Enkephalin activates the phospholipase C/Ca²⁺ system through cross-talk between opioid receptors and P₂-purinergic or bradykinin receptors in NG 108-15 cells

A permissive role for pertussis toxin-sensitive G-proteins

Fumikazu OKAJIMA,* Hideaki TOMURA and Yoichi KONDO

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

In an NG 108-15 neuroblastoma × glioma hybrid cell suspension, extracellular ATP (via P₂-purinergic receptors) and bradykinin stimulated Ins(1,4,5)P₃ formation, which was accompanied by an increase in the cytosolic Ca²⁺ concentration ([Ca²⁺]_i). Leucine enkephalin (EK) also slightly increased [Ca²⁺]_i in the absence, but not in the presence, of apyrase, which hydrolyses extracellular ATP and ADP to AMP. When the cells were stimulated by P₂-agonists or bradykinin prior to the application of EK, EK induces a remarkable rise in [Ca²⁺]_i. This P₂-agonist- or bradykinin-assisted EK action was also observed in single cells on a coverslip. A decrease in the extracellular Ca²⁺ concentration only slightly lowered the EK-induced rise in [Ca²⁺]_i, but treatment of the cells with thapsigargin, an agent which depletes Ca²⁺ in the Ins(1,4,5)P₃-sensitive pool, almost

completely abolished EK action. The observed permissive stimulation by EK of Ins(1,4,5)P₃ formation induced by a P₂-agonist or bradykinin may be a primary event for the EK-induced [Ca²⁺]_i rise. These actions of EK were antagonized by naloxone and completely reversed by prior treatment of the cells with pertussis toxin, whereas the toxin hardly affected the actions of P₂-agonists and bradykinin themselves. Thus EK can induce phospholipase C activation and subsequent Ca²⁺ mobilization, provided that the cells have been previously or are simultaneously stimulated by endogenous adenine nucleotides or by externally applied P₂-agonists or bradykinin. In this cross-talk mechanism between opioid receptors and these Ca²⁺-mobilizing agonist receptors, pertussis toxin-sensitive G-proteins play a permissive role.

INTRODUCTION

Opioids are generally known to inhibit neurotransmitter release or electrophysiological actions of cells, which is a cause of their analgesic effects [1,2]. These inhibitory actions have been shown to be mediated by an increase in membrane K⁺ conductance [3] or a decrease in Ca²⁺ conductance [4–6], which induces hyperpolarization or shortening of the Ca²⁺ component of the action potential leading to a decrease in Ca²⁺ influx in presynaptic nerve cells. However, a paradoxical hyperalgesic effect of exceedingly low doses of systemic morphine has been reported [7] and, in relation to this, opioids have been shown in some cases to evoke excitatory effects such as prolongation of the action potential [8]. Such opioid-induced excitatory effects have been proposed to be of physiological and pathological significance [9], but the mechanism remains unclear.

NG 108-15 neuroblastoma × glioma hybrid cells [10] possess δ-type opioid receptors, an agonist of which, leucine enkephalin (EK), inhibits a voltage-dependent Ca²⁺ channel via pertussis toxin-sensitive G-proteins [4,5]. This homogeneous cell line has therefore been used as a neural cell model in order to gain an understanding of the signal transduction mechanism of opioid and other neurotransmitter receptors [10].

We have previously found that, even though adenosine alone has no effect on phospholipase C activity or Ca²⁺ metabolism, it can (via an A₁-type P₁-receptor) induce enzyme activation and Ca²⁺ mobilization, provided that the cells are previously or simultaneously stimulated by Ca²⁺-mobilizing receptor agonists such as P₂-purinergic agonists [11,12], α₁-adrenergic agonists [13] or thyrotropin (TSH) [14] in FRTL-5 thyroid cells. The adenosine

effect was completely reversed by pertussis toxin treatment, suggesting the involvement of pertussis toxin-sensitive G-proteins in the cross-talk mechanism. Thus stimulation of the P₂-receptor and other receptors of Ca²⁺-mobilizing agonists allowed adenosine, via the pertussis toxin-sensitive G-proteins, to activate the phospholipase C/Ca²⁺ system. In NG 108-15 cells, as in FRTL-5 cells, P₂-purinergic receptors seem to couple to the phospholipase C/Ca²⁺ system [15]. These findings led us to examine whether EK, the receptors for which are coupled to pertussis toxin-sensitive G-proteins [5], acquires the ability to increase the cytosolic Ca²⁺ concentration ([Ca²⁺]_i) in association with activation of phospholipase C under conditions where the P₂-receptors of NG 108-15 cells are stimulated. We found that EK can induce the activation of phospholipase C and subsequent Ca²⁺ mobilization in a naloxone- and pertussis toxin-sensitive manner in the cells, provided that the phospholipase C/Ca²⁺ system of the cells had already been stimulated by P₂-purinergic agonists or by another Ca²⁺-mobilizing agonist, bradykinin [16–18]. The present results not only show the synergism between opioid receptors and Ca²⁺-mobilizing agonist receptors, such as P₂-purinergic and bradykinin receptors, but may also indicate a novel mechanism to account for some aspects of opioid-evoked excitatory effects in nervous systems.

EXPERIMENTAL

Materials

EK, bradykinin, naloxone, apyrase (EC 3.6.1.5) and thapsigargin were purchased from Sigma (St. Louis, MO, U.S.A.). Ins(1,4,5)P₃ assay kit was from Du Pont–New England Nuclear (Boston,

Abbreviations used: EK, leucine enkephalin; p[NH]ppA, adenosine 5'-[βγ-imido]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; PGE₁, prostaglandin E₁; [Ca²⁺]_i, cytosolic Ca²⁺ concentration.

* To whom correspondence should be addressed.

MA, U.S.A.). Pertussis toxin was generously provided by Dr. Michio Ui (Tokyo University, Tokyo, Japan). Reagents for the radioimmunoassay of cyclic AMP were a gift from Yamasa Shoya Co. (Choshi, Japan). The sources of all other reagents used for cell culture and other purposes were as described in previous papers [11–15,19].

Cell culture

NG 108-15 neuroblastoma × glioma hybrid cells [10] were cultured for 7–9 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and HAT (100 μ M hypoxanthine, 1 μ M aminopterin and 16 μ M thymidine) in an atmosphere of 5% CO₂/95% air (1:19) at 37 °C. Where indicated, pertussis toxin 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) in all experiments except for those shown in Figure 3, where they were grown on coverslips (Matsunami Glass, Tokyo, Japan).

Measurements of [Ca²⁺]_i, cyclic AMP content and Ins(1,4,5)P₃ content in cell suspensions

The cells were harvested from the dishes in Ca²⁺ and Mg²⁺-free PBS containing 4 mM EDTA. After centrifugation at 500 *g* for 5 min, the cells were resuspended in Ham's 10 medium containing 5% (v/v) calf serum and 20 mM Hepes (pH 7.4), and were then incubated for 20 min at 37 °C. The cells were again sedimented at 500 *g* for 5 min and then used for the following experiments.

For measurement of [Ca²⁺]_i, the cells were resuspended in Ham's 10 medium containing 0.1% (w/v) BSA (fraction V) and incubated for 20 min in the presence of 1 μ M fura-2/AM at 37 °C. After centrifugation at 500 *g* for 5 min and resuspension in Hepes-buffered medium [10 mM Hepes (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.0 mM CaCl₂, 2.5 mM NaHCO₃, 5 mM glucose and 0.02% (w/v) BSA (fraction V)], the cells were incubated with adenosine deaminase and/or apyrase for 5 min, then [Ca²⁺]_i was measured as described previously [19].

For measurement of the cyclic AMP response, the cells were treated with fura-2/AM and with adenosine deaminase and/or apyrase in the same way as for measurement of [Ca²⁺]_i. The cell suspension (1.5 ml) was incubated with the agents to be tested in Hepes-buffered medium. At the appropriate time, 100 μ l of cell suspension was transferred to a tube containing 100 μ l of 0.2 M HCl. The cyclic AMP content in the acid extract was measured by radioimmunoassay as described previously [20].

For measurement of the Ins(1,4,5)P₃ response, the cells were resuspended in Hepes-buffered medium and were washed once by repeating the sedimentation and resuspension steps in the same medium. In some experiments (shown in Figure 5), where reactions were performed in the absence of extracellular Ca²⁺, the cells were first resuspended in Ca²⁺-free Hepes-buffered medium containing 0.1 mM EGTA, and were washed with the same Ca²⁺-free medium. The cells were incubated with adenosine deaminase and/or apyrase for 5 min and with the agents to be tested for 15 s at 37 °C in a final volume of 200 μ l. The reaction was terminated by adding 10% perchloric acid (50 μ l) followed by centrifugation at 1500 *g* for 10 min. The supernatant was neutralized with 2 M KOH/100 mM Hepes, and the Ins(1,4,5)P₃ content was measured using an assay kit obtained from Du Pont–New England Nuclear.

Measurement of [Ca²⁺]_i in single cells

The cells on coverslips were treated with 2 μ M fura-2/AM for 20 min at 37 °C. The coverslip was mounted in a chamber and perfused continuously (bath volume 200 μ l; flow rate 3 ml/min) with Hepes-buffered medium at room temperature (about 25 °C) on the stage of an inverted epi-fluorescence microscope. The fluorescence image was obtained by illumination with light of wavelength 340 and 380 nm, displayed on a video screen and analysed by means of a digital image processor (ARGUS 100; Hamamatsu Photonic) as previously described [18,21].

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as representatives or as means $\pm$ S.E.M. of more than three different batches of cells unless otherwise specified.

RESULTS

EK increases [Ca²⁺]_i in a naloxone- and pertussis toxin-sensitive manner in cells sensitized by P₂-purinergic agonists

Figure 1 shows changes in [Ca²⁺]_i estimated from the fluorescence change in a fura-2/AM-loaded NG 108-15 cell suspension. In agreement with previous findings [15], externally applied ATP clearly increased [Ca²⁺]_i (Figure 1a, trace B). EK added alone also induced a small, but significant, increase in [Ca²⁺]_i in the cells (Figure 1a, trace A). When EK was applied after ATP application, the EK-induced rise in [Ca²⁺]_i was remarkably enhanced and the [Ca²⁺]_i reached that obtained with ATP alone (Figure 1a, trace B). ADP, but not AMP or adenosine up to 1 mM, transiently increased [Ca²⁺]_i by itself and allowed EK added after the nucleotide to increase [Ca²⁺]_i (Figure 1a, traces C–E). It is unlikely that a phosphorylation reaction [22] is involved in the ATP-dependent enhancement of the EK-induced rise in [Ca²⁺]_i, since two hydrolysis-resistant ATP derivatives [adenosine 5'-[β - γ -imido]triphosphate (p[NH]ppA)] and adenosine 5'-[γ -thio]triphosphate (ATP[S)] can mimic the action of ATP (Figure 1a, traces F and G). Thus these actions of adenine nucleotides seem to be mediated by P₂-purinergic receptors [23].

The EK-induced [Ca²⁺]_i increase in either the presence or the absence of P₂-agonists was almost completely reversed by prior treatment of the cells with naloxone, an opioid receptor antagonist (Figure 1a, trace H), or pertussis toxin (Figure 1b), whereas these agents hardly affected the rise in [Ca²⁺]_i induced by ATP alone. These results show that the action of EK is mediated by opioid receptors and pertussis toxin-sensitive G-proteins [24], and was enhanced by ATP when the nucleotide activated the P₂-receptor.

The increase in [Ca²⁺]_i induced by EK alone was unexpected, since previous studies on NG 108-15 cells showed that EK inhibited a voltage-dependent Ca²⁺ channel [4,5], which would be expected to produce a decrease in [Ca²⁺]_i. In relation to this, we noticed in our preliminary studies that both [³H]ATP and [³H]ADP were spontaneously released from cells prelabelled with [³H]adenine during incubation of the cell suspension. This suggests that adenine nucleotides released from broken cells enabled EK to induce a rise in [Ca²⁺]_i in the same way as does exogenously added ATP. To confirm this, we added to the medium apyrase, which hydrolyses both ATP and ADP to AMP. As expected, the apyrase treatment dramatically reduced the [Ca²⁺]_i rise induced by EK alone (Figure 1c, trace A). The ATP-induced [Ca²⁺]_i rise was also drastically decreased, but a small peak indicated that some rise in [Ca²⁺]_i was still observed. This

Figure 1 Effect of EK and P_2 -purinnergic agonists on $[\text{Ca}^{2+}]_i$

Time courses of the change in $[\text{Ca}^{2+}]_i$ are shown for the cells that were untreated (a and c) or treated (b) with pertussis toxin. In (c), the $[\text{Ca}^{2+}]_i$ change was monitored in the presence of apyrase (3 units/ml) which had been added to the reaction medium 5 min before fluorescence monitoring. At the arrows the following were applied, as indicated: EK (1 μM), ATP (30 μM), ADP (300 μM), AMP (1 mM), adenosine (Ado, 1 mM), p[NH]ppA (300 μM), the indicated dose of ATP[S], naloxone (Nal, 10 μM), EGTA (2.2 mM) and KCl (50 mM). The reaction medium contained 0.5 unit/ml adenosine deaminase, except for the experiment where adenosine was used. A representative of at least four experiments is shown.

was probably due to ATP present during the very early period of the incubation. EK added after ATP did not induce any $[\text{Ca}^{2+}]_i$ rise (Figure 1c, trace B). Hydrolysis-resistant ATP[S] (30 μM) remained fully active (see Figure 1a, trace G and Figure 1c, trace D). In contrast to ATP, the extent of the $[\text{Ca}^{2+}]_i$ rise induced by a low dose of ATP[S] (3 μM) was very small, while the secondary $[\text{Ca}^{2+}]_i$ rise induced by EK was quite obvious (Figure 1c, trace C). In addition, this effect of ATP[S] on EK action lasted for at least

Figure 2 Effect of thapsigargin on EK- and P_2 -agonist-induced rises in $[\text{Ca}^{2+}]_i$

Experimental conditions were the same as those for Figure 1(c), except that thapsigargin (TG, 100 nM), ATP[S] (30 μM) or EK (1 μM) was applied as indicated. A representative from these separate experiments is shown.

10 min after the nucleotide-induced $[\text{Ca}^{2+}]_i$ transient had ceased (results not shown). The results show that EK itself does not induce a rise in $[\text{Ca}^{2+}]_i$, but the continuous stimulation of P_2 -receptors by ATP or its derivatives is responsible for the action of EK added after the nucleotides. On the other hand, the transient rise in $[\text{Ca}^{2+}]_i$ itself is not the cause of the enhanced EK action.

In previous studies, the inhibitory actions of EK have been considered to relate to the decrease in the influx of extracellular Ca^{2+} [1–6]. Therefore we examined the source of the increased intracellular Ca^{2+} resulting from EK action (Figure 1c, traces E–G). When the extracellular Ca^{2+} concentration was decreased, a KCl-evoked $[\text{Ca}^{2+}]_i$ rise due to Ca^{2+} influx was almost completely abolished, whereas the ATP[S]-induced $[\text{Ca}^{2+}]_i$ rise remained unchanged (Figure 1c, traces D and G); peak rises of $[\text{Ca}^{2+}]_i$ in response to ATP[S] (30 μM) in the presence of 2 mM Ca^{2+} and in the low- Ca^{2+} conditions were 97 ± 24 nM and 102 ± 12 nM respectively. The EK-evoked $[\text{Ca}^{2+}]_i$ rise after application of ATP[S] was also preserved, but in this case a slight inhibition (30%) of the $[\text{Ca}^{2+}]_i$ rise was observed (202 ± 30 nM and 140 ± 26 nM in the regular and low- Ca^{2+} conditions respectively). These values are means $\pm$ S.E.M. of results obtained from four observations.

The source of Ca^{2+} was further examined by using thapsigargin, an inhibitor of microsomal Ca^{2+} -ATPase [25]. This tumour-promoting sesquiterpene lactone has been shown to cause a depletion of Ca^{2+} from the intracellular store sensitive to Ca^{2+} -mobilizing agonists in many types of cells [25–27]. In NG 108-15 cells also, this agent induced a rise in $[\text{Ca}^{2+}]_i$ (Figure 2), probably reflecting the inhibition of Ca^{2+} uptake via Ca^{2+} -ATPase on surface membranes of the Ca^{2+} pool and the depletion of Ca^{2+} from the pool. In the thapsigargin-treated cells both the ATP[S]-induced and the EK-induced $[\text{Ca}^{2+}]_i$ rises were markedly attenuated, probably as a result of prior exhaustion of the intracellular Ca^{2+} pool by thapsigargin (Figure 2).

Based on the two experiments mentioned above, we conclude that the ATP- and EK-induced transient $[\text{Ca}^{2+}]_i$ rises were mainly due to Ca^{2+} release from the intracellular pool (Figure 2), although the participation to some extent of extracellular Ca^{2+} cannot be completely ruled out for the EK-induced Ca^{2+} transient, as shown in Figure 1(c), traces D and G.

ATP- and EK-induced $[\text{Ca}^{2+}]_i$ rises occur in the same single cells

The EK-induced rise in $[\text{Ca}^{2+}]_i$ observed in the cell suspension was also observed in single cells grown on a coverslip. The

Figure 3 Prior application of ATP enables EK to increase $[Ca^{2+}]_i$ in single cells

A typical tracing is shown of the fluorescence ratio (340/380 nm) of a fura-2/AM-loaded cell in response to successively added ATP (300 μ M) and EK (1 μ M). Inset: means $\pm$ S.E.M. of the fluorescence ratios of 88 cells in the same microscope field.

Figure 4 Prior application of bradykinin enables EK to increase $[Ca^{2+}]_i$

Cells were not treated (a, and $\circ$ in c) or were treated with pertussis toxin (b, and $\bullet$ in c) and were incubated with adenosine deaminase (0.5 units/ml) and apyrase (3 units/ml) for 5 min, then the $[Ca^{2+}]_i$ change was monitored. In (a) and (b), EK (1 μ M) or bradykinin (BK, 0.1 nM) was applied as indicated. In (c) the cells were first incubated with BK (0.1 nM) for 1.5 min, then the indicated dose of EK was applied. A peak rise in $[Ca^{2+}]_i$ induced by EK is plotted.

mean $\pm$ S.E.M. values of the fluorescence ratio (340/380 nm), reflecting the $[Ca^{2+}]_i$ of 88 cells analysed in a single typical experiment, are shown in Figure 3 (inset). This pattern is very similar to those obtained in populations of about 5×10^6 cells in

suspension, as shown in Figure 1(a), trace B. Figure 3 shows a typical pattern whereby a dual rise in $[Ca^{2+}]_i$ was observed in a single cell by the application of ATP followed by EK. A similar pattern was observed in 32 out of 88 cells (36%). The second rise in $[Ca^{2+}]_i$ was not due to Ca^{2+} oscillations, since in this type of cell, no Ca^{2+} oscillations were observed during ATP perfusion for at least 3–4 min in a different series of experiments (results not shown). In contrast to the cell suspension, EK alone induced no significant rise in $[Ca^{2+}]_i$ in more than 200 single cells, even though apyrase treatment was omitted, confirming that EK itself was unable to mobilize Ca^{2+} . The cells grown on coverslips probably remained intact, and so no artifactual adenine nucleotide release occurred.

Bradykinin and AlF_4^- enable EK to increase $[Ca^{2+}]_i$

A similar induction of the EK-induced rise in $[Ca^{2+}]_i$ was also observed when P_2 -agonists were replaced with bradykinin, another Ca^{2+} -mobilizing agonist [16–18] (Figure 4a). This EK-induced rise in $[Ca^{2+}]_i$ was dose-dependent and was completely reversed by pertussis toxin treatment, whereas the toxin hardly affected the action of bradykinin alone, similar to the case with P_2 -agonists (Figure 4). This suggests that bradykinin and P_2 -agonists activate a common signal transduction process which is affected by EK via pertussis toxin-sensitive G-proteins.

We also examined the effect of NaF. This agent, in the presence of $AlCl_3$, forms AlF_4^- which has been shown to non-selectively activate G-proteins and to cause activation of phospholipase C in many types of cells [28]. Although we could not detect any significant rise in $[Ca^{2+}]_i$ induced by NaF plus $AlCl_3$ in NG 108-15 cells, EK clearly evoked a rise in $[Ca^{2+}]_i$ when it was applied 5 min after NaF addition; peak $[Ca^{2+}]_i$ rises caused by EK were 9 ± 3 nM for control cells (with only 10 μ M $AlCl_3$) and 88 ± 21 nM for 5 mM NaF (plus 10 μ M $AlCl_3$)-treated cells ($n = 5$). On the other hand, further increasing the dose of NaF did not result in a further significant rise in $[Ca^{2+}]_i$ and in fact reduced the Ca^{2+} response to EK, probably reflecting the toxic effect of NaF.

EK enhances ATP[S]- or bradykinin-induced $Ins(1,4,5)P_3$ formation

As mentioned in a previous section, we found that the EK-induced rise in $[Ca^{2+}]_i$ which occurred under the influence of P_2 -agonists or bradykinin was mainly due to Ca^{2+} mobilization from an intracellular pool. Usually, agonist-induced intracellular Ca^{2+} mobilization is mediated by $Ins(1,4,5)P_3$ produced as a result of phospholipase C activation. Therefore we examined the effects of EK and bradykinin on phospholipase C activity (Figure 5 and Table 1). In accordance with a previous report [17], bradykinin stimulated $Ins(1,4,5)P_3$ formation, reflecting phospholipase C activity in a dose-dependent manner (Figure 5a). Even though EK alone had no significant effect, this opioid co-operated with bradykinin resulting in an increase in phospholipase C activity to a level higher than that obtained with relatively low doses (1–10 nM) of bradykinin. The addition of EK did not influence the action of bradykinin when the bradykinin concentration was higher than 100 nM. As a result the bradykinin dose-response curve was shifted to the left by about half an order of magnitude in the presence of EK (Figure 5a). Phospholipase C activation due to the co-operation of EK and bradykinin was completely reversed by pertussis toxin treatment of the cells, whereas activation of the enzyme by bradykinin alone remained unchanged on toxin treatment (Figure 5b). The inset of Figure 5(a) shows the activation of phospholipase C under conditions of low extracellular Ca^{2+} .

Figure 5 Effects of bradykinin and EK on Ins(1,4,5)P₃ formation

Cells were not treated (a) or were treated with pertussis toxin (b). They were first incubated for 5 min with adenosine deaminase (0.5 unit/ml) and apyrase (3 units/ml), and then incubated for 15 s with the indicated dose of bradykinin (BK) in the absence (○) or presence (●) of 1 μM EK. Inset: cells were incubated for 15 s with EK (1 μM) and/or BK (3 nM) in the absence of extracellular Ca^{2+} as described in the Experimental section. Results are means $\pm$ S.E.M. of six values from three separate experiments and are expressed as percentages of basal values obtained in the absence of these agonists. These basal values (pmol/10⁶ cells) were: 6.47 ± 0.66 (a) and 4.28 ± 0.48 (inset; without extracellular Ca^{2+}) for control cells and 5.54 ± 0.55 (b) for pertussis toxin-treated cells.

Table 1 Effects of EK and ATP[S] on Ins(1,4,5)P₃ formation

Cells which had been treated without (Control) or with pertussis toxin (PTX) were incubated with EK (1 μM) and/or ATP[S] (300 μM). Other experimental conditions were the same as those for Figure 5. Results are presented as the means $\pm$ S.E.M. of twelve values from four separate experiments. * Significantly different from ATP[S] alone ($P < 0.01$ by Student's *t* test).

	Ins(1,4,5)P ₃ formation (pmol/10 ⁶ cells)			
	No addition	+ EK	+ ATP[S]	+ ATP[S] + EK
Control	8.7 ± 0.7	9.3 ± 1.2	14.9 ± 1.0	$19.9 \pm 1.3^*$
PTX	9.1 ± 0.8	9.9 ± 0.5	14.0 ± 0.8	12.8 ± 0.8

Table 2 Effects of PGE₁ and forskolin on the cyclic cAMP and $[\text{Ca}^{2+}]_i$ responses to EK

Experimental conditions for Ca^{2+} response were similar to those for Fig. 1(c), trace D, except that the indicated dose of PGE₁ or forskolin was applied 1 min before ATP[S] application. Results are peak rises in $[\text{Ca}^{2+}]_i$ induced by EK and are presented as the means $\pm$ S.E.M. of four separate experiments. For the cyclic AMP response, experimental conditions were the same as those for the Ca^{2+} response except that contents of cyclic AMP at 15 s after EK application were measured and presented as the means $\pm$ S.E.M. of six values from three separate experiments. * Significantly different from unstimulated cells ($P < 0.01$).

	Cyclic AMP response (pmol/10 ⁶ cells)		EK-induced rise in $[\text{Ca}^{2+}]_i$ (nM)
	No addition	+ EK	
Control	40 ± 1	$32 \pm 2^*$	193 ± 34
PGE ₁ (0.1 μM)	$77 \pm 1^*$	$55 \pm 2^*$	160 ± 32
PGE ₁ (1 μM)	$171 \pm 9^*$	$132 \pm 3^*$	140 ± 20
Forskolin (1 μM)	$824 \pm 29^*$	$691 \pm 16^*$	134 ± 26

Removal of extracellular Ca^{2+} barely influenced the effect of bradykinin alone or the co-operative effect of EK plus bradykinin.

ATP[S] also activated phospholipase C in a pertussis toxin-insensitive manner (Table 1), consistent with a previous finding [15]. As expected, EK enhanced ATP[S]-induced phospholipase C activation, although EK alone did not influence the enzyme action. This effect of EK was again reversed by pertussis toxin treatment (Table 1).

Changes in cyclic AMP metabolism are not responsible for the pertussis toxin-sensitive rise in $[\text{Ca}^{2+}]_i$

EK has been reported to inhibit adenylate cyclase activity, leading to a decrease in cyclic AMP content, through pertussis toxin-sensitive G-proteins [24,29]. This raises the possibility that a decrease in the cyclic AMP content may cause a further rise in the $[\text{Ca}^{2+}]_i$ already elevated by the Ca^{2+} -mobilizing agonist receptor stimulation. If this were the case, an increase in the cyclic AMP content by treatment with prostaglandin E₁ (PGE₁) or forskolin would reverse the action of EK on $[\text{Ca}^{2+}]_i$. In accordance with previous studies [24,29], EK slightly but significantly inhibited the accumulation of cyclic AMP both in unstimulated cells and in cells stimulated by PGE₁ or forskolin (Table 2). This inhibition of the accumulation of cyclic AMP was completely reversed by pertussis toxin treatment (results not shown). However, when EK was applied to PGE₁- or forskolin-stimulated cells, the cyclic AMP level was still higher than that in the unstimulated cells. Under the conditions of high cyclic AMP levels, the ATP[S]-dependent EK-induced rise in $[\text{Ca}^{2+}]_i$ was still noticeable, although it tended to decrease slightly with the increase in cyclic AMP content (Table 2). These results suggest that a change in the cyclic AMP content is not responsible for the EK-induced rise in $[\text{Ca}^{2+}]_i$.

DISCUSSION

We have found that, even though EK alone has no ability to increase $[\text{Ca}^{2+}]_i$, it induces a rise in $[\text{Ca}^{2+}]_i$ in NG 108-15 cells provided that the cells have been previously stimulated with P₂-purinergic agonists or bradykinin. In the absence of apyrase, EK alone evoked a slight rise in $[\text{Ca}^{2+}]_i$ in a cell suspension, but not in cells on a coverslip, suggesting that adenine nucleotides were artifactually released from the cells under the conditions of suspension, probably as a result of cell damage during harvesting or shaking. The EK-dependent part of the $[\text{Ca}^{2+}]_i$ rise resulting from the co-operative actions of EK and P₂-agonists or bradykinin was antagonized by an opioid receptor antagonist, naloxone, and was reversed by pertussis toxin treatment of the cells, indicating that opioid receptors and pertussis toxin-sensitive G-proteins mediate this action of EK. However, it still remains unclear as to which subtype of the pertussis toxin-sensitive G-proteins mediates this effect.

The P₂-agonist- or bradykinin-dependent EK-induced rise in $[\text{Ca}^{2+}]_i$ was shown to be mainly due to Ca^{2+} mobilization from an intracellular pool, although some contribution of extracellular Ca^{2+} cannot be completely ruled out (Figures 1c and 2). As expected from this result, EK enhanced phospholipase C activation in co-operation with P₂-agonists or bradykinin (Figure 5 and Table 1). The results suggest that signals arising from a system composed of an opioid receptor and pertussis toxin-sensitive G-proteins stimulate phospholipase C and the subsequent production of Ins(1,4,5)P₃, which in turn mobilizes Ca^{2+} from the intracellular pool.

Although the molecular mechanism of P₂- and bradykinin receptor-mediated phospholipase C activation has not been well

characterized in NG 108-15 cells, it is reasonable to assume that a G-protein(s) (G_p) [30] mediates the communication between a receptor and phospholipase C, since guanine nucleotides activate phospholipase C and modulate bradykinin binding in the membranes of NG 108-15 cells [31], and P_2 -agonists activate phospholipase C obtained from various types of cells in G-protein-dependent manner [32–34]. Both P_2 -agonists- and bradykinin-induced activation of phospholipase C were barely affected by pertussis toxin treatment of NG 108-15 cells (Figure 5 and Table 1), suggesting that P_2 -agonists and bradykinin activate the enzyme through a pertussis toxin-insensitive G_p .

Ca^{2+} -mobilizing agonists activate protein kinase C, resulting in elevations in $[Ca^{2+}]_i$ and protein kinase levels. On the other hand, Ca^{2+} and protein kinase C modulate phospholipase C [35,36]. This suggests that phospholipase C is sensitized to opioid signal transduction by cytosolic Ca^{2+} and/or protein kinase C, both of which are up-regulated by the prior addition of the Ca^{2+} -mobilizing agents. This, however, seems unlikely, since our preliminary studies showed that even when protein kinase C was activated by the prior application of phorbol myristate acetate, and when Ca^{2+} uptake was induced by ionomycin, EK did not increase $[Ca^{2+}]_i$. Moreover, a hydrolysis-resistant ATP derivative (ATP[S]) at 3 μ M clearly induced an EK-dependent $[Ca^{2+}]_i$ rise without having any appreciable effect by itself (Figure 1c, trace C). However, in the presence of apyrase, previous additions of ATP did not facilitate an EK-induced increase in $[Ca^{2+}]_i$, even though a small but significant rise in $[Ca^{2+}]_i$ was observed immediately after ATP addition (Figure 1c, trace B). These results suggest that the prior increase in $[Ca^{2+}]_i$ or activation of protein kinase C is not responsible for the EK-evoked rise in $[Ca^{2+}]_i$ and hence phospholipase C activation.

AlF $_4^-$, a non-selective activator of G-proteins, also enabled EK to increase $[Ca^{2+}]_i$ in a similar manner to P_2 -agonists and bradykinin. The results suggest that the induction of the rise in $[Ca^{2+}]_i$ by EK does not always require the stimulation of receptors for Ca^{2+} -mobilizing agonists. Another possible site which is influenced by stimulation of the opioid signal transduction system in G_p , which mediates activation of phospholipase C by Ca^{2+} -mobilizing agonists. In relation to this, a study on turkey erythrocytes [37] is very interesting. The reconstitution of the $\beta\gamma$ subunits of pertussis toxin-sensitive G-proteins with the turkey erythrocyte membranes enhanced the guanine nucleotide-dependent P_2 -receptor-mediated activation of phospholipase C, probably through the interaction of the $\beta\gamma$ subunits with the α subunit of G_p [37]. A similar mechanism may underlie the opioid-receptor-mediated activation of the enzyme in co-operation with P_2 - or bradykinin receptors. In any event, further experiments are necessary to clarify the mechanism in detail.

Similar cross-talk or synergism between two receptor mechanisms has been reported previously [11–14,38]. Adenosine (via an A_1 -adenosine receptor in FRTL-5 thyroid cells) [11–14] and somatostatin (via its receptor in striatal astrocytes) [38] induce a rise in $[Ca^{2+}]_i$ in association with phospholipase C activation, provided that the cellular receptor mechanisms have been stimulated by Ca^{2+} -mobilizing agonists, e.g. P_2 -purinergic agonists, α_1 -adrenergic agonists or thyrotropin in the former cells, and α_1 -adrenergic agonists in the latter cells. In these systems also, pertussis toxin-sensitive G-proteins exert a permissive role in the activation of the enzyme. Such a cross-talk between two receptor systems through pertussis toxin-sensitive G-proteins thus seems to be a universal mechanism for the regulation of phospholipase C activity.

In neurones, ATP is located in synaptic vesicles and is released as a co-transmitter from nerve endings by nerve stimulation [39]. The released ATP can be rapidly hydrolysed to adenosine. Much

attention has been focused on the modulation of neural cell activities by the adenosine thus produced [40]. Previous findings, however, show that ATP itself stimulates neurotransmitter release via an increase in $[Ca^{2+}]_i$ in the neural cells [41]. This suggests that ATP is an important extracellular messenger in the regulation of neuronal activity. The present finding that ATP enables EK to induce a rise in $[Ca^{2+}]_i$ may represent a novel mechanism of ATP action on the neuronal activity.

As mentioned in the Introduction, EK generally decreases $[Ca^{2+}]_i$ and thereby inhibits neurotransmitter release from neurones. This inhibitory effect of the opioid is believed to be the mechanism underlying its analgesic effects [1,2]. However, in some cases opioids have excitatory effects on neurones (see ref. [9] for review). The present finding of the Ca^{2+} -mobilizing activity of EK under the influence of extracellular ATP or bradykinin may account for some of the paradoxical actions of opioids previously reported.

In conclusion, EK can activate phospholipase C and Ca^{2+} mobilization provided that the P_2 -receptor- or bradykinin-receptor-mediated phospholipase C system is pre-activated by the respective agonist. Pertussis toxin-sensitive G-proteins play a permissive role in this synergism or cross-talk mechanism.

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