Regulation of cytosolic calcium and insulin secretion by galanin and ATP receptors: interactions of pertussis-toxin-sensitive and -insensitive signalling pathways

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In a previous study it was found that the expression of the exogenous fMet-Leu-Phe-receptor (NFPR) in the insulin-secreting cell line RINm5F mediates inhibition of hormone release and additionally raises cytosolic calcium concentration ($[Ca^{2+}]_i$) by activating phospholipase C (PLC) in a pertussis-toxin (PTX)-sensitive manner. We investigated whether an endogenous receptor could elicit similar effects and examined the interaction with PTX-insensitive signalling pathways. The hormone galanin inhibited insulin release at subnanomolar concentrations and increased $[Ca^{2+}]_i$, mainly by a PTX-sensitive mechanism with an EC_{50} (50 nM) comparable with that for hyperpolarization of membrane potential. The effect of galanin or fMet-Leu-Phe on $[Ca^{2+}]_i$ was inhibited by pre-activation of the P_2 -receptor by ATP, which mobilizes calcium in a PTX-insensitive fashion. Simultaneous activation of the P_2 - and peptide receptors caused

additive increases in $[Ca^{2+}]_i$, saturating at a calcium concentration corresponding to the optimal ATP response. This suggests a specific convergence of PTX-sensitive and -insensitive pathways. In contrast, galanin and FMLP inhibited the insulin secretion induced by ATP (1–100 μ M), but only when added prior to the nucleotide. In permeabilized cells, FMLP added after the calcium stimulus still inhibited secretion, indicating that the inefficacy observed in intact cells was not due to the rapid ATP-evoked rise in $[Ca^{2+}]_i$. Thus, (i) insulin-secreting cells possess an endogenous PTX-sensitive pathway mobilizing $[Ca^{2+}]_i$, (ii) inhibitory hormones preferentially activate different effectors depending on the agonist concentration and (iii) activation of NFPR or galanin receptor reveals an unusual dissociation between $[Ca^{2+}]_i$ rises and insulin secretion, pointing towards an overriding inhibitory control of exocytosis.

INTRODUCTION

Several neurotransmitters and hormones such as ATP, carbachol and [arginine]vasopressin (AVP), activate phospholipase C (PLC) upon binding to their respective receptors, causing the generation of diacylglycerol and inositol phosphate, leading to calcium mobilization in insulin-secreting cells [1,2]. Until recently, only pertussis toxin (PTX)-insensitive pathways leading to activation of PLC have been described in these cells, implicating G_q/G_{12} -like heterotrimeric G-proteins as transducers between receptors and this enzyme family [3]. However, we have recently described that the receptor for f-Met-Leu-Phe from human neutrophils (NFPR) conserves its capacity to activate PLC in a PTX-sensitive manner upon expression in insulin-secreting RINm5F cells [4]. Although this exogenous receptor thus seems to reproduce faithfully a transduction mechanism known from its cell of origin, i.e. activation of PLC via PTX substrates, we could not exclude the possibility that this response is provoked by large overexpression of the foreign protein. Indeed, several effects, such as membrane hyperpolarization, inhibition of insulin secretion or of adenylyl cyclase, were already fully present at nanomolar levels of ligand, whereas maximal coupling to PLC required micromolar concentrations. The existence of a comparable endogenous mechanism would strengthen the concept

that receptor coupling by PTX-sensitive mechanisms to different effectors depends on the receptor occupancy [5,6]. Furthermore this would demonstrate a physiological dissociation between rises in cytosolic calcium and insulin secretion. For this purpose we searched for an endogenous receptor in RIN cells exhibiting characteristics similar to the NFPR. Among the ligands for inhibitory receptors present in RINm5F cells, galanin was a potential candidate to fulfill this criterion. In pancreatic islets this neuropeptide is located in adrenergic nerve terminals [7]. Its receptor has been found to couple to a PTX-sensitive G-protein [8] and to mediate potent inhibition of insulin secretion from pancreatic islets and derived cell lines [9,10]. This action is thought to involve several effectors, including the inhibition of adenylyl cyclase with an EC_{50} value of 0.3 nM [11], membrane hyperpolarization by activation of the ATP-sensitive potassium channels (K_{ATP}) [12] and a direct action on the site of exocytosis [13].

In the present study the effects of galanin on several effector systems were compared with those of FMLP using two different clones of the rat insulinoma RIN cells: RINm5F and RIN9815, a RINm5F cell clone which stably expresses the human FMLP receptor [4]. This model system was also employed to delineate the interaction of PTX-sensitive and -insensitive pathways with respect to regulation of $[Ca^{2+}]_i$ and insulin secretion.

Abbreviations used: α_1 -AR, the α_1 -subtype of the adrenergic receptor; AVP, [arginine]vasopressin; $[Ca^{2+}]_i$, cytosolic calcium concentration; cAMP, cyclic AMP; FMLP, N-formylmethionyl-leucylphenylalanine; G-proteins, guanine-nucleotide-binding regulatory proteins; G_i/G_o , the pertussis-toxin-sensitive heterotrimeric G-proteins; K_{ATP} , the ATP-sensitive potassium channel; NFPR, the human neutrophil receptor for FMLP; P_2 -receptor, the P_2 -subtype of the nucleotide receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; V_1 -receptor, the V_1 -subtype of the receptor for [arginine]vasopressin; PTX, pertussis toxin.

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MATERIALS AND METHODS

Materials

FMLP was obtained from Sigma (Buchs, Switzerland) and dissolved at 30 mM in dimethyl sulphoxide. Synthetic rat galanin was purchased from Bachem (Bubendorf, Switzerland) and dissolved in double-distilled water.

Cell culture

RINm5F and RIN9815 cells (stably expressing the FMLP receptor) were cultured in RPMI 1640 [4]. For the measurement of membrane potential, $[Ca^{2+}]_i$ and insulin secretion from cell suspensions, cells were detached from the substratum and maintained for 3 h in spinner culture [4].

Measurement of membrane potential and $[Ca^{2+}]_i$ in cell suspensions

Average membrane potential was measured by exposing cells to the fluorescent probe bisoxonol (100 nM). For determination of $[Ca^{2+}]_i$, cells were incubated after spinner culture for 30 min at 37 °C in RPMI 1640 containing 5 mM $NaHCO_3$, 25 mM Hepes, 1% fetal-calf serum and 1 μ M fura-2 acetoxymethyl ester [4]. The loaded cells were washed and kept at room temperature until use. Fluorescence was recorded in Krebs–Ringer bicarbonate Hepes buffer (KRBH) containing (in mM) 136 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 , 5 $NaHCO_3$ and 25 Hepes, pH 7.4. Excitation and emission wavelengths were set to 340 and 505 nm respectively. $[Ca^{2+}]_i$ was calibrated as described previously [14].

Insulin secretion

Cells in suspension were washed and subsequently perfused for 45 min with KRBH followed by a 30 min stimulation period. Insulin was determined by radioimmunoassay using rat insulin as standard [4]. For static secretion experiments, cells cultured in microtitre plates were washed twice with KRBH supplemented with 0.1% BSA. The cells were then incubated for 15 min in the presence of the indicated drugs and subsequently supernatants were removed, centrifuged at 4 °C and kept at –20 °C until insulin assay. Cell permeabilization was performed as described previously [4,15]. Briefly, after spinner culture, cells were washed in Ca^{2+} -free KRBH and resuspended in cold mannitol buffer (270 mM mannitol/10 mM potassium glutamate/5 mM $MgATP$ /0.4 mM EGTA/20 mM Hepes, pH 7.0). Cells were exposed to high voltage discharge ($10 \times 30 \mu$ s with an electrical field of 3 kV/cm), immediately diluted with cold glutamate buffer (140 mM potassium glutamate/5 mM NaCl/7 mM $MgSO_4$ /5 mM Na_2ATP /0.4 mM EGTA/20 mM Hepes/creatine kinase (21 units/ml)/2 mM phosphocreatine, pH 7.0), centrifuged, and resuspended in the same buffer. GTP (100 μ M) was present in all conditions and changed neither basal nor Ca^{2+} -evoked secretion.

SDS/PAGE/Immunoblots

Cells were disrupted on ice by brief sonication (10 s) in homogenization buffer [20 mM Hepes (pH 7.4)/250 mM sucrose/0.5 mM EGTA/1 mM dithiothreitol/0.6 mM phenylmethane-

sulphonyl fluoride/20 μ g/ml aprotinin]. After 2 min of centrifugation at 300 g, the supernatant was centrifuged at 30000 g for 30 min. The supernatant was taken as cytosol and the pellet resuspended in homogenization buffer. Membranes were enriched in plasma-membrane markers by centrifugation through a Percoll gradient [4]. SDS/PAGE and immunoblotting were performed as described previously [16], except that enhanced chemiluminescence (Amersham, Zurich, Switzerland) was used for detection. Generation of antisera against peptides specific for PLC- β isotypes has been described [17,18].

Statistical analysis

Results are presented as means $\pm$ S.E.M. from experiments performed on at least three different cell preparations. Statistical analysis was performed by Student's two tailed *t* test for unpaired data (*P*).

RESULTS

Regulation of $[Ca^{2+}]_i$ by galanin

Figure 1 depicts the effect of galanin (1 μ M) on $[Ca^{2+}]_i$ in RIN9815 cells in suspension. The peptide induces a rapid increase in $[Ca^{2+}]_i$ followed by a transient decrease below the baseline. Pretreatment with PTX at 10 ng/ml for 3 h markedly reduced the observed increase in $[Ca^{2+}]_i$, although a small effect was still observed (see also Table 1). Prolonged exposure to the toxin for up to 24 h did not lead to any further inhibition as compared with the effect of FMLP, which was fully sensitive to pretreatment with PTX (Figure 1).

To characterize further the nature of the galanin-induced rise in $[Ca^{2+}]_i$, several pretreatments were employed (Table 1). A part of the galanin effect was still present after chelation of extracellular Ca^{2+} by the addition of EGTA, suggesting that intracellular calcium mobilization is implicated in the effect. In contrast, in cells pretreated with PTX, the addition of EGTA completely abolished any rise in $[Ca^{2+}]_i$ by galanin (Table 1), indicating that calcium mobilization is coupled to the toxin-sensitive action of the peptide. Additionally the phorbol ester phorbol 12-myristate 13-acetate, known to interfere with PLC-mediated events, completely prevented any subsequent action of galanin. Similarly preincubation of RIN9815 cells with thapsigargin, which empties intracellular calcium stores [19], abolished the rise in $[Ca^{2+}]_i$ due to galanin. KCl at 24 mM, added after thapsigargin and galanin, was still capable of increasing $[Ca^{2+}]_i$ by 315 ± 62 nM above basal ($n = 3$), demonstrating that the fluorescent indicator was not saturated. The voltage-sensitivity of the influx component was tested by the use of the hyperpolarizing agent diazoxide, which opens K_{ATP} channels [20]. This compound effectively reduced the response to galanin (Table 1). Pretreatment of cells with the activators of adenylyl cyclase, forskolin [4] or cholera toxin, attenuated profoundly the effect of galanin on $[Ca^{2+}]_i$ (Table 1), but did not change the effect of the peptide on membrane potential (see Figure 4). Interestingly the PTX-insensitive part of the rise in $[Ca^{2+}]_i$ did not demonstrate any cyclic AMP (cAMP)-dependent regulation (Table 1; PTX versus PTX + forskolin). Thus the responses to both peptides, galanin and FMLP [4], share similar features, suggesting the use of common pathways. Indeed, when given simultaneously, the effects of the peptides were not additive with regard to the peak increase in $[Ca^{2+}]_i$ above basal values [162 ± 7 nM ($n = 4$) for galanin 1 μ M and 175 ± 4 nM ($n = 3$) for FMLP 1 μ M alone, versus 191 ± 15 nM ($n = 4$) for both peptides added simultaneously]. Note that the characteristics of galanin-induced

Table 1 Effect of various pretreatments on galanin-induced increase in [Ca²⁺]_i in RIN9815 and RINm5F cells in suspension

[Ca²⁺]_i was measured by the use of Fura-2, and the difference between basal and peak levels is given. Pretreatments were conducted 2 min before the addition of galanin 0.3 µM, except for toxins, which were added for 3 h during the spinner culture (pertussis toxin, 0.1 µg/ml; cholera toxin 1 µg/ml). *2P < 0.05 as compared with the absence of pretreatment; nd, not determined.

Pretreatment	Δ[Ca ²⁺] _i (nM)					
	RIN9815			RINm5F		
	Mean ± S.E.M.	n	2P	Mean ± S.E.M.	n	2P
None	149 ± 6	17		134 ± 9	8	
PTX	26 ± 4	16	*	32 ± 14	3	*
EGTA (3 mM)	28 ± 1	4	*	15 ± 6	3	*
PTX + EGTA (3 mM)	0 ± 0	3	*	0 ± 0	3	*
Diazoxide (200 µM)	37 ± 12	3	*	nd		
PMA (100 nM)	0 ± 0	3	*	0 ± 0	2	*
Thapsigargin (100 nM)	0 ± 0	3	*	0 ± 0	3	*
Cholera toxin	40 ± 4	7	*	31 ± 8	3	*
Forskolin (1 µM)	37 ± 8	4	*	25 ± 11	3	*
PTX + forskolin (1 µM)	35 ± 5	3	*	nd

Figure 1 Effect of PTX on FMLP- and galanin (GAL)-evoked changes in [Ca²⁺]_i

RIN9815 cells (expressing the FMLP receptor) were preincubated for 3 h in the presence of PTX (0.1 µg/ml for 3 h) or absence of the toxin (CTR) and stimulated with galanin (1 µM) or FMLP (1 µM) as indicated by arrows. The traces are representative of at least six observations in each case. Changes in [Ca²⁺]_i were measured after loading of cells with fura-2.

alterations in [Ca²⁺]_i were also apparent in native RINm5F cells upon exposure to the neuropeptide (Table 1).

We next investigated the concentration-dependence of the galanin-mediated increase in [Ca²⁺]_i in RINm5F as well as NFPR-bearing RIN9815 cells. In addition, these results were compared with the peptide action on membrane potential and inhibition of alanine-induced insulin secretion. The peptide started to raise [Ca²⁺]_i at 10 nM and displayed similar EC₅₀ values in RIN9815 and RINm5F cells (Figure 2, circles). Hyperpolarization of the plasma membrane by galanin is thought to reflect the activation of the ATP-sensitive potassium channel,

Figure 2 Dose-response curve for effects of galanin on [Ca²⁺]_i, membrane potential and insulin secretion

Values are given as percentage of maximal effect on peak increase of [Ca²⁺]_i above basal (circles), membrane hyperpolarization (squares) and inhibition of alanine-induced insulin secretion (triangles) in RIN9815 cells (open symbols) or RINm5F cells (closed symbols). Data represent the means for 4–12 observations.

K_{ATP} [12]. This effect, as measured by monitoring the fluorescence of bisoxonol in suspensions of RIN9815 cells (Figure 2, squares), occurred between 0.1 and 1 µM of galanin (EC₅₀ 30 nM). These values differ greatly from the efficacy of the peptide for the inhibition of insulin release. EC₅₀ values of 0.1 and 0.2 nM were apparent in the case of alanine-induced insulin secretion in native and NFPR-bearing RIN cells (Figure 2, triangles).

Interaction between PTX-sensitive and -insensitive pathways on [Ca²⁺]_i

Recent observations indicate that certain signalling pathways may display a greater sensitivity to the activation of a given receptor if the cells are prestimulated by activation of another receptor type [21]. Activation of the P₂-receptor by ATP (100 µM) increases peak levels of [Ca²⁺]_i in RIN9815 cells in a PTX-

Figure 3 Pre-activation of the ATP receptor abolishes the response to galanin or FMLP on $[Ca^{2+}]_i$ in RIN9815 cells

Shown are representative traces obtained from fura-2-loaded cells in suspension. Arrows indicate the addition of FMLP 1 μ M (FMLP), galanin 1 μ M (GAL), ATP 100 μ M (ATP), AVP (10 μ M) (AVP) or KCl 24 mM (KCl).

Table 2 Characterization of the P_2 -receptor-mediated effect on galanin or FMLP-induced rise in $[Ca^{2+}]_i$ in RIN9815 cells

For experimental conditions, see Table 1. * $2P < 0.05$ as compared with the absence of pretreatment. Abbreviations: [β -S]ADP, adenosine [β -thio]diphosphate; [β -CH]ATP, adenosine [β -methylene]triphosphate.

Agonist	Pretreatment	$\Delta[Ca^{2+}]_i$		
		Mean $\pm$ S.E.M.	n	2P
FMLP (1 μ M)	None	157 $\pm$ 13	7	
	ATP (100 μ M)	0 $\pm$ 0	4	*
Galanin (1 μ M)	None	152 $\pm$ 8	11	
	ATP (100 μ M)	34 $\pm$ 7	7	*
	PTX, ATP (100 μ M)	44 $\pm$ 10	4	*
	UTP (100 μ M)	27 $\pm$ 17	3	*
	[β -S]ADP (10 μ M)	31 $\pm$ 10	3	*
	[β -CH]ATP (100 μ M)	137 $\pm$ 11	3	
	Staurosporine (100 nM), ATP (100 μ M)	28 $\pm$ 14	3	
Galanin (30 nM)	None	78 $\pm$ 5	3	
	ATP (100 μ M), wash	69 $\pm$ 8	3	
Galanin (1 μ M)	AVP (10 μ M)	142 $\pm$ 19	3	
	Adrenaline (100 μ M)	144 $\pm$ 14	3	
	Carbachol (100 μ M)	147 $\pm$ 17	3	

insensitive manner [306 ± 31 nM above basal levels ($n = 3$) versus 322 ± 20 nM ($n = 3$) in PTX-treated cells]. However, prior challenge of the cells with ATP (100 μ M) completely inhibited any subsequent increase in $[Ca^{2+}]_i$ by FMLP and markedly reduced the response to galanin by some 70% (Figure 3 and Table 2). A similar reduction of galanin action was observed in RINm5F cells [131 ± 12 nM $[Ca^{2+}]_i$ above basal ($n = 4$) versus 29 ± 11 nM after pretreatment with ATP 100 μ M ($n = 3$)}. Note that, in contrast with the effect of ATP on the subsequent stimulation by galanin, galanin (100 nM) given first did not alter the calcium

Figure 4 ATP or prestimulation of PKA does not alter the effect of galanin on membrane potential

Using 100 nM bisoxonol, the membrane potential of RIN9815 cells in suspension was determined; GAL, galanin (100 nM); FORS, forskolin (1 μ M); ATP, ATP (100 μ M). Addition of KCl (24 mM) is indicated by the vertical line. The traces are representative for at least three observations each in three independent experiments.

response induced by 100 μ M ATP [367 ± 36 ($n = 3$) versus 338 ± 24 ($n = 4$)]. This apparent heterologous desensitization of the galanin-induced effect by ATP was mimicked by UTP or adenosine [β -thio]diphosphate, but not by adenosine [β -methylene]triphosphate, a presumed agonist of P_{2x} receptors [22] (Table 2). Constant presence of the nucleotide is required, since washing of cells after exposure to ATP restored the efficacy of galanin. The PTX-insensitive component of galanin-induced rise in $[Ca^{2+}]_i$ was not subject to regulation by the P_2 -purinergic receptor (Table 2). The regulation of the response to galanin by calcium-mobilizing agents was specific for ATP receptors, since maximal prestimulation of several other receptors acting via PTX-insensitive pathways was ineffective (Figure 3 and Table 2). As the kinase inhibitor staurosporine (100 nM) failed to alter the effect of ATP on the galanin response, PKC does not seem to be involved (Table 2). To ensure that the effect of ATP on the galanin-mediated rise in $[Ca^{2+}]_i$ is not merely explained by the magnitude of the effect or by the calcium influx, the consequence of membrane depolarization was considered (Figure 3). KCl at 24 mM induced an ample increase in $[Ca^{2+}]_i$ by opening voltage-dependent calcium channels without altering the subsequent response to galanin.

Next we investigated whether the action of the P_2 -purinergic receptor on the rise in $[Ca^{2+}]_i$ by galanin or FMLP is exerted at the level of the receptor(s) or downstream in the signalling pathway. To this end we examined the interplay of these agents on membrane potential (Figure 4). Although ATP caused membrane depolarization, galanin was subsequently still able to hyperpolarize the cell to the same extent as when given alone. In addition, the nucleotide did not change the response to galanin in RINm5F or to FMLP in RIN9815 cells (results not shown). Hence the P_2 -purinergic receptor does not interfere with galanin or FMLP at the receptor level.

It is intriguing that ATP increased $[Ca^{2+}]_i$ and reduced the effect of the two peptides in RIN9815 cells with similar EC_{50} values (2.5 and 4.6 μ M respectively), suggesting a close re-

Figure 5 Effect of ATP and simultaneously added FMLP, AVP or carbachol on peak increase in $[Ca^{2+}]_i$ in RIN9815 cells

Increasing concentrations of ATP were added alone (open circles) or simultaneously with FMLP 100 nM (closed circles), AVP 1.0 μ M (closed squares) or carbachol 100 μ M (closed triangles) to fura-2-loaded RIN9815 cells. Values given represent the difference between basal and peak levels of $[Ca^{2+}]_i$, and are derived from at least three observations for each point from three independent experiments.

Figure 6 Effect of galanin and FMLP on insulin secretion stimulated by ATP, alanine or AVP

RIN9815 cells seeded in microwells (10^5 cells/well) were stimulated for 15 min with vehicle (CON), alanine (10 mM) (ALANINE), ATP (100 μ M) (ATP) or AVP (1 μ M) (AVP), and the insulin content of the supernatant was determined. FMLP (1 μ M, hatched bars) or galanin (1 μ M, black bars) was added simultaneously with the different stimuli. $n = 9-32$; $^*2P < 0.01$.

relationship for the two actions. Consequently, we studied if a common rate-limiting step in the signal transduction downstream from GTP-binding proteins might be involved. Since only the NFPR couples exclusively to PTX-sensitive pathways, we chose 100 nM FMLP and measured its action on $[Ca^{2+}]_i$ at increasing concentrations of ATP (Figure 5). Clearly, the effect of FMLP was fully additive at low concentrations of simultaneously added ATP, but both agents together were unable to increase $[Ca^{2+}]_i$ beyond 300 nM above basal levels. As can be seen in Figure 5, this coincided with the maximal stimulation achievable by the nucleotide. In contrast, the simultaneous addition of maximal doses of ATP to AVP or carbachol was still capable of eliciting a larger response, implying that receptor-dependent mobilization of $[Ca^{2+}]_i$, *per se* is not exhausted.

These observations suggest a convergence of the signalling from the P₂-purinergic receptor with that of the NFPR and the galanin receptor. A common target could be constituted by PLC isotypes regulated by PTX-sensitive and -insensitive pathways

Figure 7 Effect of galanin and FMLP on ATP-induced insulin secretion in perfused RIN9815 cells

Cells were perfused at 37 °C, and ATP (100 μ M) was added as indicated by the horizontal bar, either alone (a) or together with galanin (30 nM; b, open circles) or FMLP (1 μ M; c, open squares). The effect of galanin (30 nM; b, closed circles) or FMLP (1 μ M; c, closed squares) added 3 min prior to stimulation by ATP is also given; $n = 4-6$ from three independent experiments.

[3,23]. However, using antibodies against PLC- β_1 , PLC- β_2 and PLC- β_3 , no immunoreactivity could be detected in the corresponding molecular-mass range in membranes or cytosol derived from RIN9815 and RINm5F cells (results not shown).

Interaction between PTX-sensitive and -insensitive pathways on insulin secretion

In view of the interaction between galanin and ATP receptors on $[Ca^{2+}]_i$, we investigated their interplay in insulin secretion. As shown for static incubations in Figure 6, ATP (100 μ M) increased insulin release threefold in RIN9815 cells. Galanin (1 μ M) or FMLP (1 μ M) were incapable of counteracting this stimulation. The effect of the peptides on the stimulation by ATP was compared with that of two other stimuli: alanine (10 mM) and AVP (1 μ M) acting by membrane depolarization with subsequent Ca^{2+} influx [24] or activation of PLC followed by mobilization of calcium [25] respectively. In both cases the two peptides, galanin and FMLP, reduced the stimulated secretion. We investigated further if the inhibitory mechanism induced by the two peptides may be operative providing its activation before the stimulation by ATP. In a perfusion system which allows dynamic measurement of secretion, ATP (100 μ M) stimulated insulin release up to ten-fold above basal levels (Figure 7a). Galanin (30 nM) was inactive when given together with the nucleotide (Figure 7b). This was independent of the magnitude of the stimulation, since

Table 3 Effect of FMLP on Ca^{2+} -induced insulin secretion in electroporameabilized RIN9815 cells

Permeabilized RIN9815 cells were equilibrated during 15 min preincubation on ice with the indicated substances. Cells were transferred to 37 °C, and 10 s later the indicated additions were made. After incubation for 10 min, samples were transferred on ice, centrifuged, and aliquots drawn for determination of insulin. ATP was present at 5 mM throughout. ^aAs compared with 0.1 μM Ca^{2+} ; ^bas compared with 1.0 μM Ca^{2+} ; ^cas compared to FMLP present during the preincubation.

Preincubation on ice	Incubation at 37 °C	Insulin secretion (% of content)		
		Mean $\pm$ S.E.M.	<i>n</i>	<i>P</i>
0.1 μM Ca^{2+}	0.1 μM Ca^{2+}	0.28 $\pm$ 0.03	11	—
1.0 μM Ca^{2+}	1.0 μM Ca^{2+}	0.78 $\pm$ 0.02	11	< 0.01 ^a
1.0 μM Ca^{2+} , 1 μM FMLP	1.0 μM Ca^{2+} , 1 μM FMLP	0.40 $\pm$ 0.07	11	< 0.01 ^b
1.0 μM Ca^{2+}	1.0 μM Ca^{2+} , 1 μM FMLP	0.47 $\pm$ 0.08	11	< 0.01 ^b , > 0.05 ^c

even the effect of 1 μM ATP was not altered by simultaneously added galanin 30 nM [$3.45 \pm 0.46\%$ of insulin content released above basal ($n = 3$) by ATP alone versus 3.02 ± 0.26 in the presence of galanin ($n = 3$)]. However, the peptide reduced the ATP-induced secretion by 65% if it was added 3 min before the stimulus (Figure 7b). Comparable results were obtained with FMLP (Figure 7c).

These findings raise the possibility that ATP induces a very rapid elevation in $[\text{Ca}^{2+}]_i$ which precedes any action mediated by the peptide receptors. This hypothesis was tested by examining the effect of FMLP on secretion from electroporameabilized cells. The permeabilized cells were equilibrated on ice with 0.1 or 10 μM Ca^{2+} in the presence of FMLP or with Ca^{2+} only. In the latter case the chemotactic peptide was added 10 s after the transfer to 37 °C, conditions under which the action of calcium precedes the receptor-induced events. As shown in Table 3, FMLP inhibited effectively the Ca^{2+} -induced secretion under both experimental conditions.

DISCUSSION

Studies on receptor-mediated events are often hampered by the presence of isotypes or the expression of exogenous receptors in cells endowed with only a few physiological responses. The insulin-secreting cell clone RIN9815 offers the advantage of allowing the comparison of actions between endogenous receptors and a defined exogenous receptor, i.e. the NFPR [4]. We do indeed not know if the observed effects of galanin are mediated by a single type of receptor. Note that, in contradistinction to FMLP, galanin is also known to activate PTX-insensitive pathways in RINm5F cells [26]. As this may indicate the presence of galanin receptor subtypes, the comparison with the NFPR was felt to be crucial. This comparison was also appropriate, since measuring various parameters no difference could be detected between RINm5F and RIN9815 cells except for the expression of the NFPR.

A galanin-mediated rise of $[\text{Ca}^{2+}]_i$ in RINm5F cells was reported previously; albeit no further characterization was provided [27]. We now demonstrate that this effect persists after chelation of extracellular calcium by EGTA and can be abolished by phorbol 12-myristate 13-acetate, a compound known to interfere with PLC-mediated events [2], or by thapsigargin, which empties intracellular calcium-stores [19]. In addition the effect of galanin is voltage-sensitive, as shown by using the hyperpolarizing agent diazoxide [20]. Therefore galanin, as had been shown for FMLP [4], mobilizes calcium from intracellular stores, leading to Ca^{2+} influx in RIN cells. In an additional similarity to the NFPR, the PTX-sensitive effect of galanin can be modulated by activation of the P_2 -receptor or alteration of

cAMP levels ([4]; the present study). The rise of $[\text{Ca}^{2+}]_i$ requires rather high concentrations of ligands. However, they fall in the concentration range known to activate another effector of physiological importance, the K_{ATP} channel [12], assessed here by measurement of membrane potential. In contrast, the inhibition of insulin secretion displayed an EC_{50} value as low as 0.1 nM, which is similar to EC_{50} values of 0.3–1 nM reported for inhibition of adenylyl cyclase [11]. Such distinct concentration-dependencies for the activation of these distinct effectors parallels those observed for transfected NFPR [4], a bimodal pattern of coupling that had been described for several transfected receptors [5]. Our observation extends this concept to an endogenous receptor. Furthermore, it excludes simple overexpression of exogenous receptors, such as the NFPR, in a new host cell line as being responsible for the observed PTX-sensitive coupling to PLC [4]. Thus our findings emphasize the astonishing similarity of receptor-transducer coupling for the NFPR and the galanin receptor, despite the fact that both are expressed within the same cell at levels differing by two orders of magnitude [4,10]. In view of the comparable ligand concentrations required to obtain maximal effects for both receptors ([4]; and present study), it appears that not the amount of receptor molecules expressed, but other elements of the signal-transduction pathway, constitute the limiting step(s).

An unexpected finding of this study was the demonstration of the convergence of the PTX-sensitive and -insensitive pathways leading to calcium mobilization. Pre-activation of P_2 -receptors specifically abolished the PTX-sensitive action of the two peptides, FMLP and galanin, on $[\text{Ca}^{2+}]_i$ without altering the toxin-insensitive pathways from galanin receptors, V_1 -type AVP receptors [25], α_1 -AR [24,28] or muscarinic receptors [14,24]. The PLC activator ATP [2] seems not to act via PKC, since (i) its inhibitor staurosporine did not alter the ATP effect on galanin and (ii) AVP, an activator of the enzyme [25], did not mimic the action of ATP. The requirement of continuous P_2 -receptor occupancy indicates a direct and dynamic interaction between the toxin-sensitive and -insensitive pathway. It is unlikely that metabolism of ATP to adenosine with subsequent activation of adenylyl cyclase [22] could explain their cross-talk, since UTP and adenosine [β -thio]diphosphate, a poorly hydrolysable analogue of ATP, fully mimicked the nucleotide effect. Interactions between the PTX-sensitive and -insensitive pathways on PLC and $[\text{Ca}^{2+}]_i$ have been observed in RINm5F and other cell lines [29,30]. However, in contrast with our observations, prestimulation of PTX-insensitive pathways were required to elicit or amplify a G_i/G_o -mediated effect, and no saturation was observed. Furthermore, the PTX-sensitive increase in $[\text{Ca}^{2+}]_i$ was not modulated by cAMP [29].

The saturation observed in our system indicates the use of a

common rate-limiting step and the apparent heterologous desensitization of the PTX-sensitive transmission after preactivation of P₂-receptors can best be explained by prolonged stimulation of the signalling cascade [2]. The inefficacy of galanin in reducing the subsequent action of ATP on [Ca²⁺]_i may be due to the smaller amplitude and transient nature of the peptide effect. Indeed, kinetic data suggest that the existence of a maintained receptor–G-protein–effector complex in the presence of agonist [31] imposes refractoriness to further stimuli. The convergence of PTX-sensitive and -insensitive pathways may be located at different levels. It is noteworthy that a subgroup of PLC, i.e. PLC- β , has been characterized which displays a graded response to PTX-sensitive and -insensitive G-proteins [3,23]. Although this PLC family would constitute a good candidate, at least three of their isoenzymes are apparently absent, or only expressed in minute amounts, in the two RIN cell lines. Alternatively the convergence of PTX-sensitive and -insensitive pathways may be located beyond PLC. The [Ca²⁺]_i raising actions of galanin and FMLP are extremely sensitive to chelation of extracellular calcium as previously shown for ATP [2], which is in contrast with the other PLC activators AVP [2] and carbachol [14]. This suggests that the interaction between PTX-sensitive and -insensitive signalling, leading to mobilization of Ca²⁺, converges in the final step(s) beyond PLC.

In contrast with the PTX-insensitive activation of PLC and rise in [Ca²⁺]_i, which is commonly associated with stimulation of insulin secretion [1,14,25], the toxin-sensitive pathway is dissociated from secretion. In fact, both galanin and FMLP inhibited secretion evoked either by Ca²⁺ influx or due to Ca²⁺ mobilization by PLC activation, as shown for alanine [24] or AVP [25] respectively. This suggests that the inhibition of exocytosis overrides the stimulation of PLC and the subsequent [Ca²⁺]_i rise. Interestingly, secretion elicited by activation of the P₂-receptor could not be counteracted by simultaneous activation of the NFPR or the galanin receptor. This cannot be explained by the rapidity or magnitude of the nucleotide-evoked [Ca²⁺]_i rise, as demonstrated in intact and permeabilized cells. Nonetheless, pre-activation of the inhibitory peptide receptors produced the expected inhibition of ATP-stimulated insulin secretion.

ATP, which is stored and released from parasympathetic nerve endings in the pancreas [32], is generally viewed as a modulator of insulin secretion [33]. Our results suggest that the nucleotide not only enhances secretion, but might additionally prevent any simultaneous inhibition by the endogenous hormone galanin. Such a hypothesis will need to be tested in pancreatic β -cells. Taken together, these results imply a hierarchical organization of inhibitory and stimulatory pathways controlling exocytosis. Whether this function is exerted by a heterotrimeric G-protein acting directly on the site of exocytosis [13,15] is under current investigation.

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