Reinforcement of Signal Generation at $B_2$ Bradykinin Receptors by Insulin, Epidermal Growth Factors, and Other Growth Factors*

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Insulin and various growth factors (epidermal growth factor (EGF), insulin-like growth factor, fibroblast growth factor, and transforming growth factor $\alpha$), which fail to modify the resting $[Ca^{2+}]$ i, in PC12 rat pheochromocytoma and SKNBE human neuroblastoma cells when administered alone, became capable of inducing $[Ca^{2+}]$ i increases when administered a few (4-20 min after another agent, bradykinin. The latter peptide, working through a $B_2$ receptor, caused hydrolysis of polyphosphoinositides and a large, biphasic $[Ca^{2+}]$ i transient (an initial (1-2 min spike, or originated primarily from intracellular stores, followed by a steady-state elevation dependent on $Ca^{2+}$ influx). Priming by bradykinin of the growth factor effects was quickly dissipated by the addition of a $B_2$ blocker. Activation of other receptors coupled to polyphosphoinositide hydrolysis: muscarinic and purinergic (in PC12 and SKNBE cells); bombesin and vasoressin receptors (in Swiss 3T3 cells), was without effect in priming. Bradykinin-primed, growth factor-induced $[Ca^{2+}]$ i rises in PC12 cells appeared after a 20-30-s delay; they were relatively small, but persistent; their concentration dependence was similar to that of other effects of the factors; and they included both release of $Ca^{2+}$ from intracellular stores and stimulation of $Ca^{2+}$ influx, preceded (in PC12 cells) by a transient increase of polyphosphoinositide hydrolysis. Thus the effect of growth factors (possibly dependent on the tyrosine kinase activity of their receptors) consisted in the reinforcement of the transmembrane signaling at $B_2$ receptors. This is the first direct demonstration of a $B_2$ agent, bradykinin (BK). The $[Ca^{2+}]$ i effects of GF were specific for the transduction machinery of the BK receptor of the $B_2$ type. In fact, they were blocked by a $B_2$ antagonist and failed to appear when the cells were pretreated with agents at the level of signal generation, in particular of $[Ca^{2+}]$; control, has been investigated to a smaller extent (5, 16, 18). In the present work, a study along these lines has been carried out in various cell lines by the use of insulin, EGF, and other GF. UF unable to raise $[Ca^{2+}]$ i when administered alone were found to cause a positive modulation (reinforcement) of the PPI hydrolysis and $[Ca^{2+}]$; increase (6, 12, 13); with insulin and IGF-I this lack of effect was reported in all cells investigated (14-17).

Most of our knowledge on transmembrane signaling at GF receptors comes from studies carried out by treating in vitro serum-starved cells with one GF administered alone. In a multicellular living organism, however, cells are simultaneously exposed to a variety of stimuli, whose intracellular effects might interfere extensively with one another. A host of examples have already been reported in the literature demonstrating the synergism of various GF, and even of a GF with other, apparently unrelated, agents, with respect to cell growth (5, 15, 19). In contrast, the interaction of these various agents at the level of signal generation, in particular of $[Ca^{2+}]$; control, has been investigated to a smaller extent (5, 16, 18). In the present work, a study along these lines has been carried out in various cell lines by the use of insulin, EGF, and other GF. UF unable to raise $[CA^{2+}]$ i when administered alone were found to cause a positive modulation (reinforcement) of the PPI hydrolysis and $[Ca^{2+}]$; responses induced by the previous application of an unrelated peptide agent, bradykinin (BK). The $[Ca^{2+}]$; effects of GF were specific for the transduction machinery of the BK receptor of the $B_2$ type. In fact, they were blocked by a $B_2$ antagonist and failed to appear when the cells were pretreated with agents other than BK, working either via the activation of PPI hydrolysis or by other mechanisms.

Experimental Procedures

Cell Culture—PC12 cells (20) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, and antibiotics (penicillin, 100 units/ml; streptomycin, 0.1 mg/ml). They were subcultured weekly in a split ratio of 1:4. SKNBE human neuroblastoma cell monolayers were cultured as PC12 but horse serum was omitted from the media. The cells were split 1:6 weekly by treatment with trypsin as previously described (6, 21). Mouse Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium, with antibiotics and 10% fetal calf serum. The cells were split 1:5 weekly and main-

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The abbreviations used are: GF, growth factors; EGF, epidermal growth factor; PDI, platelet-derived growth factor; IGF-I, insulin-like growth factor-I; PPI, polyphosphoinositide; Ins-1,4,5-P3, inositol 1,4,5-trisphosphate; Ins-Fs and F2, Ins-1,4,5-P3 and 1,2-sn-diaclyglycerol (4-9). Binding of Ins-1,4,5-P3 to a specific intracellular site causes the release of $Ca^{2+}$ from a microsomal store, with consequent rise of the cytosolic free $Ca^{2+}$ concentration, $[Ca^{2+}]$ i (10). Concomitantly, the $Ca^{2+}$ conductance across the plasma membrane is stimulated (10, 11). These effects, however, are not general for all GF. In various cell types EGF itself has been found unable to cause detectable PPI hydrolysis and $[Ca^{2+}]$; increase (6, 12, 13); with insulin and IGF-I this lack of effect was reported in all cells investigated (14-17).

The generation of transmembrane signals triggered by the interaction of growth factors (GF) with their specific receptors has been intensely investigated. Receptors for epidermal GF (EGF), platelet-derived GF (PDGF), insulin, and insulin-like GF-I (IGF-I) are known to be endowed with an intrinsic tyrosine protein kinase activity, which is stimulated upon ligand binding (1-3). In addition, PDGF and EGF have been reported to stimulate the hydrolysis of membrane polyphosphoinositides (PPI) with generation of two second messengers, inositol-1,4,5-trisphosphate (Ins-1,4,5-P3) and 1,2-sn-diaclyglycerol (4-9). Binding of Ins-1,4,5-P3 to a specific intracellular site causes the release of $Ca^{2+}$ from a microsomal store, with consequent rise of the cytosolic free $Ca^{2+}$ concentration, $[Ca^{2+}]$ i (10). Concomitantly, the $Ca^{2+}$ conductance across the plasma membrane is stimulated (10, 11). These effects, however, are not general for all GF. In various cell types EGF itself has been found unable to cause detectable PPI hydrolysis and $[Ca^{2+}]$; increase (6, 12, 13); with insulin and IGF-I this lack of effect was reported in all cells investigated (14-17).

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tained in a subconfluent state. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂, 95% air.

**Measurement of [Ca²⁺].**—PC12 cells were detached from the dish by gently streaming culture medium over the dish of means of a Pasteur pipette. Detached cells were washed twice and resuspended in the incubation medium (KRH) containing (in millimolar/liter): NaCl, 140; KCl, 5; CaCl₂, 2; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 6; Hepsel, 25 (pH 7.4). Final cell concentration was 10⁶/ml. An aliquot of 10⁶ unloading cells was used for autofluorescence measurements. Loading of cells with fura-2 (21, 22) was carried out by a 30-min incubation with 5 μM fura-2/acetoxymethylester (fura-2/AM) at 37 °C in a water bath with occasional shaking. Suspensions were then diluted 1:5 with warm KRH, incubated for a further 15 min at 37 °C and washed. Aliquots (10⁵ cells/measurement) in Eppendorf tubes were stored on ice until the beginning of the assays. Before each assay the cells were pelleted by centrifugation (2 x 10³ x g) and resuspended in 1.5 ml of warm KRH. The suspension was transferred to a fluorometer cuvette housed in a thermostatted holder. Fluorescence readings were taken in a Perkin-Elmer LS-5B fluorimeter, at excitation and emission wavelengths of 345 and 490 nm, respectively, with slits of 5 nm. At the end of each measurement, fura-2 fluorescence changes were calibrated in terms of [Ca²⁺] as described by a single wavelength protocol similar to that reported by Arslan et al. (23) for quin-2. The K₅₀ of the fura-2/Ca²⁺ interaction was taken to be 225 nM (25). [Ca²⁺] measurements were routinely corrected for cell autofluorescence and dye leakage. This same protocol of [Ca²⁺] measurements was used with both 3T3 fibroblasts and SKNBE neuroblastoma cells after treatment of the cells by trypan blue as described in Ref. 8.

**Ca²⁺ Influx.**—For the measurement of unidirectional Ca²⁺ influx, PC12 cells were harvested, washed twice with warm KRH, and resuspended in the same medium (5 x 10⁶ cells/ml). The cells were preincubated at 37 °C for 3 min before addition of either BK (100 nM) or vehicle. After a further 6 min at 37 °C, [Ca²⁺] (10 μCi/ml) was added together with [H]sucrose (1 μCi/ml, as a marker for extracellular space) with or without GF. Aliquots (200 μl) of the incubated samples were withdrawn at the indicated time points, mixed with EGTA and ruthenium red to instantaneously block influx, and rapidly spun through a mineral oil layer (24).

**PP1 Hydrolysis.**—PC12 cell monolayers were incubated for 24 h with 3–5 μCi/ml of myo-[³H]inositol in basal Eagle’s medium (inositol-free) supplemented with dialyzed fetal calf serum (10%) and horse serum (5%). Before use, cells were detached from the dish, washed twice with warm KRH, and preincubated at 37 °C in KRH with or without 10 mM LiCl for 10 min, after which the various agents were added. Two different experimental protocols were investigated. In the first, insulin or EGF were added to the cells, and the incubations terminated 120 s later. In the second set of experiments BK was added first, followed 6 min later by 30–120-s treatments with insulin, IGF-I, or EGF. In all cases, the experiments were terminated by the addition of trichloroacetic acid (7.5%, final concentration) and the preparations were stored on ice for 30 min. Total inositol phosphates (Ins-P) generated during cell incubation were recovered by anion exchange chromatography (25). For the separation of the various Ins-P, the HPLC procedure described by Batty et al. (26) was used in ether-washed trichloroacetic acid supernatants supplemented with a phytic acid hydrolysate to improve Ins-P recovery (27). Radioactivity of total and separated Ins-P was counted in a Beckman LS 7500 spectrometer. Trichloroacetic acid-insoluble material was solubilized in 500 μl of 0.2 N NaOH, and protein assayed (28) using bovine serum albumin as the standard. Experiments were carried out in quadruplicate.

**DNA Synthesis.**—Swiss 3T3 fibroblasts were plated in 24-well plates at 50,000 cells/well. Two days thereafter, when cells were confluent, the media was removed and substituted with serum-free Dulbecco’s modified Eagle’s medium. Thirty h later, BK and/or GF were added to cultures. A [³H]thymidine (3 μCi/well) pulse was administered during the last 6 h of a 24-h incubation with the different agents. Monolayers were then washed twice with cold phosphate-buffered saline, and acid-insoluble radioactivity extracted and counted as described (29).

**Materials.**—IGF-I was the generous gift of Dr. R. E. Humbel (University of Zürich), Arg²-[Hyp³, Thi⁵, d-Phe⁶]BK of Dr. D. Regoli, University of Sherbrooke, and pertussis toxin of Dr. R. Rappuoli, Schlavo Institute, Siena. Carbachol, bombesin, vasopressin, ATP, verapamil, indomethacin, BK, des-Arg²[Leu⁵]BK, and porcine insulin were from Sigma; EGF, receptor grade, and FGF from Collaborative Research; rat TGFP was from Peninsula Laboratories; TGFβ from Behring Diagnostics; sera and culture media were from Flow Laboratories; Dowex ion exchange resin and chemicals for the measurement of Ins-P from Fluka; [³H]Ca²⁺; myo-[³H]inositol and [³H]Ins-P standards were from Amersham Corp. NGF was the generous gift of Dr. P. Calissano (Institut of Cell Biology, Rome).

**RESULTS**

**[Ca²⁺], in PC12 Cells.**

Effects of GF with and without BK—Figs. 1–3 illustrate the [Ca²⁺], effects induced in PC12 cell suspensions loaded with fura-2 by various GF, administered either alone or in combination with the natural nonapeptide BK. As can be

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FIG. 1. [Ca²⁺], effects of GF administered to PC12 cell suspensions alone (A) or after administration of an optimal dose of BK (100 nM, B–F). Suspensions of cells were loaded with 5 μM fura-2/AM for 30 min at 37°C, and analyzed in a thermostatted cuvette under continuous stirring. The concentration of cells ranged from 3 x 10⁵ to 10⁶/ml. GF were administered where indicated by the arrows at the following final concentrations: insulin and IGF-I, 50 nM; EGF, 20 nM; TGFα, 10 nM; FGF, 10 nM.
seen in Fig. 1A, when insulin, IGF-I, or EGF were given alone (concentration range: 1-100 nM), no change of the resting [Ca\(^{2+}\)](o), (on the average, 132 ± 6 nM, mean ± S.E., n = 54) was observed (see also Ref. 13). Likewise, no change of [Ca\(^{2+}\)](o) was observed with either TGF\(_\alpha\) and TGF\(_\beta\) (10 and 0.8 nM, data not shown and Fig. 3E, respectively) or basic FGF (not shown). A completely different situation was observed, however, when GF were administered to PC12 cells previously challenged with BK. The [Ca\(^{2+}\)](o) effect of this peptide is due to a dual mechanism: release of Ca\(^{2+}\) from intracellular store(s) mediated by Ins-1,4,5-P\(_3\), and stimulation of Ca\(^{2+}\) influx across the plasma membrane (27, 30, 31). The first such mechanism is responsible for a large part of the [Ca\(^{2+}\)](o) spike during the initial 60-120 s, whereas the second mechanism accounts for the subsequent, prolonged steady-state [Ca\(^{2+}\)](o) elevation (Figs. 1–3). Insulin, IGF-I, EGF, FGF, and TGF\(_\alpha\) (but not TGF\(_\beta\), not shown), administered during the [Ca\(^{2+}\)](o) steady-state induced by BK, were found able to trigger further increases of [Ca\(^{2+}\)](o). Such increases became appreciable after a lag of 20-40 s from the GF application, and remained visible for several minutes thereafter (Fig. 1, B–F, and see below).

In order to clarify the source(s) of the [Ca\(^{2+}\)](o), increases induced by GF, experiments were carried out in which the Ca\(^{2+}\) concentration in the medium, [Ca\(^{2+}\)](o), was switched from millimolar to very low levels by the use of EGTA (Fig. 2, A–C). When excess EGTA was added to the cells before BK, the initial [Ca\(^{2+}\)](o) peak induced by the peptide was largely maintained, whereas the subsequent steady-state plateau and the GF-induced transients were lost (Fig. 2A). In contrast, the GF-induced [Ca\(^{2+}\)](o) transients were still observed when either Ca\(^{2+}\) was reintroduced into the medium before GF addition (Fig. 2B) or EGTA was administered after BK, shortly before GF (Fig. 2C). The effect of GF on Ca\(^{2+}\) homeostasis in BK-primed cells was also investigated by unidirectional \(^{45}\)Ca\(^{2+}\) flux experiments. \(^{45}\)Ca\(^{2+}\) was administered to the cells (with or without a GF) 6 min after priming with BK, i.e. during the [Ca\(^{2+}\)](o) steady-state phase induced by the peptide. As can be seen in Fig. 3, at 60-120 s the tracer accumulation was found to be significantly greater in the cells treated with either EGF or IGF-I with respect to controls. Taken together, the results of Figs. 2 and 3 demonstrate that, similar to the BK-induced [Ca\(^{2+}\)](o), increases, also the GF-induced transients include two components, one originated from intracellular Ca\(^{2+}\) stores, which is maintained even after [Ca\(^{2+}\)](o) chelation (Fig. 2C), the other from extracellular Ca\(^{2+}\), which is responsible for both the [Ca\(^{2+}\)](o) increase after Ca\(^{2+}\) reintroduction into the medium (Fig. 2B) and the increased \(^{45}\)Ca\(^{2+}\) accumulation (Fig. 3). The intracellular store activated by the GF was apparently depleted (or otherwise inactivated) within minutes from the application of BK in the EGTA-containing medium, and required therefore the chelator and GF to be administered in rapid sequence in order to be revealed (compare Fig. 2, A and C).

When insulin or another GF was administered to PC12...
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Fig. 4. [Ca\(^{2+}\)], effects of two successive administrations of some or different GF, given to PC12 cell suspensions before or after BK (100 nM). Concentrations of cells and GF as described in the legend to Fig. 1, except for TGF\(\beta\), that was given at 0.8 nM.

A

B

C

D

E

Fig. 5. Concentration dependence of the [Ca\(^{2+}\)], rise induced by insulin (□), IGF-I (▲), and EGF (●) administered to PC12 cells 4 min after the administration of BK (100 nM). Values given (averages of two measurements from a representative experiment that was repeated twice) are percent [Ca\(^{2+}\)], increase with respect to the BK-induced steady-state level before the GF addition.

cells before BK, no appreciable effect on the [Ca\(^{2+}\)], transients induced by the peptide (range of BK concentrations used 0.1-100 nM) was observed. However, the pre-BK administration of saturating concentrations of a GF inhibited the [Ca\(^{2+}\)], increase expected from a second, post-BK administration of the same factor (Fig. 4A and data not shown). In contrast, the effect of the post-BK administration was maintained, although only in part, when the pre-BK treatment was made with a different GF (compare Fig. 4, A, C, and D). Likewise, when saturating concentrations of different GF were administered in sequence after BK, each of them induced measurable responses (Fig. 4B), but their sum was smaller than the sum of the responses elicited by the same concentration of GF in separate cell aliquots. In contrast, when the same GF was administered twice to the same aliquot of BK-pretreated cells, the second administration was ineffective (not shown in Figures). Among the GF used, only TGF\(\beta\) was unable to induce the post-BK [Ca\(^{2+}\)], transient in PC12 cells. In this respect, however, it is worth mentioning that the complement of receptors for this GF has been recently shown to be very low in this cell line (32).

Concentration and Time Dependence of the [Ca\(^{2+}\)], Effects of GF—EGF proved to be the most potent (apparent EC_{50}: 1 nM with respect to 6 and 10 nM for IGF-I and insulin, respectively) and efficacious (approximately 90 and 40% rise over the BK-induced steady-state plateau with maximal concentrations of EGF versus insulin and IGF-I, respectively) stimulator of post-BK [Ca\(^{2+}\)], transients (Fig. 5). The transients were also found to differ in their kinetics depending on the nature and concentration of the factors employed (Fig. 6). With EGF the lag phase was shorter and the [Ca\(^{2+}\)], rise steeper with respect to insulin and IGF-I (Fig. 6, A and B). [Ca\(^{2+}\)], transients by EGF, on the other hand, were distinctly shorter lived (on the average 5.0 ± 0.3 min, \(n = 12\)) with respect to those induced by insulin and IGF-I (7.4 ± 0.4 and 7.1 ± 0.7 min; \(n = 29\) and 12, respectively) (Fig. 6B). TGF\(\alpha\) closely resembled EGF in its effects on [Ca\(^{2+}\)].

The [Ca\(^{2+}\)], Increases by GF Are Strictly Dependent on B\(_2\) Receptor Activation—The dependence of the GF-induced
Mechanism of the GF-induced [Ca\textsuperscript{2+}]\textsubscript{i}, Increases

High [Ca\textsuperscript{2+}]\textsubscript{i}, and PPI Hydrolysis Are Not Sufficient to Prime PC12 Cells—The possibility that the priming effect of BK was the mere consequence of the [Ca\textsuperscript{2+}]\textsubscript{i}, increase induced by the peptide was excluded by experiments in which [Ca\textsuperscript{2+}]\textsubscript{i}, was increased by a different mechanism, i.e. high K\textsuperscript{+} depolarization (34). Treatment with 50 mM KCl caused [Ca\textsuperscript{2+}]\textsubscript{i} to increase approximately to the steady-state level achieved after 50 nM BK, yet insulin administered 4 min after K\textsuperscript{+} was without effect on [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 8A). This latter result was not due to a voltage-dependence of the GF effect because administration of 50 mM K\textsuperscript{+} after BK did not inhibit the [Ca\textsuperscript{2+}]\textsubscript{i}, rise induced by a subsequent administration of insulin (Fig. 8B). The negative results with high K\textsuperscript{+} exclude the involvement in the GF effect of voltage-gated Ca\textsuperscript{2+} channels, i.e. the channels responsible for the [Ca\textsuperscript{2+}]\textsubscript{i}, effects of depolarizing agents (35, 36). This latter conclusion is supported also by experiments with blockers of voltage-gated Ca\textsuperscript{2+} channels, such as verapamil. At a concentration (10 \mu M) known to block the K\textsuperscript{+}-induced [Ca\textsuperscript{2+}]\textsubscript{i}, increase (34, 36), this drug failed to modify appreciably both the BK-induced and the subsequent GF-induced [Ca\textsuperscript{2+}]\textsubscript{i}, transients (not shown in Figures).

Activation of muscarinic receptors by carbachol is known to induce in PC12 cells [Ca\textsuperscript{2+}]\textsubscript{i}, transients due to mechanisms similar to those triggered by BK, i.e. PPI hydrolysis with Ca\textsuperscript{2+} release from internal stores accompanied by stimulated Ca\textsuperscript{2+} influx (37, 38). Carbachol (100 \mu M), however, failed to mimic the priming effect of BK on the insulin and EGF-induced [Ca\textsuperscript{2+}]\textsubscript{i}, effects (Fig. 8C). Negative results similar to those with carbachol were obtained with NGF (Fig. 8D) and ATP (not shown). Both these agents are able to induce [Ca\textsuperscript{2+}]\textsubscript{i}, increases in PC12 cells within seconds from their administration (Ref. 19).

Prolonged (1–2 weeks) treatment with NGF causes PC12 cells to modify their phenotype by sprouting long neurites and acquisition of various receptors and channels (20). Other receptors, however, such as those for EGF, have been found recently to be decreased by the NGF-induced differentiation (39). Accordingly, the [Ca\textsuperscript{2+}]\textsubscript{i}, effects of the various agents were differently modified by NGF-induced differentiation. The response to BK was unchanged, but the subsequent responses to both insulin and EGF were distinctly smaller (24 ± 11% and 39 ± 17% of controls, mean ± S.D., n = 3, for insulin and EGF, respectively) than in NGF-untreated cells (compare Figs. 1E and 8E).

Another treatment that modified the GF-induced response was that with phorbol esters. Phorbol 12-myristate 13-acetate (100 nM), administered before or after BK, caused the [Ca\textsuperscript{2+}]\textsubscript{i}, transients induced by insulin and IGF-I to be partially inhibited (72 ± 19%, n = 4, and 69 ± 12%, n = 3, respectively) and those by EGF to be completely blocked (Fig. 8, F and G). Analogous results were obtained by using 250 nM phorbol 12,13-dibutyrate (not shown). Under the same conditions the [Ca\textsuperscript{2+}]\textsubscript{i}, transients by BK were only moderately inhibited, as reported in detail in Ref. 27. In contrast, no changes of insulin- and EGF-induced [Ca\textsuperscript{2+}]\textsubscript{i}, transients were observed in PC12 cells pretreated with the blocker of the cyclooxygenase path-

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2 A. Pandiella and J. Meldolesi, unpublished results.
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**Fig. 7.** [Ca^{2+}]_i effects of insulin in PC12 cell suspensions: dependence on the concentration of BK and effects of the Bz receptor antagonist. The traces A, B, and C were obtained with different concentrations of BK (20, 0.5, and 0.1 nM, respectively) followed by the same concentration of insulin (200 nM). A direct comparison of the time course of the insulin-induced [Ca^{2+}]_i increases with the first two [BK] (20 and 0.5 nM) is shown in the inset (averages of three determinations). Traces D and E show the effects of the specific Bz receptor antagonist, Arg^2-[Hip3,Thy5,8,D-Phe^7]BK (I-Bz, 10 μM) administered either before (D) or immediately after (E) IGF-I. Identical results were obtained using EGF or insulin. Concentrations of cells are as described in the legend to Fig. 1.

**Fig. 8.** Effect of various treatments on the [Ca^{2+}]_i increases induced by insulin in PC12 cell suspensions. Treatments were given where indicated at the following final concentrations: insulin, 200 nM; KCl, 50 mM; BK, 50 nM; NGF, 100 ng/ml; carbachol (CCh), 100 μM; EGF, 30 nM; phorbol 12-myristate 13-acetate (PMA), 100 nM. The cells shown in E had been pretreated with NGF (60 ng/ml) for 10 days in order to induce a neuron-like phenotype.
whether the administration of GF after BK results in a further increase of 
proteins of the Go and Gi class (40).

be reinforced by GF are known to be triggered via the acti-
mentation because of a role of \([\text{Ca}^{2+}]\) in the transduction of 
membrane potential (hyperpolarization; activation of voltage-
ated 30, 80, and 120 s. The reactions were quenched 
with ice-cold trichloroacetic acid, and neutralized extracts analyzed 
by anion exchange HPLC as in Ref. 26. The results are expressed as 
percent of BK-stimulated Ins-P production (mean \(\pm\) S.D. of duplica-
to exist also in the other two unrelated cell types we 
had been found to induce large increases of \([\text{Ca}^{2+}]\). In the 
case of insulin, a rise of \([\text{Ca}^{2+}]\) was predicted based on indirect 
evidence (41) but the prediction was never documented. In the 
present study we have found that the combination of BK with 
either insulin or EGF is able to trigger a marked mito-
genetic effect in Swiss 3T3 fibroblasts, although the peptide 
(and also EGF) was devoid of any effect on cell growth when 
given alone.

**DISCUSSION**

The results herewith described demonstrate an up to now 
unreported effect of insulin, EGF, and various other GF, \(i.e.\) the 
reinforcement of the PPI hydrolysis and \([\text{Ca}^{2+}]\), increase 
effects elicited by the nonpeptide BK through the activation of \(B_2\) receptors. This new type of cross-talk was studied 
that yields maximal \([\text{Ca}^{2+}]\), effects) or EGF administered alone, 
whereas insulin triggered an approximately 3-fold increase 
over basal. On the other hand, the combination of BK with 
either one of the two GF caused a marked increase of the 
incorporation: over 3-fold for insulin, and almost 4-fold for 
EGF.

**Table I**

Growth Factor Reinforcement of \(B_2\) Receptor Signals

| Time (s) | BK (100 ± 5) | BK + EGF (100 ± 6) | BK + IGF-I (100 ± 5) | BK + EGF (100 ± 10) | BK + IGF-I (80 ± 10) | BK + EGF (95 ± 10) | BK + IGF-I (85 ± 14) |
|----------|--------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| 30       | 122 ± 3      | 117 ± 1            | 121 ± 5            | 127 ± 1            | 127 ± 2            | 127 ± 2            | 127 ± 2            |
| 80       | 104 ± 5      | 106 ± 6            | 100 ± 5            | 95 ± 10            | 110 ± 11           | 133 ± 9            | 114 ± 10           |
| 120      |              |                    |                    |                    |                    |                    |                    |
| BK       | 100 ± 1      | 100 ± 2            | 100 ± 4            | 100 ± 4            | 100 ± 4            | 100 ± 4            | 100 ± 4            |

**Effects of GF Alone, with BK, and with Other Stimulators of PPI Hydrolysis**

The effects of GK on \([\text{Ca}^{2+}]\), were inves-
tigated in two additional cell lines related to PC12: the 
mouse Swiss 3T3 fibroblasts and human neuroblastoma 
SKNBE lines, with results (Fig. 9) highly consistent with 
those described in the pheochromocytoma cell line. Thus, 
EGF caused no reproducible \([\text{Ca}^{2+}]\), increase when adminis-
tered alone (Fig. 9, A and E), but was effective when adminis-
tered after BK (Fig. 9, B and F). Similar results were 
observed with insulin and IGF-I (not shown). In SKNBE 
neither high \(K^+\) (not shown), nor carbachol and ATP (Fig. 9, 
G and H), induced any priming effect with respect to GK. As 
in PC12, these treatments increase \([\text{Ca}^{2+}]\), in SKNBE cells,

**Fig. 10.** Incorporation of \([\text{H}]\)thymidine into DNA was not 
significantly modified by either BK (at a concentration that 
yields maximal \([\text{Ca}^{2+}]\), effects) or EGF administered alone, 
whereas insulin triggered an approximately 3-fold increase 
over basal. In the other hand, the combination of BK with 
either one of the two GF caused a marked increase of the 
incorporation: over 3-fold for insulin, and almost 4-fold for 
EGF.
Mechanisms of GF-induced B2 Receptor Reinforcement -

The BK-primed [Ca²⁺], rises induced by insulin, IGF-I, and EGF appear to be generated independently, with each GF working through the activation of its specific receptor. In fact, (i) the concentration dependence of the [Ca²⁺] rises was found to be similar to that of other physiological effects of the GFs; (ii) the concentration dependence of the insulin and IGF-I-induced responses were almost identical, while receptor sharing of these two GF implies different binding affinities (14, 15); and (iii) at least part of the responses were maintained in cells exposed to another GF before the application of BK, compared to the disappearance observed when the same GF was used for both, pre- and post-BK administrations. On the other hand, the responses triggered by successive administrations of two GF after BK were less than additive. These results suggest that, although originated at different receptors, the signals triggered by the various GF converge intracellularly to activate partially overlapping mechanisms. The nature of these mechanisms has been elucidated only in part.

The PPI hydrolysis experiments revealed that GF effects can be triggered when the generation of Ins-1,4,5-P₃ (and of its phosphorylation product, Ins-P₄) is already stimulated by BK, and consists in an appreciable increase of the Ins-1,4,5-P₃ generation process. The occurrence of reinforcement over a large spectrum of [BK] seems to exclude that the GF effect consists in an increase of B2 receptor affinity. Rather, the process could consist in the transient recoupling of B2 receptors to the PIP₂-specific phospholipase C, with consequent increase of [Ca²⁺], via both release from intracellular stores and activation of plasma membrane conductance. Receptors for the GF competent for reinforcement, in particular insulin, EGF, and IGF-I, are known to possess an intrinsic tyrosine kinase activity, which is activated following receptor occupancy (1-3). Tyrosine phosphorylation of some cellular proteins is stimulated also by FGF (42). The apparent specificity for the B2 receptor of the reinforcement process suggests the possibility that this receptor is, directly or indirectly, modified by the tyrosine kinase activity of GF receptors. Alternatively, the target of the GF receptors could not be the B2 receptor but the G protein that couples this (and possibly others, but not all) receptor to phospholipase C (40, 43, 44). In conclusion, the present results demonstrate that stimulation of PPI hydrolysis and increased [Ca²⁺], are part of the array of intracellular signals that can be elicited by not only PDGF, but also other GF and even insulin, in various cell types, via a reinforcement of transmembrane signaling. Whether the rein-

\[ \text{Growth Factor Reinforcement of B2 Receptor Signals} \]
forcing effect of GF can occur exclusively through the B2, or concerns other receptors as well, remains to be investigated.

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