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Quantitative and Statistical Analysis of the Shape of Amperometric Spikes Recorded from Two Populations of Cells

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Abstract: Previously used methods of comparing amperometric spike characteristics from two separate groups of cells have entailed pooling all the values for a spike characteristic from each group of cells and then statistically comparing the two samples. Although this approach has indicated that there are significant differences between the spike characteristics from coloboma and control mouse chromaffin cells, the results are not consistent between experiments. We have reexamined the assumptions of the statistical tests used as well as the variability inherent in amperometric data measured from two groups of cells. Our findings indicate that when comparing amperometric spike characteristics between groups of cells, it is more appropriate to compare samples of mean spike values. This method consistently indicates that there is no difference between coloboma and control amperometric spikes. These results have been validated by using samples of mean spike characteristics to detect changes in the shape of amperometric spikes from both mouse chromaffin cells at 37°C and PC12 cells previously exposed to 50 μM L-3,4-dihydroxyphenylalanine and by the use of an additional analysis method, the nested ANOVA. Together, these results indicate that pooled samples of amperometric spike characteristics can give results that may confound the interpretation of amperometric data. **Key Words:** Amperometry—Single cell—Exocytosis—Mouse chromaffin cells—PC12 cells—Statistics.
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In the last few years, amperometry has proven to be a very powerful method for investigating exocytosis at the level of a single cell. Single-cell amperometric measurements are performed by placing a small carbon fiber electrode (held at constant potential) in close proximity to an isolated cell. Following cell stimulation, brief current spikes can be observed as electroactive transmitters released from the cell are oxidized at the surface of the carbon fiber electrode. These individual current spikes have been attributed to the detection of electroactive neurotransmitters released from single vesicles within the cell (Wightman et al., 1991; Chow et al., 1992).

In this study we have focused on developing a useful cell model of exocytosis to be used in combination with amperometry, for investigating how genetic factors affect exocytosis at the single-cell level. Toward this goal we have used cultures of isolated mouse chromaffin cells. Although other studies have used amperometry to measure neurotransmitter release from single mouse mast cells (Alvarez de Toledo et al., 1993) and pancreatic β-cells (Smith et al., 1995), we have chosen to use the chromaffin cell as a model system because these cells do not require preloading with electroactive neurotransmitters and are developmentally and biochemically similar to sympathetic neurons (Douglas, 1968). Also, mouse chromaffin cells can be readily identified in culture (Moser and Neher, 1997*a*), and it is possible to obtain a reasonably large number of easily detectable amperometric events from a single mouse chromaffin cell (Moser and Neher, 1997*b*).

In developing this mouse model, we have chosen to investigate exocytosis at single chromaffin cells from the mouse mutant coloboma. The coloboma mouse has a deletion mutation that encompasses genes for the presynaptic protein SNAP-25 (Hess et al., 1992), and it has recently been shown that there are deficiencies in neurotransmitter release within selected brain regions of the coloboma mouse (Raber et al., 1997). Because SNAP-25 is present in chromaffin cells (Roth and Burgoyne, 1994; Gutierrez et al., 1995), we have used amperometry to determine if the coloboma mutation affects the quantal release of neurotransmitters from this cell type.

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Abbreviations used: CV, coefficient of variation; L-DOPA, L-3,4-dihydroxyphenylalanine; i_{max}, spike maximal height; $t_{1/2}$, spike width at half its height.

Several experimental designs have been used in amperometric experiments to investigate how exocytosis at a single cell can be modulated. Two of the most common experimental protocols consist of recording from a single cell before and after an experimental manipulation (Jankowski et al., 1994; Pihel et al., 1996; Borges et al., 1997; Pothos et al., 1998*a*) or recording from two separate populations of cells (Jankowski et al., 1994; Pihel et al., 1996; Pothos et al., 1996, 1998*b*; Walker et al., 1996*a*). When investigating cells from animals with two different genetic backgrounds, the protocol is limited to recording from two separate populations of cells.

To investigate the effect of an experimental manipulation, several properties of amperometric data can be analyzed. One particular area of interest is the shape of the amperometric spikes. The most commonly measured spike characteristics include the spike area, the spike maximal height (i_{max}), and spike width at half its height ($t_{1/2}$). The area of individual amperometric spikes is directly related to the number of molecules of neurotransmitter released from a single vesicle (Wightman et al., 1991). The values of i_{max} and $t_{1/2}$ have been shown to be dependent on several factors, including the number of molecules released (Pothos et al., 1996, 1998*a*), the degranulation and extrusion of transmitter from the vesicle lumen (Wightman et al., 1995; Pihel et al., 1996; Borges et al., 1997), and the type and magnitude of filtering used during the recording.

In previous studies using two separate populations of cells, samples for each spike characteristic have been pooled from each group of cells and compared statistically to draw conclusions about how a given manipulation affects release at the vesicular level (Jankowski et al., 1994; Pihel et al., 1996; Pothos et al., 1996, 1998*b*; Jaffe et al., 1998). To test for possible differences between control and coloboma mouse chromaffin cells, exocytosis was measured at groups of both cell types and pooled samples of area, $t_{1/2}$, and i_{max} values were compared statistically. As will be shown, this method indicates that there are statistically significant differences in the shape of amperometric spikes recorded from control and coloboma chromaffin cells. However, the results from this approach are inconsistent, making it difficult to determine if the coloboma mutation affects release from chromaffin cells at the vesicular level.

In this article, we propose the use of an alternative statistical method for comparing the shape of amperometric spikes measured from two populations of cells. This method consists of creating samples from each group of cells by taking one value (the mean) for a spike characteristic from each cell in a group and then comparing the two samples of mean values. Unlike comparing samples created by pooling all of the data, this method meets the assumptions of statistical tests for two unrelated samples, takes into account the variability in the number of events observed at individual cells, and gives consistent results from experiment to experiment. Using this method, we show that there are not significant differences in the shape of amperometric spikes measured from wild-type and coloboma chromaffin cells. To verify that means of spike characteristics can be used to detect changes in the shape of amperometric spikes from two separate groups of cells, this method has also been used to identify changes in the characteristics of spikes measured from mouse chromaffin cells at 37°C and PC12 cells following exposure to 50 μM L-3,4-dihydroxyphenylalanine (L-DOPA). Here we compare statistical results obtained using pooled samples and samples containing mean spike characteristics and describe potentially confounding factors associated with pooled samples of amperometric data that have not been previously identified.

MATERIALS AND METHODS

Cell culturing

Mouse chromaffin cells were obtained from 9–18-week-old coloboma (*Cm*/+) mice on a C3H/HeSnJ background. Coloboma mutants were originally purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and subsequently bred at Pennsylvania State University College of Medicine. Mutant (*Cm*/+) mice were identified at weaning primarily by head bobbing, but also by the presence of sunken, small eyes. The coloboma mutants can also be distinguished at this time by their hyperactivity and smaller size (Hess et al., 1992).

The methods used for isolating and preparing primary cultures of mouse chromaffin cells have been adapted from those described elsewhere (Guo et al., 1996; Moser and Neher, 1997*b*). Adrenal glands were removed from mice and placed directly into chilled dissection medium [M199 medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with penicillin/streptomycin]. Excess fat was removed from each gland, and medullary fragments were isolated from cortical tissue in chilled dissection medium with the aid of a dissection microscope. All medullary fragments were pooled and stored on ice before enzymatic digestion. Dissection medium was removed by gentle centrifugation (100 *g*, 10 min), and medullary fragments were resuspended in Hanks' balanced salt solution (Life Technologies) containing collagenase (collagenase A; catalytic activity, ~0.15 U/mg; Boehringer Mannheim, Indianapolis, IN, U.S.A.) at a concentration of 20 mg/ml and DNase I (Sigma, St. Louis, MO, U.S.A.) at a concentration of 0.1 mg/ml, for a total of 1 h in a 37°C water bath. Thirty minutes into the digestion, trypsin with EDTA (Life Technologies) was added (diluted from a 10× stock), and throughout the course of the digestion, medullary fragments were triturated approximately every 15 min. At the end of the digestion, enzymes were removed, and cells were resuspended in warm culture medium [M199 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 1 mg/ml bovine serum albumin, and penicillin/streptomycin]. Cells were plated with 4 ml of culture medium onto poly-D-lysine-coated culture dishes (Becton Dickinson, Bedford, MA, U.S.A.) and incubated at 37°C with 5% CO_2. Cells were used for experiments 24–48 h after plating. Cultures were generated using four to six animals (eight to 12 adrenal glands) for each genotype. Techniques used to prepare mouse chromaffin cultures were approved by the Penn State University Institutional Animal Care and Use Committee.

Electrode preparation and experimental setup

Carbon fiber microelectrodes (5 μm in diameter) were constructed as described previously (Pothos et al., 1998*b*) and

back-filled with 3 *M* KCl. Electrode tips were polished at a 45° angle on a diamond dust-embedded micropipette beveling wheel (model BV-10; Sutter Instrument Co., Novato, CA, U.S.A.). Before an experiment, electrodes were soaked in 2-propanol for 15 min. Cyclic voltammograms were generated for each electrode in a nitrogen-saturated 0.1 m*M* dopamine solution (in 0.1 *M* phosphate-buffered saline, pH 7.4), and only electrodes with stable *I–E* curves were used.

Cells were prepared for an experiment by removing the culture medium and adding physiological saline (150 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgCl_2$, 5 m*M* glucose, 10 m*M* HEPES, and 2 m*M* $CaCl_2$, pH 7.4) and then placing them onto the stage of an inverted microscope (IM-35; Carl Zeiss, Thornwood, NY, U.S.A.). The working electrode was gently lowered onto a single cell using a piezomicropositioner (PCS-750/1000; Burleigh Instruments, Fishers, NY, U.S.A.). The close proximity of the electrode to the cell surface was confirmed by a slight deformation in the outline of the cell.

Exocytosis was stimulated approximately every 30 s using a 5-s pressure (20 psi) pulse (Picospritzer II; General Valve, Fairfield, NJ, U.S.A.) of physiological saline with 60 or 100 m*M* K^+ from a micropipette cut to ~10 μm and positioned 55–70 μm from a cell. The concentration of NaCl was adjusted for each K^+ solution to maintain isotonicity. All cells were visually inspected after recording to verify that the cell had not been damaged. For experiments at 37°C, culture dishes were warmed using a solid-state Peltier heating device (Bionomic System; 20/20 Technology, Wilmington, NC, U.S.A.). All other recordings were carried out at room temperature (24–25°C).

Data acquisition

Electrodes were held at 0.650 V versus a locally constructed sodium-saturated calomel reference electrode using a commercially available patch-clamp instrument (Axopatch 200B; Axon Instruments, Foster City, CA, U.S.A.) configured as described previously (Borges et al., 1997). The output was digitized at 5 kHz and filtered at 2 kHz using an internal four-pole lowpass Bessel filter. Data were displayed in real time (Axoscope 1.1.1.14; Axon Instruments) and stored to computer with no subsequent filtering. To control for changes in the release properties of our chromaffin cells over time, recordings from both control and experimental cells were carried out during the same day. During the day of an experiment the same K^+ solution was used.

Data analysis

Exocytotic spikes were identified, and area (pC), $t_{1/2}$ (ms), and i_{max} (pA) values for each spike were determined using a multipass algorithm described previously (Schroeder et al., 1992). Signals were designated as spikes if their i_{max} values were eight times the SD (typically 0.6 pA) of a 1-s portion of stable baseline recorded before the first stimulation. Using these criteria, the smallest spikes detected had i_{max} values of ~4–5 pA (Moser and Neher, 1997*b*). All peaks identified by the program were visually inspected, and overlapping peaks were manually excluded from the data sets. Also, if a spike had a zero correlation with an exponentially modified Gaussian function, it was excluded (Jankowski et al., 1994; Schroeder et al., 1996). For the data presented in this article, this was on average ~2% of the well-resolved spikes. Only cells that released a minimum of 15 well-resolved events following six or seven stimulations were included for analysis.

Data were tested for statistically significant differences using the Mann–Whitney rank sum test (SigmaStat, version 2.03; SPSS, Chicago, IL, U.S.A.), the unpaired *t* test (SigmaStat), or the Kolmogorov–Smirnov two-sample test (GB STAT, version 6.5.4; Dynamic Microsystems, Silver Spring, MD, U.S.A.). For statistical comparisons using the nested ANOVA, the general linear model function in Minitab (version 12.2; Minitab, State College, PA, U.S.A.) was used with the factors groups and cells set as fixed and random, respectively. Resampling (Van der Kloot, 1996) was performed in Microsoft Excel; the worksheet for performing resampling is available by e-mail (ds43@columbia.edu) and can be easily reformulated for other resampling analyses. Results for all tests were considered significant if associated *p* values were <0.05. Correlation coefficients were calculated using Excel (version SR-1), and all box plots were created using SigmaPlot (version 4.00; SPSS). For the box plots shown, the ends of the boxes define the 25th and 75th percentiles, whereas error bars define the 10th and 90th percentiles of the data. All outliers (values outside of the 10th and 90th percentiles) are shown as black circles when present. For the PC12 data presented, cell culturing, data acquisition, and spike analysis were performed as described previously (Pothos et al., 1998*b*). All values are reported as mean ± SD.

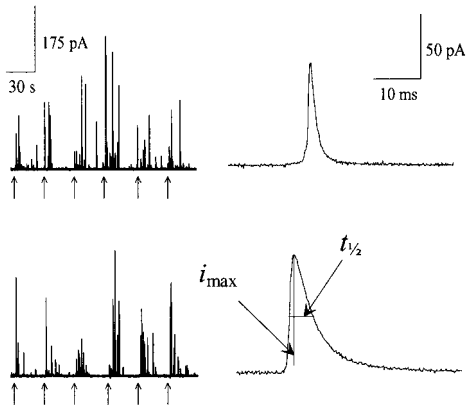

FIG. 1. Representative amperometric traces from a control (**top**) and coloboma (**bottom**) chromaffin cell. **Left:** Arrows under each trace denote when cells were stimulated with 60 m*M* K^+. **Right:** Individual current spikes from the first stimulation of each cell shown on a smaller time and current scale. Associated scale bars are shown on the left and the right. i_{max} and $t_{1/2}$ values are defined for the single coloboma spike. The area of an individual spike is defined as the time integral of the current measurements.

RESULTS

Comparing pooled samples of spike characteristics from mouse chromaffin cells gives inconsistent statistical results

To investigate if the coloboma mutation disrupts vesicular release at the single-cell level, three experiments were performed in which amperometry was used to record from separate groups of coloboma and control cells. Amperometric spikes from both types of cells can be characterized by a fast rise and slow decay and are similar in shape to those previously reported for bovine and mouse chromaffin cells (Jankowski et al., 1994; Moser and Neher, 1997*b*) (Fig. 1). To compare coloboma and control spikes, samples of area, $t_{1/2}$, and i_{max} values

TABLE 1. *Statistical analysis of pooled spike characteristics from mouse chromaffin cells*

	Mouse line				
	+/+	*Cm*/+	*T*	Statistical trends	
Experiment 1					
Area (pC)	0.26 ± 0.39	0.43 ± 0.56	58,173.5	*Cm*/+ > +/+[a]	
$t_{1/2}$ (ms)	4.89 ± 5.77	5.11 ± 5.72	61,064.0	*Cm*/+ = +/+	
i_{max} (pA)	49.9 ± 79.9	68.8 ± 98.4	58,844.5	*Cm*/+ > +/+[b]	
Events (no.)	249	266			
Cells (no.)	6	6			
Experiment 2					
Area (pC)	0.39 ± 0.61	0.27 ± 0.27	59,873.0	*Cm*/+ = +/+	
$t_{1/2}$ (ms)	7.27 ± 4.47	4.68 ± 3.88	48,272.5	*Cm*/+ < +/+[a]	
i_{max} (pA)	32.4 ± 36.1	38.0 ± 39.1	64,191.0	*Cm*/+ = +/+	
Events (no.)	253	249			
Cells (no.)	6	6			
Experiment 3					
Area (pC)	0.54 ± 0.70	0.56 ± 0.91	24,935.0	*Cm*/+ = +/+	
$t_{1/2}$ (ms)	5.83 ± 4.93	7.92 ± 6.17	29,032.0	*Cm*/+ > +/+[b]	
i_{max} (pA)	77.8 ± 102	41.5 ± 57.1	21,827.5	*Cm*/+ < +/+[a]	
Events (no.)	291	123			
Cells (no.)	7	5			

Data are mean ± SD values for pooled samples of area (pC), $t_{1/2}$ (ms), and i_{max} (pA) from groups of control (+/+) and coloboma (*Cm*/+) cells from three separate experiments. In column *T* are the sum of the ranks for the sample with the smaller number of events. Drawings to the right of each set of data represent statistical trends for the spike characteristics. Spikes represented by a dashed line indicate the relative mean change for coloboma values when the samples are significantly different.

[a] $p < 0.001$, [b] $p < 0.01$, significant difference by Mann–Whitney rank sum test.

were created from both groups of cells by pooling all the values of each spike characteristic from all cells in a group. The two pooled samples for each characteristic were then compared statistically using the Mann–Whitney rank sum test (see Discussion for an explanation of this test). Although the method of comparing pooled samples indicated that there were statistically significant differences between the shape of coloboma and control amperometric spikes, the differences were not consistent from experiment to experiment. When comparing pooled $t_{1/2}$ values for three separate experiments for example, coloboma spikes were found to be statistically equal to, faster than, and slower than those measured from control cells (statistical trends for these experiments are summarized numerically and schematically in Table 1). These results made it difficult to determine if the coloboma mutation disrupted vesicular release.

Spike characteristics from mouse chromaffin cells are cell-dependent

An underlying assumption of the statistical test used to compare characteristics of spikes recorded from control and coloboma cells is that independent random samples have been generated from the two populations (Ott, 1993). According to the definition of a random sample, every member of a population must have an equal chance of being included in the sample, and each measurement should have no effect on or relationship with any other measurement, that is, each measurement within a random sample should be mutually independent from the next (Leach, 1979; Casella and Berger, 1990).

However, pooled samples of a spike characteristic contain values from several cells. To investigate if spike characteristics within a cell were related, area, i_{max}, and $t_{1/2}$ values from individual cells were ranked in order of their increasing mean values and separately plotted. An example of this type of analysis is shown in Fig. 2, where spike area values from 13 control cells have been separately plotted as box plots and ranked from left to right in terms of their increasing mean area value. Clearly, cells

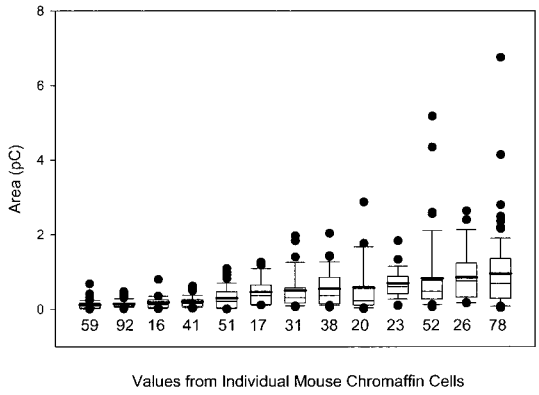

FIG. 2. Relationship between spike area values and the number of events detected at individual mouse chromaffin cells. Area values for 13 cells are ordered from left to right in terms of their increasing mean values. Mean and median values are shown in each box plot as horizontal lines. The mean of each data set is represented by the thicker line. Values were taken from the control cells used in experiments 2 and 3 (Table 1), and numbers under each box plot represent the number of events analyzed at each cell. All cells were stimulated with 100 m*M* K^+.

with smaller mean area values have a small deviation in measurements, whereas cells with larger mean area values possess a proportionately larger deviation in their measurements. Similar trends were observed when values for $t_{1/2}$ and i_{max} were plotted for individual cells in terms of their increasing mean value (data not shown).

Analysis of the relationship between the mean and SD of area, $t_{1/2}$, and i_{max} values within individual mouse chromaffin cells can be quantitatively assessed by calculating the coefficient of variation (CV) for each spike characteristic from each cell. When the CV is calculated for all coloboma and control cell spike characteristics measured at room temperature, the average CV values are 1.046 ± 0.299, 0.706 ± 0.207, and 1.110 ± 0.297 for area, $t_{1/2}$, and i_{max}, respectively (n = 47 cells). The combined average for these three CV values is 0.954 ± 0.217. These results demonstrate that when the mean of a spike characteristic changes between cells, there is a proportional change in the SD. Therefore, spike characteristics from single mouse chromaffin cells are related in magnitude and are cell-dependent.

The number of events recorded from a mouse chromaffin cell is not correlated with the mean of the spike characteristics measured at that cell

In addition to the variability in the mean area, $t_{1/2}$, and i_{max} measurements observed at individual mouse chromaffin cells, there is also a wide variability in the number of events. For the 13 cells used for Fig. 2, for example, the number of events recorded and analyzed per cell ranged from 16 to 92. A cursory examination of the data in Fig. 2 suggests that within a cell there is no relationship between the number of events and the mean of spike area values. To investigate quantitatively the relationship between the number of events and the mean of spike characteristics measured from individual cells, R^2 values were calculated for these variables. Because it was possible that experimental manipulations could mask the true relationship between these variables, only values from control cells obtained at room temperature with 100 m*M* K^+ were used. For this analysis, R^2 values were 0.0577, 0.0863, and 0.0272 for mean area, $t_{1/2}$, and i_{max} values, respectively (n = 24 cells). Clearly, there is no relationship between the mean of a spike characteristic and the number of events recorded from a mouse chromaffin cell.

Inconsistent statistical results comparing pooled samples are due to an unequal representation of different cells within the sample

The results presented above suggest that the inconsistent statistical results observed when comparing pooled samples of spike characteristics are due to an over- or underrepresentation of different cells within a group. Specifically, by pooling all the values of a spike characteristic from cells in a group, the sample from that group could be biased toward cells with a larger number of events. Likewise, cells with fewer events could be underrepresented in the sample.

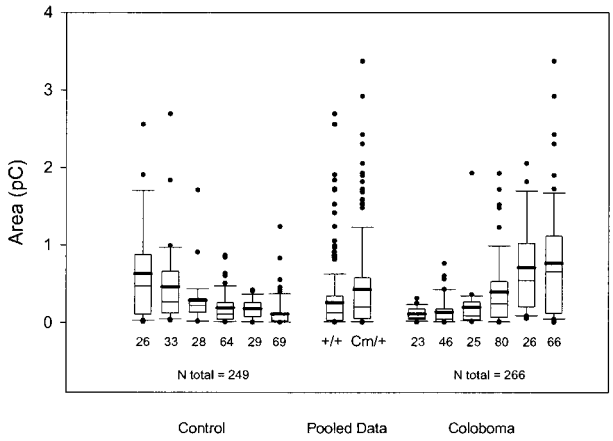

FIG. 3. Comparison of pooled and mean area values from control and coloboma chromaffin cells. Area values from six control (**left**) and six coloboma (**right**) cells are shown. The **middle two** box plots represent combined area values taken from all control (+/+) and coloboma (*Cm*/+) cells. Numbers under each box plot represent the number of events analyzed at each cell, and the numbers at the bottom of the graph indicate the total number of spikes analyzed for each group of cells. Mean and median values are shown in each box plot as horizontal lines. The mean of each data set is represented by the thicker line.

To test this hypothesis, data sets in which pooled samples gave statistically significant differences were plotted as shown in Fig. 3. In Fig. 3, spike area values from six control cells are individually plotted on the left in order of their decreasing mean area value, and area values from six coloboma cells, obtained the same day, are plotted on the right in terms of their increasing mean area values. The middle two box plots represent all of the combined area values taken from each group of control (left) and coloboma (right) cells.

For both the coloboma and control cells shown in Fig. 3, there is a wide variability in the number of events detected from each cell. In this particular experiment, two control cells that have relatively smaller mean area values (those with 64 and 69 events) make up ~53% of the total number of control area measurements. Similarly, two coloboma cells with relatively larger mean area values (those with 80 and 66 events) make up ~55% of the total number of coloboma area values. The apparent difference between pooled samples of coloboma and control area values results from the fact that cells with smaller area values are overrepresented in the control sample, whereas cells with larger area values are overrepresented in the coloboma sample.

Because there is no correlation between the mean of a spike characteristic and the number of events recorded from a cell, pooled samples of spike characteristics can be biased from experiment to experiment in an unpredictable manner. Indeed, in every instance where the use of pooled samples indicated a statistically significant difference and the data were plotted as shown in Fig. 3, differences could be attributed to an overrepresentation (or equally an underrepresentation) of cells within each group (data not shown).

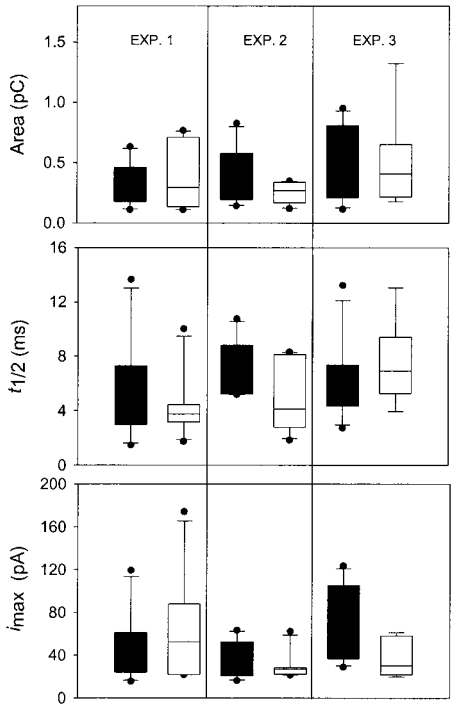

FIG. 4. Comparison of mean coloboma and control spike characteristics. Box plots summarize mean area, $t_{1/2}$, and i_{max} values measured from individual control and coloboma mouse chromaffin cells for three separate experiments. Solid and open box plots represent control and coloboma values, respectively. The number of cells used to create each box plot can be found in Table 1. The sum of the ranks for mean coloboma area values are 42.0, 30.0, and 31.0 for experiments 1, 2, and 3, respectively. The sum of the ranks for mean coloboma $t_{1/2}$ and i_{max} values are 36.0, 27.0, and 37.0 and 42.0, 36.0, and 22.0 for experiments 1, 2, and 3, respectively. When shown, the thin horizontal line in the box plots represents the median of the mean values. For all comparisons of mean values, p values were >0.05 by Mann–Whitney rank sum test.

Samples created from the means of spike characteristics

Once the problems with comparing pooled samples of spike data were realized, alternative methods for analyzing spike characteristics using statistical tests for two unrelated samples were considered. Ideally, an alternative method would generate samples from each group of cells in such a way that measurements within each sample are independent from each other and so that cells within a group are not over- or underrepresented within the sample. One alternative that meets these requirements is to create a sample from each group of cells by taking the mean for a spike characteristic from each cell in a group.

Unlike the results obtained comparing pooled samples, comparisons of mean spike characteristics consistently indicate that coloboma and control amperometric spikes do not significantly differ in their area, $t_{1/2}$, and i_{max} values. Results from these tests are summarized in Fig. 4. It should be noted that samples of mean values are created by "pooling" all of the mean values of a spike characteristic from a group of cells. However, we will refer to this method as the method of comparing means for clarity.

The method of comparing means can be used to detect changes in the shape of amperometric spikes recorded from mouse chromaffin cells

One of the main disadvantages of comparing samples of mean spike characteristics is that there is a lower number of measurements within each sample. To determine if the method of comparing means could be used to detect differences in spike characteristics from two groups of mouse chromaffin cells, two experiments were performed in which release was measured from a group of cells at room temperature and at 37°C. The results from these two experiments are summarized in Fig. 5. At

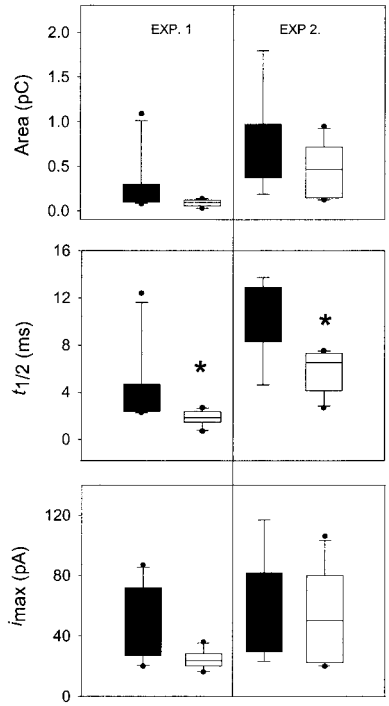

FIG. 5. Effect of elevated temperature on the means of spike characteristics measured from separate groups of mouse chromaffin cells. Box plots summarize mean area, $t_{1/2}$, and i_{max} values measured from individual mouse chromaffin cells at room temperature (solid box plots) and at 37°C (open box plots) for two separate experiments. For experiment 1, values were taken from six cells at room temperature and at 37°C. For experiment 2, values were taken from five and six cells at room temperature and 37°C, respectively. The sum of the ranks for mean values from room temperature cells are 49.0 and 33.0, 53.0 and 41.0, and 50.0 and 32.0 for area, $t_{1/2}$, and i_{max} values in experiments 1 and 2, respectively. In experiment 1, 320 events were measured from cells at room temperature, and 1,312 events were measured from cells at 37°C. In experiment 2, 263 and 635 events were measured from cells at room temperature and at 37°C, respectively. When shown, the thin horizontal line in the box plots represents the median of the mean values. Mean values that are significantly different (by Mann–Whitney rank sum test) from those measured at room temperature are marked: $*p < 0.05$ for experiment 1; $p = 0.052$ for experiment 2 (see text).

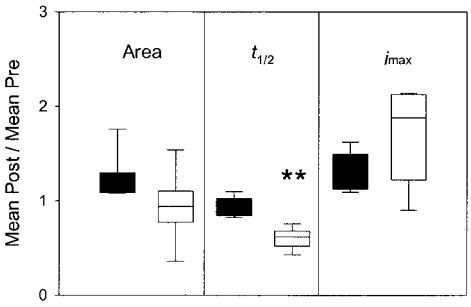

FIG. 6. Effect of elevated temperature on spike characteristics measured from the same mouse chromaffin cell. Ratios of mean spike characteristics (post/pre) were created from individual cells in which the temperature had been elevated to 37°C during the recording (open box plots) and from control cells in which the temperature was not changed (solid box plots; see text for further explanation of the method). Only control cells that had a total number of events similar to the total measured from cells in which the temperature had been elevated were used. Ratios were created from these cells by calculating mean values for each half of the data set. The thin horizontal line in the box plots represents the median of the ratio values. Data were taken from five control and experimental cells. The sum of the ranks for room temperature ratios are 36.0, 40.0, and 22.0 for area, $t_{1/2}$, and i_{max}, respectively. The average number of events used to calculate post/pre ratio values is 43/39 and 83/35 for room temperature and 37°C cells, respectively. Ratios that are significantly different (by Mann–Whitney rank sum test) than those measured from cells with no change in temperature are marked: ** $p < 0.01$.

37°C, the only consistent difference in spike shape appears to be a decrease in the mean $t_{1/2}$ values, although when comparing mean $t_{1/2}$ values in experiment 2 the associated p value is 0.052. This value is slightly greater than the traditionally accepted value of 0.05.

Because the variability in mean spike characteristics at different cells could mask effects due to an increase in temperature, a separate set of experiments was performed in which release was measured at the same mouse chromaffin cell before and after the temperature of the culture dish was changed. For these experiments, a single mouse chromaffin cell was stimulated repeatedly both before and after the temperature of the culture dish was raised to 37°C. Mean values for area, $t_{1/2}$, and i_{max} were calculated from a cell both before (preelevation) and after (postelevation) the temperature was elevated. Ratio values for each characteristic were then created for a cell by dividing the postelevation mean value of a characteristic by its corresponding preelevation mean value (mean postelevation/mean preelevation). Ideally, if there was no change in a spike characteristic, the ratio value(s) should be close to 1. To account for variability in the technique, ratio values of spike characteristics were also created from control cells in which the temperature was not changed. Ratio values for each characteristic from these two types of cells were compared statistically. Results from these experiments are summarized in Fig. 6 and also indicate that an increase in temperature decreases amperometric spike $t_{1/2}$ values from mouse chromaffin cells.

Spike characteristics from PC12 cells are cell-dependent

To investigate if the properties of mouse chromaffin cell spike characteristics were present in other cell types, amperometric spikes recorded from individual PC12 cells were also examined. Shown in Fig. 7 are the i_{max} values recorded from 13 PC12 cells. These data have been adapted from results reported previously (Pothos et al., 1998*b*) and plotted in the same manner as the mouse chromaffin spike area values shown in Fig. 2. The i_{max} values from PC12 cells also appear to be cell-dependent. To assess quantitatively the relationship between the mean and SD of spike characteristics at individual PC12 cells, the CV for these characteristics has been calculated from several cells. The average CV for untreated PC12 cells is 1.091 ± 0.498, 0.883 ± 0.404, and 1.026 ± 0.457 for area, $t_{1/2}$, and i_{max} values, respectively (n = 34 cells). The combined average for these three CV values is 1.000 ± 0.106. These results demonstrate that spike characteristics for single PC12 cells are also related in magnitude and are cell-dependent.

The number of events recorded from a PC12 cell is not correlated with the mean of the spike characteristics measured at that cell

As with mouse chromaffin cells, there is a significant variability in the number of events detected at different PC12 cells (see numbers below box plots in Fig. 7). On examining the data in Fig. 7, there again appears to be no relationship between the number of events and the mean of a spike characteristic measured at a single PC12 cell. To determine quantitatively the relationship between the number of events and the mean of spike characteristics measured from individual cells, correlation coefficients were calculated for these variables. To prevent an unequal number of stimulations at different cells from confounding the relationship between these variables,

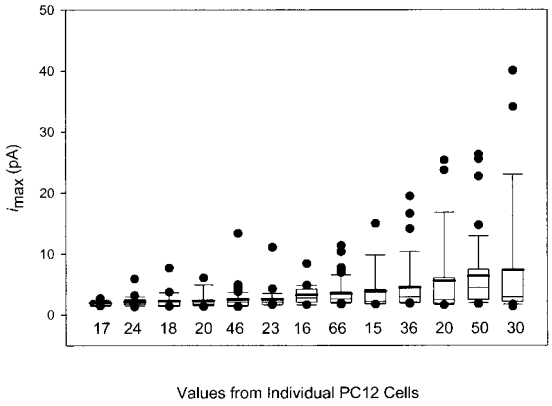

FIG. 7. Relationship between spike i_{max} values and the number of events detected at individual PC12 cells. The i_{max} values from 13 PC12 cells are separately plotted and ordered from left to right in terms of their increasing mean values. The mean of each data set is represented by the thicker line. Numbers under each box plot represent the number of spikes analyzed from each cell. Only spikes within the first 25 s of the first stimulation were counted (see text).

TABLE 2. *Statistical analysis of pooled and mean spike values from PC12 cells*

	PC12 cells			
	Control	L-DOPA	*T*	Statistical trends
Pooled values				
Area (pC)	0.06 ± 0.14 (2,262)	0.14 ± 0.26 (1,329)	3,123,260.5	L-DOPA > control[a]
$t_{1/2}$ (ms)	6.62 ± 8.27 (2,010)	6.91 ± 6.61 (1,230)	2,014,981.0	L-DOPA = control
i_{max} (pA)	5.36 ± 8.62 (2,266)	9.80 ± 19.0 (1,337)	3,036,905.5	L-DOPA > control[a]
Mean values				
Area (pC)	0.06 ± 0.06 (34)	0.09 ± 0.07 (25)	948.0	L-DOPA > control[b]
$t_{1/2}$ (ms)	6.58 ± 2.19 (34)	7.00 ± 1.78 (25)	798.0	L-DOPA = control
i_{max} (pA)	6.46 ± 4.88 (34)	8.72 ± 4.55 (25)	905.0	L-DOPA > control[c]

Data are mean ± SD values of samples created using the means of (bottom) or all (top) area (pC), $t_{1/2}$ (ms), and i_{max} (pA) values measured from PC12 cells either with or without prior exposure to 50 μM L-DOPA. For the program used to analyze these data, some area and/or $t_{1/2}$ values were difficult to define owing to the complex nature of the signal, and spurious, i.e., negative, values were discarded. The numbers of measurements used to compare spike characteristics from control and L-DOPA-treated cells are given in parentheses. In column *T* is the sum of the ranks for the sample with the smaller number of measurements.

[a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$, significant difference by Mann–Whitney rank sum test.

only data from the first 25 s of the first stimulation were used. Also, only values from untreated PC12 cells were considered. R^2 values for the relationship between mean area, $t_{1/2}$, and i_{max} values and the number of events measured from single PC12 cells are 0.1206, 0.0676, and 0.0003, respectively (n = 34 cells). From these results it can be concluded that there is no relationship between the mean of a spike characteristic and the number of events recorded from a single PC12 cell.

The method of comparing means can detect changes in the shape of amperometric spikes measured from two groups of PC12 cells

To determine if the method of comparing means can detect changes in spike characteristics measured from two groups of PC12 cells, mean area, $t_{1/2}$, and i_{max} values were compared from control cells and cells treated with 50 μM L-DOPA for 30 min before recording. Mean area, $t_{1/2}$, and i_{max} values are summarized in Table 2 along with relevant statistical values. Statistical comparison of mean spike characteristics indicates that treatment with 50 μM L-DOPA for 30 min significantly increases the i_{max} and area values of amperometric spikes recorded from PC12 cells. For comparison (see Discussion), statistical results using the pooling method and the method of comparing means are both shown in Table 2.

DISCUSSION

We have used amperometry to investigate alterations in individual quantal characteristics from separate groups of mouse chromaffin and PC12 cells. Previously used methods for comparing amperometric spike characteristics between two separate groups of cells have entailed pooling all of the values for a spike characteristic from one group of cells and comparing this sample statistically to a pooled sample created from the other group of cells (Jankowski et al., 1994; Pihel et al., 1996; Pothos et al., 1996, 1998*b*). As a first step in comparing area, $t_{1/2}$, and i_{max} values between coloboma and control mouse chromaffin cells, pooled samples for each characteristic have been compared statistically using the Mann–Whitney rank sum test. For this test, measurements from both samples are combined, and all observations are jointly ranked from lowest to highest. A test statistic, *T*, is then created by summing the ranks for one of the samples (generally the sample with the smaller number of observations), and this value is used to test the null hypothesis that the two samples were drawn from identical populations. The Mann–Whitney rank sum test is sometimes referred to as the Wilcoxon rank sum test (Leach, 1979) and is equivalent to the statistical test used previously to compare pooled samples of amperometric spike characteristics measured from two separate groups of cells (Ott, 1993; Jankowski et al., 1994; Pihel et al., 1996). The Mann–Whitney rank sum test is a nonparametric, or distribution-free, test that does not assume that the populations from which the samples are drawn are normally distributed (for a more detailed discussion of this test, see Ott, 1993). Nonparametric tests have consistently been used to compare nontransformed spike characteristics because these measurements are severely skewed to the right (Jankowski et al., 1994; Pihel et al., 1996; Jaffe et al., 1998; Pothos et al., 1998*b*).

As shown in Table 1, the use of pooled samples gives inconsistent statistical results from experiment to experiment, making it difficult to determine if the coloboma mutation disrupts release at the vesicular level. In an effort to draw more reliable statistical and experimental conclusions, the underlying assumptions of the statistical tests used as well as the structure of amperometric data recorded from two populations of cells have been reexamined.

When spike characteristics are pooled from two groups of sampled cells, there are only two samples to compare for each characteristic. One of the underlying assumptions of the Mann–Whitney rank sum test is that

independent random samples have been created from coloboma and control chromaffin cells (Ott, 1993). The nature of creating samples from two distinct groups of cells insures that the two samples are independent. According to the definition of a random sample, spike characteristics from a group of cells can be pooled to create a random sample provided the measurements within the sample are not related (Leach, 1979; Casella and Berger, 1990). Intuitively, one would not expect spike characteristics from one cell to be related to those from the next. However, CV values of spike characteristics from individual mouse chromaffin cells have values close to unity. This demonstrates that as the mean of a spike characteristic changes from individual cells, there is a proportional change in the SD. Therefore, spike characteristics from single mouse chromaffin cells are related in magnitude and are cell-dependent. Thus, when values of a spike characteristic are combined from a group of mouse chromaffin cells to create a pooled sample, measurements within the sample are not independent from each other but depend on the cell from which they were recorded, and the assumptions of a random sample are not met. Although we have referred to spike characteristics as being "cell-dependent," it is important to note that spike characteristics could be site-dependent, varying systematically with different positions of the electrode on the same cell. This hypothesis, however, was not tested in this study.

Another factor that can account for the inconsistent statistical results observed when pooled samples of spike characteristics are compared is that the number of events recorded from individual mouse chromaffin cells is highly variable. Also, there is no correlation between the mean of a spike characteristic and the number of events recorded from a cell. As a result, pooled samples of these values are subject to unpredictable bias between experiments.

To circumvent both the problems of independence and over- and underrepresenting different cells, an alternative statistical method has been used to compare spike characteristics from two separate groups of cells. This method consists of creating a sample for a spike characteristic by combining all the mean values for that characteristic from all the cells in a group. Although the mode and the median of spike characteristics from individual cells in a group could have been used to create samples, only the means of subsets can be combined to determine the mean of the complete data set (Ott, 1993). The two samples of means for each spike characteristic are compared statistically using the Mann–Whitney rank sum test. We continue to use this test (as opposed to the *t* test) when comparing samples of mean values because samples of means are generally small and assumptions of normality cannot be reliably tested.

The method of comparing means is more ideal than comparing samples of pooled spike values for several reasons. First, by taking only one value from each cell in a group, the measurements within the sample are independent. Also, by taking one value from each cell in a group, the characteristics of amperometric spikes recorded from any cell are not under- or overrepresented within the sample. Unlike the method of comparing pooled samples, the method of comparing mean values of spike characteristics recorded from coloboma and control chromaffin cells gives consistent results from experiment to experiment. The overall advantage of creating a sample from a group of cells using the mean of a spike characteristic from each cell can be seen in Fig. 3. Although samples created by pooling the data indicate that control cells have smaller area values (see results for experiment 1 in Table 1), the mean area values between these two groups of cells are essentially identical.

To demonstrate that the method of comparing means can detect changes in spike characteristics measured from mouse chromaffin cells, two experiments have been performed in which release was measured from separate groups of cells at room temperature and at 37°C. For these experiments, there was a significant decrease in the mean $t_{1/2}$ values of spikes measured from cells at 37°C. These results were confirmed by also analyzing how an increase in temperature affects release at the same cell. Experiments at the same cell should be more sensitive to the effects of an increase in temperature because changes are less likely to be masked by cell-to-cell variability.

For experiments at the same cell, postelevation/preelevation mean ratio values (see above) were calculated for each spike characteristic from individual cells in which the temperature was either kept constant or elevated to 37°C during the recording. Ratio values for each characteristic from both types of cells were compared statistically. Results from experiments at the same cell also indicate that an increase in temperatures decreases $t_{1/2}$ values at mouse chromaffin cells (Fig. 6). These results support the conclusions drawn from comparing samples of mean spike characteristics and confirm that the method of comparing means can be used to detect differences in spike values when they are measured from two separate groups of mouse chromaffin cells.

Recently, two studies have independently examined how temperature affects the characteristics of amperometric spikes detected at individual cells (Pihel et al., 1996; Walker et al., 1996*b*). Our results are consistent with these studies in that both reported a significant decrease in the widths of amperometric spikes when release was measured at 37°C. These two reports, however, differ significantly in other aspects. Specifically, results reported by Pihel et al. (1996) indicate that an elevation in temperature also increases the area of individual current spikes and the number of events detected at single bovine chromaffin and mast cells. These results apparently conflict with those reported by Walker et al. (1996*b*), who found that elevated temperatures only decrease the rise and decay times of amperometric spikes recorded from bovine chromaffin cells. Our results partially agree with both of these studies. Although none of the measurements made from mouse chromaffin cells at 37°C suggests that an increase in temperature increases the area of amperometric events detected from these

cells, we did consistently notice an increase in the number of events (see legends to Figs. 5 and 6).

To investigate if comparing pooled samples of amperometric spike characteristics could be problematic for other cell types, the properties of amperometric spikes recorded from single PC12 cells were also examined. Like those from mouse chromaffin cells, amperometric spike characteristics from PC12 cells are cell- (or possibly site-) dependent, and the number of events detected at a single PC12 cell is unrelated to the mean of spike characteristics measured at that cell. These results suggest that pooled samples of PC12 spike characteristics can also vary unpredictably from experiment to experiment and that the method of comparing mean values of spike characteristics may also be more appropriate for this cell type. To illustrate that the method of comparing means can detect changes in PC12 spike characteristics, samples of mean area, $t_{1/2}$, and i_{max} values have been compared from a group of control PC12 cells and a group of PC12 cells exposed to 50 μM L-DOPA. As shown in Table 2, differences in spike characteristics from two groups of PC12 cells can be detected using this method.

In past studies comparing amperometric spike characteristics between two groups of cells, the Mann–Whitney rank sum test has not been the only statistical test used to compare pooled samples. Some investigators have taken the cubed root of area values and then compared pooled samples of these values from two groups of cells using the *t* test for unrelated samples (Pihel et al., 1996). Likewise, others have used the Kolmogorov–Smirnov two-sample test to compare untransformed pooled spike characteristics (Jaffe et al., 1998; Pothos et al., 1998*a*,*b*). Both of these methods also give inconsistent results when comparing coloboma and control spike characteristics from experiment to experiment (data not shown). These results can be explained by the fact that both of these tests also assume that two independent random samples have been generated from each population (Ott, 1993; Daniel, 1995) and that pooled samples of even transformed values are subject to bias. It should also be noted that although most studies have compared the shape of amperometric spikes from two groups of cells by comparing pooled data, one study has compared samples of mean values (Walker et al., 1996*a*).

Recently it has been shown that different statistical methods can also lead to different experimental conclusions when analyzing two pooled samples of quantal release events derived from postsynaptic recordings at the neuromuscular junction (Van der Kloot, 1996). To overcome the inconsistencies obtained using previous tests, it was proposed that an alternative statistical method, resampling (see Materials and Methods), be used. Unfortunately, this approach also yielded inconsistent statistical results for the mouse chromaffin cell data presented here. Presumably these inconsistencies resulted from the fact that pooled samples were also used for these comparisons (Van der Kloot, 1987).

When the means of spike characteristics are compared from two groups of cells, the power of the statistical test is decreased simply owing to the smaller size of the sample taken from the two populations of cells. An alternative statistical method that can be used to compare the characteristics of spikes recorded from two populations of cells is the nested or hierarchic ANOVA test. One advantage of this statistical test is that it allows all the spike characteristics from two groups of cells to be compared without pooling the data from each group. As a result, this test is more powerful than comparing samples of mean spike values (for a more detailed description of the nested ANOVA method, see Sokal and Rohlf, 1969; Ott, 1993).

To examine if the nested ANOVA test gives statistical results similar to those found when comparing the means of spike characteristics, amperometric spike values from mouse chromaffin cells have also been analyzed using this method. Unlike the Mann–Whitney rank sum test, there are normality and equal variance assumptions associated with the nested ANOVA (Sokal and Rohlf, 1969; Ott, 1993). Amperometric spike characteristics are extremely skewed to the right, and to meet the assumptions of the ANOVA method some type of transformation of the data is required. Because there is a direct correlation between the mean and SD of spike characteristics within each cell (see Fig. 2), spike values have been transformed using the log function (Sokal and Rohlf, 1969; Ott, 1993), and tests for significant differences have been performed using these transformed values. Results using the nested ANOVA agree with those found by comparing means of spike values. This test consistently indicates that there is no significant difference between coloboma and control spike characteristics and consistently detects a decrease in $t_{1/2}$ values for cells analyzed at 37°C (data not shown).

For one of the comparisons of area values and two comparisons of i_{max} values between coloboma and control chromaffin cells, normal probability plots of the residuals indicated that the assumptions of the ANOVA method were, for reasons unknown, not being met. Results from these three comparisons would have been more reliable if an appropriate transformation could have been found. Although the nested ANOVA is undoubtedly the more powerful test to compare spike characteristics measured from two groups of cells, one disadvantage to this approach is that spike values have to be transformed to meet the assumptions of the test. One major advantage to using the Mann–Whitney rank sum test to compare mean values of spike characteristics is that this test has no underlying assumptions about the structure of the data. Although this method may be more conservative than the nested ANOVA, results from this test can also be more easily interpreted because the analysis is based on ranks.

When a significant difference in a spike characteristic between two groups of cells is found using the method of comparing means, the same statistical difference is also found using the pooling method. Compare, for example,

the statistical results when both methods were used to compare i_{max} and area values from control and L-DOPA-treated PC12 cells (Table 2). These methods are also in agreement when they are used to compare $t_{1/2}$ values from separate groups of mouse chromaffin cells analyzed at room temperature and at 37°C (pooled results not shown). These results indicate that when the change is large enough to overcome the cell-to-cell variability, both the pooling method and the method of comparing means can be used to detect a change in a spike characteristic. However, as has been shown in this study, pooled samples of spike values can give highly significant statistical results that are not found when more appropriate methods such as the method of comparing means or the nested ANOVA are used. Therefore, the major disadvantage of comparing pooled samples is that there is an increased probability of finding a difference between two groups of cells when it is not present, i.e., committing a type one error.

Conclusion

In conclusion, results presented here demonstrate that comparing samples of pooled spike characteristics from separate groups of cells may lead to inconsistent statistical results that make it difficult to interpret amperometric data. The main difficulty with comparing pooled samples is that the variability between cells is overlooked. Furthermore, pooled samples do not meet the requirements of two-sample statistical tests. We propose alternative methods for comparing amperometric spike measurements made at two separate groups of cells. These methods are more appropriate as they take into account cell-to-cell variability and the assumptions of the statistical tests being used and give consistent results from experiment to experiment.

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