PKC δ-isofrom translocation and enhancement of tonic contractions of gastrointestinal smooth muscle

Daniel P. Poole and John B. Furness

Department of Anatomy and Cell Biology and Centre for Neuroscience,
University of Melbourne, Parkville, Victoria, Australia

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Poole DP, Furness JB. PKC δ-isofrom translocation and enhancement of tonic contractions of gastrointestinal smooth muscle. Am J Physiol Gastrointest Liver Physiol 292: G887–G898, 2007. First published December 7, 2006; doi:10.1152/ajpgi.00222.2006.—PKC is involved in mediating the tonic component of gastrointestinal smooth muscle contraction in response to stimulation by agonists for G protein-coupled receptors. Here, we present pharmacological and immunohistochemical evidence indicating that a member of the novel PKC isoforms, PKC-δ, is involved in maintaining muscarinic receptor-coupled tonic contractions of the guinea pig ileum. The tonic component of carbachol-evoked contractions was enhanced by an activator of conventional and novel PKCs, phorbol 12,13-dibutyrate (PDBu; 200 nM or 1 μM), and by an activator of novel PKCs, ingenol 3,20-dibenozoate (IBD; 100 or 500 nM). Enhancement was unaffected by concentrations of bisindolylmaleimide I (BIM-I; 22 nM) that block conventional PKCs or by a PKC-δ-specific inhibitor peptide but was attenuated by higher doses of BIM-I (2.2 μM). Relevant proteins were localized at a cellular and subcellular level using confocal analysis. Immunohistochemical staining of the ileum showed that PKC-δ was exclusively expressed in smooth muscles distributed throughout the layers of the gut wall. PKC-ε immunoreactivity was prominent in enteric neurons but was largely absent from smooth muscle of the muscularis externa. Treatment with PDBu, IBD, or carbachol resulted in a time- and concentration-dependent translocation of PKC-δ from the cytoplasm to filamentous structures within smooth muscle cells. These were parallel to, but distinct from, actin filaments. The translocation of PKC-δ in response to carbachol was significantly reduced by scopolamine or calphostin C. The present study indicates that the tonic carbachol-induced contraction of the guinea pig ileum is mediated through a novel PKC, probably PKC-δ.

MEMBERS of the PKC family of phospholipid-dependent serine/threonine kinases are expressed in all cell types, where they have numerous functional roles, including the transduction of responses to G protein-coupled receptor (GPCR) activation. The consequences of activation of different isoforms of PKC have been characterized in a variety of cell types (31). In gastrointestinal smooth muscle, PKC is involved both in the initial contraction following GPCR activation and in the sustained, tonic contraction that follows. Pretreatment with PKC-activating phorbol esters leads to either an augmentation or a diminution of the initial contraction to many agents (24, 41). Phorbol esters can also enhance or reduce the associated tonic component of the contraction (15, 41). Muscarinic agonists acting through M₃ receptors contract gastrointestinal smooth muscle through a PKC-dependent mechanism (9). However, the specific PKC isoforms involved in the contraction of the guinea pig ileum in response to these agonists have yet to be determined.

Tonic contraction is a unique feature of smooth muscles and enables organs to maintain tension against a load (5). Mechanisms underlying the sustained tonic contraction of intestinal smooth muscle include prolonged myosin light chain phosphorylation, the phosphorylation of cytoskeleton filaments and associated proteins, alterations in Ca²⁺ influx, and the increased sensitivity of contractile elements to Ca²⁺. PKC plays a major role in mediating all of these changes (5, 26). Current hypotheses suggest that the movement of PKC to smooth muscle filaments or to filament-associated proteins has an important role in mediating the tonic contraction of gastrointestinal smooth muscle and therefore in controlling gut motility (5). However, PKC translocation to filaments has yet to be demonstrated in the guinea pig ileum, the tissue on which the majority of pharmacological investigations have been performed.

The PKC isoforms responsible for mediating the tonic contractions of intestinal smooth muscle have been investigated in a variety of species (1, 16, 27). There appear to be interspecies differences, with evidence for PKC-α, -β, and -ε mediating contractile responses of smooth muscle cells from the dog and guinea pig intestine (1, 27–29), whereas sustained smooth muscle contractions of the rabbit colon appear to be largely mediated through the activation and translocation of PKC-α (6, 16, 37, 44, 45). Our previous investigations (39, 40) into the expression of PKC isoforms in the guinea pig ileum have not supported the direct involvement of PKC-ε in the contractile responses described. Immunoreactivities (IRs) for conventional PKCs and PKC-ε have not been readily detected in smooth muscle cells of the guinea pig ileum, whereas strong labeling was observed in myenteric neurons, glia, and interstitial cells of Cajal (39, 40, 46). In contrast, IR for a novel PKC, PKC-δ, was very prominent in smooth muscle but was completely absent from myenteric ganglia (39). In this respect, the reported expression patterns of the different PKC isoforms in the guinea pig small intestine do not appear to correlate with the physiological evidence for conventional PKC and PKC-ε involvement in sustained smooth muscle contraction (27–29).

For this reason, we investigated the translocation of PKC-δ in response to muscarinic receptor activation and stimulation by PKC activators. Contractility experiments were performed and indicated the involvement of novel PKC isoforms other than PKC-ε in the sustained tonic component of the contraction.

Address for reprint requests and other correspondence: D. P. Poole, Dept. of Anatomy and Cell Biology, Univ. of Melbourne, Parkville, Victoria 3010, Australia (e-mail: d.poole@unimelb.edu.au).

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Another member of the novel PKCs, PKC-δ, was also detected in smooth muscle cells using immunohistochemical methods. As the enhancement of the phasic component by phorbol esters appears to be of less physiological relevance than the sustained contraction (41), and to allow direct comparison with similar studies, we focused on the subcellular events that occur during the tonic phase of contraction.

The distribution of PKC and the intracellular locales to which PKC translocates are often isoform specific and may have important implications for substrate selectivity between isoforms (7, 8, 13, 25, 43). Translocation of PKC isoforms is generally regarded to be indicative of their activation. Thus, the sites to which PKC isoforms are translocated can be used to predict their functional roles. The present study used this to implicate PKC-δ in the generation of tonic contractions of intestinal smooth muscle, based on its rapid association with the cytoskeleton following muscarinic receptor stimulation or exposure to PKC activators.

MATERIALS AND METHODS

Animals

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.

Contraction Studies

Segments of the distal ileum (at least 10 cm oral to the ileocaecal junction) were rapidly removed and placed in physiological saline solution [composed of (in mM) 118 NaCl, 4.8 KCl, 2.5 NaHCO₃, 1.0 NaH₂PO₄, 1.2 MgSO₄, 11.1 t-glucose, and 2.5 CaCl₂]. The contents of the segments were washed out, and the mesenteric attachment was removed. Whole wall strips (−1.5 cm in length) were prepared in the orientation of the longitudinal muscle layer. These were mounted vertically in 5-ml organ baths and held at a resting tension of 1 g. Baths were filled with physiological saline solution, which was maintained at 37°C and bubbled with 95% O₂ and 5% CO₂.

Contractile activity was detected using isotonic force transducers (Harvard Apparatus, Kent, UK), and the output was digitized using an analog-to-digital converter (Biopac MP100A, Biopac Systems, SDR Clinical Technology, Middle Cove, Australia). Changes in smooth muscle tension were recorded and analyzed using AcqKnowledge 3.2.4 software (Biopac Systems).

Tissue strips were allowed at least 60 min to equilibrate, during which time the bathing solution was replaced every 15 min. Following this period, preparations were exposed to carbachol (10 µM) to evoke smooth muscle contraction. Only tissues that responded reproducibly to this agonist were used for experiments involving PKC activators.

The effects of PKC activators on tonic contractions of the longitudinal smooth muscle were determined using the following protocol. Tissue strips were contracted with a single concentration of carbachol (10 µM). At the peak of this contraction, PKC activators were administered directly into the organ bath. The PKC activators used were phorbol 12,13-dibutyrate (PDBu; a conventional and novel PKC activator) and ingenol 3,20-dibenzoate (IDB; a novel PKC activator). Contractile activity was recorded for at least 10 min after the drug addition. Inhibitors were added directly into the bath at least 2 min prior to the use of any agonists or activators unless otherwise stated.

Data were analyzed by measuring the heights of the contractions in response to carbachol (10 µM) relative to the baseline tension. Analysis was performed at 1-min intervals after the initial addition of carbachol (10 µM), and data were collected for the following 10-min period. If phasic oscillations in tension were present at the measurement points, the baseline was estimated by extrapolation. Data were expressed as percentages of the maximal contraction amplitude to carbachol (10 µM).

Statistical data analysis. Student’s t-test or one-way ANOVA was used for statistical analysis. P values of <0.05 were considered to be significantly different to the null hypothesis of no difference at the 95% confidence level.

Drugs. The following drugs were used: carbamylcholine (carbachol), PDBu, scopolamine hydrochloride, and nicardipine (all from Sigma-Aldrich, Sydney, Australia); bisindolylmaleimide I (BIM-I, Calbiochem, La Jolla, CA); calphostin C (Wako, Osaka, Japan); IDB (LC Laboratories, Woburn, MA); myristoylated PKC-ε translocation inhibitor peptide (N-myristoyl-EAVSLKPT, PKC-ε inhibitor; BioMol Research Laboratories, Plymouth, PA); and TTX (Alomone Laboratories, Jerusalem, Israel). All drugs were dissolved in either distilled water or DMSO and were added to the bath in volumes not exceeding 0.5% of the total bath volume. The vehicles in which drugs were dissolved did not significantly affect responses by tissue strips to carbachol and had no effect when administered on their own.

Immunohistochemical Studies

Translocation. Segments of the ileum were transferred into physiological saline solution containing nicardipine (1 µM) to inhibit tissue contraction. The tissue was then pinned flat under moderate tension onto Silicone elastomer-lined culture dishes and treated with drugs as previously described (32). Following fixation, preparations were dissected into external muscle myenteric plexus (EM-MP) whole mounts (40). Proteins of interest were labeled by indirect immunofluorescence (40). Preparations were analyzed by confocal microscopy as previously described (32).

Image analysis. Single 1,024 × 1,024-pixel images (0.5-µm optical section) captured using the ×63 objective lens were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Density plots were prepared by analyzing pixel density profiles across the short transverse axis of smooth muscle cells immunolabeled for PKC-δ. Peak amplitudes were measured and expressed as a ratio relative to basal pixel density. Analysis was performed on 2–3 smooth muscle cells/image, and the mean ratio was determined for each cell. Data from at least three separate animals were pooled and expressed as mean values ± SE.

Primary antisera. The following primary antisera were used: antibodies against PKC-δ (1:250), PKC-ε (1:500), PKC-η (1:200), PKC-λ (1:200), and diacylglycerol kinase θ (DGK-θ; 1:250) (all mouse monoclonal antibodies; BD Transduction laboratories, Lexington, KY); actin (1:200, rabbit polyclonal antibody; Sigma); and phosphoserine PKC substrate (1:400, rabbit polyclonal antibody; Cell Signaling Technology, Beverly, MA).

Secondary antisera. The following secondary antisera were used: goat anti-mouse antibody coupled to Alexa 594 (1:200) and goat anti-rabbit antibody coupled to Alexa 488 (1:400, both from Molecular Probes, Eugene, OR).

Western Blot Analysis

EM-MP preparations were treated with drugs in the same manner as the EM-MP whole mounts for immunohistochemistry as described above (see Translocation). Following treatment, preparations were placed in cellytic mammalian tissue lysis/extraction buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and PMSF (5 µM). Protein samples were prepared according to previously described methods (39).

Lysates were separated into cytosolic, Triton X-100-soluble particulate, and Triton X-100-insoluble/SDS-soluble cytoskeletal fractions to determine the subcellular localization of proteins of interest (32).
Protein (20 μg total protein) was resolved by SDS-PAGE using 10% gels under reducing conditions. Western blot analysis was performed as previously described (39). Densitometric analysis of blots was performed on scanned images using the ImageJ gel plotting macro (30). Only within-blot comparisons were made, and changes were expressed as ratios relative to control values, which were designated as 1. All Western blots were performed in triplicate.

RESULTS

Contraction Studies

Effect of PKC activators and PKC inhibitors on basal tone and contractile activity. When administered on their own, neither PDBu (200 nM or 1 μM) nor IDB (100–500 nM) had any effect on the basal tone of the tissue strips, even after a 30-min exposure period. The only detectable response to these PKC activators was the initiation or facilitation of oscillatory contractions. In previously quiescent tissues, these contractions generally appeared 5–15 min after the addition of an activator. In preparations already exhibiting phasic contractile activity, contractions were increased in amplitude for prolonged periods in response to PKC activators. PKC inhibitors [BIM-I (2.2 μM), calphostin C (1 μM), and PKC-ε inhibitor (1 μM)] had no effect on either the basal smooth muscle tone or frequency or amplitude of spontaneous contractions.

Potentiation of carbachol-induced contractions by PKC activators. Carbachol caused a rapid contraction of the muscle, which reached a peak at ~30 s and declined rapidly over a further 120 s to ~30% of the initial amplitude (the tonic component of the response). This was followed by a sustained contraction that lasted 10 min or more (the tonic part of the response). The tonic component slowly declined (Fig. 1A). There were sometimes oscillations in tension superimposed on the tonic contraction.

The addition of PDBu (200 nM or 1 μM) at, or just after, the peak of the phasic component of the carbachol-evoked contraction generally led to an enhancement and a prolongation of the tonic component (Fig. 1, B and C). This response to PDBu was maximal at 200 nM, with no statistically significant increase in effect at the higher concentration (1 μM; Fig. 1E). The enhancement of tonic contractions caused by PDBu was still observed up to 1 h after its addition. PDBu was not readily washed out, and tissues also exhibited increased responses to carbachol (10 μM) following exposure to PDBu. In some cases, tonic contractile activity was reduced by PDBu (n = 6 of 24 experiments). Among these, it was rarely observed that the phasic contraction declined to baseline after PDBu (n = 1 of 24 experiments; Fig. 1D). Smaller decreases toward baseline followed by prolonged elevations in tone were also relatively uncommon (n = 5 of 24 experiments). Oscillatory contractions were sometimes observed in conjunction with the tonic enhancement of carbachol’s action by PDBu (e.g., Fig. 1D).

IDB (100 nM, n = 6, or 500 nM, n = 6), added at or just after the peak of the initial response, caused contractile responses, similar to those caused by PDBu, in carbachol-precontracted preparations (Fig. 2). IDB-evoked changes were dose dependent, with greater augmentation of tonic contractions at the higher dose (500 nM; Fig. 2D). IDB effects were of a shorter duration relative to those caused by PDBu, with greater declines in tone toward basal levels observed during the treatment period (10 min; Fig. 2). Both a decline to baseline (n = 1) and oscillatory contractions (n = 7) were also observed following the addition of IDB to the bathing medium (n = 13 experiments).

Effects of PKC inhibitors on PKC activator-enhanced tonic contractions. The PKC-specific inhibitor BIM-I (also called Gö-6850 or GF-109203X), at 2.2 μM, slightly reduced the phasic contraction to carbachol and prevented the enhancement of tonic contractions caused by PDBu (200 nM, n = 8), with some preparations exhibiting no increase in the basal tone of the smooth muscle in the presence of the inhibitor (Fig. 3, A and B). A lower concentration (22 nM) was ineffective (n = 7; Fig. 3, C and D). However, oscillatory contractions that occurred in some preparations were not diminished, even by the higher concentration of this compound (Fig. 3A).

The inclusion of a specific inhibitor peptide for the novel PKC isomser PKC-ε [PKC-ε inhibitor (1 μM), 10–15 min prior to carbachol] in the bathing medium did not reduce the
The phasic response to carbachol (10 μM) and failed to reduce the effects of the novel PKC isoform stimulant IDB on the tonic phase (100 nM, n = 9; Fig. 3, E and F). There were no changes in the appearance of the IDB-enhanced changes in tone in these preparations.

**Effect of PKC inhibitors on carbachol-evoked contractions.** The phasic contractile responses to carbachol (10 μM) were greatly reduced or abolished by calphostin C (1 μM, 55.2 ± 16.3% of control, n = 5; Fig. 4, A and B) but were largely unaffected by BIM-I, even at an inhibitor concentration (2.2...
μM) that was greater than that predicted to block conventional PKCs (20) (Fig. 4, C and D). However, BIM-I reduced phasic contractions in response to a lower dose of carbachol (1 μM, 84.8 ± 4.3% of control, n = 4; Fig. 4, E and F). The PKC-ε inhibitor peptide (1 μM) had no effect on carbachol-evoked contractions.

Immunohistochemical Investigations

PKC-δ, GENERAL OBSERVATIONS. IR for PKC-δ was detected in smooth muscle cells of both longitudinal and circular muscle layers of the muscularis externa, in the muscularis mucosae, and in bands of muscle within the villi (Fig. 5, A and B). PKC-δ IR was diffusely distributed throughout the cytoplasm of all smooth muscle cells, with no subcellular regions exhibiting greater labeling. No nuclear localization or enhanced perinuclear PKC-δ IR were detected (Fig. 6A). PKC-δ IR was completely absent from myenteric ganglia and interstitial cells of Cajal, consistent with our previous observations (39, 40). The distribution of PKC-δ IR did not change over time in preparations maintained in physiological saline solution at 37°C for 1 h without drug treatment.

EFFECT OF PKC ACTIVATORS ON PKC-δ LOCALIZATION. Treatment with PDBu resulted in an increase in PKC-δ IR associated with filamentous structures within the cytoplasm of smooth muscle cells (Fig. 6F). PKC-δ-IR filaments ran parallel to the length of the cell. They had widths of ~2 μm and a periodicity of 3–4 μm (Fig. 6J). These PKC-δ-IR filaments often passed over the nucleus of the cells. Increased IR located at the plasma membrane was also observed. The changes in PKC-δ localization in response to PDBu were both time and concentration dependent. The lowest PDBu concentration required to give detectable PKC-δ translocation after a 10-min exposure period was 10 nM. Maximal responses were observed with higher doses of PDBu (200 nM or 1 μM depending on the preparation examined). Time-course experiments using 200 nM PDBu indicated that the association of PKC-δ with the filaments was maximal after 5 min (Fig. 7). Image analysis showed that there was a significant increase in relative peak intensity 1 min post-PDBu addition (Fig. 7) and that there were no significant differences between responses at 5 and 10 min.

Treatment with the novel PKC activator IDB (500 nM, 10 min) also resulted in an increase in PKC-δ association with cytoskeletal structures (Fig. 6G). These effects were not as pronounced as those in response to PDBu, even though a higher concentration of this compound was used (500 nM compared with 200 nM). As PDBu and IDB appeared to have identical qualitative effects on PKC-δ translocation, no quantitative image analysis was performed on IDB-treated preparations.

EFFECT OF CARBACHOL ON PKC-δ LOCALIZATION. Muscarinic receptor stimulation by carbachol (1–10 μM, 10 min) also resulted in the redistribution of PKC-δ IR from the cytoplasm to filamentous structures and to the plasma membrane of smooth muscle cells (Fig. 6C). The carbachol-evoked changes in the localization of PKC-δ IR were comparable with those caused by PDBu but were generally more pronounced follow-

Fig. 4. PKC inhibitors effectively diminish contractile responses of the guinea pig ileum to CCh. A: longitudinal smooth muscle contraction following treatment with CCh (10 μM). B: contractile responses of the same preparation to a second dose of CCh (10 μM) were attenuated by a high concentration of calphostin C (CalC; 1 μM). C and D: examples where the PKC-selective inhibitor BIM-I (2.2 μM) failed to significantly reduce the contraction to CCh (10 μM). E and F: BIM-I (2.2 μM) effectively reduced contractions to a lower concentration of CCh (1 μM). These results demonstrate that effective PKC inhibition by these compounds was achieved in our preparations.

Fig. 5. Immunohistochemical localization of PKC-δ in the guinea pig ileum. A: cross section of a CCh-treated (10 μM, 10 min) ileum immunolabeled for PKC-δ. PKC-δ was localized to the longitudinal (LM) and circular (CM) muscle layers of the muscularis externa and to the muscularis mucosae (MM) and muscle cell within the villi (V). B: double labeling of the same section using an antiserum specific for actin. Scale = 50 μm.
ing stimulation with the muscarinic agonist. The translocation of PKC-δ/H9254 in response to carbachol (10 μM, 10 min) was rapid, with statistically significant changes observed after 1 min of treatment (Figs. 6B and 7). These initial changes were evident as increased PKC-δ IR at patches along filamentous structures within the cytoplasm of smooth muscle cells. Maximal responses were detected 5 min posttreatment and were maintained beyond 10 min. In these preparations, PKC-δ/H9254 IR was localized to filamentous structures and the plasma membrane, with many cells exhibiting a complete absence of detectable IR in the adjacent cytoplasm (Fig. 6C). Muscarinic receptor blockade by scopolamine (50 μM) significantly reduced the effects of carbachol (10 μM) on PKC-δ distribution (Figs. 6D and 7). PKC inhibition by calphostin C (1 μM) also significantly reduced PKC-δ translocation by carbachol (10 μM) and reduced its association with filaments (Figs. 6E and 7). The distribution of PKC-δ/H9254 in the inhibitor-pretreated tissues closely resembled the distribution seen following short (1 min) treatments with carbachol (10 μM). PKC-δ was now present as smaller patches of IR on filaments rather than the expected even distribution along these structures (compare Fig. 6C with Fig. 6E). Treatment of preparations with the inhibitors alone had no effect on the subcellular localization of PKC-δ IR.

Double labeling of carbachol-treated preparations (10 μM, 10 min) using antisera specific for PKC-δ and actin indicated that there was no overlap between these two markers. This result suggests that activated PKC-δ does not translocate to filamentous actin or to closely associated structures following treatment with carbachol (Fig. 8).

**EFFECT OF CARBACHOL AND PDBU TOGETHER ON PKC-δ LOCALIZATION.** The inclusion of PDBu (200 nM) during carbachol treatment (10 μM) appeared to alter the kinetics of PKC-δ/H9254 translocation (compare Fig. 6F with Fig. 6B). Treatment of preparations with both carbachol (10 μM) and PDBu (100 nM) increased the rate at which PKC-δ/H9254 translocated to the cytoskeletal relative to CCh or PDBu alone (1-min exposure, compare with B).
translocate to actin filaments in response to CCh. B*)-immunoreactive (IR) peaks labeled. This indicates that PKC- H cells within 1 min after PDBu addition (Fig. 6 associated with the cytoskeletal filaments of smooth muscle A exposure to CCh.}

**PKC-IR was localized to myenteric plexus (MP) and submucosal plexus (SMP) neurons, with no labeling detected in LM or CM muscle layers.**

PKC-IR was largely absent from the external muscle of the ileum. Prominent PKC-ε staining was detected in myenteric and submucosal neurons in cross sections and whole mounts (Fig. 9). To demonstrate the effective entry of PKC-ε inhibitor to target cells, the ability of this peptide to block IDB-evoked translocation in myenteric neurons was assayed. As reported previously (32), IDB exposure (1 μM, 10 min) stimulated the translocation of PKC-ε from the cytoplasm to the plasma membrane of myenteric neurons (Fig. 9B). This response was effectively reduced by preincubation of the preparations with PKC-ε inhibitor (1 μM) for 20 min prior to IDB addition (Fig. 9C). These results showed that PKC-ε IR in external smooth muscle layers is almost undetectable and that we can effectively inhibit translocation of this isoform using a specific peptide inhibitor.

**PKC-η.** Diffuse PKC-η IR was present in the cytoplasm of smooth muscle cells. This staining was generally very faint, and no nuclear or perinuclear immunolabeling were observed (Fig. 10A). Weak IR was also observed in a subset of neurons of the myenteric plexus, consistent with our previous report (39). Due to the relatively weak IR observed for this isoform, no quantitative immunohistochemical analysis was performed for PKC-η.

Treatment with carbachol (10 μM, 10 min) caused an increase in PKC-η IR at the plasma membrane of smooth muscle cells (Fig. 10B). Initial translocation events were detected following 1–2 min of exposure to carbachol (10 μM). The most marked labeling of the plasma membrane occurred after a treatment period of 10 min. This increase in membrane labeling was associated with a concomitant decrease in cytoplasmic PKC-η IR.

Exposure to carbachol and PDBu resulted in an increase in the intensity of PKC-η IR in smooth muscle cells, particularly at their plasma membranes. These changes did not appear to be more prominent than those in response to carbachol alone. The kinetics of PKC-η translocation did not appear to be altered by PDBu, with the initial responses first observed 1–2 min after carbachol addition. As with carbachol alone, the maximal effects were detected 5–10 min following the initial carbachol exposure.

**PKC-λ.** We investigated the translocation of PKC-λ (a representative member of atypical PKCs) in EM-MP whole mounts. Atypical PKCs can be indirectly activated via DGK-α (19, 50), whose translocation is described below. Although PKC-λ IR was not detected in smooth muscle of the guinea pig

**Fig. 8. Evidence that PKC-δ does not associate with actin filaments following exposure to CCh. A:** pixel density plot with major actin (†) and PKC-δ IR peaks labeled. This indicates that PKC-δ did not translocate to actin filaments in response to CCh. B and B’: PKC-δ and actin-IR filaments did not colocalize when examined immunohistochemically. Selected PKC-δ-IR filaments are indicated (arrows) to illustrate this. Scale = 25 μm. Pixel density analysis was performed on both images (solid lines), and the resulting line profile plots are shown in A.

**Fig. 9.** Immunohistochemical localization of PKC-ε in the guinea pig ileum. A: cross section of a PDBu-treated (100 nM, 10 min) ileum immunolabeled for PKC-ε. IR was localized to myenteric plexus (MP) and submucosal plexus (SMP) neurons, with no labeling detected in LM or CM muscle layers. B: translocation of PKC-ε from the cytoplasm to the plasma membrane of MP neurons treated with IDB (1 μM, 10 min). Representative neurons exhibiting PKC-ε translocation are indicated (arrows). C: this translocation was greatly reduced by pretreatment with a PKC-ε-specific inhibitor peptide (1 μM, 20 min), indicating that effective inhibition of this isoform was achieved in our preparations. Examples of PKC-ε-IR neurons are indicated (arrows). Scale = 50 μm.
ileum in a previous investigation (39), translocation and the resulting increased concentration of IR or altered conformation of the target protein can make PKC-λ labeling more prominent in cells (51). PKC-λ IR in control preparations was readily detected in myenteric neurons but was absent in smooth muscle cells (Fig. 10C). Treatment with carbachol (10 μM, 10 min) resulted in increased PKC-λ IR in fusiform cells at the level of the deep muscular plexus and in PKC-λ translocation in a subset of myenteric neurons (Fig. 10, D and E). PKC-λ IR was never detected in smooth muscle cells, even after treatment with carbachol (10 μM, 10 min). As atypical PKCs are insensitive to phorbol esters (35), no experiments were conducted to investigate the effects of PDBu on the distribution of PKC-λ.

**DGK-θ.** GENERAL OBSERVATIONS. The distribution and translocation of DGK-θ, the DGK isoform specifically activated by PKC-η and PKC-ε (49), was examined in EM-MP whole mount preparations. DGK-θ IR was localized to both smooth muscle cells and myenteric neurons, consistent with our previous report (39). Although immunohistochemical staining of smooth muscle cells was weak in control preparations, DGK-θ IR could be observed throughout the cytoplasm. No nuclear DGK-θ IR was present in muscle cells (Fig. 10F). DGK-θ was diffusely distributed in the cytoplasm of myenteric neurons, with no evidence that the expression of this enzyme was restricted to a particular functional subclass of neuron.

**EFFECT OF PDBU ON DGK-θ LOCALIZATION.** Exposure to PDBu resulted in a small increase in DGK-θ IR association with the plasma membrane of both smooth muscle cells and myenteric neurons. These changes were rapid, with evidence for translocation in preparations treated for a 1-min period. A corresponding reduction in cytoplasmic DGK-θ IR was also observed.

**EFFECT OF CARBACHOL ON DGK-θ LOCALIZATION.** Following treatment with carbachol (10 μM, 10 min), DGK-θ IR was more prominent at the plasma membranes of both smooth muscle cells (Fig. 10G) and myenteric neurons. The inclusion of scopolamine (50 μM) abolished the carbachol-stimulated translocation of DGK-θ in smooth muscle cells (Fig. 10H), indicating that this redistribution was a result of muscarinic receptor activation. PKC inhibition using calphostin C (1 μM) appeared to alter, but not abolish, the effects of carbachol. DGK-θ IR in these preparations was more punctate, and there was some evidence for labeling of filamentous cytoskeletal structures (Fig. 10I).

Carbachol treatment also resulted in an increase in DGK-θ IR at the plasma membrane of a subset of myenteric neurons. These neurons presumably express muscarinic receptors. This observation suggests that analysis of the translocation of different DGK isoforms in similar preparations, in conjunction with the use of specific neurochemical markers, may provide a means through which functional GPCRs can be identified in myenteric ganglia.

**Evidence for Translocation by Western Blot Analysis**

PKC-δ was detected as three bands (~30, 50, and 75 kDa, respectively) in EM-MP lysates. This has been reported previously (39) and may reflect proteolytic degradation at the hinge region separating the regulatory and catalytic domains of PKC-δ (12, 17, 18).

To confirm the immunohistochemical observations demonstrating PKC-δ translocation, Western blot analysis was performed on subcellular fractions from control and carbachol-treated EM-MP preparations (10 μM, 10 min). PKC-δ was mainly located in the cytosolic fraction of control samples, with weakly PKC-δ-IR bands detected in both the particulate and cytoskeletal fractions. Following treatment with carbachol, the relative intensities of the PKC-δ-IR bands for the three fractions were altered. Quantitative analysis was performed on the ~50-kDa PKC-δ-positive band. There was a significant reduction in PKC-δ in the cytosol and an associated increase in the cytoskeletal fraction. Densitometric analysis indicated that this increase was significantly altered relative to control levels (Fig. 11A). Although apparent increases in relative PKC-δ density were observed in the particulate fraction following treatment with carbachol, these changes were not significant at the 95% confidence level (Fig. 11A). These data confirm the immunohistochemical observations and indicate that PKC-δ translocates to the cytoskeleton of smooth muscle cells in response to muscarinic receptor activation.

**Evidence for Phosphorylation of Cytoskeletal Proteins**

Major PKC substrates were detected by Western blot analysis and through a comparison between control and treated samples. This method relied on an antiserum specific for substrates phosphorylated at the PKC consensus sequence (34). Treatment with carbachol (10 μM, 10 min) resulted in an
increase in the intensity of three major protein bands (<21, 35, and 53 kDa) in the cytoskeleton-enriched, Triton X-100-insoluble fraction (Fig. 11B).

Immunohistochemical analysis of IDB- or carbachol-treated preparations using the anti-phosphoserine antibody showed that nuclear and filamentous cytoplasmic structures were phosphorylated by PKC in smooth muscle cells (data not shown). In contrast, labeling of PKC-phosphorylated substrates in untreated control tissues was present as a weak, diffuse cytoplasmic staining. No detectable increase in IR was observed at the plasma membrane of smooth muscle cells in carbachol- or IDB-treated preparations. The intensity of this staining was generally weak, and no quantitative analysis was performed.

**DISCUSSION**

**Pharmacological Evidence That Novel PKCs Contribute to the Enhancement of Carbachol-Induced Contractions**

The contractility studies showed that PKC activators by themselves did not cause intestinal muscle to contract, confirming previous reports (4, 15, 41), but that both PDBu, which activates conventional and novel PKCs, and IDB, which activates novel PKCs (PKC-δ, -ε, -η, and -θ), enhanced and prolonged tonic contractions caused by carbachol when applied after this agonist. An inhibitor of conventional PKCs, BIM-I, at a concentration (22 nM) that is selective for these isoforms (20, 48), did not reduce the contraction caused by carbachol or the enhancement caused by PDBu, supporting the assumption that the effect is through a novel PKC. Our results with BIM-I indicate that conventional PKCs are unlikely to be involved in either the initial contraction to carbachol or in the sustained response following exposure to PKC activators. This conclusion is supported by the absence of conventional PKC (PKC-α, -βI, and -γ) IR in smooth muscle cells of the guinea pig ileum (Ref. 11, 39, and 40 and the present study). The broad-spectrum PKC inhibitor calphostin C substantially reduced the phasic and tonic contractions caused by carbachol. The specific inhibitor of PKC-ε (PKC-ε inhibitor) did not reduce the effects of the novel PKC activator IDB (2, 3). Thus, the isoforms that are likely from this pharmacological analysis to contribute to the sustained enhancement of contraction are PKC-δ, PKC-η, and PKC-θ. We found that IR for PKC-δ was strong and IR for PKC-η was weak in the muscle cells. No PKC-θ IR was present in smooth muscle, but prominent labeling was present in interstitial cells of Cajal (40).

In contrast with a previous investigation (27), the present study suggests that neither PKC-ε nor conventional PKCs have much influence on smooth muscle contractility of the guinea pig ileum (see above). It is unlikely that we used insufficient PKC-ε inhibitor peptide as the concentration and exposure time that we used can attenuate PKC-ε translocation in neurons of the guinea pig myenteric plexus, which is located within the muscularis externa (32). Furthermore, PKC-ε IR is largely restricted to enteric neurons, glia, and epithelium of the gut (32, 39, 40).

**The Contractile Apparatus as a Target for Novel PKCs Involved in Contraction Enhancement**

A previous investigation (26) has indicated that PKC interactions with the contractile apparatus can have significant effects on the tonic contraction of smooth muscle cells. Components of the contractile apparatus of gastrointestinal smooth muscle that interact with PKC include tropomyosin and thin filament-associated proteins such as calponin (38).

The enhancement of carbachol-induced contractions, without an induction of contraction in unstimulated muscle, suggests that exposure to PKC activators results in phosphorylation of a protein or proteins associated with the contractile apparatus to enhance tonic contraction. We found that PKC-δ, but not PKC-η, translocates from the cytoplasm to the cytoskeleton of smooth muscle cells in response to muscarinic receptor stimulation and exposure to PKC activators, whereas PKC-η translocated to the surface membrane. Moreover, Western blot analysis showed an increase in PKC-δ associated with cytoskeletal elements and an increase in phosphorylation in the cytoskeletal fraction from tissue exposed to carbachol. Our results thus strongly suggest that PKC-δ has a role in modifying the contractile response of the guinea pig ileum to muscarinic agonists. It is possible that PKC-η also has a role, but at the membrane, where it could possibly act on ion channels or surface receptors.

Other studies (4, 27) have also implicated PKC in sustained tonic contractions of gastrointestinal smooth muscles. However, to our knowledge, ours is the first investigation to identify a potential role for PKC-δ in smooth muscle of the guinea pig ileum. This is important, as the majority of pharmacological investigations into the role of PKC on tonic contractions have used the guinea pig ileum as an experimental model (4, 15, 23,
41, 52). Moreover, PKC-δ is the dominant isoform in smooth muscle of the human intestine (11).

Functional Implications

Translocation of PKC-δ occurred with PDBu alone, but this was not associated with contraction. Therefore, the translocation of PKC-δ and phosphorylation of substrates in response to PDBu are not sufficient to cause contraction. There must also be GPCR activation and probably an accompanying increase in the intracellular Ca\(^{2+}\) concentration. Carbachol alone stimulated PKC-δ translocation, but the sustained contraction (tonic phase) in response to this agonist was of relatively low amplitude. Thus, PKC-δ may need to be irreversibly activated by PKC activators for this response to be augmented. The carbachol-induced translocation of PKC-δ was significantly reduced by calphostin C, implying that binding to filaments is dependent on diacylglycerol binding or on access to the C1 cysteine-rich region of the regulatory domain, as calphostin C binds irreversibly to the C1 region of PKCs (14).

Our results show that, under normal conditions, activation of muscarinic GPCRs located on smooth muscle cells of the ileum leads to changes in the localization of PKC-δ. The translocation of PKC-δ from the cytoplasm to cytoskeletal structures and to the plasma membrane of these cells suggests that this isoform is most probably involved in the normal excitation-contraction coupling between muscarinic agonists and M3 muscarinic receptors expressed by smooth muscle cells (1).

The basal tone of ileal smooth muscle does not appear to be under the control of PKC. We have shown that the administration of PKC-specific inhibitors (BIM-I and calphostin C) has no effect on basal tone in our system, consistent with the results of Xu et al. (52). A previous study (15) has shown that phorbol esters do not have any significant effect when administered on their own, in agreement with our data.

As many of our experiments have used phorbol and ingenol esters to evoke exaggerated responses, our results may not reflect the events that occur under normal physiological conditions. These compounds can, however, give an indication of the role of PKC in the normal excitation-contraction coupling of smooth muscle and the effects of neurotransmitters released from the enteric nervous system. Our data suggest that these PKC activators have similar effects on the redistribution of PKC isoforms as GPCR activation with respect to the subcellular locations to which these translocate. This is supported by the similarities between PKC-δ translocation in response to PKC activators and to carbachol. In this regard, we can have confidence that our results reflect normal physiological responses.

Potential Targets of PKC Action in Guinea Pig Ileal Smooth Muscle

At present, very few substrates of PKC-δ have been identified (47). The present study suggests that potential downstream direct or indirect substrates of PKC-δ in gastrointestinal smooth muscle may be L-type Ca\(^{2+}\) channels and cytoskeletal proteins. Increased PKC-δ IR and PKC-η IR were detected at the plasma membrane following carbachol treatment, where they may regulate L-type Ca\(^{2+}\) channels. Ca\(^{2+}\) entry through these channels is necessary for muscle contraction, and channel opening can be modified directly by phosphorylation by PKC (22, 53). PKC-δ may also be involved in the phosphorylation of the myosin light chain phosphatase inhibitor protein CPI-17, resulting in prolonged myosin light chain phosphorylation and an associated sustained contractile response to agonists (10, 29).

PKC-δ Interactions With the Cytoskeleton

The proteins that exhibited changes in their phosphorylation states following exposure of preparations to carbachol probably include smooth muscle cytoskeletal proteins that have been phosphorylated by PKC-δ. We base this conclusion on the following evidence: PKC-δ appeared to be the only PKC isoform that translocated to the cytoskeleton in response to carbachol stimulation in smooth muscle cells and the majority of detectable proteins in our lysates are probably of smooth muscle cell origin (21). The major phosphoproteins were enriched in the Triton X-100-insoluble (cytoskeletal) fraction from EM-MP lysates, indicating that they were associated with the cytoskeleton. PKC-δ IR was detected in the cytosolic, particulate, and cytoskeletal fractions of EM-MP lysates, consistent with our immunohistochemical observations.

We have not identified the protein filaments with which PKC-δ is associated. However, our evidence suggests that these structures are neither actin nor desmin filaments. The distribution of PKC-δ differed to that of desmin filaments, which were comparatively thin in diameter and were more abundant (D. P. Poole, unpublished observations). This suggests that PKC-δ was not associated with desmin intermediate filaments following activation by phorbol esters or diacylglycerol.

Our results are consistent with the hypothesis that sensitization of the contractile elements to Ca\(^{2+}\) occurs following PKC activation in smooth muscles (24, 33, 36, 42). We demonstrated that PKC-δ translocates to the cytoskeleton following exposure to phorbol esters and that these compounds do not evoke smooth muscle contractions. This suggests that an additional signal, such as an increase in intracellular Ca\(^{2+}\) concentration, is required for these to occur.

Comparisons With Other Investigations

Our study is the first to implicate a role for PKC-δ in the tonic contraction of gastrointestinal smooth muscle. A previous report (27) has indicated that PKC-ε is a major isoform involved in the tonic contraction of the guinea pig small intestine. Our present findings appear to contradict this report, as we neither detected significant expression of PKC-ε IR in smooth muscle of the ileum nor successfully diminished tonic contractions of the ileum using a PKC-ε-specific inhibitor. These results suggest that PKC-δ may play a more substantial role in smooth muscle contraction than PKC-ε. There are a number of reasons why our results may differ from those previously published. We used intact segments of the ileum, whereas other groups used isolated smooth muscle cells (e.g., Refs. 16 and 28). There are likely to be significant interspecies differences in the expression and distribution of PKC isoforms in the gastrointestinal tract (e.g., Ref. 11). This should be taken into account when comparisons between species are made. There are also likely to be regional differences along the length of the gut. For this reason, we worked exclusively with the guinea pig ileum, the region in which most pharmacological
experiments have been conducted and in which the expression of the various PKC isoforms is best characterized (39, 40).

**Summary**

We demonstrated the functional coupling of muscarinic receptor activation to the translocation of PKC-δ to smooth muscle filaments. This observation fits well with the likely cellular functions of this isoform. The results presented in this study show that the immunohistochemical analysis of PKC isoform-specific translocation in response to pharmacological agents can be used to predict the functional roles of these kinases.

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**REFERENCES**


**G897 PKC-8 TRANSLATION AND SUSTAINED CONTRACTION**


