Dual-Fluorescence Flow Cytometric Analysis of Membrane Potential and Cytoplasmic Free Ca\(^{2+}\) Concentration in Embryonic Rat Hippocampal Cells

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ABSTRACT. We have demonstrated simultaneous measurement of the membrane potential and cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) by utilizing a dual-laser flow cytometer in embryonic rat hippocampal cell suspensions. Veratrine, a Na\(^{+}\) channel activator, induced both membrane depolarization and elevation of [Ca\(^{2+}\)]. These actions of veratrine were all reversed by the presence of tetrodotoxin (TTX). These findings suggest that Na\(^{+}\) channels are functionally expressed in the cells and the activation of Na\(^{+}\) channels increases [Ca\(^{2+}\)]. The usefulness of the flow cytometric analysis in elucidating the expression of membrane functions in the embryonic central nervous systems (CNS) is discussed.

The development of electrical membrane properties has been investigated in neurons from several species (9). The details of excitable membrane properties in cultured and isolated neurons have been revealed using electrode recording methods (2, 8). However, analysis of membrane functions using early embryonic neurons is difficult because of the heterogeneity of the cell population, co-existence of non-neuronal cells and different stages in development of cells (1, 7). Fluorescence flow cytometric analysis with voltage-sensitive dyes is expected to be useful for the study of membrane excitability to acutely dissociated cells from embryonic CNS (3). In the present study we have demonstrated the expression of the TTX-sensitive Na\(^{+}\) channels and [Ca\(^{2+}\)] increase dependent on the activation of the channels by using the dual-fluorescence flow cytometric technique in embryonic rat hippocampal cells.

MATERIALS AND METHODS

Hippocampal cells were dissociated by trypsinization from 18–20-day embryonic rat cerebra (5). For determination of the membrane potential and [Ca\(^{2+}\)], freshly isolated hippocampal cells were washed with Hepes-buffered Krebs medium (in mM: 124 NaCl, 5 KCl, 1.24 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 1.23 CaCl\(_2\), 10 glucose, 10 Hepes/Tris, pH 7.4) and incubated with 5 \(\mu\)M 1-(2-Amino-5-[2, 7-dichloro-6-hydroxy-3-oxo-9-xanthenyl] phenoxy)-2-(2'-amino-5-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid, and acetoxymethyl ester (Fluo-3-AM) (4) in the medium at 37°C for 60 min. The cells were centrifuged at 500 \(\times\) g for 5 min.

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to remove residual dyes and incubated in fresh medium with 1 µM bis (1,3-dibutylbarbituric acid) pentamethine oxonol (oxonol) (6) at 37°C for 30 min. The final concentration of cells for the assay was 1–2 x 10⁶ cells/ml. Flow cytometric analysis of the cell suspensions was performed on a dual-laser flow cytometer (EPICS 753, Coulter, Hialeah, FL) at 35°C. A 515-nm argon ion laser (Coherent, Palo Alto, CA) operating at 337 mW was used to excitate Fluo-3. The dye laser using rhodamine 6G, which is excitated by a second argon ion laser operating in all-line mode, was tuned 590 nm at 300 mW for excitation of oxonol. The approximate spectral characteristics of the optical filters used in this study are shown in Figure 1. The cell suspensions were usually analyzed at 300 events per second with data being collected on 50,000 events per sample. Data acquisition programs and single- and joint-parameter histograming programs were commercially available (MDADS and EPICS CYTOLOGY, Coulter, Hialeah, FL).

RESULTS AND DISCUSSION

We have examined the effects of veratrine (100 µM) on the relative frequency as function of their oxonol and Fluo-3 fluorescences generated by dual-laser flow cytometric analysis (Fig. 2). The sample population of 50,000 events was generally assumed to contain around 35,000 Fluo-3-loaded and 15,000 unloaded cells (Fig. 2, A). Both the frequency distributions of the oxonol and Fluo-3 fluorescence intensities shifted to higher values with veratrine in Fluo-3-loaded cells (Fig. 2, B). The cell population exhibited a tail extending toward high values in the two parameters. The differential of the frequencies for the two fluorescences over the control was 6,000 of a total of 50,000 events. The Fluo-3-unloaded cells were almost insensitive to veratrine in the fluorescences and this cell population was generally stained with ethidium bromide (data not shown), a indicator of dead cells (3). Therefore it is thought that a parallel increase of both oxonol and Fluo-3 fluorescences due to veratrine is attributable to live cells.

Recognition of the effect of veratrine on the Fluo-3 and oxonol fluorescences was

Fig. 1. Emission spectra and filter acceptances of the fluorescent dyes used. The argon ion and dye laser wavelength (515 nm and 590 nm, respectively) are marked with arrows.
Fig. 2. Joint distribution of Fluo-3 and oxonol fluorescences. The ordinate of each histogram shows the relative frequency of events which had the Fluo-3 and oxonol fluorescence intensities plotted along the respective coordinates indicated on the horizontal plane. I, Fluo-3-loaded cell population; II, Fluo-3-unloaded cell population. A, Control; B, Cells treated with 100 µM veratrine.

Fig. 3. Single-parameter histograms of Fluo-3 (A) and oxonol (B) fluorescences. The ordinate is the relative frequency. The abscissa is fluorescence intensity in arbitrary units from 0 to 255. Curves within for each dye are the same in the number of total events. The shift to the right of the frequency distribution of the Fluo-3 and oxonol fluorescences corresponds to the general increase of $[\text{Ca}^{2+}]$ and membrane depolarization, respectively. For Fluo-3 the mean values of fluorescence of control, veratrine and veratrine + TTX were 56.7, 62.0 and 55.7, respectively. For oxonol they were 47.2, 58.6 and 49.0, respectively. Veratrine, 100 µM; TTX, 1 µM.
carried out by using TTX, a specific Na\(^+\) channel blocker (Fig. 3). TTX completely blocked the effect of veratrine on both the Fluo-3 and oxonol fluorescence signals (Fig. 3, A and B). The fraction of Fluo-3-unloaded cells was unaffected by the Na\(^+\) channel blocker.

The present results show that TTX-sensitive Na\(^+\) channels are functionally expressed in the cells and \([\text{Ca}^{2+}]\), increase due to veratrine requires activation of the Na\(^+\) channels, although it has not been confirmed here whether the voltage-sensitive Ca\(^{2+}\) channels are activated through the depolarizing action of veratrine or Ca\(^{2+}\) transport systems such as the Na\(^+\)/Ca\(^{2+}\) exchanger are activated through increase of intracellular Na\(^+\) concentration by veratrine.

Dual-fluorescence flow cytometry was affirmed to have several advantages in analysis of suspended cells of neurons for their \([\text{Ca}^{2+}]\), and membrane potential, especially in the following points: 1) The response of individual cells of a heterogeneous population can be evaluated. Non-responsive cells can be excluded for statistic calculation. 2) Correlation of cellular events can be analyzed if appropriate fluorescence dyes are available. Therefore, dual-fluorescence flow cytometric analysis is useful in elucidating the expression of membrane functions, such as ion channels and neurotransmitter receptors, in early differentiated neurons. Especially, a specific subpopulation can be analyzed if the technique is combined with immunofluorescence analysis for the surface marker of a celltype.

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