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SOURCES OF ACTIVATOR Ca²⁺ FOR GALANIN-INDUCED CONTRACTIONS OF RAT GASTRIC FUNDUS, JEJUNUM AND COLON

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Galanin (Gal) evoked reproducible contractions of isolated rat gastric fundus, colon and jejunum longitudinal strips in concentrations ranging from 1 nM to 3 μM. EC_{50} of Gal equalled 12.63, 23.27 and 56.02 nM, respectively. Hill's coefficients were not different from unity in any of the tissues examined. Experiments have been performed in the presence of protease and peptidase inhibitors, a variety of specific antagonists and tetrodotoxin (TTX) to exclude the non-specific stimulatory or inhibitory action of Gal. Gal-evoked contractions were attenuated by diminished extracellular Ca^{2+} concentration and by diltiazem. Gal activity in gastric fundus and colon, but not in jejunum was inhibited by depleting intracellular Ca^{2+} stores, thapsigargin, dantrolene, ryanodine, TMB-8, neomycin and U-73122. Our data confirmed that Gal contracts rat fundus, jejunum and colon by stimulating specific receptors, which are coupled both to Ca^{2+} influx through the voltage-dependent calcium channels and intracellular Ca^{2+} release from ryanodine- and IP_3-sensitive stores (stomach and colon) or the extracellular Ca^{2+} influx only (jejunum). Phosphatidylinositol-specific phospholipase C (PI-PLC) plays a crucial role in the former but not in the latter signal transduction cascade.

K e y w o r d s: *galanin, smooth muscle, contraction, rat.*

INTRODUCTION

Gal is a 29—(30 in humans) amino acid peptide isolated for the first time from the extracts of pig upper intestine, based on its C-terminal amidated structure (1). Although human Gal has a 90% cross-species homology and Gal-like immunoreactivity is widely distributed in several tissues, the molecule has little in common with other neuropeptides, indicating an exclusive membership in a new peptide family (2—3).

Gal is a ubiquitous peptide transmitter which stimulates at least three different types of G-protein coupled receptors, subsequently activating several intracellular signalling pathways regulating diverse physiological processes (4). Although both N- and C-terminal ends of Gal affect smooth muscle activity, relatively little is known on how the peptide initiates its action at the intracellular level (5—8). We previously found out that Gal contracted isolated rat fundus strips by acting at receptors linked to pertussis toxin-sensitive G proteins. Besides, it seemed that myogenic action of peptide was not only due to the extracellular Ca^{2+} influx, but also to Ca^{2+} ions mobilization from the intracellular stores (9). Similar phenomenon has been observed in the mouse isolated colon (10), whereas activation of Gal receptors in guinea-pig or pig ileum, rat jejunum and dog colon was coupled to the extracellular Ca^{2+} influx only (8, 11, 12).

The goal of our current investigations was to describe the putative differences in mechanism(s) by which Gal contracts rat gastric, jejunal and colonic smooth muscle with a special emphasis on determining the sources of Gal-evoked increase in intracellular Ca^{2+} concentration in various parts of the gut. Furthermore, we aimed at defining whether phospholipase C (PLC) is involved in the signal transduction cascade elicited by the activation of Gal receptors in stomach and intestines.

MATERIALS AND METHODS

Animals and tissue preparation

All procedures were designed in accordance with the generally accepted ethical standards and the guidelines established by the Ethical Committee of the Medical University of Gdańsk.

Our experiments have been performed according to the previously described methods (9). Briefly, male Albino-Wistar rats were fasted overnight, retaining a free access to tap water. Rats were sacrificed, the longitudinal smooth muscle strips were prepared (9--10; 13—14). The tissues were mounted vertically in isolated organ chambers for the isotonic registration of mechanical activity. Tissues were allowed to equilibrate for 60 min in carbogen-gassed Tyrode solution (37°C) at a resting tension of 2.0 g. The buffer was changed every 5 min, except for the contact time of the tested agent(s) with the tissues. Viability and contractility of each strip were examined by the addition of carbachol (100 nM). The composition of Tyrode solution (pH 7.2—7.4) was (mM): NaCl 136.9, KCl 3.35, $CaCl_2$ 1.46, $MgCl_2$ 1.03, $NaHCO_3$ 11.9, NaH_2PO_4 0.48, glucose 5.0.

Characterisation of the stimulatory effects of Gal on rat gastric fundus, jejunal and colonic strips

In order to ensure that Gal-induced contractions were due to a direct myogenic effect experiments have been performed in the presence of variety of antagonists (blockers) and tetrodotoxin (TTX). Conventional concentration-contraction curves were constructed in

a non-cumulative manner. The contact time of the peptide with muscle strips ranged from 1 to 3 min. To avoid tachyphylaxis to Gal, the peptide was applied at 20—30 min intervals. No more than two complete concentration-response curves were done on each strip.

Determination of activator Ca^{2+} source

The action of Gal (300 nM) was tested on the stomach, jejunum and colon both in the absence and in the presence of diltiazem (10 μM, incubation time 20 min), a Ca^{2+} — deficient (0.9 mM $CaCl_2$) or Ca^{2+} — free Tyrode solutions (0 mM $CaCl_2$ + EGTA 0.1 mM). In the latter protocol the gut segments were then rinsed thrice, for 2 min each, with Ca^{2+} — free Tyrode to remove any EGTA and challenged with Gal. To determine the role of intracellular Ca^{2+}, the tissues were perfused with a Ca^{2+} — free Tyrode + EGTA (2 mM, 15 min), at which time carbachol was added producing a transient contractile effect. After the length of the strips returned to basal levels, the tissues were rinsed thrice with Ca^{2+} — free Tyrode containing EGTA (2 mM) and subsequently exposed to carbachol. The second exposure to carbachol did not elicit a contraction in any of the examined strips. They were washed thrice with Ca^{2+} — free Tyrode (without EGTA) bathed in the standard $CaCl_2$ — containing Tyrode and exposed again to Gal. Since EGTA can enhance the extracellular membrane permeability another way to deplete the intracellular Ca^{2+} was introduced. Following 30 min of incubation with Ca^{2+} — free Tyrode solution containing thapsigargin (10 μM), the strips were challenged with carbachol (100 nM). The strips in which carbachol did not elicit a contraction have been exposed to standard $CaCl_2$ — containing Tyrode and then to Gal (300 nM). Finally the action of Gal was tested in the absence and presence of dantrolene (10 μM, incubation time 15—20).

In the last series of experiments half-maximum effective concentrations (EC_{50}) of TMB-8, ryanodine, neomycin and U-73122 were calculated (pre-treatment 15—20 min). The EC_{50} of each blocker represents concentration of the agents reducing the contractile effect of Gal by 50 %.

Drugs

Gal was synthesised by Rekowski, Ruczyński and Szyk (15). Tetrodotoxin (TTX Crystalline 3X) was purchased from Sankyo Co., Ltd. (Tokyo, Japan). Diltiazem hydrochloride was a generous gift of Polpharma (Starogard Gdański, Poland). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Phosphoramidon, U-73122, PMSF, ryanodine, thapsigargin and indomethacin were dissolved in a small volume of absolute ethanol. Amastatin, TTX were dissolved in 100 mM acetic acid and EGTA in 10 % NH_4OH. All agents were adjusted to the desired volume with Tyrode solution. Other drugs were solubilized in Tyrode buffer only.

Statistical analysis of the acquired data

Results were expressed as the percentage of the maximum response induced by Gal. The responses to Gal in the presence of all active treatments were expressed as a percentage of control. Statistical comparisons were performed using two-tailed Wilcoxon signed rank, Mann-Whitney or one-way analysis of variance (ANOVA) plus Bonferroni post-ANOVA tests where appropriate.

EC$_{50}$s and parallelism of the concentration-contraction curves were determined using Pharmacological Calculation System (Pharm/PCS) version 4 computer program (16). EC$_{50}$s, slopes of the concentration-contraction curves were expressed as means with 95% confidence limits. Hill's coefficients were calculated using a computer program (17).

The responses in the presence of active treatment expressed as percentage of appropriate controls and the values of Hill's coefficients were calculated as means of all experiments $\pm$ SEM (standard error of mean). Experimental groups included minimum 6—12 animals. $P < 0.05$ was taken to indicate a significant difference.

RESULTS

Gal evoked reproducible concentration-dependent contractions of isolated gastric fundus, colon and jejunum at 1 nM with maximum effects at 1 or 3 μM respectively. In stomach EC$_{50}$ of Gal equalled 12.63 nM (5.33—25.22), in jejunum 56.02 nM (27.92—109) and in colon 23.27 nM (10.03—54.95). Hill's coefficients obtained from the appropriate concentration-response curves were 1.02, 1.00 and 0.87 each. (*Table 1*). The concentration-response curves of Gal constructed in colon or jejunum, were to the right, but almost parallel to that of Gal in the stomach and their slopes were not markedly different. In contrast EC$_{50}$ of Gal in jejunum differed significantly from those in gastric fundus and colonic strips (*Fig. 1*). Hill's coefficient values obtained from the respective concentration-contraction curves were not considerably different from unity in any of the examined tissues.

Table 1. A comparison of some pharmacological variables of Gal calculated from the respective concentration-response curves in different parts of the rat gut.

	EC$_{50}$ (nM)	Slopes of concentration-response curves	Hill's coefficient	Number of experiments
Gastric fundus	12.63 (5.33—25.22)	42.27 (29.54—58.65)	1.02 ± 0.12	8
Jejunum	56.02[a] (27.92—109)	47.18 (34.78—58.13)	1.00 ± 0.1	12
Colon	23.27[b] (10.03—54.95)	49.63 (37.50—83.18)	0.87 ± 0.05	12

All data were calculated from the appropriate concentration-contraction curves. They are expressed as means with 95% confidence limits or in the case of Hill's coefficient as a mean $\pm$ SEM. Ranges are given in parentheses. Variables were compared using One-Way Analysis of Aariance (ANOVA) plus Bonferroni post-ANOVA tests [a]$P < 0.001$ jejunum vs. gastric fundus; [b]$P < 0.01$ jejunum vs. colon.
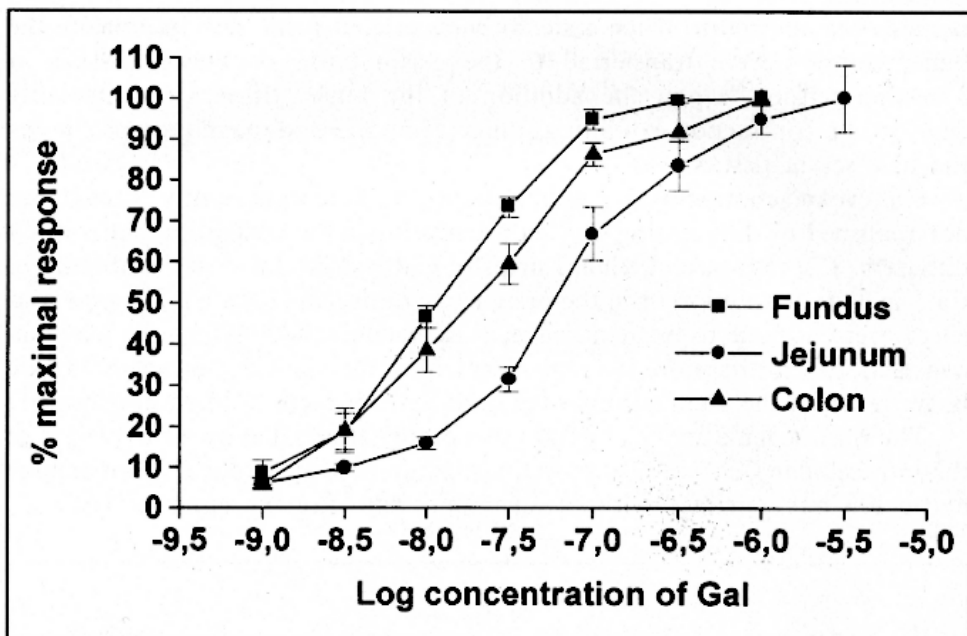
Fig. 1. Non-cumulative concentration-response curves of gastric fundus, jejunal and colonic smooth muscle exposed to Gal. Data were normalised as percentage of the maximal response to peptide and plotted against log Gal concentration. Data are presented as means ± SEM for at least 8—10 different tissues of each type.

Atropine, guanethidine, propranolol, phentolamine and indomethacin and naloxone, mepyramine, cimetidine, saralasin and TTX (10 μM each) did not affect contractions induced by Gal in stomach, jejunum and colon. In our experiments, the contractions developing in response to Gal were not maintained over time, but the force-displacement curves fell to a lower yet still suprabasal values (*Fig. 2*).

In order to determine, whether the detected phenomenon was a typical physiological response of gut tissues to Gal or a result of proteolytic peptide degradation two batches of large volumes of Tyrode buffer containing maximally effective concentrations of Gal (1 or 3 μM respectively) were prepared. One half of each sample was used to contract several strips of stomach, colon or jejunum respectively. The other half was kept at 37°C being constantly gassed with carbogen. Following the development of the steady state level of contractile response by the tissues, the bathing solution was replaced with the fresh, carbogenated and warmed Tyrode containing Gal. No change in the level of force displacement was noticed in any of the observed tissues. Subsequently, we contracted gastric, colonic and jejunal strips with Tyrode buffer samples containing maximally effective Gal concentrations. The

tissues were allowed to reach a steady state of contractile response before the buffer samples were transferred to the organ baths containing tissues at a resting tension. Upon the addition of the buffer tissue strips instantly developed a contraction exhibiting similar temporal and magnitude profiles as the first set of tissues.

Gal-evoked contractions of fundus and colon were significantly affected, but not abolished by diminishing Ca^{2+} concentration in the incubation buffer or by diltiazem. The response of jejunal strips to Gal was inhibited in the absence of Ca^{2+} in Tyrode solution or in the presence of diltiazem (*Table 2*). The observed effects were not due to tissue damage, as carbachol could still invoke maximal contractions. Furthermore the replenishment of Ca^{2+} in Ca^{2+}-deficient Tyrode buffer reversed the attenuation of Gal-induced contractions (data not shown).

The spasmogenic activity of Gal was notably attenuated by the depletion of the intracellular Ca^{2+}-stores or by thapsigargin and dantrolene in rat fundus and colon but remained without any noticeable effect in jejunum (*Table 2*).
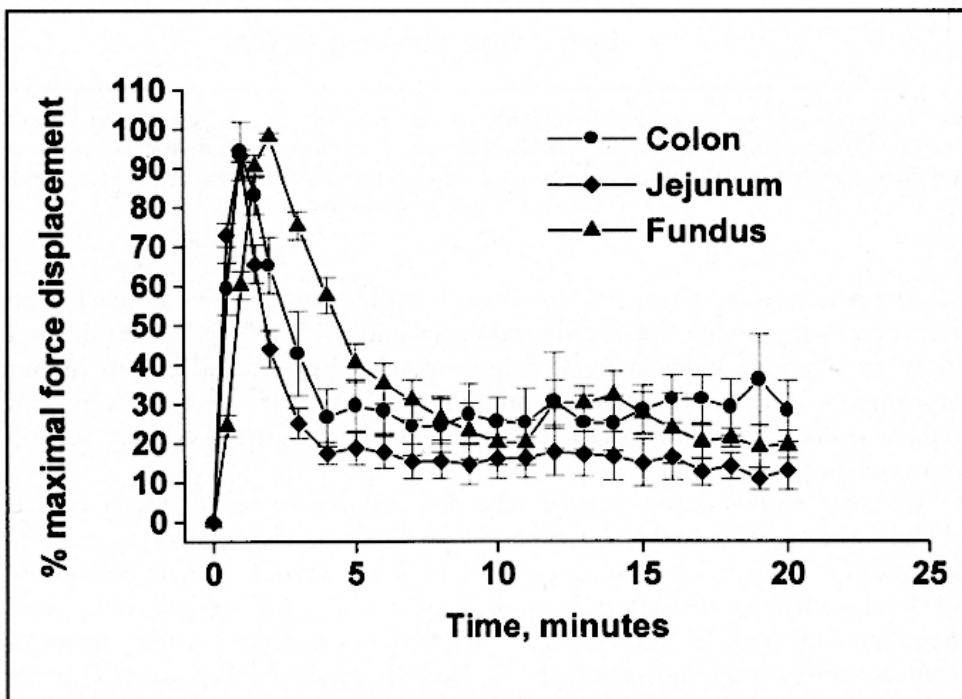

Fig. 2. Time course of force displacement during galanin-induced contraction of rat gastric fundus, colon and jejunum. Tissues were contracted with maximally effective concentrations of Gal, 1 or 3 μM respectively. As seen in the figure addition of Gal to the organ baths induced a rapid spasmogenic response falling to a lower but yet suprabasal values. Data are presented as means ± SEM for at least 10—12 different tissues of each type. Only one time course of force displacement curve was performed in each strip.

Table 2. Source of activator Ca^{2+} for Gal-induced contraction of isolated gastric fundus, jejunal and colonic strips.

Tissue contractions in the presence of treatment (%)			
	Gastric fundus	Jejunum	Colon
Diltiazem (10 μM)	13.12 ± 1.46% *	0 *	19.63 ± 1.35% *
Ca^{2+}-deficient Tyrode	31.56 ± 4.74% *	10.11 ± 2.25 *	25.00 ± 1.01% *
Ca^{2+}-free Tyrode	8.33 ± 2.65% *	0 *	12.67 ± 2.36% *
Thapsigargin (10 μM)	30.74 ± 3.46% *	95.22 ± 9.69%	41.64 ± 4.07 *
Depletion of intracellular Ca^{2+}-stores	70.24 ± 6.87% *	90.88 ± 10.98%	80.78 ± 5.99 *
Dantrolene (10 μM)	61.17 ± 5.59% *	98.62 ± 6.29%	67.52 ± 4.97% *

Strips of gastric fundus, colon or jejunum were contracted with 1 or 3 μM of Gal (controls). After the wash-out they were subsequently subjected to the appropriate treatment before being exposed for the second time to Gal (active treatment). The results were calculated as means of respective controls ± standard error of the mean. They were compared using paired, non-parametric Wilcoxon signed-rank test. * $P < 0.05$ vs. respective controls.

TMB-8 inhibited Gal-evoked contractions of rat fundus and large intestine in a concentration-dependent manner, with EC_{50} equal to 28.43 μM (13.79—58.63) and 77.03 μM (50.88—116) respectively. Ryanodine counteracted the stimulatory effects of Gal in fundus and colon with EC_{50} amounting to 1.07 μM (21.95 nM − 52.20 μM) and 57.44 μM (25.82—128) each.

Neomycin decreased concentration-dependently the contractile effects of Gal in stomach and colon reaching EC_{50} of 230 μM (100—490) and 551 μM (249 μM − 1.22 mM). Similarly U-73122 diminished the action of Gal concentration-dependently, EC_{50} values were 352 μM (90.74 μM − 1.37 mM) and 130 μM (106—160) respectively. On the contrary TMB-8, rynaodine, neomycine and U-73122 remained without noticeable effects on Gal-induced contractions of jejunum up to the concentration of 30 μM each.

DISCUSSION

We confirmed that Gal-induced contraction of rat gastric fundus, jejunum and colon longitudinal smooth muscle strips result from a direct myogenic activity of the peptide and not from a modulation of excitatory or inhibitory neuronal output (5, 9, 18). Several arguments support this hypothesis as Hill's coefficients were not significantly different from unity and Gal concentration-response curves showed characteristic sigmoid shapes with

fall-down effects at the supra-maximal concentrations (data not shown) (19—20). The excitatory influence of Gal in gut smooth muscle preparations were not modified by any of antagonists excluding a role of acetylcholine, catecholamines, opioid peptides, prostanoids, 5-hydroxytryptamine, histamine, angiotensin II in Gal-induced contraction of rat fundus, jejunum or colon. However, further work is required to elucidate the precise molecular characteristic and the type of Gal receptor responsible for the action of the peptide in different segments of gut smooth muscle. Characterisation of the changes in the time course of the force-displacement curve during Gal-evoked contraction suggests that a decline of the contractile response to suprabasal values result most probably from a desensitization of the contractile apparatus to Gal action rather than a proteolytic degradation of the peptide. This hypothesis has already been proved in rat jejunum (8). Diminished Ca^{2+} concentration or the block of L-type Ca^{2+} channels attenuated Gal-evoked contractions by approximately the same magnitude, indicating that Ca^{2+} influx through voltage-dependent channels can fully account for the dependence of Gal evoked contraction on the extracellular Ca^{2+} (5, 9—10, 18). On the other hand, myogenic effects of Gal in stomach or colon but not in jejunum were markedly reduced by depleting intracellular Ca^{2+} stores or pretreatment of tissues with thapsigargin, TMB-8, ryanodine and dantrolene. Thapsigargin increases the release and/or blocks the uptake of IP_3-sensitive and insensitive Ca^{2+} from the endoplasmic reticulum, whereas TMB-8 impedes the release of Ca^{2+} by the contractile stimuli (21). Ryanodine augments the loss of intracellular Ca^{2+} and reduces the participation of intracellular Ca^{2+} in the contractile response (22).

Several transmitters evoke cellular responses by stimulating phosphatidylinositol-specific phospholipase C (PI-PLC), which generates inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes intracellular Ca^{2+} release by activating specific receptors in the endoplasmic reticulum, whereas DAG stimulates protein kinase C (PKC), which in turn phosphorylates intracellular proteins involved in the contraction of smooth muscle cells (23).

Data obtained in our experiments suggest that Gal-stimulated contractions of gastric fundus and colon result at least partly from PI-PLC activation as it was inhibited in a concentration-dependent manner by neomycin and U-73122. Neomycin attenuates IP_3-dependent Ca^{2+} release and contraction in several smooth muscle preparations including rat urinary bladder, guinea-pig ileum and circular muscle of cat lower esophageal sphincter (24—26). One can presume that the action of U-73122 is selective for phospholipase C in our experiments, because this agent did not affect phospholipase A_2 and protein kinase C activities, secretin-stimulated cAMP production in micromolar or milimolar concentrations (25, 27—30). The role of PI-PLC and IP_3 in

Gal-induced contraction of gastric fundus is further advocated by the fact that in α-toxin permeabilized fundus strips, Gal-evoked contractions were inhibited by the IP_3 receptor antagonist heparin (31). These data support the notion that the contractile action of Gal in rat fundus and colon requires Ca^{2+} release from IP_3- and ryanodine-sensitive intracellular calcium stores, which is in opposition to the receptor-mediated events in rat jejunum (8). The latter phenomenon has been observed in the rabbit intestine as well (32).

Murthy *et al.* have demonstrated that receptor-G protein mediated processes in circular smooth muscle of guinea-pig intestine are coupled to IP_3-sensitive release of intracellular Ca^{2+}, whereas those in longitudinal smooth muscle are coupled to influx of the extracellular Ca^{2+} (33). Our results, remain partly consistent with those studies, pointing out that agonist-induced contraction of gut longitudinal smooth muscle can be coupled either to the influx and/or intracellular Ca^{2+} release, depending on the agonist(s) and receptor(s) involved. The potential interactions between two signalling pathways may provide a cell with intracellular co-ordination in order to enable it to achieve precise functional responses.

Based on the current and previously published results, we observed that Gal activates specific, pertussis toxin sensitive G-protein coupled receptor(s) leading either to Ca^{2+} influx through the voltage-dependent Ca^{2+} channels or additionally to Ca^{2+} release from both ryanodine- and IP_3-sensitive intracellular stores, depending on the tissue examined. PI-PLC is an important element of the signal transduction set in motion by the activation of Gal receptors in fundus and colon but not jejunum.

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