Endocrine Journal 1997, **44** (2), 283–288

Galanin Exerts Dual Action on Inositol-Specific Phospholipase C Activity in Isolated Pancreatic Islets

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Abstract. The intracellular mechanism whereby the neuropeptide galanin inhibits insulin secretion is not established, since the peptide affects several signal pathways, including intracellular messengers such as calcium and cyclic AMP. In this study, we have assessed the effect of galanin on the inositol-specific phospholipase C (iPLC) activity in isolated rat pancreatic islets. The iPLC activity was measured as the generation of inositol 1,4,5-trisphosphate and its metabolite inositol 1,3,4-trisphosphate from the hydrolysis of polyphosphoinositides. Inositol phosphates were measured by anion-exchange fast protein liquid chromatography (FPLC) analysis of extracts from islets prelabelled with myo-^{3}H-inositol. Galanin (1 to 100 nM) significantly increased the glucose-induced (12 mM) accumulation of inositol 1,4,5-trisphosphate after 2 min, but this stimulation of iPLC activity was followed by a significant suppression after 15 min. In the absence of extracellular calcium, both the stimulatory and inhibitory effects of galanin on the iPLC activity vanished. We therefore conclude that galanin initially stimulates iPLC in a calcium-dependent manner, followed by a secondary inhibitory effect. The secondary inhibition of iPLC activity might contribute to the insulinostatic action of the neuropeptide.

Key words: Galanin, Inositol phosphate metabolism, Insulin secretion, Phospholipase C, Pancreatic islets, Signal transduction

(*Endocrine Journal* **44**: 283–288, 1997)

THE neuropeptide galanin is localized to adrenergic nerve terminals in pancreatic islets and strongly inhibits insulin secretion [1]. It has therefore been hypothesized that galanin is an adrenergic neurotransmitter involved in the sympathetic regulation of islet hormone release [2].

The cytoplasmic free [Ca^{2+}] is known to be an important determinant for glucose-induced insulin secretion [3]. Galanin inhibits the glucose-induced increase in cytoplasmic [Ca^{2+}] in B-cells from the ob/ob mouse [4], and this is supposed to be one mechanism underlying the inhibitory action of galanin on insulin secretion (for review see [1]). The influence of galanin on the cytoplasmic [Ca^{2+}] is, however, complex since it was recently demonstrated that galanin in RINm5F cells induced a brief transient increase followed by a reduction in cytoplasmic [Ca^{2+}] [5–7], although this has not been observed in islets [8]. The transient increase in cytoplasmic [Ca^{2+}] may be due to inositol phosphates formed by phosphoinositide hydrolysis, which mobilize Ca^{2+} from intracellular stores [7, 9].

Activation of inositol-specific phospholipase C (iPLC) leads to generation of inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) and its metabolites inositol 1,3,5-trisphosphate (Ins 1,3,5-P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4). Ins 1,4,5-P_3 mobilizes Ca^{2+} from intracellular stores [10]. Although extensively studied, the exact role of iPLC in the stimulus-secretion coupling in pancreatic islets is not fully understood [3]. Furthermore, the effect of galanin on the iPLC

Received: May 13, 1996
Accepted: November 14, 1996
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activity in pancreatic islets is unknown.

In the present study, we have evaluated the effect of galanin on the glucose induced iPLC activity in isolated rat pancreatic islets with special emphasis on the glucose and calcium requirements on iPLC activity to improve our understanding of the intracellular mechanisms induced by galanin.

Materials and Methods

Animals

Male Wistar rats (150–250 g) were used. They were kept at 20 °C on a standard pellet diet and water *ad libitum*.

Chemicals

Percoll was kindly donated by Pharmacia (Uppsala, Sweden), and collagenase, type P, was purchased from Boehringer Mannheim Corp Biochem Div (Indianapolis, IN); rat galanin from rat (Lot 80H05811) and bovine serum albumin (BSA) from Sigma Chemical (St Louis, MO); D-myo-(2-^{3}H)-inositol, D-myo(2-^{3}H)-inositol-1-phosphate, D-myo-(2-^{3}H)-1,4-inositolbisphosphate, D-myo-(2-^{3}H)-1,4,5-trisphosphate and D-myo-(2-^{3}H)-inositol-1,3,4-trisphosphate from DuPont (Dreieich, FRG); Protosol® from NEN Research Products (Boston, MA); and Instagel II from Packard (Groeningen, Netherlands) were used. The other chemicals were of regular laboratory grade.

Isolation of pancreatic islets

Rats were anesthesized with pentobarbital intraperitoneally (0.08 mg per gram body weight), and the pancreas was removed. Islets were isolated from rat pancreata by collagenase digestion followed by separation and purification by discontinuous Percoll gradient centrifugation [11].

Labelling of islets with ^{3}H-inositol

Islets were labelled with ^{3}H-inositol following a previously described method [12]. Pooled islets (2.000–2.400) were washed in N-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-Krebs buffer pH 7.4 containing (in mM) 25 HEPES, 115 NaCl, 24 $NaHCO_3$, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$ and 0.1% BSA (saturated with air/CO_2, 19:1) with 3.3 mM glucose, placed in a 13 × 100 silanized borosilicated tube, and labelled with 200 μl of the Krebs-HEPES buffer with 25 mM glucose and 100 μCi of ^{3}H-inositol for 135 min in a shaking water bath at 37 °C. Islets were washed three times with Krebs-HEPES-3.3 mM glucose, then picked randomly under a stereo-microscope and placed in vials with 100 islets in each tube.

Inositol phosphates release in pancreatic islets

Release of inositol phosphates was measured following a previously described method [12]. One hundred islets were preincubated for 30 min at 37 °C in the same Krebs-HEPES buffer with 10 mM LiCl and 5.5 mM glucose added. The islets were then incubated for 2, 5 or 15 min in the same Krebs-HEPES buffer with 3.3 or 12 mM glucose together with galanin in concentrations from 1 to 100 nM. Control islets were incubated in 3.3 or 12 mM glucose alone. The reaction was stopped by adding 1 ml ice-cold 15% trichloroacetic acid (TCA). Samples were thereafter vortexed for 15 sec and sonicated in an Astrason Ultrasonic Cleaner from Ultrasonic (Farmingdale) for 30 min. Samples were then centrifugated for 20 min at 5,000 g at 4 °C. TCA was extracted twice from the supernatants with 3 ml diethyl ether, neutralized with Tris-base to pH 7.0, and then kept at −20 °C until FPLC analysis. The pellets were dissolved with 200 μl Protosol® at 20 °C for 60 min, neutralized by adding 20 μl glacial acetic acid and transferred to counting vials.

FPLC analysis of inositol phosphates

Samples (2 ml with 1 μmol EDTA) were analyzed by FPLC on a Pharmacia Mono Q HR 5/5 anion-exchange column from Pharmacia (Uppsala, Sweden) calibrated with the radiolabelled inositol phosphates standards. The solvent program is a previously described method [18] which uses gradient of water/ammonium formate (1.7 mM) pH 3.7. Ins-1,3,4-P_3 and Ins-1,4,5-P_3 were separated by isocratic elution of 25–30% ammonium formate with a flow rate of 1.5 ml/min and fraction volume of 0.3 ml. The fractions collected were counted for 2 min after the addition of 4 ml Instagel II

scintillation fluid. Results for ^{3}H-inositol phosphates are expressed as the accumulated radioactivity (in cpm) in the peak of Ins-1,3,4-P_3 and of Ins-1,4,5-P_3 as a percentage of the total radioactivity in each tube including the counts from the labelled phospholipids in the pellets.

Statistics

The data were analyzed by one-way analysis of variance followed by multiple comparisons of means by using the pooled variance estimate based on Scheffe's method. $P<0.05$ was considered statistically significant. The results are expressed as the mean ± SEM based on 4–17 observations in 8 different experiments.

Results

In the first series of experiments, the effect of different concentrations of galanin at 3.3 and 12 mM glucose on the PLC-activity was assessed by measuring levels of inositl-1,4,5-trisphosphate (Ins 1,4,5-P_3) and inositol-1,3,4-trisphosphate (Ins 1,3,4-P_3) in the medium after two min of incubation (Table 1). Following two min incubation of islets in a medium at 12 mM glucose, the concentration of Ins 1,4,5-P_3 was 0.12 ± 0.03, expressed as the accumulated radioactivity as percentage of the total radioactivity. The addition of galanin at 1 nM more than doubled the level of Ins 1,4,5-P_3 to 0.27 ± 0.03 ($P<0.05$). No further stimulation was obtained by galanin at 100 nM, 0.21 ± 0.04 ($P<0.05$), indicating a maximal effect at 10 nM. In contrast, at 3.3 mM glucose, galanin did not affect the level of 1,4,5-P_3. Furthermore, the concentration of 1,3,4-P_3 was not affected by either galanin or glucose.

The second experimental series was performed at 12 mM glucose in the absence of extracellular Ca^{2+} in the medium (Table 2). Under this condition, the galanin-induced stimulation of 1,4,5-P_3 at 12 mM glucose was abolished. Thus, after 2 min of incubation, the concentration of 1,4,5-P_3 in the medium reached 0.09 ± 0.01 with or without galanin.

In the third series of experiments, the levels of inositol phosphates were measured following 2, 5 and 15 min incubation in a medium containing 12 mM glucose (Table 3). In contrast to the

Table 1. Effect of 0, 1 and 100 nM galanin

Galanin	Glucose	Ins 1,3,4-P_3	Ins 1,4,5-P_3	N
0 nM	3.3 mM	0.50 ± 0.04	0.09 ± 0.01	6
100 nM	3.3 mM	0.45 ± 0.01	0.09 ± 0.01	5
0 nM	12 mM	0.65 ± 0.07	0.12 ± 0.03	10
1 nM	12 mM	0.75 ± 0.02	0.27 ± 0.03*	4
100 nM	12 mM	0.54 ± 0.06	0.21 ± 0.04*	7

The effect of a 2 min incubation of pacreatic islets with 1 and 100 nM galanin on the phospholipase C activity in the presence of 3.3 and 12 mM glucose. The concentration of inositol-1,3,4-trisphosphate (Ins 1,3,4-P_3) and inositol-1,4,5-trisphosphate (Ins 1,4,5-P_3) expressed as the accumulated radioactivity in percent of the total radioactivity in each tube. All data are expressed as mean ± SEM. *represents significant difference *vs.* control without galanin ($P<0.05$).

Table 2. The dependence of calcium

Time	Galanin	Ins 1,3,4-P_3	Ins 1,4,5-P_3	N
2 min	0 nM	0.66 ± 0.06	0.09 ± 0.01	12
2 min	100 nM	0.63 ± 0.05	0.09 ± 0.01	11
15 min	0 nM	0.58 ± 0.06	0.14 ± 0.04	10
15 min	100 nM	0.53 ± 0.07	0.11 ± 0.02	8

The effect of 100 nM galanin on glucose-stimulated (12 mM) phospholipase C activity in the absence of extracellular calcium. The concentration of inositol-1,3,4-trisphosphate (Ins 1,3,4-P_3) and insoitol-1,4,5-trisphosphate (Ins 1,4,5-P_3) expressed as the accumulated radioactivity in percent of the total radioactivity in each tube. All data are expressed as mean ± SEM.

Table 3. Time-dependence

Time	Galanin	Ins 1,3,4-P_3	Ins 1,4,5-P_3	N
2 min	0 nM	0.56 ± 0.06	0.11 ± 0.02	16
2 min	100 nM	0.50 ± 0.03	0.19 ± 0.04*	17
5 min	0 nM	0.55 ± 0.03	0.12 ± 0.03	11
5 min	100 nM	0.51 ± 0.05	0.13 ± 0.03	12
15 min	0 nM	0.54 ± 0.09	0.14 ± 0.03	9
15 min	100 nM	0.49 ± 0.03	0.07 ± 0.01*	9

The effect of 100 nM galanin on glucose-stimulated (12 mM) phospholipase C activity. The concentration of inositol-1,3,4-trisphosphate (Ins 1,3,4-P_3) and insoitol-1,4,5-trisphosphate (Ins 1,4,5-P_3) expressed as the accumulated radioactivity in percent of the total radioactivity in each tube. All data are expressed as mean ± SEM. *represent significant difference *vs.* control ($P<0.05$).

stimulatory action of PLC-activity caused by galanin following two min incubation of the islets, the levels of 1,4,5-P_3 were unchanged after 5 min-incubation (0.12 ± 0.03 without galanin *vs.* 0.13 ± 0.03 with galanin at 100 nM) but following 15 min of incubation, galanin decreased the concentration of 1,4,5-P_3 from 0.14 ± 0.03 to 0.07 ± 0.01 ($P<0.05$) (Table 3). In the absence of extracellular Ca^{2+}, this reduction was not observed (Table 2). Thus, after 15 min incubation a reduced PLC-activity caused by galanin was observed.

Discussion

In the present study, we show that galanin significantly increases the intracellular concentration of Ins-1,4,5-P_3 after 2-min incubation of isolated islets in the presence of 12 mM glucose and found that galanin exerts this effect already the low concentration of 1 nM. Several previous reports have shown that galanin affects insulin producing cells at low levels [6, 12–14], which suggests the existence of a high-affinity receptor for galanin. In fact, a study in hamster beta cell has identified a population of receptor sites with a very high (K_d 1.5 nM) affinity for galanin [15], which is consistent with our observation. The increase in Ins 1,4,5-P_3 was glucose-dependent, since the iPLC activity remained unchanged when the pancreatic islets were exposed to 100 nM galanin in the presence of only 3.3 mM glucose (Table 1).

Galanin may stimulate the iPLC activity either directly or indirectly by a primary alteration of the cytosolic free [Ca^{2+}]. To evaluate whether the initial increase in iPLC activity could be secondary to changed calcium-fluxes over the plasma membrane, we incubated the islets in calcium-free buffers with 12 mM glucose supplemented with EGTA. The stimulation of the iPLC-activity caused by galanin was then abolished, suggesting that this effect of galanin is dependent on extracellular calcium. In the RINm5F rat insulinoma cell line, galanin has been reported to induce direct inhibition of Ca^{2+} channel currents [16]. By contrast, in islet B cells, galanin seems to be without direct effect on a Ca^{2+} channel permeability, but rather closes voltage sensitive Ca^{2+} channels through hyperpolarization [12, 13, 17]. It is therefore unlikely that the peptide activates iPLC by initially increasing the Ca^{2+} uptake. Instead, it is more likely that galanin activates iPLC by a direct action which is dependent on extracellular Ca^{2+}. Whether the stimulated iPLC activity increases cytoplasmic free Ca^{2+} in islets is not established although galanin seems to only reduce the cytoplasmic calcium in normal B cells [7]. Interestingly, however, galanin increases cytoplasmic calcium in RINm5F cells [5–7].

Previous studies have demonstrated the association between iPLC activation and insulin secretion [9, 18]. The stimulation of iPLC activity by galanin could therefore be expected to augment insulin secretion but the administration of galanin to perifused pancreatic islets, was followed by inhibition only [17]. Mechanisms other than the transient iPLC activation therefore seem thus to be of major significance for the action of galanin on the insulin secretory process.

In contrast to the amplification of glucose-induced iPLC activity by galanin after 2 min of incubation, we found a significant suppression of iPLC activity by galanin after 15 min of incubation. This inhibitory action is a so far unknown mode of action by galanin in pancreatic islets. Again, the question arises whether this is a direct receptor mediated inhibition of iPLC at the plasma membrane or/and an indirect inhibition of iPLC due to other actions of galanin such as the lowering of cytoplasmic [Ca^{2+}]. However, our observation so far, including the kinetics of the galanin-induced inhibition of iPLC activity, favour the latter probability. Stimulatory glucose concentrations (12 mM) increase free $[Ca^{2+}]_{ic}$ via effects on the ATP-regulated K^+ channels [19]. We therefore suggest that galanin inhibits the calcium influx through the same mechanism, and therefore explains the lack of glucose-induced iPLC activation.

Although our experiments were performed with parallel controls, it might be argued, that the decrease in Ins-1,4,5-P_3 formation could be a consequence of depletion of the labelled substrate, i.e. ^{3}H-phosphatidylinositol bisphosphate (^{3}H-PIP_2), rather than the reduction in phospholipase C. Nevertheless, the galanin-mobilized radioactivity after 2 min was only 0.19 ± 0.04%, whereas another well characterized iPLC activator, cholecystokinin (CCK) in equimolar concentrations mobilized 0.25–0.30% of the total radioactivity [18]. Judged by

this, and by observations in previous time-dependency studies, where after 10 min CCK still induced an increase of approximately 400% in Ins-1,4,5-P_3 [18], it is reasonable to assume more labelled ^{3}H-PIP_2 to be present.

Ins 1,4,5-P_3 is generated via iPLC activation and metabolized by inositol-3-kinase to Ins 1,3,4,5-P_4 or by 5-phosphatases to IP_2 [20]. It might be claimed, that the observed variations of intracellular Ins 1,4,5-P_3 not necessarily reflect altered iPLC-activity but in our study we quantified all inositol metabolites (data not shown) and could not detect any increase in Ins 1,3,4,5-P_4, IP_2, or Ins-1,3,4-P_3. We also included 10 mM LiCl, which inhibits 5-phosphatase. Taken together, this reduces the probability of a possible phoshatase or kinase activation, and the observed changes in the cytosolic Ins-1,4,5-P_3 concentrations are most likely a consequence of altered iPLC activity.

Galanin is known to exert at least three important cell biological actions in insulin producing cells: lowering of the intracellular content of cAMP [17, 21], activation of the ATP-regulated K^+ channels which causes hyperpolarization and reduction of the cytoplasmic concentration of Ca^{2+} [12, 19, 22], and direct inhibition of the exocytosis [23]. Our present finding suggests a forth mechanism of galanin, the inhibition of iPLC. Galanin therefore induces a multitude of actions which together might be responsible for the potent insulinostatic action.

Acknowledgements

We appreciate the skilful technical assistance of Line Wilsgaard, Evy Johnsen and Kirsti Johnsen. The present work was supported by the Norwegian Research Council for Science and the Humanities (NAVF), The Norwegian Diabetes Assosiation, The Nordic Insulin Foundation, The Norwegian Cancer Society, AaKres Fond, Norway, The Swedish Medical Research Council (grant no 14X-6834), Albert Påhlson Foundation, the Swedish Royal Society of Medicine and the Medical Faculty, Lund University, Lund, Sweden.

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