Intracellular *p*H of Mitogen-Stimulated Lymphocytes

Abstract. *Mitogenic stimulation of mouse lymphocytes results in two sequential intracellular alkalinizations. The first shift of intracellular pH from 7.18 to 7.35 coincides with early biochemical events following mitogenic stimulation. The second alkalinization begins 12 hours after stimulation and rises in parallel with the rate of thymidine incorporation. The results suggest that intracellular alkalinization following stimulation may play a key role in the enhancement of cellular activation and mitogenesis.*

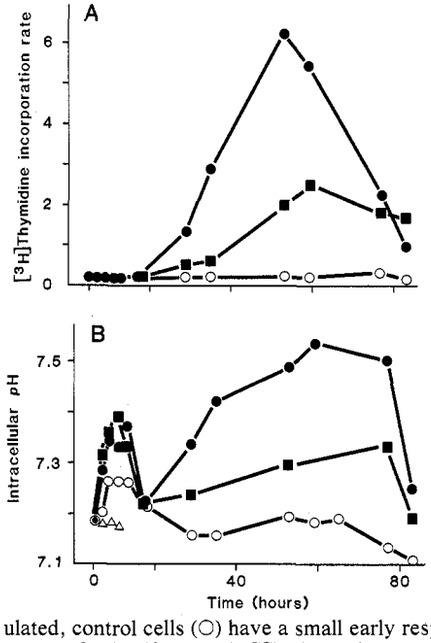
Lymphocytes remain in a quiescent state for long periods of time unless activated. This activation is the first step in an immune response. A large proportion of lymphocytes can be activated by polyclonal mitogens such as concanavalin A (Con A) or bacterial lipopolysaccharide (LPS). Since the time course after polyclonal stimulation is very well defined, mitogen-stimulated lymphocytes are well suited for the study of the biochemical correlates of both the early activation step and rapid proliferation.

In several types of cells, appropriate stimulation is followed by an intracellular alkalinization. This can happen when eggs are fertilized, spores germinate, cultured cells are diluted and re-fed, or secreting cells are triggered (*1*). These reports, and the observation that thymocyte membrane vesicles become alkaline after exposure to Con A (*2*), prompted us to study the intracellular *p*H of mitogenically stimulated mouse lymphocytes. We find that the intracellular *p*H of both B and T lymphocytes rises abruptly after mitogenic stimulation and reaches a maximum in 6 to 8 hours. This early transient intracellular alkalinization subsides in 12 hours and is followed by a second, protracted alkalinization, which reaches a maximum 48 hours after stimulation.

Because the small size of resting lymphocytes precludes the routine use of *p*H electrodes, we measured the intracellular *p*H of mitogen-stimulated lymphocytes by using the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO) (*1, 3*) labeled with ^{14}C. The method is based on a large differential in the permeability of the cell membrane to the ionized and associated forms of DMO; the associated form equilibrates across the cell membrane, and the ionized form is relatively impermeant. Thus, the total concentration of [^{14}C]DMO in the cell depends on the intracellular *p*H. The method has been described in detail (*1, 3, 4*), and the procedure is outlined in the legend to Fig. 1.

Splenic lymphocytes from BALB/c mice were treated with Con A (5 μg/ml) to stimulate T cells or with bacterial LPS (50 μg/ml) to stimulate B cells. Intracellular *p*H and the rate of [^{3}H]thymidine incorporation were measured periodically during the following 72 hours (Fig. 1). The intracellular *p*H of resting lymphocytes was 7.18, which was in good agreement with other measurements (*5*). After stimulation of either T or B cells in the spleen lymphocyte population, intracellular *p*H rises for 6 to 8 hours, declines to *p*H 7.2 by 12 hours, then rises again as mitotic activity begins, reaching the maximum 48 hours after stimulation. The rate of DNA synthesis—incorporation rate of [^{3}H]thymidine—is low for 24 hours; it rises sharply until about 48 hours after stimulation, and then declines. The maximum intracellular *p*H observed with LPS stimulation is always less than the maximum observed with Con A stimulation. This corresponds directly with the [^{3}H]thymidine incorporation rate per 10^6 live cells in the respective spleen cell cultures (Fig. 1A), suggesting that it is the dividing cells in the population which have a high intracellular *p*H (*4*).

The transient alkalinization between 0 and 12 hours in T lymphocytes stimulated with Con A coincides with the first stage of lymphocyte activation (*6*). During this stage, a number of biochemical changes occur, including phospholipid methylation and synthesis, RNA mobilization and synthesis, and the initiation of synthesis of T cell and B cell growth factors. Cell division, the second stage of activation, requires the presence of T cell growth factor. A similar process may occur in LPS-stimulated B lymphocytes (*7*). The slight alkalinization of unstimulated, control cells (○) have a small early response but no late response in the presence of 10 percent fetal calf serum (FCS); the early response of control cells is eliminated by reduction of the FCS concentration to 0.5 percent (△). Intracellular *p*H was determined by the [^{14}C]DMO-Microfuge method (*5, 6*). Cells were incubated with [^{14}C]DMO (6.0 μ*M*, New England Nuclear) for 30 minutes and, for each sample, uptake of $^{3}H_2O$ and [^{14}C]hydroxymethylinulin (0.14 m*M*, Amersham) were also measured to determine intracellular volume and extracellular space. In experiments with the [^{14}C]DMO technique, it was found that for trypan blue–admitting cells intracellular *p*H was the same as that of the growth medium, and a correction was made for the percentage of such cells (1 to 10 percent) in all experiments. Experiments with additional unlabeled DMO indicated that binding of DMO was low. The [^{14}C]DMO results are the average intracellular *p*H of the entire population of living cells. The values for times below 12 hours are the averages of 12 experimental determinations and those for times above 12 hours are the averages of 9 to 15 determinations. One standard error of the mean was between 0.02 and 0.04 *p*H unit.



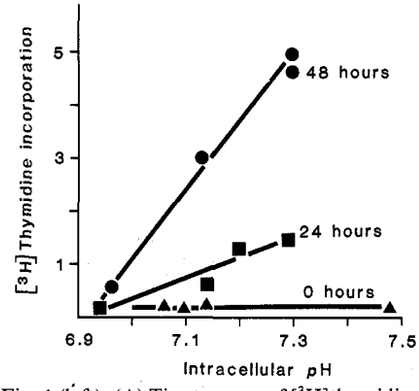


Fig. 1 (left). (A) Time course of [^{3}H]thymidine uptake during a 1-hour pulse of 4 n*M* [^{3}H]thymidine. (B) Time course of intracellular *p*H after mitogenic stimulation of mouse spleen lymphocytes with either Con A (●) or LPS (■). Initial cell concentrations were 1×10^6 and 5×10^5 per milliliter, respectively, in Iscove's modified Dulbecco's medium (Gibco 785220) supplemented with 10 m*M* Pipes, Hepes, and Tricine (Sigma, *p*H 7.30). Unstimulated, control cells (○) have a small early response but no late response in the presence of 10 percent fetal calf serum (FCS); the early response of control cells is eliminated by reduction of the FCS concentration to 0.5 percent (△). Intracellular *p*H was determined by the [^{14}C]DMO-Microfuge method (*5, 6*). Cells were incubated with [^{14}C]DMO (6.0 μ*M*, New England Nuclear) for 30 minutes and, for each sample, uptake of $^{3}H_2O$ and [^{14}C]hydroxymethylinulin (0.14 m*M*, Amersham) were also measured to determine intracellular volume and extracellular space. In experiments with the [^{14}C]DMO technique, it was found that for trypan blue–admitting cells intracellular *p*H was the same as that of the growth medium, and a correction was made for the percentage of such cells (1 to 10 percent) in all experiments. Experiments with additional unlabeled DMO indicated that binding of DMO was low. The [^{14}C]DMO results are the average intracellular *p*H of the entire population of living cells. The values for times below 12 hours are the averages of 12 experimental determinations and those for times above 12 hours are the averages of 9 to 15 determinations. One standard error of the mean was between 0.02 and 0.04 *p*H unit. Fig. 2 (right). Relation between [^{3}H]thymidine incorporation and intracellular *p*H in spleen lymphocytes stimulated with Con A. At the indicated times after addition of Con A, cells were transferred from medium of *p*H 7.3 to ones of *p*H 7.0 (●), *p*H 7.3 (■), or *p*H 7.9 (▲) for 2 hours. The cells were then returned to medium of *p*H 7.3 for measurement of intracellular *p*H (30 minutes) and [^{3}H]thymidine incorporation. Values are the averages of two experiments, each performed in triplicate. Qualitatively identical results were obtained when measurements were made at the *p*H used for incubation and when intracellular *p*H was altered with lipophilic acids or bases.

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lated spleen lymphocytes 2 to 6 hours after culturing was shown to be an effect of serum on B lymphocytes: a low serum concentration (0.5 percent) eliminates the effect (Fig. 1B); purified B lymphocytes show this effect, but purified T lymphocytes do not (*8*).

Other experiments demonstrated that intracellular alkalinization occurs only in the mitotically responding cells. Populations of spleen lymphocytes containing only functional B cells (that is, lymphocytes from nude mice or preparations in which T cells were removed by monoclonal α-θ antibody and complement) incorporated thymidine and became alkaline in response to LPS, but not to Con A. In purified T lymphocytes and the lymphocytes from C3H/HeJ mice, which are unresponsive to LPS (*9*), mitogenesis and alkalinization were found only after Con A administration (*4*). In all cases the time course and the magnitudes of the intracellular *p*H changes closely resembled the results shown in Fig. 1.

The rate of DNA synthesis is strongly correlated with intracellular *p*H from the time at which growth begins (12 to 24 hours after stimulation) until senescence of the cells (*4*). This correlation could arise either from independent increases in DNA synthesis and intracellular *p*H, from an effect of DNA synthesis on intracellular *p*H, or from an effect of intracellular *p*H on DNA synthesis. To distinguish between these possibilities, we need to manipulate intracellular *p*H and DNA synthesis independently.

To study the relation between intracellular *p*H and DNA synthesis rate, two types of experiments were performed: (i) 48-hour Con A blasts were incubated with inhibitors of DNA synthesis (1 or 5 m*M* hydroxyurea, 2.5 or 7.5 m*M* thymidine, or 50 μg of mitomycin C per milliliter) which reduced the rate of DNA synthesis by about 30-fold but did not alter the intracellular *p*H; and (ii) the intracellular *p*H of spleen lymphocytes was changed by transferring cells for 2 hours to fresh media with various *p*H values from 6.7 to 7.9. Each sample was then returned to a medium with *p*H 7.3 (to eliminate effects of growth medium *p*H on uptake rates) for the 1/2 hour needed for measuring both intracellular *p*H and [^{3}H]thymidine incorporation rate. As expected, the average intracellular *p*H of these cells, over the time of measurement, was shifted toward the *p*H of the incubation medium. The rate of DNA synthesis increased with intracellular *p*H in dividing populations (24 or 48 hours) but not in unstimulated populations (Fig. 2). Similar results were obtained either when the cells were not returned to the medium with *p*H 7.3 or when *p*H equilibrium across the cell membrane was altered with lipophilic weak acids or bases (*8*).

The kinetics of lymphocyte blastogenesis have allowed discrimination between alkalinizations associated with the biochemical stimulation of resting cells and with DNA synthesis. The early alkalinization follows the stimulation of resting cells and is associated with a burst of metabolite, RNA, and protein synthesis. As the cells enter the mitotic cycle there is a second alkalinization, which is strongly correlated with the rate of DNA synthesis. In both situations many kinase-dependent reactions occur, and for many of these, higher *p*H not only increases the rate of reaction but also shifts the equilibrium constant toward product formation.

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Inability of Oxytocin to Activate Pyruvate Dehydrogenase in the Brattleboro Rat

Abstract. *Oxytocin has insulin-like activity in that it stimulates lipogenesis and increases pyruvate dehydrogenase activity. However, in adipocytes from homozygous Brattleboro rats oxytocin is incapable of stimulating lipogenesis or pyruvate dehydrogenase activity, although insulin stimulation of both processes is normal and the antilipolytic activity of oxytocin is normal. Thus, the Brattleboro rat provides a new genetic model for the study of oxytocin action, wherein recognition of the chemical mediator is partially defective.*

When glucose enters rat epididymal adipocytes, it is immediately phosphorylated to glucose-6-phosphate (*1*). Subsequently, the sugar may be converted to glycogen or fatty acyl triglycerides, or it may be oxidized to CO_2 (*1*). The rate of formation of these metabolic products increases in the presence of insulin (*2*). Oxytocin, a neurohypophysial peptide structurally unrelated to insulin, has many insulin-like effects in isolated rat adipocytes (*3*). These effects are mediated through the binding of oxytocin to its own receptors on the plasma membrane (*4*).

In adipocytes isolated from the epididymal fat pads of rats homozygous for diabetes insipidus (Brattleboro rats) (*5*), the action of oxytocin [stimulation of glucose oxidation (*6*) or lipogenesis (*7*)] is abolished, whereas insulin-mediated stimulation of these activities is the same as in cells from control animals. Remarkably, in homozygous Brattleboro rats the oxytocin recognition site in adipocytes appears to be intact and the responsiveness of the uterus to oxytocin appears normal (*6*).

These observations suggest to us that the defect in the action of oxytocin in adipocytes from such rats is localized at a biochemical step subsequent to receptor binding but prior to the final biochemical steps involved in glucose oxidation and lipogenesis. Our interest has been focused on pyruvate dehydrogenase, a key lipogenic enzyme that also participates in the oxidation of glucose to CO_2. Insulin stimulates the activity of this enzyme.

We report (i) that oxytocin stimulates pyruvate dehydrogenase activity and lipogenesis in adipocytes from control animals but does not do so in adipocytes

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