Bombesin and thrombin affect discrete pools of intracellular calcium through different G-proteins

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In mouse NIH 3T3 cells, the mitogens bombesin and thrombin induced Ca^{2+} release from intracellular stores. Ca^{2+} release induced by bombesin was inhibited by the Ca^{2+} -ATPase inhibitor thapsigargin, while Ca^{2+} release induced by thrombin was unaffected by this agent. The Ca^{2+} -release response to bombesin was not affected by pertussis toxin, but the response to thrombin was abolished by the toxin. Stable transfectants overexpressing

the G-protein subunit type α_q showed an accentuated response to bombesin, indicating that the bombesin receptor was coupled to a G_q -like G-protein. Together, these results show that the two mitogenic receptors are coupled to distinct G-proteins that affect functionally different pools of Ca^{2+} . Organization of signalling pathways in this manner may allow cells to differentially encode information from different signals.

INTRODUCTION

Calcium is released from intracellular stores in response to various agonists and modulates diverse physiological processes. A variety of receptors coupled to heterotrimeric G-proteins activate phospholipase C (PLC), and the consequent production of inositol 1,4,5-trisphosphate (IP_3) evokes the release of intracellular Ca^{2+} [1–5]. Since the induced messenger, Ca^{2+} , is the same, but the physiological responses to different agonists vary, it is not clear how the cell discriminates between different extracellular signals. It is known that the intracellular Ca^{2+} pool is heterogeneous [5–7]. One possibility is that different receptors affect discrete pools of Ca^{2+} . This would lead to localized variations in the free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), with different physiological consequences for the cell.

To determine the extent of specificity in G-protein-mediated coupling of receptors to intracellular Ca^{2+} stores, we have examined Ca^{2+} release stimulated by two mitogenic receptors endogenous to a non-excitabile fibroblast-derived cell line, NIH 3T3. These two receptors are activated either by bombesin or by thrombin, and are known to have structures containing seven membrane-spanning domains, similar to other G-protein-coupled receptors [8,9]. There is also evidence indicating that the thrombin and bombesin receptors are coupled to G-proteins in fibroblast cell lines [10,11]. While both bombesin and thrombin induce mitogenesis, they also have various and distinct physiological effects on mammalian cells [12,13]. The experiments here attempt to delineate the nature of the circuitry that connects receptor, G-protein and intracellular Ca^{2+} store in the case of two mitogenic signalling pathways.

MATERIALS AND METHODS

Materials

NIH 3T3 cells were from the American Type Culture Collection (A.T.C.C. CRL-1658). Eagle's medium, Hank's balanced salt solution and calf serum were from Gibco-BRL (Gaithersburg, MD, U.S.A.). Bombesin, thrombin, nifedepine, lanthanum,

thapsigargin and pertussis toxin were from Sigma (St. Louis, MO, U.S.A.). The PLC inhibitor U-73122 and the inactive analogue U-73343 were purchased from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.). Fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). *myo*-[2- ^3H]inositol (633 GBq/mmol; 17.1 Ci/mmol) was from Amersham. All other reagents were from Sigma.

Solutions of U-73122 and analogue U-73343 were prepared as follows. The reagents were dissolved in chloroform, dried under a stream of nitrogen, stored at 4 °C and redissolved in 0.3 % DMSO in Krebs–Ringer's buffer (KR buffer) just before use. Thapsigargin was dissolved in DMSO.

Cell culture, selection of G_q α -subunit (α_q) transfectants and detection of α_q expression

NIH 3T3 cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 /95 % air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) calf serum. The selection of clones expressing α_q and the detection of protein expression have been described before [14]. Since the α_q introduced into these cells is under the control of an inducible promoter [14], α_q expression was induced with dexamethasone. The transfectants were cultured in 5 % (v/v) bovine serum containing 180 $\mu\text{g}/\text{ml}$ G418.

Measurement of changes in $[\text{Ca}^{2+}]_i$ in NIH 3T3 cells

For the determination of changes in $[\text{Ca}^{2+}]_i$, the culture medium was replaced with calcium/magnesium-free Hank's buffer with 0.02 % EGTA. Cells were then collected and washed three times with KR buffer containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 5 mM glucose, 20 mM Hepes, 0.5 % BSA, pH 7.4. Washed cells were incubated in KR buffer containing 5 μM fura-2/AM for 30–45 min at room temperature. After loading with fura-2, cells were washed three times with KR buffer. All the experiments were performed by adding cell

Abbreviations used: α_q , α -subunit of G-protein G_q ; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; IP_3 , inositol 1,4,5-trisphosphate; KR buffer, Krebs–Ringer buffer; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

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suspensions [1.5 ml, containing approx. $(2-3) \times 10^5$ cells] to a cuvette containing KR buffer. The cuvette holder was equipped with a magnetic stirrer. Aliquots of 15–100 μ l of individual drugs in KR buffer were added. Volume changes did not affect the ratiometric determination of $[Ca^{2+}]_i$. Thapsigargin was dissolved in DMSO and then diluted in KR buffer to appropriate concentrations. Fura-2 fluorescence of the cell suspension was monitored at room temperature (22–25 °C). Fluorescence was measured on a PTI spectrofluorimeter or a Hitachi F2000 instrument (excitation at 340 and 390 nm alternated at 100 Hz; emission at 500 nm) [15]. $[Ca^{2+}]_i$ was calculated from the ratio of observed fluorescence intensity of intracellular fura-2 as described previously [16]. To determine the values for maximum and minimum fluorescence, 5–10 μ M ionomycin was added, allowing Ca^{2+} entry and saturation of fura-2. Subsequently 20 mM EGTA was added to obtain the zero calcium level.

Measurement of inositol phosphates

NIH 3T3 cells (2×10^5 /35 mm dish) were labelled with *myo*- $[^3H]$ inositol (1 μ Ci/ml) in inositol-free DMEM and dialysed calf serum for 48 h, and then serum-starved for 24 h. The cells were incubated in serum-free DMEM with 10 mM LiCl at 37 °C for 30 min and then stimulated with the agonists, which were diluted in the same medium, for various time periods. At the end of the incubation, the plates were placed on ice, the medium was aspirated and the plates were rinsed with ice-cold PBS. Ice-cold 10 mM formic acid (1 ml) was added and the plates were kept on ice for 30 min. The cells were scraped and centrifuged (16000 g, 5 min). The supernatant (inositol phosphates) was saved and the pellet [phosphatidylinositol bisphosphate (PIP_2)] was washed with ice-cold PBS and resuspended in 1 M NaOH. The supernatant was fractionated through a Dowex column (200–400 dry mesh). Either the total inositol phosphates were eluted with 1.0 M ammonium formate in 100 mM formic acid or the individual isomers were eluted with 0.2 M, 0.4 M and 1.0 M ammonium formate in 100 mM formic acid. The radioactivity in the fractions and in the PIP_2 pellet was determined. The amounts of inositol phosphates were expressed as c.p.m./ 10^5 c.p.m. of PIP_2 .

RESULTS AND DISCUSSION

NIH 3T3 cells were loaded with fura-2 and assayed fluorimetrically. Both bombesin and thrombin induced a transient increase in $[Ca^{2+}]_i$. The peak response was not significantly affected by the presence of lanthanum, a calcium-channel blocker, indicating that the increase in $[Ca^{2+}]_i$ was due to release from intracellular stores (Figure 1A). EGTA and nifedepine, an L-type calcium-channel inhibitor, similarly did not affect the response (results not shown). Dose–response relationships showed that maximal stimulation was achieved at approx. 100 nM bombesin and 5 units/ml thrombin (Figure 1B). Experiments described below were performed at these concentrations of the mitogens.

To examine the nature of the Ca^{2+} stores from which thrombin and bombesin induce Ca^{2+} release, cells were exposed to increasing concentrations of bombesin in the presence of saturating concentrations of thrombin (Figure 1C). When the mitogens were added together, the Ca^{2+} -release response was additive. One mechanism that could contribute to such an additivity in the response is induction of Ca^{2+} release from two separate stores by the different mitogens. To examine the basis of this additivity, Ca^{2+} -release responses in the presence of thapsigargin, a Ca^{2+} -

ATPase inhibitor, were examined. In the presence of thapsigargin, Ca^{2+} that leaks out of a pool cannot be sequestered, leading to gradual increase in $[Ca^{2+}]_i$ with consequent depletion of the pool [5,17]. When NIH 3T3 cells were treated with thapsigargin, $[Ca^{2+}]_i$ increased gradually due to the inhibition of Ca^{2+} uptake into the stores (Figure 2). Thapsigargin treatment for ~3 min at a concentration of 150 nM inhibited Ca^{2+} release induced by bombesin (Figure 2), consistent with previous results obtained from the examination of Swiss 3T3 cells [17a,18]. Surprisingly, however, thapsigargin had no effect on the response to thrombin at the same concentration or at much higher concentrations of inhibitor (e.g. 10 μ M for ~5 min.) (Figure 2). It is extremely unlikely that the effect of thapsigargin on the Ca^{2+} release induced by bombesin is due to desensitization of the bombesin receptor, for the following reason. In Swiss 3T3 cells thapsigargin treatment results in the inability of the bombesin receptor to activate Ca^{2+} release, but the bombesin receptor is fully active since it can still stimulate normal levels of tyrosine phosphorylation of a focal adhesion kinase [19]. Thus this simplest interpretation for the effect of thapsigargin is that the two mitogens, bombesin and thrombin, induce Ca^{2+} release from functionally distinct intracellular pools in NIH 3T3 cells, one sensitive and another insensitive to thapsigargin.

There is considerable evidence that most of the thapsigargin-sensitive Ca^{2+} pool is IP_3 -sensitive [5]. As expected, therefore, there was a significant increase (~3 fold) in inositol phosphate production induced by bombesin when we examined the synthesis of inositol phosphates in the presence and absence of bombesin (Table 1). Moreover, the aminosteroid U-73122, which is thought to inhibit PLC-mediated pathways by decreasing the availability of substrate phosphoinositides [20,21], significantly (~90%) inhibited bombesin-induced Ca^{2+} release (results not shown). In contrast, a significant change in the production of inositol phosphates was not detected in the presence of thrombin under the conditions used here (Table 1). We examined the response to thrombin 5 s after its addition, because it has been shown that thrombin induces inositol phosphate production rapidly (IP_3 within 10 s), followed thereafter by a progressive but distinct decrease (e.g. [22,23]). We also examined inositol phosphate production after longer periods of time (1 and 30 min), but no significant change in production was seen after these time periods (results not shown and Table 1). Although thrombin-induced changes in inositol phosphates were not detected, the inhibitor U-73122 decreased the Ca^{2+} response to thrombin by ~90% (Figure 3). An inactive analogue, U-73343, did not have any effect on the thrombin-induced response (Figure 3). U-73122 reduced the thrombin response by ~60% at a concentration of 1 μ M in comparison with control cells treated with U-73343 (results not shown). Although the experiments with the inhibitor indicate that the thrombin-induced release of Ca^{2+} is mediated by IP_3 , it is unclear why there is no significant increase in inositol phosphate production induced by thrombin. One possibility is that IP_3 synthesis is localized and transient but sufficient to activate the thapsigargin-insensitive pool. Another possibility is that the inhibitor U-73122 has indirect effects on signalling pathways that have not been clearly documented and that thrombin acts through a pathway independent of PLC.

To identify the G-proteins coupled to the mitogenic receptors, NIH 3T3 cells were treated with pertussis toxin and then examined for their responses to thrombin and bombesin. Pertussis toxin ADP-ribosylates some G-protein α -subunit types and prevents interaction of the G-protein with the receptor. Activation of PLC through pertussis toxin-sensitive pathways is thought to be mediated by the $\beta\gamma$ complex of G-proteins, while PLC activation through pertussis toxin-insensitive G-proteins

Figure 1 Changes in NIH 3T3 cell [Ca²⁺]_i in response to bombesin and thrombin

(A) Induction of Ca²⁺ release by bombesin and thrombin in the absence of Ca²⁺ influx. Lanthanum (L; 5 μ M) was added 4 min before the addition of agonists [100 nM bombesin (Bm) or 5 units/ml thrombin (Tb)]. Traces are representative of five similar experiments. Arrows indicate time of addition. (B) Dose-response curves for the induction of [Ca²⁺]_i increase by bombesin and thrombin. The data shown are the maximum values of [Ca²⁺]_i after addition of the indicated concentrations of bombesin or thrombin. (C) Plot of response to various concentrations of bombesin plus a saturating concentration of thrombin (5 units/ml) (●) superimposed on the dose-response curve for bombesin alone (■). Points in (B) and (C) are means $\pm$ S.E.M. of values from two or more independent experiments.

occurs through the α -subunit types that belong to the α_q subfamily [1,2,24]. After pertussis toxin treatment of NIH 3T3 cells, the Ca²⁺ response to thrombin was almost completely abolished, while the response to bombesin was unaffected (Figure 4).

Pertussis toxin-treated cells which failed to respond to thrombin did respond to bombesin when the latter was added subsequently (Figure 4C), indicating that pertussis toxin was specific in its action.

Figure 2 Influence of thapsigargin on bombesin- and thrombin-induced changes in $[Ca^{2+}]_i$ in NIH 3T3 cells

Traces of changes in $[Ca^{2+}]_i$ resulting from the sequential addition of 150 nM thapsigargin (Tgn) and 100 nM bombesin (Bm) (A), or 10 μ M thapsigargin and 5 units/ml thrombin (Tb) (B). Traces are typical of those obtained with two or more cell suspensions.

Table 1 Agonist-induced production of inositol phosphates

Incubations were carried out for 30 min. Experiments were performed twice, each in triplicate. Results are means $\pm$ S.E.M.

Treatment	Total inositol phosphates (c.p.m./ 10^5 c.p.m. of PIP_2)
Control	3207 $\pm$ 183
Thrombin (5 units/ml)	3312 $\pm$ 297
Bombesin (100 nM)	8151 $\pm$ 634

Figure 3 Effects of the PLC inhibitor U-73122 and the analogue U-73343 on the thrombin-stimulated rise in $[Ca^{2+}]_i$

Fura-2-loaded NIH 3T3 cells were pretreated with the inactive analogue U-73343 (upper panel) or the active inhibitor U-73122 (lower panel) for 2 min and then exposed to 5 units/ml thrombin. Traces are representative of two experiments in which the concentration of U-73343 was 1 μ M or 5 μ M and the concentration of U-73122 was 5 μ M.

To confirm that the bombesin receptor is coupled to a G-protein, and also to examine the nature of the G-protein, NIH 3T3 cells stably transfected with the α_q subunit were tested for their Ca^{2+} response to bombesin. This particular subunit type was tested since it is known to be pertussis toxin-insensitive and to mediate PLC activation [2,14,24]. In these stably transfected cells, the α_q subunit is expressed under the control of a promoter that is sensitive to dexamethasone [14]. The α_q clones that we used were selected based on significantly increased serum-stimulated PLC activity [14]. If the bombesin receptor induced Ca^{2+} release through G_q and PLC, the stable transfectants expressing α_q would be expected to show an enhanced Ca^{2+} response. To test this, we induced Ca^{2+} release from these cells after addition of dexamethasone (Table 2). The magnitude of Ca^{2+} release from α_q transfectants was significantly greater than in untransfected cells. These results indicate that the bombesin receptor in NIH 3T3 cells is coupled to a G-protein, and that the α -subunit of this G-protein is α_q or a related α -subunit type. Thus, in NIH 3T3 cells, the thrombin receptor is coupled to G_i or a related pertussis toxin-sensitive G-protein, while the bombesin receptor is coupled to a member of the G_q family.

Networks created by specific interactions between receptors, G-proteins and their effectors are a characteristic property of G-protein-mediated signalling [25,26]. Together, the above results show that modulation of $[Ca^{2+}]_i$ by different G-protein-coupled receptors can be specific to a degree whereby different receptors are coupled to distinct G-protein that regulate separate pools of Ca^{2+} . In pancreatic acinar cells, acetylcholine and cholecystokinin evoke different patterns of Ca^{2+} spikes [27]. Two different mammalian muscarinic receptors overexpressed in *Xenopus* oocytes induce dissimilar spatiotemporal patterns of Ca^{2+} release [28]. If Ca^{2+} pools are spatially separate, or if their kinetics of Ca^{2+} release are different, induction of Ca^{2+} release could activate different sets of effectors. The experiments described here show that receptors native to a cell can be coupled to different G-proteins in that cell and evoke Ca^{2+} release from distinct intracellular pools. One mechanism underlying these patterns of Ca^{2+} release may be the heterogeneous response of Ca^{2+} -release channels that control release from intracellular Ca^{2+} stores. This heterogeneity may result from the differential localization of receptors, G-proteins, effectors and channels. There is so far no evidence for such compartmentalization. Alternatively, the Ca^{2+} -release channels may be differentially sensitive to IP_3 . Such heterogeneity has been shown in pancreatic acinar cells [29]. The

Figure 4 Effects of pertussis toxin on bombesin- and thrombin-induced release of Ca²⁺

NIH 3T3 cells were incubated for 6 h in DMEM in the presence of 100 ng/ml pertussis toxin. The cells were then challenged with 100 nM bombesin (Bm) or 5 units/ml thrombin (Tb). Arrows indicate the point of addition of the agonist.

Table 2 Effect of α_q overexpression on bombesin-induced Ca²⁺ release

Intracellular Ca²⁺ was induced with 5 units/ml thrombin or 100 nM bombesin. Cells stably transfected with α_q were pretreated with dexamethasone (1 μ M) for 48 h. Values are means $\pm$ S.E.M. from several samples (number of samples assayed is in parentheses). Wild-type and transfectants were assayed together in some experiments and separately in others. The basal [Ca²⁺]_i was approx. 100 nM.

	[Ca ²⁺] _i (nM)	
	Thrombin	Bombesin
Wild-type	393 $\pm$ 15 (6)	610 $\pm$ 7 (7)
α_q transfectant	401 $\pm$ 9 (8)	1226 $\pm$ 132 (6)

present results indicate that one mechanism underlying such heterogeneous responses to different receptors is differential coupling of these receptors to distinct G-proteins and their selective action on discrete pools of Ca²⁺. Such 'hard wiring' can result from selective interactions at various levels, e.g. among G-protein subunit types [30,31], between receptor and G-protein subunit types [32–35], between G-protein subunit types and effectors [36] and, finally, effects of intracellular messengers and receptors on the Ca²⁺ pools [29].

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