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Synergism in cytosolic Ca^{2+} mobilization between bradykinin and agonists for pertussis toxin-sensitive G-protein-coupled receptors in NG 108-15 cells

Fumikazu Okajima and Yoichi Kondo

Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

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Bradykinin (BK) induced a transient and pertussis toxin (PT)-insensitive increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in NG 108-15 neuroblastoma × glioma hybrid cells, whereas leucine-enkephalin (EK), somatostatin, norepinephrine or carbachol showed a weak but PT-sensitive action. When any one of the latter agonists was applied to the cells treated with low doses of BK, however, the level of $[Ca^{2+}]_i$ rise caused by the agonist was remarkably increased in a PT-sensitive manner. The decreasing of extracellular Ca^{2+} only slightly influenced the actions of these agonists. Thus, synergism between a BK receptor and PT-sensitive G-protein-coupled receptors results in marked intracellular Ca^{2+} mobilization by the latter agonists.

Enkephalin; GTP-binding protein; Pertussis toxin; Cytosolic Ca^{2+}; NG 108-15 cell

1. INTRODUCTION

NG 108-15 neuroblastoma × glioma hybrid cells (NG 108-15 cells) possess a variety of neurotransmitter receptors [1,2], e.g. opioid, α_2-adrenergic, muscarinic and somatostatin receptors coupled to adenylate cyclase in an inhibitory manner [3–5], opioid and somatostatin receptors coupled to Ca^{2+} channels in an inhibitory manner [6,7] and a bradykinin (BK) receptor coupled to a phospholipase C–Ca^{2+} system in a stimulatory manner [8,9].

We have previously shown [10–13] that the simultaneous activation of two different receptors resulted in synergism in the responses to their agonists, e.g. adenosine, even though it has almost no Ca^{2+}-mobilizing activity by itself, cooperated with one of the agonists with Ca^{2+}-mobilizing activity such as P_2-purinergic agonists, α_1-adrenergic agonists and thyrotropin, resulting in a remarkable potentiation of phospholipase C stimulation followed by Ca^{2+} mobilization in FRTL-5 thyroid cells. These results suggest the presence of a similar cross-talk mechanism in nervous system, where the neuronal activity would be under the control of a variety of neurotransmitters.

Investigating the regulation of Ca^{2+} metabolism in NG 108-15 cells, we have noticed that leucine-enkephalin (EK), an opioid receptor agonist, induced a transient and slight increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and this EK effect became quite obvious when it was applied after BK application, suggesting the cooperative action of two agonists. Here, we characterized the synergism between EK and BK in a $[Ca^{2+}]_i$ rise and found it to be pertussis toxin (PT)-sensitive. In addition, BK cooperated similarly with several agonists for receptors coupled to PT-sensitive G-proteins.

Abbreviations: EK, leucine-enkephalin; BK, bradykinin; PT, pertussis toxin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; G-proteins, GTP-binding proteins.

Correspondence address: F. Okajima, Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan. Fax: (81) (272) 34 1788.

2. MATERIALS AND METHODS

2.1. *Materials*

EK, BK, somatostatin, carbachol and naloxone were purchased from Sigma. PT was generously provided by Dr. Michio Ui of Tokyo University (Tokyo). The sources of all other reagents were those described in [10–14].

2.2. *Cell culture*

NG 108-15 cells, neuroblastoma × glioma hybrid cells [1] (provided by Dr. Haruhiro Higashida of Kanazawa University (Kanazawa, Japan)) were cultured for 7–9 days as described in [14]. Where indicated, PT treatment of the cells was performed by adding the toxin (10 ng/ml) to the medium 18 h before experiments. The cells were grown on 10-cm culture dishes (Costar).

2.3. *Measurement of $[Ca^{2+}]_i$ in cell suspension*

The cells were harvested from the dishes with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline containing 4 mM EDTA. After centrifugation at 500 × *g* for 5 min, the cells were resuspended in Ham's 10 medium containing 5% calf serum and 20 mM HEPES (pH 7.4), and incubated for 20 min at 37°C. The cells were again sedimented at 500 × *g* for 5 min, then resuspended in Ham's 10 medium containing 0.1% bovine serum albumin (Fraction V), and incubated for 20 min with 1 μM fura-2 AM at 37°C. $[Ca^{2+}]_i$ was measured as described in [10,15].

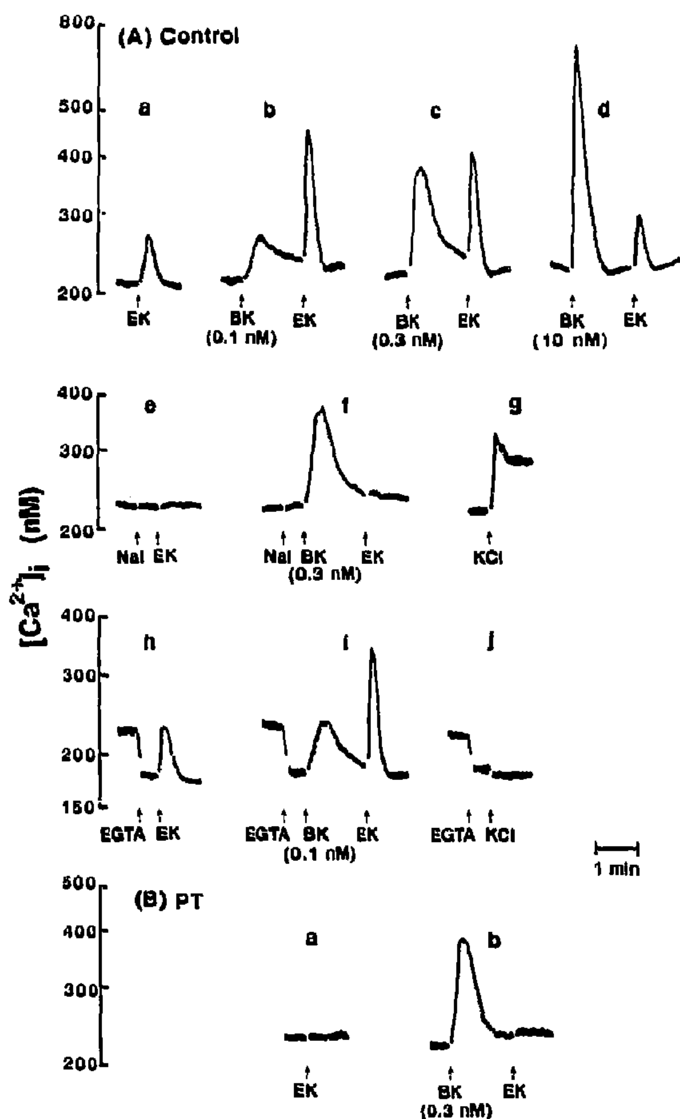

Fig. 1. Effect of EK on $[Ca^{2+}]_i$ in a cell suspension. The cells were treated without (A) or with (B) PT. At arrows, EK (1 μM), the indicated dose of BK, naloxone (Nal, 10 μM), KCl (50 mM) and EGTA (2.2 mM) were applied. The representative of at least four separate experiments is shown.

3. RESULTS AND DISCUSSION

In accordance with previous results [9], BK increased $[Ca^{2+}]_i$ in a dose-dependent manner (Figs. 1A(b–d), and 2A). The activity of 1 μM EK was as low as that of 0.1 nM BK (Fig. 1A(a and b)). When EK was applied following the addition of the lower doses of BK, however, the level of EK-evoked $[Ca^{2+}]_i$ increase was greatly increased (Fig. 1A(a–c)). On the other hand, at the higher BK concentration the potentiation of the EK activity was reduced; at 10 nM, the BK effect on the EK action disappeared (Fig. 1A(a and d)). The half-maximal effective dose for the EK action was around 30 nM both in the cells treated and untreated with BK (Fig. 2B and C).

The EK-dependent $[Ca^{2+}]_i$ increase was almost completely reversed by prior treatment of the cells with naloxone, an opioid receptor antagonist (Fig. 1A(e and f)) and with PT (Fig. 1B and Fig. 2B and C), whereas these agents hardly affected the $[Ca^{2+}]_i$ response to BK alone (Fig. 1A(c and f), and 2A). Thus, the EK action seems to be mediated by opioid receptors and PT-sensitive G-proteins.

Under the lower Ca^{2+} conditions where the KCl-induced $[Ca^{2+}]_i$ increase due to Ca^{2+} influx through voltage-dependent Ca^{2+} channels was completely abolished (Fig. 1A(g and j)), the BK-induced $[Ca^{2+}]_i$ rise remained unchanged (Fig. 1A(b and i), and Table I). This result is consistent with a previous finding that the major Ca^{2+} source which responded to BK was an intracellular Ca^{2+} pool [9]. The enhancement of the EK-induced action was still observed, though the degree of the enhancement was slightly reduced, under the lower Ca^{2+} conditions (Fig. 1A(h and i), and Table I). Thus, both BK and EK stimulate Ca^{2+} mobilization from the intracellular store, although some participation of Ca^{2+} influx across the plasma membrane cannot be completely ruled out especially for the EK action.

In Fig. 3, we examined the effect of agonists which have been shown, similarly to EK, to activate PT-sensitive G-protein-coupled receptors [3–7]. Norepinephrine, somatostatin or carbachol alone induced a small $[Ca^{2+}]_i$ rise (Fig. 3A and C). When any one of these agonists was applied after the BK stimulation, the $[Ca^{2+}]_i$ increase in the second stimulation caused by the agonist was much greater than that without prior BK stimulation (Fig. 3A and C). The norepinephrine (1 μM) action was inhibited markedly by 10 μM yohimbin, but only slightly by the same dose of prazosin (data not shown), indicating that norepinephrine acted through an α_2-adrenergic receptor [4]. The $[Ca^{2+}]_i$ increases, both in the cells treated and untreated with BK, induced by norepinephrine, somatostatin and carbachol were completely reversed by the PT-pretreatment of the cells (Fig. 3B and C).

Table I

Effect of extracellular Ca^{2+} on BK and EK-induced $[Ca^{2+}]_i$ rise

	BK (Δ nM)	EK (Δ nM)	
		Alone	After BK
	(A)	(B)	(C)
Regular Ca^{2+}	47 ± 6	63 ± 10	188 ± 11*
Low Ca^{2+}	62 ± 5	71 ± 6	137 ± 9*

Experimental conditions were essentially the same as those in Fig. 1A(a and b) for 'Regular Ca^{2+}' and Fig. 1A(h and i) for 'Low Ca^{2+}', respectively. BK, 0.1 nM; EK, 1 μM. The peak rise in $[Ca^{2+}]_i$ obtained by BK alone (A), EK alone (B), or EK following BK (C) is shown. The results are the means ± SE of 8 values from four separate experiments.

*Significantly different from Alone (P<0.01).

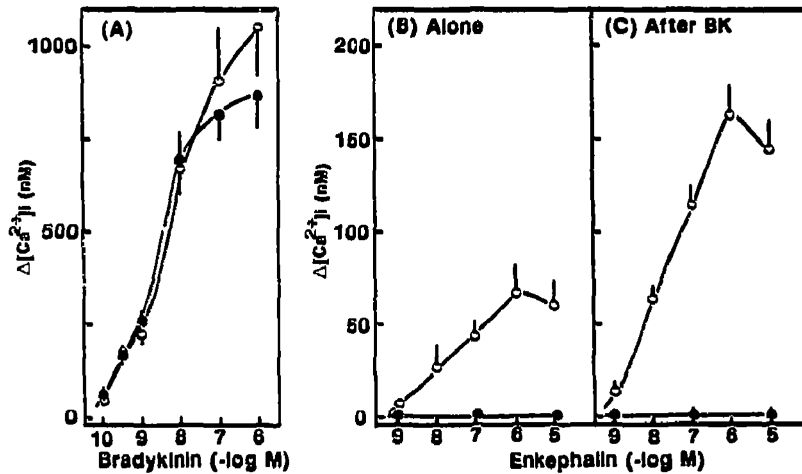

Fig. 2. Effect of PT on dose-response curves of BK and EK-induced $[Ca^{2+}]_i$ rise. The cells were treated without (○) or with (●) PT. Experimental protocols were similar to those for Fig. 1A(a and b) except for the dose of BK or EK. Peak rises in $[Ca^{2+}]_i$ induced by the indicated dose of BK alone (A), EK alone (B), or EK following BK (0.1 nM) (C) are plotted. The results are the means ± SE of four separate experiments.

EK and somatostatin have been reported to inhibit Ca^{2+} channels in NG 108-15 cells [6,7], suggesting a possible decrease in $[Ca^{2+}]_i$. α-Receptors have not been shown to couple to the phospholipase C–Ca^{2+} pathway. These observations conflict with our present results in which these agonists slightly but appreciably induced $[Ca^{2+}]_i$ rises without the cooperation of BK. In relation to this discrepancy, it is noted that, in our preliminary study, the prior application of exogenous adenine nucleotides such as ATP and ADP in place of BK enhanced the EK-induced $[Ca^{2+}]_i$ rise. The results raise the possibility that adenine nucleotides which may be released from damaged cells during cell harvesting and washing allowed EK and other agonists to increase in Ca^{2+}. This possibility is now under investigation.

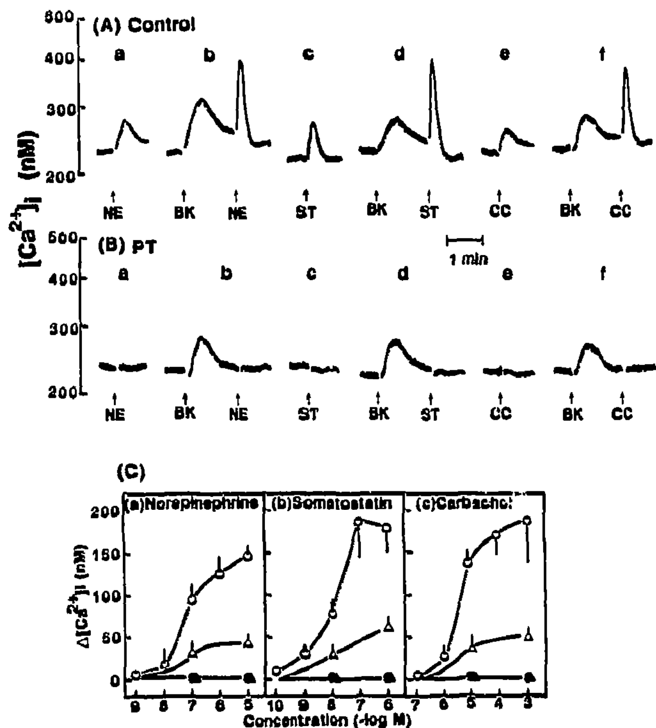

Fig. 3. Effect of norepinephrine, somatostatin and carbachol on $[Ca^{2+}]_i$. The cells were treated without (A and ○, △ in C) or with (B and ●, ▲ in C) PT. Experimental conditions were the same as those for Figs. 1 and 2. In A and B, at arrows, EK (1 μM), BK (0.1 nM), norepinephrine (NE, 1 μM), somatostatin (ST, 100 nM) and carbachol (CC, 100 μM) were applied. The representative of at least four separate experiments is shown. In C, peak rises in $[Ca^{2+}]_i$ induced by the indicated dose of norepinephrine (a), somatostatin (b) or carbachol (c) alone (△,▲) or these agonists following BK (○,●) are plotted. The results are the means ± SE of four separate experiments.

BK has been reported to increase $[Ca^{2+}]_i$ in association with phospholipase C activation [8,9]. As shown in Fig. 1A and Table I, Ca^{2+} seems to be mainly derived from the intracellular store in response to BK and also EK, which may account for the small $[Ca^{2+}]_i$ increase caused by EK after the higher dose (10 nM) BK application (Fig. 1A(d)); the higher dose of BK probably leads to the shortage of Ca^{2+} in its pool. The results also suggest that EK may activate phospholipase C in the BK-stimulated cells. Indeed, this was true in our preliminary results, suggesting that the primary effect of BK and EK synergism was the enhancement of the phospholipase C stimulating mechanism.

A similar cross-talk or synergism between two receptor systems, i.e. one leading to the activation of the phospholipase C–Ca^{2+} pathway and the other to the inhibition of the adenylate cyclase pathway mediated by PT-sensitive G-proteins, has recently been reported in FRTL-5 thyroid [10–13] and astrocytes [16]. In these systems as well, PT-sensitive G-proteins are involved in the cross-talk. Thus, such a synergistic $[Ca^{2+}]_i$ rise through PT-sensitive G-proteins might be one of the universal mechanisms regulating Ca^{2+} metabolism.

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