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Biochemistry

Bradykinin stimulates phospholipid methylation, calcium influx, prostaglandin formation, and cAMP accumulation in human fibroblasts

(arachidonic acid/methyltransferase inhibition/phospholipase inhibition)

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ABSTRACT **The biochemical events that lead to bradykinin stimulation of cAMP accumulation in human fibroblasts were examined. Treatment of human fibroblasts with bradykinin increases phospholipid methylation, Ca^{2+} influx, arachidonic acid release, prostaglandin formation, and cAMP content. The dose–response curves of bradykinin for the increase in the above changes were similar. In human fibroblasts, exogenous arachidonic acid was mainly incorporated into phosphatidylcholine, followed by phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol. Bradykinin caused a release of arachidonic acid from methylated phospholipids (phosphatidylcholine) and phosphatidylinositol. 3-Deazaadenosine, a methyltransferase inhibitor, almost completely inhibited bradykinin-stimulated phospholipid methylation and Ca^{2+} influx and partially reduced arachidonic acid release and prostaglandin formation but had no effect on cAMP formation. Mepacrine, a phospholipase inhibitor, blocked bradykinin-induced arachidonic acid release, prostaglandin release, and cAMP accumulation. Indomethacin, a cyclooxygenase inhibitor, blocked the effect of bradykinin on cAMP accumulation. Prostaglandins E_1 and E_2, but not $F_{2\alpha}$, increased accumulation of cAMP. These observations indicate that bradykinin generates cAMP via arachidonic acid release and subsequent formation of prostaglandins. Our findings suggest that arachidonic acid can arise from either phosphatidylcholine synthesized by the methylation pathway or phosphatidylinositol.**

Bradykinin increases cAMP content in several tissues and cultured fibroblasts (1). The peptide does not directly activate adenylate cyclase but stimulates a cascade of events involving phospholipid turnover, release of arachidonic acid, and production of prostaglandins, which then activate adenylate cyclase (2–4). Previous work (5, 6) suggested that interaction of several types of effectors with their cell surface receptors initiates a train of events including phospholipid methylation and leading to enhanced Ca^{2+} influx and release of arachidonic acid from methylated phospholipids and subsequent metabolism of free arachidonic acid by lipoxygenase and cyclooxygenase pathways. We examined whether a similar series of events might be involved in bradykinin-induced changes in cAMP content of human fibroblasts. In addition, the sequence of events between the interaction of bradykinin with its receptor and stimulation of adenylate cyclase were studied. We found that bradykinin produces increases in phospholipid methylation, calcium flux, release of arachidonic acid, prostaglandin formation, and cellular accumulation of cAMP and a decrease in the amount of arachidonic acid in phosphatidylinositol. Inhibition of bradykinin-induced changes in phospholipid methylation and Ca^{2+} flux partially blocked the effects of this peptide on arachidonic acid release and prostaglandin production but did not decrease cAMP accumulation.

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METHODS

Cell Cultures. Human foreskin fibroblasts (passages 6–12) were grown in monolayer in 250-ml flasks in Eagle's basal medium supplemented with Earle's salts/10% fetal calf serum/2 mM glutamine as described (7). Cells were removed from flasks by a brief (<3 min) treatment with 0.125% trypsin in Dulbecco's Ca^{2+},Mg^{2+}-free phosphate-buffered saline. Subcultures in 35-mm wells were initiated with 6–7 × 10^4 cells in 2 ml of growth medium and incubated at 37°C in a Forma Scientific water-jacketed CO_2 incubator (Marietta, OH) with 95% air/5% CO_2 and 97% humidity. Medium was changed on the 4th day. Experiments were carried out on day 6 or day 7, when cells were confluent.

cAMP Determination. After removal of growth medium, cells were washed twice with Hanks' medium and then incubated for 30 min at 37°C in 2 ml of Hanks' medium containing drugs. Bradykinin or prostaglandin was then added and, after further incubation as indicated, the medium was quickly aspirated; 1 ml of 5% trichloroacetic acid was added to each well and cAMP was isolated for assay as described (8).

Phospholipid Methylation. Cells were incubated with 1 ml of Dulbecco's minimal essential medium/4.5 mM glucose/4 mM glutamine containing 10 μCi of L-[*methyl*-^{3}H]methionine (12–15 Ci/mmol; 1 Ci = 37 GBq) for 30 min at 37°C and then bradykinin was added. After further incubation, the medium was aspirated and 1 ml of 10% trichloroacetic acid containing 1 mM unlabeled methionine was added to each well. The contents were carefully scraped from the wells with a rubber policeman and transferred to small plastic tubes. Bovine serum albumin was added to form a firmer pellet, and tubes were centrifuged (27,000 × *g*, 10 min). Supernatants were discarded and the pellets were extracted with 3 ml of chloroform/methanol, 2:1 (vol/vol) as described (9) for determination of phospholipid methylation. Drugs were added with the [^{3}H]methionine—i.e., 30 min before bradykinin. Inhibition of bradykinin-stimulated phospholipid methylation was calculated by comparing stimulated and unstimulated values at the same drug concentration although only 3-deazaadenosine affected the unstimulated in-

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corporation of methyl groups into phospholipid.

Calcium Flux. Cells were incubated with or without drugs in 1 ml of Dulbecco's minimal essential medium/4.5 mM glucose/4 mM glutamine/1.8 mM $^{45}CaCl_2$ (5 μCi/ml) for 30 min at 37°C. Bradykinin was added and, after further incubation as indicated, the medium was aspirated; each well was immediately washed twice with 1 ml of cold 0.9% NaCl/5 mM $CaCl_2$/10 mM Hepes, pH 7.4. After addition of 1 ml of 5 mM $CaCl_2$, cultures were frozen, thawed, and transferred to small plastic tubes. The tubes were centrifuged (20,000 × *g*, 10 min) and samples of the supernatants were transferred to vials for radioassay (10).

Release of Arachidonic Acid and Metabolites. Cells were incubated in 1 ml of Dulbecco's minimal essential medium/4.5 mM glucose/4 mM glutamine/40 nM [^{3}H]arachidonic acid (120 Ci/mmol)/0.5% ethanol for 30 min at 37°C. (Incubation with [^{3}H]arachidonic acid for up to 24 hr did not increase the amount of arachidonic acid release induced by bradykinin.) Cells were washed twice with 1 ml of arachidonic acid-lacking medium and incubated with bradykinin, A23187, or with drugs for 30 min at 37°C, and then bradykinin was added. Incubations were terminated by transferring the medium to tubes on ice. Tubes were immediately centrifuged (10 min, 27,000 × *g*) to remove cell debris; a sample of each supernatant was taken for radioassay and, in some experiments, separation of arachidonic acid metabolites. For assay of the latter, 0.5 ml of supernatant was adjusted to pH 3 with 1 M formic acid and extracted with 6 vol of ethyl acetate. The ethyl acetate extract was concentrated in a desiccator by using a vacuum pump and chromatographed on a silica gel H plate developed in the upper phase with ethyl acetate/2,2,4-trimethylpentane/glacial acetic acid/water, 90:50:20:100 (vol/vol). This solvent system separates prostaglandins A, B, E_2, and F, hydroxyicosatetraenoic acid, and arachidonic acid (10, 11); it does not separate 6-ketoprostaglandin $F_{1\alpha}$ from prostaglandin $F_{2\alpha}$.

Separation and Identification of Phospholipids. To identify the specific phospholipids into which arachidonic acid was being incorporated and from which it was released, cells were incubated with [^{3}H]arachidonic acid followed by bradykinin and trichloroacetic acid-precipitated pellets were prepared and extracted with chloroform/methanol, 2:1 (vol/vol) as described above. The chloroform extract was dehydrated overnight with anhydrous sodium sulfate and concentrated under a stream of N_2. The labeled phospholipids and standards were subjected to TLC on silica gel H. Silica gel plates were heat activated just before using (12) and were developed in the first dimension with chloroform/methanol/water, 65:25:4 (vol/vol) and in the second dimension with *n*-butanol/glacial acetic acid/water, 60:20:20 (vol/vol). This procedure separates phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin, and neutral lipids.

Data Presentation. Because of variations in the day-to-day responses of fibroblasts to bradykinin, particularly with cells of different passage numbers, data from representative experiments are presented as means of duplicate or triplicate samples, which usually did not vary by more than 10%. All experiments were repeated at least two or three times. Dose–response curves for inhibition were obtained by comparing bradykinin-stimulated and unstimulated samples at the same drug concentrations, although in most cases the drugs had little or no effect on unstimulated values.

Materials. L-[*methyl*-^{3}H]Methionine (12.0 and 15.0 Ci/mmol) and $^{45}CaCl_2$ (0.5 to 3 Ci/mmol) were purchased from New England Nuclear; [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (120 Ci/mmol) were from Amersham; all media and balanced salt solutions were from GIBCO; bovine phospholipid standards were from Applied Science (State College, PA); mepacrine (quinacrine), indomethacin, and prostaglandins A, B, E_2, and $F_{2\alpha}$ were from Sigma; bradykinin was from Beckman; 3-deazaadenosine was from Southern Research Institute (Birmingham, AL); A23187 was from Eli Lilly; plastic ware was from Falcon; and silica gel H chromatography plates were from Analtech (Newark, DE).

RESULTS

Bradykinin Stimulation and Phospholipid Methylation, Calcium Influx, Release of Arachidonic Acid, and cAMP Content. The temporal relationship between phospholipid methylation, calcium flux, release of arachidonic acid, and cAMP after treatment of human fibroblasts with bradykinin was examined (Fig. 1). Incubation of human fibroblasts with bradykinin produced an increase in phospholipid methylation that peaked within 15 sec and then declined over the ensuing 3–5 min. Calcium influx was highest within 30 sec after addition of bradykinin. By 5 min, the amount of $^{45}Ca^{2+}$ in cells was less than that of control cells, probably due to efflux. Release of [^{3}H]arachidonic acid and metabolites from cells and intracellular cAMP content was apparent within 1 min after addition of bradykinin. Release of [^{3}H]arachidonic acid and metabolites was maximal within 3 to

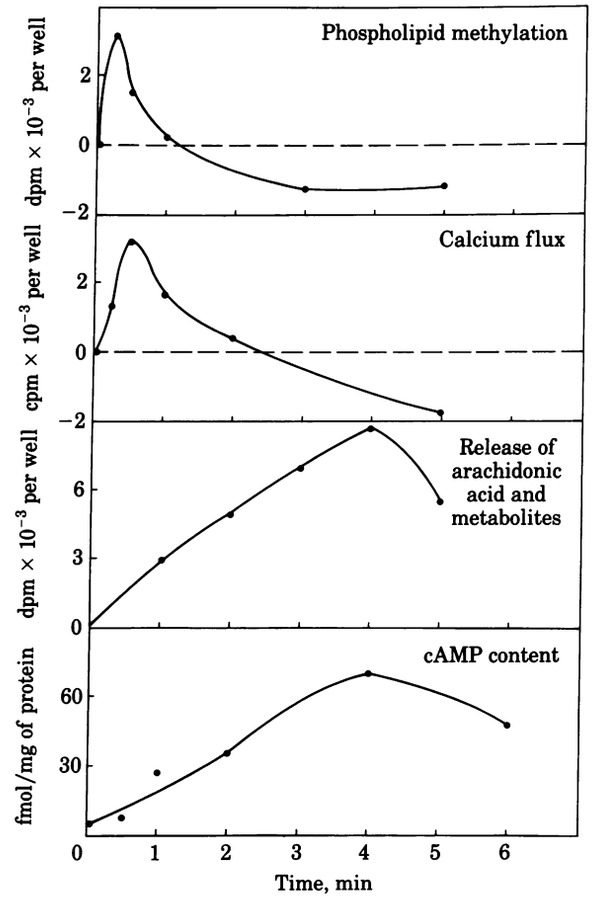

FIG. 1. Time course of the effects of bradykinin on phospholipid methylation, calcium flux, release of arachidonic acid and metabolites, and cAMP content. In the absence of bradykinin (0 time), [^{3}H]-methylphospholipid content was 2,000 dpm per well; $^{45}Ca^{2+}$ content, 4,020 cpm per well; medium [^{3}H]arachidonic acid and metabolites, 1,200 dpm per well; and cAMP content, 18 pmol/mg of protein. These values, except for cAMP content, have been subtracted from means of data from two or three replicate samples of cells incubated with bradykinin (0.05 μg/ml) for the indicated time; i.e., data presented are changes produced by bradykinin.

4 min; cAMP content increased steadily for 4 to 5 min and then decreased. In subsequent experiments, phospholipid methylation was measured at 15 sec, calcium influx at 30 sec, release of arachidonic acid and metabolites at 3.5 min, and cAMP content at 5 min after the addition of bradykinin.

The stimulation of phospholipid methylation, calcium influx, arachidonic acid release, and cAMP formation were examined at various bradykinin concentrations (Fig. 2). Bradykinin exerted a maximal effect on all of the above processes at concentrations around 0.1 μg/ml. The dose–response curves for increased phospholipid methylation, calcium influx, and release of arachidonic acid and metabolites in response to bradykinin were similar; stimulation of cAMP formation was apparent at somewhat lower concentrations.

Incorporation of [^{3}H]Arachidonic Acid in Phospholipids and the Effect of Bradykinin. Incubation of fibroblasts with [^{3}H]arachidonic acid resulted in its incorporation into several phospholipids (Table 1). The largest amount of [^{3}H]arachidonic acid was found in phosphatidylcholine, followed by that of phosphatidylserine and phosphatidylethanolamine, with a small amount present in phosphatidylinositol. Bradykinin produced a decrease in the amount of [^{3}H]arachidonic acid in phosphatidylinositol and phosphatidylcholine but no apparent change in the amounts of [^{3}H]arachidonic acid in phosphatidylserine or phosphatidylethanolamine. There was a slight decrease in [^{3}H]-phosphatidylcholine that was not statistically significant because of the high amount of radioactivity in phosphatidylcholine, not responding to the stimulation.

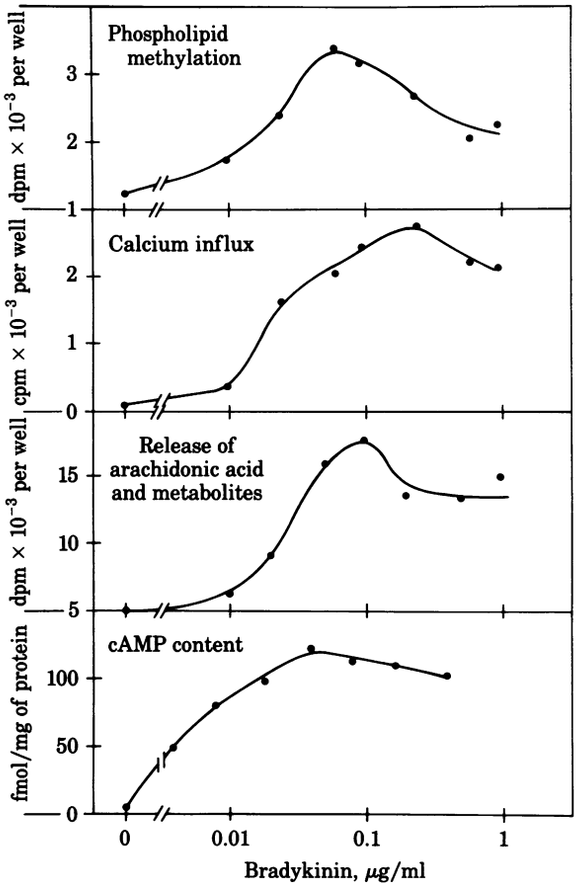

FIG. 2. Dose–response curve for bradykinin stimulation of phospholipid methylation, calcium flux, release of arachidonic acid and metabolites, and cAMP accumulation. Cells were incubated without or with bradykinin at the indicated concentrations and assayed for incorporation of [^{3}H]methyl into phospholipid (15 sec), $^{45}Ca^{2+}$ influx (30 sec), release of [^{3}H]arachidonic acid and metabolites (3.5 min), and cAMP content (5 min). Data are means of values from two or three replicate samples. At 0 time, [^{3}H]methylphospholipid content was 1,200 dpm per well; $^{45}Ca^{2+}$ content, 4,020 cpm per well; medium [^{3}H]arachidonic acid and metabolites, 5,000 dpm per well; and cAMP content, 50 pmol/mg of protein.

Table 1. Effect of bradykinin on [^{3}H]arachidonic acid content of phospholipids from fibroblasts

Phospholipid	^{3}H in phospholipids, dpm × 10^{-3} per well: Without bradykinin	With bradykinin
Phosphatidylcholine	134	127
Phosphatidylserine	91	114
Phosphatidylethanolamine	60	70
Phosphatidylinositol	20	10
Total lipid	540	603

Cells were incubated with [^{3}H]arachidonic acid for 30 min, washed, and incubated with or without bradykinin for 3.5 min, after which phospholipids were extracted and separated. Data are means of values from triplicate incubations.

[^{3}H]Arachidonic Acid Release and Metabolism. [^{3}H]Arachidonic acid and its metabolites released from fibroblasts after stimulation with bradykinin, the calcium ionophore A23187, or both were extracted from incubation media, separated by TLC, and quantified. Both bradykinin and the ionophore caused the release of [^{3}H]arachidonic acid and stimulated the production of [^{3}H]prostaglandins (the PGE_2 and PGF series) (Table 2). The amount of [^{3}H]prostaglandins that accumulated relative to [^{3}H]-arachidonic acid was higher from cells incubated with bradykinin than from untreated cells or cells stimulated by ionophore A23187. These data suggest that release of labeled arachidonic acid was more closely coupled to prostaglandin formation in cells exposed to bradykinin.

Effect of Prostaglandins and Bradykinin on cAMP Content. Exogenous prostaglandins E_1 (1 μg/ml) and E_2 (1 μg/ml) increased cAMP content of human fibroblasts 35-fold and 23-fold, respectively. Prostaglandin F_2 (1 μg/ml) had no effect. Bradykinin (0.05 mg/ml) increased cAMP content 39-fold. These observations are consistent with the view that increased production of prostaglandin E_2 from arachidonic acid contributes to the bradykinin-induced increase in cAMP (Figs. 1 and 2).

Table 2. Effects of bradykinin and A23187 on release of [^{3}H]arachidonic acid and metabolites

Addition	Radioactivity, dpm × 10^{-3} per well: PGE_2	$PGF_{2\alpha}$	Arachidonic acid	Total released
None	0.77	1.07	2.16	8.12
Bradykinin (0.1 μg/ml)	2.41	3.32	2.41	15.5
A23187 (1 μM)	1.68	1.77	6.65	18.5
Bradykinin (0.1 μg/ml)/ A23187 (1 μM)	2.24	5.77	7.56	24.8

Cells were incubated with [^{3}H]arachidonic acid for 30 min, washed, and incubated with or without additions for 3.5 min, and medium was analyzed for [^{3}H]arachidonic acid and [^{3}H]prostaglandins. Values represent means of duplicate determinations from a single experiment. Recovery of radioactivity in ethyl acetate extracts was 50–60%. Stimulation of release of radioactivity by bradykinin was 65–90%. PGE_2 and $PGF_{2\alpha}$, prostaglandins E_2 and $F_{2\alpha}$.

Effect of Metabolic Inhibitors. To confirm that increased content of cAMP is a consequence of prostaglandin formation, the effect of indomethacin, an inhibitor of cyclooxygenase, on bradykinin-stimulated cAMP formation was examined. Indomethacin (0.1 μM) almost completely reduced bradykinin-stimulated cAMP formation and partially inhibited the release of arachidonic acid and its metabolites. Neither stimulation of phospholipid methylation and calcium influx by bradykinin nor stimulation of cAMP accumulation by isoproterenol was affected by indomethacin (1 μM), suggesting that indomethacin does not inhibit adenylate cyclase, Ca^{2+} channels, or phosphatidylethanolamine methyltransferase.

Mepacrine, an inhibitor of phospholipase A_2 (13), also blocked the bradykinin-induced release of arachidonic acid and its metabolites in a dose-dependent manner. At a concentration of 0.5 mM, mepacrine completely blocked bradykinin-induced cAMP accumulation and inhibited release of [^{3}H]arachidonic acid and its metabolites by 75%. Mepacrine had no effect on bradykinin-stimulated calcium influx or phospholipid methylation.

3-Deazaadenosine is an inhibitor of 3-adenosylmethionine-requiring methyltransferases, including phospholipid methyltransferases (14). 3-Deazaadenosine (50 μM) inhibited bradykinin-induced increases in phospholipid methylation, calcium influx, and release of arachidonic acid and metabolites to various degrees but had little effect on cAMP accumulation (Fig. 3). When arachidonic acid and its metabolites were separated as described in *Methods*, bradykinin-induced release of prostaglandins E_2 and $F_{2\alpha}$ and arachidonic acid were decreased 50–60% by 30 μM 3-deazaadenosine. These results and those reported in Table 1 were consistent with the observation that the arachidonic acid released comes from both phosphatidylcholine and phosphatidylinositol. After inhibition of phospholipid methylation, the amount of arachidonic acid derived from phosphatidylinositol appears to be sufficient to form prostaglandins that enhance cAMP content.

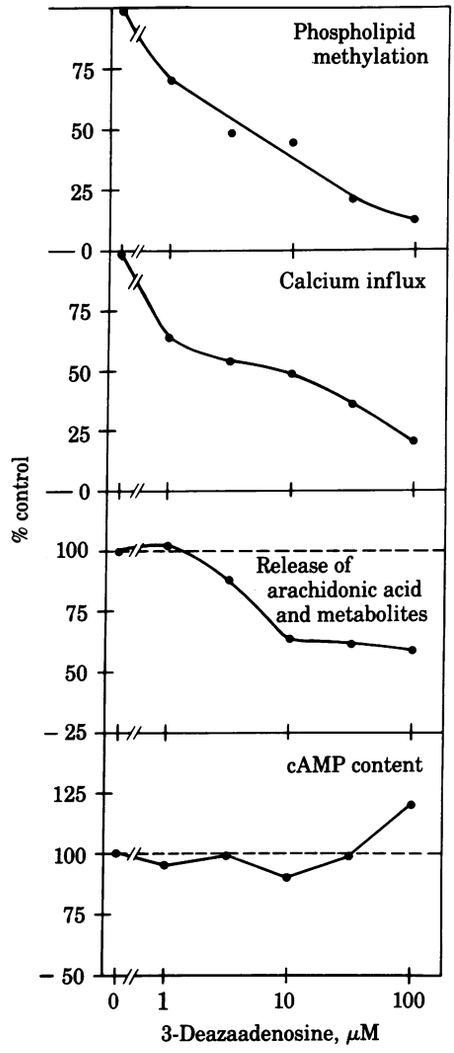

FIG. 3. Effects of 3-deazaadenosine on bradykinin-stimulated phospholipid methylation, calcium influx, release of arachidonic acid and metabolites, and cAMP accumulation. Cells were incubated for 30 min with 3-deazaadenosine at indicated concentrations and then with or without bradykinin at 0.1 μg/ml to assess incorporation of [^{3}H]methyl into phospholipid (15 sec), $^{45}Ca^{2+}$ content of cells (30 sec), release of [^{3}H]arachidonic acid and metabolites (3.5 min), and cAMP accumulation (5 min). Data are means of values from two or three replicate samples with changes produced by bradykinin in the presence of deazaadenosine expressed as a percentage of that observed in its absence. In the absence of 3-deazaadenosine, increments produced by bradykinin were [^{3}H]methyl incorporation into phospholipid, 1,200 dpm per well; $^{45}Ca^{2+}$ content, 4,000 cpm per well; [^{3}H]arachidonic acid and metabolites released into the medium, 4,200 dpm per well; and cAMP content, 750 pmol/mg of protein.

DISCUSSION

Bradykinin in cultured human fibroblasts produces rapid increases in phospholipid methylation, Ca^{2+} influx, release of arachidonic acid from phospholipids, prostaglandin production, and accumulation of cAMP. Various types of metabolic inhibitors were used to determine whether any or all of these processes are linked in a sequential cascade of events triggered by interaction of bradykinin with its cell surface receptor and leading to an increase in cAMP content.

Indomethacin, an inhibitor of prostaglandin synthesis, decreased bradykinin-induced increases in cAMP. Mepacrine, a phospholipase inhibitor, blocked the effects of bradykinin on arachidonic release, prostaglandin formation, and cAMP accumulation. These findings are consistent with the hypothesis that bradykinin affects cAMP via a sequence of events that includes activation of phospolipases with the concomitant release of arachidonic acid, a precursor for prostaglandins (Fig. 4). The prostaglandins in turn activate adenylate cyclase and increase cAMP content. Neither indomethacin nor mepacrine affected bradykinin-induced changes in phospholipid methylation. 3-Deazaadenosine, an inhibitor of methyltransferases, markedly decreased bradykinin-induced changes in phospholipid methylation and Ca^{2+} influx. However, this drug only partially inhibited (by 50–60%) effects of bradykinin on arachidonic acid release and prostaglandin production and had no effect on bradykinin-stimulated increases in cAMP. These results suggest that, although phospholipid methylation led to the formation of arachidonic acid and prostaglandins, these phospholipids are not the only source of the prostaglandins responsible for the bradykinin-induced increases in cAMP. Recent studies have indicated that bradykinin stimulates turnover of phosphatidylinositol in cultured cells (15, 16). In human fibroblasts, bradykinin produced a decrease in the [^{3}H]arachidonic content of phosphatidylinositol. In fibroblasts, bradykinin-stimulated turnover of phosphatidylinositol might also be coupled with arachidonic acid release, prostaglandin formation, and activation of adenylate cyclase.

The model presented in Fig. 4 outlines alternative pathways for receptor-mediated arachidonic acid release, through which bradykinin could increase production of the prostaglandins that activate adenylate cyclase. In one pathway, bradykinin stimulates the sequential methylation of phosphatidylethanolamine

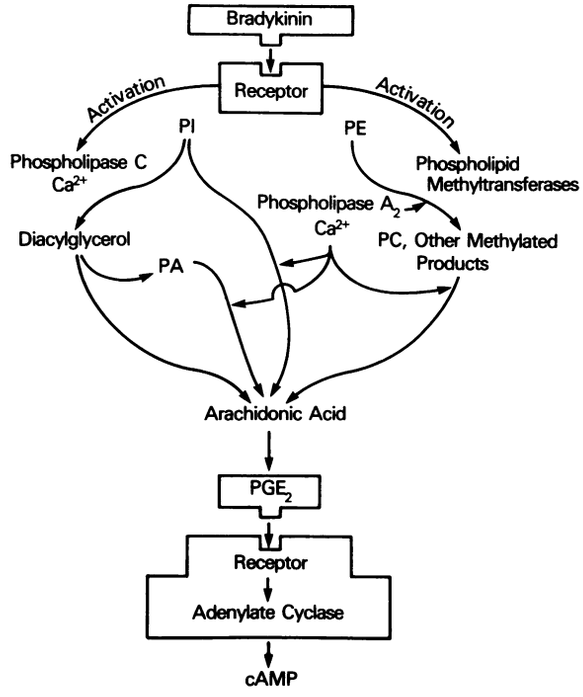

FIG. 4. Possible pathways for bradykinin stimulation of cAMP formation in cultured human fibroblasts. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PGE_2, prostaglandin E_2.

to form phosphatidylcholine that is enriched in arachidonic acid (17). Methylation of phospholipids has been found to increase membrane fluidity (18) and induce changes in Ca^{2+} influx (19). The increased availability of Ca^{2+} activates phospholipase A_2, which catalyzes release of arachidonic acid from the phosphatidylcholine produced via the methylation pathway. As depicted on the left of Fig. 4, bradykinin could also stimulate degradation of phosphatidylinositol by phospholipase A_2 (16) or phospholipase C (or both) to produce diacylglycerol from which arachidonic acid could be released by the action of diacylglyceride lipase (20). Alternatively, diacylglycerol might be converted to phosphatidic acid from which arachidonic acid could be released by a phosphatidic acid-specific phospholipase A_2 (21). Arachidonic acid, formed by either or both pathways, would be converted to prostaglandins that would in turn activate adenylate cyclase and increase cAMP content.

This dual scheme would explain the effects of the various inhibitors on bradykinin-induced increases in cAMP. Indomethacin directly inhibits prostaglandin production; mepacrine could limit availability of arachidonic acid by inhibition of phospholipases. 3-Deazaadenosine would inhibit arachidonic acid production from phosphatidylcholine synthesized by the methylation pathway but not from phosphatidylinositol. Despite the reduction of arachidonic acid release by the methyltransferase inhibitor, enough of the fatty acid might be available from the hydrolysis of phosphatidylinositol to produce prostaglandins. In this model, cAMP is only one indirect mediator of bradykinin action and physiological responses to bradykinin could be related to the production of any of several "second messengers," including Ca^{2+}, arachidonic acid, prostaglandins, phosphatidic acid, etc. The mechanisms outlined in Fig. 4 might also be applicable to other peptides whose biological actions are related to activation of the dual arachidonic acid cascade and production of prostaglandins. In turn, the concept of a dual arachidonic acid cascade offers the potential of multiple flexible points for regulation of tissue responsiveness to bradykinin and other peptides.

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