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VARIATION OF CALCIUM CURRENT DURING THE CELL GROWTH CYCLE IN MOUSE HYBRIDOMA LINES SECRETING IMMUNOGLOBULINS

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SUMMARY

1. Ca currents of the cell membrane were recorded during a four-day culture period using the whole-cell variation of the patch electrode voltage clamp on a mouse myeloma cell line (S194, non-secreting) and on two mouse hybridoma cell lines (MAb2-1 which secretes immunoglobulin G (IgG) and MAb7B which secretes immunoglobulin M (IgM)).

2. The density of Ca current increased during the four-day culture period after cells were transferred into fresh medium. The average value of the maximum inward current per cell reached 57 ± 15 pA (mean $\pm$ S.D.) by the fourth day in hybridomas secreting immunoglobulins.

3. The density of Ca current in hybridomas secreting IgG and IgM was greater than that in non-secreting myeloma throughout the four days of the culture cycle.

4. The cell density showed a sigmoidal increase during the culture period and the rate of increase in the density showed no significant correlation with the density of Ca current.

5. The immunoglobulin level in the medium increased over the culture period, as did the estimated immunoglobulin secretion per cell per day. The time course of the increase of estimated individual cell secretion resembles that of the increase of density of Ca current.

INTRODUCTION

Few studies have been carried out on the electrical membrane properties of lymphocytes and their relationship to immunological function. Using fluorescent probes, information concerning the membrane potential (Rink, Montecucco, Hesketh & Tsien, 1980) and the intracellular Ca concentration (Tsien, Pozzan & Rink, 1982*a*, *b*; Pozzan, Arslan, Tsien & Rink, 1982) has been obtained. However, the direct measurement of the membrane potential with intracellular electrodes was difficult because of the small sizes of lymphocytes (Taki, 1970). The recent introduction of the patch electrode voltage-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) has overcome this difficulty.

Using this technique, we demonstrated the presence of a Ca current in the membrane of mouse myeloma cell line S194 (Fukushima & Hagiwara, 1983). During this study, we were impressed by the large variation in the maximum Ca current observed in different cells. In fact, from the majority of cells only a vestige of Ca current was observed. This myeloma cell line synthesizes but does not secrete immunoglobulin (Hyman, Ralph & Sarkar, 1972). Hybridomas secreting immunoglobulins were constructed by fusion of S194 myeloma cells and splenic B lymphocytes (Kohler & Milstein, 1975, 1976). We observed significantly larger Ca currents in these hybridomas. This observation led to our interest in investigating the variation in the Ca current in relation to possible functional significance.

The maximum peak inward Ca current per cell showed a typical increase during the four days following the transfer of hybridoma cells into fresh culture medium. For convenience we will use the term 'culture cycle' for this four-day period. The cell density and the content of immunoglobulin in the culture medium were also observed, and we examined possible correlations between the density of Ca current, cell proliferation and the immunoglobulin-secreting activity.

METHODS

Materials

The myeloma cell line (S194) and two hybridoma cell lines (MAb2-1 and MAb7B) were maintained in suspension in RPMI 1640 medium (Irvine Chemical, Santa Ana, CA, U.S.A.) supplemented with 10 % fetal calf serum. Culture medium was renewed every fourth day. The cell number was adjusted to start at about 20×10^4 cells/ml. One-twentieth of the spent medium was always transferred together with cells into the fresh medium. When the cell density was counted by haemocytometer, Trypan Blue exclusion was used to identify viable cells.

Recording

The whole-cell variation of the patch electrode voltage-clamp technique was used to study the electrical properties of the cell membrane. The patch electrode was filled with a solution containing (in mM): KCl, 150; $MgCl_2$, 1; K salt of EGTA, 5; $CaCl_2$ (pCa = 7), 2·5; glucose, 20; and HEPES (pH = 7·4), 10. The bath solution was composed of (in mM): $CaCl_2$, 25; $MgCl_2$, 1; NaCl, 116; KCl, 5; glucose, 7; and HEPES (pH 7·4), 10. The junction potential of the internal solution relative to the bath solution was −5·5 mV. Correction of junction potentials was made as previously described (Hagiwara & Ohmori, 1982).

A droplet of cell suspension was placed in the experimental bath and the bath solution was continuously perfused after cells had settled on the bottom. These cells were renewed hourly because the cell condition gradually deteriorated in the experimental bath. The bath temperature was 24–26 °C. The resistance of electrodes ranged between 3 and 10 MΩ. The seal resistance was 3–20 GΩ. Cells of input resistance greater than 1 GΩ were selected for recording the membrane current, because the Ca current diminished rapidly in leaky cells. The voltage clamp was performed with a holding potential of −96 mV. The output of the current–voltage converter was filtered through a low-pass filter at 1 kHz (24 dB/octave). The linear leakage component of the membrane current was subtracted electronically. Membrane current after leakage subtraction was digitized at a sampling interval of 0·4 ms and stored in a computer for later analysis. Current-clamp measurements were performed by a feed-back loop that connected the current measuring output via an integrator to the command input for the pipette potential (Fenwick, Marty & Neher, 1982).

Measurement of immunoglobulin secretion

S194 myeloma cells synthesize immunoglobulin A (IgA) but do not secrete immunoglobulins into the medium (Hyman *et al.* 1972). MAb2-1 and MAb7B hybridoma cells secrete IgG and IgM, respectively. Immunoglobulin levels of the culture medium were measured by a sandwich

radioimmunoassay as described previously (Irie, Sze & Saxton, 1982). We could measure only cumulative immunoglobulin levels in the culture supernatant. We estimated the secretion of immunoglobulin during one day by calculating the difference between the levels for two successive days.

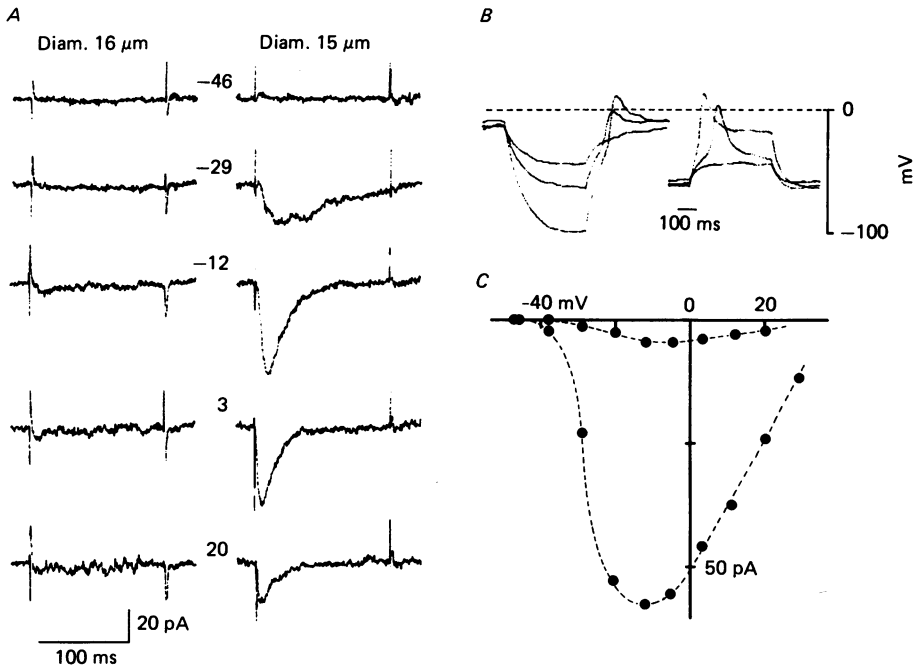

Fig. 1. *A*, inward Ca currents from two cells of almost identical size (15 and 16 μm). Both cells were from hybridoma cell line secreting IgG (MAb2-1). Figures adjacent to traces indicate the membrane potential in millivolts. Holding potential is −96 mV. *B*, current-clamp records from the cell shown on the right side of *A*. *C*, the current–voltage relation at the peak of the inward current obtained from records shown in *A*.

RESULTS

Ca current in hybridomas secreting immunoglobulin

When the membrane currents were recorded from hybridoma cells of 12–17 μm in diameter, the maximum observed amplitude of the Ca current varied considerably between different cells. Membrane currents measured during various voltage pulses from two hybridoma cells of almost identical size are shown in Fig. 1 *A*. Only a trace of inward current was seen in one cell (left), while the peak inward current reached 56 pA at −12 mV in the other cell (right). The inward current of these hybridoma cells was shown to be a Ca current as was the case with myeloma cells (Fukushima & Hagiwara, 1983). A summary of the properties of the current in these hybridomas is: (1) the inward current disappeared when the external Ca^{2+} was replaced with Mn^{2+}; (2) the amplitude of the inward current increased with an increasing $[Ca^{2+}]_o$; (3) Sr^{2+} and Ba^{2+} also carried the inward current; (4) the decaying process of the current and the steady-state inactivation were determined to be mainly voltage dependent.

From cells having a significant Ca current, action potentials were observed in the current-clamp condition (Fig. 1*B*). The left part of this trace shows the off-response produced by an inward current pulse at the zero-current potential. Action potentials were evoked by outward current pulses when the cell had been hyperpolarized with d.c. current (Fig. 1*B*, right). Spontaneous action potentials were not observed from the zero-current potentials (−10 to −30 mV), probably because the Ca channel was inactivated at these membrane potentials. It is likely that seal leakage made the zero-current potential substantially less negative that the actual resting potential, which was estimated as about −60 mV in lymphocytes with the fluorescent probe (Rink *et al.* 1980). Spontaneous action potentials might occur in intact cells.

The peak amplitudes of the inward Ca currents for the two cells used to obtain records in Fig. 1*A* were plotted against membrane potential (Fig. 1*C*). For each cell the maximum peak inward current ($I_{\text{Ca max}}$) was noted for later analysis (Figs. 2, 3 and 4). In 90% of the cells studied the outward currents were negligible in the potential range more negative than +30 mV. In 10% of the cells, outward current started to be activated at a potential about 20 mV more positive than that for the inward currents. The time course of the outward current was similar to that in T lymphocytes (Fukushima, Hagiwara & Henkart, 1984), $I_{\text{Ca max}}$ was slightly underestimated in these cells with outward current; however, this did not affect the analysis seriously since the outward current was still relatively insignificant at the membrane potential of the maximum inward current.

Ca current and tissue culture growth curve

The cell density in the culture medium showed a sigmoidal increase with time after the transfer of cells into fresh culture medium for S194 myeloma and the two hybridomas (Fig. 2*A*). Although all cultures started at a cell density of about 20×10^4 cells/ml, the cell density at the saturation level was 50% higher with S194 myeloma than with the hybridoma. $I_{\text{Ca max}}$ is also plotted against time for myeloma (Fig. 2*B*) and the hybridomas (Fig. 2*C*). In order to avoid any bias all data obtained with cells which had diameters of 12–17 μm and input resistances greater than 1 GΩ were plotted. This is also the case for Figs. 3 and 4. In non-secreting myeloma $I_{\text{Ca max}}$ was predominantly in the narrow range between 0 and 20 pA throughout the four-day cycle. In only six out of forty-seven cases was it greater than 20 pA, and these were found on the third and fourth days. The average of $I_{\text{Ca max}}$ in the hybridomas secreting IgG and IgM was greater than that of the myeloma for each of the four days. The average of $I_{\text{Ca max}}$ in the hybridomas during the third and fourth days was dramatically increased from the level of the first and second days. The average value reached 57 ± 15 (S.D.) pA by the fourth day. The results suggest that the increase of the Ca current is related to the culture cycle.

The diameter of the cells ranged between 12 and 17 μm. When results obtained during all four days were combined, no significant correlation was found between $I_{\text{Ca max}}$ and the diameter in either myeloma or hybridoma. When the data obtained on the third or fourth day were examined in the hybridomas, a weak positive correlation was found.

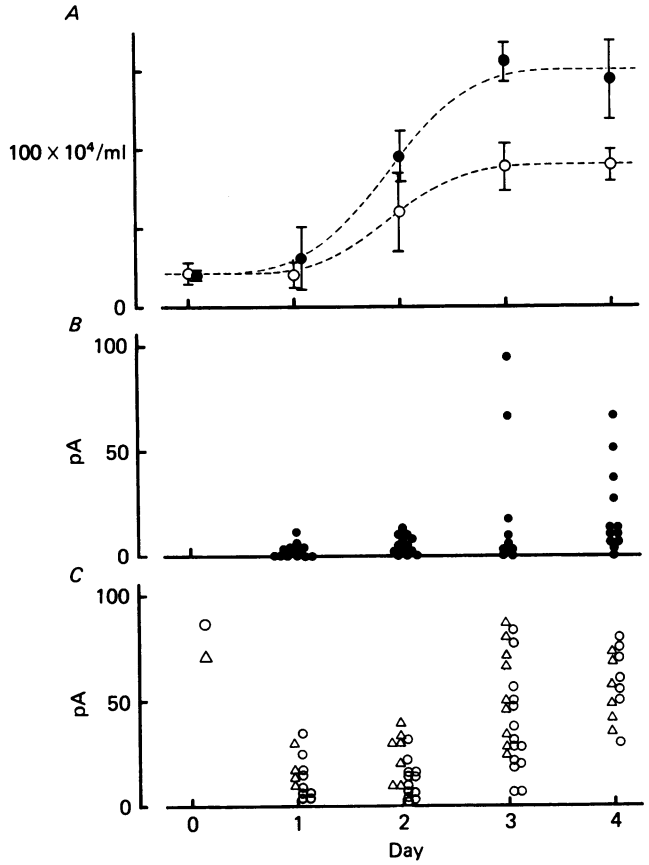

Fig. 2. *A*, cell proliferation curve. Circles and bars indicate the mean and the standard deviation of the cell density at each day in the culture ($n = 3$ for S194, filled circles; and $n = 5$ for hybridomas, open circles). Cells were transferred into the fresh culture medium at day 0. The data for two hybridomas secreting IgG ($n = 3$) and IgM ($n = 2$) were averaged together because they showed essentially similar time courses. *B*, each circle indicates the maximum peak inward current of each cell of S194 myeloma (non-secreting type). *C*, the maximum inward current in hybridomas secreting IgG (MAb2-1, open circles) and IgM (MAb7B, open triangles).

Cell proliferation and immunoglobulin secretion

We examined the possible correlation between density of Ca current to cell proliferation or to immunoglobulin secretion. The cell density (Fig. 3*A*), the immunoglobulin level in the culture medium (Fig. 3*B*) and values of $I_{\text{Ca max}}$ (Fig. 3*C*, filled circles) of all observed cells having diameters of 12–17 μm and input resistances greater than 1 GΩ were obtained from the same culture flask, and plotted against time after cells were transferred into the fresh medium. Comparison of parts *A* and *C* of Fig. 3 indicates that the increase in $I_{\text{Ca max}}$ had no correlation with the rate of increase in the cell density, i.e. the cell proliferation.

The immunoglobulin level in the culture medium increased with time. The

immunoglobulin secretion per day was calculated as the difference of the immunoglobulin levels between two successive days (shown by columns in Fig. 3*B*). The secretion per day stayed at low levels the first and second days and then gradually increased up to the fourth day. In this case, the culture started at the cell density of 22×10^4 cells/ml. The cell density increased sigmoidally and saturation was reached the third day. The increase of the secretion level per day, therefore, was not explained

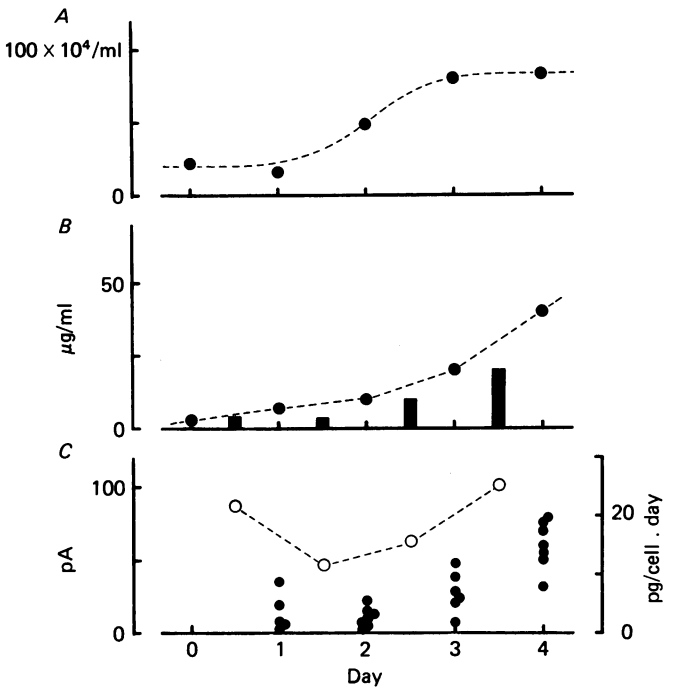

Fig. 3. Ca current and immunoglobulin secretion in hybridoma secreting IgG measured after renewal of the culture medium. *A*, cell proliferation curve. The culture started at the cell density of 22×10^4 cells/ml. *B*, circles indicate the immunoglobulin level in the supernatant of the culture medium at each day. The differences of level between two successive days are shown by the bars, which thus indicate the immunoglobulin secretion for one day. *C*, filled circles indicate the maximum observable Ca current in each cell. Open circles indicate the immunoglobulin secretion per cell per day, which was calculated by dividing the immunoglobulin secretion for one day (columns in *B*) with the average cell density of two successive days.

by the increase in the cell density alone. The immunoglobulin secretion per cell per day was, then, obtained by dividing the secretion per day by the average of the cell densities of two successive days and plotted against time in Fig. 3*C* (open circles). This shows that the average secreting activity per cell increased during culture even though the secreting activity may not be uniform among cells. This curve closely resembles the time course of the increase in $I_{Ca\,max}$.

The change of the Ca current density showed slightly different patterns among different culture cycles. The variation appeared to be related to the pattern of the

cell proliferation, which varied slightly among different culture cycles. Fig. 4 illustrates a similar experiment to that just described when the culture started at higher cell density (46×10^4 cells/ml) in order to modify the cell proliferation time course. The cell density was already saturated by the second day (Fig. 4*A*). The immunoglobulin secretion for the third day was as high as that for the fourth day (Fig. 4*B*). The secretion per cell per day became maximum between the second and

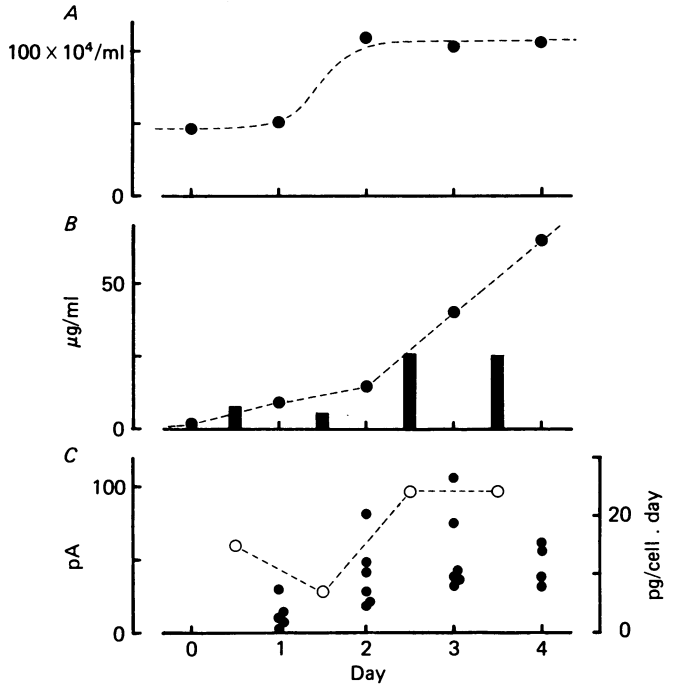

Fig. 4. The Ca current and the immunoglobulin secretion in hybridoma secreting IgG when the culture started at a relatively higher cell density. *A*, cell proliferation curve when the culture started at a cell density of 46×10^4 cells/ml. *B*, the immunoglobulin level in the supernatant (circles) and the immunoglobulin secretion per day (columns). *C*, the maximum observed Ca current (filled circles) and the immunoglobulin secretion per cell per day (open circles).

third day and then stayed almost constant. The secretion per cell per day again paralleled the change in $I_{\text{Ca max}}$. Somewhat larger maximum inward Ca currents appeared as early as the second day. The distribution of $I_{\text{Ca max}}$ showed almost no change between the third and fourth days. The rate of secretion per cell per day was larger the first day than the second day in both cases illustrated in Figs. 3 and 4. Measurements of cell density would be expected to contain larger relative errors at the period of low cell densities. However, we think that the high secreting activity the first day may be authentic, because the cell population at the start of the culture was essentially the same as that at the fourth day, when the high secreting activity was observed. From both examples (Figs. 3 and 4) we can conclude that the maximum Ca currents and the secreting activity change in parallel during the culture period.

DISCUSSION

The maximum amplitude of the peak inward Ca current varied widely from cell to cell in two hybridomas secreting IgG and IgM, as well as in S194 myeloma cells. The correlation between the Ca current and the culture cycle was obscure in S194 myeloma cells, because the majority of myeloma cells had only a trace of Ca current. The Ca current of hybridomas secreting IgG and IgM was larger than the Ca current of non-secreting S194 myeloma throughout the culture cycle, and the change in the Ca current showed a clear correlation with the culture cycle or the time after the cells were transferred into the fresh culture medium.

We have some speculations concerning the mechanism underlying the changes in the Ca current during the culture cycle. The cell density showed a sigmoidal proliferation curve and was saturated by the third and fourth days in cultures. At the saturation period of the cultures most cells may be arrested in a certain stage of the cell cycle due to lack of some nutrients in the culture medium. Therefore, it is likely that the higher density of Ca channels is linked to a certain blocked stage of the cell cycle which accumulates during the saturated period of the culture. It is also likely that immunoglobulin-secreting activity is high at this stage.

It is reported that calmodulin levels are elevated 2-fold at late G1 and/or early S phases during the growth cycle of CH0-K1 cells (Chafouleas, Bolton, Hidaka, Boyd & Means, 1982). Our culture was not synchronized and we do not have information about the distribution of the cell stages in the cell populations at each period of the culture. Further analyses are obviously necessary to elucidate the mechanism underlying the change of the Ca current during the culture cycle.

Parallel changes in the density of Ca current and the immunoglobulin secretion during the culture cycle are interesting. At the present time, however, it seems too early to draw any significant functional link between the two events. Obviously further studies are required. In spite of the lack of any positive conclusion we report this phenomenon since this may stimulate studies along this line. One way of further exploration is to determine the effects of specific Ca channel blockers such as D600 or nifedipine on the immunoglobulin secretion. Unfortunately the Ca channel of the hybridomas used here was rather insensitive to these agents. D600 at 10 μM had no observable effect on the Ca current, the cell proliferation or immunoglobulin secretion. At 100 μM the Ca current was reversibly reduced by about 37%. This concentration of D600 suppressed both cell proliferation and immunoglobulin secretion. Although the suppression of the latter was significantly greater, it is not strong evidence to support the link between the Ca channel and immunoglobulin secretion.

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