THE RATE OF DIFFUSION OF CA^{2+} AND BA^{2+} IN A NERVE CELL BODY

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ABSTRACT A spectrophotometric method was developed to directly measure the diffusion rate of Ca^{2+} and some other ions in nerve cell bodies, using pulsed ionophoretic injections and an optical microprobe to record locally absorbance changes of the dye arsenazo III. We report here that Ca^{2+} and Ba^{2+} diffuse at approximately the same rate in nerve soma cytoplasm, having effective diffusion coefficients in the range of 7–12 $\times 10^{-7}$ cm^2/s, while identical measurements conducted in an electrolytic solution yielded values of 5.2 $\times 10^{-6}$ cm^2/s for Ca and 5.4 $\times 10^{-6}$ cm^2/s for Ba. The results are discussed in relation to the mechanisms that regulate the intracellular concentration of free Ca.

The transient rise in intracellular free Ca^{2+}, which occurs in many nerve cells with excitation of the plasma membrane, has been shown to function in the regulatory schemata of a number of important physiological processes. Among these Ca-mediated processes are transmitter release from nerve terminals (Katz and Miledi, 1967; Llinás and Nicholson, 1975) and the control of ion movement through channels in the nerve soma membrane (Meech, 1978: Tillotson, 1979). The time-varying Ca^{2+} concentration at any point in a nerve cell body is determined by a number of factors: (*a*) the size of the Ca^{2+} load and the locus where it is applied; (*b*) the cytoplasmic mechanisms responsible for regulating the free-Ca concentration in the intracellular compartment; and (*c*) the rate of diffusion of Ca^{2+} in the cytoplasm. The validity of predictions of local Ca^{2+} changes associated with membrane excitation depends on having a reasonable estimate for each of these factors. It has long been known that the mobility of Ca in squid giant axons is greatly reduced (Hodgkin and Keynes, 1957) compared with what should be expected in a saline solution. This fact has been interpreted as a reflection of the binding of Ca to intracellular sites. It can be shown that the presence of a distributed, high-capacity population of immobile binding sites that react rapidly with the diffusant would affect a diffusion process essentially by simply reducing the effective diffusion coefficient (Crank, 1975). The intimate relation between the diffusion coefficient and buffering by the cytoplasm makes it particularly interesting to measure in vivo the diffusion of Ca and other target ions.

The in vivo experiments described below were performed on selected neuron cell bodies of the abdominal ganglion of *Aplysia californica* (cells R15, L2, L3, L4, L6; see Frazier et al., 1967) bathed in artificial seawater at ~20° C. To measure the arsenazo III (Az III) absorbance changes arising from a narrow region of the cell under study, our microspectrophotometer (described in Gorman and Thomas, 1978) required a number of modifications. Most important among these was that the light-accepting fiber-optic probe was reduced in diameter to ~25 μm using an electrode puller and a microforge (the diameter of the probe normally used is ~150 μm, as the cell bodies have diameters >200 μm). In addition, the optical assembly (which includes the light delivery and light accepting probes) was mounted on a Huxley-type micromanipulator that allowed fine control of movement in three dimensions (~1-μm resolution). Finally, a xenon-arc light source replaced our quartz halogen source, since the small size of the light-collecting probe required a high light flux density to produce an acceptable signal-to-noise ratio.

The experimental arrangement for measuring the diffusion of ions is shown in Fig. 1. The selected nerve soma is held under voltage clamp and is pressure injected with purified Az III. The cell is then penetrated with a multibarreled microelectrode containing $CaCl_2$ and $BaCl_2$ solutions (100 mM) in separate barrels. This allows ionophoretic injections of each ion to be made at the same point within the nerve cell. The optical micro-probe assembly is positioned so that a small test injection of Ca^{2+} produces a maximal absorbance change signal. This position is considered to represent zero distance between the source and the optically sampled region. After a series of Ca^{2+} and Ba^{2+} injections are presented and the resulting absorbance changes recorded, the whole optical probe assembly is moved in precise steps, and the test injections are repeated.

The same apparatus was used to record absorbance changes by ion injections in vitro. In these experiments

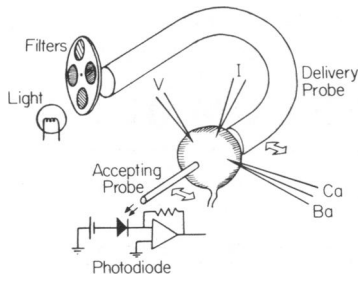

FIGURE 1 Schematic depiction of the experimental arrangement for the in vivo measurements. The cell soma is positioned between the two fiber optics probes, which are mounted on a Huxley-type micromanipulator, and can be displaced as a monolithic assembly in any direction. Two standard microelectrodes are used to hold the cell under voltage clamp (one of them serving also to pressure inject the metallochromic indicator Az III), and a third, multibarreled microelectrode is employed to apply minute ionophoretic injections of either Ca^{2+} or Ba^{2+}. The probe assembly is displaced while test injections are administered, until the evoked Az III signal reaches a maximum amplitude, presumably when the tip of the injecting electrode is located near the center of the measuring beam. This point is taken as the zero-distance reference point, and the probes are then moved in precise steps away from it, repeating the injections and measuring the resulting absorbance signal at each position.

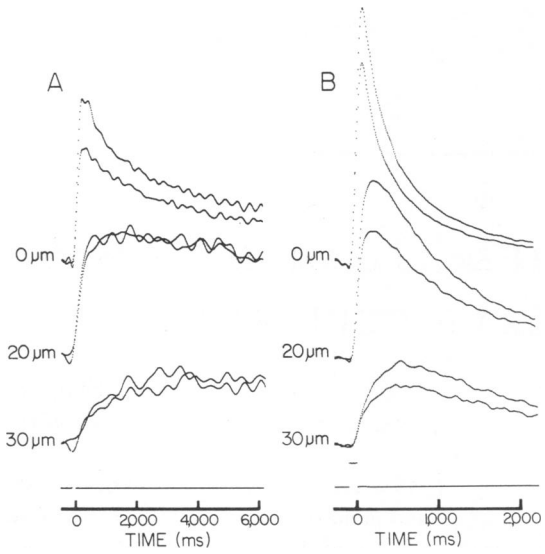

FIGURE 2 (*A*). Differential absorbance signals (660–690 nm) recorded in vivo at three distances from the ionophoretic microelectrode tip (indicated near each pair of traces), in response to injections of Ca and Ba. Each trace represents the average of 16 records, and was low-pass filtered at a cut-off frequency of 2 Hz with a digital algorithm with zero-phase characteristics to minimize distortion in the peak of the signal. The records corresponding to 20 and 30 μm distance from the ionophoretic electrode tip are scaled by a factor of five compared with the zero-distance record. The cell had been previously pressure injected with the dye to an estimated concentration of 1 mM. (*B*) Absorbance signals in response to Ca and Ba injections in vitro. The procedure is essentially identical to the way the in vivo measurements were performed, except that the probe assembly was placed in a small volume bath (~500 μl) containing 200 μM Az III, 100 mM KCl, 10 mM Tris, pH 7.7. The cut-off frequenty for the low-pass digital filter was 6 Hz. The two lower pairs of records are scaled by a factor of 2.5, with respect to the top pair.

both the probe assembly and the injecting microelectrode were placed in a small volume of saline solution containing Az III. The injecting tip-to-probe separation experiment described above could thus be repeated under conditions of unimpeded diffusion. A comparison of the mobility in the cell and in free solution can then be used to estimate the impediments to diffusion within the cytoplasm for different test ions that can be detected with Az III, with the aim of separating nonspecific factors from buffering.

Data from an in vivo Ca^{2+}/Ba^{2+} comparison experiment are shown in Fig. 2 *A*. Absorbance change signals produced by a 100 nA, 100-ms duration, Ca^{2+} and Ba^{2+} injections into cell L-4 are illustrated. Each trace represents the ensemble average of 16 raw records, digitally low-pass filtered at 2 Hz. Averaging and filtration were found to be necessary to obtain a sufficiently high signal-to-noise ratio that would permit implementing an objective, computerized protocol to determine the time at which the maximum absorbance change occurred. It can be seen that as the distance between the ion-injecting tip and the measuring beam increased the amplitude of the signals decreased and the time-to-peak of the signals increased, as expected from diffusion theory (Crank, 1975). An in vitro experiment using similar injections of Ca^{2+} and Ba^{2+} (50 nA for 100 ms) generated the averaged and filtered data shown in Fig. 2 *B*.

The peak of the absorbance signal measured in these experiments can be regarded as occurring at the moment in time when the total free Ca^{2+} in the restricted measuring region reached its maximum. Further, the relationship between the time of the peak concentration and the distance between the source and the measuring beam is uniquely defined by the coefficient of diffusion. Qualitatively, one would expect that the higher the diffusion rate, the shorter the time-to-peak at a given distance. Fig. 3 shows a plot of the time-to-peak of Ca^{2+}-Az III and Ba^{2+}-Az III signals as a function of the optical probe-injecting tip separation for in vivo and in vitro data. As expected, the diffusion rate is significantly lower in the cell than in solution. The smooth curves represent the result of least-square fitting the theoretical relation between distance from a point source and the expected time-to-peak, in order to determine the best estimate for the diffusion coefficient for each set of data. The effective diffusion coefficients found in this experiment were 8.3×10^{-7} cm^2/s for Ca^{2+} and 9.2×10^{-7} for Ba^{2+} in the cell. In vitro the corresponding values were 5.2×10^{-6} cm^2 and 5.4×10^{-6} cm^2 s, respectively. The measurements in vitro were very consistent in different experiments, with a variability of <5% ($n = 3$), while a larger degree of variability was observed in cell somas, covering a range of $7–12 \times 10^{-7}$ cm^2/s ($n = 4$).

At least two factors contribute to the reduction in the rate of diffusion of Ca^{2+} in the nerve cell body as compared

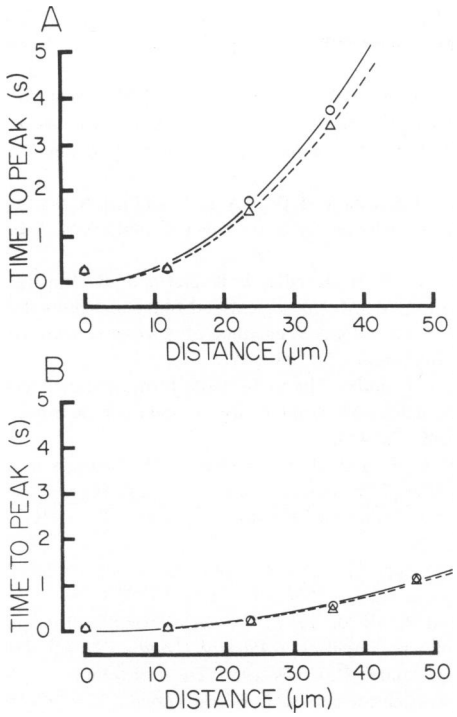

FIGURE 3 (*A*) Time-to-peak (in vivo) of the Az III signal following an injection of Ca (open circles) or Ba (open triangles) by means of a multibarreled microelectrode. The injections with the two ions were therefore administered at exactly the same point within the cell. The measurements were corrected for the time shift induced by the linear phase anti-aliasing analog filter used before the analog-to-digital conversion. The two lines were calculated by maximizing the instantaneous point-source solution of the diffusion equation in three dimensions after having integrated it along an axis orthogonal to the direction of displacement of the optical probe assembly. The approach serves as a reasonable approximation for the fact that the spectrophotometer records a signal proportional to the total free calcium that is found along the beam sampled by the light-accepting microprobe. This requires only the assumption that the changes in absorbance can be regarded as a linear function of Ca for a fixed concentration of Az III (Palade and Vergara, 1983) but is entirely independent from any specific knowledge about the absolute calcium concentration, the extinction coefficient, or the stoichiometry of the Ca-Az III reaction. The resulting D value was 8.3×10^{-7} cm^2/s for Ca (continuous line) and 9.2×10^{-7} cm^2/s for Ba (dashed line). (*B*) Time-to-peak for the Az III signal in vitro. The procedure was similar to the in vivo conditions, and yielded a value of the diffusion coefficient of 5.2×10^{-6} cm^2/s for Ca (filled circles, continuous line) and 5.4×10^{-6} cm^2/s for Ba (filled trianbles, dashed line). Both in the in vivo and the in vitro experiments, a finite time lag before the occurrence of the peak is observed, even at the zero-distance point. This appears to reflect momentary local saturation of the dye in the vicinity of the injecting tip shortly after the pulse, in such a way that the recorded absorbance signal continues to rise as the injected ions diffuse away from the saturated region. Such interpretation is consistent with the fact that only at the zero-distance point does the time to peak display a prominent lengthening as injection intensity is increased (unpublished observation). Furthermore, such high local concentrations of the diffusant could momentarily saturate the endogenous buffer of the cell, thereby resulting in a concentration-dependent diffusion coefficient near the injecting source. The time-to-peak analysis would therefore not be valid at such short distances. The excellent fit of the measured times by a simple diffusion model at all points but the one nearest to the source, however, suggests that this potential complication does not pose a serious problem.

with free solution. One is viscosity of the cytoplasm and the physical impediment or tortuosity imposed by immobile structures such as organelles and cytoskeleton (for an analysis of how diffusion theory can handle path tortuosity, provided that it refers to a small distance scale, as compared with the scale of the diffusion measurements, see, for example, Nicholson and Phillips [1981]). The second and perhaps more important factor is reversible binding of Ca^{2+} to relatively immobile intracellular sites. Our method for measuring Ca^{2+} diffusion gives no direct information about the relative contribution of these two factors, although a comparison of the diffusion rate in vivo vs. in vitro for Ca and other ions that are not thought to bind to cytoplasmic constituents could provide some insight into the question.

We and others have reported that Ba^{2+} is less well buffered than Ca^{2+} (Connor et al., 1981; Tillotson and Gorman, 1983). This was based in part on the observation that whole-cell Ba-Az III signals decay much more slowly than Ca-Az III signals associated with ionophoretic injections or with influx through the plasma membrane (Tillotson and Gorman, 1983). If such difference in buffering reflected a difference in reversible binding of Ca^{2+} and Ba^{2+} to intracellular sites, we would predict that the reduction in mobility in the cell as compared with a free solution should be more pronounced than for Ba. The similarity between the effective diffusion coefficients found for these ions both in vitro and in vivo suggests that Ca^{2+} and Ba^{2+} are buffered (in the sense of rapidly equilibrating, reversible binding) to the same extent. This further suggests that the difference in buffering noted above is likely to be due to slower processes of uptake and/or extrusion. Those processes would appear to be more strongly selective for Ca^{2+} over Ba^{2+} than the reversible binding. This underscores the importance of distinguishing between reversible binding and uptake/extrusion mechanisms when considering neuronal regulation of Ca^{2+}. The fact that the diffusion coefficient measured in the free solution was quite consistent within a series of experiments and is in reasonable agreement with the value of $\sim 7 \times 10^{-6}$ cm^2/s, obtained by Wang (1953) in a $CaCl_2$ solution, as well as the theoretical value of 6×10^{-6} cm^2/s, calculated for the limiting case of infinitely diluted solutions (Hodgkin and Keynes, 1957), suggests that our method is valid. If the assumption is made that the 6–10-fold reduction that has been observed in cytoplasm can be attributed entirely to reversible binding to a high capacity buffer system of immobile sites (Blaustein and Hodgkin, 1969; Smith and Zucker, 1980), this would indicate that cytoplasmic free calcium is 10–15% of the total intracellular calcium. Such an estimate appears to be substantially higher than the ratio of free Ca to total Ca in squid axons (Keynes and Lewis, 1956; Di Polo et al., 1976; Brinley et al., 1977) and indicates that the assumption of the immobility of the binding sites is likely to be an oversimplification; this is

consistent with the fact that the mobility of Ca in squid axoplasm as compared with free solution is reduced much more dramatically than the self-diffusion coefficient (Hodgkin and Keynes, 1957).

The method we have developed and employed to estimate the diffusion coefficients has significant advantages over those used in the past where the spread of an injected patch of radioactively labeled Ca^{2+} was measured (Hodgkin and Keynes, 1957). (*a*) The optical microprobe method measures only Ca^{2+} that is free to diffuse, whereas the 45 Ca experiments also measured Ca^{2+} sequestered in organelles. (*b*) In vivo-in vitro comparisons are possible under very similar experimental conditions with the present method. (*c*) Side-by-side comparison of the diffusion of different ions under identical conditions is feasible by the use of multibarreled injecting microelectrodes. (*d*) The diffusion of relatively minute quantities of Ca^{2+} and Ba^{2+} can be measured in the current method (as a rough estimate, assuming a transport number of 0.3 [Gorman and Hermann, 1979], a 100-ms injection at 50 nA implies 7.8×10^{-15} mol of Ca being injected into the cell). (*e*) The spread of the applied load is measured in real time in vivo and the nature of the technique makes it applicable to cells whose size is beyond the spatial resolution that can be attained with the measure of radiosotope. These factors lead us to conclude that the diffusion-rate estimate reported here may represent an improvement over those made previously. In addition, our experiments were performed on identified nerve cell somas. Since intraneuronal Ca^{2+} regulation mechanisms (particularly reversible binding) may significantly affect the diffusion rate, we believe it important to have obtained the diffusion estimate in a cell that, during its normal functioning, experiences and regulates large transients of Ca_i. Such a cell may require Ca^{2+} buffering mechanisms, that are more powerful than those of a cell that experiences relatively little Ca^{2+} influx with excitation (e.g., axon tissue).

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