Endogenous Calcium Buffering Capacity of Substantia Nigral Dopamine Neurons

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Foehring RC, Zhang XF, Lee JCF, Callaway JC. Endogenous calcium buffering capacity of substantia nigral dopamine neurons. J Neurophysiol 102: 2326–2333, 2009. First published August 12, 2009; doi:10.1152/jn.00038.2009. Dopamine (DA)-containing cells from the substantia nigra pars compacta (SNc) play a major role in the initiation of movement. Loss of these cells results in Parkinson’s disease (PD). Changes in intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) elicit several events in DA cells, including spike afterhyperpolarizations (AHPs) and subthreshold oscillations underlying autonomous firing. Continuous Ca\(^{2+}\) load due to Ca\(^{2+}\)-dependent rhythmicity has been proposed to cause the death of DA cells in PD and normal aging. Because of the physiological and pathophysiological importance of [Ca\(^{2+}\)]\(_i\) in DA cells, we characterized their intrinsic Ca\(^{2+}\)-buffering capacity (K\(_S\)) in brain slices. We introduced a fluorescent Ca\(^{2+}\)-sensitive exogenous buffer (200 μM fura-2) and cells were tracked from break-in until steady state by stimulating with a single action potential (AP) every 30 s and measuring the Ca\(^{2+}\) transient from the proximal dendrite. DA neurons filled exponentially with a \(\tau\) of about 5–6 min. [Ca\(^{2+}\)]\(_i\), was assumed to equilibrate between the endogenous Ca\(^{2+}\) buffer and the exogenous Ca\(^{2+}\) indicator buffer. Intrinsic buffering was estimated by extrapolating from the linear relationships between the amplitude or time constant of the Ca\(^{2+}\) transients versus [fura-2]. Extrapolated Ca\(^{2+}\)-transients in the absence of fura-2 had mean peak amplitudes of 293.7 ± 65.3 nM and \(\tau\) = 124 ± 13 ms (postnatal day 13 [P13] to P17 animals). Intrinsic buffering increased with age in DA neurons. For cells from animals P13–P17, K\(_S\) was estimated to be about 110 (n = 20). In older animals (P25–P32), the estimate was about 179 (n = 10). These relatively low values may reflect the need for rapid Ca\(^{2+}\) buffering, e.g., to allow activation of sK channels, which shape autonomous oscillations and burst firing. Low intrinsic buffering may also make DA cells vulnerable to Ca\(^{2+}\)-dependent pathology.

INTRODUCTION

Dopamine (DA) cells are important for the initiation of movement and the loss of these cells results in Parkinson’s disease (PD; Dauer and Przedborski 2003). In the mammalian midbrain, DA-containing cells are primarily found in the ventral tegmental area and the substantia nigra pars compacta (SNc). They typically fire action potentials (APs) tonically at low rates (<10 Hz) in vivo, in either regular or irregular patterns (Grace and Bunney 1983b, 1984a; Hylund et al. 2002; Overton and Clark 1997; Tepper et al. 1995; Wilson et al. 1977). Burst firing is a third pattern, superimposed on the background of tonic firing and characterized by a high rate of firing within the burst (Celada et al. 1999; Grace and Bunney 1984b; Hylund et al. 2002; Tepper et al. 1995). Burst firing of DA cells is temporally locked to reward prediction error (Schultz 2002), allowing these cells to control reinforcement learning (Dayan and Balline 2002; Schultz 2002). In vitro, DA cells typically fire autonomously and tonically in a regular pattern (Chan et al. 2007; Fujimura and Matsuda 1989; Grace and Bunney 1984a,b; Harris et al. 1989; Kita et al. 1989; Lacey et al. 1987; Nedergaard and Greenfield 1992; Puopolo et al. 2007).

Many aspects of firing behavior in DA neurons are Ca\(^{2+}\)-dependent and several types of Ca\(^{2+}\) channels are expressed in DA neurons (Cardozo and Bean 1995; Durante et al. 2004; Nedergaard et al. 1993; Wolfart and Roeper 2002), including Ca\(_{\text{v}1.3}\) L-type channels (Cahn et al. 2007; Takada et al. 2001), which activate at relatively negative potentials (vs. Ca\(_{\text{v}1.2}\) channels; Koschak et al. 2001; Scholze et al. 2001; Xu and Lipscombe 2001). The autonomous firing of mature DA cells from SNc reflects underlying oscillations mediated by the low-threshold calcium current through Ca\(_{\text{v}1.3}\) L-type channels (Fujimura and Matsuda 1989; Grace and Onn 1989; Harris et al. 1989; Kang and Kitai 1993a,b; Mercuri et al. 1994; Wilson and Callaway 2000).Although subthreshold activation of L-type channels results in activation of an amamin-sensitive sK-mediated current (Ping and Shephard 1996; Shephard and Bunney 1991; Wilson and Callaway 2000), spike-induced afterhyperpolarizations (AHPs) are coupled to N-type as well as L-type Ca\(^{2+}\) channels (Papuolo et al. 2007). Blockade of this sK current leads to development of long-duration Ca\(^{2+}\)-dependent plateau potentials (Johnson and Wu 2004; Shephard and Bunney 1991). Ca\(^{2+}\)-dependent oscillations in dendrites combine to account for tonic firing in DA neurons (coupled oscillator model; Medvedev et al. 2003; Wilson and Callaway 2000). Addition of N-methyl-D-aspartate conductance to the model allows burst firing (Kuznetsov et al. 2006). Ca\(^{2+}\)-dependent sK currents play a key role during burst firing by removing inactivation of Na\(^+\) currents to allow high-frequency firing (Kuznetsov et al. 2006).

Because of the physiological and pathophysiological importance of intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) and Ca\(^{2+}\)-dependent currents in DA cells from SN, we characterized their endogenous Ca\(^{2+}\)-buffering capacities by addition of an exogenous Ca\(^{2+}\) indicator/buffer that alters native [Ca\(^{2+}\)]\(_i\) dynamics in predictable ways (Helmen et al. 1996; Neher et al. 1992). We found that intrinsic Ca\(^{2+}\) buffering in DA cells increased with postnatal age, although buffering remains exceptional at both age ranges, potentially placing these cells at risk for Ca\(^{2+}\)-dependent pathophysiology.

METHODS

Brain slice preparation

Thin coronal brain slices containing the SNc were made from brains of Sprague-Dawley rats (postnatal day 13 [P13] to P17 or

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The rats were deeply anesthetized with an intraperitoneal injection of a mixture of 87 mg/kg ketamine and 13 mg/kg xylazine and perfused intracardially with cold cutting solution. This solution contained (in mM): 250 sucrose, 25 KCl, 1 NaH2PO4, 11 glucose, 4 MgSO4, 0.1 CaCl2, and 15 HEPES (pH 7.3–7.4; 300 mM). Their brains were then removed and the brain was sliced at 200–300 μm (P25–P32) or 300 μm (P13–P17). Slices were maintained in an artificial cerebrospinal fluid (aCSF) mixture of (in mM) 124 NaCl, 2.5 KCl, 2.0 CaCl2, 2.0 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 d-glucose (bubbled with 95% O2-5% CO2, pH 7.4). Slices were stored at room temperature prior to recording. Recordings were obtained at 32 ± 1°C because Ca2+-dependent oscillations were much more robust at temperatures approximating those in vivo.

**PATCH-CLAMP RECORDING.** Slices were transferred to a mesh surface in a chamber containing aCSF at room temperature for ≥1 h. The brain slice was placed in a recording chamber on the stage of an Olympus BX50WI upright microscope (in carbogenated aCSF at 2°C). Stacks were transferred to a mesh surface in a chamber containing aCSF at room temperature prior to recording. Recordings were obtained at 30-s intervals beginning from cell break-in, a single AP was elicited by a 10-ms suprathreshold current injection and the fluorescence correction was performed by subtraction of measured autofluorescence of a nearby region of the slice from the measured initial value of F.

**Measurement of fura-2 filling**

After membrane breakthrough, the loading of fura-2 was followed by measuring the isosbestic fluorescence (λ = 360 nm) over time. Measurements were made at the main proximal dendrite where it connects to the soma. Autofluorescence was measured every 30 s until steady-state filling of the dye fura-2 was observed. The time course of fura-2 filling was then fitted with a single exponential using a macro in Igor. Concentration of fura-2 in the cell was assumed to be zero just before breaking into the cell and 200 μM (concentration in electrode) at steady state. The concentration at any point in time could then be extrapolated from the filling curve.

**Ca2+ binding capacity**

The Ca2+ transient is buffered by endogenous mechanisms (Ca2+-binding proteins, internal stores, extrusion mechanisms, etc.) and by the exogenous indicator/buffer (in this case, fura-2). Neher (1995) measured the Ca2+ transient in chromaffin cells in response to different exogenous Ca2+-indicator/buffer concentrations. By extrapolation to zero exogenous buffer, the endogenous component of Ca2+ buffering was estimated (“added buffer method”). Using a single-compartment model of Ca2+ transient buffering (Helmchen et al. 1996; Neher and Augustine 1992), the [Ca2+] transient evoked by an action potential can be described by the balance in partitioning of free Ca2+ among different Ca2+ buffers and Ca2+ removal mechanisms, expressed together collectively as the endogenous Ca2+ buffering capacity Ks.

In general, the differential Ca2+ binding capacity (or binding ratio) of Ca2+ buffer X is defined as the ratio of buffer bound Ca2+ to total free Ca2+: Xs = δ[X]/[Ca2+]. An incremental Ca2+ binding capacity KX can also be defined for significant changes from resting Ca2+.

\[
\kappa_X = \frac{K_X [X_s]}{K_D + [Ca^{2+}]_{resting} (K_D + [Ca^{2+}]_{peak} + K_D)}
\]

where [X] = [Ca2+] is the change in [Ca2+] and KX, Pe, and endogenous buffer capacity Ks. The incremental buffering capacity of Ca2+ (Ks) was calculated using the following equation

\[
K_s = [B] [K_s]/([Ca^{2+}]_{resting} + K_D ([Ca^{2+}]_{peak} + K_D))
\]

where (B) and K are the concentration and dissociation constant for fura-2, respectively (Helmchen et al. 1996). [Ca2+]peak is the difference between resting [Ca2+] and peak [Ca2+] due to an AP.

We evoked a single AP every 30 s after break-in and measurements of fura-2 fluorescence were made at 380 nM to allow estimation of [Ca2+]. The Ca2+ transient evoked by an AP can be described as an instantaneous step function with amplitude A and an exponential decay time constant τ for the change in [Ca2+]. If AP-induced Ca2+
influx is constant over time during the recording, the time integral of the Ca\(^{2+}\) transient should remain constant and be independent of K\(_B\). Assuming that the Ca\(^{2+}\) influx from the AP and the endogenous Ca\(^{2+}\) buffering capacity do not change over time, any changes in the Ca\(^{2+}\) transient will be due to the increasing concentration of the exogenous Ca\(^{2+}\) buffer over time. A and \(\tau\) can then be related to the buffering capacity of the endogenous buffer (K\(_S\)) by Eqs. 3 and 4 (Helmchen et al. 1996)

\[
A = \Delta[Ca^{2+}]_2/(1 + K_S + K_B) \tag{3}
\]

\[
\tau = (1 + K_S + K_B)/\gamma \tag{4}
\]

where \(\Delta[Ca^{2+}]_2\) is the increase in total Ca\(^{2+}\) (free and bound) and \(\gamma\) is the Ca\(^{2+}\) extrusion rate. Both \(\tau\) and the inverse of A depend linearly on K\(_B\), allowing estimates of endogenous Ca\(^{2+}\) binding ratio (K\(_S\)) as the negative X intercept of plots of 1/A or \(\tau\) versus K\(_B\) (or K\(_B\); Helmchen et al. 1996).

Using a modification of the multivariate curve-fitting method of Jackson and Redman (2003), the concentration of the endogenous buffer [B\(_T\)] and its dissociation constant K\(_B\) can be estimated (assuming a single, lumped buffer). In this experiment, the amplitude of the Ca\(^{2+}\) transient is dependent on the concentration of exogenous buffer (fura-2) introduced into the cell and the endogenous buffering capacity remains constant (K\(_S\))

\[
\frac{1}{A} = \frac{1}{A_T} = \frac{1}{A_T} = \frac{1}{A_T} = \frac{1}{A_T} + \frac{[B]_T K_B}{([Ca^{2+}]_{resting} + K_B) ([Ca^{2+}]_{peak} + K_B)} \tag{5}
\]

From the experiment, the values of A, A\(_T\), [Ca\(^{2+}\)\(_{resting}\)], and [Ca\(^{2+}\)\(_{peak}\)] are known. Therefore, the only free parameters are [B], and K\(_B\). Using a multivariate fit by minimizing the sum-of-squares error, solutions to [B] and K\(_B\) were obtained. We compared solutions to our estimate of K\(_S\) from the previous analysis and only solutions that provide a similar estimated K\(_S\) were accepted.

Similarly,

\[
\tau = \tau_T + \tau_S (K_S) + \tau_T + \tau_S \frac{[B]_T K_B}{([Ca^{2+}]_{resting} + K_B) ([Ca^{2+}]_{peak} + K_B)} \tag{6}
\]

The results of \(\tau\), \(\tau_T\), [Ca\(^{2+}\)\(_{resting}\)], and [Ca\(^{2+}\)\(_{peak}\)] are known. Again, the only free parameters are [B], and K\(_B\). The previous analysis for \(\kappa_S\) provides an estimate of the acceptable \(\kappa_S\) solution space.

The multivariate fits and estimates of errors in the fitting parameters for [B], and K\(_B\) were obtained using Origin. Summary data are presented as means ± SE. Linear curve fitting was performed using Kaleidograph. Summary statistics and unpaired t-tests were performed using Prism and Excel.

**RESULTS**

All recordings were obtained from DA cells in slices from SNc of Sprague–Dawley rats (P13–P17 or P25–P32) using internal solutions that included 200 μM fura-2. DA cells fire autonomously in slices (Fig. 1D). In mature neurons, the subthreshold oscillations underlying this firing persist after blockade of Na\(^+\) channels with tetrodotoxin (TTX; 1 μM; Fig. 1D) and are due to Ca\(^{2+}\) entry through L-type channels (Chan et al. 2007; Nedergaard et al. 1993; Wilson and Callaway 2000). This subthreshold Ca\(^{2+}\) entry also activates sK channels, leading to an apamin-sensitive medium afterhyperpolarization (mAHP) (Ping and Shephard 1996; Puopolo et al. 2007; Shephard and Bunney 1991; Wilson and Callaway 2000; Wolfart and Roeper 2002; Wolfart et al. 2001). In this study, all cells were hyperpolarized to about −60 mV with DC current and APs were elicited with brief (10-ms) current injection (APs: Fig. 1). Mean values for “resting” membrane potential, AP amplitude, AP width at half-amplitude (from resting membrane potential) for the sample of neurons at P13–P17 or P25–P32 are included in Table 1. Following a single AP, DA cells expressed a prominent AHP (Fig. 1B; Table 1).

Our initial experiments were on young animals (P13–P17) to take advantage of the lack of myelin and advantageous optical conditions. This facilitated imaging at early times after break-
in, when fura-2 concentrations in the cell are low. Every 30 s, we measured single AP-induced changes in fura-2 fluorescence (excitation at 380 nM). Prior to the AP, data were also obtained at the isosbestic wavelength (360 nm). Figure 1A shows typical transient fura-2 responses corresponding to a single AP. Records at 380 nM reflect \( \text{Ca}^{2+} \)-dependent quenching of fura-2 fluorescence (Fig. 1, A and C). All records were corrected for autofluorescence (see Methods) and data are presented as \( \% \Delta F/F \) to correct for the intensity of F just prior to the stimulus. These data were converted to estimates of \([\text{Ca}^{2+}]_i\), using Eq. 1 (Methods: Grynkiewicz et al. 1985; Wilson and Callaway 2000). \( \% \Delta F/F \) was measured at a basal dendritic location (<25 \( \mu \)m from soma; boxes in Fig. 1C).

Figure 1 also shows changes in the intensity of fura-2 fluorescence, \( \% \Delta F/F \), and the AHP with time during the recording (P17 animal). Just after initial break-in, fura-2 concentration was low and fluorescence (measured at 360 or 380 nM) weak (Fig. 1C, top). Fura-2 fluorescence increased with time after break-in (Fig. 1C, bottom). There were also changes in \( \% \Delta F/F \) (Fig. 1A). In this cell, the response at 1 min shows a sharp peak and exponential decay. At 24 min, the peak response was attenuated but the decay was prolonged. AP amplitude and half-width changes little over this time (Fig. 1B). In contrast, the AHP was reduced in amplitude at 24 min (Fig. 1B).

Fluorescence at the isosbestic wavelength (360 nM) was used to estimate the rate of filling of the cell with fura-2 (Fig. 2). The amplitude of the 360-nM response increased with time and these data were well fit by a single-exponential function. Figure 2 shows a representative cell (filling \( \tau = 3.69 \) min) and the histogram summarizes data from 27 cells (\( \tau = 4.8 \pm 0.4 \) min: P13–P17). Deviations from exponential filling may be due to changes in pipette access or cell leakage; thus cells exhibiting such deviations were not studied further. Concentration of fura-2 in the cell was assumed to be zero just before breaking into the cell and 200 \( \mu \)M (concentration in electrode) at steady state. The concentration at any point in time could then be extrapolated from the filling curve.

We used the methods of Helmchen et al. (1996) to estimate \( K_S \) by extrapolation of plots of \( K_S \) (determined from estimated \([\text{Ca}^{2+}]_i\); Methods). Figure 3 shows determination of \( K_S \) in a representative cell (P15). Fura-2 transients (\( \% \Delta F/F \)) in response to a single AP changed with time after break-in (Figs. 1A and 3A). These transients were fit with a single-exponential function to determine \( \tau_{\text{decay}} \) and amplitude (extrapolate to \( t=0 \): Fig. 3A). Similar estimates of \( K_S \) were obtained from transient amplitude obtained by extrapolation or measured directly from the peak of the transient. \( K_S \) can be estimated from either the change in the amplitude of \( \% \Delta F/F \) (Fig. 3B) or from the decay \( \tau \) (Fig. 3C). We plotted either the reciprocal of the amplitude (1/\( \text{A} \)) or \( \tau_{\text{decay}} \) against \( K_S \) (determined for each time from the filling curve; see preceding text). The negative of the X intercept corresponds to \( K_S \) (Methods).

For the cell in Fig. 3, the plots of 1/\( \text{A} \) versus \( K_S \) estimated \( K_S \) to be about 65 and the estimate from \( \tau_{\text{decay}} \) was about 104. Typically there was closer agreement between these two measures in a given cell (P13–P17) and overall there were no significant differences between estimates of \( K_S \) from 1/\( \text{A} \) (110 \pm 12, \( n = 20 \) cells) and \( \tau_{\text{decay}} \) (117 \pm 21, \( n = 11 \) cells). We also obtained similar values for \( K_S \) from 1/\( \text{A} \) (101 \pm 13; \( n = 10 \)) and \( \tau \) (96 \pm 5; \( n = 3 \)) for layer II/III neocortical pyramidal cells (P13–P17; data not shown).

Extrapolations also permitted estimates of the amplitude and \( \tau_{\text{decay}} \) that would be expected in DA cells in the absence of exogenous buffer. We estimated transient amplitude in the absence of exogenous buffer to be 269 \pm 32 nM (\( n = 20 \)) and \( \tau_{\text{decay}} \) as 124 \pm 13 ms (\( n = 19 \)). These values are similar to those obtained for pyramidal neurons (Helmchen et al. 1996; Kaiser et al. 2001; Maravall et al. 2000) and neocortical bifuited interneurons (Kaiser et al. 2001). We used multivariate curve-fitting methods (Methods; Jackson and Redman 2003) to estimate values of total buffer (\( B_T \)), buffer \( K_B \), and resting \([\text{Ca}^{2+}]_i\). For \( B_T \) we estimated 2.8 \pm 0.5 \( \mu \)M from transient amplitude (\( n = 10 \)) and 2.1 \pm 0.5 \( \mu \)M from \( \tau_{\text{decay}} \) (\( n = 7 \)). Estimates for \( K_B \) were 271 \pm 64 nM from amplitude (\( n = 10 \)) and 197 \pm 75 nM from \( \tau_{\text{decay}} \) (\( n = 7 \)).

An assumption with this method is that \( \text{Ca}^{2+} \) entry and changes in \([\text{Ca}^{2+}]_i\) are constant with time (constant stimulus so that changes with time reflect only changes in exogenous buffer; Helmchen et al. 1996). To test this, we measured the integral of \([\text{Ca}^{2+}]_i\)(A \( \times \) t) and plotted this against \( K_B \) (Fig. 3D). This slope of this relationship was not significantly

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**TABLE 1. Data for membrane potentials and action potentials**

<table>
<thead>
<tr>
<th></th>
<th>RMP, mV</th>
<th>AP, mV</th>
<th>AP Half-Width, ms</th>
<th>AHP Amplitude, mV</th>
<th>AHP Duration, ms</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13–P17</td>
<td>-63 ± 0.9</td>
<td>70 ± 2.0</td>
<td>2.1 ± 0.1</td>
<td>10 ± 1.0</td>
<td>622 ± 99</td>
<td>24</td>
</tr>
<tr>
<td>P25–P32</td>
<td>-67 ± 1.3</td>
<td>73 ± 1.8</td>
<td>1.8 ± 0.1</td>
<td>12 ± 2.1</td>
<td>522 ± 62</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are means ± SE. AP and AHP amplitude were measured from “resting” potential to the peak voltage change. AP half-width was measured at half the amplitude (measured from rest).
different from zero in any cell measured \((n = 21)\), indicating that \(\text{Ca}^{2+}\) entry did not change significantly over time.

For technical reasons (see preceding text), most of our data were obtained from very young rats (P13–P17). Because \(\text{Ca}^{2+}\)-dependent autonomous firing is developmentally labile in DA cells (Chen et al. 2007) and adult DA cells become impaired and die in PD, we tested whether intrinsic \(\text{Ca}^{2+}\) buffering changes with age in DA neurons. We thus recorded from DA cells from animals at P25–P32. Attempts to study older animals were limited by the development of large myelinated axons adjacent to and above the DA neurons in SNc. The increased light scatter in animals >P32 resulted in our being unable to detect early stages of cell filling with fura-2 and limited resolution of cell dendrites. We were able to obtain filling curves from 10 cells in the older age group, with \(\tau_{\text{filling}} = 6.0 \pm 0.7 \text{ min} \) (Fig. 2, inset). From plots of \(1/\Lambda\), our estimate of \(K_S\) was 179 ± 33 \((n = 10)\) for the older animals (Fig. 3B, inset). This was significantly greater than that in P13–P17 animals \((P < 0.02\), unpaired \(t\)-test). We could obtain reliable measurements of only \(\tau_{\text{decay}}\) to determine \(K_S\) in four cells \((120 \pm 44)\). There was no relationship between \(K_\text{BH}\) and \(A \times \tau\) in the older animals (not shown).

**Discussion**

In DA neurons from SNc, changes in \([\text{Ca}^{2+}]_i\) elicit several \(\text{Ca}^{2+}\)-dependent events, including AHPs and subthreshold oscillations. Notably, the tonic firing of mature DA cells reflects underlying pacemaker oscillations mediated by a low-threshold calcium current through L-type channels (Fujimura and Matsuda 1989; Grace and Önn 1989; Harris et al. 1989; Wilson and Callaway 2000) and subsequent activation of an apamin-sensitive sK-mediated current (Shephard and Bunney 1991; Wilson and Callaway 2000). \(\text{Ca}^{2+}\)-dependent oscillations in dendrites combine to account for tonic firing in DA neurons (Medvedev et al. 2003; Wilson and Callaway 2000) and \(\text{Ca}^{2+}\)-dependent sK currents play a key role in removing inactivation of \(\text{Na}^+\) currents to allow high-frequency firing during bursting (Kuznetsov et al. 2006).

Because of the physiological and pathophysiological importance of \([\text{Ca}^{2+}]_i\) in DA cells (Surmeier 2007), we characterized the effectiveness of intrinsic \(\text{Ca}^{2+}\) buffers by calculating the intrinsic \(\text{Ca}^{2+}\)-binding ratio \((K_S)\) in brain slices of the SN by the “method of added buffer.” We introduced a \(\text{Ca}^{2+}\)-sensitive indicator/buffer at a known concentration \((200 \ \mu\text{M fura-2})\). DA neurons filled exponentially with fura-2, with a time constant of around 5–6 min. The concentration of fura-2 at any time during the recording was then estimated from the filling curve. \(K_S\) was estimated by extrapolating from the linear relationships between the amplitude or time constant of the \(\text{Ca}^{2+}\) transients versus combined exogenous and endogenous buffering. At P13–P17, \(K_S\) was 110–117. At P25–P32, \(K_S\) was significantly greater \((-179)\), but still modest. Thus despite the potential for a sustained \(\text{Ca}^{2+}\) load due to subthreshold \(\text{Ca}^{2+}\) entry and autonomous firing, DA cells display a modest level of intrinsic \(\text{Ca}^{2+}\) buffering, similar to many cell types that do not exhibit \(\text{Ca}^{2+}\)-dependent pacemaker firing (see following text; Helmchen et al. 1996; Kaiser et al. 2001; Neher and Augustine 1992; Powell et al. 2008; Regehr and Tank 1992).

**Measurements of intrinsic \(\text{Ca}^{2+}\) buffering**

\((\text{Ca}^{2+}\)-binding ratio\)

Only a small percentage of \(\text{Ca}^{2+}\) entering the cytosol remains as free \(\text{Ca}^{2+}\) (Berridge et al. 2000; Gorman and Thomas 1980; Tank et al. 1995) because multiple mechanisms collectively regulate \([\text{Ca}^{2+}]_i\) within narrow limits. There is a bewildering number of possible \(\text{Ca}^{2+}\)-binding reaction partners (including mitochondria, internal stores, calcium binding proteins), thus an overall estimate of \(K_S\) is a reasonable initial step toward understanding the role of buffering of \(\text{Ca}^{2+}\) in different types of cells (Neher 1995). The basic strategy of extrapolating relationships between exogenous buffers plus endogenous \(\text{Ca}^{2+}\) buffering, similar to many cell types that do not exhibit \(\text{Ca}^{2+}\)-dependent pacemaker firing (see following text; Helmchen et al. 1996; Kaiser et al. 2001; Neher and Augustine 1992; Powell et al. 2008; Regehr and Tank 1992).
about 1.3–2.5% of Ca\(^{2+}\) ions that enter, remain free. Helmchen et al. (1996) and Lee et al. (2000) found excellent agreement between estimates of \(K_S\) derived from many cells, each with a single [buffer] to estimate using a single-cell method where the dye-filling curve was used to estimate [dye] (see also Kaiser et al. 2001).

\(K_S\) provides an estimate of the ability of a cell to handle \(Ca^{2+}\) loads during physiological and pathophysiological activation, with high \(K_S\) values associated with greater ability to handle a \(Ca^{2+}\) load. Estimates of \(K_S\) vary nearly 50-fold across the cell types tested to date. We found that despite the potential for nearly continual \(Ca^{2+}\) entry in vivo, DA cells do not have especially high intrinsic buffering capacity \(K_S = 110\) (P13–P19) or 179 (P25–P32). That is, 0.5–1% of \(Ca^{2+}\) that enters remains free at steady state. Several other neuron types that do not exhibit \(Ca^{2+}\)-dependent autonomous firing have similar intrinsic \(Ca^{2+}\)-buffering capacity to DA cells (\(K_S = 100–200\)). These include neocortical pyramidal cells (our results; Helmchen et al. 1996; Kaiser et al. 2001), basal forebrain neurons (Tatsumi and Katayama 1993), and hippocampal granule cells (Stocca et al. 2008). In contrast, motoneurons (Lips and Keller 1998; Palacek et al. 1999) and CA1 hippocampal pyramidal neurons had \(K_S\) values of 30–60 (Lee et al. 2000; Maravall et al. 2000; Powell et al. 2008; Sabatini et al. 2002; but see Helmchen et al. 1996). Other neuron types have higher intrinsic \(Ca^{2+}\)-buffering capacity. \(K_S\) estimates were 200–300 in cortical GABAergic interneurons (Aponte et al. 2008; Kaiser et al. 2001; but see Lee et al. 2000) and about 500–600 in snail neurons (Belan et al. 1993; Muller et al. 1993), crayfish neuromuscular junction (Tank et al. 1995), and mammalian sympathetic neurons (Wanaverbecq et al. 2003). The highest value measured to date was for Purkinje cells (~2,000; Fiero and Llano 1996).

**Basis for \(K_S\)**

The \(K_S\) measurement is thought to be dominated by fixed buffers because the value does not decrease substantially, even during long-lasting dialysis of the cell by whole cell recording (Helmchen et al. 1996; Neher and Augustine 1992; Steunkel 1994). The buffer also shows little sign of saturation over the range of \([Ca^{2+}]\) tested. In addition, comparison of wash-in and washout of two different concentrations of fura-2 in a single cell (two successive patch recordings) provides similar estimates of \(K_S\), suggesting no washout of endogenous buffer (Helmchen et al. 1996; Lee et al. 2000). Popular candidates for fixed buffers include various calcium-binding proteins (CaBPs; e.g., calmodulin, calbindin, calretinin, and parvalbumin) that are distributed in a cell-type–specific manner in the nervous system (Baimbridge et al. 1995). Neher and Augustine (1992) favored calmodulin as the endogenous buffer in chromaffin cells. Calbindin has been suggested as an important buffer in DA neurons, we also recorded from cells at P25–P32. We found that buffering capacity increased significantly with age, but not parvalbumin (Alfahel-Kakunda and Silverman 1997; McRitchie et al. 1996). Calbindin is absent from ventrally located SCa DA cells (Gerfen et al. 1987; Gonzalez-Hernandez and Rodriguez 2000; Nemoto et al. 1999). With PD in humans, there is relative sparing of DA melanin-negative cells containing calbindin (Yamada et al. 1990). The bulk of our recordings were from cells ventral and medial within the SCa.

Surmeier and colleagues proposed that DA cells are at risk for \(Ca^{2+}\)-dependent mitochondrial failure and cell death (e.g., in Parkinson’s disease and aging) because of the persistent \(Ca^{2+}\) load from subthreshold \(Ca^{2+}\) entry (Chan et al. 2007; Surmeier 2007). An intriguing possibility is that mitochondria play an important role in determining \(K_S\). Mitochondrial polymorphisms are associated with PD (Kazuno et al. 2006) and loss of mitochondrial function has been proposed as a mechanism for \(Ca^{2+}\)-dependent cell death in DA cells in PD (Chan et al. 2007; Surmeier 2007).

**Technical limitations**

Several assumptions are required to estimate \(K_S\). First, these methods assume instantaneous \(Ca^{2+}\) entry, which is approximated by the brief, steep-rising transients elicited by a single AP (Helmchen et al. 1996). The decay of \([Ca^{2+}]\), was well fit by a single exponential. \(Ca^{2+}\) entry in response to an AP must also be constant with time. This was confirmed by the lack of relationship between the integral of \(Ca^{2+}\) entry versus time or \(A \times \tau\) versus \(K_S\). The concentration of the intrinsic buffer is also assumed constant. We found no evidence for loss of highly mobile buffers lost within the first 10–20 min after break-in. In some cells at longer times (>20 min), we observed deviations from a linear relationship between either \(A\) or \(\tau\) versus \(K_S\). These deviations may reflect slow washout of buffers. All of our estimates were therefore confined to data taken during the time before such deviations. A recent study suggests that nearly all of the calbindin in CA1 pyramidal cells is mobile but washes out with a \(\tau\) of about 10 min (Muller et al. 2005), compared with a \(\tau\) of roughly 5–6 min for wash-in of fura-2 in DA cells. The \(K_S\) estimates correspond to steady-state \(Ca^{2+}\), a condition that may never fully exist in cells with multiple \(Ca^{2+}\) reaction partners differing in affinity or kinetics (Markram et al. 1998).

**Development of \(K_S\)**

Most of our data were obtained from rats aged P13–P17 to facilitate imaging of DA neurons; however, \(K_S\) has been shown to be age sensitive in other cell types (Fiero and Llano 1996; Maravall et al. 2000; Murchison and Griffith 1998; Stocca et al. 2008). To test whether \(K_S\) was developmentally labile in DA neurons, we also recorded from cells at P25–P32. We found that buffering capacity increased significantly with age, but remains modest in DA neurons. Chan et al. (2007) reported that in mice, the \(Ca^{2+}\)-dependence of subthreshold oscillations and autonomous firing was developmentally regulated, with DA cells from P12–P17 mice showing \(Na^{+}\)-dependent and P28–P32 animals showing \(Ca^{2+}\)-dependent pacemaking behavior. Thus the increase in \(K_S\) with age may correspond to a change in the basis for pacemaking behavior.
Functional consequences

Given the autonomous firing and low threshold for Ca\(^{2+}\) entry in DA cells from SNC, the modest K\(_{S}\) in these cells would be expected to make these cells vulnerable to Ca\(^{2+}\)-dependent pathophysiology, such as occurs with aging and in PD (Surmeier 2007). This low safety factor may be necessitated by the need for sufficient activation of the sK channels important to the oscillations underlying autonomous firing (Chan et al. 2007; Wilson and Callaway 2000) as well as to prevent Na\(^{+}\) inactivation to allow burst firing (Medvedev et al. 2003). The unknown molecules contributing to K\(_{S}\) would play a major role in determining the dimensions of microdomains for Ca\(^{2+}\)-dependent activation of sK channels (Abel et al. 2004; Fakler and Muller 2008; Muller et al. 2005). All else being equal, a low K\(_{S}\) for a given Ca\(^{2+}\) influx would result in faster Ca\(^{2+}\) dynamics (large amplitude, fast decay, local), which would be advantageous for rhythmic oscillatory activity. This has been previously proposed for the very low (~40) K\(_{S}\) in motoneurons (Lips and Keller 1998; Palecek et al. 1999). This is also consistent with observations that blockade of sK current with apamin or intracellular EGTA leads to development of bursting and prolonged Ca\(^{2+}\)-dependent plateau potentials (Johnson and Wu 2004; Shepard and Bunney 1991).

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