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BRADYKININ STIMULATES DNA SYNTHESIS IN COMPETENT Balb/c 3T3 CELLS AND ENHANCES INOSITOL PHOSPHATE FORMATION INDUCED BY PLATELET-DERIVED GROWTH FACTOR

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Abstract—Both platelet-derived growth factor (PDGF) and bradykinin were found to induce a growth response in Balb/c 3T3 cells. However, whereas PDGF brought about a five-fold increase in the incorporation of [^{3}H]thymidine into DNA, the response to bradykinin was never more than 50%. When bradykinin was present simultaneously with sub-optimal concentrations of PDGF the response was about 15% greater than with PDGF alone. In contrast, if the cells were made competent by a 5 hr pre-incubation with PDGF which was then washed away, subsequent addition of bradykinin induced a more than two-fold increase in incorporation of [^{3}H]thymidine into DNA compared with competent cells subsequently incubated with serum-free medium alone. Bradykinin also acted synergistically with insulin when the two agents were added simultaneously to competent cells. PDGF induced marked increases in the concentration of inositol phosphates at 30 min after stimulation, but by this time point any effect of bradykinin had disappeared. However, the simultaneous presence of PDGF and bradykinin induced increases at 30 min that were 50–100% greater than with PDGF alone. It is concluded that the pathways by which PDGF and bradykinin initiate a growth response in BALB/c 3T3 cells only partly overlap. Their actions on the synthesis of inositol phosphates exhibit distinctive temporal characteristics, but can be co-operative at 30 min and at earlier time intervals. This effect was found to be time-dependent, and developed over the first 5 min.

The nonapeptide bradykinin is released during the inflammatory response, and may participate in late events of inflammation [1] such as wound repair, involving cell proliferation. In this regard, bradykinin has been found to stimulate DNA synthesis in lymphocytes [2], human skin fibroblasts [3], NIL8 hamster cells [4] and A431 cells [5], and also in human foetal lung fibroblasts [3, 4]. However, the degree of stimulation is not large. In contrast, platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin and, in distinction to a number of other growth factors, can stimulate DNA synthesis in the absence of other agents [6]. PDGF can also induce in some cell types a metabolic state which has been termed "competence" [7] and which is also induced by overexpression of the *src* oncogene [8]. Quiescent Balb/c 3T3 fibroblasts exposed to PDGF for a few hours replicate their DNA and divide. If exposure to PDGF is of short duration, cells do not progress efficiently through the cell cycle into the S phase unless they are exposed to a second set of growth factors of which EGF and insulin-like growth factors (somatomedins) are examples [9, 10]. Singh *et al.* [11] have shown, using anti-PDGF antibodies, that the PDGF-induced competent state is not an artefact, but results from an intracellular accumulation of relatively stable PDGF-regulated gene products.

It has also been shown [12–15] that one action of PDGF on 3T3 cells is to stimulate the breakdown of phosphatidylinositol 4,5-bisphosphate with formation of inositol 1,4,5-trisphosphate, which may function as a second messenger to mobilize intracellular Ca^{2+}. Diacylglycerol, which activates protein kinase C is also formed. These functions are associated with growth stimulation in several systems [16]. Bradykinin has also been shown to bring about phosphoinositide breakdown in a variety of cell types, including A431 cells [5], a neuroblastoma × glioma cell line [17], Manin–Darby canine kidney cells [18] and PC12 phaeochromocytoma cells [19]. However, bradykinin failed to induce phosphoinositide hydrolysis in a parental line of NIH 3T3 cells, although *ras*-transformed NIH 3T3 cells exhibited a pronounced phosphoinositide response [20]. The main effect of bradykinin on phosphoinositide metabolism occurs during the first min [5, 17], whereas in the case of PDGF the response is much more prolonged [12, 13, 15, 21].

We investigated whether bradykinin acts as a secondary growth factor, enabling Balb/c 3T3 cells made competent by PDGF to progress into the S phase. We report here the evidence that this is the case. Attempts to demonstrate that stimulation of phosphoinositide metabolism is involved in this action of bradykinin led to the observation that when bradykinin and PDGF were present together, the levels of inositol phosphates were two-fold greater than found in the presence of PDGF alone, a time-dependent effect that developed over the first 5 min of PDGF action.

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MATERIALS AND METHODS

Chemicals and reagents. Bradykinin, lys-bradykinin and insulin were purchased from the Sigma Chemical Co. (Poole, U.K.). Highly purified porcine PDGF was purchased from Bioprocessing Ltd (Durham, U.K.). [^{3}H]Thymidine and *myo*-[2-^{3}H]inositol were obtained from Amersham International (Amersham, U.K.).

Cells. Balb/c 3T3 cells (clone A31) were obtained from Flow Laboratories (Irvine, U.K.). Cells were cultured in polystyrene flasks with 25 and 75 cm^2 growth areas (Falcon Tissue Culture Plastics). Growth medium consisted of HEPES-buffered (2 mM) MEM (Eagle) containing glutamine (2 mM) and supplemented with 10% (v/v) foetal bovine serum (Imperial Laboratories, U.K.), penicillin (19,000 units/100 mL) and streptomycin (19,000 units/100 mL). Cells were grown in polystyrene flasks at 37° in a 98% air: 2% CO_2 atmosphere and routinely subcultured by trypsinization.

DNA synthesis assays. Cells were seeded in 24-well multiwell plates (Falcon Tissue Culture Plastics) at a density of 2×10^4 cells/well. After 3 days, when the cells were sub-confluent, they were washed and incubated in serum-free MEM for 20–24 hr prior to the addition of the growth factors to be tested. At this stage [^{3}H]thymidine (1 μCi/mL) was added to the medium and the cells incubated for a further 24 hr. The cells were then washed five times with phosphate buffered saline (PBS), incubated for 30 min in cold 5% TCA, and washed three more times with PBS. The cells were dried and extracted with 1 N NaOH (250 μL/well) by overnight incubation at 37°. An aliquot of the NaOH extract was dispersed in scintillation fluid and counted in a Beckman liquid scintillation counter to determine incorporation of [^{3}H]thymidine into DNA.

Induction of competence. Cells were made competent by incubation for 5 hr with PDGF (45 ng/well). The cultures were then washed three times with serum-free medium after which serum-free medium containing [^{3}H]thymidine with or without growth factors was added.

Inositol phosphate assays. Cells were seeded at 5×10^4 cells/mL in 2 mL of growth medium in 30 mm diameter triple vent tissue culture dishes (Sterilin Ltd). After 3 days, when the cells were near confluence, they were washed twice with inositol-free MEM (Gibco) and incubated for 24 hr in 2 mL/dish of inositol-free MEM containing 20 μCi/dish of *myo*-[2-^{3}H]inositol. After incubation the radio-isotope-containing medium was removed by aspiration and the cell monolayers were rinsed three times with inositol-free MEM. The cells were then incubated for 10 min in 1 mL inositol-free MEM containing LiCl at a final concentration of 10 mM. After a further incubation for 30 min (unless otherwise stated) with or without growth factors the medium was removed and 1 mL of cold 5% TCA was added for 30 min to extract the water-soluble inositol phosphates. The extract was transferred to a test tube and the cells were washed twice with 1 mL of distilled water. The washings were combined with the original acid extract and TCA was removed with diethyl ether. The final extract was neutralized with 1 N NaOH. The HPLC analysis was carried out on a 25 × 0.5 cm column of Partisil SAX-10 preceded by a 5 × 0.5 cm guard column. A flow rate of 1.3 mL/min and a 1 min fraction collection interval were used. A linear gradient over 60 min from 0 to 1 M $(NH_4)_2HPO_4$ buffered to pH 3.7 with orthophosphoric acid was used. The column eluates were collected and radioactivity of aliquots of these fractions was measured. [2-^{3}H]Inositol 1-phosphate, [2-^{3}H]inositol 1,4-bisphosphate, [2-^{3}H]inositol 1,4,5-trisphosphate (all from Amersham International) and [1-^{3}H]inositol 1,3,4-trisphosphate (from NEN/Du Pont) were used as standards to calibrate the HPLC system.

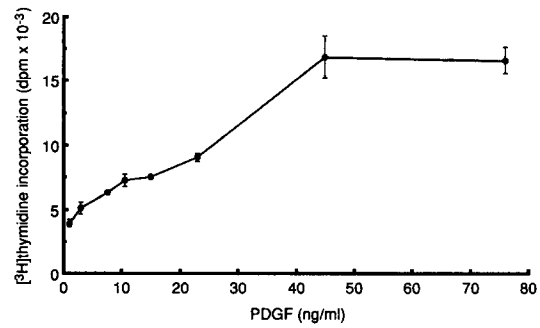

Fig. 1. Dose–response curve for the effect of PDGF on DNA synthesis in quiescent cultures of Balb/c 3T3 cells. Results are means ± SEM of quadruplicate determinations.

RESULTS

Stimulation of DNA synthesis

Effect of bradykinin and PDGF on DNA synthesis. When DNA synthesis was induced by bradykinin in the absence of serum the maximal effect was obtained at 10^{-7} M, but bradykinin brought about no more than 40% stimulation (about 1/20 of that seen after exposure to 10% serum—data not shown). Lys-bradykinin, an agonist of the B_2 subset of bradykinin receptors [22] gave only 12% stimulation at 10^{-6} M under similar conditions suggesting that at least some of the effect is mediated by B_1 receptors (data not shown). By contrast, in the case of PDGF the maximal stimulation of incorporation of [^{3}H]thymidine was approximately four-fold higher than the basal level (Fig. 1), corresponding to about 45% of that achieved by 10% serum (data not shown). It is known that the effects of growth factors can combine to give enhanced DNA synthesis [23, 24]. We investigated the combined effect of bradykinin and PDGF at two concentrations of PDGF, a low concentration (15 ng/mL) which did not give maximal stimulation, and one (45 ng/mL) at which the maximal [^{3}H]thymidine incorporation attainable in the presence of PDGF was achieved (Fig. 1). In both cases the results showed that the addition of bradykinin resulted in greater stimulation than when PDGF alone was used. At 15 ng/mL PDGF, 10^{-4} M bradykinin increased the overall stimulation of DNA synthesis by 12% and at 45 ng/

Table 1. Stimulation of DNA synthesis by bradykinin and PDGF added simultaneously to Balb/c 3T3 cells

	Bradykinin (M)	PDGF (ng/mL)	[^{3}H]Thymidine incorporation (dpm × 10^{-3})	% Stimulation
Expt 1	0	45	35.8 ± 1.7	—
	10^{-10}	45	35.8 ± 1.92	0
	10^{-4}	45	39.1 ± 0.8	9.2
Expt 2	0	15	68.7 ± 1.5	—
	10^{-10}	15	69.6 ± 0.7	1
	10^{-8}	15	72.7 ± 2.1	6
	10^{-7}	15	80.2 ± 2.7	17
	10^{-6}	15	80.7 ± 0.5	17
	10^{-4}	15	77.1 ± 1.8	12

Results are means ± SEM of quadruplicate determinations.

mL PDGF there was a 9.2% increase (Table 1).

Effect of bradykinin on cells made competent by pre-exposure to PDGF. When bradykinin was added to cells made competent by prior exposure to PDGF, a two-fold increase in [^{3}H]thymidine incorporation resulted, in contrast to a 20% stimulation when bradykinin was added to cells that had been incubated in serum-free MEM without PDGF (Fig. 2).

Effect of insulin on competent cells. The response to bradykinin was compared to that of insulin, which is known to enhance the effect of other growth factors on DNA synthesis [23], although it has been reported to exhibit only a modest effect on Balb/c 3T3 cells [3]. In our hands the addition of insulin greatly increased [^{3}H]thymidine incorporation after the cells had been made competent. Two concentrations of insulin were studied, 1 and 10 μg/mL. Both concentrations gave approximately five-fold stimulation. The addition of bradykinin with the insulin gave a further enhancement to approximately eight-fold stimulation (Fig. 2).

The data illustrated in Fig. 2 emphasize the importance of the induction of "competence" in the response to secondary growth factors. The differential response of competent and non-competent cells is particularly marked in respect to the action of insulin. However, examination of the ratio of the stimulatory effects on competent and non-competent cells (Table 2) demonstrates that bradykinin has a greater competence-dependent action (1.8) than insulin at both concentrations tested (1.1). This competence-dependent action is still detectable in the co-presence of insulin (see Fig. 2).

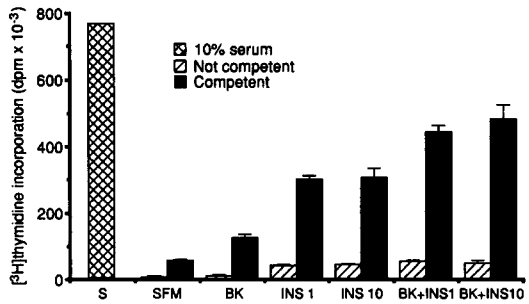

Fig. 2. Effect of bradykinin and insulin on cells made competent by prior exposure to 45 ng/mL PDGF for 5 hr. The histogram shows the effect on DNA synthesis of various additions as follows: S = 10% foetal bovine serum; SFM = serum-free medium; BK = bradykinin (10^{-4} M); INS 1 = insulin (1 μg/mL); INS 10 = insulin (10 μg/mL); BK + INS 1 = bradykinin (10^{-4} M) plus insulin (1 μg/mL); BK + INS 10 = bradykinin (10^{-4} M) plus insulin (10 μg/mL). (▨) Cells not made competent; (▩) cells made competent. Results are means ± SEM of quadruplicate determinations.

Table 2. Comparison of stimulatory effects on DNA synthesis in competent and non-competent cells

Agent(s)	Ratio stimulated/serum-free medium: Comp	Ratio stimulated/serum-free medium: Non-comp	Ratio (comp/non-comp)
A.			
BK	2.17	1.18	1.8
Ins 1	5.20	4.66	1.1
Ins 10	4.32	4.98	1.1
Serum	—	84.9	—
B.	BK + Ins/Ins		
BK (Ins 1)	1.48	1.3	1.14
BK (Ins 10)	1.57	1.1	1.43

A. The percentage stimulation (see Fig. 2) of [^{3}H]thymidine incorporation by various agents compared with the equivalent control cultures in serum-free medium is shown for untreated cells (non-comp) and cells made competent (comp) by prior treatment with PDGF (for details see test) expressed as a ratio.

B. The stimulation (see Fig. 2) of [^{3}H]thymidine incorporation by bradykinin in the presence of two concentrations of insulin compared with the cultures stimulated with insulin alone for competent and non-competent cells, expressed as a ratio.

Table 3. Effect of bradykinin (10^{-6} M) on the release of inositol phosphates in Balb/c 3T3 cells

Time of exposure (min)	Ins(1)P (dpm × 10^{-3})	Ins(4)P (dpm × 10^{-2})	Ins(1, 4)P_2 (dpm)	Ins(1, 3, 4)P_3 (dpm)
0	18.6 ± 0.52	9.3 ± 0.3	72 ± 2	18 ± 4
5	13.2 ± 0.17	13.4 ± 3.3	78 ± 6	0 ± 0
30	15.2 ± 2.6	13.4 ± 1.9	105 ± 13	15 ± 4

Results are means ± SEM of triplicate determinations.

Table 4. Stimulation of inositol phosphate formation by bradykinin and PDGF in Balb/c 3T3 cells for 30 min

Assay conditions	Ins(1)P (dpm × 10^{-3})	Ins(4)P (dpm × 10^{-2})	Ins(1, 4)P_2 (dpm)	Ins(1, 3, 4)P_3 (dpm)
Control	46 ± 3.3	9.5 ± 1.3	205 ± 35	65 ± 18
Bradykinin	54.3 ± 4.2	15.4 ± 6.2	325 ± 22	50 ± 30
PDGF	67.7 ± 2.8	41.3 ± 0.8	1040 ± 50	205 ± 35
Bradykinin + PDGF	52.8 ± 1.7	97.7 ± 2.7	2230 ± 95	295 ± 39

Results are means ± SEM of triplicate determinations.

Short-term stimulation of inositol phosphate formation

Table 3 shows that 30 min after addition of 10^{-6} M bradykinin there was only a slight effect on the level of inositol phosphates in quiescent Balb/c 3T3 cells. In contrast, 30 min after addition of PDGF (45 ng/mL), inositol phosphate levels were considerably elevated (Table 4). When cells were treated with both bradykinin (10^{-6} M) and PDGF, the level of inositol phosphates was increased compared with that seen with PDGF alone (Table 4). Inositol 1-monophosphate levels were essentially unchanged, but PDGF induced large increases in the levels of inositol 4-monophosphate, 1,4-bisphosphate and 1,3,4-trisphosphate, which were further increased about two-fold when bradykinin was present together with PDGF. The levels of inositol 1,3,4-trisphosphate in stimulated cells were always much higher than those of inositol 1,4,5-trisphosphate, which showed only small changes that were not significant at these time intervals. The ability of bradykinin to increase inositol phosphate levels induced by PDGF could be seen at earlier time intervals and was dependent on the length of exposure of the cells to the growth factors (Table 5). After 2 min of stimulation no significant effect of bradykinin on PDGF-stimulated $InsP_3$ levels was observed, but by 5 min bradykinin had brought about a five-fold increase in the response to PDGF.

DISCUSSION

We have demonstrated here that bradykinin stimulates DNA synthesis in Balb/c 3T3 cells that have been made competent by prior addition of PDGF. After cells have been made competent the effect of bradykinin is much more pronounced, in contrast to the effect of insulin which is equivalent for competent and non-competent cells (Table 2). Competence-dependent stimulation is greater than that when bradykinin is added simultaneously with PDGF (Table 1), the predominant effect in this case being due to the action of PDGF.

In human fibroblasts bradykinin can activate protein kinase C and thereby induce expression of the *myc* oncogene [25] and in Swiss 3T3 cells it transiently activates protein kinase C [26]. However, the fact that bradykinin is only weakly mitogenic demonstrates that it lacks the ability to activate some additional process essential for a full mitogenic response. The actions of bradykinin and PDGF appear to overlap with regard to this additional process in cells previously exposed to PDGF. In the continuous presence of PDGF, little extra response can be induced by bradykinin whereas when PDGF is absent bradykinin effectively stimulates progression into the S phase.

Our results show that in otherwise unstimulated cells, bradykinin has little influence on inositol phosphate levels at the longer time intervals that we have studied (30 min). This is in agreement with results previously reported, which have indicated that the bradykinin effect on phosphoinositides occurs mainly in the first min [5, 17], in contrast to PDGF which exerts a more prolonged response [12, 13, 15, 21]. We have now shown, however, that when added simultaneously with PDGF, bradykinin can increase by about two-fold the levels of inositol phosphates present 30 min after stimulation. That this does not bring about a commensurate increase in DNA synthesis may indicate that in this situation the response to inositol phosphates has reached saturation. A similar increase can be observed at

Table 5. Time course of the effect of simultaneous addition of bradykinin (10^{-6} M) and PDGF (45 ng/mL) on the levels of inositol phosphates in Balb/c 3T3 cells

Assays conditions	Ins(1)P (dpm × 10^{-3})	Ins(4)P (dpm × 10^{-2})	Ins(1, 4)P_2 (dpm)	Ins(1, 3, 4)P_3 (dpm)
Control (0 min)	16.3 ± 2.85	21.90 ± 3.6	180 ± 15	21 ± 6
PDGF (2 min)	16.3 ± 1.80	36.60 ± 4.8	750 ± 36	210 ± 21
PDGF + bradykinin (2 min)	18.8 ± 0.27	42.90 ± 3.6	900 ± 150	165 ± 00
PDGF (5 min)	17.0 ± 1.44	81.90 ± 11.1	1110 ± 54	180 ± 30
PDGF + bradykinin (5 min)	22.4 ± 1.35	102.00 ± 12.0	1740 ± 57	1000 ± 45

The experiments were carried out as described in Materials and Methods, except that TCA was added for the extraction of inositol phosphates after the times stated. Results are means ± SEM of triplicate determinations.

earlier time intervals. One of the most interesting findings of this study is that this effect of bradykinin is time-dependent, which indicates that one of the agonists is inducing, over a period of 2–5 min, an increased capacity for the formation of inositol phosphates by phospholipase C. This modification could be mediated by an effect at the G-protein level. While it would be valuable to ascertain the effect of bradykinin on inositol phosphate levels in competent cells, such measurements would present the difficulty that since it is necessary to incubate the cells for long periods with radioactive inositol in order to label the lipid precursors to equilibrium, the perturbation of phosphoinositide metabolism brought about by PDGF would prevent the establishment of a clear baseline. Thus it remains to be established whether a stimulation of the phosphoinositide pathway is involved as a second messenger in the effect of bradykinin on competent cells. The effect of bradykinin in acting as a secondary growth factor suggests that one possible physiological action of bradykinin is to prolong the effects of a transient rise in the concentration of PDGF.

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