Stimulation of the neurokinin 3 receptor activates protein kinase Cε and protein kinase D in enteric neurons

D. P. Poole,1,2 S. Amadesi,2 E. Rozengurt,3 M. Thacker,1 N. W. Bunnett,2 and J. B. Furness1

1Department of Anatomy and Cell Biology and Centre for Neuroscience, University of Melbourne, Parkville, Victoria, Australia; 2Departments of Surgery and Physiology, University of California, San Francisco, San Francisco, California; and 3Center for Ulcer Research and Education, Department of Medicine, University of California, Los Angeles, California

Submitted 9 November 2007; accepted in final form 22 February 2008

Poole DP, Amadesi S, Rozengurt E, Thacker M, Bunnett NW, Furness JB. Stimulation of the neurokinin 3 receptor activates protein kinase Cε and protein kinase D in enteric neurons. Am J Physiol Gastrointest Liver Physiol 294: G1245–G1256, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00521.2007.—Tachykinins, acting through NK3 receptors (NK3R), contribute to excitatory transmission to intrinsic primary afferent neurons (IPANs) of the small intestine. Although this transmission is dependent on protein kinase C (PKC), its maintenance could depend on protein kinase D (PKD), a downstream target of PKC. Here we show that PKD1/2-immunoreactivity occurred exclusively in IPANs of the guinea pig ileum, demonstrated by double staining with the IPAN marker Neu N. PKCε was also colocalized with PKD1/2 in IPANs. PKCε and PKD1/2 trafficking was studied in enteric neurons within whole mounts of the ileal wall. In untreated preparations, PKCε and PKD1/2 were cytosolic and no signal for activated (phosphorylated) PKD was detected. The NK3R agonist senktide evoked a transient translocation of PKCε and PKD1/2 from the cytosol to the plasma membrane and induced PKD1/2 phosphorylation at the plasma membrane. PKCε translocation was maximal at 10 s and returned to the cytosol within 2 min. Phosphorylated-PKD1/2 was detected at the plasma membrane within 15 s and translocated to the cytosol by 2 min, where it remained active up to 30 min after NK3R stimulation. PKD1/2 activation was reduced by a PKCε inhibitor and prevented by NK3R inhibition. NK3R-mediated PKCε and PKD activation was confirmed in HEK293 cells transiently expressing PKCε and green fluorescent protein-tagged PKCε, PKD1, PKD2, or PKD3. Senktide caused membrane translocation and activation of kinases within 30 s. After 15 min, phosphorylated PKD had returned to the cytosol. PKD activation was confirmed through Western blotting. Thus stimulation of NK3R activates PKCε and PKD in sequence, and sequential activation of these kinases may account for rapid and prolonged modulation of IPAN function.

Tachykinin receptors couple to the pertussis toxin-insensitive-G-proteins Gq/G11, the activation of which results in phosphoinositide hydrolysis, the production of inositol triphosphate and diacylglycerol (DAG), and the activation of protein kinase C (PKC) (19). The receptors can also couple to Gs and Gq/G11 (4, 55). However, because phospholipase C and PKC inhibition block the depolarization of enteric neurons to the NK3R agonist senktide (4), the predominant effects are concluded to be mediated through Gq/G11.

A role of protein kinase D (PKD, also known as PKCμ) in these changes cannot be excluded, since PKD is also activated by DAG analogs (47) and its activity can be suppressed by PKC inhibitors, both because PKD is commonly downstream of PKC (35, 38) and because certain PKC inhibitors also inhibit PKD (52). PKD, together with PKCε, can phosphorylate the transient receptor potential/vanilloid channel of nociceptive neurons, resulting in hyperalgesia to thermal stimuli (5, 53). A similar role of PKD in controlling IPAN excitability is of particular interest, since this kinase is capable of sustained activation and of amplifying receptor-mediated signals (26). PKD could thus play an integral role in mediating the increased IPAN excitability associated with the sEPSP or sustained slow postsynaptic excitation elicited in response to low-frequency stimulation of interganglionic connectives (6, 32). Long-term increases in IPAN excitability may have a role in the pathological alterations in gastrointestinal function that occur after the gut is inflamed (22, 23). However, nothing is known about the localization and regulation of PKD in the enteric nervous system.

The three major tachykinin (neurokinin) receptors, NK1R, NK2R, and NK3R, are differentially expressed throughout the gastrointestinal tract. The principal endogenous tachykinins of enteric neurons are substance P and neurokinin A, neurokinin B being absent from enteric neurons (14). A major functional role of the NK2R in the gastrointestinal tract is to mediate noncholinergic slow excitatory postsynaptic potentials (sEPSPs) in myenteric intrinsic primary afferent neurons (IPANs) of the guinea pig ileum, thereby augmenting the excitability of these neurons (4). Similar transmission occurs in other regions of the gut, including the stomach, duodenum, and gallbladder (29, 42), suggesting a general functional role of NK2R in the enteric nervous system. Although NK3R has been shown to be involved in generating sEPSPs, NK3R antagonists fail to alter peristaltic contractions of the guinea pig small intestine (16). It has been suggested that NK3R may only be functionally significant under conditions where the gut is stressed, such as “supraphysiological” stimuli or localized inflammation to bacterial toxins (40). Thus NK3R may mediate altered gut functions in pathology (40). However, the mechanisms by which the NK3R regulates excitability of enteric neurons are unknown.

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In the present study, we characterized the distribution, translocation, and activation of PKD in myenteric neurons of the guinea pig ileum. We report that the neuronal expression of PKD1/2 is restricted to IPANs and that PKD1/2 is activated...
PKD in the Enteric Nervous System

Table 1. Antibodies and neuronal labels used in this study, with respective concentrations

<table>
<thead>
<tr>
<th>Targets</th>
<th>Host Species (for Antibodies)</th>
<th>Source or Reference</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin</td>
<td>Rabbit</td>
<td>Swant</td>
<td>1:1000</td>
</tr>
<tr>
<td>GGC8</td>
<td>Rabbit</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Isocitric B4-FITC</td>
<td></td>
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<td>1:400</td>
</tr>
<tr>
<td>NPY</td>
<td>Sheep</td>
<td>9</td>
<td>1:400</td>
</tr>
<tr>
<td>Porin/VDAC1</td>
<td>Mouse</td>
<td>Abcam</td>
<td>1:500</td>
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<td>Rabbit</td>
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<td>1:200</td>
</tr>
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<td>Phosphoserine (PKC substrate)</td>
<td>Rabbit</td>
<td>CST</td>
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</tr>
<tr>
<td>PKD1/2 (PKCε)</td>
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<td>Santa Cruz</td>
<td>1:200</td>
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<td>Rabbit</td>
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<tr>
<td>PKD phosphorylated at Ser744/748</td>
<td>Rabbit</td>
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<td>1:250</td>
</tr>
</tbody>
</table>

Suppliers were Abcam, Abcam, Cambridge, MA; BD Biosciences (BD), North Ryde, NSW, Australia; Chemicon, Chemicon International, Temecula, CA; Cell Signaling Technology (CST), Beverly, MA; Sigma-Aldrich Biotechnology (Santa Cruz), Santa Cruz, CA; Sigma-Aldrich, Sydney, Australia and St. Louis, MO; Swant, Bellinzona, Switzerland; Upstate Biotechnology (Upstate), Charlottesville, VA.

through a PKCe-dependent mechanism in response to NK3R activation. We confirmed coupling of NK3R with PKCe and PKD1–3 using transfected cell lines.

MATERIALS AND METHODS

Preparation of intestinal segments. Guinea pigs of either sex (180–250 g) were stunned by a blow to the head and killed by severing their carotid arteries and spinal cord. All procedures conformed to National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. Every effort was made to minimize the number of animals used. Segments of distal ileum were severed their carotid arteries and spinal cord. All procedures conformed to National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. Every effort was made to minimize the number of animals used. Segments of distal ileum were removed and placed in physiological saline (in mM: 118 NaCl, 4.8 KCl, 25 NaHCO3, 1.0 NaH2PO4, 1.2 MgSO4, 11.1 d-glucose, 2.5 CaCl2) containing nicardipine (1 μM, Sigma-Aldrich, Sydney, Australia) to inhibit tissue contraction. Segments were cleaned of contents, opened along the mesenteric attachment, and pinned flat, mucosa down under moderate tension, onto silicone elastomer-lined culture dishes. Preparations were allowed to equilibrate in physiological saline (37°C, bubbled with 95% O2-5% CO2) for at least 30 min before experiments began. Preparations were then exposed to drugs before treatment was terminated by using ice-cold fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.2) followed by overnight fixation at 4°C.

Immunostaining of myenteric neurons. Preparations were cleared of fixative and stored as described (34). External muscle-myenteric plexus whole mounts were prepared and labeled for proteins of interest by indirect immunofluorescence (34). In brief, whole mounts were incubated in 10% normal horse serum plus 1% Triton X-100 in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) for 30 min prior to incubation with primary antibodies for 48 h at 4°C. Details of the antisera and markers used in the present study are summarized in Table 1. Whole mounts were washed (3 × 10 min, PBS), then incubated with secondary antibodies (1 h, room temperature). Preparations were washed (3 × 10 min in PBS) and mounted (Dako, Carpinteria, CA). Preparations were analyzed by confocal microscopy on a Bio-Rad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan2 microscope. Single 1,024 × 1,024 pixel images (nominal 0.5-μm optical section thickness) were captured (×63 objective, NA 1.4). Single optical sections are shown, and the contrast and brightness of all images have been adjusted by use of CorelDraw X3 and Corel PhotoPaint software.

Image analysis. Translocation was defined as a shift in protein kinase immunoreactivity from the cytoplasm to another subcellular site, especially the plasma membrane. The subcellular distributions of PKCe-immunoreactive (IR) and phospho-PKD916-IR were quantified from captured confocal images using ImageJ software (http://rsb.info.nih.gov/ij/) as described (31). Analysis of PKCe translocation was restricted to IPANs, which were identified by using the IPAN-specific markers calbindin, isocitric B4 (IB4), or NeuN (13, 17, 49). Nuclear fluorescence was used to set the background threshold for quantifying specific staining, since no nuclear PKCe-IR or phospho-PKD916-IR was detectable. Pixels were designated as either above threshold (255) or below threshold (0). Fluorescence in the total cell and cytoplasm was measured by counting the number of pixels above threshold. The outlines of cells and the plasma membrane were identified visually, aided by using neurochemical markers to indicate the periphery of the neurons analyzed. The total cell was defined as the number of positive pixels within the outline of the cell. The counting area was set inside the membrane to measure the number of positive pixels within the cytoplasm. The membrane fluorescence was calculated by subtracting the cytoplasmic pixel counts from the total cell pixel count. The membrane-associated fluorescence was expressed as a ratio relative to the cytoplasmic fluorescence. The percentage of phospho-PKD916-positive pixels relative to total pixels was calculated for the combined membrane and cytoplasm. This was defined as the total phospho-PKD916 produced in the cell.

Analyses were made from preparations from three or more animals with >30 neurons analyzed per treatment. Differences between

Fig. 1. Localization and translocation of protein kinase D (PKD) 1/2 in myenteric intrinsic primary afferent neurons (IPANs), double stained for PKD1/2 and NeuN. A and A': cytoplasmic PKD1/2 was detected in the majority of neurons with cytoplasmic NeuN (NeuNcyt)-immunoreactive (IR) (IPANs; arrow) but was not found in other types of enteric neurons. About 15% of NeuNcyt-IR neurons were not immunoreactive for PKD1/2; an example is indicated by the arrow with asterisk. B and B': PKD1/2 translocated from the cytoplasm to the plasma membrane of IPANs in response to phorbol 12,13-dibutyrate (PDBu, 100 nM, 10 min; arrows). PKD1/2 immunoreactivity was also detected in smooth muscle cells (asterisk), particularly in close apposition to the muscle cell nucleus. Scale bar = 50 μm.
treated and control groups were determined by one-way ANOVA comparisons using Minitab 13 software (Minitab, Sydney, Australia), with $P$ values of $\leq 0.05$ and $\leq 0.01$ deemed as statistically significant at the 95 and 99% confidence levels, respectively.

**Cell lines and treatments.** HEK-FLP cells (Invitrogen, Carlsbad, CA) were maintained in DMEM, 10% FBS, and Zeocin (100 µg/ml). Cells were transiently transfected with 1–2 µg of PKD1, -2, or -3 coupled to green fluorescent protein (GFP) or with PKCe-EGFP using Lipofectamine 2000 (Invitrogen). Cells were also cotransfected with NK3R. At 48 h after transfection, cells were incubated in low-serum medium (0.5% FBS) for 16–18 h and then treated with senktide (1 µM) or vehicle for various times before immunostaining or Western blotting. The human PKD1, -2, and -3-GFP transcripts have been described (25, 36, 37), as has the rat NK3R with NH2-terminal FLAG epitope (43). Human PKCe-EGFP transcript was a gift from Dr. Daria Mochly-Rosen (Stanford University) (41).

**Immunostaining of cell lines.** Cells were washed, fixed with 4% paraformaldehyde (20 min, 4°C), and incubated with PBS, 1% normal goat serum, and 0.1% saponin (30 min). Cells were incubated with the following primary antibodies: anti-phospho-PKD916 or anti-phospho-PKD744/748, and anti-FLAG (16–18 h, 4°C; Table 1). Cells were washed and incubated with secondary antibodies (1 h, room temperature). Cells were mounted and observed by using a Zeiss Axiovert and LSM 510 META laser scanning microscope with Zeiss Plan Apo ×100 (NA 1.3) objective. Images were collected at a zoom of 1.2, colored to represent the appropriate fluorophores, and processed to adjust contrast and brightness using Adobe Photoshop CS2 9.0 (Adobe Systems, Mountain View, CA).

**SDS-PAGE and Western blotting.** Cells were washed and lysed in 50 mM Tris·HCl (pH 7.4, 50 mM NaF, 1 mM Na3VO4, 1% SDS, and protease inhibitors), boiled, and centrifuged. Lysates (40 µg) were separated by SDS-PAGE (8% acrylamide), transferred to polyvinylidene difluoride membrane (Immobilon FL, Millipore), and incubated with blocking buffer (LI-COR, Lincoln, NE). Membranes were incubated in blocking buffer with primary antibodies anti-phospho-PKD744/748 (1:1,000) or anti-phospho-PKD916 (1:1,000) and anti-GFP (1:10,000) (overnight, 4°C). Membranes were washed and then incubated with secondary antibodies coupled to either Alexa Fluor 680 or IRDyeTM 800 (1:10,000, 1 h, room temperature). Immunoreactive proteins were viewed by using an Odyssey infrared imaging system (LI-COR). For densitometric analysis, the phospho-PKD744/748 signal and the GFP signal (used as loading control) were calculated, expressed as a ratio, and compared with unstimulated cells (0 min).

**Materials.** The following pharmacological agents were used: phorbol 12,13-dibutyrate (PDBu) (Sigma-Aldrich), ingenol 3,20-dibenzoate (IDB; LC Laboratories, Woburn, MA), calphostin C (Wako, Osaka, Japan), myristoylated PKCe translocation inhibitor peptide (PKCeI; N-myristoyl-EAVSLKPT; BioMol Research Laboratories), phosphatase inhibitor cocktail, (Sigma-Aldrich), SB-235375 (12) (kindly supplied by Dr. Gareth Sanger, GlaxoSmithKline Laboratories, Harlow, UK), senktide (Auspep, Parkville, Australia, and Calbiochem, La Jolla, CA), and tetrodotoxin (Alomone Laboratories).

Fig. 2. PDBu-evoked PKD phosphorylation and PKCe translocation in myenteric IPANs. A and A’; PKCe-IR was cytoplasmic, and activated PKD phosphorylated at serine916 (phospho-PKD916) was absent in untreated controls (arrows). B and B’; exposure to PDBu (100 nM, 10 min) resulted in prominent phospho-PKD916 labeling at the plasma membrane of IPANs, identified by their cytoplasmic NeuN-IR (arrows). C and C’; PKD activation by PDBu was always associated with PKCe translocation to the plasma membrane. PKD activation in response to PDBu was dependent on the time of exposure (D) and the concentration of PDBu (E). Scale bar = 50 µm; histograms, means ± SE, $n = 7$ animals.
PKD is localized to IPANs of the myenteric plexus. The distribution of PKD-IR was examined in longitudinal muscle-myenteric plexus whole mounts, by use of an antibody that recognizes both PKD1 and PKD2 (PKD1/2). PKD1/2-IR was detected in a subset of myenteric neurons and in intestinal and vascular smooth muscle (Fig. 1). Labeling was prominent in the cytoplasm of large myenteric neurons, but no immunoreactivity was associated with the nuclei or axons of these neurons (Fig. 1A). Almost all neurons that expressed PKD1/2 (99 ± 1%, means ± SE, 111 neurons from 4 animals) also showed cytoplasmic NeuN-IR (NeuNcyt-IR), confirming their identity as IPANs (49). Conversely, most NeuNcyt-IR neurons (Fig. 1, A and A'), indicating that a large proportion of this functional subclass of neuron expresses PKD. Since practically all PKD1/2-IR neurons were identified as IPANs, no double labeling with neurochemical markers for other functional subtypes of myenteric neuron was necessary. Smooth muscle cells also expressed PKD1/2-IR. Immunoreactivity was largely localized in close apposition to the nucleus of these cells, possibly associated with the Golgi apparatus (Fig. 1B). No detectable PKD1/2-IR was observed in interstitial cells of Cajal of the myenteric region or in enteric glia. These results indicate that the neuronal expression of PKD1/2 is restricted to a single functional subclass of myenteric neuron. This restricted expression may reflect a specific functional role for this kinase in the enteric nervous system.

DAG analogs promote membrane translocation of PKD in IPANs. Since PKD translocates in response to DAG and DAG analogs (25), we examined the effects of DAG analogs on the subcellular distribution of PKD1/2 in myenteric IPANs. Exposure to phorbol or ingenol esters resulted in the translocation of PKD1/2 from the cytoplasm to the plasma membrane of NeuNcyt-IR neurons (Fig. 1, B and B'). This movement was dependent on time and concentration. The association of PKD1/2 with the plasma membrane was observed at a PDBu concentration of 10 nM and was maximal at 1 μM. Translocation was first noticeable within 2–5 min of initial PDBu exposure. No association of PKD1/2 with the nucleus was observed. These results indicate that PKD1/2 is targeted to the plasma membrane of IPANs in response to increased DAG.

DAG analogs activate PKD in IPANs. PKD is activated in a DAG-dependent manner (27). Activation of PKD1/2 in response to DAG analogs was analyzed in IPANs with respect to location, time, and concentration. We used an antiserum specific for PKD phosphorylated at serine916 (phospho-PKD916) to assay PKD1/2 activation in myenteric neurons, because phosphorylation of PKD at serine916 is indicative of PKD autophosphorylation and activation (27, 28). In unstimulated preparations (control), phospho-PKD916-IR was undetectable in neurons, including those immunoreactive for PKCe (Fig. 2, A and A'). This is consistent with previous reports that used isolated cells (25, 26). PDBu induced prominent membrane-associated phospho-PKD916 labeling. Phospho-PKD916-IR was only observed in NeuNcyt-IR IPANs of the myenteric plexus (122 neurons from 7 animals; Fig. 2, B and B'), consistent with PKD1/2 expression. An accurate determination of the proportion of NeuN-positive neurons that were also phospho-PKD916-IR was not possible because some neurons were very weakly stained. However, some NeuN-IR IPANs did not express detectable PKD1/2-IR (Fig. 1, A and A'). Membrane

![Phospho-PKD916](image)

**Fig. 3.** PKD activation in myenteric IPANs is a PKCe-dependent process. A and A': treatment with ingenol 3,20-dibenzoate (IDB, 1 μM, 10 min) resulted in the translocation of PKCe and activation of PKD. B and B': both the translocation of PKCe and the activation of PKD were significantly reduced by PKCe-specific inhibition (PKCeI, 1 μM). C: quantitation of the ratio of membrane associated to cytoplasmic phospho-PKD916 in control, after IDB, and after IDB plus PKCeI (1 μM). Values are means ± SE, n = 5 animals. **P < 0.001. Scale bar = 50 μm.**
association of phospho-PKD$^{916}$-IR in PDBu-treated preparations was always associated with PKCe translocation to the plasma membrane (Fig. 2C and C'). PDBu (1 μM) activated PKD in a time- and concentration-dependent manner. Phospho-PKD$^{916}$-IR was first detected at 5 min, was maximal at 10 min, and was not significantly reduced after 30 min (Fig. 2D). PKD1/2 activation was dependent on PDBu concentration. The initial induction of phospho-PKD$^{916}$ production was observed at 100 nM PDBu, and activation was maximal at 1 μM PDBu (10 min exposure period; see Fig. 2E). Phospho-PKD$^{916}$ production was prevented by calphostin C (1 μM), a specific PKC inhibitor (11). Thus PKD1/2 is activated at the plasma membrane of IPANs in response to DAG analogs.

PKCe mediates PKD activation. Although the activation of PKD is not dependent on phosphorylation by any one specific PKC isoform, preferential activation of PKD through a novel PKC (nPKC)-dependent mechanism has been suggested (31). Treatment with the nPKC activating ingenol ester, IDB, resulted in marked phospho-PKD$^{916}$ immunolabeling in IPANs (Fig. 3, A and A'). This effect was significantly attenuated after preincubation with a PKCe-inhibitor peptide (PKCeI, 1 μM, 20 min preincubation, Fig. 3, B, B', and C). Phospho-PKD$^{916}$ production was always associated with a concomitant translocation of PKCe. Phospho-PKD$^{916}$ immunoreactivity was completely colocalized with PKCe (100%, 137 neurons from 10 animals). The concentration and time dependency of PDBu- and IDB-evoked PKCe translocation in myenteric IPANs has previously been characterized (31). Treatment with either PDBu or IDB gave rise to prominent PKC-dependent phosphorylation (phosphoserine-IR) at the plasma membranes of myenteric neurons, including NeuNcyt-IR IPANs. Together, these findings indicate that PKD1/2 is activated in a PKCe-dependent manner in IPANs.

GPCR coupling to PKCe and PKD. IPANs express several GPCR types, many of which activate PKC (10). Agonists of several GPCRs were screened to determine whether the receptors couple to PKCe and PKD in myenteric IPANs. Treatment with carbachol (10 μM, 10 min), histamine (10 μM, 5–10 min), 5-hydroxytryptamine (10 μM, 5–10 min), or PGE$_2$ (10 μM, 2–10 min) did not induce PKCe translocation or phospho-PKD$^{916}$ production. ATP (1 μM, 1 min) stimulated the translocation of PKCe from the cytoplasm to the plasma membrane of a subset of myenteric neurons. This change in distribution was less pronounced after a 5-min treatment and largely absent after 10 min, suggesting that it was a transient, receptor-mediated, effect. All neurons that responded to ATP were immunoreactive for nitric oxide synthase (NOS; 33 neurons from 3 animals). No phospho-PKD$^{916}$-IR was detected in these neurons, consistent with the localization data showing that PKD1/2 is not expressed in inhibitory motor neurons and descending interneurons. Treatment with protease-activated receptor 2 activating peptide (SLIGRL-NH$_2$, 50 μM, 2–10 min) resulted in the weak production of phospho-PKD$^{916}$ in a few large myenteric neurons. All phospho-PKD$^{916}$-IR neurons in preparations treated with activating peptides also exhibited increased PKCe association with the plasma membrane.

NK$_3$R activates PKCe and PKD in myenteric neurons. The NK$_3$R-selective peptide agonist, senktide (1 μM, 10 s to 10 min) resulted in the translocation of both PKCe and PKD and the production of phospho-PKD$^{916}$-IR in myenteric neurons.

PKCe translocation. The neuron types exhibiting PKCe translocation were identified by using neurochemical markers

Fig. 4. PKCe translocation in response to NK$_3$ receptor activation (senktide, 1 μM, 10 s) occurred in different neurochemically identified subtypes of myenteric neuron; preparations double-stained for PKCe and specific markers. A and A': PKCe translocation was detected in isoleucin B4 (IB4)-positive IPANs (arrows). B and B': PKCe translocation in nitric oxide synthase (NOS)-IR neurons was restricted to a subpopulation of smaller diameter neurons (arrows), whereas PKCe-IR was generally associated with the Golgi apparatus in the larger diameter NOS-IR neurons (arrow with asterisk). C and C': PKCe translocation was observed in calretinin-IR neurons. D and D': neurons that were strongly immunoreactive for neuropeptide Y (NPY) showed translocation in response to senktide. Scale bar = 50 μm.
for the major functional classes of myenteric neurons. PKCε translocation was detected in calbindin-IR and IB4-positive IPANs (Fig. 4, A and A’), NOS-IR inhibitory interneurons and motor neurons (Fig. 4, B and B’), calretinin-IR ascending interneurons and motor neurons (Fig. 4, C and C’), and neuropeptide Y (NPY)-IR secretomotor neurons (Fig. 4, D and D’). Some large NOS-IR neurons did not exhibit PKCε translocation: these were not identified with other markers and we

![Image of PKCε translocation and PKD activation in myenteric neurons in response to NK1 receptor activation by senktide (1 μM). A and A’: PKCε-IR was cytoplasmic and PKD was not phosphorylated in untreated controls. B and B’: prominent PKCε labeling and PKD activation (phospho-PKD916) occurred at the plasma membrane within 15 s. C and C’: After 2 min, both PKCε-IR and phospho-PKD916 returned to the cytoplasm. D and D’: PKD activation was prolonged and phospho-PKD916-IR was still detectable after 10 min. PKCε translocation was time (10 s–10 min; E) and concentration (0 nM–1 μM; F) dependent. G: PKD activation was similarly time dependent. PKD activation occurred at the plasma membrane from 10 s to 1 min. H: PKD activation was sustained; maximal total activated PKD was detected after 2 min. Values are means ± SE, n = 3 animals; *P < 0.05; **P < 0.001, compared with no senktide exposure. Scale bar = 50 μm.

Fig. 5. Time course and concentration sensitivity of PKCε translocation and PKD activation in myenteric neurons in response to NK1 receptor activation by senktide (1 μM). A and A’: PKCε-IR was cytoplasmic and PKD was not phosphorylated in untreated controls. B and B’: prominent PKCε labeling and PKD activation (phospho-PKD916) occurred at the plasma membrane within 15 s. C and C’: After 2 min, both PKCε-IR and phospho-PKD916 returned to the cytoplasm. D and D’: PKD activation was prolonged and phospho-PKD916-IR was still detectable after 10 min. PKCε translocation was time (10 s–10 min; E) and concentration (0 nM–1 μM; F) dependent. G: PKD activation was similarly time dependent. PKD activation occurred at the plasma membrane from 10 s to 1 min. H: PKD activation was sustained; maximal total activated PKD was detected after 2 min. Values are means ± SE, n = 3 animals; *P < 0.05; **P < 0.001, compared with no senktide exposure. Scale bar = 50 μm.
PHOSPHATASE INHIBITORS. Initial phospho-PKD916 production was 10–30 min after senktide addition, even in the absence of treatment (Fig. 5, time and was exclusively cytoplasmic within 2 min after Phospho-PKD916 production was observed in a subset of neu- relative to cytoplasmic labeling, is summarized in Fig. 5 proved too difficult to quantify.}

The translocation of PKCe from the cytoplasm to the plasma membrane after treatment with senktide was rapid and transient (Fig. 5). PKCe translocation occurred in IPANs and other functional classes of myenteric neurons. PKCe was predominantly associated with the plasma membrane within 10 s of NK3R activation. There was a continued association with the plasma membrane (10 s to 1 min; Fig. 5B and B’), followed by a complete return to the cytoplasm (2 min; Fig. 5C and C’). Between 2 and 10 min after treatment, PKCe was associated with vesicle-like structures within the cytoplasm (Fig. 5, D and D’). Double labeling of these preparations using antisera against PKCe and porin/VDAC1, a mitochondrial marker (56), showed no colocalization between PKCe and mitochondria. Colocalization between PKCe and IB4 was evident in IPANs, suggesting that the target of PKCe translocation was the Golgi apparatus (45). This was confirmed through double labeling with a specific marker of the trans-Golgi network, GCC88 (24) (not shown).

On neurochemically identified IPANs, significant transloca- tion of PKCe occurred between 10 s and 1 min after NK3R activation (Fig. 5E). The ratio of membrane-associated to cytoplasmic PKCe did not differ significantly at 2 and 10 min after NK3R activation (Fig. 5E). PKCe translocation was dependent on senktide concentration (30-s exposure period). Statistically significant PKCe translocation was first detected at 10 nM and maximal PKCe translocation occurred at 1 μM senktide (Fig. 5F).

PKD activation. Phospho-PKD916-IR was detected at the plasma membrane of IPANs after exposure to senktide (1 μM). This membrane association was most prominent at 10–30 s, and phospho-PKD916 was completely cytoplasmic by 2 min. Phospho-PKD916 production was observed in a subset of neu-rons: double labeling of senktide-treated (2 min) preparations with NeuN showed that all phospho-PKD916-IR neurons were IPANs (100%, 196 neurons from 10 animals). However, only a subpopulation of IPANs exhibited PKD activation (69 ± 9%, 284 neurons from 10 animals), consistent with the distributions of NK3R and PKD.

Activation of PKD1/2 was most prominent after a 2-min exposure period and was enhanced by phosphatase inhibition. Phospho-PKD916-IR was first detected after 10 s and was maximal by 2 min. Phospho-PKD916-IR was still present 10–30 min after senktide addition, even in the absence of phosphatase inhibitors. Initial phospho-PKD916 production was first observed at the plasma membrane within 10 s after treatment. Maximal association with the plasma membrane occurred within 15 s of senktide addition (Fig. 5, B and B’). Phospho-PKD916 labeling was increasingly cytoplasmic over time and was exclusively cytoplasmic within 2 min after treatment (Fig. 5, C and C’). The time-dependent shift in the ratio of phospho-PKD916-IR associated with the membrane, relative to cytoplasmic labeling, is summarized in Fig. 5G.

Quantitative analysis of pixels positive for phospho-PKD916 relative to total pixels indicated that maximal PKD activation occurred within 2 min of the initial stimulus (Fig. 5H). PKD activation was prolonged and prominent labeling was detectable at 10 min (Fig. 5, D’ and H) and up to 30 min after...
senktide was added (29 ± 2% of total pixels were positive, 30 neurons from 3 animals).

There was a direct relationship between the total amount of activated PKD and PKCε translocation. Phospho-PKD916 production at the plasma membrane closely paralleled PKCε translocation in that maximal membrane PKD1/2 activation occurred slightly after maximal PKCε translocation (15 s compared with 10 s). All neurons with membrane associated phospho-PKD916-IR also had PKCε translocation, further supporting the involvement of PKCε in the PKD activation mechanism.

Thus both PKCε and PKD1/2 are activated in response to NK3R activation. PKCε translocation occurs in subclasses of myenteric neurons known to express NK3R. The translocation of PKCε and activation with PKD in IPANs are closely paralleled with respect to time and location and suggest that the two events are interlinked.

**PKCε mediates NK3R-induced activation of PKD.** The effects of various inhibitors on senktide-evoked PKD activation (1 μM, 2 min) and PKCε translocation (1 μM, 15 s) were assayed to determine whether these events result directly from NK3R activation on IPANs and to test the PKCε-dependence of NK3R-stimulated PKD1/2 activation. Tetrodotoxin (1 μM), used to inhibit neurally mediated events, had no significant effect on PKD activation (2 min, Fig. 6, A, A’, and D) or on PKCε translocation (15 s and 2 min, not shown). Inhibition of PKCε translocation (PKCεI, 1 μM) significantly attenuated phospho-PKD916 production (Fig. 6, B, B’, and D). Weak phospho-PKD916-IR was sometimes observed in neurons that also exhibited PKCε translocation, suggesting incomplete blockade of PKCε translocation by PKCεI or partial activation of PKD through another PKC isoform. Phospho-PKD916 production was effectively abolished by the NK3R-specific antagonist SB235375 (1 μM, 1 min pretreatment, Fig. 6, C, C’, and D). Similarly, no PKCε translocation occurred in SB235375 treated preparations exposed to senktide (1 μM) for 15 s. None of the inhibitors stimulated PKD1/2 activation or PKCε translocation when administered on their own. Collectively, these results suggest that the effects of senktide on PKD1/2 activation are mediated through a direct action on NK3R expressed on responsive IPANs and that PKD1/2 activation occurs through a PKCε-dependent pathway.

**Stimulation of NK3 activates PKD1, -2, and -3 in HEK293 cells.** To further investigate the cascade of events that was suggested by the studies of myenteric neurons, we cotransfected HEK293 cells with FLAG-tagged NK3R and either PKD1, -2, or -3. Trafficking and phosphorylation of PKD-GFP were examined by immunostaining and confocal microscopy.

In unstimulated cells (0 min), NK3R was localized at the membrane as well as in the cytosol in cells expressing a high level of this protein. PKD1-, PKD2-, and PKD3-GFP were cytosolic and immunoreactivity was minimal for the phospho-PKD744/748 antibody that detects PKDs only when they are dualy phosphorylated at serines 744 and 748 (Fig. 7). Senktide (1 μM, applied for 5 min) induced internalization of NK3R into vesicles immediately beneath the plasma membrane, and, after 15 min, NK3R was found in perinuclear endosomes, as previously reported (43) (Fig. 7). Senktide (1 μM; 0.5, 1 and 5 min) induced translocation of PKD1, -2, and -3-GFP from the cytosol to the plasma membrane and stimulated the phosphorylation of all three PKDs, as indicated by the increased immunoreactivity detected by phospho-PKD744/748 (Fig. 7). After 15 min, PKD1, -2, and -3-GFP were still detected at the membrane. In contrast, PKD1, -2, and -3-GFP returned to the cytosol by 15 min in cells expressing low levels of these kinases. However, in both cases PKDs remained phosphorylated, suggesting a sustained activation (Fig. 7). In a different set of experiments, we used the phospho-PKD916 antibody that detects endogenous levels of PKD only when phosphorylated at serine916. The immunoreactivity detected with this antibody had a similar pattern to phospho-PKD744/748 in cells transfected with PKD1 and -2-GFP, with a slightly higher immunoreactivity in basal conditions (unstimulated cells). In contrast, the phospho-PKD916 antibody did not reveal phospho-PKD3-GFP; there was no detectable signal for phosphorylated PKD3-GFP in either unstimulated or treated cells. In cells not transfected with FLAG-tagged NK3R, senktide (1 μM) did not cause translocation or phosphorylation of PKD (not shown).

![Fig. 7](http://ajpgi.physiology.org/)

Fig. 7. Activation of NK3 receptors (NK3R) induced redistribution and phosphorylation of PKD1, -2, and -3 green fluorescent protein (GFP) in HEK293 cells, determined by immunostaining and confocal microscopy. In unstimulated cells (0 min) expressing NK3R (immunoreactive to the flag antibody, asterisk in right panels) PKDs were found in the cytosol and in vesicles. The level of phosphorylated kinases detected by the phospho-PKD Ser744/748 antibody (pSer744/748) was minimal (arrowheads). However, activation of NK3R with senktide (1 μM) for 5 min induced translocation of PKDs to the plasma membrane and appearance of phosphorylated kinases (arrow). After 15 min, PKDs returned to the cytosol in cells with a low level of protein expression, whereas PKDs were still found at the membrane in cells with high level of protein expression. Scale bar = 25 μm.
We confirmed these results by Western blotting. Stimulation with senktide (1 μM; 5–15 min) increased the levels of phosphorylated PKD1-GFP (~2.6 and 4-fold increases), PKD2-GFP (~5.2 and 6.9-fold increases) and PKD3-GFP (~6.3 and 9.7-fold increases) detected by using the anti-phospho-PKD744/748 antibody, suggesting activation (Fig. 8). Similarly, stimulation with senktide (1 μM, 5 to 15 min) increased levels of phosphorylated PKD1 and -2-GFP detected by the anti-phospho-PKD744/748 antibody. As indicated above, the anti-phospho-PKD744/748 antibody did not react with phosphorylated PKD3-GFP.

These findings confirm that the NK3R is functionally linked to PKD and indicate a high degree of spatial and temporal similarity between PKD translocation and activation in cell lines and myenteric IPANs. NK3R activates PKD1, PKD2, and PKD3.

Stimulation of NK3R activates PKCε-EGFP expressed in HEK293. We used immunostaining and confocal microscopy to investigate whether stimulation of NK3R induces redistribution of PKCε-EGFP expressed in HEK293-NK3R. In unstimulated cells, NK3R was found at the membrane and internalized after stimulation with senktide (1 μM; 15 min). Also, in unstimulated cells, PKCε-EGFP was localized in the cytosol. Treatment with senktide (1 μM; 1–5 min) induced trafficking of PKCε-EGFP from the cytosol to the plasma membrane, and after 15 min PKCε-EGFP returned to the cytosol (Fig. 9). Thus we confirm that NK3R activation leads to transient PKCε translocation to the plasma membrane.

**DISCUSSION**

To our knowledge, this is the first investigation to identify which enteric neurons express PKD and the first demonstration that NK3R activates PKD. In a previous study (20), a weak immunoreactivity for PKCε-IR (the previous designation of PKD1) was found in enteric neurons of the mouse ileum, but the authors did not identify the neuron types expressing this kinase. We observed prominent PKD1/2 labeling of large myenteric neurons and many submucosal neurons. In 99% of cases, the myenteric neurons with PKD1/2 also had cytoplasmic NeuN labeling. Such labeling has been shown to be exclusively expressed by Dogiel type II neurons in the guinea pig small intestine (49), and analysis of the responses of these neurons to physiological stimuli shows that they are IPANs (10). In addition to PKD1/2 immunoreactivity being confined to IPANs, the production of phospho-PKD916 after exposure to

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**Fig. 8.** Activation of NK3R induced phosphorylation of PKD1, -2, and -3 GFP in HEK293 cells, determined by Western blotting. A and B: Western blot and densitometric analysis of phosphorylated PKD1, -2, and -3-GFP in HEK293-NK3R lysates, detected with an anti-phospho-PKD744/748 antibody (pSer744/748). Treatment with senktide (1 μM) for 5 to 15 min increased the levels of phosphorylated PKD1-GFP (A and B, top), PKD2-GFP (A and B, middle), and PKD3-GFP (A and B, bottom) compared with unstimulated (0 min). *P < 0.05 compared with 0 min, n = 3 experiments.
PDBu was only observed in IPANs, despite the fact that all myenteric neurons express PKC (34).

**Link between NK3 receptors and PKC and PKD activation.**
Our experiments on myenteric neurons in their normal tissue environment (which includes their synaptic relations with other neurons and the presence of enteric glia, interstitial cells, and smooth muscle cells) and in the restricted environment of transfected HEK293 cells both indicate that agonist-mediated stimulation of NK3 tachykinin receptors activates PKD. In transfected cells, each of the PKD isoforms (PKD1, 2, and 3) were activated. The actual form in IPANs is not known, since the available antibodies do not distinguish between PKD1 and PKD2. Suitable anti-PKD3 antibodies are not available. In contrast with PKD1 and PKD2, PKD3 is not autophosphorylated at serine916 (39). Thus one or more PKD isoforms in IPANs might be activated. Both in the natural environment of the IPANs and in transfected cells, activation caused translocation and phosphorylation of PKD. In IPANs, we have investigated the relation between PKC and PKD activation, since PKD has been shown in isolated cell systems to be a downstream target of PKC (35, 38).

In IPANs, both PKCe and PKD immunoreactivities were prominent. Both PKCe and PKD translocated to the plasma membrane in response to DAG analogs or senktide, and activation of PKD, shown by its phosphorylation, occurred at the plasma membrane during the same period as PKCe appearance at this site. Ingenol, a stimulator of novel PKCs, of which PKCe is a member, led to PKD activation, which indicates that PKD is downstream of a novel PKC isoform in these neurons. The activation of PKD by senktide was significantly reduced by PKCeI (1 μM), which specifically inhibits the translocation of PKCe (30), suggesting that PKCe is an upstream kinase. However, suppression was not complete, leaving open the possibility that other PKCs expressed by IPANs, such as PKCγ (31, 34), may contribute to the response. The NK3R-dependence of the response of IPANs to senktide was confirmed by its being blocked by a specific NK3R antagonist. There was also block in cell lines in which both the receptor and PKDs were coexpressed.

**Time courses of PKCe and PKD activation: relation to electrophysiological effects on IPANs by NK3R agonists.**
Reactivation of PKCs, which is indicative of enzyme activation, is thought to target PKC catalytic activity close to potential substrates and provides a mechanism through which substrate specificity can be conferred (7). This same assumption does not apply to PKD, since translocation and activation involve separate mechanisms (38). Because activation of PKD results from phosphorylation of key residues, it is phosphorylation that provides the best index of activation. Similarly to other investigators, we have used phospho-PKD916 as a measure of PKD activation (27, 28). This site is autophosphorylated, thus requiring catalytic activity of PKD (27).

We find that senktide caused a peak PKCe translocation to the membrane at 15 s and a similarly rapid appearance of phosphorylated PKD (phospho-PKD at serine916) at the membrane. In contrast to the rapid and transient peak appearance of PKCe and phospho-PKD at the membrane, PKD activation (phospho-PKD916) in the cytoplasm of IPANs, and to a lesser extent at the membrane, is sustained. Phosphorylation of the COOH-terminal serine916 alters the conformation of PKD, leading to alterations in the duration of activation (50). The sustained activation of PKD that occurs in transfected cells (26) also occurs in normal cells. This sustained PKD activation may enable longer term cellular responses to transient receptor-mediated signals (26) or alter subsequent responses to agonist exposure. With respect to the enteric nervous system, bath-applied agonist (substance P or senktide) causes slow depolarization of myenteric neurons that can persist for several minutes (42). Thus the time course of effect is consistent with the long duration of PKD activation. It is also consistent with PKD...
being downstream of PKC, because the depolarization is blocked by inhibitors of PKC (4), although the selectivity of these inhibitors for PKC over PKD is poor (52).

PKC and PKD phosphorylate distinct motifs; thus the downstream targets of the two kinases are likely to differ (33, 47, 48). Our results suggest that differences in temporal and spatial targeting of the two kinases to subcellular sites may also contribute to the phosphorylation of distinct substrates in IPANs.

Functional implications. Sustained PKD activation in IPANs in response to NK3R agonists may significantly alter responses by these neurons to subsequent exposure to tachykinins or other GPCR agonists. PKD coupling to a number of receptors for peptides has been reported. These include angiotensin, neurotensin, endothelin, bombesin, vasopressin, PDGF, gastrin/CKK, and bradykinin (36, 46, 57). Some of these receptors, and other PKC-coupled GPCRs, are expressed by IPANs and their activation depolarizes IPANs (10); thus, there may be some degree of convergence through PKCe and PKD. Because IPANs are highly interconnected, are arranged into self-reinforcing networks, and mediate reflex responses to mechanical and chemical stimuli (10), any alteration in the activities of these neurons can potentially have profound effects on the entire circuit and ultimately on gut motility and secretion (8, 10). The dependence of sEPSPs in enteric neurons on PKC has previously been demonstrated (4). Future functional experiments to determine whether PKD coupling to PKCe is involved in the generation or modulation of sEPSPs are required.

The distribution of the NK3R in the guinea pig ileum has been assayed by using a fluorescently labeled peptide agonist (18). This study indicated that 70–80% of all myenteric neurons expressed NK3R, in accordance with electrophysiological investigations of sentkide-responsive neurons (3). The functional subclasses of neurons with PKCe translocation are consistent with the previously reported distribution of the NK3R receptor in the guinea pig ileum (18). The number of IPANs with PKCe translocation in response to sentkide is also consistent with the proportion of IPANs that express NK3R (18). The authors of that report used calbindin to identify IPANs. Calbindin is a marker of ~82% of all Dogiel type II/AH neurons (17). We have utilized markers of all IPANs (IB4 and cytoplasmic NeuN) and have therefore analyzed a greater number of neurons. However, when our percentages are corrected for the difference in total neuron number, they are consistent with those reported previously (18).

Conclusion. The present study investigated the distribution of PKD in the myenteric plexus and describes the NK3R-dependent activation of PKCe and PKD in IPANs. Our results suggest that PKD1/2 may have a role in mediating the effects of NK3R activation on these neurons.

ACKNOWLEDGMENTS

The authors thank Dr. Daria Mochly Rosen (Stanford University, CA) for the human PKCe-EGFP transcript, Professor Paul Gleeson for the GCC88 antibodies, Dr. Ian Trounce for the Porin/VDAC1 antibodies, and Professor David Williams for use of confocal microscope facilities and advice.

GRANTS

This work was funded by the National Health and Medical Research Council (Australia) grant 400019 (J. B. Furness) and by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-57840 and DK-39957 (N. W. Bunnett).

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