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Heterologous desensitization of bombesin- and vasopressin-stimulated phospholipase D activity in Swiss 3T3 fibroblasts

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Abstract Bombesin- and vasopressin-stimulated phospholipase D (PLD) activities are rapidly desensitized in 3T3 cells, in addition both agonists are subject to heterologous desensitization. Binding studies showed that homologous desensitization was partly a result of loss of cell surface receptors, whilst heterologous desensitization was independent of receptor changes. Pretreatment with either agonist reduced subsequent GTPγS-stimulated PLD activity by 50% whereas a pretreatment with GTPγS did not attenuate the response, suggesting that the G-protein or downstream effector systems were affected by receptor activation resulting in desensitization. The desensitization of receptor-stimulated PLD activation provides support for the phospholipase functioning in a key signalling pathway.

Key words: Phospholipase D; Desensitization; G-protein; Fibroblast

1. Introduction

Desensitisation is a key characteristic of second-messenger pathways and is represented by diminished responsiveness to persistent agonist stimulation. A notable feature of PtdIns-PLC-linked receptors is the very rapid homologous desensitisation to agonist (reviewed in [1]), characterised by a decrease in the rate of phosphoinositide hydrolysis, the extent of which is receptor specific [2,3]. Homologous desensitisation of bombesin-stimulated Ins(1,4,5)P_3 production in Swiss 3T3 cells is rapid, reversible, dose dependent and PKC-independent [4]. In addition heterologous desensitisation of both vasopressin and bombesin-stimulated Ins(1,4,5)P_3 has been detected (unpublished results).

Further to the well characterised hydrolysis of PtdInsP_2 by phospholipase C, the phospholipase D (PLD) catalysed hydrolysis of phosphatidylcholine in response to a range of agonists has been reported [5]. It remains incompletely defined as to how an occupied cell surface receptor stimulates PLD activity, but activation has been proposed to be downstream of protein kinase C activity, tyrosine kinase activity or changes in calcium concentration, a role for the small molecular weight G-protein ARF in directly regulating PLD has also been proposed [6,7]. Bombesin-stimulated PLD activity in Swiss 3T3 cells as determined by [^{3}H]PtdBut generation is inhibited by 50–70% by the selective PKC inhibitor Ro-31-8220 [8] which would suggest that activation is predominantly downstream of PtdInsP_2 hydrolysis. We have previously shown that pretreatment of Swiss 3T3 cells with vasopressin or bombesin completely attenuated the [^{3}H]PtdBut generation in response to a subsequent stimulation with the same agonist [9]. This homologous desensitisation was found to be PKC-independent and rapidly reversible even in the continual presence of agonist. At least some of the attenuated response was thought to be due to the rapid loss in cell-surface receptor binding. The loss of cell-surface binding was subsequent to an internalisation of the receptor, with receptor recycling to the cell surface occurring over a similar time course to resensitization of agonist-stimulated PLD activity. However, resensitization of agonist-stimulated PLD activity was less than the recovery of cell surface receptor binding suggesting that downstream signalling components were also affected. Furthermore the IC_{50} for bombesin-mediated homologous desensitisation of stimulated PLD activity was 10-fold lower than other bombesin-receptor linked responses. In order to investigate the effect of a short agonist pretreatment on downstream effector mechanisms the heterologous nature of desensitisation was therefore determined in both intact and permeabilised 3T3 cells.

2. Materials and methods

2.1. Cell culture

Swiss 3T3 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) containing 10% (v/v) newborn calf serum, 27 mg glutamine/ml and penicillin/streptomycin (250 units/ml and 250 mg/ml, respectively) at 37°C in a humidified atmosphere of air/CO_2 (19:1). Cells were grown to confluency and quiescence by serum depletion in 2% (v/v) calf serum for 24 h prior to experiment. For labelling studies ([9,10(*n*)-^{3}H]palmitic acid, 2 μCi in 0.5 ml) was included in the 2%-serum containing medium.

2.2. Assay of PLD transphosphatidylation activity in whole cells

Quiescent Swiss 3T3 cells labelled with [^{3}H]palmitic acid were washed in 0.5 ml Hanks buffered saline solution, pH 7.4, containing 10 mM-glucose, 1% (w/v) BSA and 20 mM HEPES (HHBG) for 20 min at 37°C prior to incubation for a further 5 min in 0.5 ml of HHBG containing 30 mM butan-1-ol (0.3%, v/v). 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, [^{3}H]phosphatidylbutanol was separated by t.l.c. as described previously [8].

2.3. Cell permeabilisation

Quiescent Swiss 3T3 cells grown in 24-well plates and labelled with [^{3}H]palmitic acid as described above for the PLD assay in intact cells, were washed in 0.5 ml HHBG for 20 min at 37°C prior to addition for 5 min of streptolysin O (0.6 i.u./ml final) in permeabilisation buffer

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Abbreviations: PLD, phospholipase D; PKC, protein kinase C; PtdBut, phosphatidylbutanol; AVP, arginine vasopressin.

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containing 20 mM HEPES, pH 7.2, 120 mM KCl, 8.49 mM MgCl, 61 μM CaCl, 2 mM KH_2PO_4, 0.1 mM EGTA, 0.1% BSA and 2.5 mM ATP (150 nM Ca^{2+} final) as previously described [10]. This was followed by washing twice with 150 μl of permeabilisation buffer over 10–15 s and stimulation with 150 μl of the test reagent in permeabilisation buffer containing 30 mM butanol at the concentrations and times indicated. Incubations were terminated by direct addition of 0.5 ml methanol and lipids extracted in chloroform. The lipid extracts were then treated exactly as described for the PLD transphosphatidylation assay in intact cells.

2.4. Binding of [^{3}H]AVP and [^{3}H]AVP antagonist to Swiss 3T3 cells

Binding to intact cells was performed as described [11]. Quiescent cells were washed three times over 20 min at 37°C in DMEM supplemented with glutamine, 10 mM HEPES, pH 7.4, and 0.2% (w/v) bovine serum albumin. Pretreatments were performed at 37°C for the times indicated with the test reagent at the required concentration in DMEM supplemented with glutamine, 10 mM HEPES, pH 7.4, 0.05% (w/v) bovine serum albumin, 10 mM glucose and 2 mM bacitracin. After washing incubations were commenced by replacing the medium for the times indicated with incubation buffer containing [^{3}H]vasopressin antagonist [β-mercapto-β,β-cyclopenta-methylenepropionyl^1,*O*-Et-Tyr^2, Val^4,Arg^8]vasopressin ([^{3}H]AVP antagonist) or [8-Arginine][Phe-3,4,5-H(*n*)]vasopressin ([^{3}H]AVP) at the required concentration. The medium was then removed and the cells washed 4 times with ice-cold DMEM supplemented with glutamine, 10 mM HEPES, pH 7.4, and 0.1% (w/v) bovine serum albumin, solubilised for 30 min at room temperature in 0.5 M NaOH, 1% (w/v) SDS and 10 mM HEPES, neutralised with 0.5 M HCl and the radioactivity determined by liquid-scintillation counting. Non-specific binding was determined as the radioligand not displaced by a 200-fold excess of unlabelled antagonist or agonist as appropriate. Cell number was determined using one well per plate which was treated in the same way, except that no agonist pretreatment or radiolabel was added and after the final wash, cells were removed from the well with trypsin and counted using a haemocytometer.

3. Results and discussion

The nature of agonist-desensitised PLD activity was investigated, by pretreating cells with maximal concentrations of either bombesin or vasopressin for 1 min in the absence of butanol, washing the cells briefly, then stimulating with the other agonist for 1 min in the presence of butanol. Fig. 1 shows that pretreatment with 100 nM bombesin or vasopressin completely prevented a subsequent generation of [^{3}H]PtdBut, in response to the same agonist as previously reported [9]. In addition when the alternative agonist was used for the stimulation of PLD activity a reduction in that detected in the control cells was also observed, however, in contrast to homologous desensitization, between 20–30% of the agonist-stimulated [^{3}H]PtdBut generated attained in the absence of any pretreatment remained. Thus agonist-stimulated PLD activation can be desensitized in a heterologous as well as a homologous manner.

Since the homologous desensitization of bombesin-stimulated PLD activity is in part mediated through a reduction in cell surface receptors, vasopressin receptor number was examined. Firstly, the vasopressin receptors upon Swiss 3T3 cells were characterised, the number of vasopressin receptors per cell was determined by measuring the specific binding of a [^{3}H]vasopressin antagonist at 4°C as a function of ligand concentration. Equilibrium binding of the [^{3}H]vasopressin antagonist was reached after 1 h and Scatchard analysis generated a K_d of 2.5 nM and a B_{max} of approximately 50,000 receptors per cell (Fig. 2). This value is similar to that reported by Collins and Rozengurt [12]. Table 1 shows that a 1 min pretreatment with a maximal dose of bombesin at 37°C had no effect upon the binding of either [^{3}H]AVP or the [^{3}H]AVP antagonist at 4°C, whereas a 1 min pretreatment with unlabelled AVP at 37°C reduced subsequent [^{3}H]vasopressin antagonist binding at 4°C by 70%. This suggested that, as we have previously reported for bombesin, homologous desensitization of AVP-stimulated PLD activity was due to a reduction in cell surface vasopressin receptor number. In contrast heterologous desensitisation was

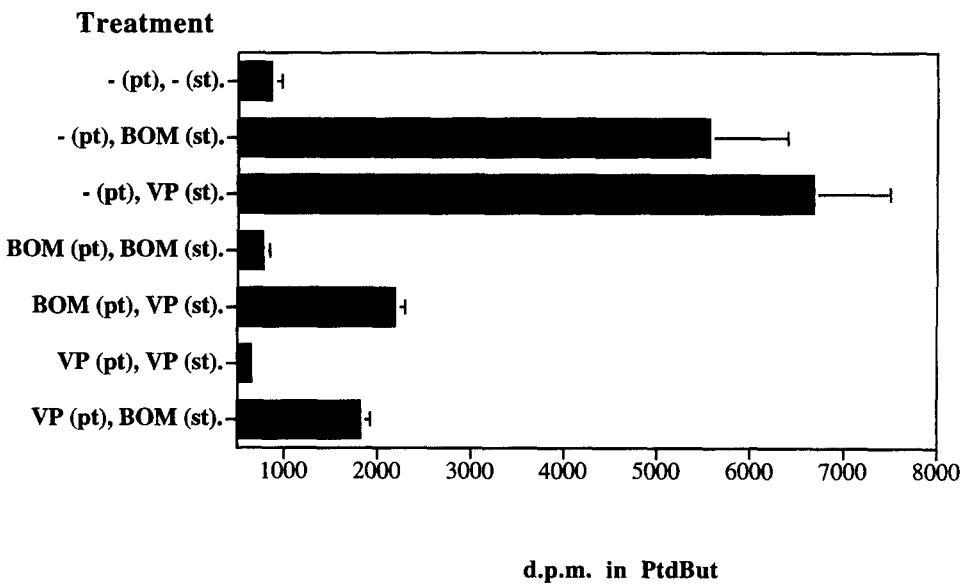

Fig. 1. Heterologous desensitisation of agonist-stimulated PLD activity. [^{3}H]Palmitate-labelled cells were pretreated for 1 min with buffer, 100 nM vasopressin or 100 nM bombesin in the absence of butanol, then washed for 3 × 30 s with buffer. Cells were stimulated for 1 min in the presence of 30 mM butanol with buffer, 100 nM bombesin or 100 nM AVP. Incubations were terminated with ice-cold methanol, chloroform extracts prepared and the generation of [^{3}H]PtdBut quantified. Results are expressed as radioactivity in [^{3}H]PtdBut (mean d.p.m. ± S.D., n = 3) and are from a single experiment typical of three. −(pt), cells pretreated with buffer; −(st), cells stimulated with buffer + butanol; BOM (pt), cells pretreated with 100 nM bombesin; BOM (st), cells stimulated with 100 nM bombesin + butanol; VP (pt), cells pretreated with 100 nM AVP; VP (st), cells stimulated with 100 nM AVP + butanol.

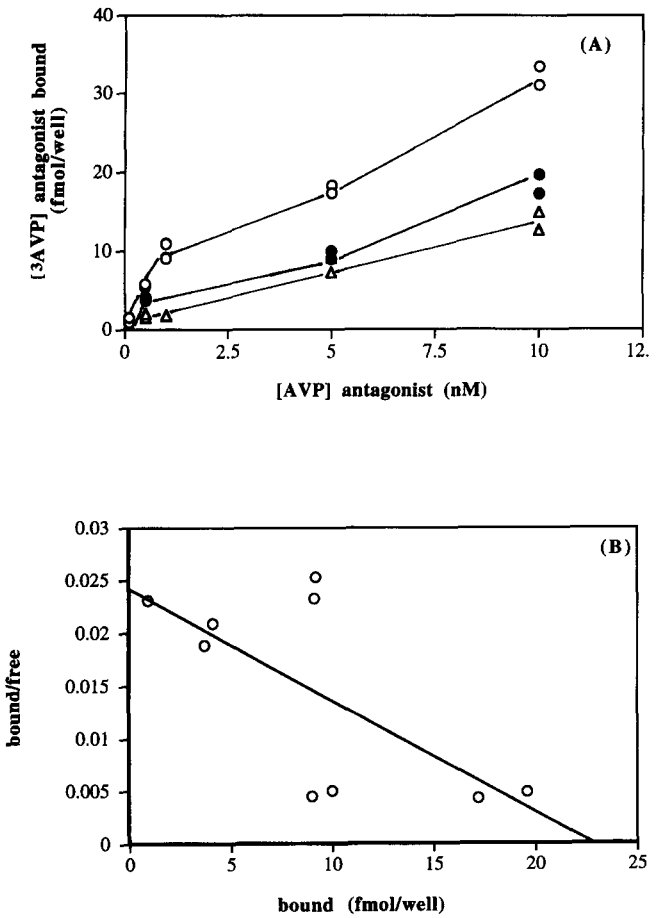

Fig. 2. Scatchard analysis of [^{3}H]AVP antagonist binding to intact Swiss 3T3 cells at 4°C. Swiss 3T3 cells were grown in 6-well plates, washed and incubated with [^{3}H]AVP antagonist at concentrations indicated for 1 h at 4°C, cell associated radioactivity was then determined as in section 2. Non-specific binding at each concentration was determined in the presence of a 200-fold excess of AVP antagonist and was subtracted from total binding to give the specific binding shown. Results are expressed as: (A) a saturation isotherm of total (○), specific (●) and non-specific (△) binding, of [^{3}H]AVP antagonist in fmol/well, (B) the derived Scatchard plot where, B_{max} = 24 fmol, K_d = 2.5 nM. Each point was performed in duplicate and results were taken from a single experiment representative of three. The number of cells per well was approximately 2.8×10^5.

not a consequence of a reduction in cell surface receptors. The heterologous desensitization of bombesin-stimulated PLD activity was also not due to a reduction in receptor number since a 1 min pretreatment with AVP was without effect upon the binding of [^{125}I]gastrin releasing peptide (which is an agonist for bombesin receptors) over 1 min at 37°C (results not shown). Indeed prolonged AVP pretreatment has been reported not to alter the number, affinity or internalisation capacity of bombesin-receptors in Swiss 3T3 cells [13].

Bombesin and vasopressin receptors in Swiss 3T3 cells couple through the pertussis-toxin insensitive G-protein, G_q to the activation of phospholipase Cβ1. We have previously reported that bombesin-stimulated PLD activity was also regulated by guanine-nucleotides, though whether a G-protein other than G_q was involved was not determined [14]. However there is evidence that the small molecular weight G-proteins ARF [6,7] and rho [15] can regulate activity. Experiments were thus performed to investigate whether a short agonist exposure could affect the stimulation of PLD activity through G-protein activation alone. Permeabilised cells were pretreated for 1 min with bombesin (Fig. 3A), or vasopressin (Fig. 3B), washed briefly and stimulated for 5 min with 30 μM GTPγS. Both 3 nM bombesin and 30 nM vasopressin partially reduced the subsequent generation of [^{3}H]PtdBut in response to GTPγS in the presence of butanol, to approximately 50% of that attained in the absence of pretreatment. Fig. 3A also shows that a 1 min pretreatment with 30 μM GTPγS did not reduce subsequent GTPγS stimulated [^{3}H]PtdBut accumulation. This suggested that agonist-induced desensitisation of PLD activity is not due solely to receptor/G-protein uncoupling which has been proposed to be the site of PMA-mediated inhibition of bombesin-stimulated Ins(1,4,5)P_3 generation in Swiss 3T3 cells [16]. Therefore a component of the signalling pathway at, or downstream of the G-protein is also affected.

The agonist-induced reduction in G-protein regulated PLD activity may account for the attenuation of stimulated PLD activity observed in response to a second agonist (Fig. 1). This may occur if the receptors for both agonists use the same pool of G-proteins to activate PLD as proposed for the α_1-adrenoceptor and the AVP receptor in hepatocytes [17]. The finding that GTPγS pretreatment of cells did not decrease a subsequent GTPγS-stimulation of PLD activity suggested that activation of the G-protein itself, is insufficient for desensitisation but that receptor activation and coupling to the G-protein is essential. This contrasts with desensitisation of the β-adrenergic receptor where stimulation of adenylyl cyclase through the activation of G_s was not affected despite loss of responsiveness to the agonist and alterations in the function of G_s did not affect desensitisation of β-adrenergic receptors [18].

As agonist-stimulated PLD activity is reduced by 50–70% following pretreatment with the protein kinase C inhibitor Ro-31-8220 [8] and heterologous desensitisation of stimulated PLD activity resulted in such a large attenuation of the response, it proved impossible to demonstrate the involvement of PKC in

Table 1
Effect of a 1 min pretreatment at 37°C with 30 nM vasopressin or 100 nM bombesin on [^{3}H]AVP antagonist binding

Treatment	Specific [^{3}H]AVP antagonist bound (fmol/well)	% of [^{3}H]AVP antagonist bound in the absence of pretreatment
No pretreatment, 0 min wash	15.9 ± 0.6	100 ± 4
1 min AVP pretreatment, 0 min wash	5.2 ± 0.1	33 ± 1
1 min bom pretreatment, 0 min wash	14.9 ± 1.7	94 ± 9

Quiescent cells were washed and pretreated for 1 min with 30 nM AVP, 100 nM bombesin or incubation buffer alone at 37°C. After washing the binding of [^{3}H]AVP antagonist was determined as described in section 2. Non-specific binding was determined at each time point, by including a 200-fold excess of unlabelled AVP antagonist (1 μM) in the binding medium. Results are expressed as: specific bound [^{3}H]AVP antagonist in fmol/well, after subtracting non-specific binding (mean ± S.D., n = 2); also as % of [^{3}H]AVP antagonist bound, where incubation buffer was used during the pretreatment period (mean ± S.D.%, n = 2). Results are taken from a single experiment representative of two. The number of cells per well was approximately 1.8×10^5.

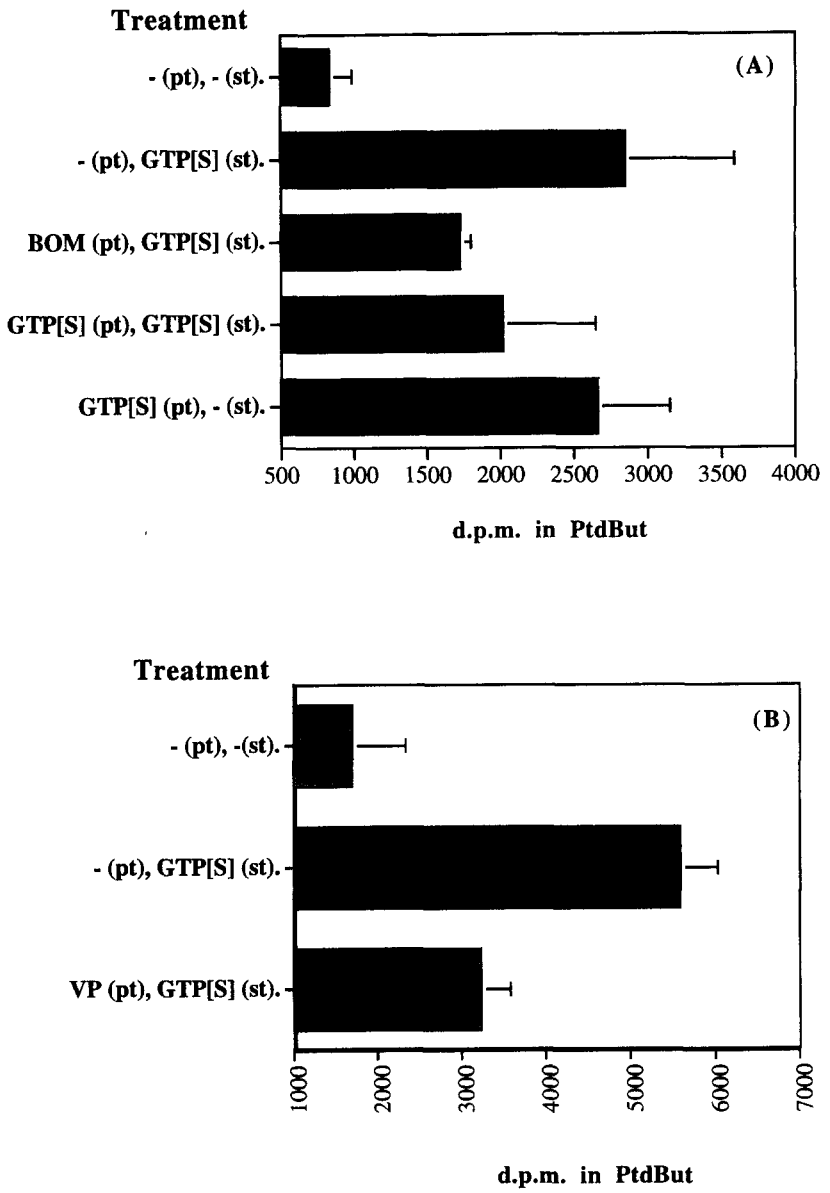

Fig. 3. Effect of agonist pretreatment on GTPγS-stimulated PLD activity. [^{3}H]Palmitate-labelled cells were permeabilised with streptolysin-O (0.6 U·ml^{-1}), then pretreated for 1 min in the absence of butanol, with buffer or; (A) 3 nM bombesin or GTPγS; (B) 30 nM vasopressin. Cells were washed stimulated for 5 min with vehicle or 30 μM GTPγS in the presence of 30 mM butanol and the generation of [^{3}H]PtdBut determined. Results are expressed as radioactivity in [^{3}H]PtdBut (mean d.p.m. ± S.D., n = 3) from a single experiment representative of three. −(pt), cells pretreated with buffer; −(st), cells stimulated with buffer + butanol; BOM (pt), cells pretreated with 100 nM bombesin; VP (pt), cells pretreated with 100 nM vasopressin; GTP[S] (pt), cells pretreated with GTPγS; GTP[S] (st), cells stimulated with GTPγS + butanol.

he phenomenon. However we have previously shown that a 1 min pretreatment with bombesin had no effect on a subsequent PMA stimulation of PLD activity [9], suggesting that agonist pretreatment of cells did not affect the stimulation of PLD induced by the activation of PKC.

A number of studies have suggested a role for an increase in $Ca^{2+}]_i$ in the regulation of agonist-stimulated PLD activity. In relation to this carbachol-mediated desensitisation of α-thrombin-stimulated PLD activity in Chinese hamster lung fibroblasts has been proposed to be due to the depletion of intracellular Ca^{2+} stores [19]. Heterologous desensitisation of agonist-stimulated Ca^{2+} mobilisation in astrocytoma cells [20] and in bombesin-stimulated human small cell lung cancer cell lines [21] and has been suggested to be due to emptying of the Ins(1,4,5)P_3 sensitive Ca^{2+} pools. It was therefore considered possible that changes in $[Ca^{2+}]_i$ may be involved in heterologous desensitization of PLD activity in Swiss 3T3 cells.

Arguing against a role for Ca^{2+} are the observations that changes in $[Ca^{2+}]_i$ play only a minimal role in bombesin-stimulated PLD activity [8] and that treatment of cells with the Ca^{2+} ionophore A23187 which elevates $[Ca^{2+}]_i$ induces a small desensitization of bombesin-stimulated PLD [9]. Further in the experiments examining the effect of GTPγS upon PLD activity in permeabilised cells, in which a desensitization was or was not observed depending upon pretreatment, a Ca^{2+}-EGTA buffer which maintained the $[Ca^{2+}]$ at 150 nM was utilised.

Since neither effects upon $[Ca^{2+}]_i$ or PKC activity appear to be involved in heterologous desensitization of agonist-stimulated PLD activity, the involvement of other second messengers must be considered. Bombesin is among several peptide agonists known to induce the rapid tyrosine phosphorylation of a number of proteins in Swiss 3T3 cells [14,22]. The finding that genistein partially inhibited bombesin-stimulated PLD activity suggested that tyrosine kinases may be involved in the PKC-independent pathway of agonist-stimulated [^{3}H]PtdBut generation. It is conceivable that phosphorylation on tyrosine of a component of the signalling pathway downstream of the receptor/G-protein interaction may prevent the full activation of PLD by another agonist. Furthermore phosphorylation by a serine/threonine kinase such as MAP kinase which is activated by mechanisms involving tyrosine phosphorylation could also be involved. The product of PLD activation, phosphatidate (PtdOH) might also exert a regulatory feedback effect. PtdOH has been reported to have multiple second messenger effects (reviewed in [23]) including inhibition of adenylyl cyclase, activation of phosphatidate-dependent kinases [24] and regulation of small molecular weight G-protein guanine nucleotide state [25]. Full elucidation of the mechanism underlying the loss of stimulated PLD activity in a homologous or heterologous manner awaits purification of the enzyme and thus reconstitution with the possible components of the stimulatory pathway. Nevertheless the identification of both homologous and heterologous desensitization pathways in the regulation of agonist-stimulated PLD activity emphasises the potential importance of this phospholipase as a second messenger generating enzyme.

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