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Galanin reduces PDBu-induced protein phosphorylation in rat ventral hippocampus

Caterina La Porta[a], Roberto Bianchi[a], Silvano Sozzani[a], Tamas Bartfai[b] and Silvana Consolo[a]

[a]*Istituto di Ricerche Farmacologiche 'Mario Negri', Milano, Italy* and [b]*Department of Biochemistry, Arrhenius Laboratories, Stockholm University, S-10691, Stockholm, Sweden*

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The effect of galanin (GAL) on basal and phorbol-12,13-dibutyrate (PDBu) induced protein phosphorylation in rat ventral hippocampal miniprisms was investigated. GAL (0.5, 1 and 2 μM) inhibited PDBu stimulation in a concentration-dependent manner without altering basal protein phosphorylation. This inhibitory effect was prevented by the GAL antagonist galantide. GAL did not affect either the activity of protein kinase C (PKC) from rat brain or basal phosphorylation in ventral hippocampal hippogenates, suggesting that it did not directly modulate PKC activity. Depolarization of miniprisms from ventral hippocampi by 18 mM K^+ prevented the effect of GAL on PDBu-induced phosphorylation. The results indicate that GAL indirectly regulates neuronal protein phosphorylation by a GAL receptor-mediated action.

Protein kinase C; Neuropeptide; Second messenger; Galanin antagonist

1. INTRODUCTION

Since its discovery [1], galanin (GAL) a 29-amino-acid long C-terminal-amidated peptide, has been shown to have numerous physiological and pharmacological actions in consonance with its widespread distribution in the endocrine, peripheral and central nervous systems [2–5]. In rats and monkeys, GAL-like immunoreactivity has been located, among other brain regions, in the septal area where GAL coexists with acetylcholine (ACh) in a subpopulation of cholinergic cell bodies projecting to the hippocampus and in their terminals in the ventral hippocampus [6–8]. In addition, autoradiographic and equilibrium binding studies with ^{125}I-labeled GAL show a high density of putative high-affinity GAL receptors in the ventral hippocampus [9].

As a functional correlate of the coexistence of ACh and GAL, it was demonstrated that GAL acted as an inhibitory modulator of ACh function specifically in the ventral hippocampus; presynaptically, GAL prevented acetylcholine release both in vivo and in vitro [10], and postsynaptically it inhibited the effect of the muscarinic agonist carbachol on turnover of inositol phosphates (PI) [11]. The inhibitory effect of GAL on signal transduction appears to be specific, since GAL did not affect either basal and forskolin-stimulated cyclic AMP accumulation [9], or K^+ depolarization-induced cGMP accumulation in the ventral hippocampus [12].

Because stimulation of PI turnover is associated with activation of Ca^{2+} and phospholipid-dependent protein kinase C (PKC), modulation of PI turnover in the ventral hippocampus by GAL might be expected to affect the phosphorylation of endogenous protein substrates. Therefore we investigated the effect of GAL on basal and phorbol-12,13-dibutyrate (PDBu)-induced PKC activity [13] in rat ventral hippocampus. The results suggest a possible role for GAL in controlling protein phosphorylation.

Correspondence address: S. Consolo, Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy. Fax: (39) (2) 356277.

2. MATERIALS AND METHODS

2.1. *Phosphorylation of endogenous protein in ventral hippocampal miniprisms*

Ventral hippocampi from male CD-COBS rats (Charles River, 150–200 g body weight), were chopped into 350 × 350 μm miniprisms on a McIlwain chopper and preincubated in oxygenated Krebs–Hepes bicarbonate buffer (KHB) (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.7 glucose, pH 7.4) for 45 min at 37°C. Free Ca^{2+} concentration in the incubation buffer was adjusted to 1 μM by addition of 0.9 mM calcium EGTA/0.2 mM EGTA [14]; 50 μl of gently packed tissue preparation (corresponding to 0.8–1 mg of tissue) were added to flat-bottomed plastic vials containing 2 μCi inorganic ^{32}P ($^{32}P_i$) (10 mCi/ml, [^{32}P]H_3PO_4, Amersham) in 310 μl of KHB. The vials were incubated in a shaking water bath at 37°C for 1 h. When indicated, drugs were added and the reaction was stopped after 1 min by addition of 1 ml ice-cold 25 mM Tris-HCl (pH 7.4). The miniprisms were washed three times with 1 ml of 25 mM Tris-HCl (pH 7.4) and homogenized in 400 μl of buffer A (25 mM Tris-HCl, pH 7.4, 1 mM EGTA, 10 μg/ml leupeptin and 10 μg/ml aprotinin) with 0.2% sodium dodecylsulfate (SDS), final concentration. An aliquot was taken and protein was measured by the method of Lowry et al. [15]. One ml of 20% trichloroacetic acid (TCA) and 50 μg BSA were added to 100 μl of homogenate. The proteins were collected onto Millipore membrane filters (Ø 0.45 μm) and the filters were washed eight times with 1 ml 20% TCA. Radioactivity was measured by liquid scintillation spectrometry. The results are ex-

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pressed as nmoles $^{32}P_i$ incorporated/min/mg protein or as the percentage of phosphorylation above basal values.

2.2. *Phosphorylation of endogenous proteins in ventral hippocampal homogenate*

Ventral hippocampi were homogenated in buffer A and the protein concentration was measured. Phosphorylation was carried out for 1 min at 37°C in 50 μl of a reaction mixture containing (final concentration): 0.05% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$ and 10 μM [^{32}P]ATP (2μCi) in 35 μM Tris buffer, pH 7.4 and 40 μg of protein, with or without GAL (1 μM). This mixture contained 1 μM free Ca^{2+} in a calcium EGTA/EGTA buffer [14]. The reaction was stopped by addition of 1 ml 20% TCA. The protein were collected as described in section 2.1. $^{32}P_i$ incorporation was expressed as nmoles/min/mg protein.

2.3. *PKC activity*

PKC was purified from rat brain essentially as described by Murais et al. [16]. PKC activity was evaluated as the incorporation of $^{32}P_i$ into histone type III (40 μg/tube) using a final ATP concentration of 50 μM [17]. The enzyme was incubated at 30°C in the assay mixture (35 mM Tris-HCl, pH 7.5, 10 mM 2-ME, 0.2 mM phenylmethylsulfonylfluoride, 0.05% Triton X-100, 0.4 mM EGTA, 10 mM $MgCl_2$, 0.6 mM $CaCl_2$, 20 μg/ml phosphatidylserine, 6 μg/ml diolein) and the reaction was stopped after 20 min by the addition of 1 ml 25% TCA. The precipitates were collected and processed as described in section 2.1. $^{32}P_i$ incorporation was expressed as pmol/min.

2.4. *Statistics*

Statistical analysis was performed by analysis of variance (ANOVA), 2 × 2 factorial analysis and Tukey's test.

3. RESULTS

In ventral hippocampal miniprisms PDBu (1 μM) stimulated protein phosphorylation by 120% at 1 μM free [Ca^{2+}] in the extracellular medium. GAL reduced in a concentration-dependent manner the PDBu stimulation of protein phosphorylation (Fig. 1). The concentration curve of the inhibitory effect was steep,

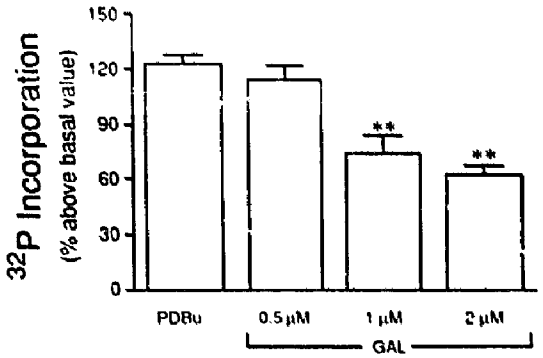

Fig. 1. Dose-dependent inhibition by GAL on 1 μM PDBu-induced $^{32}P_i$ incorporation in miniprisms of ventral hippocampus. Miniprisms were preincubated for 45 min and labeled for 1 h in the presence of $^{32}P_i$ and then incubated with 1 μM PDBu and/or GAL for 1 min. GAL did not alter basal $^{32}P_i$ incorporation. Results are expressed as percentage above the basal value. Data are the mean ±S.E. of three experiments carried out in duplicate. The significance of the interaction was as follows: $^{**}P < 0.01$ vs. PDBu, by two-way ANOVQA (2 × 2) and Tukey's test for unconfounded means.

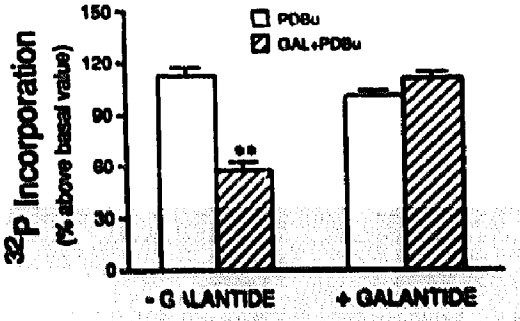

Fig. 2. Effect of galantide on inhibition by GAL's effect on PDBu-induced $^{32}P_i$ incorporation in miniprisms from ventral hippocampus. Miniprisms were labeled for 1 h with $^{32}P_i$ and then exposed to PDBu and/or GAL for 1 min. Galantide (10 μM) added 10 min before 1 μM PDBu and GAL (1 μM), did not alter the basal value. Results are expressed as percentage above the basal value. The data are the mean ±S.E. of three experiments done in duplicate. The significance of interaction was a follows: $^{**}P < 0.01$ vs. PDBu, by two-way ANOVA (2 × 2) and Tukey's test for unconfounded means.

with no effect at 0.5 μM and a plateau at 1 and 2 μM with 40 and 50% of inhibition, respectively. GAL by itself did not alter basal phosphorylation, the $^{32}P_i$ incorporation in GAL-treated ventral hippocampal miniprisms and control ones being 5.9±1 and 5.4±0.9 nmol/min/mg protein, respectively.

Galantide, the recently described GAL receptor-antagonist [18,19], at the concentration of 10 μM, did not affect either basal (5.4±0.9 and 4.9±1 nmol/min/mg protein for control and galantide, respectively) or PDBu-induced protein phosphorylation (Fig. 2). However, when galantide was added 10 min before GAL and PDBu, it completely reversed GAL's inhibition of PDBu stimulation (Fig. 2).

The in vitro effect of GAL on protein phosphorylation in homogenates from ventral hippocampus and on rat brain purified protein kinase C activity (PKC) was investigated. GAL (1 μM) did not alter basal phosphorylation of ventral hippocampal homogenate incubated in the presence of 1 μM free [Ca^{2+}] (13.2±0.5 and 14.0±0.7 nmol/min/mg protein for control and GAL, respectively). Similarly, GAL at concentrations up to 10 μM did not affect the enzyme's ability to phosphorylate a saturating concentration of histone type III, the $^{32}P_i$ incorporation being 14.5±0.7 and 14.4±1.3 pmol/min for control and GAL (10 μM), respectively.

The possibility that GAL inhibits PDBu-induced phosphorylation by lowering Ca^{2+} influx was investigated after K^+ depolarization. Tissue miniprisms from ventral hippocampus were depolarized by raising the K^+ concentration from 6 to 18 mM. In the medium with higher K^+ concentration, basal protein phosphorylation rose from 5.4±0.9 to 12.0±1.2 nmol/min/mg protein; i.e. 222%. PDBu stimulation at 6 and 18 mM was 24.3±2.7 and 22.0±2.0 nmol/min/mg protein after subtraction of respective basal values. Addition of 1 μM GAL to the

6 mM K^+ medium caused the usual 60% reduction of protein phosphorylation ($P < 0.01$ vs. PDBu), but when GAL was added to 18 mM K^+ medium it had no effect.

4. DISCUSSION

The present study shows that the neuropeptide GAL caused a concentration-dependent reduction of PDBu-stimulated protein phosphorylation in rat ventral hippocampus miniprisms. GAL did not exert this inhibitory effect when added to homogenates of ventral hippocampus or to purified rat brain PKC, suggesting that the peptide effect requires the integrity of the neuronal cells or network; importantly, GAL did not act by directly modulating PKC activity. The effect of GAL on protein phosphorylation was presumably mediated through binding to specific GAL-receptors since it was completely prevented by the GAL-receptor antagonist, galantide, a chimeric peptide composed if the N-terminal part of GAL and C-terminal part of substance P [GAL (1–12)-Pro-Substance P (5–11)] [18,19]. Galantide binds with high affinity (IC_{50}=0.1 nM) to GAL binding sites in membranes from ventral hippocampus, midbrain and rat spinal cord [18] and antagonizes several effects of GAL, such as the inhibition of evoked release of ACh from the hippocampus in vivo and the hyperpolarization of locus coeruleus neurons in tissue slices [19].

The mechanism underlying the inhibitory effect of GAL on PDBu-induced protein phosphorylation is still uncertain. However, previous results suggest an involvement of intracellular second messengers which in turn regulate the activity of PKC. In fact GAL inhibited muscarinic agonist-induced phospholipase C (PLC) activation by lowering Ca^{2+}influx through voltage-dependent Ca^{2+} channels (VSCC) [20]. PI-specific PLC activation is closely related to PKC activity since one of the products of the PLC reaction, DAG, is an activator of the PKC.

Thus, under physiological conditions, GAL might inhibit PKC activity by blocking Ca^{2+} entry through plasma membrane channels. This would lead to a decrease of DAG formation and, as a consequence, to inhibition of PKC activity. Indeed this theory was supported by the finding that a rise in cytosolic Ca^{2+} concentration by K^+ depolarization, which increases Ca^{2+} influx through VSCC channels, abolished the inhibitory effect of GAL on protein phosphorylation induced by PDBu, which binds and activates PKC by mimicking DAG [13]. The partial inhibition of protein phsophorylation by GAL could be explained by the finding that DAG (and PDBu) increase the affinity of the enzyme for Ca^{2+}, by lowering the Ca^{2+} requirement and allowing enzyme activation at a suboptimal Ca^{2+} concentration [21].

In conclusion it thus appears that GAL, by indirectly inhibiting PKC activity, might exert an important physiological regulation on neuronal function. The exact identity of the proteins whose phosphorylation is affected by GAL is to be determined.

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