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Automatic analysis for amperometrical recordings of exocytosis

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Abstract

Amperometry is a widely used technique for monitoring the secretion of catecholamines (CA) by exocytosis. The use of carbon fibre microelectrodes allows the on-line recording of CA released from a single secretory vesicle. Amperometric signals are generated by oxidation of the quantally released CA close to the electrode tip. Each event of exocytosis is called a secretory spike. Here we describe a program written for IGOR (Wavemetrics, Lake Oswego, OR, USA), which may be used to analyze amperometric signals off-line. The procedures allow, (i) digital filtering and analysis of the current noise, spike identification and calculation of spike kinetic parameters; (ii) spike review; (iii) pooling spikes and data to create galleries, tables and histograms of measured parameters which can be exported to a graphic format or files for further analysis.

Keywords: Adrenaline; Catecholamines; Chromaffin cells; Digital filtering; IGOR macros; Secretion; Spike analysis

1. Introduction

Chromaffin cells release CA by exocytosis, a calcium-dependent process that entails the fusion of a secretory vesicle and the cell membrane. Single vesicle exocytosis can be monitored using amperometric techniques combined with carbon fibre microelectrodes placed on the cell surface (Wightman et al., 1991). This approach allows the direct observation of the time–course of single secretory events and has been successfully used to study the late phase of exocytosis (Jankowski et al., 1993; Chow et al., 1994; Schroeder et al., 1996).

A carbon fibre microelectrode, acting as an amperometrical working electrode, detects the CA exocytosis of a chromaffin granule. The oxidation of a molecule of CA produces two electrons which are taken up by the positively charged working electrode, thereby creating a current directly proportional to the CA concentration surrounding the electrode tip (Kawagoe et al., 1993). A potentiostat maintains a fixed potential and amplifies the current producing an output voltage signal, which can be acquired on-line.

Amperometrical recording experiments commonly produce over a hundred megabytes of raw data whose analysis requires the help of well-structured programs. Several programming languages like Basic have been used to make locally-written software (Schroeder et al., 1996) although they are very slow to analyze large series of raw data. Commercially available acquisition/analysis software for spike analysis eg AxoScope® (Axon Instruments, Foster City, CA), on the other hand have not been specifically conceived for amperometry (Graham and Burgoyne, 2000). IGOR is a software specially designed to work with data arrays. It offers its own programming environment with extensive libraries of built-in functions, which can be complemented with user-written macros to create procedures for specific purposes. These advantages have been used in the past to create small programs for secretory spike analysis (Chow et al., 1992, 1994; Elhamdani et al., 1998, 1999).

Due to large variability on secretory spikes and characteristics obtained upon cell stimulation, even from a single cell, it becomes necessary to conduct statistical studies on populations of several hundreds of events. One important feature of an analysis program for amperometry should be its versatility. Researchers

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should be able to choose the type and parameters of filters used and the spike identification criteria. In addition, the program should perform an automatic processing of previously selected data files and manual supervision and correction of results. Furthermore, final results should be exportable to graphic or statistical programs for further analysis.

Here, we present a series of macros written for IGOR for the full analysis of exocytotic events detected by amperometry. All of these macros are freely available (see below).

2. Material and methods

2.1. Chromaffin cell cultures

Bovine adrenal chromaffin cells, enriched in adrenaline through a single-step Urografin® gradient, were prepared as described elsewhere (Moro et al., 1990). Cells were plated on 12 mm-diameter glass coverslips contained in 24-well culture plates at an approximate density of 5×10^5 cells per well. Cells were maintained at 37°C in a water-saturated and 5% CO_2 environment and were used at room temperature between 1 and 5 days after isolation.

2.2. Amperometric detection of exocytosis

Carbon fibre microelectrodes were prepared as described (Kawagoe et al., 1993; Cahill et al., 1996). Carbon fibres of 5 μm radius (Thornel P-55; Amoco Corp., Greenville, SC, USA) were a kind gift of Professor R.M. Wightman, Chapel Hill, NC, USA. Electrodes were backfilled with 3 M KCl to connect to the headstage and they were tested with a flow-injection system using noradrenaline as a standard solution. Electrochemical recordings were performed using an EI-400 potentiostat (Ensman Inst. Bloomington, IN, USA) or an Axopatch 200B (Axon Instruments, Foster City, CA, USA). A fixed potential of +650 mV was maintained between the carbon fibre electrode versus an Ag/AgCl pellet reference electrode.

Glass coverslips with adhering cells were placed in a perfusion chamber mounted on the stage of an inverted microscope (DM-IRB Leica, Weltzlar, Germany). Cells were incubated in a Krebs–HEPES solution containing (in mM), NaCl (140); KCl (5); $MgCl_2$ (1.2); $CaCl_2$ (2), HEPES (10) Glucose (11) pH 7.35 (NaOH). Carbon electrodes were gently placed onto the cell membrane. Cell secretion was stimulated by pressure ejection of 5 mM $BaCl_2$ 5 s from a glass micropipette situated 40 μm from the cell.

2.3. Amperometry data acquisition

Amperometric signals were low-pass filtered at 1 kHz, sampled at 4 kHz through a PCI-1200 card (National Instruments, Austin, TX, USA) and collected using locally-written software (Labview 5.0 for Macintosh, National Instruments). This program (i) configures A/D card, port identification and sets sampling rate; (ii) displays data on screen and (iii) saves data to the hard disk. Each experiment is saved in a format recognized by IGOR.

Since the sampling rate used for data acquisition of biological signals is generally slow (1–10 kHz), sequential cards may be used satisfactorily. These are economical and allow pseudo-simultaneous data acquisition from several channel entries.

2.4. Programs for data treatment and analysis

IGOR is an integrated software specifically created for visualizing and analyzing large amounts of numeric data. The term 'wave' is used to describe the IGOR object that contains an array of numbers, which usually consists of thousands of values. Our acquired waves usually have 500 Kpts with 32 bits single precision floating point numeric type (i.e. ≈ 2 min at 4 kHz). It takes ≈ 2 Mb of hard disk space.

The automatic data analysis includes (i) digital filtering, (ii) remainder noise analysis, (iii) spike identification, (iv) location of the initial, maximum and final points of identified spikes, (v) extraction of kinetic parameters and (vi) histogram creation.

3. Results

3.1. File location and destination

A flow diagram used for data analysis is illustrated in Fig. 1. Program asks for the folder where raw data were stored. Once this path has been defined, the automatic analysis macro can be run.

3.2. File finding and opening

The program loads the file into RAM in the form of a wave.

3.3. Data scale adjustment

User introduces calibration scale to convert signal from voltage to current (i.e. 100 pA/V).

3.4. Digital filtering

User selects the most suitable filter settings for reduction of line- and high frequency-noise which are present with signal. Program calculates the FFT and proceeds to apply filters.

The macro allows the use of several types of filters that can be combined.

Notch filters: the maximum noise component is line frequency, 50/60 Hz, and its odd harmonics (i.e. 150, 250, 350, ...). The number of harmonics and the window width can be chosen. Filtering is carried out, in the frequency domain, by the suppression of the desired notch window, through an interpolation line from each window limits. For instance, if a 50 Hz and two odd harmonics with 1 Hz notch window width are selected, the macro will filter all the frequencies between 49 and 51, 149 and 151, 249 and 251 Hz.

Rolling-off low-pass filter: this is a low-pass filter which gradually filters between two selected frequencies. For instance, if user chooses 400 and 1000 Hz, the macro will attenuate progressively from 400 Hz until the total suppression of frequencies over 1000 Hz.

Smoothing filter: this is an IGOR built-in function. This smoothing algorithm computes the output value for a given point using an equal number of neighboring points before and after the point being smoothed, acting as a low-pass filter. Smoothing is particularly useful in attenuating signal oscillations caused by white noise.

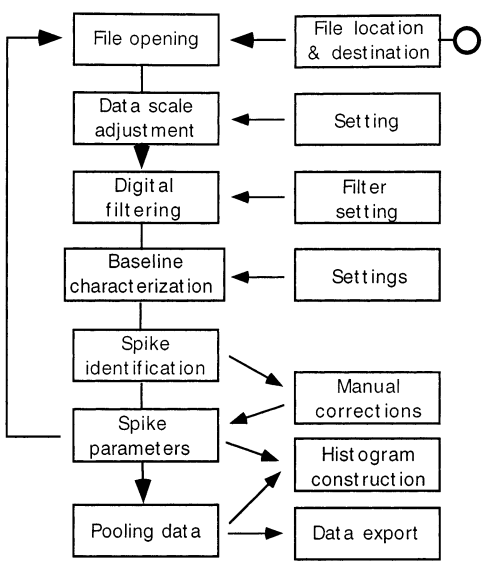

Fig. 1. Program structure. Main parts of the program are shown. On the left is situated the automatic analysis and on the right the interaction with the researcher: program settings and manual corrections.

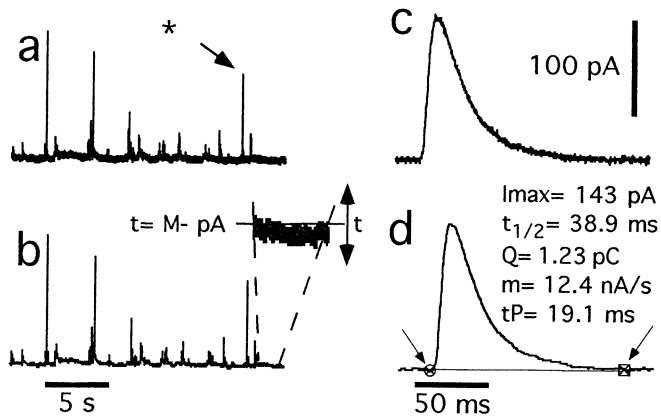

Fig. 2. Spike identification and processing. (a) Trace of raw unfiltered data. Asterisk indicates the secretory spike magnified in c and d. (b) Filtered data. The effect of digital filtering of trace a. Noise threshold (t) is set by a subtraction of a fixed current value (pA) from the averaged current of the whole file (M). (c) Enhanced spike from trace a. (d) Enhanced spike from trace b. Program displays the parameters extracted from the spike. See text and Fig. 4 for details.

Customized filter: IGOR allows the use of convoluting functions to design several digital FIR (finite impulse response) filters which may be customized by providing a wave of filter coefficients (please see to Wavemetrics IGOR Manual).

Fig. 2 shows the effect of digital filtering. Four notch filters (50, 150, 250 and 350 Hz, 1 Hz window width) and a rolling-off filter (from 400 to 1000 Hz) were used. As can be seen, no important changes in the I_{max} are observed between the raw and filtered data, indicating that there was no over filtering.

Filter condition settings require some pre-test experiments to establish which are the major components of noise present within the file. This can easily be done providing IGOR allows the FFT, on the condition that an even number of data is used. After analogic low-pass filtering is carried out by the potentiostat, usually with a Bessel filter, line noise, usually 50 Hz, is the most abundant component in the frequency domain. Odd-harmonics of line noise are usually also present. Rolling-off allows the selective removal of high frequencies, which do not contribute to the signal component of a secretory spike.

3.5. Baseline identification

Fig. 2b shows how the program locates a segment of the baseline free of current spikes to analyze noise. The average current of the whole file is calculated (M), then an initial arbitrary threshold (t) is fixed by subtraction of a fixed amount of current (usually 2 pA) from the average current (t = M-2 pA), as indicated by the horizontal line. Program searches for a 2 s segment below the threshold value, starting from the beginning of the

file and advancing 0.5 s until a segment is located. If not found, the threshold value is increased by 0.5 pA, and the search repeated. If the threshold reaches an excessive value (i.e. M + 2 pA) the search is restarted using a shorter segment. The program returns an error message when all of these strategies fail to locate the baseline. It usually occurs in less than 1% of the files analyzed.

3.6. Spike identification

First the program calculates the numerical first derivative of the whole record. Then a smoothing filter is applied to the derivative wave in order to improve the signal/noise ratio. The use of the first derivative is particularly useful because it enhances the raising slopes of oscillations that supposedly are greater in spikes than in noise. In addition, slow oscillations of the baseline are not evident within the first derivative trace.

The noise level, present within the signal, is estimated using the standard deviation of the baseline segment already located (s_B) and its first derivative (s_S,). Both s_B and s_S are calculated with the usual estimator of S.D.

$$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$$

where x_i, signal point values; N, the number of data points; $\bar{x} = (1/N)\Sigma_{i=1}^{N} x_i$ (the average of point values).

A multiple-pass algorithm is used for spike identification. (i) Program finds the maxima of the spikes. In the first derivative wave, it looks for peaks over a threshold, defined as k times s_S. User can modify k. This local maximum, sited at the ascending slope of the original spike, is defined as t_a (Fig. 3). (ii) The program then looks for t_b, the first point with the same value than t_a, towards its right. (iii) The maximum of the spike is found between t_a and t_b. (iv) The minimal values between peaks are used to calculate the spike height. (v) The program deletes those spikes whose heights are under a threshold, defined as k' times s_B (k' can be modified by user), since these are considered noise. (vi) The beginning and ending points of the spikes are located. The program calculates the mode of values situated within the 300 ms previous to the peak. This strategy is used because mode defines better than mean what the middle line of the noise band is. (vii) The program localizes the first point with a value under the mode going backwards from the maximum of spike (max). This point will correspond to the beginning of the spike. (viii) The same strategy is used to localize the end of spike but using the mode calculated within 600 ms from the max.

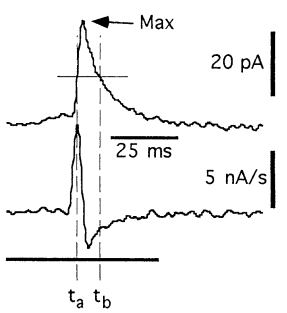

Fig. 3. Algorithm followed for spike identification. Upper graph shows a filtered trace and the lower graph its first derivative. Note the different scales in the calibration vertical bars. See the text for further explanations.

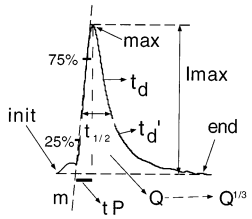

Fig. 4. Spike parameters. See text.

3.7. Spike parameters

Fig. 4 describes the parameters measured from each secretory spike (Jankowski et al., 1993).

Once the automatic analysis has been performed, the researcher is able to examine the results. To facilitate the reviewing of spikes, the whole wave and individual spikes are shown. The researcher can move easily along the record and make changes such as addition or deletion of spikes and corrections on the initial/final points. Once an experiment has been checked, the kinetic parameters (I_{max}, m, tP, …) can be displayed as histograms.

3.8. Pooling results

Due to the large number of secretory events required to perform a statistical analysis, it is necessary to accumulate spikes from different experiments/files. Once data are grouped, histograms and tables can be made of kinetic parameters from hundreds/thousands of spikes. These data can easily be exported in graphical or statistical formats.

4. Discussion

Amperometric recording analysis requires the assistance of computer programs capable of dealing with files with a large quantity of data. Several programming languages (BASIC, FORTRAN, C or PASCAL) have been used to write analysis procedures (Schroeder et al., 1996; Zhou et al., 1996; Borges et al., 1997; Marszalek

et al., 1997). IGOR has become very popular for off-line analysis of biological signals and macros for spike analysis have been made by our group and others (Criado et al., 1999; Zhou et al., 1996; Moser and Neher, 1997).

We have improved the analytical procedures for amperometrical data by developing a program with three macros called: (i) *Spike-analysis*, which includes full automatic noise analysis and spike finding; (ii) *Spike-view*, that is intended for spike overviewing and (iii) *Galleries*, used for pooling data. The consecutive use of these three macros permits the conversion of raw data, usually a binary or text file, into tables of spike kinetic parameters, which can be used to create graphics and perform statistical analysis. We currently use this program to study the effects of drugs on the late step of exocytosis (Machado et al., 2000) as well as to validate mathematical models of the secretory phenomenon.

Spike parameters provide valuable information about the secretory process and about the conditions of CA storage prior to release. For instance, net granule charge (Q, $Q^{1/3}$) reveals the amount of CA present within the granules. Under conditions where chromaffin cells have not been treated or altered, $Q^{1/3}$ roughly indicates the granule sizes (Jankowski et al., 1993; Schroeder et al., 1996). The pre-spike feature 'foot' is thought to reflect the CA liberation from granules through an undilated fusion pore (Chow et al., 1992; Alés et al., 1999). In addition, other parameters extracted from spikes have been used to infer the release kinetics related to the exocytotic process (Schroeder et al., 1996). Hence, Imax indicates the highest concentration of CA reaching the electrode tip. Chromaffin cell is a model for neurotransmission and the electrode tip will detect CA secretion as a post synaptic cell. Other parameters like $t_{1/2}$, tP and m may indicate the speed of CA release during the fusion pore expansion and/or the slow dissociation of adrenaline from intragranular matrix (Marszalek et al., 1997).

We used to obtain two spike fade measurements, t_d and t'_d (Fig. 4), as the time required by the current to fall to specific values, $I_{max} - I_{max}/e$ and I_{max}/e, respectively, taken from the adjusted decay exponential. These two time values should be proportional, because spike fade can be adjusted to a single exponential, unless another spike occurs during the spike decay. We used the $t_d - t'_d$ ratio as a quick test to detect the overlapped spikes. Since they are not very useful during normal spike analysis, they are not longer present in our macros, although they are free shareware upon request.

The program described here adds several improvements to currently available software for the analysis of secretory spikes. For instance, the filtering conditions and spike identification criteria can be modified by the user. Full automation results in an appreciable time saving (Criado et al., 1999; Machado et al., 2000). For instance, a typical day of experiments produces 50–70 files of 2 Mb of raw data. Manual analysis would be almost completely impossible. A semi-automatic analysis, which includes file by file digital filtering and spike finding, requires over a week of hard work. However, the automatic analysis described here last 15–20 min (Power Macintosh G3/266 MHz) and the further manual corrections can be assessed within 4–5 h. The histograms produced after the analysis allow comparison of several populations including hundreds/thousands of exocytotic events obtained under different experimental conditions. The program is user-friendly and is accompanied by a user manual.

These procedures would facilitate studies concerning the nature of the exocytotic process and how a drug treatment or a given physical condition (temperature, pH, ionic environmental, etc.) can affect this process. For instance, if the incubation with a drug results in a decrease in the net charge (Q), it would indicate a reserpine-like process, whereas a reduction of the I_{max} and increase in the $t_{1/2}$ would point towards an effect on the fusion pore or on the dissociation of CA from the intragranular matrix. Considering that chromaffin granules and sympathetic dense cored vesicles are very similar organelles and the later is responsible for the noradrenaline releasing from sympathetic varicosities, studies carried out on secretory spike kinetics of chromaffin cells offer a valuable information about the synaptic neurotransmission.

The program and its user manual (Mac and PC compatible) are available as free shareware at the web address: http://webpages.ull.es/users/rborges/

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