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## Interplay between nitric oxide and VIP in CCK-8-induced phasic contractile activity in the rabbit sphincter of Oddi

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### INTRODUCTION

The sphincter of Oddi (SO) plays an important role in delivery of bile into the duodenum. In animals like rabbits, opossums and guinea pigs, the partially extraduodenal sphincter operates like a peristaltic pump that actively squeezes bile into the duodenum<sup>[1]</sup>. Thus, contraction and relaxation mechanisms are of equal importance in controlling normal sphincter function. Regulation of the relaxation function of this sphincter is mainly executed by non-adrenergic, non-cholinergic (NANC) nerves<sup>[2]</sup>. With regard to the neurotransmitters involved, evidence favors a role for vasoactive intestinal polypeptide (VIP)<sup>[2]</sup> and nitric oxide (NO) in various species including guinea pigs<sup>[3]</sup>, rabbits<sup>[4]</sup>, and humans<sup>[5]</sup>. In the rabbit SO the NANC relaxation is completely blocked by N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO-synthase<sup>[4]</sup>. The inhibitory effect of L-NAME can be reversed by concurrent incubation with L-arginine but not D-arginine indicating the response to be essentially nitrenergic. It is widely accepted that NO evokes smooth muscle relaxation through formation of cyclic GMP within muscle cells<sup>[6]</sup>. Nevertheless, NO has been shown to stimulate the release of VIP from enteric nerve terminals, an effect to enhance or contribute to its 'per se' relaxant effect<sup>[7]</sup>.

Much less is known about the influence of endogenous substances that promote the peristaltic pump-like sphincter function through generating phasic contractions. Cholecystokinin (CCK) is generally regarded as the major hormone regulating postprandial SO motility. CCK has been shown to initiate VIP and acetylcholine release at both pre- and post-junctional sites in enteric nervous system through CCK<sub>A</sub> receptors, whereas CCK<sub>B</sub> receptors seem to mediate NO release at post-junctional sites<sup>[8]</sup>. Nevertheless, the contribution of NO and VIP release to CCK-induced stimulation of phasic contractile activity of the SO has not been explored. The present work was therefore concerned with the possibility that an interplay between NO and VIP would contribute a significant degree to CCK-induced phasic contractions in the SO of rabbits.

### Abstract

**AIM:** The sphincter of Oddi (SO) plays an important role in delivery of bile into the duodenum. To establish whether vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) were involved in phasic contractile activity of the rabbit SO stimulated by cholecystokinin-octapeptide (CCK-8).

**METHODS:** Isolated SO muscle rings were cleaned of fat and mounted horizontally on two small L-shaped hooks one of which was connected to a force transducer for the measurement of isometric tension. The experiments were carried out in a thermostatically controlled (37±0.2 °C) organ bath (5 mL) containing Krebs solution. The organ fluid was gassed with 95% O<sub>2</sub> and 50 mL/L CO<sub>2</sub> to keep the pH at 7.40±0.05. Contractile responses to CCK-8 (1 µmol/L) were evaluated in the presence and absence of N<sup>G</sup>-nitro-L-arginine (L-NAME), an inhibitor of NO synthase (100 µmol/L), and (*p*-chloro-D-Phe<sup>6</sup>-Leu<sup>17</sup>)-VIP (VIPa, 30 µmol/L), a VIP receptor antagonist.

**RESULTS:** CCK-8 stimulated the phasic activity of the SO. NO synthase inhibition increased the frequency and amplitude of contractions with a slight increase in developed tension. Pre-incubation with VIPa also attenuated this CCK-8 effect. The combined application of L-NAME and VIPa abolished the phasic activity of the muscle rings with a marked increase in tension in response to CCK-8.

**CONCLUSION:** VIP and NO together contribute to an increase in phasic activity of SO.

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**Key words:** Sphincter of Oddi; CCK; VIP; NO; L-NAME

### MATERIALS AND METHODS

#### Ethics

The experiments in the present work conformed to European

Guiding Principles for Care and Use of Experimental Animals. In addition, the experimental protocol applied was approved by the local ethical board of University of Szeged and University of Debrecen, Hungary.

### Isometric tension measurements

These measurements were described in detail elsewhere<sup>[4]</sup>. Biliary SO muscle rings of approximately 6 mm in length from adult male New Zealand white rabbits weighing 3 500–4 000 g were prepared. The papilla Vateri was eliminated and the ampullary part of the muscle rings of approximately 3 mm in length were mounted horizontally on two small L-shaped glass hooks of which one was connected to a force transducer (SG-O2, Experimetria, Budapest, Hungary) attached to a six channel polygraph (R61 6CH, Mikromed, Budapest, Hungary) for measurement and recording of isometric tension as described<sup>[4]</sup>. One muscle ring was prepared from one animal. The experiments were carried out in an organ bath (5 mL) containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, MgSO<sub>4</sub> 1.0, KH<sub>2</sub>PO<sub>4</sub> 1.0, CaCl<sub>2</sub> 2.5, NaHCO<sub>3</sub> 25.0, glucose 11.1) which was maintained at 37 °C and aerated continuously with carbogen (50 mL/L CO<sub>2</sub> in oxygen, Ph. Eur. III.). The initial tension was set at 10 mN and the rings were allowed to equilibrate for over an hour. Cholecystokinin octapeptide (CCK-8, 0.01–10 µmol/L), atropine (1 µmol/L), tetrodotoxin (TTX, 1 µmol/L), (*p*-chloro-D-Phe<sup>6</sup>, Leu<sup>17</sup>)-VIP (VIPa, 30 µmol/L), and N<sup>G</sup>-nitro-L-arginine (LNNA, 100 µmol/L) were added directly to the organ bath in 50–150 µL volume.

### Experimental protocol

The muscle rings underwent brief experimental protocols as follows. Protocol 1: the preparations with a resting tension of 10 mN were exposed to cholecystokinin-octapeptide (CCK-8) (0.1 µmol/L, the EC<sub>50</sub> for the peptide in this preparation) subsequent to an equilibration period of over 60 min. After a stable contractile response was obtained, the preparations were washed until tension returned to previous baseline level. Protocols 2 and 3 were to study the effect of atropine and TTX (1 µmol/L for both) on CCK-8-induced increase in phasic activity. Atropine and TTX were applied either prior to CCK-8 or at maximum increase in phasic contractions, respectively. Protocols 4 and 5 were to study the effect of NO synthase inhibition (100 µmol/L LNNA) and neutralization of VIP action on CCK-8-induced responses. The preparations were pre-incubated with either

100 µmol/L LNNA or 30 µmol/L VIPa for over 20 min, and then CCK-8 (0.1 µmol/L) was given. In protocol 6, LNNA and VIPa were applied together prior to CCK-8. Except for protocol 3, baseline contractile patterns were re-gained subsequent to washout; in case of TTX, however, the decrease in baseline contractile amplitudes could not be overcome even by extensive washout.

### Drugs and chemicals

Atropine, TTX, LNNA, and CCK-8 were obtained from Sigma Chemical Company (St. Louis, USA). Polyclonal VIPa was a generous gift from Joseph Nemeth (Department Pharmacology, Medical University of Pecs). The compounds were dissolved in Krebs solution and added directly to the organ bath except for LNNA which was dissolved in ethanol and then diluted with Krebs solution.

### Data and statistical analysis

Parameters producing the data for evaluation were as follows. The *amplitude of contractions* (mN) was referred to as the difference between peak contractions and relaxations. The averages of the amplitudes were calculated for every minute (results are expressed as mean±SE, *n* = number of frequencies in a minute). The *frequencies of contractions* (cpm) were calculated for every minute. Statistical analysis was performed for every 10 min of the experiments using either Student's *t*-test (when the data consisted of two groups) or ANOVA (when three or more data groups were compared). Results were expressed as mean±SE, *n* = 5, *P* < 0.05 was accepted as statistically significant.

## RESULTS

### Effect CCK-8 on isolated SO motility

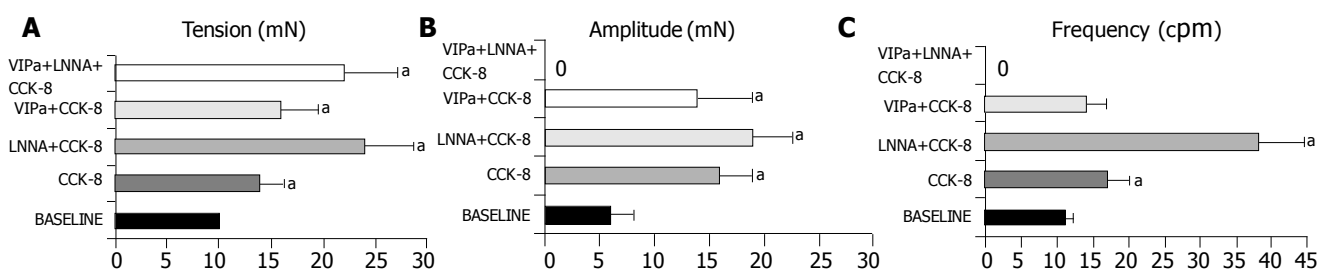
CCK (0.1 µmol/L) increased frequency and amplitude of contractions, and elevated the developed tension of the muscle rings (Figures 1A–C).

### Effect of atropine and TTX on CCK8-stimulated SO contractions

CCK-8-stimulated SO contractions were blocked by TTX (1 µmol/L). Atropine in the same concentration (1 µmol/L) also abolished agonist-induced contractile activity.

### Effect of LNNA and VIPa on CCK-8-stimulated SO motility

NO synthase inhibition by LNNA markedly increased the frequency and amplitude of contractions with a slight increase



**Figure 1** Effects of LNNA and VIPa on CCK-8-stimulated mechanical activity of rabbit SO *in vitro*. **A:** Summary of the effect of LNNA, VIPa and combined application of these substances on CCK-8-induced change of tension. Data are mean±SD, *n* = 6, \**P* < 0.05 vs baseline. **B:** Summary of the effect of LNNA, VIPa

and combined application of these substances on CCK-8-induced change of amplitudes. Data are mean±SD, *n* = 6, \**P* < 0.05 vs baseline. **C:** Summary of the effect of LNNA, VIPa and combined application of these substances on CCK-8-stimulated contractile frequency. Data are mean±SD, *n* = 6, \**P* < 0.05 vs baseline.

in developed tension. Pre-incubation with polyclonal VIPa attenuated each CCK-8 effect. Combined application of LNNA and VIPa significantly increased the tension of CCK-8, but abolished the phasic contractile activity of the muscle rings (Figures 1A-C). Separate application of LNNA and VIPa potentiated the effect of CCK-8-induced phasic contractions, while combined application of these substances completely abolished the phasic activity of the muscle rings with a marked increase in tension in response to CCK-8. These results indicated that VIP and NO together are mediators of the CCK-8-stimulated phasic contractions in rabbit SO.

## DISCUSSION

The results showed that the CCK-8-induced increase in phasic contractile activity characterized by an amplification of frequency of contractions superimposed on an increase in tension was augmented by NO synthase inhibition. The amplitude of contractions, exhibited only a tendency to increase when the effect of CCK-8 on rings pre-exposed to LNNA was studied. Neutralization of VIP yielded an increase in tension and a reduction of contractile amplitude with no effect on frequency of contraction. NO synthase inhibition together with neutralizing VIP completely blocked any phasic activity after CCK-8 with a substantial increase in tension i.e., this combined treatment converted the phasic activity stimulating effect of CCK-8 to a pure increase in tonic contraction.

As far as the mechanism of action of CCK on the SO is concerned, previously it has been postulated that CCK at least in the dog sphincter exerts the majority of its effect through stimulation of receptors on smooth muscle cells<sup>[9]</sup>. Nevertheless, results by Behar and Biancani<sup>[10]</sup> revealed that blockade of action potential propagation by TTX abolishes the inhibitory effect of CCK on sphincter mechanics converting its action to stimulation. This evokes the concept of the involvement of neural inhibitory mechanisms in the CCK effect that interact with stimulatory impulses targeted to smooth muscle receptors. Regarding neural inhibition on SO of rabbits, we have shown that it is primarily of nitrgenic origin<sup>[4]</sup>. NO besides producing smooth muscle relaxation may enhance the release of VIP, which in turn may further stimulate NO formation<sup>[7]</sup>. Moreover, CCK A receptors have been found to stimulate VIP and acetylcholine release from enteric nerve terminals at pre-and post-junctional sites, whereas CCK B receptors seem to elicit NO release at post-junctional sites<sup>[8]</sup>. The results that neither LNNA nor VIPa was able to block the phasic activity stimulating effect of CCK-8 but combined application of these substances resulted in conversion of CCK-8 effects to a full contractile one seem to support the assumption that an interplay between VIP and NO is of crucial importance in the development of phasic activity by CCK-8 in the rabbit SO. Moreover, since both TTX and atropine could block the effect of CCK-8, the majority of the effects of CCK-8 seem to be mediated by cholinergic impulses.

To the best of our knowledge this report is the first to describe that the integrity of neural nitrgenic and VIP-

mediated processes are pre-requisites for the ability of CCK-8 to stimulate sphincter motility. Since in rapacious animals, the peristaltic pump-like sphincter activity actively squeezes bile into the duodenum, and this sphincter activity is underlain by neurotransmitter release from either intrinsic or sensory neurons, it is not surprising that diseases that deteriorate these nerves impair sphincter function as well<sup>[11,12]</sup>. Of course, based on the present results it is not possible to estimate whether or not the neural responses participate in CCK-8-induced responses. However, that VIP and NO together comprise the principle neural effectors of relaxation interrupting tonic contractions evoked by CCK-8 in the rabbit SO seem to be supported by the data obtained. Similarly, the present work has not elucidated the precise role of cholinergic impulses implicated in either contractile or relaxant effects of CCK-8. Nevertheless, since similar to that seen with TTX, atropine also blocked the CCK-8 effects, thus it is possible that cholinergic nerves besides eliciting contractions may facilitate relaxation through releasing NO or VIP or other currently undefined relaxants.

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