Calcium handling in porcine coronary endothelial cells by gastrin-17

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Abstract

In porcine coronary artery endothelial cells (PCAEC), gastrin-17 has recently been found to increase nitric oxide (NO) production by the endothelial NO synthase (eNOS) isoform through cholecystokinin 1/2 (CCK1/2) receptors and the involvement of protein kinase A (PKA), PKC and the β_2 -adrenoreceptor-related pathway. As eNOS is the Ca^{2+} -dependent isoform of the enzyme, we aimed to examine the effects of gastrin-17 on Ca^{2+} movements. Thus, experiments were performed in Fura-2-acetoxymethyl-ester-loaded PCAEC, where changes of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) caused by gastrin-17 were analysed and compared with those of CCK receptors and β_2 -adrenoreceptors agonists/antagonists. In addition, some experiments were performed by stimulating cells with gastrin-17 in the presence or absence of cAMP/PKA activator/inhibitor and of phospholipase C (PLC) and Ca^{2+} -calmodulin dependent protein kinase II (CaMKII) blockers. The results have shown that gastrin-17 can promote a transient increase in $[\text{Ca}^{2+}]_c$ mainly originating from an intracellular pool sensitive to thapsigargin and from the extracellular space. In addition, the response of cells to gastrin-17 was increased by the adenylyl cyclase activator and the β_2 -adrenoreceptor agonists and affected mainly by the CCK2 receptor agonists/antagonists. Moreover, the effects of gastrin-17 were prevented by β_2 -adrenoreceptors and CaMKII blockers and the adenylyl cyclase/PKA and PLC inhibitors. Finally, in PCAEC cultured in Na^+ -free medium or loaded with the plasma membrane Ca^{2+} pump inhibitor, the gastrin-17-evoked Ca^{2+} transient was long lasting. In conclusion, this study shows that gastrin-17 affected intracellular Ca^{2+} homeostasis in PCAEC by both promoting a discharge of an intracellular pool and by interfering with the operation of store-dependent channels through mainly CCK2 receptors and PKA/PLC- and CaMKII-related signalling downstream of β_2 -adrenoreceptor stimulation.

Key Words

- CCK receptors
- endothelial cells
- gastrin-17
- intracellular stores

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Introduction

The gastrointestinal peptide hormone gastrin-17 has been recently found in anaesthetized pigs to cause vasodilation through mechanisms related to nitric oxide (NO) effects (Grossini *et al.* 2011). NO measurement and protein analysis performed in porcine coronary artery endothelial cells (PCAEC) have shown an acute and dose-dependent endothelial NO synthase (eNOS)-related NO production

in response to gastrin-17, which was modulated by β_2 -adrenoreceptor agonists/antagonists and was mainly related to a cAMP/protein kinase A (PKA) pathway. Moreover, although both cholecystokinin (CCK) receptors were found to be involved, the subtype 2 of CCK receptors was shown to play the most important role in such effects (Grossini *et al.* 2012).

Changes of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) levels are of primary importance in the regulation of NO production. It is well known that the constitutive isoform of NOS present in endothelial cells (eNOS) is Ca^{2+} dependent (Lowenstein *et al.* 1994, Mizuno *et al.* 2000). eNOS is inactive until $[\text{Ca}^{2+}]_c$ increases, the Ca^{2+} -binding protein binds to Ca^{2+} and the Ca^{2+} -calmodulin complex activates eNOS (Moncada *et al.* 1991). The dynamic steady state of Ca^{2+} in the cytosol is the result of the balance between active and passive fluxes through the cell membranes and is strictly regulated (Munaron 2006). Transient fluctuations in $[\text{Ca}^{2+}]_c$ may occur following inhibition of Ca^{2+} ATPases located in the plasma membrane or in the membrane of non-mitochondrial stores and activation of the release from intracellular stores may occur whether or not it is dependent on inositol-1,4,5-triphosphate-phosphate (IP3) generation. Also, the release of intracellular Ca^{2+} coupled to subsequent Ca^{2+} entry (Putney 1990), known as 'capacitative Ca^{2+} entry', is a mechanism widely reported to affect Ca^{2+} homeostasis in response to both receptor-mediated stimuli (Putney 1990, Schilling *et al.* 1992, Fasolato & Nilius 1998) and receptor-independent emptying of intracellular stores by agents such as thapsigargin, cycloviobuxine D and urocortin II (Putney 1990, Schilling *et al.* 1992, Fasolato & Nilius 1998, Grossini *et al.* 2005, 2010). In addition, the restoration of basal intracellular Ca^{2+} levels would play an important role by the activation of the plasma membrane Ca^{2+} -ATPase (PMCA) pump and of $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX; Moccia *et al.* 2002, Wang *et al.* 2002).

Changes of $[\text{Ca}^{2+}]_c$ have been reported to be also crucial for the response of gastrointestinal cells to CCK receptor agonists. Hence, Ca^{2+} is an essential signalling molecule in gastrin-17-stimulated cell proliferation (Olszewska-Pazdrak *et al.* 2004) and is involved in gastrin-induced histamine secretion (Shibata *et al.* 2006). In addition, in isolated pig parietal cells, both gastrin-17 and the synthetic peptide pentagastrin increased $[\text{Ca}^{2+}]_c$ in a dose-dependent manner (Cabero *et al.* 1989). Also, the CCK receptor agonists, CCK58 and CCK8, have been found to either cause transient oscillatory $[\text{Ca}^{2+}]_c$ increases or sustained responses depending on whether CCK receptor agonists were used in physiological or supra-physiological concentrations in murine isolated pancreatic acinar cells (Criddle *et al.* 2009). The above effects on Ca^{2+} movements have been generally reported to be related to either Ca^{2+} entry from the extracellular space through CCK receptor stimulation (Criddle *et al.* 2009) and/or PKC and IP3-dependent opening of intracellular

stores (Zanner *et al.* 2002, Olszewska-Pazdrak *et al.* 2004). In addition, involvement of MEK1/2 and ERK1/2 pathways has been reported (Olszewska-Pazdrak *et al.* 2004).

Thus, with the above considerations and as an extension of earlier works from our group showing an effect of gastrin-17 on NO production, the aim of this study was to investigate in PCAEC the effects of gastrin-17 on $[\text{Ca}^{2+}]_c$ handling and the mechanisms involved.

Materials and methods

Experiments were performed in primary culture cells, PCAEC, which were isolated from coronary arteries of anaesthetized pigs, as described previously (Grossini *et al.* 2009). The cells were plated into 0.1% gelatin-coated culture dish by endothelial growth media-2 (EGM-2; EGM-2 BulletKit (CC-3124); Lonza, Inc., Basel, Switzerland) with the addition of human epidermal growth factor, hydrocortisone, gentamycin-amphotericin-B 1000, 2% foetal bovine serum (FBS), vascular endothelial growth factor, human fibroblast growth factor-basic, recombinant analogue insulin-like growth factor 1-human, ascorbic acid, heparin (Lonza) and 1% penicillin-streptomycin-glutamine (Sigma-Aldrich) and maintained in incubator at 37 °C and 5% CO_2 .

After 72 h, 10 ml fresh medium was added and the incubation continued for 48 h. After this time, the cells formed a monolayer and were subcultured. The cells used for the experiments were obtained from passages 3 to 5.

Cells (1×10^5) were plated in 0.1% gelatin-coated 24-well plates in complete medium for 24 h and then maintained with DMEM (Sigma) 0% FBS supplemented with 1% penicillin-streptomycin-glutamine (Sigma) without red phenol (starvation medium) for 4–6 h.

Some experiments were repeated in human coronary artery endothelial cells (HCAEC; Lonza) cultured in EGM-2. Experiments were performed with cells from passages 5 to 8. At confluence, cells were serum starved for 6 h in endothelial cell basal medium (Lonza). HCAEC (1×10^5) were plated as described for PCAEC and then maintained in starvation medium for about 6 h.

Fura-2 fluorescence measurement of $[\text{Ca}^{2+}]_c$

In the first set of experiments, the measurement of $[\text{Ca}^{2+}]_c$ was performed in cells grown to confluence, washed twice with sterile PBS $1 \times$ (Sigma) and incubated with Fura-2/acetoxymethyl (AM) ester (5 μM final concentration, Sigma) for 30 min in the dark in DMEM (Sigma) 0% FBS and without red phenol. In some samples, cells

were incubated in zero Na^+ -poly(sodium styrene sulphonate) (PSS) (*N*-methyl-D-glucamine 126 mM, KCl 1.5 mM, MgCl_2 1.2 mM, HEPES 10 mM, D-glucose 10 mM and CaCl_2 1 mM, Sigma). After further washing with DMEM (Sigma), the coverslips were mounted in agitation at 37 °C in thermostated quartz cuvette in a Hitachi F-4500 Fluorescence Spectrometer (Hitachi High-Technologies Corporation) operating continuously for 300 s at the wavelength pair 340 nm excitation/510 nm emission. Gastrin-17 (Tocris Bioscience, Bristol, UK) was added to Fura-2/AM-loaded cells, in the presence or absence of Ca^{2+} in the incubated medium (obtained with 50 mM EGTA, Sigma). In some experiments, the effects of gastrin-17 were compared with those elicited by the CCK1 receptor agonists, sulphated CCK8 (Tocris) and A71378 (Tocris), or the CCK2 receptor agonist, pentagastrin (Sigma), which were administered either alone or in co-stimulation with gastrin-17. The above agents were given at the final concentration of 10 pM or, in the dose-response study, at final concentrations of 10^{-14} – 10^{-6} M. In some experiments, the effects of gastrin-17 were compared with those elicited by thapsigargin (1 μM , Sigma), ATP (10 μM , Sigma) and thrombin (0.5 U/ml, Sigma), which were administered either before or after gastrin-17. Moreover, in some samples, agents were given before gastrin-17; they included the adenylyl cyclase blocker 2'/5'-dideoxyadenosine (1 μM , Sigma; for 15 min), the selective cAMP-dependent PKA inhibitor H89 (1 μM , Sigma; for 15 min), the phospholipase C (PLC) γ inhibitor U73122 (1 μM , for 30 min, Sigma), the CCK1 receptors inhibitor lorglumide (50 μM , Sigma; for 30 min), the CCK2 receptor inhibitor CAM-1028 (15 μM , Sigma; for 30 min), the Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93 (1 μM , Sigma; for 15 min), the PMCA pump blocker, carboxyeosin diacetate (25 μM , Invitrogen; for 15 min) and the β_2 -adrenoreceptor antagonist, butoxamine (100 μM , Sigma; for 15 min). Finally, in some experiments, the effects of the adenylyl cyclase activator, forskolin (1 μM , Sigma), and the β_2 -adrenoreceptor non-selective and selective agonists isoproterenol and zinterol (10 μM , Sigma) respectively, given alone or in co-administration with gastrin-17 on $[\text{Ca}^{2+}]_c$, were analysed.

In the second set of experiments, the effects of gastrin-17 on the 'capacitative' Ca^{2+} entry through the plasma membrane Ca^{2+} channels were examined. Briefly, the cells on coverslips pre-treated with EGTA (50 mM, Sigma) were subsequently exposed to thapsigargin (1 μM) and gastrin-17 (1 μM) alone or in combination for 5 min. Finally, 60 mM CaCl_2 was added to the different samples and the effects on 'capacitative' Ca^{2+} entry were analysed.

Quantification of $[\text{Ca}^{2+}]_c$ was obtained conventionally, as previously reported (Gryniewicz *et al.* 1985, Grossini *et al.* 2010) using the following equation:

$$(\text{Ca}^{2+}) = K_d ((R - R_{\min}) / (R_{\max} - R)).$$

The K_d of Fura-2/AM for Ca^{2+} was taken as 224. $R_{\min}$ and $R_{\max}$ were the minimum and the maximum values of fluorescence ratio obtained in Ca^{2+} -free (EGTA 0.1 M) and Ca^{2+} -saturated conditions. The fluorescence intensities obtained were corrected for cell autofluorescence at the wavelengths employed (Gryniewicz *et al.* 1985).

Statistical analyses

All results were obtained from five different cell cultures for each experimental protocol. Student's paired *t*-test was used to examine the effects of each agent within each sample. Two-way ANOVA followed by Bonferroni's *post hoc* test were used for comparison among different experiments. A simple regression analysis was performed to examine the correlation between the concentration of administered agents and the observed $[\text{Ca}^{2+}]_c$ effects in the dose-response study. All data are presented as mean $\pm$ s.d. A value of $P < 0.05$ was considered statistically significant.

Results

The addition of gastrin-17 (10^{-14} – 10^{-6} M) to a suspension of Fura-2/AM-loaded PCAEC (or HCAEC) in the presence of Ca^{2+} in the medium of incubation (Fig. 1) caused a rapid and transient dose-related increase in $[\text{Ca}^{2+}]_c$ reaching a peak effect after about 40 s. The effects of gastrin-17 were lower than those caused by equimolar sulphated CCK8 and A71378 and particularly pentagastrin ($P < 0.05$; Figs 1A and 2A), which induced the highest response. In addition, the effects of gastrin-17 were increased when given in co-stimulation with either sulphated CCK8, A71378 or pentagastrin ($P < 0.05$; Figs 1A and 2B). A linear relationship was observed between the administered concentrations of CCK receptor agonists and the effects on $[\text{Ca}^{2+}]_c$ ($P < 0.05$). In PCAEC, therefore, gastrin-17 alone or in the presence of pentagastrin, sulphated CCK8 or A71378 respectively increased the $[\text{Ca}^{2+}]_c$ to 126.4 ± 4.8 , 144.2 ± 6.9 , 134.8 ± 5.7 and 134 ± 4.5 nM ($P < 0.05$) from control values of about 111.4 ± 2.4 nM (Fig. 2B).

Moreover, as shown in Fig. 2C, the effects of gastrin-17 alone or in co-stimulation with equimolar pentagastrin, sulphated CCK8 or A71378 were significantly reduced

Figure 1

Concentration-dependent effects of gastrin-17 and other CCK receptor agonists on $[Ca^{2+}]_c$ in Fura-2/AM-loaded coronary endothelial cells. In (A) and (B), the effects of gastrin-17, sulphated CCK8, pentagastrin and A71378 alone or in co-stimulation in PCAEC and HCAEC, are shown respectively. Reported data are means $\pm$ s.d. ($n=5$); CCK8, sulphated CCK8. In (C), an example showing the dose-response effects of gastrin-17 in PCAEC taken from one of the five different experiments for each experimental protocol.

in PCAEC cultured in Ca^{2+} -free medium ($P<0.05$). In PCAEC treated with EGTA, gastrin-17 alone or in the presence of pentagastrin, sulphated CCK8 and A71378 respectively increased the $[Ca^{2+}]_c$ to 105 ± 2.8 , 111.2 ± 4.6 ,

108.8 ± 2.5 and 108 ± 2.1 nM ($P<0.05$) from control values of about 100.2 ± 1.5 nM. These results demonstrated that gastrin-17 mobilizes Ca^{2+} by both an extracellular milieu and intracellular stores (as suggested by the persistence of Ca^{2+} signal even in Ca^{2+} -free medium).

As the effects of sulphated CCK8 were not different from those caused by A71378, in all successive experiments, sulphated CCK8 was used. Furthermore, for all successive experiments, the above agents were used at 10 pM, which was a similar concentration to the one that gradually increased NO release in PCAEC (Grossini *et al.* 2012). It is also noteworthy that this concentration was similar to serum gastrin-17 levels found in normal subjects (Grossini *et al.* 2012).

Analysis of Ca^{2+} stores mobilized by gastrin-17

In order to obtain more details on the nature of the intracellular store affected by gastrin-17, experiments were performed using extracellular ATP and thrombin, which mobilize Ca^{2+} from IP3-sensitive pools (Patel *et al.* 1999), and also thapsigargin, which is a powerful inhibitor of non-PMCA (Thastrup *et al.* 1990, Gamberucci *et al.* 1994). As shown in Fig. 3A and B, in PCAEC, the amount of Ca^{2+} mobilized by either gastrin-17 (10 pM and 1 μ M) or ATP (10 μ M) was significantly reduced when the agents were given in succession both in the absence or presence of EGTA ($P<0.05$). By contrast, the amount of Ca^{2+} mobilized by either gastrin-17 (1 μ M) or thrombin (0.5 U/ml) did not significantly differ irrespective of the sequence of addition (Fig. 3C; $P>0.05$). Similar results were found in HCAEC (Supplementary Figure 1, see section on supplementary data given at the end of this article).

As depicted in Fig. 4, in PCAEC, the administration of thapsigargin (1 μ M) caused a persistent increase in $[Ca^{2+}]_c$ to levels of 155.8 ± 3.2 nM ($P<0.05$) from control values of 110.8 ± 2.3 nM. When gastrin-17 was administered before thapsigargin or in co-administration with thapsigargin, the kinetics of $[Ca^{2+}]_c$ fluctuations promoted by thapsigargin was markedly changed. Hence, as shown in Fig. 4, when gastrin-17 (10 pM and 1 μ M) was administered before thapsigargin, the $[Ca^{2+}]_c$ reached peak values of 149.4 ± 1.9 and 130.4 ± 7.6 nM respectively, from similar control values as those reported earlier. Similar results were observed when gastrin-17 was given in co-stimulation with thapsigargin. The effects of thapsigargin in the presence of gastrin-17 were statistically different from the one caused by thapsigargin alone ($P<0.05$). Also in HCAEC, gastrin-17 (1 μ M) changed the response of cells to thapsigargin in a similar way to that

Figure 2

Effects of gastrin-17 and other CCK receptors agonists on $[Ca^{2+}]_c$ in Fura-2/AM-loaded PCAEC. In (A) and (B), gastrin-17 (10 pM), sulphated CCK8, pentagastrin and A71378 (10 pM) were administrated alone or in co-stimulation respectively. In (C), gastrin-17, sulphated CCK8, pentagastrin and A71378 were administrated alone or in co-stimulation, in the presence of EGTA (50 mM). In (A), (B) and (C), examples were taken from one of the five different experiments for each experimental protocol. The abbreviations are as in Fig. 1.

observed in PCAEC (Supplementary Figure 2A, see section on supplementary data given at the end of this article). These findings strongly support the conclusion that gastrin-17 mobilizes Ca^{2+} from a store that is depleted by thapsigargin treatment.

Extracellular Ca^{2+} implications in gastrin-17-induced Ca^{2+} mobilization

The depletion of thapsigargin-sensitive intracellular stores activates channels in the plasma membrane, which are permeable to Ca^{2+} (Fasolato *et al.* 1994, Berridge 1995). This phenomenon can be easily investigated through experiments aimed to analyse 'capacitative' Ca^{2+} entry. In PCAEC-treated gastrin-17 alone, the effects on Ca^{2+} handling observed after Ca^{2+} re-admission were not different from those previously observed. Moreover, in PCAEC treated with thapsigargin alone or in the presence of gastrin-17, the re-admission of Ca^{2+} in the incubation

medium caused a Ca^{2+} increase amounting to 545 ± 4 and 543 ± 3.4 nM ($P < 0.05$) respectively, from control values of 102.8 ± 0.8 and 101.8 ± 2.4 nM. The Ca^{2+} increase caused by the concomitant administration of the two substances was not statistically different from the one caused by thapsigargin alone. An example of these findings taken from one of the five different experiments performed in PCAEC is depicted in Fig. 5A and B. Similar results were found in HCAEC (Supplementary Figure 2B).

As the NCX and PMCA have been shown to contribute to lower $[Ca^{2+}]_c$ in vascular endothelial cells (Moccia *et al.* 2002, Wang *et al.* 2002), experiments were performed to abolish their contribution to Ca^{2+} extrusion. As depicted in Fig. 5C, in PCAEC cultured in Na^+ -free medium, the gastrin-17 (10 pM and 1 μ M)-evoked Ca^{2+} increase was lower and had not returned to control values at 5 min from the start of gastrin-17 administration; the peak

Figure 3

Analysis of intracellular Ca^{2+} stores mobilized by gastrin-17 in Fura-2/AM-loaded PCAEC. In (A) and (B), gastrin-17 (1 μ M and 10 pM) was given before/after 10 μ M ATP either in the absence or presence of EGTA. In (C), gastrin-17 (1 μ M) was given before/after 0.5 U/ml thrombin either in the absence or presence of EGTA. In (A), (B) and (C), examples were taken from one of the five different experiments for each experimental protocol.

Figure 4

Modulation of thapsigargin-dependent $[Ca^{2+}]_c$ mobilization by gastrin-17 in Fura-2/AM-loaded PCAEC. Gastrin-17 (10 pM and 1 μ M) was given before or in co-administration with thapsigargin (1 μ M). Examples were taken from one of the five different experiments for each experimental protocol. Thap, thapsigargin.

effect of gastrin-17 on $[Ca^{2+}]_c$ amounted to 121.6 ± 3.5 and 139.2 ± 3.1 nM ($P < 0.05$) from control values of 111.4 ± 2.7 nM. In PCAEC cultured in Na^+ -free medium and in the presence of the PMCA pump blocker carboxyeosin (25 μ M), the $[Ca^{2+}]_c$ increase induced by gastrin-17 (10 pM and 1 μ M), after peak effects of 120.4 ± 2.3 and 140 ± 1.6 nM ($P < 0.05$) respectively from control values of 110 ± 1.6 nM, reached a plateau (Fig. 5C; $P < 0.05$). Similar results were found in HCAEC (Supplementary Figure 2C).

Mechanisms of action of Ca^{2+} mobilization

To verify the involvement of the CCK1 and CCK2 receptors, β_2 -adrenoreceptors, cAMP/PKA, PLC γ and CaMKII pathways in Ca^{2+} movements caused by gastrin-17, experiments were performed in the presence

of specific inhibitors/activators. In particular, PCAEC and HCAEC were treated with the CCK1 receptor antagonist lorglumide (50 μ M), the selective CCK2 receptor antagonist CAM-1028 (15 μ M), the β_2 -adrenoreceptor non-selective and selective agonists, isoproterenol (10 μ M) and zinterol (10 μ M), and antagonist, butoxamine (100 μ M), the adenylyl cyclase activator, forskolin (1 μ M), and blocker, 2'/5'-dideoxyadenosine (1 μ M), the selective cAMP-dependent PKA inhibitor, H89 (1 μ M), the PLC γ inhibitor, U73122 (1 μ M) and the CaMKII blocker, KN-93 (1 μ M). All the above agents were used in doses similar to those previously shown to affect the response of PCAEC to gastrin-17 (Grossini *et al.* 2012). Moreover, 50 μ M lorglumide and 15 μ M CAM-1028 were able to prevent the effects of 1 μ M sulphated CCK8 and 1 μ M pentagastrin on Ca^{2+} movements in preliminary experiments performed in PCAEC.

The effects of various agents given alone on Ca^{2+} movements are reported in Table 1. As depicted in Fig. 6A, the response of PCAEC to gastrin-17 (1 μ M) was increased by isoproterenol, zinterol and forskolin by about 22, 23 and 32% respectively. It is also noteworthy that zinterol increased the effects of gastrin-17 when given in co-stimulation with pentagastrin to an extent greater than that with sulphated CCK8. In PCAEC treated with gastrin-17/pentagastrin/zinterol, the $[Ca^{2+}]_c$ amounted to 206 ± 11.9 nM from control values of 109 ± 3.4 nM, whereas in the absence of zinterol, it amounted to 165.4 ± 6.1 nM from similar control values. In cells treated with gastrin-17/CCK8/zinterol, the $[Ca^{2+}]_c$ amounted to 174 ± 7.4 nM from control values of 108.2 ± 3.2 nM, whereas in the absence of zinterol it amounted to 152.4 ± 6.7 nM from similar control values. In addition, CAM-1028 and lorglumide, which were able to decrease basal $[Ca^{2+}]_c$ (Table 1), caused a significant reduction of the response of cells to gastrin-17 when given separately and prevented the effects of gastrin-17 when given in co-stimulation (Fig. 6A).

Similarly, the effects of gastrin-17 were prevented by butoxamine, 2'/5'-dideoxyadenosine, H89, U73122 and KN-93 (Fig. 6A and C). Similar results were obtained in HCAEC (Fig. 6B and D).

Discussion

This is the first study showing that gastrin-17 can cause transient increases in $[Ca^{2+}]_c$ in endothelial cells through the activation of CCK receptors and the involvement of the β_2 -adrenoreceptor pathway and of cAMP/PKA-, PLC γ - and CaMKII-related signalling.

Figure 5

Effects of gastrin-17 on 'capacitative' Ca^{2+} entry, NCX and PMCA in Fura-2/AM-loaded PCAEC. In (A), cells pre-treated with EGTA were stimulated with gastrin-17 (1 μM). The effects on Ca^{2+} movements were examined also with CaCl_2 re-admission. In (B), cells pre-treated with EGTA were stimulated with thapsigargin (1 μM) alone or in co-stimulation of gastrin-17 (1 μM). The effects on Ca^{2+} movements were examined also with CaCl_2 re-admission. Example was taken from one of the five different experiments for each experimental protocol. Abbreviation is as in Fig. 4. In (C), effects of gastrin-17 (10 pM and 1 μM) on $[\text{Ca}^{2+}]_c$ in PCAEC incubated in PSS- Na^+ in the absence or presence of carboxyeosin (25 μM). Example was taken from one of the five different experiments for each experimental protocol. Car E, carboxyeosin.

The dynamic steady state of $[\text{Ca}^{2+}]_c$ is the result of the balance between active and passive fluxes that affect the cytosol, intracellular stores and the extracellular environment. Ca^{2+} enters the cytosolic compartment by direct influx from the extracellular milieu through a variety of plasma membrane channels or from intracellular stores. Free $[\text{Ca}^{2+}]_c$ is maintained very low by Ca^{2+} pumps like the NCX located in the plasma membrane and by active pumps in the membrane of either mitochondrial or non-mitochondrial stores such as the endoplasmic reticulum that extrude Ca^{2+} from the cytosol by direct ATP consumption (Munaron 2006).

Intracellular Ca^{2+} concentration in endothelial cells plays an important role in regulating a variety of cellular functions, some of which are related to the production of NO by the constitutive Ca^{2+} -dependent isoform of NOS, eNOS (Lowenstein *et al.* 1994). Many agents known to induce NO release by endothelial cells stimulate eNOS by increasing the $[\text{Ca}^{2+}]_c$, either by promoting Ca^{2+} release from intracellular stores and/or by enhancing Ca^{2+} influx from the extracellular compartment (Newby & Henderson 1990, Buga *et al.* 1991, Moncada *et al.* 1991). It is noteworthy that the present results have shown for the first time that β_2 -adrenoreceptor stimulation, which has been reported to increase endothelial NO release (Grossini *et al.* 2012), also augmented $[\text{Ca}^{2+}]_c$ in both human and porcine coronary endothelial cells.

In PCAEC, gastrin-17, at concentrations similar to those that increased NO production in the same cells (Grossini *et al.* 2012), was shown to cause a dose-dependent increase in $[\text{Ca}^{2+}]_c$, which peaked at about 40 s and almost returned to basal level in about 60 s. The fact that the response of cells to gastrin-17 was changed in the presence of EGTA and ATP but not by thrombin suggests that Ca^{2+} mobilized by gastrin-17 is partly of extracellular origin and partly from the same intracellular IP₃-regulated pool mobilized by ATP, but different from that mobilized by thrombin. In addition, the findings obtained in the absence of EGTA would suggest the absence of effects of gastrin-17 on the Ca^{2+} -dependent stimulated Ca^{2+} influx from extracellular space, which has been reported to be caused by both ATP and thrombin (Ahmed *et al.* 2004, Aromolaran & Blatter 2005). Similar findings were also obtained in HCAEC, which confirmed the above results in PCAEC.

Table 1 Effects of various agents on Ca^{2+} movements in PCAEC. Values are means $\pm$ s.d. (%) of five independent experiments for each experimental protocol

Sample	$[\text{Ca}^{2+}]_c$ (nM)
Control	106.6 $\pm$ 3.7
Gastrin-17 (1 μM)	137.4 $\pm$ 3.2
Zinterol (10 μM)	136.8 $\pm$ 4*
Isoproterenol (10 μM)	134.6 $\pm$ 3.4*
Forskolin (1 μM)	136.2 $\pm$ 2.3*
CAM-1028 (15 μM)	94.2 $\pm$ 2.6*
Lorglumide (50 μM)	100 $\pm$ 3.1*,†
Butoxamine (100 μM)	101 $\pm$ 1.6*
2'5'-Dideoxyadenosine (1 μM)	99.8 $\pm$ 1.3*
H89 (1 μM)	100.6 $\pm$ 1.3*
U73122 (1 μM)	103 $\pm$ 3.7
KN-93 (1 μM)	103.8 $\pm$ 4.5

* $P < 0.05$ vs control and † $P < 0.05$ vs CAM-1028.

Figure 6

Effects of gastrin-17 and other CCK receptor agonists on $[Ca^{2+}]_i$ movements in the absence or presence of various agonists/antagonists. In (A) and (B), gastrin-17 was given alone or in co-administration with isoproterenol, zinterol, forskolin, butoxamine, CAM-1028 and lorglumide in PCAEC and HCAEC respectively. G-17, gastrin-17 (1 μ M); ISO, isoproterenol (10 μ M); ZIN, zinterol (10 μ M); F, forskolin (1 μ M); butoxamine, butoxamine

(100 μ M), CAM-1028, CAM-1028 (15 μ M); Lor, lorglumide (50 μ M). $P < 0.05$: b, c, d, e and f vs a. In (C) and (D), gastrin-17 was given alone or in co-administration with 2'5'-dideoxyadenosine, H89, U73122 and KN-93 in PCAEC and HCAEC respectively. 2'5'-Dideoxy, 2'5'-dideoxyadenosine (1 μ M); H89, H89 (1 μ M); U73122, U73122 (1 μ M); KN-93, KN-93 (1 μ M). Abbreviations are as in (A) and (B). Reported data are means $\pm$ s.d. ($n = 5$).

The present results also showed that the intracellular pool depleted by gastrin-17 was the same as that of thapsigargin, the Ca^{2+} -ATPase inhibitor that is able to deplete the endoplasmic reticulum Ca^{2+} pool (Thastrup *et al.* 1990, Gamberucci *et al.* 1994). By contrast, gastrin-17 did not affect the sustained increase in $[Ca^{2+}]_i$ promoted by thapsigargin, thus excluding the modulation of the so-called 'capacitative' opening of plasma membrane Ca^{2+} channels caused by thapsigargin (Toshima *et al.* 2000, Kwan *et al.* 2001).

The effects of gastrin-17 on Ca^{2+} movements were less than those elicited by the CCK2 receptor-specific agonist

pentagastrin, which potentiated the response of cells to gastrin-17 to a higher extent than both non-selective and selective CCK1 receptor agonists, sulphated CCK8 and A71378 respectively (Grossini *et al.* 2012). It is also noteworthy that pre-treatment of cells with the CCK receptor inhibitors, particularly CAM-1028, caused a reduction of basal $[Ca^{2+}]_i$, which confirmed a role for mostly CCK2 receptor in modulation of calcium movements in PCAEC. Moreover, in PCAEC and HCAEC pre-treated with lorglumide and above all CAM-1028, the effects of gastrin-17 were significantly reduced, being abolished by co-administration of the above blockers.

These findings also confirmed the prevalent involvement of CCK2 receptors over CCK1 ones in the mechanism of action at the basis of gastrin-17-dependent $[Ca^{2+}]_c$ increase as well as previously reported observations (Rihab 1999, Grossini *et al.* 2011, 2012).

As shown by experiments performed in the presence of adenylyl cyclase agonist/antagonist, PKA/PLC and CaMKII inhibitors, the effects of gastrin-17 were found to be related to cAMP–PKA–PLC and CaMKII-dependent signalling. Hence, the effects of gastrin-17 were increased by forskolin and abolished by pre-treatment of PCAEC and HCAEC with 2′5′-dideoxyadenosine, H89, U73122 and KN-93, which were given at similar concentrations to those previously used in the same cells (Grossini *et al.* 2012). These findings are in agreement with previous data showing that G protein-coupled CCK receptor agonists activate cAMP/PKA and PLC/PKC intracellular cascades (Noble *et al.* 1999, Kombian *et al.* 2006, Grossini *et al.* 2012).

In addition, the present results are in line with previous reports regarding the effects of CCK receptor agonists on Ca^{2+} movements and their related mechanisms of action. Hence, in isolated pig parietal cells, both gastrin-17 and pentagastrin increased $[Ca^{2+}]_c$ in a dose-dependent manner over the concentration range 10^{-9} – 10^{-6} M (Cabero *et al.* 1989). Also CCK8 increased $[Ca^{2+}]_c$ in porcine chief cells, mainly through the involvement of CCK1 receptors (Heim *et al.* 1995). Furthermore, in various cell lines, gastrin-17 stimulated the production of cAMP, phosphatidylinositol hydrolysis and mobilization of intracellular Ca^{2+} (Ishizuka *et al.* 1994).

In this context, it is noteworthy that the removal of extracellular Ca^{2+} in small-cell lung cancers did not affect the increase in $[Ca^{2+}]_c$ activated by gastrin, suggesting Ca^{2+} release from internal stores (Rihab 1999), which partly confirms findings obtained in this study.

Also the results obtained in the presence of specific β_2 -adrenoreceptors agonists/antagonists are in agreement with findings previously observed in coronary endothelial cells (Grossini *et al.* 2012). Hence, the response of PCAEC to gastrin-17 was increased by the β_2 -adrenoreceptors non-selective and selective agonists, isoproterenol and zinterol, and abolished by the specific inhibitor butoxamine; this confirmed the involvement of a β_2 -adrenergic-related signalling in the intracellular pathway activated by gastrin-17. Although the role of the β -adrenergic system in the regulation of Ca^{2+} movements in myocytes is widely accepted (Kurokawa *et al.* 2002), the finding of a modulation of $[Ca^{2+}]_c$ in vascular endothelial cells of either porcine or human origin is quite new. In addition, these observations

would suggest a rather preferential association of CCK2 over CCK1 receptors and β_2 -adrenergic-related pathway in modulation of $[Ca^{2+}]_c$ mobilization in PCAEC and confirm previous findings about the existence of a preferential cross-reaction between the CCK2 receptor signalling cascade and β_2 -adrenergic pathway in the same cellular model (Grossini *et al.* 2012). Hence, the response of cells to gastrin-17 plus pentagastrin was more augmented by co-stimulation with zinterol than that observed by gastrin-17 plus sulphated CCK8 in co-stimulation with zinterol. Although not clearly stated yet, the involvement of intracellular mediators downstream of CCK receptor and β_2 -adrenoreceptor activation could be at the basis of the observed effect. Further experiments will be necessary to address this issue.

The return of $[Ca^{2+}]_c$ to control values was shown to be related to the activation of the PMCA pump and NCX activation, which are widely reported to restore the basal intracellular Ca^{2+} levels in endothelial cells (Moccia *et al.* 2002, Wang *et al.* 2002). Hence, in PCAEC and HCAEC cultured in Na^+ -free medium, the decay phase of the gastrin-17-evoked Ca^{2+} transient was significantly longer. Moreover, in PCAEC and HCAEC cultured in Na^+ -free medium and loaded with carboxyeosin, the specific PMCA pump inhibitor, the effects of gastrin-17 on $[Ca^{2+}]_c$ transient reached a plateau. Thus, the results obtained showed that during the decay phase of the Ca^{2+} transient, Ca^{2+} was extruded by both the PMCA pump and by NCX. Blocking the Na^+ -dependent and Na^+ -independent Ca^{2+} extrusion mechanisms has made the cells unable to extrude the Ca^{2+} released by the thapsigargin-sensitive and IP3-dependent pool following gastrin-17 stimulation.

In conclusion, in porcine and human coronary endothelial cells, gastrin-17 used in similar doses as those recently reported to cause eNOS activation through CCK receptors and cAMP/PKA, PLC and CaMKII-dependent signalling, transiently increased $[Ca^{2+}]_c$. At the basis of such an effect, there could be the CCK receptors cAMP/PKA- and PLC-dependent depletion of the thapsigargin-sensitive and IP3-dependent Ca^{2+} pool and $[Ca^{2+}]_c$ increase. In such signalling, the involvement of intracellular factors downstream of β_2 -adrenoreceptor activation would play a role in modulating the response of PCAEC to gastrin-17. Moreover, the activation of both NCX and PMCA by gastrin-17 would be implicated in restoring the basal $[Ca^{2+}]_c$.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0148>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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