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Pharmacological Investigations of the Cellular Transduction Pathways Used by Cholecystokinin to Activate Nodose Neurons

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Abstract

Cholecystokinin (CCK) directly activates vagal afferent neurons resulting in coordinated gastrointestinal functions and satiation. *In vitro*, the effects of CCK on dissociated vagal afferent neurons are mediated via activation of the vanilloid family of transient receptor potential (TRPV) cation channels leading to membrane depolarization and an increase in cytosolic calcium. However, the cellular transduction pathway(s) involved in this process between CCK receptors and channel opening have not been identified. To address this question, we monitored CCK-induced cytosolic calcium responses in dissociated nodose neurons from rat in the presence or absence of reagents that interact with various intracellular signaling pathways. We found that the phospholipase C (PLC) inhibitor U-73122 significantly attenuated CCK-induced responses, whereas the inactive analog U-73433 had no effect. Responses to CCK were also cross-desensitized by a brief pretreatment with m-3M3FBS, a PLC stimulator. Together these observations strongly support the participation of PLC in the effects of CCK on vagal afferent neurons. In contrast, pharmacological antagonism of phospholipase A₂, protein kinase A, and phosphatidylinositol 3-kinase revealed that they are not critical in the CCK-induced calcium response in nodose neurons. Further investigations of the cellular pathways downstream of PLC showed that neither protein kinase C (PKC) nor generation of diacylglycerol (DAG) or release of calcium from intracellular stores participates in the response to CCK. These results suggest that alteration of membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) content by PLC activity mediates CCK-induced calcium response and that this pathway may underlie the vagally-mediated actions of CCK to induce satiation and alter gastrointestinal functions.

Keywords

CCK; CCK1 Receptor; PLC; Signal Transduction; Vagal Afferent

Introduction

Nutrient induced release of cholecystokinin (CCK) from I cells in the small intestine acts to facilitate efficient gastrointestinal function and promotes the process of satiation (Gibbs et al., 1973). CCK-induced satiation is attenuated or abolished by surgical (Smith et al., 1985) or chemical (Ritter et al., 1985) ablation of the afferent vagus; consistent with a local

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paracrine activation of peripheral afferent terminals following release of CCK. Both binding and autoradiographic studies have demonstrated the presence of CCK receptors on vagal afferent nerves (Broberger et al., 2001; Corp et al., 1993; Lin et al., 1992). Studies with selective antagonists and knock-out models demonstrated these peripheral effects are specifically mediated by CCK1 receptors (CCK1Rs) (Li et al., 1997; Moran et al., 1994; Reidelberger et al., 2004; Whited et al., 2006).

In vitro, dissociated vagal afferent neurons are directly activated by CCK via CCK1Rs (Lankisch et al., 2002; Simasko et al., 2002). Brief exposure to CCK results in a rapid membrane depolarization, action-potential firing, and an elevation in cytosolic calcium (Lankisch et al., 2002; Simasko et al., 2003; Simasko et al., 2002); and this occurs primarily through an increase in membrane conductance (Peters et al., 2006a). A study with pharmacological manipulations demonstrated that these effects are mediated by increased activity of the vanilloid family of transient receptor potential (TRPV) cation channels, excluding TRPV1 (Zhao et al., 2010). While progress has been made in identifying the CCK sensitive conductances in vagal afferent neurons, little is known about the intracellular signal transduction pathway by which CCK1Rs translate ligand binding to channel opening at this important physiological site.

CCK1Rs are G-protein coupled receptors (GPCR) which associate with a complex array of signal transduction pathways. In pancreatic acinar cells, CCK1Rs couple through phospholipase C (PLC) (Matozaki et al., 1989; Tsunoda et al., 1993) and phospholipase A₂ (PLA₂) (Tsunoda et al., 1993; Tsunoda et al., 1995) to produce characteristic spike or oscillating calcium signals, respectively. PLC activation often leads to an subsequent increase in inositol trisphosphate (IP₃) and diacylglycerol (DAG) produced from phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis. Additional pathways that have been shown to be activated by CCK include phosphatidylinositol 3-kinase (PI₃K), phospholipase D, small G proteins, and non-receptor tyrosine kinases (Alcon et al., 2002; Williams et al., 2002). In neurons, a variety of coupling mechanisms have been established, but details differ in various neuronal cell types (Deng et al., 2010; Peters et al., 2006a; Rogers et al., 2008; Tsujino et al., 2005; Wu et al., 1994; Wu et al., 1996; Yang et al., 2006; Yang et al., 2007). Which elements of the transduction cascade are activated by CCK1Rs on vagal afferent neurons has not been established.

In the present study, we monitored intracellular calcium concentrations in dissociated vagal afferent neurons isolated from rat nodose ganglia. An advantage of the dissociated preparation is that it eliminates indirect actions that might occur *in vivo* or in a slice preparation via neighboring cells, and thus effects of pharmacological agents can be directly attributed to an action on the neuron under study. We examined the effects of various compounds, known to activate or block particular signaling pathways, on CCK-induced responses. We conclude from our observations that the CCK-induced calcium response involves PLC activation but is independent of subsequent activation of PKC, generation of DAG, or release of calcium from intracellular stores.

Experimental Procedures

Animals

Adult male Sprague-Dawley rats (220–240 g, Simonsen) were used for tissue collection in all experiments. Animals were housed in AAALAC accredited quarters under a 12:12-h light-dark cycle with *ad libitum* access to pelleted chow and water. The Washington State University Institutional Animal Care and Use Committee approved all procedures.

Dissociation and primary culture of nodose neurons

Methods for harvesting and dissociating rat nodose ganglia are as previously described (Simasko et al., 2003). Briefly, under deep anesthetic plane (ketamine 25 mg/100 g and xylazine 2.5 mg/100 g) both nodose ganglia were isolated from the rat under aseptic conditions. Ganglia were then combined and desheathed while in Hank's balanced-salt solution (HBSS), followed by enzymatic dissociation in 3 ml of digestion buffer (1 mg/ml dispase II and 1 mg/ml collagenase type Ia in calcium and magnesium free HBSS) for 120 min at 37°C. Dispersed neurons were then washed in HEPES-buffered Dulbecco's modified Eagle's medium (HDMEM), mechanically triturated, and plated onto poly-L-lysine-coated coverslips (treated with 200 µg/ml poly-L-lysine for 30 min). Neurons were maintained in HDMEM supplemented with 10% fetal calf serum at 37°C and 5% CO₂ until used in experiments. All experiments were performed within 48 hours of isolation.

Fluorescent calcium imaging

Intracellular calcium concentrations were monitored using ratiometric fluorescence measurements with the calcium indicator fura-2 AM (Molecular Probes, Eugene, OR). Image pairs (340 and 380 nm excitation, 510 emissions) were collected every 6 sec or 15 sec and analyzed using MetaFluor Software (Universal Imaging, West Chester, PA). The ratio of the fluorescence intensities were converted to calculated calcium concentrations using a calibration curve. Coverslips containing dissociated vagal afferent neurons were loaded with fura-2 AM (2 µM) for 45 min at room temperature (~22° C) followed by a 15 min wash at 37° C and then mounted onto a closed perfusion chamber. Chambers were continuously perfused with a physiological saline solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 6 glucose, 10 HEPES, pH adjusted to 7.4 with Tris-base) at room temperature; with drug solutions applied through a common manifold upstream of the perfusion chamber. At the end of each experiment neurons were briefly depolarized by application of a 55 mM potassium (Hi-K) solution (in mM: 90 NaCl, 55 KCl, otherwise same with physiological saline) to determine cell viability. Neurons were considered healthy and included in the final analysis if they responded to the Hi-K challenge with a rapid and reversible calcium response of at least 30 nM in amplitude. These control responses are not included in the figures presented.

Chemicals

Drugs used in this study are as follows (Name, Abbreviation): From Peptides International (Louisville, KY): Cholecystokinin-octapeptide (sulphated), CCK. From Sigma (St. Louis, MO): 1-[6-[[[(17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione, U-73122; 1-[6-[[[(17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione, U-73343; m-3M3FBS, m-3M; 2-aminoethoxydiphenyl borate, 2-APB; wortmannin, WMN. From Calbiochem (Gibbstown, NJ): arachidonyl trifluoromethyl ketone, AAC; palmityl trifluoromethyl ketone, PAC; bisindolylmaleimide I, Bis; phorbol 12-myristate 13-acetate, PMA. From Tocris (Ellisville, MO): N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride, H89; Edelfosine (ET-18-OCH₃), Edel; Chelerythrine, Chel; O,O'-[1,6-Hexanediylbis(iminocarbonyl)]dioxime cyclohexanone (RHC 80267), RHC. Initial stock solutions were made in DMSO except for CCK, Bis, Edel, Chel, H89 (made in distilled water), and 2-APB (made in 95% ethanol).

Data analysis and statistics

Calcium responses were calculated as the maximum change in the intracellular calcium concentration from the baseline level immediately preceding the drug challenge. Test responses to CCK with the presence of reagents under investigation are normalized to the

initial CCK responses, expressed as percentage of control, and presented as mean $\pm$ SEM. Paired T-tests were used to determine if an agent altered CCK-induced responses. $P < 0.05$ was considered significant.

Results

Complex effects of PLC inhibitors on calcium responses elicited by CCK

We first explored the role of PLC in the calcium responses to CCK (10 nM; standard challenge concentration for all experiments) with the use of U-73122, an aminosteriod that has been widely used as a direct inhibitor of PLC (Bleasdale et al., 1990; Smith et al., 1990). At 500 nM ($n = 5$) or lower concentrations (10 nM, $n = 4$; 50 nM, $n = 7$; and 100 nM, $n = 5$), U-73122 blocked (Fig. 1A) or reduced CCK-induced responses in 13 of 21 neurons (data not shown; a reduction in response greater than 30% of the control) but had minimal effects in others (less than 30% reduction; 8 of 21; representative trace not shown). At 50 nM, U-73122 reduced the responses to CCK to about 60% of control (averaged from 7 neurons; Fig. 1C). Unfortunately, U-73122 often activated neurons by itself. This occurred in $\sim 28\%$ of neurons (12 of 43 neurons, including both CCK responders and non-responders) at U-73122 concentrations $< 1 \mu\text{M}$ and was reversible (example shown Fig. 1A), but at $10 \mu\text{M}$, a concentration required for the maximum blockade of PLC (Bleasdale et al., 1990), U-73122 alone often elicited a robust calcium response (18 of 22 neurons, data not shown). Regardless of whether or not the neuron responded to U-73122, CCK-induced responses were always irreversibly abolished by these high concentrations of U-73122 (Fig. 1B). In addition, following exposure to $10 \mu\text{M}$ U-73122, neurons that were able to respond to Hi-K challenges before would not respond to Hi-K, suggesting a disruption of voltage-dependent calcium influx pathways. However, at lower concentrations of U-73122 ($< 1 \mu\text{M}$), Hi-K-induced calcium responses were always observed. On the other hand, U-73433, the inactive analogue of U-73122, did not significantly alter CCK-induced calcium responses (trace not shown; summary data in Fig. 1C) even at $10 \mu\text{M}$, the highest concentration tested (induced direct activations in $\sim 30\%$ of neurons). Together, the observed inhibition of the CCK-induced signal by submicromolar concentrations of U-73122 that do not cause non-specific effects provides strong evidence that activation of PLC is part of the pathway activated by CCK1Rs in nodose neurons.

Cross-desensitization between responses to CCK and m-3M

To further confirm the involvement of PLC in the actions of CCK in nodose neurons, we examined the cross-desensitization between responses to CCK and m-3M, a selective PLC stimulator (Bae et al., 2003; Horowitz et al., 2005). If CCK utilizes the same pathway that m-3M activates, we reasoned that calcium responses to CCK would be attenuated if the neurons were challenged with m-3M just prior to the CCK challenge due to desensitization of the pathway. We first performed pilot experiments to determine an appropriate concentration to use for m-3M. At concentrations at or greater than $25 \mu\text{M}$, m-3M caused calcium responses in 8 of 14 neurons that were sometimes irreversible. However, as previously reported (Bae et al., 2003), we found that at least $10 \mu\text{M}$ was required to get consistent calcium responses (data not shown). Thus we settled on a concentration of $15 \mu\text{M}$ for a standard challenge. At this concentration 16 of 29 neurons responded to m-3M, of which 9 neurons were also responsive to CCK. Of these 9 neurons, 6 had reversible responses to m-3M, and values from these 6 neurons were used in the summary data presented in Fig. 2.

We found that following a challenge with m-3M the response to CCK was significantly reduced when compared with the control response (CCK prior to m-3M). After additional time was given for the cells to recover there was an incomplete yet significant recovery (Fig.

2A and 2D). The reduced response to the second CCK challenge is not simply due to the excessive calcium influx elicited by m-3M because a similar cross-desensitization of CCK-induced responses was not observed with a Hi-K pretreatment (Fig. 2B and 2D). Finally, responses to repeated Hi-K challenges were not affected by m-3M (Fig. 2C and 2D), indicating that such cross-desensitization by m-3M is likely to be specific for PLC-involving events.

Involvements of PLA₂, PKA, and PI₃K pathways in calcium responses to CCK

Besides PLC, a variety of intracellular pathways including other phospholipases or protein kinases are activated by CCK in pancreatic acinar cells (Tsunoda et al., 1993; Tsunoda et al., 1995; Williams et al., 2002). Therefore, we further investigated the potential roles of these pathways in calcium signals elicited by CCK in nodose neurons. AAC (40 or 60 μ M), a PLA₂ inhibitor (Morioka et al., 2002), and its inactive analogue PAC (0.4 or 4 μ M), had no effect on CCK-induced responses (Fig. 3A and 3D). Responses to CCK were also unaltered at the presence of 1 μ M H89 (Fig. 3B and 3D), which potently inhibits PKA (Wang et al., 2007). Wortmannin (WMN) inhibits PI₃K at concentrations of 10 nM (Okada et al., 1994); however, at the concentrations we used (100 nM and 1 μ M), WMN never suppressed CCK-induced calcium responses (Fig. 3C and 3D), demonstrating that the PI₃K pathway is not important in CCK signaling.

Involvement of PKC in calcium responses to CCK

Since PLC activation often leads to activation of PKC through the production of DAG and Ca²⁺ mobilization, we next investigated the involvement of PKC by use of specific inhibitors and activators of this pathway. We found that two different blockers of PKC, Bis (0.1 or 1 μ M) (Hong et al., 2004) and Chel (10 μ M) (Ozcan et al., 2009), did not inhibit CCK-induced calcium responses (Fig. 4A-C). In fact, Chel, at the concentration used in this study, often induced a calcium response by itself, and even potentiated the response to CCK (Fig. 4B and 4C). The phorbol ester PMA, an agent commonly used to activate PKC, elicited calcium responses in both CCK-sensitive and CCK-insensitive neurons (Fig. 4D and 4F). There were also neurons which responded to CCK but were insensitive to PMA (Fig. 4E). The lack of an apparent correlation between CCK activation and PKC activation (Fig. 4G) further supported the conclusion from the antagonist study that CCK-induced activation does not require the participation of PKC.

Involvement of DAG and IP₃ in calcium responses to CCK

DAG and IP₃ are produced through PIP₂ hydrolysis downstream of PLC activation but upstream of PKC. To assess the roles of DAG and IP₃ in the calcium increase elicited by CCK, we used reagents that either disrupt the intracellular equilibrium of these messenger molecules or interrupt their intracellular signaling. RHC (20 μ M), a well-established reagent that prevents DAG hydrolysis through inhibition of DAG lipase (DAGL) (Liu et al., 2008), did not alter CCK-induced responses (Fig. 5A). Thus we conclude that CCK-induced responses are unlikely to depend on the generation of DAG. Furthermore, as we have previously reported (Zhao et al., 2010), 2-APB, a compound commonly used as an IP₃-R inhibitor, either had no effect on CCK-induced calcium responses, or actually augmented the response (summarized results shown in Fig. 5B). This finding, together with prior studies with the use of thapsigargin or calcium-free extracellular solutions (Simasko et al., 2002), demonstrates that calcium release from the intracellular store through IP₃ signaling is not the key pathway through which CCK elicited calcium increases.

Discussion

The transduction mechanism that CCK1Rs couple to in vagal afferent neurons has not been previously identified. The results presented here suggest that activation of PLC is an important component in the CCK-induced activation of these neurons, but that the pathway is unlikely to involve the downstream mediators such as PKC, DAG, or calcium release from the internal stores. In addition, we also found that several other potential pathways, specifically, PLA₂, PI₃K, and PKA, are also not involved. These results suggest that loss of membrane PIP₂ as a result of PLC activation is likely to participate in the response to CCK.

Involvement of PLC

The involvement of PLC in CCK-induced responses is supported by the observation that nanomolar concentrations of U-73122, a PLC inhibitor, produced a moderate but significant attenuation of CCK-induced calcium responses. However, at micromolar concentrations, U-73122 often elicited robust responses on its own and also inhibited another calcium influx pathway (voltage-dependent calcium influx) that we know is not coupled to CCK-induced responses (Zhao et al., 2010). Although U-73122 has been used for years as a pharmacological tool to assess the involvement of PLC in various biological processes (Bleasdale et al., 1990), an increasing number of studies have raised concerns about the non-specificity of this reagent. Such effects include depletion of PIP₂ upon long exposure, likely due to an inhibition of lipid kinases (Vickers, 1993), alkylation of associated proteins (Horowitz et al., 2005; Jin et al., 1994), direct activation of ion channels (Mogami et al., 1997), and morphological effects such as an enhancement of nuclear envelope permeability (Horowitz et al., 2005). A prior study using patch-clamp electrophysiology revealed that U-73122 releases intracellular calcium, potentiates IP₃-mediated calcium release and directly activates ion channels in mouse pancreatic acinar cells (Mogami et al., 1997). In another study using cultured dorsal root ganglia neurons, a peripheral afferent neuronal preparation similar to ours, Jin et al reported a total block of L-type calcium channels by U-73122 at micromolar concentrations (Jin et al., 1994). These authors concluded that U-73122 is not selective at concentrations required for a maximal block of PLC. Nevertheless, U-73122 at concentrations below 1 μ M often reduced or blocked the effects of CCK without any effects by itself or with only a modest reversible action to increase cellular calcium. In addition, U-73433, an analog of U-73122 that does not inhibit PLC, had only insignificant effects on calcium responses induced by CCK compared to the effects of U-73122. These observations provide evidence that PLC is involved in the actions of CCK to induce a calcium response in nodose neurons. However, due to the non-specificity of the effects of U-73122 that we observed, we cannot exclude the possibility that another pathway exists between the CCK receptor and calcium influx that does not go through PLC, but which is inhibited by micromolar concentrations of U-73122 in some manner.

A second line of evidence that supports the conclusion that PLC is involved in activation of vagal afferent neurons by CCK comes from an observed cross-desensitization between responses to CCK and m-3M, a PLC activator. Surprisingly, m-3M did not activate every neuron tested, even at 50 μ M, a maximal concentration for the activation of PLC by m-3M (Bae et al., 2003). If every neuron expressed an m-3M sensitive PLC isoform, this observation would indicate that PLC activation does not always result in a calcium response in every nodose neuron. Thus, in some neurons the critical calcium influx pathway must either be absent, or it must be segregated from PLC, such that activation of PLC does not induce an elevation in cytosolic calcium concentration. Nevertheless, that observed selective cross-desensitization between m-3M and CCK provides additional support for our conclusion that PLC is involved in the CCK-induced responses.

Involvement of mediators downstream of PLC

Our results demonstrate that in nodose neurons the common downstream PLC-activated pathways, PKC and the generation of IP₃ and subsequent release of intracellular Ca²⁺, are not involved in the responses to CCK. PKC inhibitors (Bis and Chel) had no effect on the response. Further, prior studies (Simasko et al., 2002) have demonstrated that in nodose neurons the Ca²⁺ response to CCK is dependent on extracellular Ca²⁺ and is not altered by depletion of intracellular stores with thapsigargin. In addition, inhibition of DAG lipase with RHC did not alter the CCK-induced response. If generation of DAG was a critical component in the response, one would have expected RHC to augment the CCK-induced response. We also found that 2-APB, a compound shown to inhibit particular isoforms of IP₃ receptors (Bootman et al., 2002), failed to inhibit the responses to CCK, further demonstrating that 2-APB-sensitive IP₃ receptors are not involved in the response to CCK. We did frequently observe a calcium signal induced by 2-APB alone, and only in neurons sensitive to 2-APB was the CCK-induced response enhanced by the presence of 2-APB. Previous findings have shown that 2-APB inhibits particular subtypes of TRPC channels (Ramsey et al., 2006) but activates TRPV1-3 (Hu et al., 2004). These observations support our prior conclusion that CCK1Rs activate nodose neurons through an action to augment the activity of TRPV channels but not TRPC channels (Zhao et al., 2010).

Non-PLC pathways

In other cell types CCK1Rs have been shown to couple to a multitude of pathways (Alcon et al., 2002; Tsunoda et al., 1993; Tsunoda et al., 1995; Williams et al., 2002). In dissociated nodose neurons we found no evidence to support the involvement PLA₂, PKA, and PI₃K in the actions of CCK. Via the use of the PKA inhibitor H89, Rogers and Hermann (2008) have suggested that PKA is important for CCK-induced responses at the central terminals of vagal afferent neurons. This observation differs from our finding in the present study and those of others who have examined the effects of H89 on the responses to CCK (Herness et al., 2002; Ma et al., 2006). However, a difference between our preparation and that of Rogers and Hermann (2008) is that they studied the central terminals of the afferent vagus, while we studied actions at the soma. The actions of CCK at central terminals are likely to involve modulation of presynaptic release events as opposed to initiating action potentials at a peripheral site represented in our preparation. Indeed, Yang and colleagues (Yang et al., 2006; Yang et al., 2007) have shown different coupling mechanisms for CCK in presynaptic and postsynaptic sites in the peri-aquiductal grey. Thus, it is not unreasonable that actions of CCK within the vagal system might involve different pathways and would be dependent upon which components are trafficked to the particular sites and/or the precise relationship of effectors relative to the receptors within the site.

Comparison of CCK1Rs-coupled pathways in nodose neurons to other neurons

While it is generally accepted that the cellular actions of CCK are mediated by PLC in neurons, inconsistent results are often found regarding the downstream pathways following the activation of PLC. A number of studies (Deng et al., 2010; Herness et al., 2002; Ma et al., 2006; Wu et al., 1994; Wu et al., 1996; Yang et al., 2006), by the use of thapsigargin, BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), or 2-APB, suggest that CCK receptors (CCK1Rs or CCK2Rs) couple through PLC to generate IP₃ which in turn releases internal stores of calcium. However, other studies have found CCK-induced responses to be extracellular calcium dependent (Tsujino et al., 2005; Yang et al., 2007), including our prior study on dissociated nodose neurons (Simasko et al., 2002). The involvement of PKC is also controversial. In dorsal root ganglia neurons or hippocampal slice preparations, PKC is required for the effects of CCK as demonstrated by the use of PKC inhibitors (Deng et al., 2010; Ma et al., 2006); whereas in substantia nigra dopaminergic neurons, the activation of PKC appears not involved in the generation of

CCK-induced cationic currents (Wu et al., 1994), a finding consistent with our present studies with activators and inhibitors of PKC. In short, these results from different preparations demonstrate that the transduction pathway CCK receptors utilize to activate a neuron is unique to the specific neuronal type under study.

An issue raised by these considerations is that perhaps within nodose neurons that innervate different targets there are differences. Prior findings suggest that most CCK responsive neurons likely innervate GI targets. For example, we previously reported that 70-75% of neurons labeled from the stomach or duodenum respond to CCK (Peters et al., 2006b). Powley and colleagues (1991) have reported that ~70% of vagal afferents arise from the GI tract. Thus, in a random selection of nodose neurons the overall response rate to CCK should be ~50% if all the targets in the GI tract have response rates similar to those observed in the duodenum and stomach, and all CCK responsive neurons arise from the GI tract. Since we typically observe 35-50% response rates to CCK in randomly selected nodose neurons, it would suggest that if a neuron responds to CCK, it is highly likely that it innervates a GI structure.

It is further possible that within this population there could be different coupling mechanisms. In terms of overall pathways, we have consistently observed that the CCK-induced response is virtually completely eliminated by removing extracellular calcium, and thapsigargin has little effect to reduce the responses (Simasko et al., 2002). If intracellular stores are involved in some responses but not others, then we would expect to see some cells sensitive to thapsigargin, and others resistant to removal of extracellular calcium, but we have not observed such subpopulations. This conclusion is further supported by our observations with Bis and Chel in which the response in the presence of inhibitors is ~100% of the control response. If a subpopulation existed in which the response was dependent on PKC activity, we would expect a decrease in the population response approximately equal to the portion of neurons that utilize this pathway. A similar conclusion can be drawn for the other pathways we examined (PKA, PI₃-K, and PLA₂).

On the other hand, we have found evidence that not every nodose neuron responds to CCK in exactly the same manner. For instance, U-73122, at sub-micromolar concentrations, had variable effects on nodose neurons. Similarly, in a prior study in which we concluded that the Ca²⁺ influx pathway activated by CCK involves TRPV channels (Zhao et al., 2010) we often observed incomplete inhibition of the response with the TRPV channel blocker ruthenium red. Further, our studies with La³⁺ (unpublished data) clearly demonstrate that CCK couples to multiple ionic pathways, likely to be different TRP channel subtypes. Whether these differences involve different targets within the GI tract, or are related to different sensory modalities, remains to be determined.

Conclusions

In summary, our findings are consistent with the conclusion that activation of dissociated vagal afferent neurons by CCK involves PLC, but does not require participations of PLA₂, PI₃K, PKA, or PKC. Additionally, presence of DAG or calcium release from intracellular stores also appeared to be of minimum impact on the CCK-induced response. We postulate that PIP₂ or other lipid mediators may play significant roles in the CCK-coupled signaling. In support of this hypothesis, we have previously presented evidence that calcium responses to CCK are mediated by members of the TRPV family (Zhao et al., 2010), and lipid regulation of TRP channels has emerged as a common mechanism shared by many subtypes (Rohacs, 2007; Rohacs, 2009; Voets et al., 2007). Therefore, it is likely that loss of membrane PIP₂, or other phosphoinositides, mediates CCK-induced activation by modulating TRPV channels; however, more direct tests of this hypothesis will be needed to confirm this conclusion.

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Fig. 1. Effects of U-73122 on calcium responses elicited by CCK

In this and all subsequent figures, applications of chemicals are indicated by the labeled horizontal bars above each trace. Example of challenge to CCK in presence of 100 nM U73122 is shown in panel **A**. In this neuron U73122 induced a transient calcium response by itself and completely, but reversibly, blocked the calcium response to CCK. Example of challenge to CCK in the presence of 10 μM U73122 is shown in panel **B**. In this neuron this high concentration of U73122 did not induce a calcium response by itself, but completely blocked the action of CCK. Summarized results are shown in panel **C**. Black bars represent control responses to CCK, and gray bars represent CCK-induced responses with the presence of the reagent under investigation. Results with 50 nM U73122 are from 7 neurons. Results with 10 μM U-73122 are from 5 neurons. Results with 10 μM U73433 (the inactive analogue of U-73122) are from 10 neurons. Values are expressed relative to the mean of the control response from all neurons within each group. *Statistically different from control, $P < 0.01$.

Fig. 2. Cross-desensitization of CCK-induced responses by pretreatment with m-3M

Representative traces are shown as follows: In **A** desensitization of responses to CCK following a treatment with m-3M (15 μ M); in **B** pretreatment with Hi-K (HiK) failed to desensitize the response to CCK; and in **C** m-3M failed to desensitize responses induced by Hi-K. Summarized data are shown in **D**. **Con** (black) is the response to the first challenge. **Test** (light gray) is the response to the compound of interest after recovery from the pretreatment with m-3M or Hi-K. **Rec** (dark gray) is the response to the compound at the end of the experiment. The control value for each group is the average of the response to the first challenge for all neurons within the group. Bars labeled **CCK/m-3M**: Pretreatment with m-3M significantly reduced responses elicited by CCK, which later had a significant level of recovery (n = 6). Bars labeled **CCK/HiK**: Pretreatment with the Hi-K solution did not significantly alter the response to CCK (n = 8). Bars labeled **HiK/m-3M**: The response elicited by Hi-K was statistically insensitive to pretreatments with m-3M (n = 7).

*Statistically different from indicated comparison, P < 0.01.

Fig. 3. Effects of PLA₂, PKA, and PI₃K blockade on CCK-induced calcium responses
Representative traces of CCK-induced responses in the presences and absence of various compounds. In **A**: AAC (40 μ M; PLA₂ inhibitor); in **B**: H89 (1 μ M; PKA inhibitor), and in **C**: WMN (100 nM; PI₃K inhibitor). **D**: Summary of responses to CCK in the presence of each blocker (normalized to the control value for each neuron. Number of neurons used in the averages are: **AAC** (n = 4); **PAC** (n = 5); **H89** (n = 8); and **WMN** (n = 10). None of the blockers had significant effects.

Fig. 4. Effects of PKC activator and inhibitors on CCK-induced calcium responses

PKC inhibitors Bis (0.1 or 1 μ M) and Chel (10 μ M) did not alter CCK-induced calcium responses. Representative traces of the CCK-induced response in the presence of Bis (1 μ M) and Chel are shown in **A** and **B**, respectively. Bis was without effect at both concentrations tested, thus results were pooled for analysis. In **C**: Summary of normalized responses to CCK in the presence of Bis (n = 10) and Chel (n = 6). In **D-F**: Examples of a neuron that was sensitive to both CCK and PMA (8 of 47), a neuron only sensitive to CCK (19 of 47), and a neurons only sensitive to PMA (2 of 47), respectively. A Venn diagram demonstrating the overlap of responsive neurons to PMA and CCK is shown in **G** (total area represents 47 neurons, other areas are scaled to respective number of neurons within each category).

*Statistically different from control, $P < 0.05$.

Fig. 5. Effects of RHC and 2-APB on CCK-induced calcium responses

In **A**: representative trace showing responses to CCK with and without 20 μ M RHC (DAGL inhibitor). In **B**: summary of normalized responses to CCK in the presence of RHC (n = 10) or 2-APB (IP₃-R inhibitor; n = 9). *Statistically different from control, $P < 0.05$.