

Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes

(tetraphenylphosphonium/depolarization/hyperpolarization/concanavalin A/lipopolysaccharide)

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ABSTRACT By monitoring differences in accumulation of the lipophilic cation [³H]tetraphenylphosphonium in media containing low or high potassium concentrations [Lichtshtein, D., Kaback, H. R. & Blume, A. J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 650-654], the membrane potential of lymphocytes from various sources has been estimated. On the basis of this method, the potential of normal mouse spleen lymphocytes (T and B cells) is -65 ± 2 mV (mean \pm SEM, interior negative). During the course of mitogenic stimulation by concanavalin A, lipopolysaccharide, or fetal calf serum, the membrane potential of murine spleen lymphocytes changes systematically according to the following pattern: (i) early depolarization lasting 2-3 hr, (ii) repolarization over the next 7 hr, and (iii) a final hyperpolarization phase during the last 24-48 hr. During repolarization and hyperpolarization, moreover, there is a direct correlation between the membrane potential and DNA synthesis, as judged by [³H]thymidine incorporation. By using isolated T and B cells, it is observed that concanavalin A depolarizes T cells only, whereas lipopolysaccharide depolarizes B cells only. Thus, both mitogens exhibit the same specificity for depolarization as for mitogenic stimulation. On the basis of these observations, it is suggested that the transition of lymphocytes from a resting state to mitotic activity is initiated by depolarization of the plasma membrane.

There is a considerable body of evidence suggesting that mitogenic stimulation of lymphocytes is associated with changes in the flux of certain ions across the cell membrane (1-9). Furthermore, there is increasing interest in the notion that ion fluxes, changes in membrane potential ($\Delta\Psi$), or both play a role in regulating growth in eukaryotic cells in general (10-12). Because the $\Delta\Psi$ (interior negative) across the plasma membrane is clearly involved in the distribution of ions and other solutes across the cell membrane, the possibility arises that an important early event in mitogenic stimulation involves an alteration in $\Delta\Psi$.

This communication describes changes in the $\Delta\Psi$ of isolated lymphocytes during mitogenic stimulation as determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP⁺) (13, 14). The use of "lipophilic ions" to measure $\Delta\Psi$ across biological membranes was initiated by Skulachev and Liberman and their coworkers (15), and the synthesis of these compounds in radioactive form has extended their use considerably (13-29). Importantly, moreover, recent studies with neuroblastoma/glioma NG108-15 hybrid cells (13, 14), *Escherichia coli* giant cells (30), and chicken embryo heart cells[§] demonstrate that distribution studies with [³H]TPP⁺ yield $\Delta\Psi$ values that compare favorably with those obtained by using direct intracellular recording techniques.

Electrophysiologic measurement of $\Delta\Psi$ in lymphocytes is difficult because the cells are relatively small and because the

nucleus occupies most of the intracellular volume. Nevertheless, Taki (31), using intracellular microelectrodes with resting lymphocytes, recorded $\Delta\Psi$ values of -12 to -20 mV. More recently, Deutsch *et al.* (27) estimated the $\Delta\Psi$ of human peripheral lymphocytes from the distribution of [³H]triphenylmethylphosphonium (TPMP⁺) in the presence of tetraphenylboron⁻ and also from the distribution of [¹⁴C]thiocyanate (SCN⁻) and obtained values from -35 to -53 mV. Although it has been demonstrated that tetraphenylboron⁻ decreases the internal dipole potential of lipid bilayers, increasing cation permeation by an order of magnitude (32), the use of lipophilic anions and cations in conjunction can produce artifacts (19), and SCN⁻ distribution is relatively insensitive to changes in $\Delta\Psi$ (interior negative). In view of these considerations and because previous studies (13, 14, 30)[§] demonstrated that TPP⁺ distribution provides an excellent quantitative measure of $\Delta\Psi$ in at least three different cell types, this cation was used here.

MATERIALS AND METHODS

Materials. Lipopolysaccharide (LPS) (*E. coli* 055:B5) was purchased from Difco, concanavalin A (Con A) from Pharmacia (Uppsala, Sweden), and ³H₂O (5 mCi/g), [¹⁴C]inulin (11.3 mCi/mmol), and [³H]thymidine (2 Ci/mmol) from the Radiochemical Centre (Amersham, England) (1 Ci = 3.7×10^{10} becquerels). [³H]TPP⁺ (bromide salt; 2.5 Ci/mmol) was synthesized by the Isotope Synthesis Group of Hoffmann-La Roche, under the direction of Arnold Liebman. Unlabeled TPP⁺ was obtained from K & K Laboratories. All other materials were reagent grade obtained from commercial sources.

Mice. BALB/c, 6-8 weeks old, were obtained from the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland; BALB/c *nu/nu*, from Bomholtgaard, Denmark.

Cells. *Sheep lymphocytes*, obtained by cannulation of efferent lymph ducts in the intact animal, were provided by Iver Heron (33). *Human lymphocytes* were prepared from fresh blood by centrifugation over Ficoll (34). *Mouse spleen cells* were prepared as described by Schreier (35). Viability was determined by trypan blue exclusion and normally exceeded 90% after removal of erythrocytes by lysis with 0.8% NH₄Cl. *Mouse T cells* were prepared according to Julius *et al.* (36). *Lymphocytic cell lines* were graciously donated by the following individuals: thymoma EL-4 (C57BL/6) by H. von Boehmer; "K" (BALB/c, Abelson murine leukemia virus) (37), BM 18-4 (BALB/c bone marrow cells transformed by Abelson murine leukemia virus *in vitro*) (38), and ABL-1 (BALB/c cells

Abbreviations: $\Delta\Psi$, membrane potential; TPP⁺, tetraphenylphosphonium; TPMP⁺, triphenylmethylphosphonium; LPS, lipopolysaccharide; Con A, concanavalin A; FCS, fetal calf serum.

[§] L. J. Elsas, J. H. Krick, J. Serravezzo, and R. L. DeHaan, unpublished data.

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transformed by Abelson murine leukemia virus *in vivo* (39) by B. Weimann.

In all instances, cells were centrifuged and resuspended to a given concentration in RPMI 1640 medium containing 50 μ M 2-mercaptoethanol, 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) and 5% fetal calf serum ("low- K^+ medium") or in the same medium prepared with 102.7 mM KCl in place of 102.7 mM NaCl ("high- K^+ medium"). Where indicated, fetal calf serum (FCS) was omitted. Both media were 280 milliosmolar in salt and adjusted to pH 7.3. Cultures were incubated at 37°C in 95% air/5% CO₂.

[³H]TPP⁺ Distribution and Intracellular Volume. One milliliter of cells suspended in low- or high- K^+ medium was incubated at 37°C for 5 min and [³H]TPP⁺ (30 mCi/mmol) was added to a final concentration of 25 μ M (10 μ l of a 2.5 mM stock solution). At given times, 250 μ l of the cell suspension was pipetted into a micro reaction tube (400 μ l volume; Beckman 314320) containing 10 μ l of a solution of 10% NaCl and 0.7% Triton X-100, overlaid with 100 μ l of Versilube F-50 silicone fluid (General Electric, Waterford, NY). The tubes were then centrifuged at approximately 10,000 $\times g$ for 2.5 min in a Beckman Microfuge. After freezing of the tubes in dry ice, the tips were cut off, placed in Pico-Fluor-15 (Packard) or Insta-Gel (Packard), and assayed for radioactivity 12 hr later by liquid scintillation spectrometry.

The same methodology was used to determine intracellular volume with ³H₂O and [¹⁴C]inulin (4, 5). The external H₂O volume carried down with the cells did not exceed 5% of the total volume of the sample. Intracellular ³H₂O was shown to be a linear function of cell number. In addition, it was found that the average intracellular volume of a given cell population was about 10–15% higher in high- K^+ medium than in low- K^+ medium.

Calculation of $\Delta\Psi$ from TPP⁺ Distribution. The amount of [³H]TPP⁺ accumulated by the cells was converted into a concentration by using values for intracellular volume obtained as described above. This value was then used to calculate $\Delta\Psi$, using the Nernst equation as described by Lichtstein *et al.* (13, 14):

$$\Delta\Psi = \frac{2.3RT}{F} \log \frac{[\text{TPP}^+]_{\text{in}}^{\text{corrected}}}{[\text{TPP}^+]_{\text{out}}} \quad [1]$$

in which $2.3RT/F$ is equal to -61 mV at 37°C and $[\text{TPP}^+]_{\text{in}}^{\text{corrected}} = [\text{TPP}^+]_{\text{in}}^{\text{low-}K^+} - [\text{TPP}^+]_{\text{in}}^{\text{high-}K^+}$. In addition, in certain long-term experiments, a correction was made for the number of dead cells in the preparation by assuming that $\Delta\Psi$ in such cells is zero. The number of dead cells was estimated by trypan blue exclusion and never exceeded 15–30%.

RESULTS

TPP⁺ Distribution as a Probe for $\Delta\Psi$ in Lymphocytes. Splenic lymphocytes from BALB/c mice suspended in low- K^+ medium (i.e., 5.36 mM K^+ /102.7 mM Na^+) take up [³H]TPP⁺ rapidly for about 10 min and achieve a steady-state level of accumulation at approximately 20 min (Fig. 1). In contrast, when the cells are suspended in medium containing a high K^+ concentration (i.e., 102.7 mM K^+ /5.36 mM Na^+), the steady-state level of TPP⁺ accumulation is depressed by 50–60%. These results are similar to those obtained with neuroblastoma/glioma NG108-15 hybrid cells (13) and human peripheral lymphocytes (27) and indicate that the $\Delta\Psi$ across the plasma membrane of murine lymphocytes is also due substantially to a K^+ diffusion potential ($K^+_{\text{in}} \rightarrow K^+_{\text{out}}$).

As shown in Fig. 2, the steady-state level of TPP⁺ accumulation by lymphocytes suspended in medium of either low or high external K^+ concentration is linearly related to cell number

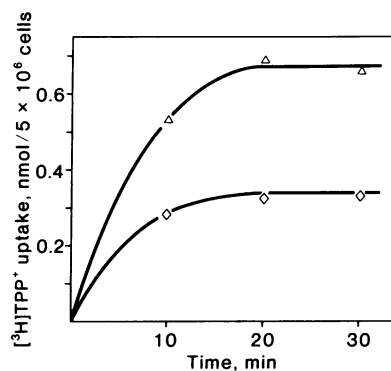


FIG. 1. Time course of [³H]TPP⁺ accumulation. Mouse (BALB/c) spleen lymphocytes (T and B cells) were centrifuged and resuspended in low- K^+ (Δ) or high- K^+ (\diamond) medium without FCS at concentrations of 5×10^6 cells per ml and assayed for [³H]TPP⁺ uptake at 37°C for the given times.

from about 2 to 5×10^6 cells per reaction mixture. Furthermore, the difference in TPP⁺ accumulation between cells suspended in low- and high- K^+ media is constant over this range of cell concentrations, as observed previously with NG108-15 neuroblastoma/glioma cells (13).

In order to calculate TPP⁺ concentration gradients (i.e., $[\text{TPP}^+]_{\text{in}}/[\text{TPP}^+]_{\text{out}}$), the intracellular volume under the experimental conditions utilized was determined (Table 1). Assuming that the $\Delta\Psi$ across the plasma membrane is negligible when the cells are suspended in high- K^+ medium (13), that component of the total accumulation due to the $\Delta\Psi$ across the plasma membrane can be approximated (i.e., $[\text{TPP}^+]_{\text{in}}^{\text{low-}K^+} - [\text{TPP}^+]_{\text{in}}^{\text{high-}K^+} = [\text{TPP}^+]_{\text{in}}^{\text{corrected}}$); and dividing by the external TPP⁺ concentration ($[\text{TPP}^+]_{\text{out}}$) yields a distribution ratio that can be inserted into the Nernst equation to calculate $\Delta\Psi$ (13, 14). Accordingly, the $\Delta\Psi$ of resting mouse lymphocytes is -65 ± 2 mV (mean \pm SEM), and essentially the same value is obtained for isolated T and B cells. Although different values are presented for thymomas, myelomas, and cell lines transformed by Abelson murine leukemia virus, these differences may reflect heterogeneity in the physiological state of the cells rather than true differences in $\Delta\Psi$ (i.e., differences in growth rates were not controlled).

Effect of Mitogenic Stimulation on $\Delta\Psi$. After mitogenic stimulation of mouse (BALB/c) spleen cells, marked systematic alterations in $\Delta\Psi$ are observed (Fig. 3). Thus, addition of LPS, FCS, or Con A results in depolarization between 1 and 4 hr after addition of mitogen. The cells then repolarize within the next 6–8 hr, and differentiation into blast cells over the last 24 hr is accompanied by hyperpolarization. Because these experiments

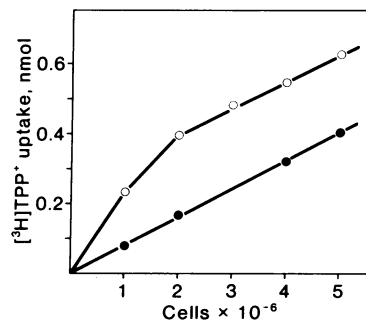


FIG. 2. Effect of cell number on [³H]TPP⁺ accumulation. Mouse (BALB/c) spleen lymphocytes (T and B cells) were centrifuged and resuspended in low- K^+ (\circ) or high- K^+ (\bullet) medium without FCS to given concentrations. [³H]TPP⁺ accumulation was assayed at 30 min.

Table 1. $\Delta\Psi$ of various lymphocyte populations as determined by TPP⁺ distribution

Lymphocytes	Source	Ref.	No. of expts.	Cell volume, $\mu\text{l}/10^6$ cells	$\Delta\Psi$, mV
Mouse					
BALB/c	Spleen (whole)	35	35	0.2–0.25	-65 ± 2
	Spleen (T cells)	36	10	0.21	-65 ± 2
BALB/c <i>nu/nu</i>	Spleen ("B" cells)	35	14	0.20	-66 ± 2
BALB/c	Spleen (small T/B)	35	2	0.18	-67
	(large T/B)		2	0.27	-74
LPS blast cells, after separation by bovine serum albumin gradient					
BALB/c	Spleen	40	5	0.70	-75 ± 4
Sheep	Efferent lymph	33	4	0.22	-67 ± 1
Human	Peripheral (T/B cells)	34	1	0.38	-67
Cell lines					
Thymoma EL4 C57BL/6			2	1.31	-50
Myeloma X63Ag8 BALB/c			2	1.31	-54
CTL clone C57BL/6			5	1.10	-63 ± 2
"K"		37	4	0.75	-56 ± 3
BM 18-4		38	4	0.47	-63 ± 4
ABLS-1		39	4	0.47	-71 ± 3

Cells were incubated in low- and high-K⁺ media at concentrations ranging from 2 to 5 × 10⁶ cells per ml. Samples were incubated at 37°C for 5 min, and [³H]TPP⁺ (30 mCi/mmol) was added to a final concentration of 25 mM. At 30 min, samples were assayed. Data are presented with the SEM (where applicable).

are performed with heterogeneous asynchronous populations in culture, it should be stressed that the $\Delta\Psi$ values obtained are not representative of any single cell, but reflect the average $\Delta\Psi$ of the population.

There is a direct relationship between the increase in $\Delta\Psi$

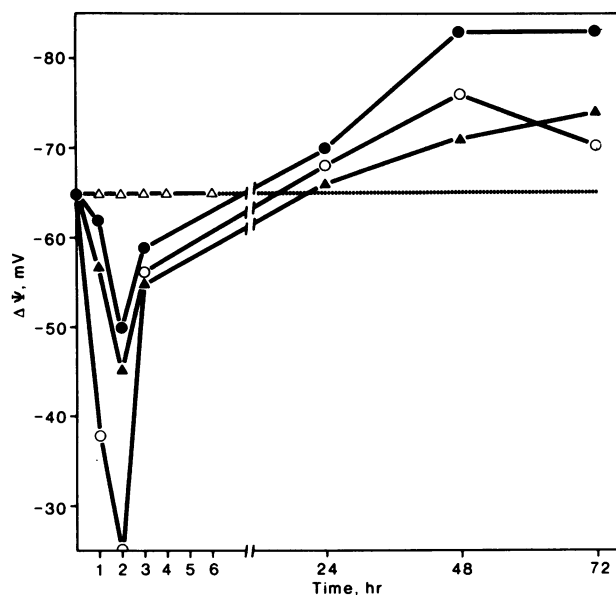


FIG. 3. Changes in $\Delta\Psi$ during mitogenic stimulation. Mouse (BALB/c) spleen lymphocytes were cultured at 37°C in an atmosphere of 95% air/5% CO₂ in the following media: Δ , RPMI 1640 without FCS; \bullet , RPMI 1640 containing 5% FCS; \blacktriangle , RPMI 1640 containing 5% FCS and LPS at 25 $\mu\text{g}/\text{ml}$; \circ , RPMI 1640 containing 5% FCS and Con A at 5 $\mu\text{g}/\text{ml}$. At given times, samples were removed, centrifuged, resuspended in low-K⁺ or high-K⁺ medium without FCS to a concentration of 2–5 × 10⁶ cells per ml, and assayed for [³H]TPP⁺ accumulation at 30 min. $\Delta\Psi$ was calculated from the differential accumulation of [³H]TPP⁺ in low- and high-K⁺ media. The data presented are the mean of at least three experiments, each of which was carried out in triplicate (standard deviation = 1–5%). The time for maximum depolarization (2 hr) was the average of at least six experiments (range, 0.5–4 hr).

observed during repolarization and hyperpolarization and the rate of DNA synthesis, as judged by [³H]thymidine incorporation (Fig. 4). However, the relationship between $\Delta\Psi$ and thymidine incorporation exhibits a different slope with FCS alone as opposed to FCS plus LPS or FCS plus Con A, indicating that the absolute increase in $\Delta\Psi$ is not directly responsible for the increased rate of DNA synthesis.

Mitogenic Specificity. The data presented in Fig. 5 provide a preliminary indication that mitogen-induced depolarization of T and B mouse lymphocytes is in accord with the accepted specificities of the corresponding mitogens (41). LPS stimulates and depolarizes B cells (Fig. 5B) but not T cells (Fig. 5A), whereas Con A stimulates and depolarizes T cells only. Because FCS seems to stimulate B cells only (data not shown) and all of the experiments must be done in the presence of FCS, Con A

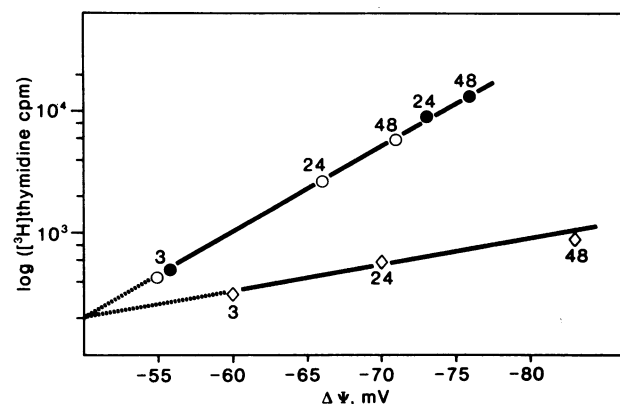


FIG. 4. Correlation between $\Delta\Psi$ and [³H]thymidine incorporation. Mouse (BALB/c) spleen lymphocytes were cultured as described for Fig. 3 in the following media: \circ , RPMI 1640 containing 5% FCS; \bullet , RPMI 1640 containing 5% FCS and LPS at 25 $\mu\text{g}/\text{ml}$; \circ , RPMI 1640 containing 5% FCS and Con A at 5 $\mu\text{g}/\text{ml}$. At the times (hr) noted on the figure, samples were removed, washed, and resuspended in low-K⁺ or high-K⁺ medium without FCS to a concentration of 2–5 × 10⁶ cells per ml, and assayed for [³H]TPP⁺ accumulation at 30 min. Parallel samples were assayed for [³H]thymidine incorporation after the addition of 1 μCi of [³H]thymidine per 10⁵ cells for 2 hr at 37°C.

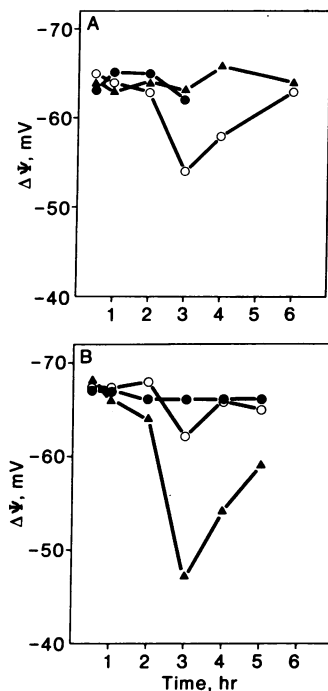


FIG. 5. Changes in $\Delta\Psi$ during mitogenic stimulation of T cells (A) and B cells (B). Mouse (BALB/c) T cells prepared according to Julius *et al.* (36) or B cells prepared from the spleens of BALB/c *nu/nu* mice were cultured as described for Fig. 3 in the following media: ●, RPMI 1640 without FCS; ○, RPMI 1640 containing 5% FCS and Con A at 5 $\mu\text{g}/\text{ml}$; ▲, RPMI 1640 containing 5% FCS and LPS at 25 $\mu\text{g}/\text{ml}$. At given times, samples were removed, centrifuged, resuspended in low- K^+ or high- K^+ medium without FCS to a concentration of $2\text{--}5 \times 10^6$ cells per ml, and assayed for $[^3\text{H}]\text{TPP}^+$ accumulation at 30 min. $\Delta\Psi$ was calculated from the differential accumulation of $[^3\text{H}]\text{TPP}^+$ in low- and high- K^+ media. The data presented are the average of two experiments carried out in triplicate (standard deviation = 1–5%).

probably causes the largest degree of depolarization in mixed populations (Fig. 3) because of the additive stimulatory effect of FCS on B cells.

The reason separated populations of T cells do not reach the same degree of depolarization as B cells presumably lies in the preparation and separation procedures used, which might render some of the cells insensitive to stimulation. It is also noteworthy that the time course of depolarization after addition of mitogens varies, starting immediately in some cases and up to 4 hr later in others. Finally, it is important that these experiments were performed with asynchronous lymphocyte populations, which could explain the relatively long time course of the depolarization phenomena.

DISCUSSION

In Jerne's "network theory" of the immune system (42), a "striking resemblance to the nervous system" is noted. Both systems receive and transmit signals that may be excitatory or inhibitory, and cells in both systems interact either directly or through chemical mediators. Both systems learn from experience and develop a memory that is not transmitted to the next generation. At the cellular level, nerve cells and lymphocytes are triggered by effectors that bind to specific receptors on the plasma membrane, and in both cell types activation is associated with changes in ion fluxes. As suggested by the experiments presented here, the analogy may be extended further, because it now appears that electrical events across the lymphocyte plasma membrane may play an important role in information transfer during initiation of the mitogenic response.

Judging from the differential distribution of $[^3\text{H}]\text{TPP}^+$ in low- and high- K^+ media, a biochemical method for estimating $\Delta\Psi$ that compares favorably with electrophysiological methods in at least two other eukaryotic cells (11, 12),⁸ mouse (BALB/c) spleen lymphocytes exhibit a "resting" $\Delta\Psi$ of -65 ± 2 mV. Although this value compares reasonably well with that obtained by Deutsch *et al.* (27), it is considerably more electro-negative than that reported by Taki (31), who used intracellular microelectrodes. On the other hand, Taki observed changes in $\Delta\Psi$ after mitogenic stimulation that are similar to those reported here. In both instances, addition of mitogens results sequentially in early depolarization (probably to close to 0 mV), followed by repolarization and hyperpolarization over longer periods of time. Although it seems likely that mitogen-induced depolarization represents an early event in the mitogenic response and that all cells capable of responding to LPS or Con A depolarize, this event is not sufficient to induce blast formation. In order to enter the subsequent phases of the response characterized by repolarization, hyperpolarization, and increased DNA synthesis, the cells apparently require a growth factor(s) that is produced by accessory cells, as shown by Larsson and Coutinho (43) for Con A stimulation of T cells. Experiments with I-A-depleted T-cell populations (H. Kiefer, unpublished results) demonstrate that the cells are depolarized by Con A but are incapable of initiating DNA synthesis without addition of growth factor. Thus, although the observations as a whole suggest that depolarization of the lymphocyte plasma membrane is an important early event in the mitogenic response, mitogen-induced depolarization, in itself, is insufficient for initiation of DNA synthesis and blastogenesis.

LPS and Con A bind to both T and B cells but stimulate one cell type or the other selectively (41). As shown here, the depolarizing effects of these mitogens exhibit similar specificities. That is, LPS depolarizes B cells, but not T cells, whereas Con A depolarizes T cells but not B cells. Clearly, therefore, the physiological response of a given cell to a particular mitogen probably involves specific interactions between the mitogen and a receptor that may be on the cell surface. Possibly, this interaction might lead to the opening of a Na^+ channel, causing depolarization, increased Na^+K^+ -ATPase activity, and enhanced K^+ influx (9), a mechanism similar to that suggested by Smith and Rozengurt (44) for the effect of FCS on 3T3 cells.

There is increasing evidence (10) that alterations in $\Delta\Psi$ play a role in the activation of other eukaryotic cells in addition to lymphocytes. In echinoderm eggs, for instance, Steinhart *et al.* (45) documented changes in $\Delta\Psi$ after fertilization that closely resemble the phenomena reported here. This similarity can also be extended to include early changes in intracellular pH (40, 46), because the average intracellular pH of mouse spleen lymphocytes exhibits a marked increase 2 hr after stimulation by Con A or LPS (D. Gerson and H. Kiefer, unpublished results). Finally, it has been suggested (12) that low $\Delta\Psi$ s are associated with high mitotic activity in somatic cells, and it should be interesting to investigate this correlation in cultured cells transformed with various carcinogens.

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