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The Mitogenic Action of Bradykinin on Thymic Lymphocytes and its Dependence on Calcium* (33753)

A. D. PERRIS AND J. F. WHITFIELD
(Introduced by Helen J. Morton)
Division of Biology, National Research Council of Canada, Ottawa 7, Canada

The mitotic activity of bone marrow and thymus tissue is stimulated by increasing the level of ionized calcium in the plasma of the rat (1–3), and is in fact directly proportional to the ambient calcium level (4). It has also been found that changes in the mitotic activity of bone marrow and thymus parallel changes in the growth rate of the rat, and are accompanied by marked and parallel shifts in the level of ionized plasma calcium (5). Thus there appears to be a direct involvement of the calcium ion, and the hormones which govern its concentration, in the control of overall growth and mitotic activity in the rat.

Although generalized changes in mitotic activity may be controlled by shifts in plasma calcium concentrations, it is unlikely that localized increases in cell division are mediated in this way. In the proliferative response to injury, for example, a general increase in plasma calcium level would affect uninjured as well as the injured tissue. Nevertheless the calcium ion can still play a role in this response. The demonstration that detergents and polyamines increased mitosis in thymocyte suspensions by sensitizing the cells to the stimulatory action of calcium (6), led us to speculate that a similar calcium-dependent mechanism might operate in the mitotic response to injury. If some substance were released in damaged tissue which could act upon the cell membrane either to displace bound calcium or to increase its permeability to calcium, this ion might then initiate the mitotic stimulation needed for repair.

To test this hypothesis the nonapeptide bradykinin was selected as a possible sensitizing agent, since it is one of several kinins responsible for the vascular dilatation and permeability changes of the inflammatory response to injury (7–11). Suspensions of rat thymocytes maintained *in vitro* were used as the test system as they provide a well-controlled, highly reproducible, and rapid assay procedure for mitogenic activity. These cell populations are physiologically realistic since they exhibit the same degree of mitotic competence over a 6-hr period as they do *in vivo* (5, 6).

Methods. Thymuses were removed under ether anesthesia from male Sprague-Dawley rats (weighing between 120 and 170 g) and minced with fine scissors in a balanced glucose–salts medium containing 5.5 m*M* glucose, 120 m*M* NaCl, 5.0 m*M* KCl, 5.0 m*M*

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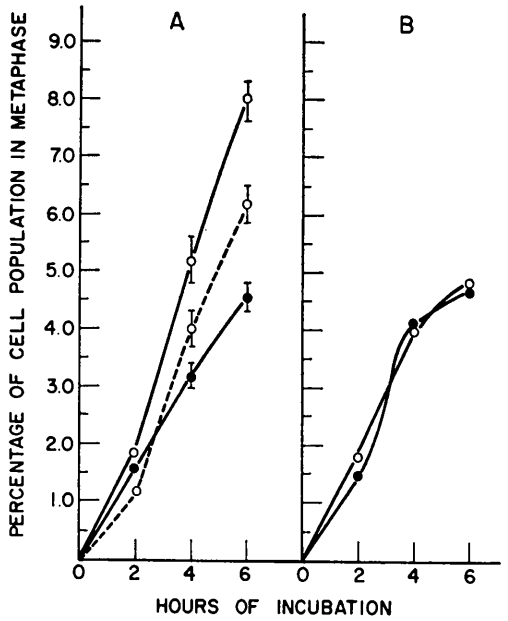

FIG. 1. Effect of bradykinin on mitotic activity of rat thymocytes: (A) Suspension cultures contain 0.6 m*M* $CaCl_2$; (B) Cultures contain 0 m*M* $CaCl_2$; (●), control suspensions; (○), 1.0 μM bradykinin; (○), 2.5 μM bradykinin; Values are means of at least 6 separate determinations ± SEM where relevant. In (A) the difference between the percentage of cells in metaphase at 6 hr in normal and bradykinin-treated (1.0 and 2.5 μM) populations is highly significant ($p < 0.001$).

Na_2HPO_4, 1.0 m*M* $MgSO_4$, 5.0 m*M* Tris (hydroxymethyl)aminomethane buffer, and $CaCl_2$ at different concentrations. All media were adjusted to pH 7.2 with HCl. The resultant cell suspension was filtered through four layers of cheesecloth and diluted with medium to give a final concentration of approximately 2×10^8 cells/ml. Bradykinin (Sigma Chemical Co., St. Louis, Mo.) was added to some of the suspensions to give a final concentration of either 1.0 or 2.5 μM. To determine the rate of entry of cells into mitosis, the metaphase blocking agent, colchicine, was present in all suspensions (0.062 m*M*) which were rotated about their long axes in stoppered tubes at 37°. Samples of the suspensions were removed at intervals, fixed in neutral formalin, and stained with hematoxylin. The percentage of metaphase cells in the population in each sample was determined by examining at least 1000 cells. Full details of the preparative procedures can be found elsewhere (12, 13).

Results. In the presence of 0.6 m*M* $CaCl_2$, thymocytes in control suspensions progressed steadily into mitosis and by 6 hr, 4.5% of the population had collected in metaphase (Fig. 1A). When bradykinin (2.5 μM) was also present in the medium a marked increase in the rate of mitosis was observed to occur between 2 and 6 hr and almost twice as many cells (8.0%) entered mitosis and reached metaphase during the same 6-hr period (Fig. 1A). A lower (1.0 μM) concentration of bradykinin also significantly stimulated mitosis, but it was less effective (Fig. 1A). The presence of calcium ions in the medium was necessary for the bradykinin-induced stimulation; in the absence of calcium no mitotic stimulation was observed (Fig. 1B). It should be noted that the omission of calcium from the medium did not depress the mitotic activity of control suspensions below that observed in 0.6 m*M* $CaCl_2$.

Although bradykinin cannot stimulate mitosis in the absence of calcium, increasing the calcium ion concentration alone can do so. When normal thymocytes were incubated in the presence of $CaCl_2$, a progressive increase in the rate of entry of cells into mitosis was observed when the concentration rose above 0.6 m*M*, the maximum accumulation of metaphase cells being obtained when the calcium concentration reached 2.5 m*M* (Fig. 2). When bradykinin (2.5 μM) was added to suspensions containing different concentrations of calcium, maximum rates of mitosis were obtained when the $CaCl_2$ concentration was only 0.6 m*M* (Fig. 2). Since bradykinin did not enhance mitotic activity in the absence of calcium it seems that the polypeptide altered the cell to make it more permeable, or more sensitive to external calcium.

To test further this mitotic susceptibility of bradykinin-treated thymocytes to external calcium, cells were first exposed to the polypeptide for 3 hrs in the absence of calcium and then transferred to a medium containing 0.6 m*M* $CaCl_2$. When calcium was finally placed in the medium (whether or not

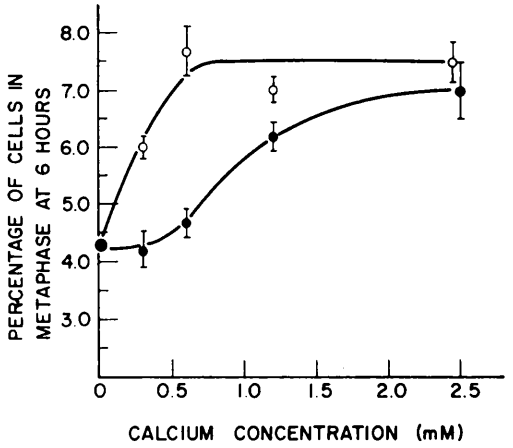

FIG. 2. Mitotic activity of rat thymocytes in presence of bradykinin and different calcium concentrations: (●), control suspensions; (○) suspensions contain 2.5 μM bradykinin; values are means of 4 separate experiments ± SEM.

bradykinin was removed or allowed to remain) a strong acceleration of the progression of the cells into mitosis was observed during the next 4-hr period (Fig. 3). If the cells had not been first exposed to bradykinin the subsequent exposure to 0.6 mM $CaCl_2$ did not cause this surge of mitosis to develop (Fig. 3). Since bradykinin did not have to be present at the same time as the calcium, prior exposure of the cells to the polypeptide must have sensitized the cells to the external calcium which was the actual effector of the mitotic response.

Discussion. Bradykinin is a member of a group of potent naturally occurring polypeptide kinins which cause vasodilatation, produce edema by increasing capillary permeability, stimulate the contraction of smooth muscle and induce pain (7–11). To this list of properties can now be added a calcium-dependent mitogenic activity. In fact bradykinin's mitogenic action and its ability to stimulate the contraction of smooth muscle could be different expressions of the same mechanism. The depolarization of the muscle cell membrane prior to contraction is associated with the release of membrane-bound calcium which then stimulates the myofilbrillar apparatus. The polypeptide neurohypophyseal hormones which like bradykinin stimulate smooth muscle (14, 15), and, as we have found (unpublished), increase mitotic activity of thymocytes, facilitate the access of the released calcium to the contractile machinery (14, 15). Since a sufficiently large increase in calcium concentration can itself stimulate mitotis, bradykinin presumably acts on the cell membrane of mitotically competent cells by facilitating the access of this ion to some calcium-sensitive mitotic process within the cell. The action of bradykinin thus parallels that of the polypeptide, parathyroid hormone, which has been shown to

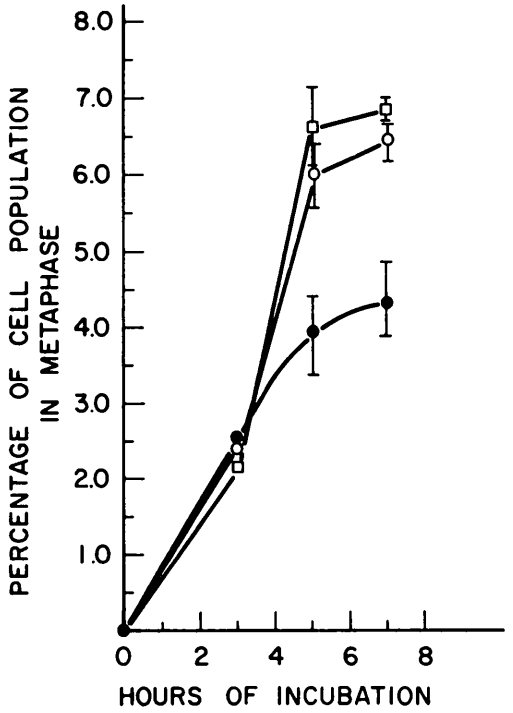

FIG. 3. Mitotic sensitization of rat thymocytes to calcium by prior exposure to bradykinin. During the first 3 hr of incubation at 37° cells were suspended in medium containing 0 mM calcium and then spun down at room temperature and resuspended in medium containing 0.6 mM $CaCl_2$ for the remaining 4 hr of incubation at 37°; (●), control suspensions with 0 μM bradykinin; (○), suspensions contain bradykinin (2.5μM) for full 7-hr incubation period; (□) suspensions contain bradykinin only for the first 3 hr of incubation; values are means of 4 separate experiments ± SEM. At 5 and 7 hr the difference between the percentage of cells in metaphase in normal and bradykinin-treated populations is highly significant ($p<0.01$).

affect the cell membrane, stimulate calcium uptake, and increase the multiplication of HeLa strain human cells (16, 17).

The increased accumulations of calcium and disturbed electrolyte movements observed in cells damaged or injured in a variety of ways (18–20) may thus be caused by the increased concentrations of kinins at sites of injury. The inflammatory response to injury, associated with the release of the kinins, extending over an area surrounding the damaged cells would thus stimulate the neighboring undamaged cells into mitotic activity and help restore the cell population to normal. Support for such a reparative function of kinins is provided by the observation that the enzyme kallikrein which releases kallidin, a decapeptide functionally similar to bradykinin (21, 22), stimulates the replacement of epithelial cells and thus promotes the healing of radiation-induced skin ulcers. Kallikrein also increases the cellularity of the bone marrow, in, and reduces the mortality of, irradiated rats (23, 24). Although these effects have been attributed to the increased supply of nutrients to the cells accompanying the vascular dilatation produced by the kallikrein (23), the present study indicates they may be due to a calcium-mediated mitogenic action of the bradykinin-like kallidin.

These experiments and previous observations (1–5) leave little doubt that calcium plays a central role in the control and induction of mitosis. However it is not yet clear how calcium might act at the subcellular level to produce such effects.

Summary. Bradykinin, a naturally occurring nonapeptide which causes an acute inflammatory response to injury, was found to be a potent, fast-acting mitotic stimulant for rat thymic lymphocytes suspended *in vitro*. This response was only obtained when calcium ions were also present in the suspension medium. It appears that bradykinin sensitizes cells to the environmental calcium and that calcium is the actual mitotic stimulator.

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