Cyclic AMP Mediation of Bradykinin-Induced Stimulation of Mitotic Activity and DNA Synthesis in Thymocytes[1] (34668)

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Bradykinin (a polypeptide) is rapidly released from certain proteins in damaged tissues and causes the edema, erythema, and pain which accompany injury (1–5). The recent demonstration that bradykinin stimulates mitotic activity in rat thymic lymphocyte (thymocyte) populations suspended *in vitro* (6) raises the possibility that this compound may also cause the postinjury stimulation of cell proliferation which is needed to repair the damaged tissues. Thus, bradykinin may be a mitogenic "wound hormone."

The mitogenic action of bradykinin is a calcium-dependent process. The kinin can interact with thymocytes in the absence of calcium and prepare them for mitotic stimulation but extracellular calcium is needed to actually set the stimulatory reaction in motion (6). This calcium-dependent mitogenic action of bradykinin closely resembles the identical mitogenic actions of growth hormone, parathyroid hormone, and vasopressin on rat thymocytes (7). These functionally (and chemically) different hormones are known to stimulate the formation of adenosine 3′5′-monophosphate (cyclic AMP) by the cells of their specific target tissues and cyclic nucleotide appears to be the common intracellular mediator of their diverse actions *in vivo* (8–12). Therefore, we suspected that cyclic AMP might also mediate the mitogenic action of bradykinin. Furthermore, if this cyclic nucleotide should be the intracellular mediator of the kinin's mitogenic action, this action must arise from a stimulation of the initiation of deoxyribonucleic acid (DNA) synthesis since cyclic AMP itself increases mitotic activity by stimulating DNA synthesis (13, 14).

Methods. Thymus tissue was removed from albino, male, specific-pathogen-free rats (bred in this laboratory) and thymocyte suspensions (containing 2×10^8 cells/ml) were prepared as previously described (7, 13). The suspended cells were incubated at 37° (while contained in roller tubes revolving at 40 rpm) in a complex tissue culture medium, MAC-1. MAC-1 consisted of the glucose–salts (GS) medium used in the previous study (6) plus all of the amino acids, vitamins, nitrogenous bases and supplementary growth factors contained in the tissue culture medium 199 (15). The GS medium contained 5.5 m*M* glucose, 5.0 m*M* KCl, 1.0 m*M* $MgSO_4$, 0.6 m*M* $CaCl_2$, 120 m*M* NaCl, 5.0 m*M* Na_2HPO_4, and 5.0 m*M* tris (hydroxymethyl)aminomethane (tris) buffer (pH 7.2). Medium 199 minus its salts and glucose was prepared for us by Difco Laboratories (Detroit) and we then combined this partial medium with the GS medium to produce MAC-1 medium.

Since only a small segment (up to a maximum of 20%) of a thymocyte population is capable of actively proliferating and rapidly responding to stimulation by mitogenic agents (16), the mitogenic capacity of bradykinin (bradykinin triacetate from Calbiochem, Los Angeles) cannot be accurately assessed from changes in the total cell concentration. However, when the MAC-1 medium contained 0.062 m*M* colchicine, cells of the proliferating subpopulation flowed into mitosis, but could not progress beyond metaphase. The rate of progression of these cells through their growth–division cycle into mitosis was then determined by simply plotting the prog-

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ressive accumulation in the population of cells in colchicine-metaphase. This was done by removing samples from the cell suspensions after various times of incubation, fixing the cells in neutral formalin and staining them with hematoxylin.

To determine whether bradykinin could affect DNA synthesis, cell populations were continuously exposed to ^{3}H-thymidine (2.0 μCi of radioactivity/ml of MAC-1 medium from labeled nucleoside with a specific activity of 15 Ci/mmole) during the first 3 hr after their suspension *in vitro*. At various times during incubation, cells were fixed by adding formaldehyde (to a final concentration of 6.6%) to a sample of the cell suspension. After a 30-min exposure to formalin, the cells were washed twice with a cold (4°) solution of 10 m*M* unlabeled thymidine in distilled water. This washing procedure combined with the formalin fixation ensured the removal of any labeled thymidine not incorporated into DNA (17). The washed cells were mounted on slides, covered with a film of Kodak nuclear track emulsion NTB-2 (Kodak, Rochester) and then stored for 48 hr. The autoradiographs were then developed and the cells underlying the emulsion were stained with nuclear fast red (18). Since treatment of the formalin-fixed cells with deoxyribonuclease completely removed cellular radioactivity, the ability of a cell to become labeled under these conditions was a valid indication that the ^{3}H-thymidine had become incorporated into DNA which, in turn, indicated that the cell must have been synthesizing DNA.

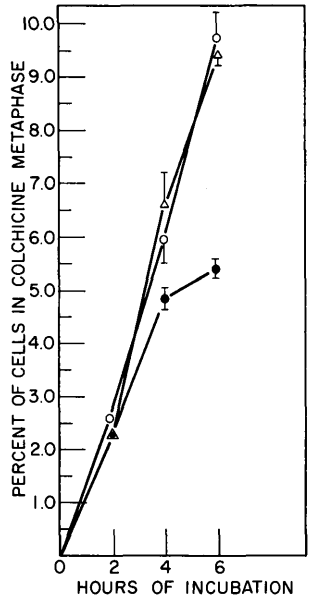

FIG. 1. Stimulation of the progression of rat thymocytes into mitosis by bradykinin. (●), thymocytes suspended in normal MAC-1 medium; (○), 1.0 μ*M* bradykinin; (△), 4.0 μ*M* bradykinin. Values are means of 4 to 12 experiments ± SEM.

Results and Discussion. When thymocyte populations were suspended in colchicine-

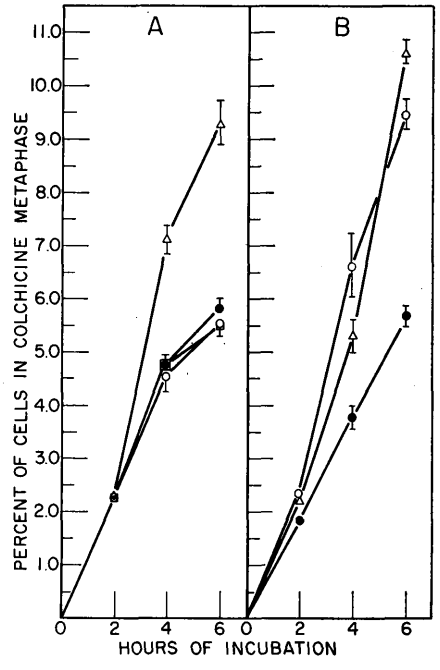

FIG. 2. A. Potentiation of the mitogenic action of bradykinin by caffeine: (□), cells suspended in normal MAC-1 medium; (●), cells suspended in medium containing 0.5 μ*M* bradkykinin; (○), thymocytes suspended in medium containing 0.4 m*M* caffeine; (△), cells suspended in medium containing 0.5 μ*M* bradykinin and 0.4 m*M* caffeine. The values are means of 12 experiments ± SEM. (B) Inhibition of the mitogenic action of bradykinin by imidazole: (○), thymocytes suspended in MAC-1 medium containing 4.0 μ*M* bradykinin; (●), cells suspended in medium containing 4.0 μ*M* bradykinin and 4.0 m*M* imidazole; (△), cells suspended in medium containing 4.0 μ*M* bradykinin, 4.0 m*M* imidazole and 0.4 m*M* caffeine. The values are the means of 4 experiments ± SEM.

containing MAC-1 medium, cells of the proliferating subpopulation continued to flow into mitosis and accumulate at metaphase (Figs. 1 and 2). Exposure of thymocyte populations to bradykinin concentrations as high as 0.5 μM did not affect the flow of cells into mitosis (Fig. 2A), but increasing the kinin concentration to 1.0 μM strongly (and maximally) stimulated this flow (Fig. 1). The stimulatory effect of the kinin did not become immediately evident; the stimulated cells began to arrive at metaphase only after 2 hr of incubation in bradykinin-containing medium (Fig. 1 and 2).

If the mitogenic action of bradykinin is mediated by cyclic AMP, it should be potentiated by caffeine and inhibited by imidazole. Caffeine inhibits the action of the cyclic AMP-degrading enzyme, phosphodiesterase, and thereby increases the effectiveness of hormones which stimulate cyclic AMP formation by raising the net intracellular yield of the cyclic nucleotide (12). Imidazole, on the other hand, increases the activity of the phosphodiesterase and thereby lowers the net yield of cyclic AMP. However, before an inhibitory effect of imidazole on the kinin's mitogenicity can be assumed to indicate an involvement of cyclic AMP, it must be shown that this inhibitory effect is suppressed by caffeine.

When thymocyte populations were suspended in MAC-1 medium containing *either* 0.5 μM bradykinin, or 0.4 mM caffeine, the flow of cells into mitosis was the same was it was in normal (untreated) populations (Fig. 2A). Caffeine (0.4 mM) and the kinin (0.5 μM) acted synergistically and the flow of cells into mitosis was then maximally stimulated (Fig. 2A). Imidazole (at a concentration of 4.0 mM) totally blocked the normally maximum stimulatory action of 4.0 μM bradykinin (Fig. 2B). However, caffeine (0.4 mM) completely suppressed the inhibitory action of imidazole and the kinin was again able to maximally stimulate mitotic activity (Fig. 2B). Thus, it appears that cyclic AMP mediates the mitotically stimulatory action of bradykinin. This conclusion is further supported by the fact that a low concentration (0.1 μM) of cyclic AMP precisely mimics bradykinin action by causing an increased flow of thymocytes into mitosis which begins only after 2 hr of exposure to the cyclic nucleotide (13, 14).

In view of the rather short period of time required for the bradykinin-stimulated cells to reach metaphase, it would seem unlikely that the mitogenic effect of the kinin could be due to a stimulation of the initiation of DNA synthesis. However, such a consideration does not apply to the present situation since proliferating thymocytes (and other lymphoblasts) have very short (5 to 7 hr) generation times (19–22). To determine whether bradykinin affects the flow of cells into the DNA-synthetic (S) phase of the cell cycle, freshly isolated thymocyte populations were continuously exposed to ^{3}H-thymidine (2.0 μCi/ml of medium) in colchicine-containing MAC-1 medium. The inclusion of colchicine in the medium would prevent any spurious increase in the proportion of DNA-synthesizing cells in the total cell population caused by the division of labeled (but not currently synthesizing DNA) members of the proliferating subpopulation. Furthermore, the starting population of G2 cells also could not complete their division and ultimately reach the S phase. Therefore, under these conditions, it would be expected that the proportion of labeled cells in normal populations would reach a certain maximum value when the flow of G1 cells into S is complete; in normal populations this maximum value was between 10 and 12% (16). Any bradykinin-induced increase in the proportion of labeled cells must arise from a stimulation of the entry of some G1 cells into the S phase which normally cannot enter this phase (or do so only very slowly). It is also possible that such an increase could arise from the stimulation of resting (or G0) cells to enter directly into the S phase.

Exposure of thymocyte populations to maximally mitogenic concentrations of bradykinin (1.0 and 4.0 μM) nearly doubled the proportion of cells which became labeled during the first hour of incubation in medium containing ^{3}H-thymidine (Fig. 3A). On the other hand, 0.5 μM bradykinin which did not affect mitotic activity (Fig. 2A) also did not

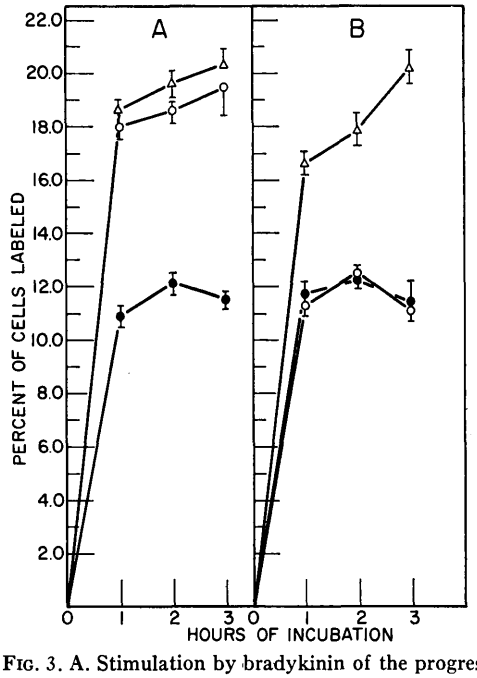

FIG. 3. A. Stimulation by bradykinin of the progression of thymocytes into the DNA-synthetic phase of the cell cycle. The cells were continuously incubated in MAC-1 medium containing colchicine and 2.0 μCi of ^{3}H-thymidine/ml: (●), untreated thymocytes; (○), 1.0 μM bradykinin; (△), 4.0 μM bradykinin. (B) Potentiation by caffeine of bradykinin's ability to stimulate the flow of thymocytes into the DNA-synthetic phase of the cell cycle: (●), thymocytes exposed to 0.5 μM bradykinin; (○), cells exposed to 0.4 mM caffeine; (△), cells exposed to 0.5 μM bradykinin and 0.4 mM caffeine. The values are means of 5 to 12 experiments ± SEM.

affect the progression of cells into the DNA-synthetic (S) phase of the cell cycle (Fig. 3B). However, as was the case with mitotic stimulation, caffeine (0.4 mM) potentiated the action of this normally ineffective level of bradykinin on DNA synthesis and the flow of cells into the S phase was then strongly stimulated (Fig. 3B). Thus, the kinin-induced stimulation of DNA synthesis is also mediated by cyclic AMP.

We conclude that the mitogenic action of bradykinin on rat thymocytes is mediated by cyclic AMP. The cyclic AMP-mediated reaction which increases the flow of the cells into mitosis involves a stimulation of the initiation of DNA synthesis. The magnitude and rapidity of this stimulatory effect of bradykinin on the entry of cells into the DNA-synthetic (S) phase suggests that it might principally affect a group of G1 cells in the actively proliferating or "cycling" subpopulation which are poised on the threshold of the S phase and normally either cannot enter this phase or do so only very slowly. Alternatively, the polypeptide may promote the rapid entry of some "noncycling" (G0) cells directly into the S phase. The biochemical mechanism by which bradykinin (through the action of its intracellular agent cyclic AMP) affects DNA synthesis is unknown.

Summary. Bradykinin increases the progression of rat thymocytes (suspended *in vitro*) into mitosis by a cyclic AMP-mediated reaction which stimulates the initiation of deoxyribonucleic acid synthesis.

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