Two Components of Voltage-dependent Calcium Influx in Mouse Neuroblastoma Cells

Measurement with Arsenazo III

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ABSTRACT N1E-115 mouse neuroblastoma cells were injected with the calcium indicator dye arsenazo III. Optical absorbance changes during voltage-clamp depolarization were used to examine the properties of the two calcium currents present in these cells. The rapidly inactivating calcium current (Moolenar and Spector, 1979b, Journal of Physiology, 292:307-323) inactivates by a voltage-dependent mechanism. The slowly inactivating calcium current is dominant in raising intracellular calcium during depolarizations to greater than -20 mV. Lowering the extracellular calcium concentration affects the two calcium currents unequally, with the slowly inactivating current being reduced more. Intracellular calcium falls very slowly (τ > 1 min) after a depolarization. The rapidly inactivating calcium current is responsible for a calcium action potential under physiological conditions. In contrast, it is unlikely that the slowly inactivating calcium current has an important electrical role. Rather, its function may be to add a further increment of calcium influx over and above the calcium influx through the rapidly inactivating calcium channels.

INTRODUCTION

Calcium movement into neurons has important short- and long-term effects. On the subsecond time scale, calcium influx constitutes an electrical current that contributes to the total electrical behavior of the cell membrane (Hagiwara and Byerly, 1981); pathological depolarization of neurons by calcium influx may initiate epileptic attacks (Prince, 1978). In contrast, elevation of the cytosolic free calcium concentration ([Ca++]i) for a period of seconds causes biochemical changes in neurons (Study et al., 1978) and may cause long-term changes in excitability and memory that can persist for hours or days (Lynch et al., 1979; Kandel, 1981; Alkon, 1984). Recent experiments suggest that the short- and long-term roles of calcium may be subserved by two or more different populations of calcium channels (Fishman and Spector, 1981; Carbone and Lux, 1984; Fox and Krasne, 1984; Armstrong and Matteson, 1985; Fedulova et al., 1985; Nowycky et al., 1985). A precise description of both types of calcium influx in terms of voltage, time course, and their relative contribution to total membrane current and raised [Ca++], in a mammalian neuron is therefore necessary for an understanding of mammalian nervous system function. Unfortunately, published
experiments that demonstrate two populations of calcium channels in vertebrate neurons have used experimental protocols that can distort one or more of these parameters. Quinidine (Fishman and Spector, 1981) can block or modify calcium channels (Hermann and Gorman, 1984; Bkaily et al., 1985; J. Brown et al., 1985). Intracellular perfusion (Carbone and Lux, 1984; Fedulova et al., 1985; Nowycky et al., 1985) selectively eliminates one population of channels (Fedulova et al., 1985) and can greatly modify the voltage dependence of channel gating (Fernandez et al., 1984). The present article characterizes two components of calcium influx in mouse neuroblastoma cells without an exchange of the intracellular ionic medium or the use of quinidine. Rather, calcium influx was measured independently of other membrane currents by measuring its effect upon \([\text{Ca}^{++}]_i\) (Smith et al., 1983). The results suggest that the two components of calcium influx have very different functional roles.

**METHODS**

**Electrophysiology**

N1E-115 cells were grown as described previously (Moolenaar and Spector, 1978) and studied after 6-19 d of differentiation in growth medium containing 2% dimethyl sulfoxide (DMSO). A coverslip with attached cells was placed in an experimental chamber of 2 ml volume on the stage of a compound microscope. When the bathing medium was to be changed during an experiment, the chamber was constantly perfused at a rate of 1.6-1.8 ml/min. The bath exchange time was determined both by dye washout and by high-K⁺ depolarization of impaled cells to be 1-2 min. The normal electrophysiological medium contained 120 mM NaCl, 5.5 mM KCl, 10 mM CaCl₂, 1 mM MgCl₂, 25 mM glucose, and 20 mM Tris, pH 7.3. Osmolarity was adjusted to 320 mosmol with sucrose. For 1.8 mM Ca⁺⁺ medium, CaCl₂ was replaced isosmotically with sucrose. CoCl₂ and tetraethylammonium (TEA) Cl were added where indicated without further adjustment. Experiments were performed at room temperature (22 ± 2°C). The cell soma was impaled by a micropipette containing 20 mM sodium arsenazo III (98% purity grade, Sigma Chemical Co., St. Louis, MO), 100 mM KCl, and 5 mM HEPES, pH 7.8. Arsenazo III was injected by applying pressure (1-5 psi) to the back of the pipette to a final absorbance of 0.03 at 660 nm. To allow for the dye to diffuse throughout the cell, measurements were made at least 10 min after dye injection. Action potentials were elicited by superimposing a 1-s depolarizing current pulse on a steady hyperpolarizing holding current. For voltage-clamp experiments, the arsenazo III pipette was used to measure membrane voltage and a second intracellular pipette containing 2 M KCl was used to pass current. The holding voltage was −80 mV in all voltage-clamp experiments. Unless otherwise noted, depolarizing pulses were separated by a period of at least 15 s at the holding voltage. Light from a quartz-halogen source was limited to a 10-nm-wavelength band by interference filters and focused onto the cell by a microscope condenser. Light passing through the cell was collected by a long-working-distance objective and measured with a photodiode. Light reaching the photodiode was limited to that passing through the cell soma by two pairs of razor blades that defined a rectangular aperture in the back focal plane of the objective. The optical pathlength through the soma was estimated by focusing first on the lower surface of the soma, and then on the upper surface, using Hoffman modulation contrast optics (Modulation Optics, Green Vale, NY). The optical signal was low-pass-filtered at 80 Hz using a four-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA). Other signals were limited only by the bandwidth (125 Hz) of the brush
recorder (Gould, Cleveland, OH). No electrical signal averaging was used. Results are expressed as means ± SEM.

**Arsenazo III In Vitro Calibration**

The absorbance spectra of free arsenazo III (AIII), magnesium arsenazo III (Mg:AIII), and calcium arsenazo III (Ca:AIII) were measured in cuvettes of 200 μm pathlength. Solutions contained 1 mM sodium arsenazo III, 10 mM HEPES, and 120 mM KCl plus one of the following: 60 mM KCl (AIII solution), 20 mM MgCl₂ (Mg:AIII solution), or 20 mM CaCl₂ (Ca:AIII solution). The pH was adjusted to 7.50 with KOH. Blanks contained the corresponding salt and buffer concentrations. Absorptivity values at 540 and 660 nm were (absorbance units, molar⁻¹ cm⁻¹): ε₅₄₀ = 3.52 ± 0.03; ε₆₆₀ = 0.37 ± 0.01; ε₅₄₀[AIII] = 2.55 ± 0.03; ε₆₆₀[AIII] = 1.50 ± 0.02; ε₅₄₀[Mg:AIII] = 2.22 ± 0.05; ε₆₆₀[Mg:AIII] = 2.82 ± 0.04. Other absorbance and difference spectra were calculated arithmetically from the three measured spectra and will be referred to as “calculated in vitro spectra.” Although the stoichiometry of Ca:AIII binding is still in doubt (H. M. Brown and Rydqvist, 1981; Thomas, 1982), the 1:1 binding model of Brown and Rydqvist was used because it provides a reliable empirical framework that allows calibration of in vitro absorbance changes in terms of changes of calcium concentration. In this work, the mean total intracellular concentration of arsenazo III was 1.14 ± 0.06 mM, so the value $K_D = 2 \mu M$, appropriate for 1 mM AIII, was used (H. M. Brown and Rydqvist, 1981). The change of $[Ca^{2+}]$ required to produce a measured optical absorbance change at 660 nm was calculated by assuming that pH and $[Mg^{2+}]$ are constant, and therefore that the ratio $[AIII]/[Mg:AIII]$ re-equilibrates and remains constant after the formation of Ca:AIII.

**RESULTS**

**Absorbance and Difference Spectra**

The absorbance spectrum of intracellular arsenazo III was similar to the calculated in vitro absorbance spectrum of a mixture of free arsenazo III (AIII) and arsenazo III bound to magnesium (Mg:AIII) (Fig. 1A). The fraction $R = [AIII]/([AIII] + [Mg:AIII])$ was calculated from the measured absorbances at 540 and 660 nm to be 0.74 ± 0.02 (51 cells), corresponding to an intracellular free magnesium concentration of 0.7 ± 0.1 mM at pH 7.24, the intracellular pH of these cells (Baylor et al., 1982a; Moolenaar et al., 1984). The absorbance of cells bathed in a medium containing 10 mM Ca²⁺ and 10 mM TEA did not change significantly during an action potential elicited before the injection of arsenazo III (10 cells). After injection of arsenazo III, absorbance increased at 610, 630, and 660 nm and declined at 540 and 580 nm during an action potential (Fig. 1, B and C) (nine cells). The absorbance change at 660 nm ($ΔA_{660}$) was greater than the absorbance change at 630 nm, which indicates that arsenazo III had bound calcium (J. E. Brown et al., 1977). Therefore, the intracellular free calcium concentration increased during an action potential. However, the complete difference spectrum and the calculated in vitro difference spectrum of calcium binding were markedly dissimilar (Fig. 1C). A similar deviation of intracellular arsenazo III absorbance changes from the in vitro difference spectrum has been observed in most other preparations studied (Ahmed and Connor, 1979; Harary and Brown, 1981; Baylor et al., 1982b). In some cases, a depression of the absorbance change at 610 nm resulted from a fall of intracellular pH.
coincident with the [Ca++] change (Ahmed and Connor, 1980; Harary and Brown, 1981; Bolsover et al., 1986). A depression at 610 nm of the magnitude seen in Fig. 1C would be produced by a cytosolic acidification of 0.01 pH unit. Changes of pH have comparatively little effect on the absorbance at 660 nm (Gorman and Thomas, 1978).

**Figure 1.** Comparison of in vivo and in vitro spectra. A cell soma (pathlength, 50 µm) was bathed in medium containing 10 mM Ca++ and 10 mM TEA and impaled by a single micropipette containing arsenazo III solution. (A) Solid circles: the absorbance increase produced by injection of arsenazo III solution was measured at five different wavelengths. Abscissa: wavelength. Ordinate: absorbance increase. Continuous line: calculated in vitro difference spectrum of arsenazo III in the presence of magnesium; total concentration [AIII] + [Mg:AIII] = 0.82 mM; \( R = [\text{AIII}]/([\text{AIII}] + [\text{Mg:AIII}]) = 0.55; \) pathlength, 50 µm. (B) Optical absorbance change at 660 nm during a single action potential after injection of arsenazo III solution. The horizontal axis is time. \( A_{660} \), absorbance at 660 nm; \( i \), membrane current; \( V \), membrane voltage. The dashed line indicates zero voltage. (C) Solid circles: the absorbance change during an action potential elicited after arsenazo III injection was measured at five different wavelengths. Abscissa: wavelength. Ordinate: absorbance change during action potentials elicited at 15-s intervals. Each point is the mean for six action potentials; error bars (±1 SEM) are smaller than the symbols employed. Continuous line: calculated in vitro difference spectrum for addition of calcium to a solution with the parameters listed in A, to a total free calcium concentration of 45.3 nM. This difference spectrum was calculated by assuming that [Mg++] and pH, are constant, so that \( R = [\text{AIII}]/([\text{AIII}] + [\text{Mg:AIII}]) \) re-equilibrates and remains constant after the formation of Ca:AIII. Thus, the calculated difference spectrum includes a component resulting from the net loss of Mg:AIII.
Subsequent results are all from cells injected to ~1 mM intracellular arsenazo III. To facilitate comparison of the present results with previously published work, it is convenient to express changes of absorbance at 660 nm in terms of the change of cytosolic free calcium concentration ($\Delta[Ca^{++}]$) that would cause such an absorbance change in vitro. For example, the increase of $A_{660} (\Delta A_{660})$ during action potentials of the cell shown in Fig. 1 corresponded to a $\Delta[Ca^{++}]$ of 45 nM. This value of $\Delta[Ca^{++}]$ was unusually large; in 38 cells bathed in medium containing 10 mM Ca$^{++}$ and 10 mM TEA, $\Delta[Ca^{++}]$ during an action potential was 7.1 ± 1.3 nM.

Absorbance Increase During Voltage-Clamp Depolarization

The majority of voltage-clamp experiments were performed in medium containing 10 mM Ca$^{++}$ to increase the magnitude of the calcium influx and 10 mM TEA to allow comparison of the current records with the results of Moolenaar and Spector (1978, 1979b). Data on the specific effects of changes of extracellular TEA and Ca$^{++}$ are presented later. $A_{660}$ increased with a simple, exponential time course ($\tau = 170 \pm 20$ ms, 11 cells) during 1-s depolarizations to −40 mV, indicating an average increase of $[Ca^{++}]$ during the 1-s depolarization of 4.7 ± 0.6 nM (49 cells) (Fig. 2A). A depolarization to −40 mV elicited the inward membrane current identified by Moolenaar and Spector (1978, 1979b) as a calcium current (Fig. 2A). This current inactivated with $\tau_i = 91 \pm 8$ ms (nine cells). In the majority of cells, $A_{660}$ during depolarization to −20 mV could also be fit by a single exponential with $\tau = 110 \pm 16$ ms (six cells); the inward membrane current inactivated with $\tau_i = 67 \pm 12$ ms (five cells).

In 3 of 44 cells, $\Delta A_{660}$ showed this simple time course over the whole of the voltage range studied. Fig. 2 illustrates results from one such cell. In these cells, $\Delta A_{660}$ during the first 0.5 s of depolarization was maximal at −40 mV and declined monotonically at more positive voltages (Fig. 2B). $A_{660}$ remained elevated for many seconds after a depolarization. The efflux of calcium from the cell must therefore have been very slow. A steady influx of calcium during depolarization would therefore have resulted in a steady increase of $A_{660}$. The exponential increase of $A_{660}$ that was actually observed during depolarization must therefore have resulted from an exponentially decaying calcium influx. On this interpretation, depolarization activates a rapidly inactivating calcium influx, so that $[Ca^{++}]$, and therefore $A_{660}$ increase; as the influx inactivates, $[Ca^{++}]$, and therefore $A_{660}$ increase at a steadily decreasing rate and reach a steady level when the influx has completely inactivated. This rapidly inactivating calcium influx occurred in the voltage range −60 mV < V < +60 mV, over which the rapidly inactivating calcium current is active (Moolenaar and Spector, 1978) (Fig. 2C); it is presumably a consequence of that current. The more negative voltage optimum of the rapidly inactivating calcium influx as compared with the peak inward calcium current is not inconsistent with this hypothesis. Moolenaar and Spector (1979b) calculated the total calcium influx into N1E-115 cells bathed in 20 mM Ca$^{++}$ by measuring the time integral of the rapidly inactivating calcium current. The maximum influx was at −33 mV, a voltage significantly more negative than the voltage at which the maximum peak inward calcium current
FIGURE 2. The rapidly inactivating component of $\Delta A_{660}$. The absorbance at 660 nm of a cell soma bathed in medium containing 10 mM Ca$^{++}$ and 10 mM TEA was measured during depolarization to a range of voltages. (A) Single typical depolarization to $-40$ mV. An upward deflection of the trace corresponds to an increase of $[\text{Ca}^{++}]$. $A_{660} = 0.030$, $R = 0.77$; thus, a $\Delta A_{660}$ of $5 \times 10^{-4}$ corresponds to a $\Delta[\text{Ca}^{++}]$ of 12.7 nM. The thin horizontal line represents the leakage current calculated by linear extrapolation of the current at $-100$ mV. The capacity transient and the inward sodium current ($\tau_i < 1$ ms; Moolenaar and Spector, 1978) were summed and slowed by the slow bandwidth (125 Hz) of the recording system to form a single "switching transient"; the distinct inward current was the calcium current described by Moolenaar and Spector (1979b). (B) The change of optical absorbance at 660 nm ($\Delta A_{660}$) during voltage-clamp depolarizations is plotted as a function of membrane voltage during the depolarization. Each point is the mean $\pm$ SEM of six depolarizations. Open circles: $\Delta A_{660}$ during the first 0.5 s of depolarization. Solid squares: $\Delta A_{660}$ during the second 0.5 s of depolarization. This cell was one of three in which $\Delta A_{660}$ during the second 0.5 s of depolarization was small at all voltages. (C) Membrane current during voltage-clamp depolarizations. Each point is the mean of six depolarizations; error bars ($\pm$ SEM) are not shown when they are smaller than the symbols employed. Open squares: minimum current (i.e., peak of inward current after leak correction). Solid circles: current immediately before termination of the 1-s depolarization. The straight lines marked "L" are linear extrapolations of the current at $-100$ mV ($\pm$ SEM) and represent the leakage current.
occurred. The $\Delta A_{660}$ that was essentially complete within 0.5 s will be referred to as the rapidly inactivating component of $\Delta A_{660}$.

In the majority of cells (41 of 44), $\Delta A_{660}$, during depolarization to voltages more positive than $-20$ mV, showed a more complex time course. In these cells, $A_{660}$ increased steadily throughout a 1-s depolarization to 0, +20, or +40 mV (Fig. 3, C and D), so that a significant $\Delta A_{660}$ occurred in the second 0.5 s of depolarization (Fig. 3F). An average $\Delta[Ca^{++}]$ of $3.2 \pm 0.3$ nM was indicated during the second 0.5 s of depolarization to +20 mV (45 cells). A plot of $\Delta A_{660}$ during the first 0.5 s of depolarization showed two peaks, one at $-40$ mV and one at 0 or +20 mV (Fig. 3F). The slowly developing calcium-dependent potassium outward current (Moolenaar and Spector, 1979b) was observed at the voltages at which $A_{660}$ increased throughout a 1-s depolarization (Fig. 3, C, D, and G).

$A_{660}$ continued to increase monotonically over 10 s at +20 mV; however, the rate at which $A_{660}$ increased declined steadily (Fig. 4A). The change of $A_{660}$ after the first 0.5 s of depolarization could be fitted very well by a single exponential (Fig. 4A, open symbols). In 18 cells, $\tau$ was $5.4 \pm 0.5$ s. The return of $A_{660}$ to baseline after a depolarization could be followed in a subset of five cells in which the signal-to-noise ratio was particularly good (Fig. 4B); in these cells, the decay of $A_{660}$ at $-80$ mV could be fitted quite well by a single exponential (Fig. 4B, open symbols) with a mean time constant of $78 \pm 18$ s.

The voltage-clamp data can be most simply interpreted in terms of two components of $\Delta A_{660}$, each of which has an exponential time course. These two components might result from the complex behavior of a single calcium influx mechanism; however, it is more likely that they correspond to the two calcium currents believed to be present in these cells. The rapidly inactivating component of $\Delta A_{660}$ is active in the voltage range $-60$ mV < $V$ < +60 mV and is maximal at $-40$ mV; since its time constant is <200 ms, the rapidly inactivating component is essentially complete within 0.5 s. It is likely that the rapidly inactivating component of $\Delta A_{660}$ is a consequence of the rapidly inactivating calcium current described by Moolenaar and Spector (1978, 1979b). A second component of $\Delta A_{660}$ is active only in the voltage range $-20$ mV < $V$ < +60 mV and is maximal at +20 mV; $\tau = 5$ s, so this component produces similar increments of $A_{660}$ in the first and second 0.5 s of a depolarization. This component of $\Delta A_{660}$ will be referred to as the slowly inactivating component. It is likely that the slowly inactivating component of $\Delta A_{660}$ is a consequence of the slowly inactivating calcium current that can be detected after block of the calcium-dependent potassium current with quinidine (Fishman and Spector, 1981). Because both components of $\Delta A_{660}$ can be active during the first 0.5 s of depolarization, the plot of $\Delta A_{660}$ vs. membrane voltage shows two peaks, one at $-40$ mV and one at 0 (eight cells) or +20 mV (four cells). Because only the slowly inactivating component can be active in the second 0.5 s of depolarization, the plot of $\Delta A_{660}$ vs. membrane voltage shows a single peak at +20 mV. The terms "rapidly" and "slowly" refer to the inactivation time course; at the resolution of the optical system (20 ms), both components of $\Delta A_{660}$ activate without detectable delay.

The rapidly inactivating component of $\Delta A_{660}$ could be studied alone as the change of $A_{660}$ during depolarization to $-40$ mV. The change of $A_{660}$ in 1 s at
FIGURE 3. Typical $\Delta A_{660}$. The absorbance at 660 nm of a cell bathed in medium containing 10 mM Ca\(^{++}\) and 10 mM TEA was measured during depolarization to a range of voltages. Single typical depolarizations were given to -40 (A), -20 (B), 0 (C), +20 (D), and +60 (E) mV. $A_{660} = 0.030$, $R = 0.523$; thus, a $\Delta A_{660}$ of $2.5 \times 10^{-4}$ corresponds to a $\Delta [\text{Ca}^{++}]$ of 15.1 nM. (F) $\Delta A_{660}$ during voltage-clamp depolarizations is plotted as a function of membrane voltage during the depolarization. Each point is the mean (± SEM) of six depolarizations. Open circles: $\Delta A_{660}$ during the first 0.5 s of depolarization. Solid squares: $\Delta A_{660}$ during the second 0.5 s of depolarization. (G) Membrane current during voltage-clamp depolarizations. Each point is the mean of six depolarizations; error bars (± SEM) are not shown when they are smaller than the symbols employed. Open squares: minimum current (i.e., peak of inward current after leak correction). Solid circles: current immediately before termination of the 1-s depolarization. The straight lines marked "L" are linear extrapolations of the current at -100 mV (± SEM) and represent the leakage current.
-40 mV was therefore used in subsequent experiments as a measure of the rapidly inactivating component. The rapidly inactivating component was exponential with $\tau < 200$ ms; therefore, the change of $A_{660}$ during the second 0.5 s of a depolarization was dominated by the slowly inactivating component. $\Delta A_{660}$ in the second 0.5 s was greatest during depolarization to +20 mV, so the $\Delta A_{660}$ in the second 0.5 s at +20 mV was used in subsequent experiments as a measure of the slowly inactivating component. As described in the Methods, cells were induced to differentiate by plating out in growth medium containing 2% DMSO (Moolenaar and Spector, 1978) and were used 6–19 d later (median age, 11 d). The relative amplitude of the rapidly and slowly inactivating components appeared to change with the number of days that the cells were maintained in 2% DMSO before the experiment. The three cells in which the slowly inactivating component was indetectable had been in DMSO for 14, 18, and 18 d. In the 43 cells analyzed, the fraction slowly inactivating component/rapidly inactivating component showed a significant negative correlation with days in DMSO (at 5%, two-tailed $t$ test; Bulmer, 1967).

**Figure 4.** 10-s-long depolarization to +20 mV. The horizontal axis is time. Cell bathed in medium containing 10 mM Ca$^{2+}$ and 10 mM TEA. $A_{660} = 0.031, R = 0.818$; therefore, a $\Delta A_{660}$ of 0.005 corresponds to a $\Delta [Ca^{2+}]$ of 101 nM. (A) The open circles represent an exponential of time constant 4.5 s. (B) The open circles represent an exponential of time constant 114 s.

**Effect of Extracellular TEA Cl**

To test for a possible effect of TEA upon the calcium influx, cells were impaled and voltage-clamped in medium containing 10 mM Ca$^{2+}$ but no TEA. $A_{660}$ during repetitive depolarization was measured as the medium bathing the cells was changed to one containing 10 mM Ca$^{2+}$ and 10 mM TEA. TEA had no significant effect upon the rapidly inactivating component (five cells) or upon the slowly inactivating component (four cells). The peak outward membrane current at +20 mV was reduced to 13 ± 2% of control by TEA.

**Effect of a Reduction of Extracellular Calcium**

$\Delta A_{660}$ during repetitive depolarizations declined within 1–2 min when the medium bathing the cells was changed from one containing 10 mM Ca$^{2+}$ and 10 mM TEA to one containing 1.8 mM Ca$^{2+}$ and 10 mM TEA (Fig. 5). The rapidly inactivating component declined to 47 ± 3% of control (four cells); the slowly inactivating component declined significantly more, to 18 ± 5% of control (five
cells). $\Delta A_{660}$ recovered when the medium containing 10 mM Ca$^{++}$ was restored (Fig. 5). Neither the voltage optima nor the time course of the rapidly inactivating or slowly inactivating components changed detectably when extracellular calcium was reduced.

**Effect of Extracellular Cobaltous Ion**

$\Delta A_{660}$ declined within 1–2 min when the medium bathing the cells was changed from one containing 10 mM Ca$^{++}$ and 10 mM TEA to one containing 10 mM Ca$^{++}$, 10 mM TEA, and 10 mM CoCl$_2$. The effect of CoCl$_2$ on the two components was not significantly different: the rapidly inactivating component declined to 1 ± 5% of control (five cells), and the slowly inactivating component declined to 5 ± 2% of control (five cells). $\Delta A_{660}$ recovered when the cobalt-free medium containing 10 mM Ca$^{++}$ and 10 mM TEA was restored.

**Inactivation and Recovery of the Rapidly Inactivating Component**

When two 1-s depolarizing pulses to −40 mV were separated by ≤2 s at the holding voltage of −80 mV, $\Delta A_{660}$ during the second pulse was less than $\Delta A_{660}$ during the first pulse, which indicates that the calcium influx that gives rise to the rapidly inactivating component of $\Delta A_{660}$ had not completely recovered from inactivation in the interpulse period (five cells) (Fig. 6). The recovery of $\Delta A_{660}$ during the interpulse period was exponential, with a mean time constant of 1.8 ± 0.2 s (five cells) (Fig. 6).

To investigate the voltage dependence of steady state inactivation of the rapidly inactivating component of $\Delta A_{660}$, a 1-s depolarization to −40 mV was immediately preceded by a 5-s prepulse to one of a range of voltages. The increment of $A_{660}$ during the test pulse to −40 mV was plotted as a function of prepulse voltage.
FIGURE 6. Recovery of the rapidly inactivating component from inactivation. Two 1-s-long depolarizing pulses to −40 mV were separated by a variable interval at the holding voltage of −80 mV. Ordinate: time interval between the pulses. Abscissa: ΔA660 during the second of the pulses. Each point is the mean (± SEM) of six measurements. The continuous line is an exponential recovery of time constant 1.35 s. Cell bathed in medium containing 10 mM Ca** and 10 mM TEA. A660 = 0.032, R = 0.706; therefore, a ΔA660 of 0.0001 corresponds to a Δ[Ca++] of 2.93 nM.

(Fig. 7). 5 s is greater than the time constants of both inactivation and the recovery from inactivation, so the plot can be regarded as equivalent to an h∞ plot, where h∞ at a particular voltage is the fraction of channels available for recruitment by a subsequent depolarization. The smooth line in Fig. 7 is a plot

FIGURE 7. Voltage dependence of rapidly inactivating component inactivation. The sketch shows the experimental protocol. After 14 s at a holding voltage of −80 mV, the membrane voltage of a cell bathed in medium containing 10 mM Ca** and 10 mM TEA was stepped to one of a range of voltages and held for a 5-s prepulse period. A 1-s-long test pulse to −40 mV followed immediately after the prepulse. Abscissa: prepulse voltage. Ordinate: further increment of A660 during the 1-s test pulse. Each point is the mean (± SEM) of six measurements. A660 = 0.031, R = 0.843; therefore, a ΔA660 of 0.0002 corresponds to a Δ[Ca++] of 3.66 nM. The smooth line is a plot of the equation ΔA660 = 2.15 × 10^−4/[1 + exp [(V + 59)/4.8]]. At the resolution of the optical system, the rapidly inactivating component of ΔA660 activates without detectable delay at −40 mV. Therefore, artifacts of the type described by Gillespie and Meves (1980) for the sodium current, in which the degree of activation during the prepulse can affect the current recorded during the test pulse, will not be a problem here.
of the equation $h = \frac{1}{1 + \exp[(V - V_h)/K_h]}$, where $K_h = 4.8$ mV (Moolenaar and Spector, 1979b) and $V_h = -59$ mV. The mean $V_h$ in five cells was $-63 \pm 2$ mV. Moolenaar and Spector (1979b) obtained the value $V_h = -56$ mV for the rapidly inactivating calcium current of cells bathed in 20 mM extracellular Ca$^{++}$. In certain preparations, inactivation of calcium currents is voltage dependent and does not require [Ca$^{++}$] to increase (Fox, 1981; Cota et al., 1984). In other preparations, inactivation of calcium currents requires an increase of [Ca$^{++}$], so depolarizations that do not increase [Ca$^{++}$] do not cause inactivation (Tillotson, 1979). The arsenazo III technique allows an unequivocal test between these two modes of inactivation to be made. In N1E-115 cells, depolarizations to +70 mV

\[ \Delta A = 0.0005 \]

\[ 20 \text{nA} \]

\[ 100 \text{mV} \]

\[ 1 \text{s} \]

\[ A_{660} \]

\[ \Delta A_{660} \]

\[ \text{DISCUSSION} \]

\textit{Origin of the Absorbance Change}

The absorbance change recorded upon depolarization of N1E-115 cells injected with arsenazo III may be the result of changes of the cytosolic concentration of both calcium and a second ion, most likely H$^+$. A pH change, if present, is probably caused by the same calcium influx that elevates [Ca$^{++}$] (Ahmed and Connor, 1980). Six pieces of evidence confirm that the $\Delta A_{660}$ recorded during depolarization of N1E-115 cells injected with arsenazo III does indeed result from calcium influx through the cell membrane, rather than either a nonspecific effect of membrane voltage or membrane current, or the result of a voltage-dependent release of calcium from an intracellular store.
(a) No significant $\Delta A_{660}$ was recorded during the depolarization of uninjected cells. (b) No significant $\Delta A_{660}$ of injected cells was recorded during the depolarizations of greatest amplitude, to +70 mV. (c) Extracellular TEA, a potassium current blocker, reduced the total membrane current 10-fold without affecting the $\Delta A_{660}$ of injected cells. (d) The $\Delta A_{660}$ of injected cells was reversibly reduced when the extracellular calcium concentration was reduced. (e) The $\Delta A_{660}$ of injected cells was reversibly reduced during extracellular application of the calcium antagonist cobaltous ion. (f) As discussed below, both components of $\Delta A_{660}$ can be matched to known inward calcium currents.

The injection of arsenazo III to 1 mM should not greatly increase the calcium-buffering capacity of the cytosol. The total rapidly equilibrating buffering capacity of N1E-115 cells injected with arsenazo III was estimated by comparing the time integral of inward current at -40 mV with the $\Delta[Ca^{++}]$, indicated by arsenazo III. The result was 4,000 ± 1,000 ions bound per free ion. In comparison, 1 mM arsenazo III alone, with $R = [AI]/([AI] + [Mg:AI]) = 0.74$, will bind 420 ions per free ion. Thus, the injection of 1 mM arsenazo III increases the intracellular calcium-buffering capacity by ~10%.

**Characteristics of the Two Calcium Currents**

Both components of $\Delta A_{660}$ show an exponential time course, that is, $A_{660}$ increases toward a steady level upon depolarization. For both components, the time constant of the $A_{660}$ increase is much faster than the time constant of the return of $A_{660}$ to baseline after depolarization ends. This result is incompatible with a model in which the steady, elevated $A_{660}$ during depolarization represents a balance between a steady calcium influx and a calcium efflux. Rather, both components of $\Delta A_{660}$ must be caused by calcium influx mechanisms that inactivate with time constants of <200 ms and 5 s, respectively. Because of the similarity of time course, voltage dependence of activation, and voltage dependence of inactivation, it is probable that the rapidly inactivating component of $\Delta A_{660}$ is a consequence of the rapidly inactivating calcium current described by Moolenaar and Spector (1978, 1979b) and that the slowly inactivating component of $\Delta A_{660}$ is a consequence of the slowly inactivating calcium current described by Fishman and Spector (1981). A problem with this identification is an apparent mismatch between $\tau_n$ of the rapidly inactivating calcium current and the time constant of the rapidly inactivating component of $\Delta A_{660}$. This mismatch does not appear to be the result of a diffusional or other delay in the arsenazo III response. If diffusion or some other intracellular process were limiting, then the rate of change of $A_{660}$ would be independent of membrane voltage. In fact, the rapidly inactivating component of $\Delta A_{660}$ is faster at -20 mV than at -40 mV, and the ratio of time constants at these two voltages ($\tau$ at -40 mV = 170 ms; at -20 mV, $\tau = 110$ ms) is very similar to the ratio of $\tau_n$ values for the current ($\tau_n$ at -40 mV, 91 ms; at -20 mV, 67 ms). It is possible that calcium currents outside the soma, which contribute to the current record but not to $\Delta A_{660}$, inactivate more rapidly than does the current in the soma.

If the rapidly inactivating component of $\Delta A_{660}$ is a consequence of the rapidly inactivating calcium current described by Moolenaar and Spector (1978, 1979b),
and if the slowly inactivating component of $\Delta A_{660}$ is a consequence of the slowly inactivating calcium current of Fishman and Spector (1981), then the following new information on calcium currents in neuroblastoma cells can be inferred from this work.

(a) The rapidly inactivating calcium current is similar to currents variously described as transient (T-type, Nowycky et al., 1985), slowly deactivating (Armstrong and Matteson, 1985), or low-voltage activated (Carbone and Lux, 1984). Like the T-type current of annelid oocytes (Fox, 1981), it inactivates by a voltage-dependent mechanism that does not require $[Ca^{++}]_i$ to increase.

(b) The slowly inactivating calcium current is similar to currents described as long-lasting (L-type, Nowycky et al., 1985) and fast deactivating (Armstrong and Matteson, 1985). In particular, the slowly inactivating calcium current inactivates with a time constant of 5 s.

(c) The slowly inactivating calcium current is dominant in raising $[Ca^{++}]_i$ at membrane voltages more positive than $-20$ mV. The slowly inactivating calcium current is thus dominant in raising $[Ca^{++}]_i$ during depolarizations that activate the calcium-dependent potassium current (Moolenaar and Spector, 1979b) and depolarizations that activate a cytosolic guanylate cyclase (Study et al., 1978).

(d) Both components of calcium current are indetectable or absent at membrane voltages of $+60$ mV or more, as predicted by the constant-field equation (Hagiwara and Byerly, 1981).

(e) TEA is widely used to increase the calcium influx during single action potentials (e.g., Neering and McBurney, 1984; Bolsover and Spector, 1986). In molluscan neurons, the enhancement of calcium influx by TEA is not a direct effect of TEA upon the calcium current. Rather, TEA increases the calcium influx during single action potentials by blocking potassium currents and thereby causing a prolongation of the action potential (Ahmed and Connor, 1979). TEA does not affect either component of calcium influx in voltage-clamped N1E-115 cells; therefore, in these mammalian cells, too, the effect of TEA upon calcium influx during single action potentials is a result of blockade of potassium channels.

(f) The reduction of extracellular calcium from 10 to 1.8 mM does not affect both components of calcium current equally. The slowly inactivating calcium current is reduced in proportion to the reduction of extracellular calcium, whereas the rapidly inactivating calcium current is reduced significantly less. This finding is similar to the result of Fedulova et al. (1985) in rat sensory neurons. In contrast, Carbone and Lux (1984) found that, of the two components of calcium current in chick sensory neurons, the rapidly inactivating current was the one that was more affected by changes of extracellular calcium. One possible explanation for a less than proportionate fall of calcium current with extracellular calcium has been presented by Hagiwara and Takahashi (1967). In this model, calcium-binding sites may be present close to the extracellular mouth of the rapidly inactivating calcium current channel, whereas no calcium-binding sites are close to the extracellular mouth of the slowly inactivating calcium current channel. If this explanation is correct, then the rapidly inactivating and slowly inactivating calcium currents must indeed represent different populations of channels, rather than different gating mechanisms operating on the same channel.
(g) $[Ca^{++}]$, falls very slowly after a depolarizing pulse. The fall of $[Ca^{++}]$, can be reasonably well fitted by a single exponential, $\tau > 1$ min, which indicates that the rate at which calcium ions are removed from the cytosol is proportional to $[Ca^{++}]$. In contrast, $[Ca^{++}]$ falls to baseline with a time constant of $<10$ s in mammalian neurons and frog sympathetic neurons (Gorman and Thomas, 1978; Smith et al., 1983). The present experiments were performed at room temperature; $[Ca^{++}]$ may fall much faster in N1E-115 cells at their physiological temperature of $37^\circ$C.

N1E-115 is a transformed mammalian sympathetic line. In contrast, frog sympathetic neurons show only a single calcium influx mechanism similar to the slowly inactivating calcium current of N1E-115 cells (Smith et al., 1983).

**Physiological Roles of the Two Components**

The rapidly inactivating calcium current is activated by depolarization to $-50$ mV or above (Moolenaar and Spector, 1979b). In contrast, the sodium current begins to activate at $-40$ mV and is fully active at $-10$ mV (Moolenaar and Spector, 1978). Thus, depolarization that is just suprathreshold will trigger sodium action potentials indirectly, by activating the rapidly inactivating calcium current. Action potentials in physiological medium last $\sim 30$ ms (Moolenaar and Spector, 1978), so each action potential will cause only partial inactivation of the rapidly inactivating calcium current. However, recovery of the rapidly inactivating calcium current from inactivation is slow ($\tau = 2$ s), so calcium inactivation will build up during a train of action potentials and may have a role in regulating the duration of the train.

The slowly inactivating calcium current is active in a voltage range where large outward currents dominate. It is therefore unlikely that the slowly inactivating calcium current has an important electrical role. I believe that the role of the slowly inactivating calcium current is rather to add a further increment of calcium influx over and above the calcium influx through the rapidly inactivating calcium channels. As a consequence of the extremely slow inactivation of the slowly inactivating calcium channels, calcium influx into neuroblastoma cells will continue throughout trains of action potentials lasting many seconds.

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