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Mass measurement of inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol in bombesin-stimulated Swiss 3T3 mouse fibroblasts

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Two specific and selective assays were used to measure changes in the mass of Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol in bombesin-stimulated Swiss 3T3 cells. The results demonstrate that the increase in Ins(1,4,5)P_3 was extremely rapid, but transient, returning to basal levels by 30 s. In contrast, the increase in *sn*-1,2-diacylglycerol was biphasic: the first phase mirrored the transient Ins(1,4,5)P_3 response, whereas the second phase was sustained and occurred in the absence of elevated Ins(1,4,5)P_3. The possible source of the second phase of diacylglycerol is discussed.

INTRODUCTION

Bombesin, a potent peptide mitogen for Swiss 3T3 cells (Rozengurt & Sinnett-Smith, 1983), binds to a single population of high-affinity receptors (Brown & Laurie, 1986), thereby stimulating the rapid hydrolysis of PtdIns(4,5)P_2 (Brown *et al.*, 1984; Heslop *et al.*, 1986; Takuwa *et al.*, 1987*a*). The products of this hydrolysis both have second-messenger roles: Ins(1,4,5)P_3 triggers the release of intracellular stores of Ca^{2+} (Berridge & Irvine, 1984), whereas *sn*-1,2-diacyglycerol (DAG) activates the Ca^{2+}- and phospholipid-dependent enzyme protein kinase C (Nishizuka, 1984).

Bombesin-stimulated inositol polyphosphate metabolism has previously been measured in Swiss 3T3 cells by prelabelling the inositol lipids with [^{3}H]inositol (Brown *et al.*, 1984, 1987; Heslop *et al.*, 1986; Takuwa *et al.*, 1987*a*; Nanberg & Rozengurt, 1988). Similarly, radioisotopic labelling of cellular phospholipids with [^{3}H]glycerol has been used to study bombesin-stimulated diacylglycerol production (Muir & Murray, 1987). However, interpretation of the results of these experiments in terms of Ins(1,4,5)P_3 and DAG mass are limited. For example, it has to be assumed that there are no distinct pools of phospholipids (Monaco, 1982; Michell *et al.*, 1988) or that the radioisotope is equally distributed throughout all pools. In addition, t.l.c. methods used to isolate radioactive diacylglycerols do not resolve *sn*-1,2-diacylglycerol from *sn*-2,3-diacylglycerol. Furthermore, the use of a radiolabelled fatty acid precludes the detection of changes in those species of diacylglycerols which remain unlabelled.

We have, therefore, used two mass assays, one specific for Ins(1,4,5)P_3 and the other for DAG, to provide the first mass measurements of both second messengers in bombesin-stimulated Swiss 3T3 cells.

MATERIALS AND METHODS

Materials

Tissue-culture materials were from Gibco, Paisley, Renfrewshire, Scotland, U.K. Bombesin was from CRB, Cambridge, U.K. [^{3}H]Ins(1,4,5)P_3 (sp. radioactivity 38–44 Ci/mmol) and [γ-^{32}P]ATP (sp. radioactivity 3 Ci/mmol) were obtained from Amersham International. DAG kinase (from *Escherichia coli*) was purchased from Lipidex, Westfield, NJ 07090, U.S.A.; cardiolipin was from Avanti Polar-Lipids, Pelham, AL 35124, U.S.A. Ins(1,4,5)P_3 was generously provided by Dr. R. F. Irvine (A.F.R.C., Cambridge, U.K.). All other chemicals were of the highest grades commercially available.

Culture of Swiss 3T3 cells

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn-calf serum at 37 °C in an air/CO_2 (19:1) atmosphere at 95% humidity. Cells were routinely passaged when subconfluent, whereas confluent quiescent cells were used in all experiments.

Measurement of Ins(1,4,5)P_3 mass

Swiss 3T3 cells were cultured in 75 cm^2 flasks as described above until confluent and quiescent. Cells were harvested mechanically into their culture medium and preincubated, in suspension, for 45 min at 37 °C. After two washes in Hanks buffered saline, pH 7.4, containing 2% (w/v) BSA (fraction V) and 10 mM-glucose (HBG), cells were resuspended in HBG at approx. 2.5×10^6 cells per sample. Cells were incubated at 37 °C for a further 15 min before the experiment.

Aliquots of cells (50 μl) were incubated for the required times at 37 °C with 25 μl of HBG or bombesin at the concentrations indicated. Incubations were terminated by addition of 25 μl of ice-cold 10% (v/v) $HClO_4$, and the samples were extracted on ice at 4 °C. After neutralization with 1.5 M-KOH/60 mM-Hepes, aliquots of the supernatant were assayed for Ins(1,4,5)P_3 by the method of Palmer *et al.* (1989). Briefly, Ins(1,4,5)P_3 of the cell extract was allowed to compete with [^{3}H]Ins(1,4,5)P_3 for the Ins(1,4,5)P_3-specific binding site of bovine adrenocortical microsomes. A standard curve based on pure Ins(1,4,5)P_3 was constructed in parallel and thus the quantity of Ins(1,4,5)P_3 in the cell extract was determined.

Measurement of DAG mass

Swiss 3T3 cells were seeded on to 60 mm-diameter culture dishes in DMEM + 10% (v/v) newborn-calf

Abbreviations used: DAG, *sn*-1,2,-diacylglycerol; HBG, Hanks buffered saline/2% (w/v) BSA (fraction V)/10 mM-glucose; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate ('TPA'); EC_{50}, concentration causing 50% of the maximum effect.

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serum. After 5 days growth, the medium was changed to DMEM + 1 % (v/v) calf serum and the cells grown for a further 48 h until confluent and quiescent. The cells were washed in HBG for 20 min and then incubated with 2 ml of HBG containing bombesin for the times indicated. Incubations were terminated by removal of the medium and the addition of 1 ml of ice-cold methanol. Samples were harvested from the dishes, combined with chloroform and the lipids extracted. Phases were split by the addition of chloroform and water. Aliquots of the organic phase were assayed for DAG mass and total phospholipid phosphorus. In some experiments, cells were prepared as described for the measurement of Ins(1,4,5)P_3 and gave similar results.

Assay of DAG mass was essentially by the method of Preiss *et al.* (1986). Samples were incubated with DAG kinase and [γ-^{32}P]ATP and the products separated by t.l.c. The band co-migrating with pure 1-stearoyl-2-arachidonoyl-*sn*-glycerophosphate (R_F 0.41) was excised and its radioactivity determined. When known amounts of *sn*-1-stearoyl-2-arachidonoylglycerol were assayed in parallel, a linear quantitative conversion into phosphatidate was obtained. Thereby the DAG content of cell extracts was calculated. DAG levels were quantified as nmol/100 nmol of phospholipid (mol %). An aliquot of the chloroform phase from cell samples was evaporated to dryness and digested in 0.6 ml of concentrated $HClO_4$ at 260 °C for 2 h. Free phosphate was assayed by the method of Bartlett (1959). In addition, cell number, determined using a haemocytometer, was used to correlate data between individual experiments.

RESULTS

Stimulation of Swiss 3T3 cells with 617 nM-bombesin caused the rapid generation of Ins(1,4,5)P_3 which reached a peak at 5 s, the earliest time point measured. In general, Ins(1,4,5)P_3 was increased by approx. 14-fold at 5 s (13.55 ± 6.5, $n = 8$ expts.). Ins(1,4,5)P_3 returned to basal values by 20 or 30 s (Fig. 1) and was maintained at this level for the remainder of the 15 min stimulation (results not shown). Pretreatment of cells with a maximal dose of bombesin for 1 min decreased Ins(1,4,5)P_3 production in response to stimulation with a subsequent maximal dose (Table 1).

In contrast, DAG production in reponse to bombesin was biphasic (Figs. 2*a* and 2*b*). DAG increased rapidly on stimulation and reached a peak at 5 s before returning towards basal levels at between 30 s and 1 min. However, DAG increased in a pronounced second phase from 1 to 15 min. Indeed, DAG levels remained elevated above control for up to 60 min (results not shown). Details varied between individual experiments: the initial peak of DAG was sometimes seen as a less pronounced shoulder, but elevated DAG was always sustained after Ins(1,4,5)P_3 had returned to pre-stimulation levels (Fig. 1) and at a time when Ins(1,4,5)P_3 generation was apparently desensitized (Table 1).

The dose-dependency of bombesin-stimulated Ins(1,4,5)P_3 and DAG generation at 5 s is shown in Fig. 3. The EC_{50} (concentration causing 50 % of the maximum effect) values, determined using a computer-assisted curve-fitting program, were similar for the generation of each second messenger: 5.88 ± 3.66 nM ($n = 3$) for Ins(1,4,5)P_3 and 1.66 ± 1.15 nM ($n = 3$) for DAG. The EC_{50} value for bombesin-stimulated DAG generation at

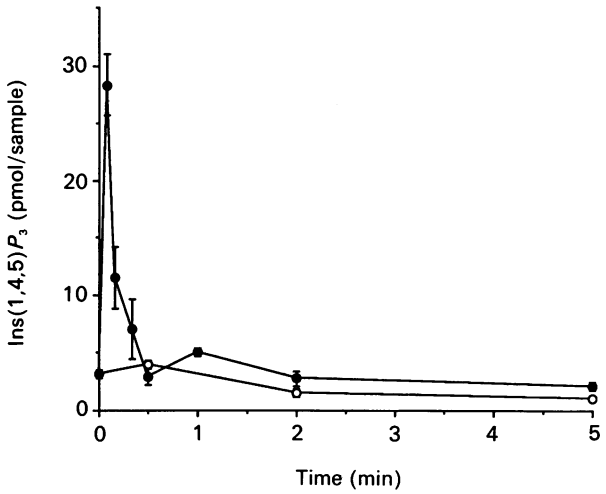

Fig. 1. Time course of Ins(1,4,5)P_3 mass changes in bombesin-stimulated Swiss 3T3 cells

Cells that had been harvested mechanically were incubated for the times indicated with 617 nM-bombesin (●) or HBG (○). Incubations were terminated by the addition of ice-cold 10% (v/v) $HClO_4$, and the supernatants were neutralized and assayed for Ins(1,4,5)P_3 mass as described in the materials and methods. The results are means ± S.D. ($n = 3$) from a single experiment, typical of three.

Table 1. Homologous desensitization of bombesin-stimulated Ins(1,4,5)P_3 production in Swiss 3T3 cells

Cells that had been harvested mechanically were incubated with 617 nM-bombesin for the times indicated. The Ins(1,4,5)P_3 mass of the cell extracts was determined as described in the Materials and methods section. Results are means ± S.D. ($n = 3$) for a single experiment typical of three.

Treatment and incubation time	Ins(1,4,5)P_3/sample (pmol)
No addition	4.9 ± 1.28
Bombesin	
for 5 s	27.33 ± 2.52
for 30 s	6.8 ± 0.61
for 60 s, then 5 s	7.06 ± 1.94

5 min was also similar (0.8 ± 0.28 nM; $n = 2$; results not shown).

Normalization of the data to mol/10^6 cells revealed striking differences in the generation of the two second messengers. A 5 s stimulation with 617 nM-bombesin produced an increase of 89 ± 32 pmol of DAG/10^6 cells ($n = 3$), whereas Ins(1,4,5)P_3 was only elevated by 28.8 ± 11.9 pmol/10^6 cells ($n = 7$).

DISCUSSION

The results reported here are the first mass measurements of Ins(1,4,5)P_3 in bombesin-stimulated Swiss 3T3 cells. Bombesin has previously been demonstrated to stimulate the generation of [^{3}H]inositol phosphates in [^{3}H]inositol-labelled Swiss 3T3 cells (Brown *et al.*, 1984, 1987). In more recent studies (Heslop

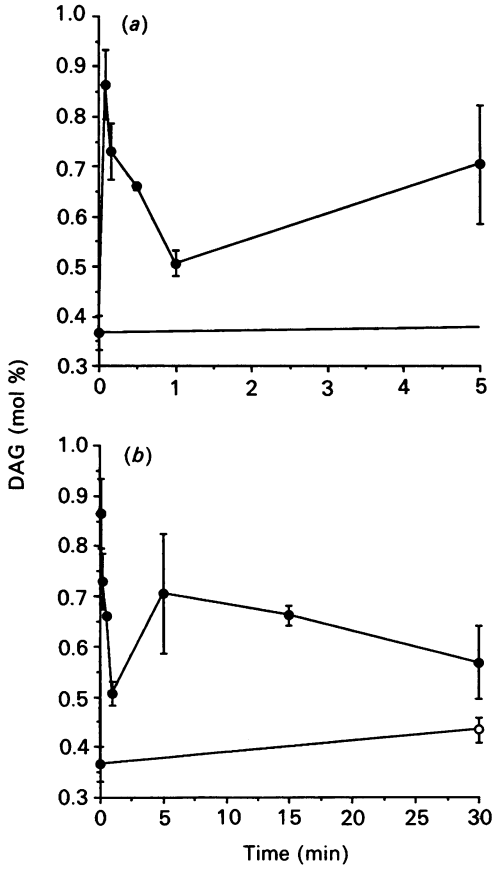

Fig. 2. Time course of DAG mass changes in bombesin-stimulated Swiss 3T3 cells

Cells, attached to 60 mm-diameter tissue-culture dishes, were incubated for the times indicated with 617 nM-bombesin (●) or HBG (○). Lipids were extracted under neutral conditions, and the DAG mass was assayed as described in the Materials and methods section. The results are means ± S.D. ($n = 3$) from a single experiment, typical of three. (*a*) 0–5 min; (*b*) 0–30 min.

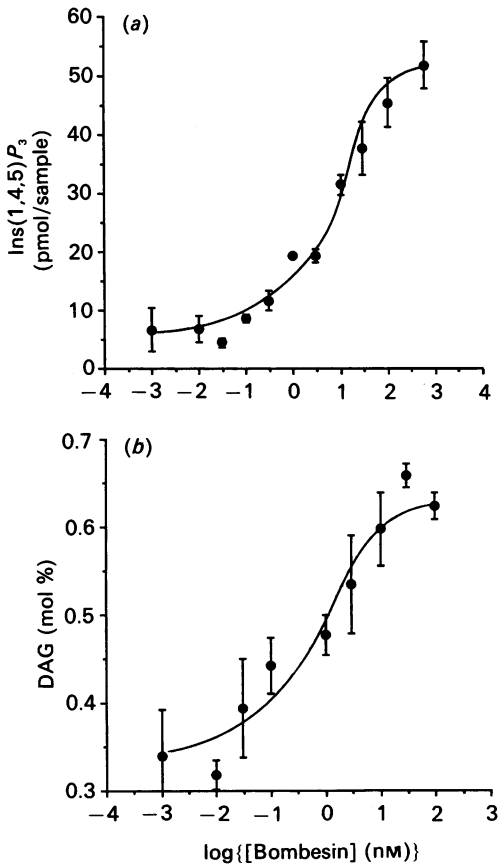

Fig. 3. Dose–response curves for bombesin-stimulated Ins(1,4,5)P_3 and DAG production

Cells, incubated as described in the Materials and methods section, were stimulated with the concentration of bombesin indicated for 5 s. Ins(1,4,5)P_3 and DAG mass were assayed as described in the Materials and methods section. The results are means ± S.D. ($n = 3$) from two separate experiments, each typical of three. (*a*) Ins(1,4,5)P_3; (*b*) DAG.

et al., 1986; Takuwa *et al.*, 1987*a*; Nanberg & Rozengurt, 1988), the inositol trisphosphate isomers have been resolved by h.p.l.c.. In all cases, the accumulation of Ins(1,4,5)P_3 was observed to be transient.

The kinetics of Ins(1,4,5)P_3 production in this study were similar to those observed by Takuwa *et al.* (1987*a*), i.e. Ins(1,4,5)P_3 levels were maximal at 5–10 s, returned to basal levels by 30 s and were maintained at this level for 10–15 min (see Fig. 1). These kinetics correlate well with those of bombesin-stimulated Ca^{2+} mobilization in Swiss 3T3 cells (Brown *et al.*, 1984; Takuwa *et al.*, 1987*a*; Nanberg & Rozengurt, 1988). These results are also similar to those reported for Ins(1,4,5)P_3-specific mass assay, in bradykinin-stimulated NIH-3T3 fibroblasts and human fibroblasts (Fu *et al.*, 1988; Seishima *et al.*, 1988).

Heslop *et al.* (1986) and Nanberg & Rozengurt (1988) observed maximal Ins(1,4,5)P_3 accumulation at 15–30 s, with a subsequent decline towards basal levels over 2–5 min. However, the two latter studies were conducted in the presence of 10–20 mM-LiCl; thus Ins(1,4,5)P_3 levels might have been sustained as a result of the feedback inhibition of Ins(1,4,5)P_3 5-phosphatase (Shears, 1989).

Bombesin-stimulated Ins(1,4,5)P_3 production will necessarily result in a substrate-induced activation of Ins(1,4,5)P_3 5-phosphatase and 3-kinase. However, the rate of Ins(1,4,5)P_3 production may be diminished as a consequence of desensitization (Table 1). In support of this, the rate of accumulation of total [^{3}H]inositol phosphates in bombesin-stimulated Swiss 3T3 cells, in the presence of 10 mM-LiCl, is reduced after 1 min (11528 d.p.m./min from 0–1 min, 2426 d.p.m./min from 1–5 min, $n = 4$; results from a single typical experiment; (S. J. Cook & M. J. O. Wakelam, unpublished work).

It is also notable that the magnitude of the Ins(1,4,5)P_3 response observed in the present study was considerably larger than that observed in studies that employed [^{3}H]inositol-labelled Swiss 3T3 cells [13.55 ± 6.5-fold ($n = 8$) and approx. 2–3 fold respectively]. This may be due to the greater sensitivity of the Ins(1,4,5)P_3 mass assay, in which changes of as little as 0.2 pmol can be detected. In addition, those studies which employed [^{3}H]inositol-labelled cells all involved a preincubation period of at least 10 min in the absence of [^{3}H]inositol: thus, the specific radioactivity of a hormone-sensitive pool of inositol lipids may be in decline at the point of stimulation and therefore the agonist-stimulated response would be severely underestimated.

Bombesin stimulation of Swiss 3T3 cells resulted in a biphasic DAG response (Figs. 2*a* and 2*b*). The first phase (0–30 s or 1 min) mirrored the transient Ins(1,4,5)P_3 response. The rapid decline in the initial DAG peak may reflect the high activity of a membrane-associated DAG kinase, selective for arachidonoyl-containing DAGs, as reported in Swiss 3T3 cells (MacDonald *et al.*, 1988). The second phase of DAG production (1 min onwards) was sustained for at least 60 min (results not shown) and occurred in the absence of elevated Ins(1,4,5)P_3 and at a time when Ins(1,4,5)P_3 generation was apparently desensitized (see Fig. 1 and Table 1). Takuwa *et al.* (1987*a*) observed a bombesin-stimulated production of DAG in Swiss 3T3 cells of similar magnitude, i.e. approx. 2-fold, which was sustained for 60 min, but which was not biphasic. However, the latter study employed a submaximal concentration of bombesin (1 nM), and the biphasic nature of the DAG response has been reported to be lost at low concentrations of agonist (Wright *et al.*, 1988). In addition, the earliest time point measured was 15 s, at which time the first phase of DAG production would have been in decline (see Fig. 2). In contrast, Muir & Murray (1987) were unable to detect significant DAG production until 5 min in bombesin-stimulated [^{3}H]glycerol-labelled Swiss 3T3 cells.

The similarity in the kinetics of the Ins(1,4,5)P_3 response and the first phase of DAG suggests that they are derived from the same source, i.e. PtdIns(4,5)P_2. However, at 5 s the net increase in Ins(1,4,5)P_3 mass was approx. 3-fold less than the net increase in DAG mass. This disparity may reflect as yet undetermined differences in the kinetic properties of the enzymes removing these second messengers, i.e. Ins(1,4,5)P_3 5-phosphatase and 3-kinase, and DAG kinase and lipase, respectively. Alternatively, the difference may reflect the generation of DAG from a source other than PtdIns(4,5)P_2. Molecular-species analysis of the DAG produced upon stimulation may resolve this question. The second phase of DAG production is, however, clearly divorced from a corresponding rise in Ins(1,4,5)P_3. Consequently the DAG may be derived from a source other than PtdIns(4,5)P_2.

A number of recent studies have suggested that lipids other than polyphosphoinositides may be involved in DAG production via phospholipase C and D mechanisms, e.g. phosphatidylcholine (Bocckino *et al.*, 1987; Pai *et al.*, 1988; Slivka *et al.*, 1988; Pessin & Raben, 1989). Indeed, Pessin & Raben (1989) analysed the molecular species of DAG in α-thrombin-stimulated IIC9 fibroblasts by gas chromatography. They found that the fatty acid composition of the DAG produced in the first 15 s of stimulation was similar to that of PtdIns, whereas DAG produced at 5–60 min stimulation was similar to phosphatidylcholine. Furthermore, we have recently shown that bombesin and phorbol 12-myristate 13-acetate (PMA) stimulate phosphatidylcholine hydrolysis in Swiss 3T3 cells (Cook & Wakelam, 1989), probably via a C-kinase dependent phospholipase D mechanism. Thus the subsequent conversion of phosphatidic acid into DAG by phosphatidate phosphohydrolase may account for the second phase of DAG production observed here. In addition Takuwa *et al.* (1987*b*) have demonstrated that PMA stimulates an increase in DAG mass in Swiss 3T3 cells with kinetics similar to the second phase generated in response to bombesin (Fig. 2*b*). This was without effect upon inositol phosphate formation, but was accompanied by the release of [^{3}H]choline metabolites and loss of [^{3}H]-phosphatidylcholine.

There is a strong correlation between bombesin- and PMA-stimulated [^{3}H]choline release (Cook & Wakelam, 1989), PMA-stimulated DAG production (Takuwa *et al.*, 1987*b*) and the second phase of bombesin-stimulated DAG production (Fig. 2*b*). This suggests that PtdIns(4,5)P_2 hydrolysis cannot be regarded as the sole source of bombesin-stimulated DAG generation, but may serve to initiate its generation from an alternative source, probably phosphatidylcholine.

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