Rapid desensitization and resensitization of bombesin-stimulated phospholipase D activity in Swiss 3T3 cells

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The kinetics of bombesin-stimulated phospholipase D (PLD) activity were examined in Swiss 3T3 fibroblasts. The stimulated activity was found to rapidly desensitize, being completely absent after 40 s. This activity then quickly, but incompletely, resensitized, with PLD being detectable after a 4.5 min wash of the desensitized cells and 75–80% of the activity being recovered after 10 min. The desensitization was dose-dependent; however, the half-maximal stimulatory concentration of bombesin was an order of magnitude lower than that required for bombesin-stimulated second messenger generation and the K_D for bombesin receptor binding. This suggested that desensitization was stimulated by a 'downstream' effect, but experiments have ruled out

changes in protein kinase C activity and Ca^{2+} concentration. Binding experiments suggested that part of the desensitization is due to receptor internalization, and the requirement for an extracellular agonist for resensitization implies that receptor recycling plays a role. Over an extended time course, cycles of desensitization and resensitization of bombesin-stimulated PLD activity were apparent which may be relevant to mitogenic signalling. These studies add further evidence for a second messenger pathway of PLD activation, and the disparity between the kinetics of diacylglycerol generation and PLD activation supports the possibility that phosphatidic acid may have a messenger role in stimulated cells.

INTRODUCTION

Bombesin, a peptide mitogen for Swiss 3T3 cells (Rozengurt and Sennett-Smith, 1983), binds to a single class of high-affinity cell-surface receptors (Zachary and Rozengurt, 1985), and through coupling to a G-protein of the G_q subfamily (Plevin et al., 1990; Smrcka et al., 1991) stimulates phospholipase C (PLC)-mediated phosphatidylinositol 4,5-bisphosphate hydrolysis (Heslop et al., 1986). This generates the two second messenger molecules inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol (DAG). $(1,4,5)P_3$ stimulates a transient increase in cytosolic Ca^{2+} concentration (reviewed by Berridge and Irvine, 1989), whilst DAG activates protein kinase C (reviewed by Bell and Burns, 1991). In addition to this widely studied pathway, bombesin is typical of an extensive range of hormones and growth factors which have been demonstrated to stimulate phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine (PtdCho) (see Billah and Anthes, 1990; Cook and Wakelam, 1991a).

In Swiss 3T3 cells bombesin stimulates two phases of DAG generation, whereas non-PtdIns(4,5) P_2 -hydrolysing stimulants, for example phorbol 12-myristate 13-acetate (PMA) or epidermal growth factor, generate only a single sustained phase (Cook et al., 1991; Cook and Wakelam, 1992). Structural analysis has suggested that the first transient phase of bombesin-stimulated DAG generation originates from PtdIns(4,5) P_2 hydrolysis, whereas the second is derived from PtdCho (Pettitt and Wakelam, 1993). Despite this probable phospholipid source, the second sustained phase cannot be fully accounted for by dephosphorylation of the phosphatidate (PtdOH) generated by PLD-mediated PtdCho hydrolysis. The inclusion of butanol to 'trap' any PtdOH being formed by PLD activation only attenuated the

sustained phase of bombesin-stimulated DAG by 30%, whereas PMA-stimulated DAG generation was diminished by 70% (Cook et al., 1991). These results demonstrated that, at least in fibroblasts, activation of PLD is not exclusively responsible for the sustained phase of DAG generation, and thus the enzyme must be involved in other signalling pathways.

The tight regulation of enzymes involved in signal transduction is a key characteristic of second messenger pathways; indeed, desensitization frequently occurs (e.g. stimulation of β_2 -adrenergic receptors; Hausdorf et al., 1990). Homologous desensitization of bombesin-stimulated Ins(1,4,5) P_3 production in Swiss 3T3 cells is rapid, reversible, dose-dependent and protein kinase C-independent (Palmer et al., 1991), though the precise molecular mechanism remains undefined. The hydrolysis of both PtdCho and PtdIns(4,5) P_2 is rapidly desensitized in vasopressin-stimulated A10 vascular smooth muscle cells (Plevin and Wakelam, 1992). In an attempt to determine the function and regulation of PLD in a mitogenic signalling system, we have investigated this desensitization phenomenon in bombesin-stimulated Swiss 3T3 cells.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum, 27 mg of glutamine/ml and penicillin/streptomycin (250 units/ml and 250 μ g/ml respectively) at 37 °C in a humidified atmosphere of air/CO₂ (19:1); they were routinely passaged when subconfluent into 24-well plates. Cells were grown to confluency and quiescence by serum depletion in 2% (v/v) calf serum for

Abbreviations used: DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; GRP, gastrin-releasing peptide; HHBG, Hank's buffered saline containing 20 mM Hepes, pH 7.4, 1% (w/v) BSA and 10 mM glucose; Ins P_n , total inositol phosphates; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol myristyl acetate; PtdBut:phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid.

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24 h prior to experiments. For labelling studies the relevant radiochemical ([9,10(n)- ^3H]palmitic acid, 4 $\mu\text{Ci/ml}$; *myo*-[^3H]inositol, 1 $\mu\text{Ci/ml}$; or [*methyl*- ^3H]choline, 2 $\mu\text{Ci/ml}$) was included in the 2%-serum-containing medium.

Assay of PLD transphosphatidylation activity in whole cells

Quiescent Swiss 3T3 cells labelled with [^3H]palmitic acid were washed in 0.5 ml of Hepes-buffered Hanks-buffered saline solution, pH 7.4, containing 10 mM glucose, 1% (w/v) BSA fraction V and 20 mM Hepes (HHBG) for 20 min at 37 °C prior to incubation in 0.5 ml of HHBG containing 30 mM butan-1-ol (0.3%, v/v) for a further 5 min. Incubations were commenced by replacing the medium with 0.2 ml of HHBG/butan-1-ol and the stimulant at the concentrations and times indicated. Incubations were terminated by removal of the medium and addition of ice-cold methanol; after extraction of lipids in chloroform, [^3H]phosphatidylbutanol was separated by t.l.c. as described previously (Cook et al., 1991).

Measurement of total inositol phosphates

Quiescent Swiss 3T3 cells grown in 24-well plates and labelled with [^3H]inositol were washed in 0.5 ml of HHBG for 2 $\times$ 10 min at 37 °C prior to incubation for a further 5 min in 0.5 ml of HHBG containing 10 mM LiCl (HHBG). Incubations were commenced by replacing the medium with 0.2 ml of the agonist in HHBG/10 mM LiCl at the times and concentrations indicated. Incubations were terminated by direct addition of 100 μl of 10% (v/v) HClO_4 . The cell extracts were neutralized and total inositol phosphate formation was determined as described previously (Cook and Wakelam, 1991b).

Assay of [^{125}I]-[Tyr 4]gastrin-releasing peptide (GRP) binding to Swiss 3T3 cells: measurement of internalized ligand

Cells were grown in 24-well plates and made quiescent as above. Binding experiments were essentially as described by Brown et al. (1987); however, they were performed under non-equilibrium conditions in order to mimic the desensitization experiments. The wells were washed in HHBG containing 1 mM KI for 20 min at 37 °C before incubation with 100 nM bombesin in HHBG/1 mM KI containing 0.1 μCi of [^{125}I]-GRP/150 μl (specific radioactivity > 2000 Ci/mmol). Following binding, the medium was replaced with ice-cold HHBG/1 mM KI and placed on ice to prevent any further internalization. If internalization was being assessed, the cells were washed for 2 $\times$ 2 min with 50 mM glycine/100 mM NaCl, pH 3, or with HHBG/1 mM KI before a further four washes in ice-cold HHBG/1 mM KI. Solubilization was with 0.5 ml of 0.5 M NaOH. Non-specific binding was determined as the radioligand that was not displaced by at least a 200-fold excess of bombesin, and varied between experiments from 20 to 50% of specific binding. Solubilized sample from each well (400 μl) was counted in a γ -radiation counter together with an aliquot of the specific and non-specific bombesin/[^{125}I]-[Tyr 4]GRP solutions. The number of cells in a well was also determined using a haemocytometer following trypsin treatment.

Measurement of total choline generation

Quiescent cells were grown in 24-well plates and labelled to equilibrium for 36–48 h with 2 $\mu\text{Ci/ml}$ [*methyl*- ^3H]choline chlor-

ide (Cook and Wakelam, 1989). Prior to the experiment the medium was replaced with 0.5 ml of unlabelled DMEM for 2 h. Cells were washed with medium containing DMEM, 1% (w/v) BSA, 10 mM glucose and 20 mM Hepes (buffer A) for three washes of 5, 10 and 30 min respectively. Stimulations were performed with 150 μl of agonist and washes were with 0.5 ml of buffer A. The incubations were terminated by direct addition of 0.5 ml of methanol. The cells were scraped and washed (0.2 ml of methanol) and then extracted by 310 μl of chloroform at room temperature for 15–20 min or overnight at 4 °C. The phases were divided by addition of 390 μl of chloroform and 480 μl of water, followed by centrifugation at 1000 *g* for 5 min. A 0.8 ml sample of the upper phase was analysed for [^3H]choline content using cation-exchange chromatography on Dowex-50W (H^+) columns as described by Cook and Wakelam (1989), except that 1 M KCl was used to elute choline from the columns (Kanoh et al., 1991).

Expression of results

All data are from at least three separate experiments unless otherwise stated. Results are expressed as means $\pm$ S.D. Statistical analysis used an unpaired Student's *t* test.

RESULTS AND DISCUSSION

Desensitization and resensitization of PLD

PLD activity was determined as the stimulated accumulation of [^3H]phosphatidylbutanol ([^3H]PtdBut) in [^3H]palmitate-labelled cells. Under the labelling conditions utilized, approx. 540 000 d.p.m. per well was incorporated into cellular lipids, of which some 65% was in PtdCho. Bombesin-stimulated [^3H]PtdBut accumulation was apparent after 15 s and increased in a linear manner until 60 s; its generation then appeared to cease (Cook et al., 1991). PMA-stimulated PLD activity, on the

Figure 1 Time course of the desensitization of bombesin-stimulated PLD activity

[^3H]Palmitate-labelled cells were pretreated with 100 nM bombesin for increasing times in the absence of butanol and then stimulated with 100 nM bombesin for 30 s in the presence of 30 mM butanol. The generation of [^3H]PtdBut was determined by t.l.c. Results are expressed as means $\pm$ S.D. ($n = 3$), and are percentages of the [^3H]PtdBut generated after a pretreatment with buffer in the absence of butanol followed by 30 s stimulation with agonist in the presence of butanol. Mean basal radioactivity (d.p.m.) obtained when vehicle was used for both the pretreatment and stimulations was subtracted before percentage values were determined. Basal radioactivity (d.p.m.): 0 s pretreatment, 768 $\pm$ 186 (used for 0, 5, 10 and 15 s points); 40 s pretreatment, 643 $\pm$ 23 (used for 20, 25, 30 and 40 s points). Stimulation at the 40 s time point (100%) was 2297 $\pm$ 335 d.p.m. in the experiment shown. Results are from a single typical experiment representative of three.

Figure 2 Time course of resensitization of bombesin-stimulated PLD activity (a) and inositol phosphate generation (b) following a 1 min bombesin pretreatment

Cells labelled with (a) $[^3\text{H}]$ palmitate or (b) $[^3\text{H}]$ inositol were pretreated for 1 min with 100 nM bombesin or buffer in the absence of (a) butanol or (b) LiCl, then washed with buffer for 3×30 s followed by a fourth wash for the times indicated. The cells were then stimulated with 100 nM bombesin or buffer in the presence of 30 mM butanol or 10 mM LiCl for 1 min, and PLD activity or inositol phosphate generation was determined. Results are expressed as means $\pm$ S.D. and are percentages of the $[^3\text{H}]\text{PtdBut}$ or $[^3\text{H}]\text{InsP}$ generation obtained following buffer pretreatment, wash and stimulation with 100 nM bombesin for 1 min. Results are from single typical experiments representative of four. Mean basal values where vehicle was used for pretreatments and stimulations were subtracted before percentages values were determined. Basal radioactivity (d.p.m.): (a) 1.5 min wash, 834 ± 223 , 11.5 min wash, 918 ± 57 , 41.5 min wash, 1020 ± 166 ; (b) 1.5 min wash, 1398 ± 268 , 41.5 min wash, 1168 ± 18 .

other hand, was only apparent after 60 s, but continued for 20 min, albeit at a rate lower than its initial rapid activity. This reduction in bombesin-stimulated $[^3\text{H}]\text{PtdBut}$ formation was suggestive of desensitization. In order to address this possibility the transphosphatidylation assay was modified. The cells were first exposed to the agonist in the absence of butanol, and the alcohol was then added in order to determine any subsequent PLD activity. Using this experimental protocol, desensitization of 100 nM bombesin-stimulated PLD activity was found to be rapid, being significant by 15 s ($P = 0.007$), and reached completion by 40 s (Figure 1). In some experiments the onset of desensitization was slightly slower and was only significant after 20 s. A similar time course of desensitization was observed with 3 nM bombesin, a concentration close to the EC_{50} for bombesin-stimulated $[^3\text{H}]\text{PtdBut}$ accumulation (results not shown).

Desensitization of bombesin-stimulated PLD activity was reversible. The rate of resensitization was examined in cells that had been pretreated for 1 min with 100 nM bombesin. These

Figure 3 Desensitization and resensitization of bombesin-stimulated choline production after a 1 min bombesin pretreatment

$[^3\text{H}]\text{Choline}$ -labelled cells were pretreated with 100 nM bombesin for 1 min and then washed for 3×30 s with buffer A followed by a fourth wash. After the times indicated the cells were treated with 100 nM bombesin (■) or DMBGH (□) for 1 min and total choline was measured as described in the Materials and methods section. $\triangle$ represents the radioactivity (d.p.m.) in $[^3\text{H}]\text{choline}$ in parallel, resting, untreated cells. Each point represents the mean $\pm$ S.D. ($n = 3$), and is from a single experiment representative of three. Controls: stimulation in the absence of bombesin pretreatment 3535 ± 461 d.p.m.; 100 nM bombesin for 1 min, 5126 ± 385 d.p.m.

were washed for increasing periods of time and the formation of $[^3\text{H}]\text{PtdBut}$ in response to a 1 min stimulation with 100 nM bombesin in the presence of butanol was determined. Resensitization of bombesin-stimulated PLD activity was found to be rapid, with $[^3\text{H}]\text{PtdBut}$ generation recommencing after a total wash time of 4.5 min ($P = 0.007$) (Figure 2a). However, resensitization was never found to reach completion within the times examined. At best only about 80% of the control response was ever recovered, even after a total wash time of 41.5 min. Incomplete resensitization was not due to depletion of $[^3\text{H}]\text{palmitate}$ -labelled PtdCho , since similar results were obtained when $[^3\text{H}]\text{palmitate}$ was included in the experimental incubation medium (results not shown).

In order to confirm that the desensitization of bombesin-stimulated $[^3\text{H}]\text{PtdBut}$ accumulation was a direct reflection of PLD-mediated PtdCho hydrolysis, the effect of a 1 min bombesin stimulation upon total $[^3\text{H}]\text{choline}$ generation was determined in cells labelled with $[^3\text{H}]\text{choline}$. Cells were washed for the times indicated after the bombesin pretreatment before addition of buffer or bombesin for a 1 min period (Figure 3). Bombesin-stimulated choline production was clearly desensitized to levels comparable with those in the control cells that were pretreated with bombesin but restimulated with buffer. Significant choline production above that of the control cells ($P = 0.01$) was always observed by 11.5 min after agonist removal, and in some experiments after 6.5 min of washing. The difference in time of resensitization between experiments measuring $[^3\text{H}]\text{PtdBut}$ and $[^3\text{H}]\text{choline}$ generation probably reflects the fact that the former is an accumulation assay, while the latter is measuring a product that can be metabolized by a number of different pathways. In this regard, choline production from the control cells decreased with increasing wash time, presumably because the choline produced from the initial bombesin stimulation was metabolized.

The dose-dependency of bombesin-stimulated PLD desensitization was determined by pretreating cells for 1 min with increasing concentrations of bombesin and then adding butanol for 2 min in the presence of 100 nM bombesin (Figure 4). The

Figure 4 Dose-response of bombesin-mediated homologous PLD desensitization

[³H]Palmitate-labelled cells were pretreated with increasing concentrations of bombesin in the absence of butanol for 1 min and then stimulated for 2 min with 100 nM bombesin in the presence of 30 mM butanol; the generation of PtdBut was determined. Results are expressed as means $\pm$ S.D. ($n = 3$), and represent the [³H]PtdBut generated after a 1 min pretreatment with buffer followed by the 2 min stimulation with 100 nM bombesin in the presence of butanol; the data are from a single experiment typical of three. Where vehicle was used for both the pretreatment and stimulations the mean basal radioactivity was 699 ± 60 d.p.m.

Figure 5 Time course of A23187-stimulated PLD activity

PLD activity was determined in [³H]palmitate-labelled cells stimulated with 5 μ M A23187 for increasing times in the presence of butanol after a 5 min preincubation with 30 mM butanol. $\square$, Control cells [0.1% (v/v) dimethyl sulphoxide]; $\blacksquare$, stimulated cells. Results are means $\pm$ S.D. ($n = 3$) and are from a single experiment representative of three.

dose-response curve generated an IC_{50} from three separate experiments of 0.2 ± 0.1 nM; this is an order of magnitude lower than the EC_{50} values for other bombesin-receptor-linked responses in Swiss 3T3 cells, e.g. DNA synthesis (Rozengurt and Sinnott-Smith, 1983), inositol phospholipid hydrolysis (Plevin et al., 1990), DAG generation (Cook et al., 1990), phospholipase A_2 activation (Currie et al., 1992) and PLD activation (Cook et al., 1991). This difference in half-maximal effective concentrations implies that desensitization of PLD activity is an event that

Table 1 Effect of elevating the Ca^{2+} concentration on bombesin-stimulated PLD activity

[³H]Palmitate-labelled cells were pretreated with vehicle, 5 μ M A23187 or 100 nM bombesin for 1 min before washing for 3×30 s with HHBG. The cells were then stimulated with vehicle, 100 nM bombesin or 5 μ M A23187 in the presence of 30 mM butanol for 1 min. All pretreatments and stimulations contained 0.1% (v/v) dimethyl sulphoxide. [³H]PtdBut was determined by t.l.c. Results are expressed as means $\pm$ S.D. ($n = 3$) and are percentages of basal activity (vehicle used in the pretreatment and stimulation). Results are from a single experiment representative of three. The mean basal radioactivity was 1527 ± 255 d.p.m.

Pretreatment	Stimulation	PLD activity (% of basal)
None	Bombesin	291 ± 78
None	A23187	200 ± 36
Bombesin	Buffer	94 ± 18
A23187	Buffer	86 ± 18
Bombesin	Bombesin	115 ± 19
Bombesin	A23187	118 ± 17
A23187	A23187	100 ± 15
A23187	Bombesin	200 ± 31

occurs downstream of receptor occupation, presumably being a result of a signalling cascade.

The role of Ca^{2+} in desensitization

The increase in intracellular Ca^{2+} stimulated by bombesin in Swiss 3T3 cells is transient (Takuwa et al., 1987; Currie et al., 1992), and thus the decline in Ca^{2+} levels could play a role in desensitization, particularly in light of the suggestion that in some cells PLD activity may be regulated by changes in intracellular Ca^{2+} concentration. Therefore the effect of maintaining an elevated intracellular Ca^{2+} level by treatment with the Ca^{2+} ionophore A23187 was determined. A23187 stimulated rapid [³H]PtdBut accumulation (Figure 5) which reached a plateau between 30 s and 1 min, i.e. it desensitized. Thus maintaining an elevated intracellular Ca^{2+} concentration could not prevent desensitization. While pretreatment with 5 μ M A23187 desensitized the stimulation of PLD activity by a subsequent addition of A23187, the ionophore only caused a 40% reduction in the stimulated response to bombesin (Table 1). However, pretreatment with 100 nM bombesin for 1 min completely abolished any subsequent bombesin- or A23187-stimulated [³H]PtdBut accumulation. Thus desensitization is via a Ca^{2+} -independent pathway; indeed, the effect of the ionophore may be a result of stimulation of the Ca^{2+} -dependent protein kinase C- α isoenzyme, since we have previously shown that the inhibitor Ro-31-8220 can inhibit much of the stimulation of PLD activity by A23187 (Cook et al., 1991).

The role of protein kinase C in desensitization

Since PMA-stimulated PLD activity does not desensitize in the same manner as in bombesin-stimulated cells, it is probable that desensitization is protein kinase C-independent. However, in order to fully assess the role of protein kinase C activity in bombesin-mediated desensitization and resensitization of PLD activity, cells were treated with the selective protein kinase C inhibitor Ro-31-8220 (Davis et al., 1989). As we have previously reported, the inhibitor reduced bombesin-stimulated PLD activity by about 70% (Cook et al., 1991). Nevertheless, the remaining PLD activity was completely abolished in the cells pretreated with bombesin in the presence of Ro-31-8220 (Table

Table 2 Effect of Ro-31-8220 on bombesin-mediated PLD desensitization (a) and resensitization (b)

(a) Cells were preincubated with vehicle [containing 0.04% (v/v) dimethyl sulphoxide] or 10 μ M Ro-31-8220 for 5 min before pretreating the cells, in the absence of butanol, with vehicle, Ro-31-8220, 100 nM bombesin alone or 100 nM bombesin plus 10 μ M Ro-31-8220 for 1 min. The cells were then stimulated for 1 min with vehicle, 10 μ M Ro-31-8220, 100 nM bombesin alone or 100 nM bombesin plus 10 μ M Ro-31-8220 each in the presence of 30 mM butanol, and [3 H]PtdBut generation was determined. All additions contained 0.04% (v/v) dimethyl sulphoxide. Results are percentages of the [3 H]PtdBut obtained in the absence of Ro-31-8220 after a vehicle pretreatment and stimulation with 100 nM bombesin for 1 min in the presence of butanol (means $\pm$ S.D.; $n = 3$), and are from a single experiment typical of three. Mean basal values where vehicle or 10 μ M Ro-31-8220 only was used in pretreatments and stimulations were subtracted before the percentage values were determined. Basal radioactivities (d.p.m.): vehicle, 1100 $\pm$ 331; Ro-31-8220, 1023 $\pm$ 238. (b) The cells were pretreated as above. They were then washed with vehicle in the absence or presence of 10 μ M Ro-31-8220 for 3 $\times$ 30 s followed by a fourth wash for 10 min, after which cells were stimulated for 1 min with vehicle, 100 nM bombesin alone or 100 nM bombesin plus 10 μ M Ro-31-8220, and [3 H]PtdBut generation was determined. Results are percentages of the [3 H]PtdBut obtained in the absence of Ro-31-8220 after a vehicle pretreatment, 3 $\times$ 30 s and 1 $\times$ 10 min washes, and stimulation by 100 nM bombesin for 1 min in the presence of butanol. The data (means $\pm$ S.D.; $n = 3$) are from a single experiment typical of three. Mean basal values where vehicle or 10 μ M Ro-31-8220 only was used in pretreatments and stimulations were subtracted before percentage values were determined. Basal radioactivity (d.p.m.): vehicle, 644 $\pm$ 51; Ro-31-8220, 694 $\pm$ 106.

Pretreatment	Stimulation	Response (% of maximum)
(a) Desensitization		
Vehicle + Ro-31-8220	Ro-31-8220 + bombesin	29 $\pm$ 17
Bombesin	Bombesin	-9 $\pm$ 14
Ro-31-8220 + bombesin	Ro-31-8220 + bombesin	-11 $\pm$ 5
(b) Resensitization		
Vehicle + Ro-31-8220	Ro-31-8220 + bombesin after 10 min wash	33 $\pm$ 8
Bombesin	Bombesin after 10 min wash	33 $\pm$ 7
Ro-31-8220 + bombesin	Ro-31-8220 + bombesin after 10 min wash	14 $\pm$ 6

2a). Therefore, preventing the stimulation of protein kinase C activity does not prevent the desensitization of agonist-stimulated PLD activity. A recovery in protein kinase C activity is unlikely to be the mechanism whereby resensitization occurs, since performing the resensitization experiments in the presence of the kinase inhibitor resulted in a similar percentage inhibition of the resensitized [3 H]PtdBut generation (Table 2b) compared with the inhibition by Ro-31-8220 of PLD activity in the non-pretreated cells (Table 2a). As expected, PMA-stimulated [3 H]PtdBut accumulation was reduced to basal levels by the presence of Ro-31-8220.

Desensitization and resensitization of inositol phosphate generation

Bombesin-stimulated PtdIns(4,5) P_2 hydrolysis in Swiss 3T3 cells is also rapidly desensitized. Generation of total labelled inositol phosphates ([3 H]Ins P_i) has been reported to be biphasic, with a rapid increase up to 1 min followed by a second phase at a reduced rate (Cook et al., 1991). The presence of 30 mM butanol in the incubation medium had no effect upon 100 nM bombesin-stimulated Ins P_i generation (results not shown). Resensitization of bombesin-stimulated [3 H]Ins P_i accumulation was examined in

a similar manner to that for [3 H]PtdBut, except that LiCl was substituted for butanol in the 1 min stimulation following the various wash times. A 1 min bombesin treatment was found to reduce subsequent bombesin-stimulated inositol phosphate generation over 1 min to 20–50% of that observed with a vehicle pretreatment. An increase in bombesin-stimulated [3 H]Ins P_i accumulation over that remaining after the bombesin pretreatment was not observed until after a wash time of 6.5 min ($P = 0.003$) (Figure 2b). Subsequently the recovery of [3 H]Ins P_i generation occurred rapidly, being essentially complete after an 11.5 min total wash time. In order to provide additional evidence for a dissociation between the resensitization of bombesin-stimulated PLD activity and PtdIns(4,5) P_2 hydrolysis, experiments were performed in cells double-labelled with [3 H]palmitate and [3 H]inositol. In these cells it was possible to determine the resensitization of both pathways in the same cell. While there was, not surprisingly, some variation between experiments, there was resensitization of PLD in all experiments and resensitization of [3 H]Ins P_i accumulation in only some experiments after a 110 s wash to a 1 min stimulation (typical data from one of three experiments is shown in Table 3). Thus both the magnitude and the kinetics of desensitization and resensitization of bombesin-stimulated PtdIns(4,5) P_2 hydrolysis differ from those of PLD activity, suggesting different mechanisms of regulation and also that the activation of PtdIns(4,5) P_2 hydrolysis is not the sole mechanism involved in the stimulation of PLD activity by bombesin in Swiss 3T3 cells.

Bombesin receptor regulation and desensitization

One mechanism thought to be involved in desensitization is the loss of cell surface receptors. GRP has the same affinity for the bombesin receptor as bombesin itself (Nagalla et al., 1992), and thus 125 I-GRP was used to investigate the effect of washing cells on the binding of bombesin to its receptor. Whilst equilibrium binding of 125 I-GRP is only achieved at 37 $^{\circ}$ C after 30 min (Zachary and Rozengurt, 1985), a binding period of 1 min was used to examine the effect of washing so as to mimic the experimental conditions used in the resensitization experiments. Initial control experiments suggested that the washing procedure had no effect on the amount of bombesin bound. Agonist-occupied receptors are rapidly internalized, a process which could be involved in desensitization; thus in order to examine the internalization of 125 I-GRP, the cells were washed with a glycine buffer at pH 3. This procedure, which removes all surface-bound ligand, has been reported to give an estimate of the extent of receptor internalization (Kuppuswamy and Pike, 1989). All of the cell-associated radioactivity remaining after washing at pH 7.4 was still present after the acid wash, implying a very rapid internalization of the bombesin receptor (mean specific c.p.m. bound/ 10^6 cells: no acid wash, 254 $\pm$ 27; with acid wash, 285 $\pm$ 65; $n = 3$; results from a single experiment typical of three).

Experiments were performed to investigate whether the internalized receptor subsequently returned to the surface. The cells were pretreated with 100 nM bombesin at 37 $^{\circ}$ C for 1 min before washing for 3 $\times$ 30 s plus a fourth wash for the indicated time. They were then incubated at 37 $^{\circ}$ C in bombesin plus 125 I-GRP for 1 min so as to mimic the resensitization experimental conditions. Whilst the results suggested that there was indeed an increase in the cell-surface-associated 125 I-GRP, it proved impossible to generate statistically valid data due to the very low levels of specific binding under these conditions. However, indirect experiments measuring PLD activation were performed to determine whether the receptors returned to the cell surface

Table 3 Resensitization of bombesin-stimulated PLD activity and inositol phosphate generation

Cells were labelled with both [3 H]inositol and palmitate as described in the Materials and methods section. They were pretreated with vehicle or 100 nM bombesin for 1 min in the absence of butanol or LiCl, then washed with 3×0.5 ml of HHBG over 20 s. They were then preincubated for 90 s with LiCl or washed for a further 2 min 40 s prior to its addition. The cells were subsequently stimulated for 1 min with 100 mM bombesin in the presence of the LiCl and 30 mM butanol. The results are means $\pm$ S.D. where $n = 3$, and are from a single experiment representative of three.

Treatment	PtdBut (d.p.m.)	InsP ₃ (d.p.m.)
Vehicle pretreatment; vehicle stimulation		
1 min 50 s wash	1272 $\pm$ 260	400 $\pm$ 16
4 min 30 s wash	1265 $\pm$ 112	377 $\pm$ 27
Vehicle pretreatment; bombesin stimulation		
1 min 50 s wash	3139 $\pm$ 83	752 $\pm$ 79
4 min 30 s wash	4320 $\pm$ 779	775 $\pm$ 161
Bombesin pretreatment; vehicle stimulation		
1 min 50 s wash	1110 $\pm$ 42	556 $\pm$ 71
4 min 30 s wash	1159 $\pm$ 251	439 $\pm$ 17
Bombesin pretreatment; bombesin stimulation		
1 min 50 s wash	1369 $\pm$ 152	554 $\pm$ 43
4 min 30 s wash	2060 $\pm$ 268	503 $\pm$ 26

Table 4 Effect of restimulation with bombesin on resensitization

Cells were pretreated with 100 nM bombesin or vehicle for 1 min in the absence of butanol, washed in HHBG for 3×30 s and then stimulated with 30 μ M butanol with or without 100 nM bombesin for 10 min, and [3 H]PtdBut was generation determined. Results are expressed as percentages of the [3 H]PtdBut generated following a vehicle pretreatment, wash and 10 min stimulation with bombesin and butanol (or a 1 min stimulation in desensitization controls), and are from a single experiment typical of three (means $\pm$ S.D.; $n = 3$). Mean basal values where vehicle was used in both the pretreatment and stimulation periods were subtracted before the percentage values were determined. The mean basal radioactivity was 2872 ± 627 d.p.m.

Pretreatment	Stimulation	Response (% of unpretreated)
Bombesin	Bombesin + butanol (10 min)	37 $\pm$ 18
Bombesin	Butanol (10 min)	0 $\pm$ 15
Bombesin	Bombesin + butanol (1 min)	0 $\pm$ 7

and also to examine whether the agonist-receptor complex was completely recycled. Cells were pretreated as above with 100 nM bombesin and unbound ligand was removed by washing for 3×30 s. The cells were then incubated in medium containing butanol with or without 100 nM bombesin for 10 min. If bombesin remained bound to its receptor then the return of the bombesin-receptor complex to the cell surface would generate [3 H]PtdBut in the presence of butanol without the need for re-occupancy of the receptors. However, Table 4 shows that no [3 H]PtdBut formation was observed unless bombesin was additionally present in the medium. Hence the receptors did recycle to the cell surface, but re-occupancy was essential for restimulation of PLD activity following desensitization.

Since resensitization required the stimulation of the cells with agonist, the effect of sustained stimulation with bombesin was examined. The cells were desensitized by stimulating with 100 nM bombesin in the absence of butanol for 1 min; the alcohol was

Figure 6 Time course of bombesin-stimulated PLD activity after a 1 min pretreatment in the continual presence of 100 nM bombesin

Bombesin-stimulated [3 H]PtdBut generation was determined in [3 H]palmitate-labelled cells that had been pretreated with 100 nM bombesin for 1 min in the absence of butanol prior to the addition of 30 mM butanol. $\square$, control cells; $\blacksquare$, stimulated cells. Each point represents the mean $\pm$ S.D. of triplicate determinations and is taken from a single experiment typical of three. The numbers refer to the significance values obtained from an unpaired *t* test comparing the following time points: 1, 4 min versus 1 min, $P = 0$; 2, 7 min versus 4 min, $P = 0.003$; 3, 16 min versus 7 min, $P = 0.009$; 4, 22 min versus 16 min, $P = 0.0012$; 5, 28 min versus 22 min, $P = 0.085$; 6, 34 min versus 28 min, $P = 0.041$; 7, 49 min versus 34 min, $P = 0.047$.

then added and the formation of [3 H]PtdBut was examined over a 1 h time course. Figure 6 demonstrates that there was a pattern of desensitization/resensitization which appeared to be repeated four times over the 1 h time period. This suggests that in a mitogenic situation for example, where continued presence of the agonist is necessary, pulses of PLD activation occur which may be important for growth-factor-mediated signalling. In this regard, we have previously observed a similar pulsing of platelet-derived growth factor-stimulated PLD activity in Swiss 3T3 cells (Plevin et al., 1991). It is probable that the pulsing of bombesin-stimulated PLD activity is a consequence of cycling of the bombesin receptor between the cell surface and the interior, as suggested by the binding experiments described above, with re-occupation of the receptor presumably leading to further desensitization.

The downstream effector of desensitization thus remains to be identified. Other signalling pathways stimulated by the occupation of the bombesin receptor include the activation of cytosolic tyrosine kinase activity (Zachary et al., 1991) and of a number of Ser/Thr kinases, in addition to protein kinase C, such as S_6 kinase, myelin basic protein kinase and casein kinase 2 (Agostinis et al., 1992). The activation of tyrosine kinase is perhaps relevant to the results described in the present paper, since the EC_{50} value for bombesin was 0.3 nM which, like that for desensitization, is an order of magnitude lower than that for other bombesin-stimulated events. However, this similarity in EC_{50} values may be coincidental, since, unlike the situation reported for the stimulated neutrophil (Uings et al., 1992), stimulated tyrosine kinase activity only plays a minor role in bombesin-stimulated PLD activity in Swiss 3T3 cells (C. P. Briscoe and M. J. O. Wakelam, unpublished work).

As stated above, the exact mechanism whereby occupation of the bombesin receptor leads to the stimulation of PLD remains incompletely defined, and indeed this may remain the case until the enzyme is purified. Nevertheless, the disparity in the time courses of desensitization and in particular resensitization for bombesin-stimulated PLD activity (Figure 2a) and inositol

phosphate generation (Figure 2b) provides further evidence for independent regulation of these two signalling pathways. The fact that bombesin pretreatment prevents subsequent stimulation of PLD activity in response to A23187 (Table 1) but not PMA also emphasizes that multiple pathways can be involved in the stimulation of the phospholipase.

The rapidity of the desensitization of bombesin-stimulated PLD activity, along with our previous observation that butanol only reduces the sustained generation of DAG by 30 % (Cook et al., 1991), argues against PLD-catalysed PtdCho hydrolysis being a pathway for the generation of DAG in a growth-factor-stimulated cell. Thus PtdOH may play a second messenger role. Indeed, the transient bursts of PtdOH produced in response to continual bombesin stimulation of PLD might act to desensitize PLD activity by feedback regulation. As regards messenger functions, a Ca^{2+} -independent phosphatidate-stimulated kinase has been reported (Bocckino et al., 1991), and PtdOH has also been demonstrated to have effects on protein kinase C (Epand and Stafford, 1990), p21^{ras} GAP (GTPase activating protein) (Tsai et al., 1989) and p21^{ras} GIP (GTPase inhibitory protein) (Tsai et al., 1990), adenylate cyclase (Murayama and Ui, 1987) and phosphatidylinositol 4-phosphate kinase (Moritz et al., 1992). PtdOH accumulation has been reported to correlate with mitogenesis in platelet-derived growth factor-stimulated Balb/c3T3 cells (Fukami and Takenawa, 1992) and to act as a mitogen in quiescent fibroblasts (Van Corven et al., 1992). Alternatively, it may be converted to lyso-PtdOH, which has mitogenic actions (Van Corven et al., 1992) and has been suggested to play a role in cytoskeletal reorganization (Ridley and Hall, 1992).

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