellular divalent cations, monitored by fura-2 fluorescence, accompanied quantal release and had a similar time course.

Exocytosis caused by Ba^{2+} in Ca^{2+}-free medium had a larger mean spike area at pH 8.2 than at pH 7.4. When Ba^{2+}-induced spikes measured at pH 7.4 were compared, the spikes in Ca^{2+}-free medium were found to be broader and shorter, but had the same area. Release induced by K^+ after exposure to Ba^{2+} was comprised of larger quantal events when compared to preceding K^+ stimulations. Finally, spikes obtained with Ba^{2+} exposure at an extracellular pH of 5.5 had a different shape than those obtained in more basic solutions. These changes in spike size and shape are consistent with the interactions between catecholamines and other intravesicular components.

Key words: chromaffin cells, catecholamine, barium induced exocytosis, fura-2, calcium independent exocytosis

Running title: Effects of extracellular ions on quantal release

INTRODUCTION

Many cells secrete chemical substances by exocytosis, whereby intracellular vesicles fuse with the plasma membrane and extrude their contents into the extracellular space (for reviews see Almers, 1990; DeCamilli and Jahn, 1990; Burgoyne and Morgan, 1993). This process has classically been viewed as an all-or-none secretory event (Martin, 1966; Viveros et al., 1969) and, as a result, is often referred to as quantal secretion (Bekkers et al., 1990). Single exocytotic events involving the release of electroactive substances can now be quantitated by oxidation with a carbon-fiber microelectrode placed adjacent to a cell (Leszczyszyn et al., 1990; Wightman et al., 1991; Chow et al., 1992; Schroeder et al., 1992; Alvarez de Toledo et al., 1993). This technique is used here to probe changes in exocytosis as indicated by the size and shape of individual current spikes.

Bovine adrenal medullary cells, which are used in this work, have played a benchmark role in numerous investigations of exocytosis (for reviews see Viveros, 1975; Pollard et al., 1985; Burgoyne, 1991). These cells secrete catecholamines which are stored in vesicles at a high concentration (>300 mM (Winkler, 1976; Schroeder, 1992)). The vesicles contain several other substances including Ca^{2+} (30 mM (Bulenda and Gratzl, 1985)) and chromogranin A (CGA, 1 mM). CGA is a highly acidic protein (Yoo and Albanesi, 1991; Helle et al., 1993; Winkler, 1976) which aggregates and binds Ca^{2+} and catecholamines at the intravesicular pH of 5.5 (Videen et al., 1992; Yoo and Lewis, 1992). As a result it has been proposed to play the role of a vesicle condensing protein (Simon and Aunis, 1989; Winkler and Fisher-Colbrie, 1992). Thus, exocytotic release must involve dissociation of the intravesicular contents as well as extrusion of the vesicle contents.

In previous work with bovine adrenal medullary cells, we have examined the characteristics of catecholamine concentration spikes resulting from individual exocytotic events induced by Ca^{2+} entry after digitonin permeabilization (Jankowski et al., 1993; Ciolkowski et al., submitted). Spike symmetry, amplitude, area, and frequency were found to be dependent on

the extracellular pH. Furthermore, at each pH value examined, the catecholamine spikes were temporally broadened relative to that expected for free diffusion of a concentration packet of catecholamines away from the cell surface (Schroeder et al., 1992). Partial release of vesicular contents has been detected with amperometric measurements of serotonin release from mast cells (Alvarez de Toledo et al., 1993). All of these observations suggest that the state of association of the vesicle contents can affect the temporal characteristics and quantal size of an exocytotic event.

In this work we investigate the shape of the amperometric spikes induced by exposure to Ba^{2+}. This secretagogue is unique in its ability to cause Ca^{2+}-independent secretion, and, therefore, allows examination of the effects of alteration of extracellular Ca^{2+} gradients on spike shape. The results show that the quantal size and time course of extrusion of the vesicular contents are affected both by the external pH and the external Ca^{2+} concentration.

EXPERIMENTAL PROCEDURES

Electrodes and Electrochemical Procedures. Carbon fibers (5-μm radius, Thornell P-55, Amoco, Corp., Greenville, SC) were used to prepare microelectrodes (Kawagoe et al., 1993) which were polished to obtain an elliptical sensing surface with an average radii of 6 μm. Electrodes were operated amperometrically with an applied potential of 0.650 V versus a saline-saturated calomel electrode.

Single Cell Stimulus-Secretion Experiments. Primary cultures of bovine adrenal medullary cells were prepared from fresh tissue and cultured as described previously (Wightman et al., 1991). Cell populations were enriched in epinephrine-containing cells by differential centrifugation with a single step Renografin gradient (Moro et al., 1990). Experiments were conducted within days 3 and 10 of culture. For release studies, the culture medium was replaced with the incubation buffer which contained 150 mM NaCl, 4.2 mM KCl, 1.0 mM Na_2HPO_4, 11.2 mM glucose, 0.7 mM $MgCl_2$, and 10 mM HEPES with the pH adjusted to the desired value using NaOH. In Ca^{2+}-containing solutions 3.0 mM $CaCl_2$ was employed. Without added Ca^{2+}, the buffer contained 2.8 μM free Ca^{2+} as determined with an ion-selective electrode (Orion Model 93-20, Cambridge, MA). To further lower the concentration of free Ca^{2+}, 0.2 mM EGTA (with 6 mM NaCl to maintain osmolarity) was added to prepare the solutions termed Ca^{2+}-free. This was done at all pH values even though EGTA has a much lower affinity for Ca^{2+} at pH 5.5. $BaCl_2$ (3.0 mM in incubation buffer without Ca^{2+} or Mg^{2+}) and KCl (60 mM in incubation buffer with 3 mM $CaCl_2$ and NaCl reduced to 94.2 mM) were applied to cells via pressure ejection from micropipettes. Experiments were conducted at room temperature on the stage of an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY). Carbon-fiber microelectrodes were positioned 1 μm from the surface of individual cells using a piezoelectric driver (PCS-250 Patch Clamp Driver, Burleigh Instruments, Fishers, NY) as previously described (Schroeder et al, 1992).

Data Analysis. Unfiltered electrode responses were recorded on 1/2" videotape. When replayed, the records were low pass filtered at 2600 Hz

(CyberAmp 320, Axon Instruments, Inc., Foster City, CA), and computer digitized at 0.2 msec/pt (Fetchex, Axon Instruments, Foster City, CA). Spikes were computer selected with locally written software (Schroeder et al., 1992) if their amplitude was five times greater than the root-mean-square noise of the record. Skew values of the spikes were calculated as described by Foley and Dorsey (1983). Because the data is inherently nonparametric, significance was evaluated by the Mann-Whitney test (Systat, Evanston, IL).

Fluorescence studies. Chromaffin cells cultured on glass coverslips (Assistent Glass, Carolina Biological Supply, Burlington, NC) were loaded at room temperature for 60 minutes with 1 µM fura-2 AM (Molecular Probes, Eugene, OR) in Krebs-Ringer buffer (145 mM NaCl, 5 mM KCl, 1.2 mM Na_2HPO_4, 10 mM glucose, 1.3 mM $MgCl_2$, and 20 mM HEPES, pH 7.4 (O'Sullivan et al., 1989)) containing 4 mM EGTA, and 0.1% bovine serum albumin. The culture plate was then rinsed twice and refilled with Krebs-Ringer buffer of the desired pH and Ca^{2+} concentration. Ionic concentrations were adjusted as in the stimulus-secretion studies. Fura-2 fluorescence measurements at single cells were made at room temperature with a 40x oil immersion objective (Fluor 40/1.3, Nikon, Melville, NY) on an inverted microscope equipped with an EMPIX Photometer System (EMPIX Imaging, Mississauga, Canada) which employs a 510 nm (40 nm bandpass) emission filter. Fluorescence intensities from alternate excitation at 340 nm and 380 nm were measured every 110 ms through a 27 µm pinhole aperture at the photomultiplier tube, corrected for autofluorescence and electrode reflectance, and then ratioed (F_{340}/F_{380}).

Fura-2 can bind to Ba^{2+} as well as Ca^{2+} but with a greater affinity for Ca^{2+} than Ba^{2+} (($K_{d,Ca}$ = 224 nM (Grynkewicz et al, 1985), $K_{d,Ba}$ = 780 nM (Schilling et al., 1989), both at 37°C). In addition, the wavelength for maximal excitation of fluorescence differ for the bound forms. This results in a difference in the maximum fluorescence ratio as measured both *in vitro* and *in situ* with our instrument. *In vitro* calibration curves were constructed in EGTA-buffered solutions which mimic the cytosol (115 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM KMOPS, pH 7.1 (Schilling et al., 1989)). The maximum ratio

(obtained with 5 mM free Ca^{2+}) was 8.68 times greater than that for Ba^{2+} (3 mM free concentration). *In situ* calibrations, obtained in single cells permeabilized with 2 μM ionomycin (Williams and Fay, 1990) showed some cell-to-cell variability, and the relative sensitivity to the two ions was only 65% of that found *in vitro*.

Reagents. Unless noted above, chemicals were obtained from Sigma (St. Louis, MO). Solutions were prepared in doubly distilled water.

RESULTS

Comparison of release caused by K^+ and Ba^{2+} at pH 7.4. At pH 7.4, a 3-s pressure ejection of 60 mM K^+ with 3 mM Ca^{2+} onto a single cell caused amperometric spikes (exocytotic release of catecholamine) with or without Ca^{2+} in the external media (Figure 1A,B). Simultaneous fluorescence measurements in cells preloaded with fura-2 showed a corresponding increase in the fluorescence ratio (increase in $[Ca^{2+}]_i$). Both responses returned to baseline values 30 to 60 s after the initial exposure. Exposure of individual cells to K^+ without Ca^{2+} did not cause amperometric spikes (Wightman et al., 1991) or a change in the fluorescence ratio.

Having established cell viability with two exposures to K^+ with Ca^{2+}, the same cells were exposed to 3.0 mM Ba^{2+} for 15 s. Amperometric spikes were observed whether or not Ca^{2+} was present in the external medium (Figure 1). Secretion lasted for at least 4 min and was accompanied by a sustained increase in the fluorescence ratio. The extended time course of both responses is in sharp contrast to the responses induced by 3-s exposure to 60 mM K^+. (K^+ application for 15 s also led to transient release whereas 3-s applications of Ba^{2+} caused release for several minutes (data not shown)).

A short time (1-2 min) after the initial rise in the fluorescence ratio and release of catecholamine following exposure of the cells to Ba^{2+}, concomitant fluctuations in the ratio and release were consistently observed at cells in Ca^{2+}-containing buffer (Figure 1B). Such cytosolic Ca^{2+} oscillations are often observed during long-lasting stimuli which alter internal Ca^{2+} stores (Berridge, 1990; Tse et al., 1993).

For experiments in media containing Ca^{2+} the cells were exposed to K^+ (3-s pressure ejection, two exposures) after the Ba^{2+}-induced release had subsided. The fluorescence ratio increased with K^+ as observed before exposure to Ba^{2+}. However, the characteristics of the spikes were altered relative to those induced by K^+ at the same cell before exposure to Ba^{2+} (Table 1). The means for the spike area (Q) and width at half-height ($t_{1/2}$) were significantly increased.

Spike characteristics as a function of pH. Amperometric spikes were also induced by Ba^{2+} from cells in buffers at pH 8.2 (Figure 2) or 5.5 (Figure 1C,D) whether or not Ca^{2+} was present in the buffer. In contrast, K^+ at pH 5.5 is unable to induce release although small changes in the fluorescent ratio were seen when the buffer contained Ca^{2+} (Figure 1D). The average characteristics (area, width at half height, and peak current (i_{max})) for Ba^{2+} induced spikes are summarized in Table 2. The spike area is particularly significant because it is a direct measure of the quantity of material detected, and thus, the mean value is the quantal size (Jankowski et al., 1992). Under all conditions, a broad distribution of Q values is observed (e.g., Figure 2B). This distribution is consistent with the variation in chromaffin cell vesicular radii (Schroeder et al., 1992).

The experimental results with the most significant differences are summarized in the following sections.

Ba^{2+}-induced spikes at pH 7.4 and 8.2 in the absence of Ca^{2+}. Release at pH 7.4 and 8.2 appears qualitatively similar (Figure 2A). However, the distribution of spike areas (Figure 2B) and their mean values (Table 2) show that the quantal size is increased at pH 8.2. In Ca^{2+}-free medium, the mean quantal size of spikes induced by Ba^{2+} is 50% greater at pH 8.2 than at pH 7.4, a significant ($p < 0.001$) change seen as a shift in the histogram.

The two spikes in Figure 2C have the average values obtained at each pH. At pH 8.2 the mean $t_{1/2}$ value is increased by 30% relative to that at pH 7.4, a significant ($p < 0.001$) change. The increase in $t_{1/2}$, which reflects the rates of dissociation and diffusion of the vesicular contents to the microelectrode surface, demonstrates that the increase in quantal amount is accompanied by an alteration in the rate of vesicular extrusion.

Ba^{2+}-induced spikes at pH 7.4 as a function of external Ca^{2+}. The mean quantal sizes following exposure to Ba^{2+} at pH 7.4 are the same regardless of the extracellular Ca^{2+} concentration (Table 2). However, in the absence of Ca^{2+} the mean amplitude of spikes induced by Ba^{2+} are significantly ($p < 0.001$) lower and the mean half widths are significantly ($p < 0.001$) wider than those

obtained in medium containing Ca^{2+} (37% decrease in mean i_{max}, 50% increase in mean $t_{1/2}$). Histograms of these values clearly show these differences (Figure 3A). When Ca^{2+} is absent from the buffer, fewer large amplitude spikes and fewer narrow spikes are observed. Spikes which have the mean characteristics of those measured during exposure to Ba^{2+} at pH 7.4 are shown in Figure 3B for solutions with and without Ca^{2+}.

Spike shape at pH 5.5. Under physiological conditions, the shape of individual spikes has been found to be similar to that predicted for diffusional dispersion from a point source (Schroeder et al., 1992; Jankowski et al., 1993; Ciolkowski et al., submitted). The shape of such a spike is characterized by a sharp rise followed by slow decay, resembling an exponentially modified gaussian (Schroeder et al., 1992). Qualitatively, a significant number of the spikes obtained in response to Ba^{2+} at pH 5.5 without extracellular Ca^{2+} appeared to be more symmetric than expected for diffusional dispersion.

To quantitatively analyze their symmetry, skew values for the spikes were determined. The measure of skew employed was originally developed to characterize the asymmetry of chromatographic peaks (Foley and Dorsey, 1983). In this analysis, a diffusion-controlled shape has a skew value (termed γ_s) of 1.8 while a purely gaussian shape has a value of 0.4. Because the method is based on the peak width at 10% of the peak height, skew values less than 1.7 also may indicate the presence of a pre-spike feature (Chow et al., 1992). Example spikes are shown in Figure 4. The spike in Figure 4A has a skew value of 0.41, and noticeably deviates from the superimposed diffusion-controlled shape. The spike in Figure 4B has a low skew value (0.77) caused by a pre-spike feature. Figure 4C shows a spike with a skew of 1.0, and in Figure 4D a spike is shown with a skew of 1.8 which correlates ($r = 0.98$) with that predicted for a diffusion-controlled shape. As these examples show, the spikes with low skew values have a greater shape symmetry or a distinct pre-spike feature.

Skew values of spikes detected at pH 5.5 were compared to those obtained

at pH 8.2 (Jankowski et al., 1993). At pH 8.2 in the absence of Ca^{2+}, the mean skew value is 1.78 and 96.7% of the spikes fall in the range of 1.7-1.9. However, at pH 5.5 without Ca^{2+}, 30.5% of the spikes have skew values less than 1.7 indicating a deviation from the diffusion-controlled shape.

DISCUSSION

In this study we establish that the release of catecholamines induced by Ba^{2+}, both in the absence and presence of Ca^{2+}, occurs as a prolonged series of sharp, amperometric spikes. This confirms that an influx of Ba^{2+} can substitute for Ca^{2+} as a trigger for exocytosis. Second, we show that the characteristics of the packets of catecholamine released by Ba^{2+} are dependent on the composition of the extracellular medium. A hypothesis which accounts for this observation is that the temporal characteristics of the individual vesicular events are dependent on the state of association of the vesicle contents prior to exocytosis.

In prior work we have shown that the area and distribution of amperometric spikes at pH 7.4 is independent of the secretagogue employed and its concentration (Wightman et al., 1991). Release induced by Ba^{2+} in the presence of Ca^{2+} at pH 7.4 shares this characteristic in that the mean characteristics of the spikes are similar to those induced by K^+ (Tables 1 and 2). However, release induced by Ba^{2+} continues for a longer time than that induced by K^+ (Figure 1) even though both secretagogues are applied transiently in the form of small droplets. The droplets are rapidly diluted by the incubation medium as a result of diffusion and convection (Leszczyszyn et al., 1991) on a time scale that is approximately that for the release of catecholamine following exposure to K^+. The Ba^{2+}-induced release continues for 3-15 minutes after its initial application even in medium containing EGTA which should dissipate it even more rapid because of complexation. This suggests Ba^{2+} is retained in the cell where it can promote release either directly, by mobilization of internal Ca^{2+} stores, or by blockade of Ca^{2+} efflux (Przywara et al., 1993).

Prior work has shown that Ba^{2+} is capable of inducing massive secretion from several cell types, including adrenal cells, that is independent of extracellular Ca^{2+} (Douglas and Rubin, 1964a; Heldman et al., 1989; Morita et al., 1990; TerBush and Holz, 1992; Przywara et al., 1993). Ba^{2+} may enter through both voltage- and receptor-gated Ca^{2+} channels (Forsberg and Pollard,

1988; Heldman and Pollard, 1989), and, once inside the cell, directly cause release of catecholamines (TerBush and Holz, 1992). Irrespective of the mechanism, the long-lasting and high-frequency occurrence of spikes following exposure to Ba^{2+} (Figure 1) establishes that Ba^{2+} can directly induce exocytosis, and can do so in the absence of extracellular Ca^{2+}.

Entry of Ca^{2+} into adrenal medullary cells is the normal trigger for exocytosis (Burgoyne, 1991). The simultaneous observations of intracellular Ca^{2+} elevation and catecholamine release in response to K^+ (Figure 1) supports a close temporal link between these two events at pH 7.4. At acidic pH values Na^+ channel activity is reduced (Rutten et al., 1989), and lower $[Ca^{2+}]_i$ changes are observed that are not accompanied by catecholamine release.

The fura-2 fluorescence measurements also show that extracellular Ba^{2+} causes an increase in intracellular divalent ions. While a portion of the measured signal is undoubtedly due to Ba^{2+} which enters the cell (Heldman et al., 1989, Terbush and Holz, 1992), the fluorescent ratio obtained in Ca^{2+} free media exceeds the $R_{max,Ba}$ obtained by *in situ* calibrations by 18 % (n = 5). Therefore, a portion of the increase must be due to liberated intracellular Ca^{2+}. However, since Ba^{2+} does not elevate the intracellular messenger IP_3 (TerBush and Holz, 1992) and has been suggested to have poor affinity for the Ca^{2+}-ATPase (Schilling, 1989), the intracellular source of Ca^{2+} does not appear to be the endoplasmic reticulum. A similar interpretation has been invoked to explain Ba^{2+} induced changes in cultured sympathetic neurons (Przywara et al., 1993). While this paper was being reviewed, fura-2 measurements were published which also indicate a contribution of both Ba^{2+} and Ca^{2+} to the Ba^{2+}-induced fluorescence ratios (von Rüden et al., 1993).

The most surprising results of these experiments is that the characteristics of spikes induced by Ba^{2+} can be altered by the external media. First, exocytosis caused by Ba^{2+} in Ca^{2+}-free medium has a larger mean spike area at pH 8.2 than at pH 7.4. The smaller mean "quanta" at pH 7.4 suggests that a significant percentage of catecholamine is either not released

upon vesicle fusion (Alvarez de Toledo et al., 1993), or is released at a very slow, undetectable rate. Second, when Ba^{2+}-induced spikes measured at pH 7.4 are compared, the areas of the spikes are independent of the presence of Ca^{2+} in the external medium, but the spikes in Ca^{2+}-free medium are broader and shorter. Furthermore, release induced by Ba^{2+} at pH 7.4 appears to alter the quantal size of spikes induced by subsequent exposure to K^+. Third, while large area spikes are obtained at an extracellular pH of 5.5, the shape of some of the spikes is quite different from those in more basic solutions. Although some of the external media employed are extreme conditions for the cells, the consistently low basal fura-2 ratios at all pH values show that the cells retain integrity with respect to Ca^{2+} entry.

A possible reason for these differences in size and shape of the spikes is the extent of aggregation or association of the vesicular matrix before and even during exocytosis. This association depends in large part on the conformation of chromogranin-A, CGA, the primary water soluble protein of the vesicles in adrenal medullary cells (Winkler and Fisher-Colbrie, 1992). Because CGA can bind Ca^{2+} (Reiffen and Gratzl, 1986; Gorr et al., 1988; Westermann et al., 1988; Yoo and Albanesi, 1990a & b; Yoo and Albanesi, 1991; Videen et al., 1992; Yoo and Lewis, 1992), the vesicles are a major storage site of Ca^{2+} in the adrenal medullary cell (Yoo and Albanesi, 1990b). Prior studies have shown that pH and Ca^{2+} affect the conformation of CGA and its ability to aggregate catecholamine (Gorr et al., 1988, Westermann et al., 1988; Yoo and Albanesi, 1990a; Yoo and Albanesi, 1991). At the intravesicular pH of 5.5, CGA exists predominantly as a tetramer with a high α-helical content, while at the physiological pH value normally encountered upon fusion, CGA is less aggregated and exists predominantly as a dimer with less α-helical character (Yoo and Lewis, 1992). CGA also has a lower binding capacity for Ca^{2+} and catecholamines at pH 7.4, although calculations suggest that even at pH 5.5 only 5.3% of the intravesicular catecholamine is chemically bound by CGA (Videen et al., 1992).

Thus, we hypothesize that the observed changes in spike characteristics

are due to the presence of concentration gradients of the intravesicular ions and molecules that are initially generated upon vesicle fusion which, when increased, promote further dissociation of the vesicular matrix. The condition which maximizes the quantity of material released by exocytosis, high pH and low Ca^{2+}, is that which maximizes the disaggregation of CGA (Gorr et al., 1988). Since the measured quantal size (Q) at pH 7.4 with or without Ca^{2+} never reaches the value found at pH 8.2, exocytosis induced by secretagogues at pH 7.4 must result in a partial or slowed release of vesicular contents. Continued attachment of some CGA to the vesicle int (Yoo, 1993) would provide a matrix for withholding catecholamines. Alternatively, partial exocytosis may result from closure of the fusion pore (Alvarez de Toledo et al., 1993). The altered response to K^+ following exposure to Ba^{2+} at pH 7.4 may indicate an alteration of intravesicular aggregation caused by Ba^{2+} entry. However, we can not eliminate the possibility that, since Ba^{2+} causes prolonged release, the population of vesicles from which release occurs during the latter exposure to K^+ may be different from the original population.

The spikes at pH 8.2 in the absence of Ca^{2+} are wider than at other pH values, and much wider than expected for free diffusion of catecholamines (Jankowski et al., 1993). CGA could behave like other vesicular proteins present in different cells (Verdugo, 1990; Chandler, M. et al., 1989; Merkle and Chandler, 1991) and swell upon fusion. Swelling, with its associated liberation of catecholamines could be kinetically limiting. Alternatively, swollen CGA could cause slow diffusion of the catecholamines from the cell surface to the microelectrode because of interaction with the prevalent anionic sites in the swollen CGA matrix (Winkler and Smith, 1975). The effect of Ca^{2+} on spike width at pH 7.4 is also consistent with these interpretations. The presence of EGTA in the external medium may promote matrix swelling by increasing the Ca^{2+} concentration gradient. An alternate factor that could affect the spike width is that the fusion pore formed upon exocytosis restricts the rates and amounts of vesicular extrusion. However,

there is no evidence that formation of the fusion pore is pH-dependent. Indeed, the fusion pore in mast cells has been shown to form with equal probability at low and physiological pH values (Monck et al., 1991).

According to the above hypothesis, exocytosis into an extracellular medium of pH 5.5, which eliminates the pH gradient, would result in little disaggregation of the matrix. Indeed, we have shown previously that the frequency of exocytotic events are greatly diminished at this pH unless a large catecholamine concentration gradient is generated by the carbon-fiber microelectrode (Jankowski et al., 1993). The spikes at pH 5.5 also tend to be narrower than at more basic pH values, and have lower skew values than predicted for diffusional processes. These differences suggest another process becomes rate limiting such as a change from slow diffusion to slow matrix dissociation or perhaps ionic effects on cell membrane dynamics. While narrow, the spikes are still wider than predicted for diffusion of catecholamines in free solution (Jankowski et al., 1993).

In summary, these investigations suggest two different ways in which the kinetics of exocytosis can be affected. First, the quantity of the released packet of catecholamine can be affected by the concentration gradient of H^+ ions which occurs upon vesicular fusion. Alteration of the extracellular medium to favor disaggregation of the matrix increases the mean quantal size of catecholamine. Second, an increase of the Ca^{2+} and pH gradients which occur upon vesicle fusion, by adjustment of the external medium, can cause an increase in the time of dissociation of the vesicular matrix as seen in the increase in half width of the individual spikes.

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FIGURE LEGENDS

Figure 1. Simultaneous measurement of fura-2 fluorescence ratio (upper traces) and amperometric release (lower traces) at single adrenal medullary cells. Arrows indicate pressure ejection of secretagogue (K^+: 3 s application of 60 mM K^+ and 3 mM Ca^{2+}; Ba^{2+}: 15 s application of 3 mM Ba^{2+}). (A) Cell in Krebs-Ringer buffer containing 0.2 mM EGTA, pH 7.4. (B) Cell in Krebs-Ringer buffer containing 3 mM Ca^{2+}, pH 7.4. (C) Cell in Krebs-Ringer buffer containing 0.2 mM EGTA, pH 5.5. (D) Cell in Krebs-Ringer buffer containing 3 mM Ca^{2+}, pH 5.5. Note that for *in vitro* calibrations, $R_{max,Ba} = 0.38$.

Figure 2. Catecholamine release from adrenal medullary cells induced by Ba^{2+} in Ca^{2+}-free media. (A) Amperometric traces recorded at two different cells at the pH values noted on the traces. At the time indicated by the bar, 3 mM Ba^{2+} was applied for 15 s by pressure ejection from a micropipette. (B) Histogram of the charge measured for individual spikes at the pH values indicated. Results are from 657 spikes collected at 5 cells at pH 7.4 and 413 spikes collected from 5 cells at pH 8.2. (C) A single spike from each of the pH values which has the mean characteristics of the data set (see Table 2). The definitions for i_{max} and $t_{1/2}$ are indicated on the spike recorded at pH 7.4.

Figure 3. Secretion of catecholamines induced by Ba^{2+} at pH 7.4 with and without extracellular Ca^{2+}. (A) Width at half height and peak current histograms. Filled bars are results from 757 spikes obtained at 5 cells with 3 mM Ca^{2+} in the medium. Open bars are results from 657 spikes obtained at 5 cells with 0.2 mM EGTA. (B) Representative spikes with the mean spike characteristics for the data sets (see Table 2) with (dashed line) and without (solid line) extracellular Ca^{2+} are superimposed.

Figure 4. Spikes detected in response to 3.0 mM Ba^{2+} at pH 5.5 with no extracellular Ca^{2+}. Superimposed on each spike (dashed line) is the exponentially modified gaussian shape for diffusional dispersion. (A) Skew = 0.41, r = 0.85. (B) Skew = 0.77, r = 0.92. (C) Skew = 1.0, r = 0.84. (D) Skew = 1.8, r = 0.98 (r is the correlation coefficient for the experimental data with the diffusion-based shape (Schroeder et al., 1992)).

TABLE 1. Mean characteristics of spikes measured at cells in pH 7.4 buffer with 3.0 mM extracellular Ca^{2+} stimulated for 3 s with 60 mM K^+ before and after a 15-s exposure to 3.0 mM Ba^{2+}. Spikes were measured with a 6-μm radius electrode placed 1 μm away from the cell surface. Data was collected at 200 μsec/pt and low pass filtered at 2600 Hz. Means are reported ± s.e.m.

Stimulus	60 mM K^+	
	Before Ba^{2+}	After Ba^{2+}
Q (pC)	1.17 ±.07	1.58* ±.09
$t_{1/2}$ (ms)	7.23 ±.38	10.1* ±.50
i_{max} (pA)	129 ±6.2	120 ±5.8
f (Hz)	1.22	1.72
# of spikes	305	276
# of cells	5	5

* Means are significantly different from 60 mM K^+ before Ba^{2+} exposure (Mann-Whitney test $p < 0.001$).

TABLE 2. Mean characteristics of spikes measured at cells exposed to 3.0 mM Ba^{2+} for 15 s in the presence and absence of 3.0 mM Ca^{2+} and at different pH values. Spikes were measured with a 6-μm radius electrode placed 1 μm from the cell surface. Data was collected at 200 μsec/pt and low pass filtered at 2600 Hz. Means are reported ± s.e.m.

pH	5.5		7.4		8.2	
$[Ca^{2+}]$	0 mM	3 mM	0 mM	3 mM	0 mM	3 mM
Q (pC)	1.87 ±.07	1.67 ±.07	1.35 ±.05	1.26 ±.05	2.02 ±.09	1.37 ±.10
$t_{1/2}$ (ms)	11.4 ±.38	11.0 ±.23	12.5 ±.30	8.34 ±.26	16.4 ±.54	12.7 ±.84
i_{max} (pA)	124 ±4.5	95.2 ±3.1	73.7 ±2.2	117 ±3.8	89.9 ±3.7	85.7 ±6.1
f (Hz)	0.78	0.97	1.14	1.22	0.61	0.77
# of spikes	557	419	657	757	413	173
# of cells	5	5	5	5	6	4

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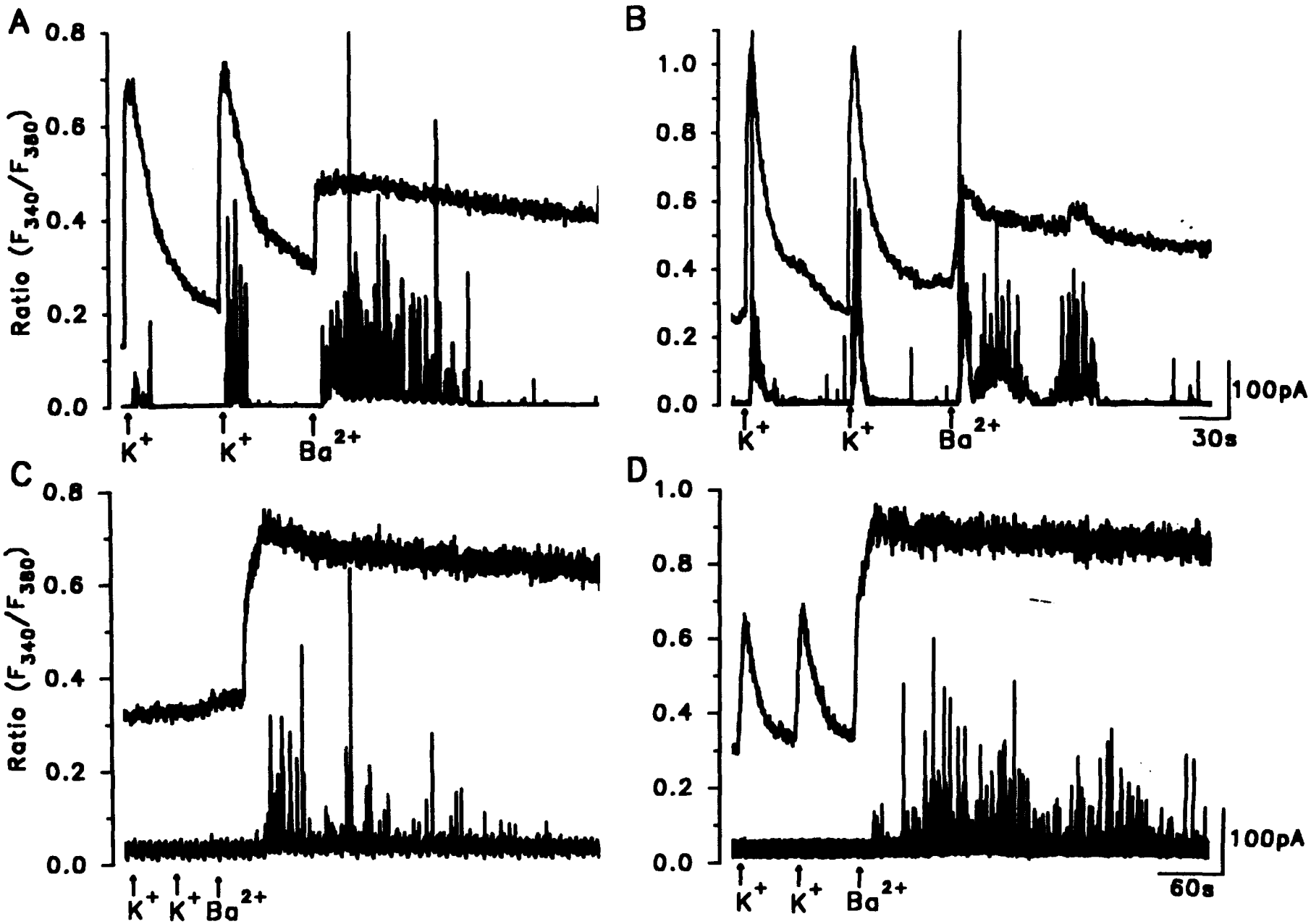
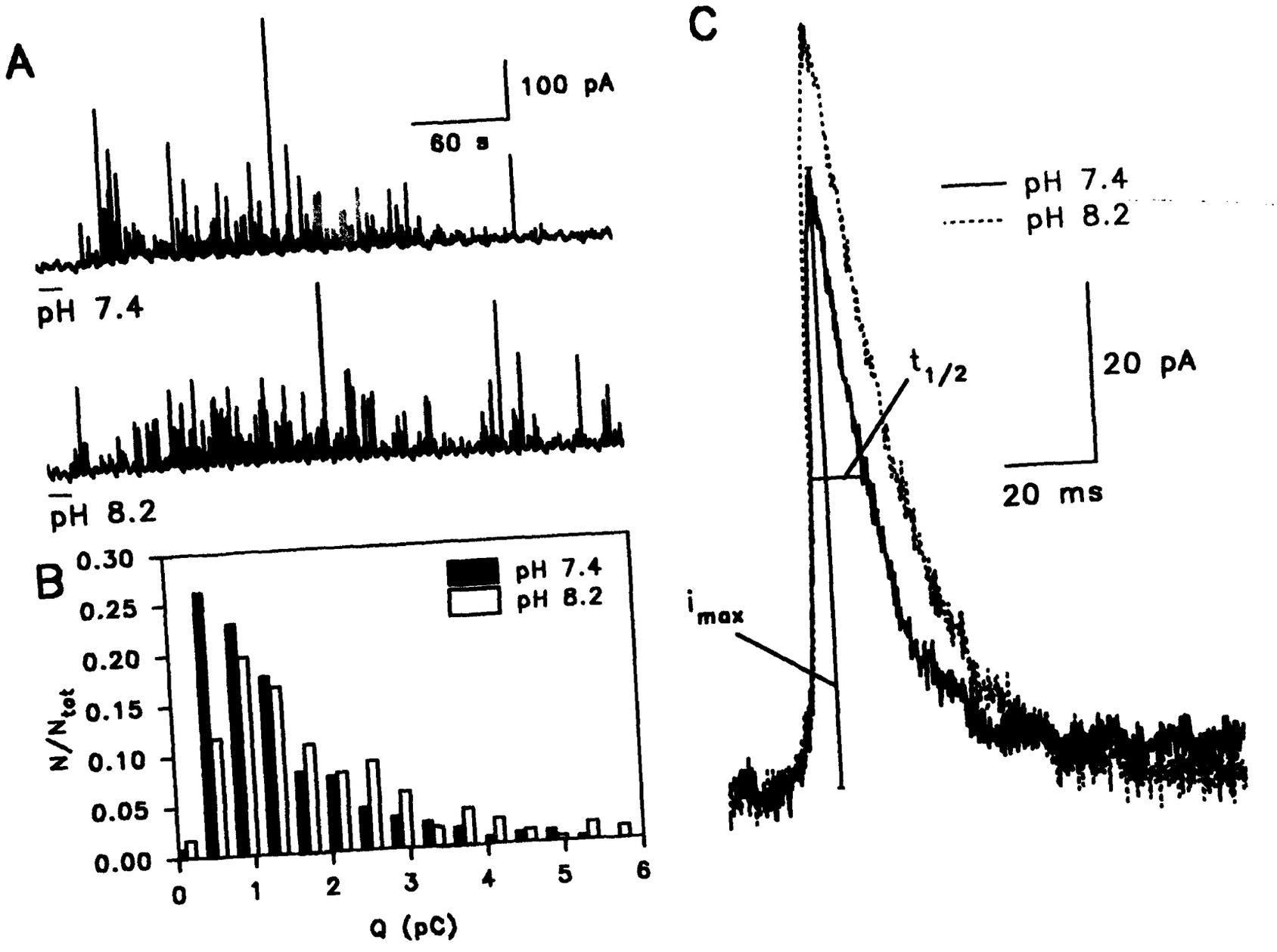


Figure 1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pH 7.4
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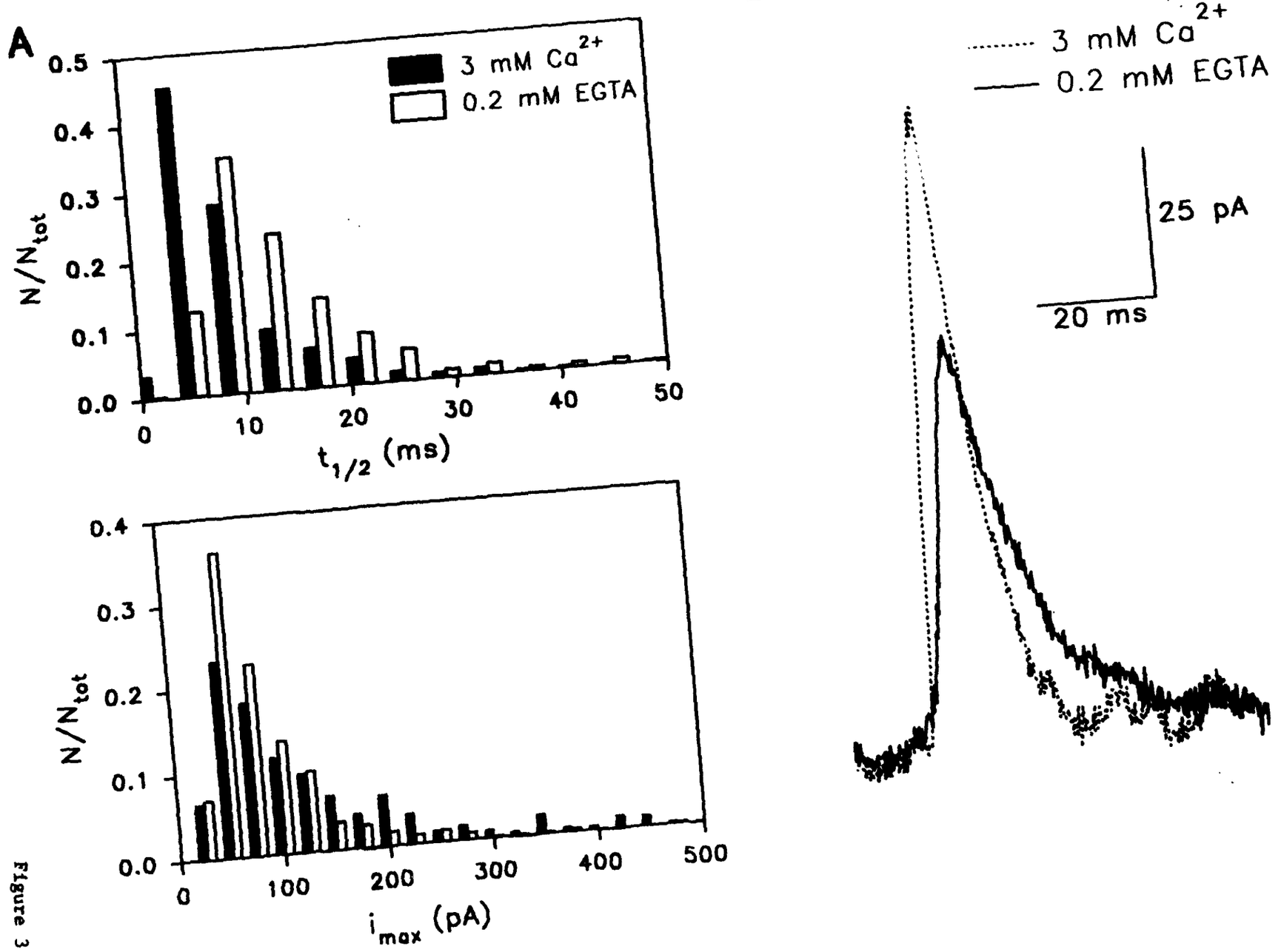
Figure 2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30
40
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t1/2 (ms)
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Figure 3

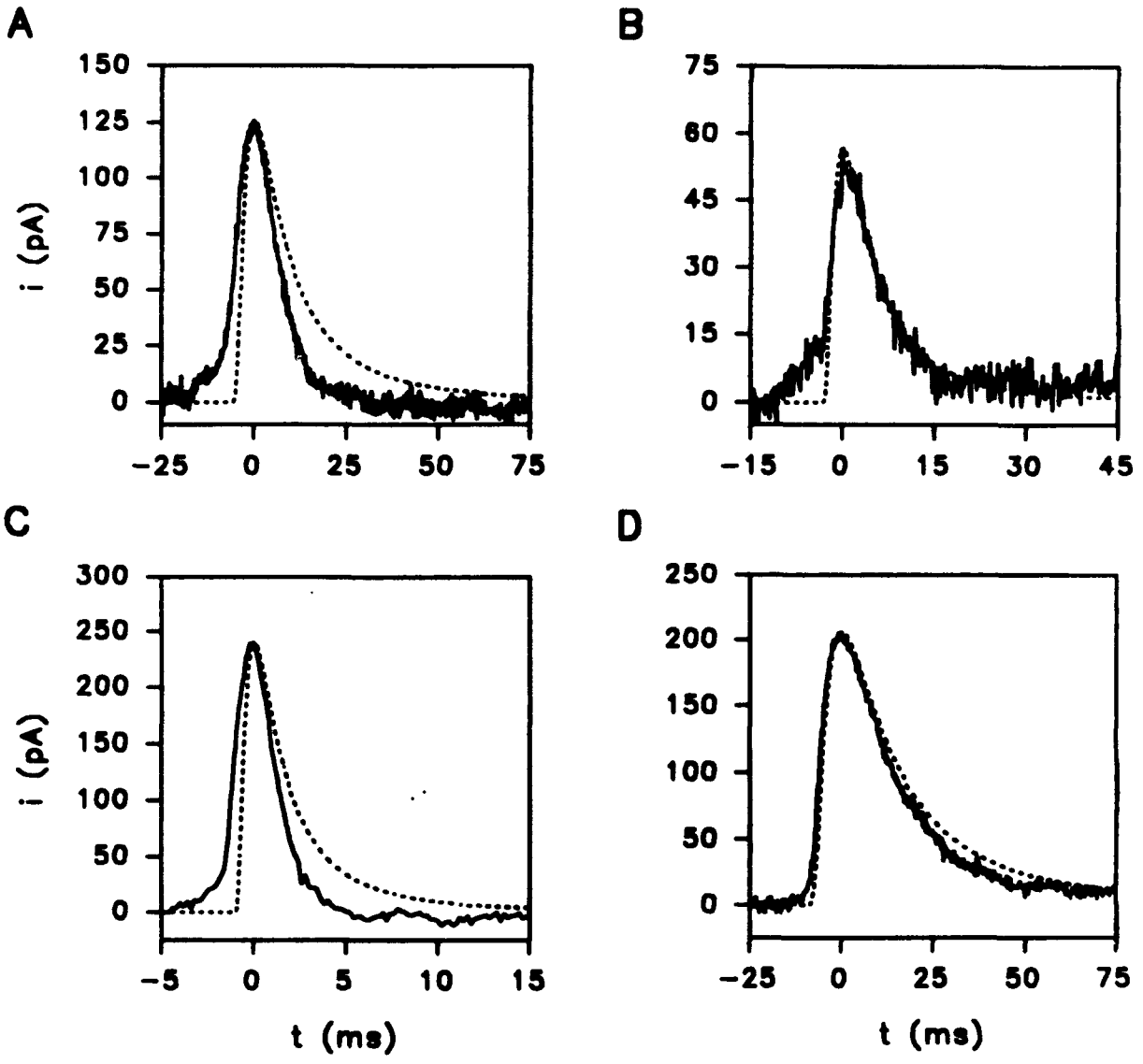
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Figure 4