Bradykinin-Induced Cyclic AMP Accumulation in Mouse Fibrosarcoma Independent of Prostaglandin E2 Formation

Masafumi FUJIMOTO and Tadashi OKABAYASHI
Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan
Accepted March 6, 1986

Abstract—The relationship between bradykinin (BK)-induced prostaglandin E2 (PGE2) and cyclic AMP syntheses in mouse fibrosarcoma cells (HSDM1C1) was investigated. Maximal BK-induced increases in cyclic AMP preceded increases in PGE2 production. PGE2 synthesis reached maximum at a much lower concentration of BK than cyclic AMP synthesis. Indomethacin completely inhibited BK-induced PGE2 production, but did not influence the cyclic AMP levels. Arachidonic acid in the medium induced PGE2 production in large quantities, but increased cyclic AMP accumulation only slightly. A high PGE2 concentration increased cyclic AMP levels only slightly. Theophylline increased basal and BK-mediated cyclic AMP levels, but did not affect PGE2 production at all. These results indicate that BK-evoked PGE2 and cyclic AMP syntheses in HSDM1C1 are not dependent upon each other.

Bradykinin (BK) can reproduce the basic symptoms of inflammation, such as increased vascular permeability, vasodilation, and production of pain, in most animal species (1). Most, if not all, of such biological effects of BK are likely to depend partially, but not totally, upon the BK-induced production and release of stimulating prostaglandins (PG's), because indomethacin, an inhibitor of PG synthesis, substantially inhibits these BK effects (1, 2). Furthermore, it has been reported that BK and PG’s exert synergistic effects on various biological systems (1–3). Such partial participation of PG’s on the BK-induced effects make it difficult to elucidate the BK actions which are independent of PG synthesis. Our studies were started to search for a BK-induced cell response independent of PG synthesis.

Many functionally and chemically different hormones and neurotransmitters are known to stimulate the formation of cyclic AMP by cells in their specific target tissues, and the cyclic nucleotide appears to be the common intracellular mediator of their diverse actions in vivo (4). BK also has been reported to increase the cyclic AMP contents in several tissues and cultured cells (5–9). In most cases, it has been suggested that the BK effect on cyclic AMP accumulation results from the production of PG’s which then activate adenylate cyclase (6, 7, 9). In this study, we examined the effects of BK on PGE2 and cyclic AMP syntheses in mouse fibrosarcoma cells (HSDM1C1) (10), which are known to release PGE2 in response to BK (11–13), to clarify whether BK-induced cyclic AMP accumulation is independent of BK-induced PGE2 synthesis.

Materials and Methods

Materials: HSDM1C1 cells were obtained from the American Type Culture Collection. Ham’s F-10 medium and fetal calf serum were purchased from Flow Laboratories, Inc., U.S.A.; horse serum from Gibco Laboratories, Grand Island, NY; and Medium 199 from Nissui Seiyaku Co., Ltd., Tokyo, Japan; PGE2, arachidonic acid, trypsin (crystallized and lyophylized), and EDTA were obtained from Sigma Chemical Co.; BK from Peptide Institute, Inc., Osaka, Japan; kanamycin sulfate from Meiji Seika Co., Tokyo, Japan; and 4-(2-hydroxyethyl)-1-piperazineethan-
sulfonic acid (HEPES) from Calbiochem., La Jolla, CA. [2,3-Proryl-3,4-3H(N)] BK (54.9 Ci/mmol) and [8-3H]cyclic AMP (26 Ci/mmol) were obtained from New England Nuclear, Boston, MA, and The Radiochemical Centre Amersham, respectively.

Cell cultures: HSDM1C1 cells were grown in monolayers in 250-ml flasks (Falcon) in 20 ml Ham's F-10 medium supplemented with horse serum (15%), fetal bovine serum (2.5%) and kanamycin (100 μg/ml) in an atmosphere of 5% CO2 and 95% air as described by Schremmer et al. (11). Cells were removed from flasks by brief (about 20 sec) treatment with 0.16% trypsin and 0.016% EDTA Na2 in phosphate-buffered saline. Subcultures in plastic tissue culture cluster dishes (22.6-mm I.D., Costar) were initiated with about 2×104 cells in 2 ml of growth medium and incubated at 37°C in a water-jacketed CO2 gas incubator (WJ-11, Hirasawa, Japan). On the 3rd day when cells were confluent, the growth medium was changed to HEPES-buffered serum-free Medium 199 prior to the experiments. Although the cell density of different subcultures varied (2-4×105 cells/well), the variation in a single experiment was <10%.

Production and assay of cyclic AMP and PGE2: After removal of the growth medium, the cells were washed with HEPES-buffered serum-free Medium 199 and then incubated for 15 min at 37°C in 1 ml of the same medium with or without indomethacin or theophylline. BK, arachidonic acid and PGE2 were then added. After further incubation as indicated, 0.2 ml of the medium was removed to measure the PGE2 contents using an [3H]PGE radioimmunoassay kit (Clinical Assays, Division of Travenol Laboratories, Inc., Cambridge, MA), and 0.1 ml of 50% trichloroacetic acid was quickly added to each well. Cyclic AMP was isolated from the cells and measured as described previously (14, 15).

Results
The time courses of basal and BK-stimulated cyclic AMP and PGE2 syntheses are shown in Fig. 1. BK-mediated PGE2 synthesis proceeded linearly for 1 min, and the maximal level lasted for at least 5 min. By contrast, BK-stimulated cyclic AMP accumulation was maximal at 30 sec and then decreased quickly to near the basal level. Figure 2 shows the concentration-response curves of BK measured at 30 sec after BK addition. PGE2 accumulation was maximal at 1 nM, while cyclic AMP accumulation was not maximal up to 10 μM. Indomethacin was used to assess the effect of inhibition of PG production on cyclic AMP synthesis (Fig. 3). At 100 nM, indomethacin completely blocked

![Fig. 1](image_url)
PGE₂ synthesis, but did not significantly alter either the basal cyclic AMP content or its BK-induced increase. At 1 μM, this drug decreased both basal and BK-stimulated cyclic AMP contents. Arachidonic acid (1 μg/ml), a precursor of PG's, caused release of a larger amount of PGE₂ than BK (1 μM), while it gave rise to a smaller increase in the cyclic AMP level than BK (1 μM) (Fig. 4). Exogenous PGE₂, at 1 μg/ml, which was 100–300 times more than that released in response to BK, increased the cyclic AMP content of HSDMC₁ cells by only 20% (Fig. 5), while BK (1 μM) increased it about 70% (Fig. 1). Theophylline (5 mM), an inhibitor of cyclic AMP phosphodiesterase.

**Fig. 2.** Dose-response curves for BK stimulation of PGE₂ release and cyclic AMP content. Cells were preincubated for 15 min at 37°C and subsequently incubated with (●) or without (○) BK for 30 sec at 37°C. Each point and vertical line represent the mean±S.E.M. of three wells.

**Fig. 3.** Effect of indomethacin on BK stimulation of PGE₂ release and cyclic AMP content. Cells were preincubated with (●, ▲) or without (○, △) indomethacin twice for 15 min at 37°C with an intermediate medium change and subsequently incubated with (▲, △) or without (●, ○) 1 μM BK for 30 sec at 37°C. Each point and vertical line represent the mean±S.E.M. of three wells.

**Fig. 4.** Time course of the effects of arachidonic acid on PGE₂ release and cyclic AMP content. Cells were preincubated for 15 min at 37°C and subsequently incubated with (●) or without (○) 1 μg/ml arachidonic acid for the indicated times at 37°C. Each point and vertical line represent the mean±S.E.M. of three wells.
increased both basal and BK-stimulated cyclic AMP accumulations, but did not affect the release of PGE₂ at all (Fig. 6).

![Graph](image)

**Fig. 5.** Time course of the effect of PGE₂ on cyclic AMP content. Cells were preincubated for 15 min at 37°C and subsequently incubated with (●) or without (○) 1 μg/ml PGE₂ for the indicated times at 37°C. Each point and vertical line represent the mean±S.E.M. of three wells.

![Graph](image)

**Fig. 6.** Effect of theophylline (5 mM) on BK stimulation of PGE₂ release and cyclic AMP content. Cells were incubated with (●) or without (○) theophylline (5 mM) for 15 min at 37°C and then challenged with 1 μM BK for the indicated times at 37°C. Each point and vertical line represent the mean±S.E.M. of three wells.

**Discussion**

Our results indicated that BK-stimulated synthesis of cyclic AMP in HSDM₁C₁ cells is not dependent upon BK-evoked PGE₂ production, because 1) cyclic AMP synthesis preceded PGE₂ synthesis, 2) BK-dependent cyclic AMP accumulation was not maximal at 1 μM, at which concentration PGE₂ synthesis reached the maximum, 3) indomethacin completely inhibited BK-induced PGE₂ synthesis at a concentration at which cyclic AMP synthesis was not affected, 4) arachidonic acid, compared with BK, led to the release of a larger amount of PGE₂ but the synthesis of a smaller amount of cyclic AMP, and 5) even a much higher concentration of PGE₂ than that released by the action of BK gave rise to a smaller accumulation of cyclic AMP than induced by BK. On the other hand, cyclic AMP did not seem to be involved in the synthesis of PGE₂, since theophylline stimulated both basal and BK-induced cyclic AMP syntheses but did not affect PGE₂ synthesis at all.

Unlike our present results with HSDM₁C₁ cells, many investigators have reported that BK-induced cyclic AMP response occurs through BK-induced PGE₂ production in several kinds of cells (6, 7, 9). One possible reason for this discrepancy is the difference in cell preparations. The other systems might have contained cell populations which were much more sensitive to PGE₂ than PGE₂-releasing cells, because lung tissues or uncloned fibroblasts were used. Another possibility is that previous studies used much higher concentrations of indomethacin (10–15 μM) than required to inhibit PG synthesis, which may have also caused inhibition of BK-induced cyclic AMP accumulation by a mechanism independent of the PG synthesis inhibition. Becherer et al. reported that the cyclic AMP level was not related to the desensitization of BK-mediated PG synthesis in HSDM₁C₁, but did not refer to the relationship between cyclic AMP and PG syntheses (13).

Recently, Zenser et al. demonstrated that BK-dependent syntheses of cyclic GMP and PGE₂ in rabbit renal inner medulla slices are not dependent of each other (16).
transient increase in cyclic AMP content in HSDM₁C₁ cells also may be a good model system for studying the PG-independent BK response. Our preliminary binding studies at 4°C suggested the existence of multiple binding sites which exhibit different affinities for BK, since inhibition of [³H]BK binding to HSDM₁C₁ cells by unlabelled BK showed a shallow curve between 0.1 nM and 1 μM. The high- and low-affinity binding sites for BK may mediate PGE₂ and cyclic AMP syntheses, respectively. It is still unknown at the present whether BK-induced PG-independent cyclic AMP synthesis is due to direct activation of the BK-coupled adenylate cyclase system, to a calcium-dependent mechanism as reported with isolated rat thymocytes (5, 17), or to other mechanisms.

References
13 Becherer, P.R., Mertz, L.F. and Baenziger, N.L.: Regulation of prostaglandin synthesis mediated by thrombin and B₂ bradykinin receptors in a fibrosarcoma cell line. Cell 30, 243–251 (1982)