Pflügers Arch – Eur J Physiol (1996) 431 : 729–735

ORIGINAL ARTICLE

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Time course of release of content of single vesicles in bovine chromaffin cells

Received: 25 April 1995 / Received after revision: 7 August 1995 / Accepted: 19 September 1995

Abstract The time course of the spontaneous current spikes produced by release of the catecholamine contents of individual vesicles was examined in bovine chromaffin cells using carbon filament electrodes. The rate of spontaneous release was enhanced by adding either $LaCl_3$ (0.01–0.5 mM) or $BaCl_2$ (2 mM) to the extracellular solution. A paucity of events of very short duration was evident from the frequency histograms of the rise and the decay times. In the scatterograms of the rise and of the decay times the regression lines are invariably positive (i.e. the longer the duration of the rise times the longer the duration of the decay times). However, the regression lines never go through the origin but intercept the ordinate (the axis of the decay times) at (± SD) 16.1 ± 6.4 ms (n = 11). On the other hand, the regression lines of paired rise and decay times for the time courses of diffusion are both linear and go through the origin. This relationship holds irrespective of whether the diffusion from an instantaneous point source was assumed to occur in an infinite plane or in an infinite volume. Therefore our experimental findings are incompatible with the model(s) assuming that diffusional broadening determines entirely the time course of current spikes. However, they can be explained, although only partially, by the possible slow speed of the electrode. They thus suggest that in chromaffin cells the duration of exocytosis of individual vesicles is much longer than in synapses.

Key words Vesicular secretion · Chromaffin cells · Carbon electrodes · Diffusional broadening · Scatterogram

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Introduction

Secretion of chemical substances – exocytosis – is a mechanism fundamental to our understanding of the nature of chemical intercellular communication. Combined biochemical, anatomical and physiological evidence argues strongly that: (1) substances involved in such a chemical intercellular communication are stored in vesicles, and (2) as a result of depolarization followed by entry of calcium, vesicles fuse with the plasma membrane and release their contents [1, 18].

In both peripheral and central synapses a release of the individual vesicles can be measured by recording spontaneous postsynaptic potentials or currents [2, 4, 7, 9, 11]. A fusion of vesicles with the plasma membrane can also be monitored in secretory cells that do not form synapses by measuring the changes of the capacitance of the cell [13]. Finally, carbon filament electrodes are now established to be sensitive enough as electrochemical detectors to measure catecholamine concentrations occurring from release of single vesicles [3, 20]. In bovine chromaffin cells the catecholamine concentrations occurring from release of single vesicles are observed as a series of current spikes when a carbon filament electrode is positioned close to the plasma membrane.

Both in peripheral and in central synapses a spontaneous release of one quantum, or evoked release of many quanta, is very rapid (< 100–200 μs) [7, 6]. However, in bovine chromaffin cells the time course of the spontaneous unitary current spikes has been found to last much longer. It was initially suggested that this could be due to the diffusional broadening [14], but based on the paucity of the current spikes of short duration, and on the comparative abundance of the spikes of long duration, it has been subsequently argued that the factors involved in the exocytotic process are not negligible [12]. In this study we try to assess how recording conditions (speed of the electrode, distance

between the release site and the tip of the electrode, diffusion conditions) influence the amplitudes, the time courses of the current spikes, and their frequency distributions. We also try to assess the duration of release of the content of individual vesicles from the relationship between the parameters describing the time course of the current spikes. A preliminary account has appeared [10].

Materials and methods

Cultures, solutions and microelectrodes

Primary cultures of bovine adrenal medullary cells were prepared from fresh tissue as described previously [17]. Experiments were performed at room temperature (21–23° C), using cells that had been in culture for 4–10 days. The glass coverslip, coated with collagen and with cells attached, was removed from the dish containing the culture medium. It was then placed in the recording chamber with Locke's solution of the following composition (mM): NaCl 154, $CaCl_2$ 2.2, $MgCl_2$ 1.2, KCl 2.5, K_2HPO_4 0.85 and dextrose 10.

Microelectrodes were prepared by aspirating a carbon fibre (Thornell T650/42, 5 μm radius, or alternatively P120 10 μm radius, Amoco, Greenville, S.C, USA) into a glass capillary. They were subsequently pulled and filled with a colloidal graphite solution (Polysciences, Warrington, Pa., USA) to create electrical continuity. The tip of the electrode was flame-etched to achieve a conical shape and a polymer (90 mM 2-allyphenol, 90 mM allyamine, 2% 2-butoxyethanol in 1:1 water:methanol) was electrochemically deposited on the surface to insulate it. The tips of the electrodes were subsequently bevelled to reveal the active carbon surface [15]. They typically had resistances ranging from 200 to 300 MΩ. All electrodes were tested for catecholamine sensitivity by pressure ejection of 100 μM of isoproterenol hydrochloride (a synthetic catecholamine, Winthrop, Lot No. MO26HR).

Recording, data acquisition and analysis

During an experiment the electrodes were pushed gently against the cell membrane until the cell membrane was deflected slightly. Oxidation currents were recorded with a patch-clamping system (Axopatc-200; Axon Instruments), with carbon electrodes held between +350 and +650 mV, and filtered with a Bessel filter at 5 kHz. Data were stored on the videotape with a VCR adapter (Model PCM 4, Medical Systems). They were subsequently replayed, anti-alias filtered at 500 Hz, and digitized at 1.5 kHz using a Labmaster data acquisition card (Scientific Solutions) and the Axotape software package (Axon Instruments). All analysis and simulation have been done using MATLAB (MathWorks), an interactive software package for scientific and engineering numeric computation on a 486 (90 MHz) PC computer. All graphics have been done using Origin (Microcal Software).

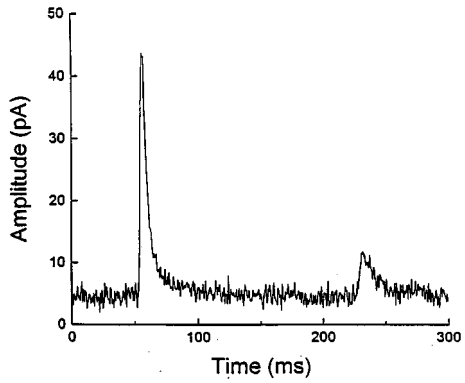
Results

Frequency histograms of amplitudes and of rise and decay times

Figure 1 gives a recording of the spontaneous current spikes obtained with a carbon filament electrode placed close to the cell membrane of a bovine chromaffin cell. We use the term spontaneous release although the release has been enhanced [by adding $BaCl_2$ (2 mM) or $LaCl_3$ (0.01–0.5 mM) to the extracellular solution] [9, 11, 16]. This is to conform with the established terminology that considers the release as spontaneous, even when elevated (using a high K^+, $LaCl_3$, $BaCl_2$,..) if the application of a secretagogue is sustained [9, 11, 16]. Evoked release is a term typically reserved for release induced by a rapid and short depolarization (produced by an action potential, a pulse of high K^+,...).

Figure 2 gives the frequency distributions of the amplitudes (Fig. 2A), the rise times (defined as the time interval needed for the spontaneous current spikes to reach from 10% to 90% of the maximal value; Fig. 2B), and the decay times (defined as the time interval needed for the spontaneous current spikes to decline from the maximal value to 1/e of the maximal value; Fig. 2C). Estimating the decay time from the time interval needed for a current spike to decrease from a maximal value to 1/e of a maximal value was felt to be justified since our attempts to fit one exponential to a decay phase have been, as a rule, highly successful. Note the skewed nature of all three frequency distributions, and the paucity of short rise and decay times. Table 1, that gives the estimates of the means, standard deviations and the skews of the amplitudes, the rise and the decay times from this and from other cases examined also shows that all histograms are skewed to the right (all skews are positive; Figs. 1–4 are all from a single cell with the electrode in one position). Finally note that: (1) there does not appear to be any relationship between the concentration of $LaCl_3$ and the amplitudes (or the rise or the decay times) of the spontaneous current spikes, and (2) both the rise and the decay times are clearly longer when the spontaneous release is enhanced by the addition of $BaCl_2$ to the bath solution (the corresponding mean durations of both the rise and the decay times are statistically different at the significance level of 0.05; at the same level of significance the mean amplitudes were not statistically different).

Fig. 1 A recording of the spontaneous current spikes obtained with a carbon filament electrode placed close to a membrane of the bovine chromaffin cell. The trace shows two current spikes from both ends of the amplitude range



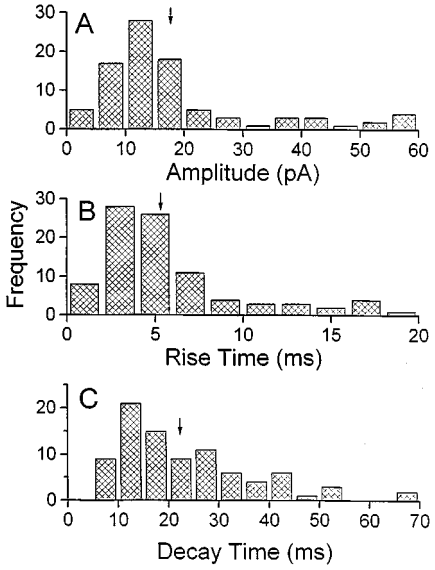


Fig. 2 Frequency distributions of **A** amplitudes, **B** rise times (τ_r), and **C** decay times (τ_d). Note the paucity of the τ_r and τ_d values of short duration. *Arrows* indicate the mean values

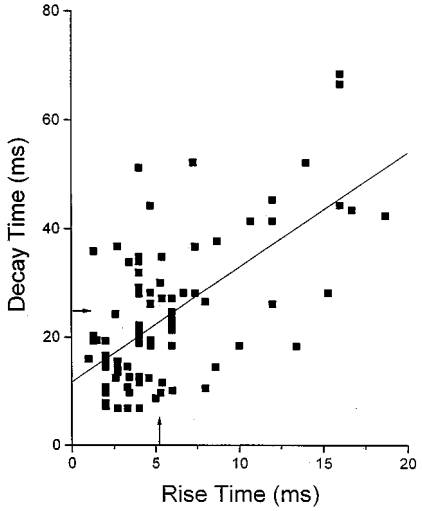


Fig. 3 Relationship between the rise times (τ_r; shown on the *abscissa* and taken as the time necessary to reach from 10% to 90% of the amplitude of spontaneous current spikes) and the decay times (τ_d *-ordinate*; taken as the time necessary for a current to decline from its maximal to 1/e of its maximal value). *Closed squares* – paired individual observations. The *line* estimated using the least-squares fitting method is: $\tau_d = 11.8 + 2.1 \times \tau_r$. *Arrows* indicate the estimates of the mean values of τ_r and τ_d. Note that the relationship is positive and that the intercept on the *ordinate* is not even close to the origin

Scatterograms of the rise and the decay times

Figure 3 gives the paired measurements of the rise times and the decay times. The best fitted line has been obtained using the least-squares method. In this, as well as in other cases, (see Table 2) the relationship has been found to be invariably positive (i.e. the longer the duration of the rise time of the current spikes, the longer the duration of their decay time), but the intercepts on the ordinate (i.e. the estimates of the durations of the decay times for the "zero durations" of the rise times) do not go through the origin, and are, as a rule, positive (see Table 2). In order to determine whether $BaCl_2$ and/or $LaCl_3$ affect the relationship between the rise and the decay times we calculated mean slopes and

Table 1 The basic statistical estimates of the amplitudes, the rise times (τ_r) and the decay times (τ_d) of the spontaneous current spikes. In each case the mean, the standard deviation and the skew of the estimated value are given in the *adjacent columns*. Measurements were made at pH = 7.2. The experiments were made either in the presence of lanthanum chloride ($LaCl_3$) or barium chloride ($BaCl_2$) in the bath to increase the rate of the spontaneous vesicular release, and the concentration is in each case indicated in the *last column*. The *first column* gives the number of observations used for the estimate

No.	Ampl (pA)	S.D. (pA)	Skew	τ_r (ms)	S.D. (ms)	Skew	τ_d (ms)	S.D. (ms)	Skew	Conc. (μM)
68	15.9	11.7	3.1	6.6	3.9	0.7	21.7	9.8	1.3	La 10
48	14.7	17.8	4.5	6.6	4.0	1.3	18.4	10.4	0.8	La 20
37	10.9	7.4	2.2	6.4	4.5	0.6	21.8	12.6	1.1	La 50
33	8.9	4.7	1.2	5.8	3.3	1.6	19.7	9.4	0.6	La 50
87	18.8	13.7	1.1	5.5	4.1	6.0	23.4	13.6	1.5	La 500
Mean ± SD	13.8 ± 4.0			6.2 ± 0.5			21.0 ± 2.0			
40	5.3	1.6	0.3	17.4	6.9	0.4	44.3	17.3	1.0	Ba 2000
23	5.0	2.1	0.1	13.9	9.3	1.2	62.7	30.2	1.3	Ba 2000
21	7.6	7.2	1.8	13.8	10.9	1.1	32.5	22.4	1.8	Ba 2000
201	15.5	9.5	1.3	14.5	9.7	1.4	49.3	27.0	1.4	Ba 2000
100	17.7	10.1	1.8	21.5	11.8	1.1	62.3	26.1	0.7	Ba 2000
173	22.3	15.7	1.7	7.9	4.1	1.2	26.0	8.6	1.1	Ba 2000
Mean ± SD	12.2 ± 7.3			14.8 ± 4.5			46.2 ± 15.1			
Overall Mean ± SD	13.0 ± 5.8			10.9 ± 5.5			34.7 ± 17.0			

Table 2 The basic statistical estimates of the best fitted regression lines to the experimental data pairs of the rise times and the decay times. The estimates of both the *y*-intercepts and of the slopes are given together with the corresponding standard deviations in the *adjacent columns*. Measurements were made at pH = 7.2. The experiments were made either in the presence of lanthanum chloride ($LaCl_3$) or barium chloride ($BaCl_2$) in the bath to increase the rate of the spontaneous vesicular release, and the concentration is in each case indicated in the *last column*. The *first column* gives the number of observations used to obtain the estimates

Number of observations	*y*-intercept (ms)	SD (ms)	Slope	SD	Concentration (μM)
68	13.2	2.0	1.3	0.3	La 10
48	13.1	2.8	0.8	0.4	La 20
37	16.3	3.5	0.9	0.5	La 50
33	8.8	2.5	1.9	0.4	La 50
87	11.8	1.9	2.1	0.3	La 500
Mean ± SD	12.6 ± 2.7		1.4 ± 0.6		
40	15.4	5.8	1.7	0.3	Ba 2000
23	28.8	7.8	2.4	0.5	Ba 2000
21	8.2	4.3	1.8	0.2	Ba 2000
201	21.9	2.5	1.9	0.1	Ba 2000
100	24.2	3.3	1.8	0.1	Ba 2000
173	15.1	1.1	1.4	0.1	Ba 2000
Mean ± SD	18.9 ± 6.5		1.8 ± 0.3		
Overall Mean ± SD	16.1 ± 6.4		1.6 ± 0.5		

intercepts separately (Table 2). However, the differences between the intercepts (or slopes) of these two groups were not statistically significant (at the level of significance of 0.05).

Amplitude dependence of the rise and the decay times

Figure 4 gives the paired measurements of the rise times and the amplitudes (Fig. 4A), and the decay times and the amplitudes (Fig. 4B). Note that for both the short rise times (A) and for the short decay times (B) the amplitudes of spontaneous current spikes are widely scattered, and also that the largest amplitudes have both short rise and short decay times.

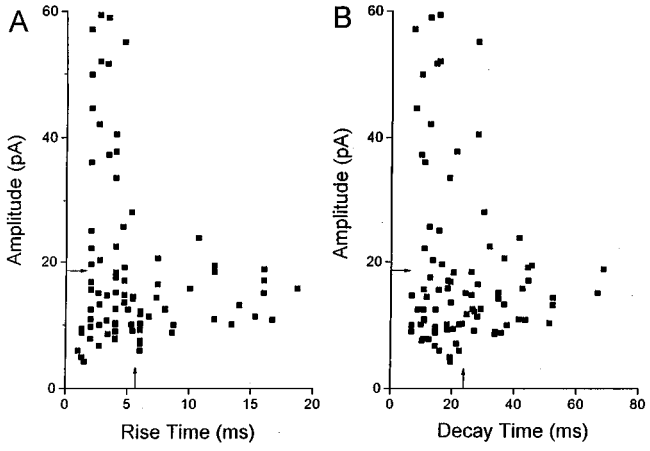


Fig. 4 Relationship between **A** the rise times (shown on the *abscissa* and taken as the time necessary to reach from 10% to 90% of the amplitude of spontaneous spikes), or **B** the decay times (also shown on the *abscissa* and taken as the time necessary for a current to decline from its maximal to 1/e of its maximal value), and amplitudes (A's; shown in both cases on the *ordinate*). *Closed squares* - paired individual observations. *Arrows* indicate the estimates of the mean values of rise times, decay times and amplitudes

Diffusion from an instantaneous point source

Figure 5 gives two families of curves of the change in the concentration of catecholamine released from an instantaneous point source in: (1) an infinite volume, or (2) an infinite plane, and recorded at distances ranging from 1 μm to 10 μm. All concentrations (C) have been normalized to their maxima for easier comparison, and have been calculated using the following formulae:

$$C = \{1/[8(\pi Dt)^{3/2}]\}\exp(-r^2/4Dt) \quad \text{infinite volume} \quad (1)$$

$$C = [1/(4\pi Dt)]\exp(-r^2/4Dt) \quad \text{infinite plane} \quad (2)$$

where D is the diffusion constant (assumed to be $6.0 \cdot 10^{-6}$ cm^2/s for adrenaline in our calculations, and r is the distance between the point where the concentration is calculated and the point source [5]. In chromaffin cell cultures 80% of cells are adrenaline-containing cells, while the remaining 20% contain noradrenaline [19]. Figure 6 gives the relationship between the duration of rise and decay times of the simulated concentration curves given in Fig. 5. Note that in both cases the relationship between rise and decay times is linear and that it goes through the origin. We take the same definition for the rise and decay times for theoretical curves as we did for the experimental data. For the rise times this poses no problem. The time course of the concentration decay however, is not monoexponential but a more complex function given in the text (Eqs. 1 and 2). We nevertheless feel that the essential argument remains the same, since the deviations from the monoexponentiality become important only when the concentrations decline to very low levels.

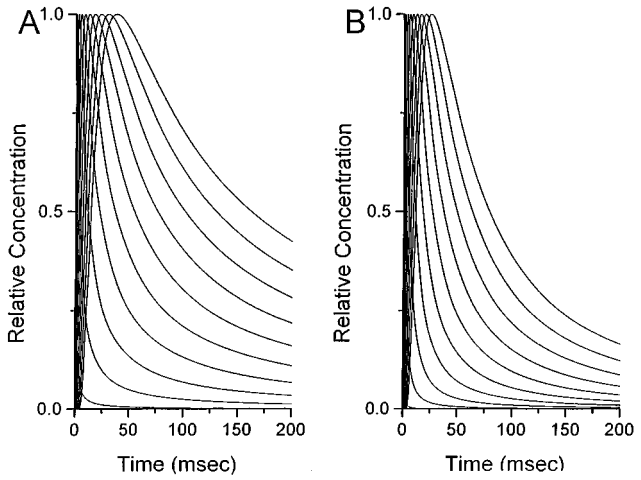


Fig. 5 A family of theoretical curves giving the time course of the relative concentration change at different distances ranging from 1 μm to 10 μm from an instantaneous point source in: **A** an infinite plane, and **B** an infinite volume. Diffusion constant of adrenaline was taken to be $6.0 \cdot 10^{-6}\,cm^2/s$

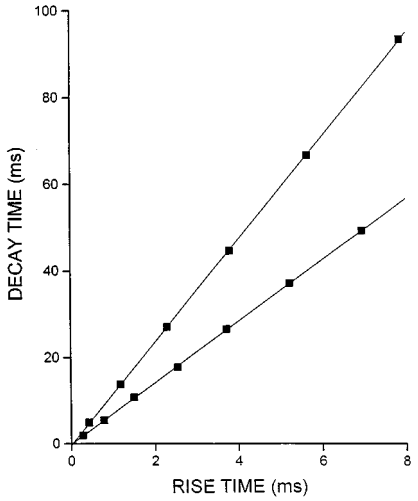
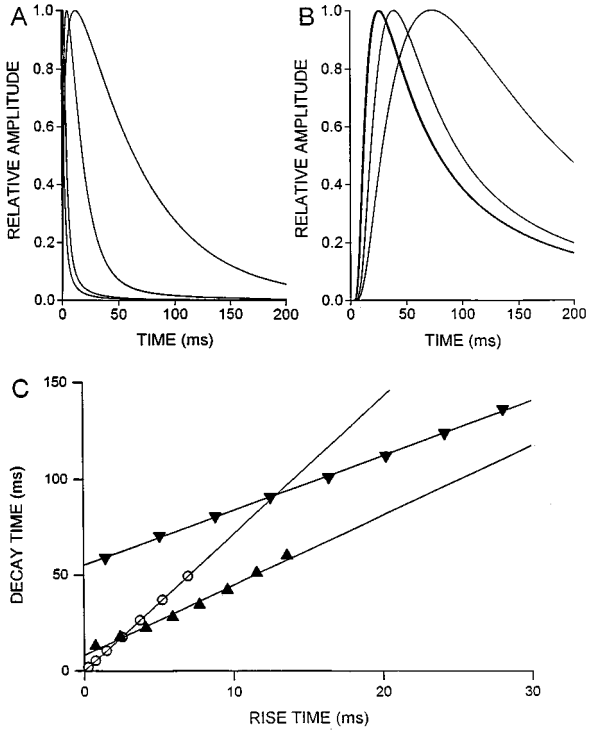


Fig. 6 A relationship between the rise times and the decay times of the time courses of the theoretical relative concentration changes of adrenaline expected to occur at different distances from the source of release. Instantaneous point source is assumed to be either in an infinite plane, or in an infinite volume. The estimates of the rise times and the decay times were made from two families of curves shown in Fig. 5 (see text). Note that the assumption of the diffusion in an infinite volume leads to shorter decay times for the same rise times (i.e. to the line with the smaller slope). The relationship is linear in both cases, with lines going through the origin

Theoretical assessment of how the speed of the recording electrode can affect the time course of the current spikes

There is very little information about the speed of the carbon filament electrodes that are presently used for recording of vesicular release. We therefore decided to make some simple theoretical calculations to determine how a low speed of the recording electrode may affect our results (Fig. 7). We simulate the electrode as an RC element, i.e. we assume that it acts as a simple low-pass filter whose time constant is 1, 10 or 50 ms. Two families of theoretical curves were calculated assuming that the release of catecholamines is an instantaneous point source in an infinite volume and that the recording electrode is positioned 1 or 5 μm away from the locus of release (Fig. 7A and B respectively). It is clear that: (1) the time course of "current spikes" is markedly different for electrodes of different speed, and (2) irrespective of the distance (and thus of the time course of the catecholamine concentration), the electrode affects their time course profoundly. The speed of the electrode also alters the relationship between the rise and the decay times. This relationship is linear and passes through the origin for an infinitely fast recording electrode. If the same catecholamine concentration changes are

Fig. 7 Two families of the current spikes that are expected to occur at 1 μm and 5 μm from the instantaneous point source (A and B respectively). These are the theoretical estimates and assume diffusion in an infinite volume. Four curves in each case are the theoretical estimates that assume that the recording electrodes are represented as simple RC-low pass filters with time constants of 50 ms, 10 ms, 1 ms or 0 ms (i.e. they are considered to be infinitely fast). Note that the theoretical curves for 0 ms and 1 ms are practically superimposed over the whole time course shown, if the distance between the instantaneous point source and the recording electrode was assumed to be 5 μm (**B**). **C** The relationship between the rise and the decay times for an electrode whose time constant is 0 ms (*open circles*), 10 ms (*filled triangles*) or 50 ms (*inverse filled triangles*). Note that the *y*-intercept does not go through the origin but is just above the value equal to the time constant of the recording electrode, and also that the slope becomes progressively smaller as the time constnat of the electrode increases



recorded with an electrode that is not instantaneous, but that has a time constant that is not negligible in comparison to the time course of the concentration changes, the current spikes will be prolonged (the rise times being faster will be prolonged comparatively more). As a result, the relationship between the rise and the decay times will not go through the origin but will intercept the *y*-axis (which depicts the decay times) just above the value equal to the time constant of the electrode (Fig. 7C). The slope on the other hand, will be reduced. The longer the time constant of the electrode, the more it dominates the time course of the current spikes and the more it is independent of the time course of the catecholamine concentration change. Since the short events are prolonged comparatively more than the long events, the rise and the decay times become not only progressively longer, but also become less variable and display a progressively greater paucity of the events of short duration.

Discussion

Secretion of individual vesicles containing catecholamines can be measured on the surface of the bovine chromaffin cells in culture using carbon fibre microelectrodes operated in the amperometric mode. The exocytosis of vesicles is observed as a series of current spikes [3, 20]. It was originally suggested on the basis of the shape of the individual current spikes (fast rise time and slower decay time), and the broad range of the spike widths and amplitudes, that the time course can be largely explained by the effects of diffusion (diffusional broadening; [14]). However, a recent study of the spontaneous quantal release in bovine chromaffin cells has shown that the frequency histograms have a marked paucity of the current spikes of short durations. This was taken to suggest that the duration of release of individual vesicles is much longer than in synapses [12]. We confirm this finding, and extend it, by showing that the frequency histograms, of both rise and decay times, display such a paucity. The large scatter of the amplitudes of the current spikes with short rise and decay times (i.e. of events whose locus of release clearly could not have been far from the recording electrode) suggests that the amount of catecholamines released in each exocytosis is very variable. This is not surprising since a moderate variability of vesicle diameters leads to a much larger variability of vesicular volumes and thus of vesicular contents. It also leads to frequency distributions of amplitudes that, as observed, are skewed to the right [8, 20]. On the other hand a presence of the small and slow events is expected to be a result of a release from spots situated at some distance from the recording electrode.

Our results also show that the time course of the current spikes is significantly slower when the rate of the spontaneous release is elevated by addition of $BaCl_2$ instead of $LaCl_3$ to the extracellular solution. We did not explore the origin of these differences but they do suggest that Ba^{2+} and/or La^{3+} alter the rate and the extent of the release of the content of the individual vesicles. This effect of Ba^{2+} and/or La^{3+} is not entirely surprising. Recent studies have shown that the rate and the extent of the release of the content of the individual vesicles is not insensitive to changes of the extracellular solution (the rate of release strongly depends on extracellular pH; [12]).

To elucidate in greater detail the problem of whether the time of release of vesicular content is much longer in bovine chromaffin cells than in synapses, we further the analysis of the factors involved in determining the time course and the amplitude of the current spikes in several respects. First of all, we examined the scatterograms of the rise and the decay times. The relationship between the rise and the decay time has been found to be invariably positive [i.e. the spontaneous current spikes that have longer decay times also have longer rise times; the slope is (± SD) 1.6 ± 0.5], but the scatter of decay times about the decay versus time rise time best fitted line is pronounced. A positive relationship between decay and rise times is in agreement with the model that assumes that diffusional broadening determines the time course of the current spikes. However, our study shows that the duration of the decay times of the current spikes of "zero duration" is not zero. The *y*-intercepts of regression lines do not go through the origin, but are regularly positive. They were (± SD): 16.1 ± 6.4 ms.

Secondly, we tried to estimate whether this relationship can be explained by the diffusion of catecholamines. The models we consider are simple. The diffusion is assumed to occur either from an instantaneous point source in an infinite plane or from an instantaneous point source in an infinite volume [5]. Both predict that the relationship should be linear and that it should go through the origin. Assuming that the release occurs from a disc instead of from a point source is not expected to alter this relationship in any significant manner since the dimensions of a disc are much smaller than the distances we are considering for diffusion or that we are likely to encounter experimentally (this is assuming that a disc diameter is as large as the vesicular diameter).

Thirdly, we also try to assess how the speed of the electrode may alter the frequency histograms and scatterograms, and show that these changes can be very complex. The most important point is that the evidence, that was previously considered as suggesting the prolonged time interval of release of the individual vesicles, can be simply due to the unaccounted effect of the slow speed of the electrode. When the speed of the electrode becomes comparable to the time course of the events to be measured, the frequency histograms of the rise and the decay times show a paucity of

short events. Furthermore, in such cases the best fitted regression lines through paired observations of the rise and the decay times do not go through the origin. However, we show that it is extremely unlikely that the paucity of the current spikes of short duration and the non-zero y-intercepts of the regression lines are due to the slow speed of the electrode. The shortest decay time of the current spikes ought to be faster than the time constant of the electrode and the shortest decay times are typically ≈ 10 ms shorter than the y-intercepts. Furthermore, the positive relationship between the rise and the decay times demonstrates that the speed of the electrode does not dominate the time course of the current spikes. The marked scatter of the rise and the decay data pairs is also contrary to such an idea. It is still, however, to be determined to what extent the positive relationship between decay and rise times is due to the contribution of diffusional broadening (at some distances between the locus of the release and the recording point, the contribution of the diffusional broadening has to be observed), or to what extent it reflects the dependence between the processes that determine the rise and the decay time of the release mechanism itself.

In conclusion, our analysis puts the earlier suggestion that the time course of release of individual vesicles in bovine chromaffin cells, unlike in synapses (in the peripheral or in the central nervous system), is not instantaneous or very short, and therefore that the exocytotic event does not last 100–200 μs, but approximately two orders of magnitude longer (i.e. > 10 ms) on much more solid ground.

Acknowledgements This research was supported by the Medical Research Council of Canada (M.I.G. and J.M.T) and by the Fonds de la Recherche en Sante du Québec (A.W.). We would like to thank Tim Schroeder for his technical advice on the methods of electrode fabrication, and Mrs. R. Tang for the preparation of the chromaffin cell cultures.

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