Differential autophosphorylation of CaM kinase II from phasic and tonic smooth muscle tissues

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Am J Physiol Cell Physiol 283: C1399–C1413, 2002. First published July 3, 2002; 10.1152/ajpcell.00020.2002.—Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) is regulated by calcium oscillations, autophosphorylation, and its subunit composition. All four subunit isoforms were detected in gastric fundus and proximal colon smooth muscles by RT-PCR, but only the γ and δ isoforms are expressed in myocytes. Relative γ and δ message levels were quantitated by real-time PCR. CaM kinase II protein and Ca2+/calmodulin-stimulated (total) activity levels are higher in proximal colon smooth muscle lysates than in fundus lysates, but Ca2+ activity levels are higher in proximal colon smooth muscles. CaM kinase II autonomous activity is higher in fundus lysates and restored its responsiveness to Ca2+.

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Ca2+/CALMODULIN-DEPENDENT PROTEIN KINASE II (CaM kinase II) mediates many cellular responses to elevated Ca2+ in a wide variety of cells and tissues (33). CaM kinase II is involved in the regulation of ion channels, cytoskeletal dynamics, gene transcription, neurotransmitter synthesis, insulin secretion, and cell division (5a). In vascular smooth muscle tissues and cultured cells, CaM kinase II is implicated in the modulation of myosin light chain kinase sensitivity to Ca2+ and in regulation of cell migration, and it is involved in the control of Ca2+ channels, sarcoplasmic reticulum Ca2+-ATPase activity, and MAP kinase activation (2, 3, 10, 24, 37). CaM kinase II is activated by physiological Ca2+ elevations in vascular smooth muscle cells, and its inhibition decreases contraction and force maintenance by inhibiting MAP kinase activation and myosin light chain (LC20) phosphorylation (2, 18, 26). In cardiac muscle phosphorylation of phospholamban Thr17 by CaM kinase II in response to β-adrenergic stimulation activates the sarcoplasmic reticulum Ca2+-ATPase, resulting in faster relaxation (31). In addition, the membrane potential and excitability of proximal colon smooth muscle cells are modulated by CaM kinase II regulation of delayed rectifier K+ currents and Ca2+-activated K+ channels (19, 20). These findings indicate that a number of proteins are substrates for CaM kinase II in smooth muscle and suggest that CaM kinase II can function at several points to enhance or attenuate the contractile response.

CaM kinase II holoenzymes are multimers composed of 6–12 kinase subunits arranged as a stacked pair of hexameric rings (33). The central core of the holoenzyme contains the COOH-terminal association domains of each subunit, with the variable domain and NH2-terminal regulatory and catalytic domains extending outward (33). Four genes encode the kinase subunit isoforms (α, β, γ, δ), and alternative splicing within the variable domain generates additional diversity (39). The α- and β-isoforms have narrow distributions, being restricted to neuronal and endocrine tissues, whereas the γ- and δ-isoforms are ubiquitously expressed within neuronal and nonneuronal tissues (8). The functional significance of many of the variable domains to the enzymatic activity of CaM kinase II is not clear. Variable domain region I regulates the cytoplasmic distribution of the β-isoform and increases its affinity for Ca2+/calmodulin (CaM) but has no effect on the affinity of the γA isoform for Ca2+/CaM (21, 40). Variable domain region III is responsible for the nuclear localization of δB CaM kinase II (25, 40).

Ca2+/CaM binding to the holoenzyme kinase subunits results in the rapid phosphorylation of Thr286.
(numbering based on the α-isof orm) on adjacent activated subunits (5a). Two consequences of Thr286 autophosphorylation are that 1) the rate of Ca2+/CaM dissociation in response to Ca2+ removal is decreased by several orders of magnitude, and 2) the kinase maintains activity toward its substrates in the absence of Ca2+/CaM (independent or autonomous activity) (5a). Thus the phosphorylation of target substrates by CaM kinase II in response to transient increases in cytosolic Ca2+ levels is sustained after the Ca2+ levels decrease. The Ca2+-independent activity of CaM kinase II plays an important role in the physiological response of cells to transient Ca2+ increases (33). Additional autophosphorylation in the CaM-binding domain at Thr305/306 following Thr286 autophosphorylation prevents subsequent CaM binding and lowers total CaM kinase II activity levels (13, 14). Thr305/306 autophosphorylation also promotes CaM kinase II dissociation from postsynaptic sites, providing a mechanism for regulating the subcellular distribution of autonomous CaM kinase II (30). The combined effects of these dual autophosphorylations result in complex activation of the holoenzyme in response to Ca2+ oscillations (7, 12, 13).

The level of autonomous activity of CaM kinase II in a tissue lysate is an indication of prior activation of the enzyme because Thr286 autophosphorylation can only occur after Ca2+/CaM binding to the holoenzyme. However, because Thr286/305/306 autophosphorylation regulates the number of kinase subunits that can bind Ca2+/CaM, the extent of Thr286 autophosphorylation is also regulated by Thr305/306 autophosphorylation (12–14). Thus identical CaM kinase II holoenzymes with different levels of Thr286 and Thr305/306 autophosphorylation will have different kinetics of CaM association and dissociation and of activation (12–14). However, the subunit composition of the CaM kinase II holoenzyme also regulates the kinetics of activation, autophosphorylation, and CaM dissociation in response to Ca2+ oscillations (5a, 7, 26). The holoenzyme structure of CaM kinase II and the effects of Thr286 autophosphorylation on enzyme activation led to the hypothesis that CaM kinase II can be activated to different levels in response to different Ca2+ oscillation frequencies (12). The in vitro study of immobilized CaM kinase II by De Koninck and Schulman provides strong evidence supporting this hypothesis (7).

The proximal colon and gastric fundus are representative phasic and tonic gastrointestinal smooth muscles, respectively, that are characterized by different Ca2+ signaling patterns and sensitivities to excitation-contraction coupling (5, 34). Because CaM kinase II activity levels are modulated by Ca2+ oscillations, we are investigating the characteristics of CaM kinase II expressed in fundus and proximal colon smooth muscle tissues to test the hypothesis that tissues with different Ca2+ signaling patterns express CaM kinase II holoenzymes with different activation properties. Because the subunit composition influences activation, we investigated the CaM kinase II isoforms expressed in fundus and proximal colon smooth muscle tissues by Western blot and real-time PCR analysis. We investigated the regulation of CaM kinase II activation by Thr286 and Thr305/306 autophosphorylation by measuring the total and autonomous CaM kinase II activity levels and comparing the kinetics of generation of CaM kinase II autonomous activity in each smooth muscle tissue. Generation of autonomous CaM kinase II activity in fundus lysates required prior alkaline phosphatase treatment. Total CaM kinase II activity levels in fundus are also increased following alkaline phosphatase treatment. Our findings indicate that gastric fundus and proximal colon are characterized by CaM kinase II holoenzymes having distinct enzymatic characteristics and may be differentially regulated by Thr286 and Thr305/306 autophosphorylation. Incubation of fundus and proximal colon smooth muscle tissues with acetylcholine (ACh) increased autonomous CaM kinase II activities. The CaM kinase II inhibitor KN-93 enhanced the generation of tone in fundus and inhibited phasic contractions in proximal colon smooth muscle tissues in response to ACh stimulation. Together, these results indicate that CaM kinase II is activated by contractile stimuli and modulates contractile force in fundus and proximal colon smooth muscle tissues in vivo.

MATERIALS AND METHODS

Materials. Alkaline phosphatase and EDTA-free protease inhibitor tablets were purchased from Roche (Indianapolis, IN). CaM was obtained from Sigma (St. Louis, MO). [γ-32P]ATP (6,000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Autocamtide-2 was obtained through United Bio Medical Research (Seattle, WA). Taq DNA polymerase, dNTPs, and SuperScript II reverse transcriptase were purchased from Life Technologies (Gaithersburg, MD). Primers were synthesized by Life Technologies. Goat anti-CaM kinase II α, β, γ, and δ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-P04-Thr286 antibody (anti-active CaM kinase II) was purchased from Promega (Madison WI). This antibody recognizes the sequence surrounding the autophosphorylated Thr286 or Thr287 in CaM kinase II γ- and δ-subunits [MHRQET(P04)VDCLKKFN]. Alkaline phosphatase-conjugated rabbit anti-goat IgG antibodies were obtained from Chemicon (Temecula, CA). l-Phenylalanine and ACh were from Sigma. KN-93 and KN-92 were purchased from Biomol (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. CD-1 mice were purchased from Charles River (Cambridge, MA).

CaM kinase II activity assays. Fundus and proximal colon smooth muscle tissues were prepared from the stomachs and colons removed from adult CD-1 mice. Briefly, mice were anesthetized with CHCl3, followed by cervical dislocation and removal of tissues as approved by the Institutional Animal Care and Use Committee. The fundus and proximal colon tissues were pinned out in a Sylgard-lined dish and washed with Ca2+-free Hanks’ buffer (125 mM NaCl, 5.36 mM KCl, 15.5 mM NaOH, 0.336 mM Na2HPO4, 0.44 mM KH2PO4, 10 mM glucose, 2.9 mM succrose, and 11 mM HEPES, pH 7.2). The mucosa and submucosa layers were removed with fine-tipped forceps. Fundus and proximal colon smooth muscle tissues were obtained as described above and pinned out in small dishes containing Krebs buffer (120 mM NaCl, 6 mM KCl, 15 mM NaHCO3, 12 mM glucose, 3 mM MgCl2, 1.5 mM Na2HPO4, and 3.5 mM CaCl2, pH 7.2). For determining the
effect of ACh on CaM kinase II activity, the tissues were equilibrated in Krebs buffer for 1 h at 37°C and then incubated at 37°C for various times in the absence or presence of 10 μM ACh or for 30 min with various ACh concentrations. KN-93 was added to the equilibrated tissues 20 min before the addition of ACh. After treatment, the tissues were collected, frozen in liquid nitrogen, and stored at −80°C until used for the CaM kinase II assays. When needed for assays, frozen tissues were homogenized at 4°C with a glass tissue grinder in lysis buffer (50 mM MOPS, 2% Nonidet P-40, 100 mM Na3P2O7, 100 mM NaF, 250 mM NaCl, 3 mM EGTA, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor tablet). Homogenates were centrifuged at 16,000 g for 15 min at 4°C. The supernatant was aliquoted and stored at −80°C until the kinase assay was performed. Kinase assays were done at least three times in triplicate from each tissue from three animals. Protein concentrations were determined by using the Bradford assay with bovine gamma globulin as standard.

CaM kinase II activity in the lysates was assayed in a total volume of 10 μL containing 50 mM MOPS (pH 7.4), 10 mM magnesium acetate, 0.2 mM [γ-32P]ATP (500–1,000 cpm/pmL), 20 μM autophosphatase-2 (a specific CaM kinase II peptide substrate): KKALRRQETVDAL, plus 600 nM CaM and 0.8 mM CaCl2 (for total activity) or 1.0 mM EGTA (for autonomous activity) (11). Reactions were initiated by the addition of 3 μg of lysate protein, allowed to proceed at 30°C for 2 min, and terminated by spotting 10 μL of the reaction onto P-81 paper. The papers were washed thoroughly in 75 mM phosphoric acid, rinsed in ethanol, and dried. The papers were added to vials of Ecoscint O (National Diagnostics), and the affixed radioactivity was quantified by scintillation counting. Kinase activity was calculated and expressed as pmol of P1 incorporated per minute per microgram of lysate protein. Autonomous activity is expressed as a percentage of the total Ca2+/CaM-dependent activity. To measure the generation of autonomous activity, tissue lysates were preincubated for either 30 s or 5 min in 50 mM MOPS (pH 7.4), 10 mM magnesium acetate, 3 mM EGTA, 4 mM CaCl2, 400 nM CaM, 0.2 mM ATP, and 15 mM 2-mercaptoethanol at 30°C. Control lysates lacked Ca2+/CaM and ATP. Reactions were initiated by the addition of lysate and terminated by the addition of 55-μL aliquots to tubes (at 4°C) containing 11 μL of 90 mM EDTA (32). Aliquots (5 μL) were assayed as described above for total and autonomous activity; however, 6 mM CaCl2 was added to the total tubes to compensate for the EDTA.

Dephosphorylation of fundus and proximal colon smooth muscle tissue lysates. Smooth muscle tissues were obtained and homogenized as described in CaM kinase II activity assays, with the exception that phosphatase inhibitors (Na3P2O7 and NaF) were omitted from the homogenization buffer. Lysates were incubated with calf intestinal alkaline phosphatase at 37°C for 40 min by using a range of 0.2–1.0 unit of enzyme per microgram of lysate. Phenylalanine was added to the samples (5 mM final concentration) to inhibit alkaline phosphatase (17). Assays for total and autonomous CaM kinase II activity were performed on control and dephosphorylated lysates as described above.

SDS-PAGE and Western blot analysis of CaM kinase II from proximal colon and fundus smooth muscle tissues. Fundus and proximal colon smooth muscle tissue lysates were obtained from CD-1 mice, and protein concentrations were determined as described in CaM kinase II activity assays. Tissue lysate proteins were separated by SDS-PAGE (7.5%) and transferred to nitrocellulose by Western blotting. The amount of lysate protein per lane is indicated. The blots were incubated with primary and secondary antibodies, washed, and processed for image detection by using the Western-Light chemiluminescence system from Tropix (Bedford, MA). The CaM kinase II antibodies were used at 1:200 dilutions, and the alkaline phosphatase-conjugated rabbit anti-goat IgG antibody was used at a 1:5,000 dilution. Protein bands were visualized with a charge-coupled device camera-based detection system (Epi Chem II; UVP Laboratory Products). The collected images were opened in Adobe Photoshop and inverted for analysis. Densitometry was carried out by using Un-Scan-It software from Silk Scientific. Δ316Ca kinase II was baculovirus-expressed and purified by CaM-Sepharose chromatography as described previously (23). Autophosphorylation and alkaline phosphatase treatment of purified Δ316Ca kinase II was carried out as described above.

RT-PCR of α, β, γ, and δ CaM kinase II isoforms from proximal colon and fundus smooth muscle tissues. Fundus and proximal colon smooth muscle tissues were obtained from CD-1 mice, and smooth muscle cells from each tissue were enzymatically dispersed and collected as described (9). Total RNA was purified by using the Epicentre MasterPure RNA purification kit. First-strand cDNA was synthesized from 4.0 μg of total RNA by using random primers and SuperScript II Moloney murine leukemia virus RNase H– reverse transcriptase (Life Technologies). PCR was performed on 2 μL of cDNA with a final concentration of 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 μM primers, and 0.025 unit of Taq DNA polymerase (Life Technologies). First-strand cDNA preparations were monitored for the absence of genomic DNA by using intron-flanking β-actin primers (9). To identify splice variants, we used primers designed to flank the variable region of each CaM kinase II isoform. The δ and γ forward primers were designed at nucleotides encoding for amino acids WDTVTPE (5'-GGGACACAGTGACACCTGAA-3' and 5'-GGGACAGTCATCCTGAA-3', respectively), the δ reverse primer was complementary to bases encoding for amino acids EALGNL (5'-CGAGGTCCTTCGGAAGGAGG-3'), and the γ reverse primer was complementary to the nucleotide sequence encoding amino acids GLN5 (5'-CTCCACAGGTTACAAACC-3'). The forward α primer corresponds to the nucleotides encoding amino acids LLASKL (5'-GGTGTCGTCCTCGAAGCTC-3'), and the α reverse primer is complementary to the nucleotide sequence encoding amino acids GLDFHR (5'-ATCGATGAAGTTCAGGCC-3'). The forward β primer corresponds to the nucleotides encoding amino acids EYLRKE (5'-CGAGGTCTCCTCAGGAGG-3'), and the β reverse primer is complementary to the nucleotide sequence encoding amino acids EALGNL (5'-GACCGAGTGCTGCCAGGCT-3'). The PCR profile for the δ primers was as follows: 30 s of denaturation at 94°C, 30 s of annealing at 63°C, and 1 min of extension at 72°C for 40 cycles, with a final 5-min extension at 72°C. The PCR profile for the α, β, and γ primers was the same as that for the δ primers, except for annealing at 55°C, 61°C, and 63°C, respectively. The PCR products were separated by 4.0% agarose gels and visualized with GelStar stain (BioWhittaker Molecular Applications; Rockland, CA.). The fragments were excised from the gel, isolated with Micropure Separators (Amicon), and cloned with the Topo II TA cloning kit (Invitrogen). Putative positive clones were identified by diagnostic PCR. The plasmids were subsequently purified by using the Quantum Prep plasmid miniprep kit (Bio-Rad), and the cloned PCR fragments were sequenced using an ABI dye terminator cycle sequencer (Applied Biosystems, Foster City, CA.).
primers). The forward primers were designed at nucleotides encoding for amino acids NIVRLH (α) (5′-CAATATGCTC-GACCTCAAC-3′), VHFRDKL (β) (5′-GCTGCTCACA-GACCTCAAG), IHQHDI (γ) (5′-ATCCACCAACAGCTGACATCG-3′), and LNGIVH (δ) (5′-CTTAAATGGCAGATGTCC-3′). The reverse primers are complementary to the bases encoding for amino acids LHCHQM (α) (5′-CATGCT-GTGACAGTGAC-3′), KPVDIV (β) (5′-CCAGGTA-CTCA-CAGGTTCACG-3′), PEVLRK (γ) (5′-CTTTCCTCAACAGCTCAGG-3′), and EVLRKDP (δ) (5′-GGATCTTTCCAGGTAC-3′). The PCR profile was as follows: an initial heating for 5 min at 94°C, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 57°C, and extension for 30 s at 72°C, with a final extension for 5 min at 72°C. The PCR products were separated in 4.0% agarose gels and visualized with GelStar stain. The fragments were excised from the gel, isolated by using MicroPure Separators (Millipore, Bedford, MA), and sequenced by using dye terminator cycle sequencing.

Real-time PCR analysis of α, β, γ, and δ CaM kinase II isoforms from proximal colon and fundus smooth muscle tissues. Total RNA was purified as described above, and the pan α, β, γ, or δ primers were used in the real-time PCR reactions. Relative quantitation of the α, β, γ, or δ CaM kinase II isoforms was performed by using SYBR green detection real-time PCR. cDNA from CD-1 mouse brain cerebellum was initially diluted 1:20 and then serially diluted 1:2 for six dilutions, with 2 μL subsequently used as template for the standard curves of the CaM kinase II isoforms and the internal standard, GAPDH. Proximal colon and fundus cDNA samples were diluted 1:20 and 1:40, with 2 μL used as template in triplicate reactions for the CaM kinase II isoforms and GAPDH. The SYBR green master mix (Applied Biosystems) and the gene-specific pan primers at final concentrations of 0.3 μM were used for amplification and detection of product on an ABI Prism 7700 sequence detection system (Applied Biosystems). The relative quantitation was then determined by the standard curve method (ABI, User Bulletin no. 2).

Mechanical responses of fundus and proximal colon smooth muscle tissues. A standard organ bath technique was employed to measure changes in isometric force provided by fundus muscle strips. The mucosa was removed from the gastric fundus by sharp dissection, and strips of muscle (~6 × 3 mm) were isolated. One end of the tissue was attached to a fixed mount, and the opposite end to a Fort 10 isometric strain gauge (WPI, Sarasota, FL). The muscles were immersed in organ baths maintained at 37 ± 0.5°C with oxygenated Krebs solution (KRB). A resting force of 400 mg was applied to set the muscles at optimum length (data not shown). This was followed by an equilibration period of 1 h, during which time the bath was continuously perfused with oxygenated KRB. Mechanical responses were recorded on a personal computer running Aeqnowledge 3.2.6 (BIOPAC Systems, Santa Barbara, CA).

RESULTS

Identification of CaM kinase II isoforms in fundus and proximal colon smooth muscle tissues. Isoform-specific primer sets that anneal to sequences common to each reported isoform splice variant were used in RT-PCR analysis to first determine which mRNAs are present for α, β, γ, and δ CaM kinase II in murine proximal colon and fundus smooth muscle tissues. As shown in Fig. 1A, PCR products were obtained from the

α, β, γ, and δ CaM kinase II primer pairs. Sequencing confirmed that the PCR products obtained with each CaM kinase II isoform-specific primer pair corresponded to α, β, γ, and δ CaM kinase II, indicating that mRNAs for all four CaM kinase II isoforms are present in murine proximal colon and fundus smooth muscle tissues. It is well established that the expression of α and β CaM kinase II is restricted to neuronal and neuroendocrine cells and that γ and δ CaM kinase II are expressed in cardiac, skeletal, and vascular smooth muscle cells (5a, 29, 32, 38). To determine which CaM kinase II isoforms are expressed in gastrointestinal smooth muscle cells, cDNA from isolated fundus and proximal colon smooth muscle cells was analyzed by RT-PCR. With the use of cDNA from collected fundus smooth muscle cells, no PCR products were obtained with the α and β CaM kinase II primers (Fig. 1C). In contrast, PCR products were obtained with the γ and δ CaM kinase II primers. These findings indicate that murine fundus and proximal colon smooth muscle cells express γ and δ CaM kinase II. These findings also suggest that the α and β CaM kinase II detected in the fundus and proximal colon smooth muscle tissue samples are most likely expressed in enteric neurons.

Relative expression of CaM kinase II isoforms in fundus and proximal colon smooth muscle tissues. Real-time PCR analysis was used to quantitate the relative message levels of the α, β, γ, and δ CaM kinase II expressed in murine fundus and proximal colon smooth muscle tissues. The PCR products were amplified by the appropriate isoform-specific pan primers, and amounts produced were measured relative to
GAPDH. As shown in Fig. 2, in fundus and proximal colon smooth muscle tissues, δ CaM kinase II message levels are more abundant than γ CaM kinase II message levels, relative to GAPDH message levels. The δ:γ ratio in fundus is ~1.66:1, whereas the δ:γ ratio in proximal colon smooth muscle tissues is ~1.3:1. Relative to GAPDH and to γ and δ CaM kinase II, the message levels of the α- and β isoforms are present at extremely low levels in fundus and proximal colon smooth muscle tissues. These findings are consistent with the RT-PCR results (Fig. 1C) showing that message for the α- and β isoforms was not amplified from isolated smooth muscle cells.

Detection of CaM kinase II isoforms in murine fundus and proximal colon smooth muscle tissues. The results of the SDS-PAGE and Western blot analysis of gastric fundus and proximal colon smooth muscle tissue lysates using CaM kinase II isoform-specific antibodies are shown in Fig. 3. Murine brain lysates were used as a positive control for α and β CaM kinase II expression (5a, 26). In agreement with previous reports, a strong signal at ~50 kDa was generated from brain lysate by using the anti-α CaM kinase II antibody (5a, 26). Similarly, a protein band at ~60 kDa was observed from brain lysates by using the anti-β CaM kinase II antibody. The β CaM kinase II staining is less intense than the α CaM kinase II staining from brain lysates. These results are consistent with previous findings that α CaM kinase II is the predominant isoform expressed in neuronal tissues (5a, 26). The anti-α CaM kinase II antibody generated a modest signal at 50 kDa from proximal colon lysates and an almost undetectable signal from fundus lysates. Protein staining of fundus and proximal colon smooth muscle tissue lysates using the anti-β CaM kinase II antibody was not detected. These results indicate that proximal colon smooth muscle tissue is characterized by a higher level of α CaM kinase II expression than fundus and suggest that β CaM kinase II is not ex-
pressed or is expressed at levels below the limits of detection in both smooth muscle tissues (see below). It should be noted here that the amount of protein from fundus and proximal colon lysates in each lane is twice the amount of the protein in the lanes with brain lysates. These results indicate that the α and β CaM kinase II expression levels in fundus and proximal colon smooth muscle tissues are lower than the expression levels found in brain.

A number of studies indicate that γ and δ CaM kinase II are the predominant isoforms expressed in nonneuronal tissues, including vascular smooth muscle and cardiac muscle (15, 32). The γ- and δ-isofoms range in molecular mass from 51 to 63 kDa (33). Rat aorta and heart homogenates were used as positive controls for the anti-γ and -δ CaM kinase II antibodies, respectively. As shown in Fig. 3, the anti-γ and -δ CaM kinase II antibodies resulted in strong staining of protein bands at ~60 kDa in fundus and proximal colon smooth muscle lysates. These results, and the RT-PCR results of Fig. 1C, indicate that γ and δ CaM kinase II proteins are expressed in murine fundus and proximal colon smooth muscle cells. Densitometric analysis indicates that the signal intensities of the γ and δ bands from proximal colon lysates are 1.5 ± 0.3- and 1.35 ± 0.2-fold higher, respectively, than the corresponding γ and δ bands from fundus lysates. These findings indicate that the γ and δ CaM kinase II expression levels are slightly higher in proximal colon smooth muscle tissues than in fundus.

Identification of CaM kinase II isoform splice variants in fundus and proximal colon smooth muscle tissues. RT-PCR analysis was carried out by using isoeform-specific primers flanking the variable region of CaM kinase II to identify the α, β, γ, and δ splice variants expressed in murine proximal colon and fundus smooth muscle tissues. After agarose gel electrophoresis, the PCR products were excised from the gels and cloned by using Topo cloning vectors. As shown in Fig. 4A, four similarly sized PCR products ranging

![Figure 4](http://ajpcell.physiology.org/)

**Fig. 4.** CaM kinase II isoform splice-variants detected by RT-PCR in murine fundus and proximal colon smooth muscle tissues. A: PCR products obtained using primers flanking the variable regions of CaM kinase II γ, δ, α, and β. Aliquots of the PCR reactions were separated by agarose gel (4%) electrophoresis and visualized by SYBER green staining: lane 1, fundus; lane 2, proximal colon. Variable domain structure and amino acid sequences of CaM kinase II γ and δ (B) and α and β (C) isoform splice variants detected in murine fundus and proximal colon smooth muscle tissues are shown.
from ~550 to 750 bp and 450 to 700 bp were obtained from fundus and proximal colon smooth muscle tissues with the γ- and δ-specific primers, respectively. Sequencing the PCR products obtained with the γ-isof orm-specific primers identified the 550-, 650-, 700-, and 750-bp PCR products as γC, γD, γB, and γA, respectively. In contrast, although four PCR products are visible in the gel, following cloning and sequencing, all five δ CaM kinase II splice variants were identified. Sequencing identified the 450-, 550-, 600-, and 700-bp PCR products from the δ-isof orm-specific primers as δC, δB/δE, δD, and δA, respectively. The predicted sizes of the δB and δE amplicons differ from each other by only nine base pairs; thus these two amplicons are unlikely to be separated by the agarose gel electrophoresis. In addition, by using the variable domain-flanking primers specific for α CaM kinase II, we amplified two PCR products of ~285 and 318 bp from fundus and proximal colon smooth muscle tissues and determined them to be α and α33 CaM kinase, respectively, by sequence analysis. Five PCR products were obtained by using the variable domain-flanking primers specific for β CaM kinase II. After cloning and sequencing, on the basis of the predicted sizes of the splice variant amplicons, the 377-, 404-, 449-, and 635-bp products correspond to βC, βE, βD, and β CaM kinase II, respectively. Sequence analysis indicates that the 318-bp amplicon corresponds to α33, indicating that the β-isof orm-specific primers also annealed to the complementary sequences of α33 CaM kinase II. The nomenclature of the α, β, γ, and δ splice variants is from Tombes and Krystal (39). γH and γL were previously identified in rabbit liver as novel splice variants and were named γH and γL, respectively (36). Our results are the first demonstration of the expression of these two γ splice variants outside the liver, indicating that they have a wider tissue distribution. Figure 4B shows a chart of the variable region domain structure of these γ and δ splice variants and their corresponding predicted amino acid sequences (39).

Total and autonomous CaM kinase II activity in fundus and proximal colon smooth muscle lysates. Because the subunit composition and levels of autophosphorylation influence the kinase activity of the CaM kinase II holoenzyme, we compared total and autonomous CaM kinase II activity from murine fundus and proximal colon smooth muscle tissue lysates using the peptide substrate autacamide-2. Addition of the specific CaM kinase II inhibitor KN-93 (1 μM) to the tissue lysates inhibited 90% of the total kinase activity toward autacamide-2 (data not shown), indicating that, similar to previous reports, the autacamide-2 kinase activity measured in the tissue lysates is due to CaM kinase II (26, 35). Total CaM kinase II activity in fundus smooth muscle lysates is threefold lower than the activity in proximal colon smooth muscle tissue lysates (1.09 ± 0.19 vs. 3.65 ± 0.74 pmol·min⁻¹·μg⁻¹, respectively). In contrast, autonomous CaM kinase II activity in fundus is 3.5-fold higher than the autonomous activity in proximal colon smooth muscle tissue (0.32 ± 0.07 vs. 0.09 ± 0.03 pmol·min⁻¹·μg⁻¹, respectively). Thus, as a percentage of total CaM kinase II activity, autonomous CaM kinase II activity is high in fundus smooth muscle tissues (28.9 ± 3.35%) but low in proximal colon smooth muscle tissues (3.6 ± 0.6%). These findings indicate that fundus smooth muscle tissue contains higher levels of autonomous CaM kinase II activity than proximal colon smooth muscle tissue.

Autonomous CaM kinase II levels in fundus and proximal colon smooth muscle tissues were also compared by Western blot analysis using anti-PO4-Thr286 antibodies (18). Increasing amounts of fundus or proximal colon smooth muscle tissue lysates were probed with anti-PO4-Thr286 antibodies. As shown in Fig. 5, the signals obtained from fundus smooth muscle lysates with the anti-PO4-Thr286 antibody were more intense than the corresponding signals from proximal colon smooth muscle tissues. These findings are consistent with the findings of threefold higher autonomous activity levels in fundus compared with proximal colon smooth muscle tissue and provide additional evidence that fundus smooth muscle tissues are characterized by higher levels of autonomous CaM kinase II activity than proximal colon smooth muscle tissue. Because autonomous activity is an indication of prior activation by Ca²⁺/CaM, these findings strongly suggest that Ca²⁺/CaM activation of CaM kinase II occurs to a greater extent in fundus smooth muscle tissue than in proximal colon smooth muscle tissue.

The initial level of Thr₂₈⁶ autophosphorylation influences the subsequent development of additional auton-

**Fig. 5.** Western blots of autophosphorylated CaM kinase II in murine fundus (A) and proximal colon tissues (B). Autophosphorylated CaM kinase II was visualized from tissue lysates by using an antibody that recognizes PO₄-Thr²₈⁶ of CaM kinase II. Lanes 1, 2, 3, and 4 contain 10, 20, 40, and 60 μg of lysate protein, respectively. Total CaM kinase II activity was detected by using an antibody that recognizes all four CaM kinase II isoforms. Results are representative of 3 Western blotting experiments from fundus and proximal colon smooth muscle tissue lysates obtained from several animals.
omous activity (7). The levels of autonomous CaM kinase II activity achieved typically range from 20 to 80% of total activity (26). Because of the differences in the levels of autonomous CaM kinase II activities from fundus and proximal colon smooth muscle tissues, we examined the generation of autonomous CaM kinase II activity in lysates of fundus and proximal colon smooth muscle tissues. As expected, incubation of proximal colon smooth muscle lysates with Ca2+/CaM readily increased autonomous CaM kinase II activity. As shown in Fig. 6, within 5 min of stimulation with Ca2+/CaM, CaM kinase II autonomous activity in proximal colon smooth muscle lysates increased from 3.8 ± 0.9 to 52 ± 4.1% of total CaM kinase II activity. In contrast, CaM kinase II in fundus smooth muscle lysates was relatively unresponsive to Ca2+/CaM-induced increases in autonomous activity. Autonomous CaM kinase II activity increased from 29.2 ± 4.9 to only 37.8 ± 4.7% of total CaM kinase II activity (Fig. 6). Total CaM kinase II activity levels were unchanged at each time point (data not shown).

**Activation of fundus smooth muscle CaM kinase II by alkaline phosphatase.** A simplified diagram of the current model of CaM kinase II regulation by autophosphorylation is diagrammed in Fig. 7. Ca2+/CaM can cause little or no additional Thr286 autophosphorylation and autonomous activity as the number of Thr286-autophosphorylated kinase subunits of a holoenzyme increases (Fig. 7, II and III). This model and our results showing that autonomous CaM kinase II activity in fundus smooth muscle lysates is 30% of the total CaM kinase II activity and is largely resistant to generation of additional autonomous activity suggest that fundus smooth muscle CaM kinase II may be almost fully Thr286 phosphorylated (Fig. 7, III). Evidence in support of this conclusion is provided by the results of the experiments comparing autonomous CaM kinase II activity before and after alkaline phosphatase treatment. We first confirmed that alkaline phosphatase dephosphorylates Thr286-autophosphorylated CaM kinase II using autophosphorylated Δ316α CaM kinase II. As shown in Fig. 8, lane 1, the anti-PO4-Thr286 antibody generated a strong signal toward autophosphorylated Δ316α CaM kinase II. However, no signal was detected following incubation of autophosphorylated Δ316α CaM kinase II with alkaline phosphatase (Fig. 8, lane 2). These results demonstrate that alkaline phosphatase dephosphorylates Thr286. Next, we examined the ability of alkaline phosphatase to dephosphorylate CaM kinase II in fundus and proximal colon smooth muscle lysates and the ability of CaM kinase II to undergo Thr286 autophosphorylation following alkaline phosphatase treatment of fundus and proximal colon smooth muscle lysates. Lysates were incubated with alkaline phosphatase, followed by phenylalanine (5 mM final concentration) to inhibit alkaline phosphatase, as previously reported (17). The lysates were then incubated CaM, CaCl2, and ATP to allow Thr286 autophosphorylation to occur. No signal was detected from alkaline phosphatase-treated fundus and proximal colon smooth muscle lysates with the anti-PO4-Thr286 antibody (Fig. 8B, lanes 1 and 4). However, strong signals were generated from the lysates that were incubated alkaline phosphatase, followed by phenylalanine, and CaM, CaCl2, and ATP (Fig. 8, lanes 2 and 3). These results demonstrate that alkaline phosphatase dephosphorylates Thr286 in fundus and proximal colon smooth muscle tissue lysates. These results also demonstrate the ability of CaM kinase II in fundus and proximal colon smooth muscle tissue lysates to undergo Thr286 autophosphorylation following inhibition of alkaline phosphatase by 5 mM phenylalanine.

Because elevated autonomous activity levels are due to increased Thr286 autophosphorylation, dephosphorylation of Thr286 should decrease autonomous activity and restore the ability of the dephosphorylated holoenzyme (Fig. 7, I) to generate autonomous activity in response to Ca2+/CaM stimulation. As shown in Fig. 9, the initial value of 23% autonomous CaM kinase II activity decreased to 7% of total CaM kinase II activity in alkaline phosphatase-treated fundus lysates. Subsequent inhibition of alkaline phosphatase with 5 mM phenylalanine and incubation of the fundus lysates with Ca2+/CaM for 5 min increased autonomous CaM kinase II activity to 32% of total CaM kinase II activity. In contrast, autonomous CaM kinase II in control fundus smooth muscle lysates remained level at 23–27% of total CaM kinase II activity (Fig. 9). These results suggest that Thr286 dephosphorylation by alkaline phosphatase caused the decrease in autonomous activity. The subsequent Ca2+/CaM-induced increase in autonomous activity from 7 to 32% provides additional evidence that alkaline phosphatase dephosphorylated Thr286, thus making more CaM kinase II subunits in fundus smooth muscle lysates available for subsequent Ca2+/CaM-induced Thr286 autophosphorylation.

The current model of CaM kinase II regulation by Ca2+/CaM and autophosphorylation also predicts that as the number of Thr286-autophosphorylated subunits increases, the number of Thr285/286-autophosphory-
lated subunits can also increase. As diagrammed in Fig. 7, Thr^{305/306} autophosphorylation can only proceed after autonomous activity has been induced by Thr^{286} autophosphorylation and Ca^{2+}/CaM dissociates from the enzyme (Fig. 7, IV). Ca^{2+}/CaM binding and Thr^{305/306} autophosphorylation are mutually exclusive (Fig. 7, V) (13, 14). As stated above, this model and our results suggest that fundus smooth muscle CaM kinase II may also be almost fully Thr^{286} phosphorylated. Because Thr^{305/306} autophosphorylation depends on prior Thr^{286} autophosphorylation, these findings suggest that CaM kinase II from fundus smooth muscle tissue lysates may also have a high level of Thr^{305/306} autophosphorylation (Fig. 7, IV). Because of its reduced ability to bind Ca^{2+}/CaM, the total activity from a CaM kinase II holoenzyme decreases with increasing Thr^{305/306} dephosphorylation (6, 13). Thus Thr^{305/306} dephosphorylation should increase total CaM kinase II activity. To determine whether fundus smooth muscle CaM kinase II is Thr^{305/306} phosphorylated, we measured total CaM kinase II activity in alkaline phosphatase-treated or untreated fundus and proximal colon smooth muscle lysates. Alkaline phosphatase treatment of fundus lysates increased total CaM kinase II activity fourfold, from 1.23 ± 0.47 to 4.85 ± 0.64 pmol·min^{-1}·μg^{-1}. In contrast, a smaller increase in total CaM kinase II activity was measured in alkaline phosphatase-treated proximal colon smooth muscle lysates (3.14 ± 0.98 to 6.99 ± 0.89 pmol·min^{-1}·μg^{-1}). These results suggest that fundus smooth muscle CaM kinase II holoenzymes contain a higher level of phosphorylated Thr^{305/306} than proximal colon smooth muscle CaM kinase II.

Alkaline phosphatase treatment of fundus smooth muscle lysates lowered autonomous activity but also increased total CaM kinase II activity fourfold. However, the decrease in autonomous activity (as a percentage of total activity) is not due just to the increase in total activity caused by alkaline phosphatase but also to a decrease in the amount of 32P incorporated into the autocamtide-2 substrate. Values of 0.32 ± 0.06 and 0.22 ± 0.04 pmol·min^{-1}·μg^{-1} were measured for
autonomous CaM kinase II activity in fundus lysates at 0 min without or with alkaline phosphatase treatment, respectively. In addition, although Ca\(^{2+}\)/CaM-induced autonomous activity levels (as a percentage of total activity) from alkaline phosphatase-treated and untreated lysates are similar (30–34% of total activity), the amount of \(^{32}\)P incorporation into autophosphatide-2 is higher from alkaline phosphatase-treated fundus lysates. Values of 0.33 ± 0.06 and 1.11 ± 0.3 pmol·min\(^{-1}\)·mg\(^{-1}\) were measured for autonomous CaM kinase II activity in fundus lysates at 5 min without or with alkaline phosphatase treatment, respectively.

Activation of CaM kinase II in fundus and proximal colon smooth muscle tissues by ACh. CaM kinase II is involved in the generation of contraction and maintenance of force in vascular smooth muscle (18, 26). In addition, the in situ Ca\(^{2+}\) dependence for CaM kinase II activation in cultured vascular smooth muscle cells falls within the range of cytosolic Ca\(^{2+}\) concentration increases induced by angiotensin II (1). However, our findings that CaM kinase II from fundus smooth muscle lysates is unresponsive to Ca\(^{2+}\)/CaM activation compared with the enzyme from proximal colon smooth muscle lysates suggest that CaM kinase II in fundus and proximal colon smooth muscles may show different sensitivities to physiological stimuli that increase cytosolic Ca\(^{2+}\) levels in these tissues. Because ACh is the neurotransmitter primarily responsible for Ca\(^{2+}\) mobilization and contraction of gastrointestinal smooth muscle tissues, we assessed the ability of ACh to activate CaM kinase II in fundus and proximal colon smooth muscle tissues. Fundus and proximal colon smooth muscle tissues were cultured in the absence or presence of 10 μM ACh, as described in MATERIALS AND METHODS. CaM kinase II activation was determined by measuring autonomous CaM kinase II activity in lysates from control and ACh-treated tissues. As shown in Fig. 10, CaM kinase II autonomous activity levels were elevated in a time- and dose-dependent manner in ACh-treated fundus and proximal colon tissues. After a 45-min incubation of fundus smooth muscle tissues with 10 μM ACh, autonomous CaM kinase II activity increased from 31 to 54% of total CaM kinase II activity. Similarly, incubation of proximal colon smooth muscle tissues with 10 μM ACh increased autonomous CaM kinase II activity from 7 to 19% of total CaM kinase II activity (Fig. 10A). In addition, autonomous CaM kinase II activity levels in both smooth muscle tissues increased with increasing ACh concentrations and plateaued between 5 and 10 μM ACh (Fig. 10B). Preincubation of the smooth muscle tissues with KN-93 (1 μM) prevented the ACh-induced increases in CaM kinase II autonomous activities (data not shown). These findings indicate that contractile stimuli activate CaM kinase II in fundus and proximal colon smooth muscle tissues.

Effects of KN-93 on contractions of gastric fundus and proximal colon smooth muscles. Because ACh activated CaM kinase II, the contribution of CaM kinase II to the contractile activity of the circular muscle layers of gastric fundus and proximal colon was determined. ACh (10 μM) caused the contractile tone of fundus smooth muscle to increase by 4.11 ± 0.8 mN (Fig. 11A). This increase in tone was reversible upon washout of ACh. Perfusion of muscle strips with KN-93
(5 μM) did not produce any resolvable change in basal tension. After a 30-min perfusion period with KN-93, the increase in contractile force produced by ACh increased to 7.9 ± 1.3 mN (Fig. 11B). Thus preincubation with KN-93 caused an ~90% increase in the amplitude of contractile force elicited by ACh. Perfusion of the proximal colon with ACh (10 μM) produced an increase in the tone of the circular muscle layer by 11.1 ± 1.7 mN and also increased the amplitudes of the phasic contractions (Fig. 11C). In contrast to the circular muscle layer of the fundus, incubation of proximal colon circular muscle with 1 μM KN-93 decreased spontaneous mechanical activity and reduced the tone produced by ACh by 2.9 ± 0.4 mN. KN-93 also reduced the steady-state amplitudes of ACh-induced phasic contractions to (Fig. 11D). The inactive KN-93 analog KN-92 (5 μM) had no effect on basal and ACh-induced contractions of fundus and proximal colon smooth muscles (data not shown). These findings indicate differential roles for CaM kinase II in regulating contractile activity of gastric fundus and proximal colon smooth muscle tissues.

DISCUSSION

The holoenzyme structure and subunit composition of CaM kinase II modulate its activation in response to Ca\(^{2+}\) oscillations (7). Although each subunit is independently activated by Ca\(^{2+}\)/CaM, the kinase subunits regulate each other by intersubunit autophosphorylation reactions on Thr\(^{286}\) and Thr\(^{305/306}\) (28, 33). The number of Thr\(^{286}\)-autophosphorylated subunits determines the sensitivity of the CaM kinase II holoenzyme to Ca\(^{2+}\)/CaM stimulation, and the level and duration of autonomous activity (7). Different frequencies and amplitudes of Ca\(^{2+}\) oscillations give rise to different levels of Thr\(^{286}\) autophosphorylation and to CaM kinase II holoenzymes with different levels of autonomous activity and sensitivities to Ca\(^{2+}\)/CaM stimulation (7). These findings suggest that tissues and cells having different Ca\(^{2+}\) oscillation patterns may express CaM kinase II holoenzymes characterized by different subunit compositions and levels of autophosphorylation. To test this hypothesis, we have initiated studies to characterize CaM kinase II in gastric fundus and proximal colon smooth muscle tissues. The fundus and proximal colon are tonic and phasic gastrointestinal smooth muscle tissues, respectively, with distinct Ca\(^{2+}\) signaling characteristics (5, 34). Tonic smooth muscles generally have higher resting Ca\(^{2+}\) levels compared with phasic smooth muscles (30). These differences in Ca\(^{2+}\) signaling underlie the cyclic depolarizations and repolarizations that determine the phasic contractile activity of the colon and also the tonic activity of the gastric fundus due to neural and hormonal regulation (16, 34).

RT-PCR analysis demonstrated the presence of message for α, β, γ, and δ CaM kinase II in fundus and proximal colon smooth muscle tissues (Fig. 1). In contrast, in isolated smooth muscle cells, the RT-PCR analysis shows only δ and γ CaM kinase II expression (Fig. 1C). These findings indicate that smooth muscle cells of the digestive tract express δ and γ CaM kinase II. These findings also suggest that α and β CaM kinase II are expressed in the enteric neurons, because it has been previously established that α and β expression is restricted to neurons (5a). A strong α CaM kinase II signal was obtained from brain lysates, whereas weaker signals were generated from fundus and proximal colon smooth muscle lysates. Although we detected β CaM kinase II in Western blots of murine brain lysates (Fig. 3), no signal was obtained from fundus and proximal colon smooth muscle tissue lysates. It is well established that CaM kinase II mes-

![Graph A: Autonomous CaM kinase II activity vs. time (min)](image)

![Graph B: Autonomous CaM kinase II activity vs. [ACh] (μM)](image)
Fig. 11. Mechanical responses of fundus or proximal colon smooth muscle to exogenous ACh before and after incubation with KN-93. ACh (10 μM) produced a sustained contraction of fundus muscle that was reversible upon washout (A). After 30-min perfusion with KN-93 (5 μM), the contractile response to ACh was significantly enhanced (B). Perfusion of proximal colon with ACh (10 μM) increased the tone and steady-state amplitudes of contractions, which was reversed upon washout (C). After perfusion with KN-93 (1 μM), tonic contraction and steady-state amplitudes of contractions were significantly decreased (D). These recordings are representative of 6 strips from 2 animals for each tissue.

SAGE levels correlate with protein levels (4). Thus the lack of a β CaM kinase II signal from fundus and proximal colon smooth muscle lysates is most likely due to a level of expression that is below the limits of detection by Western blot analysis. Indeed, the real-time PCR results indicate that the expression of CaM kinase II α and β expression is >100-fold lower than γ and δ expression in fundus and proximal colon smooth muscle tissues. In the brain, CaM kinase II mRNA is abundant and protein expression levels approach 2% of total (33). Similarly, immunohistochemical results suggest that α and β CaM kinase II may be expressed at high levels in enteric neurons (19). However, neuronal mRNA represents a small minority of the total mRNA purified from the fundus and proximal colon smooth muscle tissues, with the vast majority of mRNA purified from the smooth muscle cells.

Several subtypes of each CaM kinase II isoform are generated by alternative splicing (29, 34). Using RT-PCR analysis, we found the same two α, four β, five δ, and four γ CaM kinase II isoform splice variants expressed in murine fundus and proximal colon smooth muscle tissues: γB, γC, γI, γJ, δA, δB, δC, δD, δE, α, α33, β, β′, βc, and β′c. These results do not support the hypothesis that tissues having different Ca2+ oscillation patterns express different CaM kinase II subunit isoforms. However, the real-time PCR analysis indicates that both fundus and proximal colon have slightly different δ:γ ratios. We found δ:γ ratios of ~1.66:1 and 1.3:1 in fundus and proximal colon smooth muscle tissues, respectively. Although these differences are less than twofold, they may have significant functional implications for the CaM kinase II holoenzyme subunit compositions. For example, a heptameric CaM kinase II holoenzyme with a 1.3:1 δ:γ ratio indicates that four subunits are δ and three are γ. In contrast, a 1.66:1 δ:γ ratio suggests an octameric CaM kinase II holoenzyme with five δ and three γ subunits. We are currently carrying out coimmunoprecipitation experiments to determine the presence of δ and γ CaM kinase II heteromeric holoenzymes in fundus and proximal colon smooth muscle tissues.

Consistent with the real-time PCR results and previous reports of CaM kinase II expression in cardiac and vascular smooth muscle, Western blot analysis indicates that γ and δ CaM kinase II protein expression are also prevalent in murine fundus and proximal colon smooth muscle tissues. The apparent mass (~60 kDa) of the protein bands stained with the anti-δ CaM kinase II antibody suggest that δα is the predominant subtype expressed in murine fundus and proximal colon smooth muscle tissues. The single protein band detected with the anti-γ CaM kinase II antibody is probably due to the subtypes detected by RT-PCR in murine fundus and proximal colon smooth muscles (γB, γC, γI, γJ), which have been found to range in mass from 59 to 63 kDa (34). The densitometric analysis of the γ and δ CaM kinase II protein bands from the Western blots indicate that CaM kinase II protein expression is higher in proximal colon than in fundus.
The pattern of \( \gamma \) and \( \delta \) CaM kinase II splice variant expression is unique to fundus and proximal colon smooth muscle tissues. Astrocytes express CaM kinase II isoform splice variants \( \gamma_A, \gamma_B, \) and \( \delta_C \) (41). Aortic myocytes express CaM kinase II \( \gamma_B, \gamma_C, \gamma_E, \gamma_G, \) and \( \delta_C \) (32). Biliary epithelial cells express only CaM kinase II \( \gamma \) splice variants (22). CaM kinase II \( \gamma_B, \gamma_C, \gamma_I, \gamma_J, \delta_C, \delta_{C6}, \) and \( \delta_C;\delta_{C6} \) with alternative COOH termini are expressed in rabbit liver (36). The identification of CaM kinase II \( \gamma_I \) and \( \gamma_J \) in fundus and proximal colon smooth muscle tissues is the first demonstration of the expression of these two CaM kinase II \( \gamma \) splice variants outside the liver, suggesting that they have a wider tissue distribution.

Although the same CaM kinase II \( \alpha, \beta, \gamma, \) and \( \delta \) splice variants were identified in fundus and proximal colon smooth muscle tissue, we observed distinct differences in CaM kinase II enzymatic activity from these two tissues. Total CaM kinase II activity levels in proximal colon smooth muscle tissue lysates were approximately threefold higher than total CaM kinase II activity levels in fundus smooth muscle tissue. However, fundus smooth muscle lysates are characterized by a high level of autonomous CaM kinase II activity compared with proximal colon smooth muscle lysates (23–32% vs. 4–10% of total CaM kinase II activity). Both tissues were processed for kinase assays identically, suggesting that the high levels of autonomous CaM kinase II in fundus lysates are not due to proteolysis of CaM kinase II during tissue preparation. To demonstrate that Thr\(^{286} \) autophosphorylation is responsible for the high level of autonomous CaM kinase II activity, we treated fundus smooth muscle lysates with alkaline phosphatase to dephosphorylate Thr\(^{286} \). As shown in Fig. 8, autonomous CaM kinase II activity levels in fundus smooth muscle tissue lysates decreased from ~23 to 7% of total CaM kinase II activity. These results provide strong evidence that the high level of autonomous CaM kinase II activity in fundus smooth muscle tissue lysates is due to Thr\(^{286} \) autophosphorylation. The low level of autonomous CaM kinase II activity in proximal colon smooth muscle tissue lysates was also decreased slightly by alkaline phosphatase (data not shown). The remaining kinase activity measured in the tissue lysates following alkaline phosphatase treatment may be due to other kinases or to a limited amount of CaM kinase II proteolyzed endogenously or during tissue preparation.

In addition to differences in total and autonomous activity, CaM kinase II activity in fundus and proximal colon smooth muscle tissue lysates displayed differences in sensitivities to Ca\(^{2+} \)/CaM activation. Ca\(^{2+} \)/CaM stimulation of CaM kinase II in proximal colon smooth muscle tissue lysates increased autonomous CaM kinase II activity from 4 to 52% of total CaM kinase II activity. However, Ca\(^{2+} \)/CaM stimulation only increased CaM kinase II autonomous activity to 37% from 29% in fundus smooth muscle lysates (Fig. 6). The results in Fig. 8 showing that alkaline phosphatase decreases autonomous CaM kinase II activity indicate that the high level of autonomous CaM kinase II activity in fundus smooth muscle tissue lysates is due to Thr\(^{286} \) autophosphorylation. Little or no additional autonomous activity can be generated by Ca\(^{2+} \)/CaM activation if all (or most) of the kinase subunits of a CaM kinase II holoenzyme are already Thr\(^{286} \) autophosphorylated. Our findings that CaM kinase II in fundus smooth muscle lysates is resistant to Ca\(^{2+} \)/CaM-induced generation of autonomous activity (Fig. 6) further suggest that fundus CaM kinase II is characterized by a high level of Thr\(^{286} \) autophosphorylation.

The lower levels of total CaM kinase II activity in fundus relative to proximal colon smooth muscle tissues could be due to differences in CaM kinase II protein levels between the two tissues, different holoenzyme subunit compositions, or differences in levels of Thr\(^{305/306} \) phosphorylation. CaM kinase II subunits that are autophosphorylated on Thr\(^{305/306} \) cannot bind Ca\(^{2+} \)/CaM. Thus the total activity from a CaM kinase II holoenzyme with some (or all) subunits Thr\(^{305/306} \) phosphorylated is lower than the total activity of a CaM kinase II holoenzyme having none of its kinase subunits Thr\(^{305/306} \) phosphorylated (13, 14). To determine whether the lower level of total CaM kinase II activity in fundus smooth muscle lysates is due to autophosphorylated Thr\(^{305/306} \), we incubated fundus and proximal colon smooth muscle lysates with alkaline phosphatase to dephosphorylate any Thr\(^{305/306} \) phosphorylated CaM kinase II subunits before the addition of Ca\(^{2+} \)/CaM and the subsequent determination of total CaM kinase II activity. Alkaline phosphatase treatment of fundus lysates caused a fourfold increase in total CaM kinase II activity. In contrast, a less than twofold increase in total CaM kinase II activity was measured in alkaline phosphatase-treated proximal colon smooth muscle lysates. The findings from the alkaline phosphatase studies suggest that fundus smooth muscle CaM kinase II holoenzymes contain a high level of phosphorylated Thr\(^{305/306} \), which lowers total CaM kinase II activity. However, after alkaline phosphatase treatment, the total CaM kinase II activity levels in fundus lysates were still 1.3–1.5 times lower than the total CaM kinase II activity levels in proximal colon lysates. Furthermore, the densitometric analyses of the Western blots suggest that the \( \gamma \) and \( \delta \) CaM kinase II protein expression levels in fundus smooth muscle tissues are about 1.4-fold less than the levels in proximal colon. Together, these findings suggest that fundus smooth muscle tissue has lower levels of CaM kinase II protein expression than proximal colon smooth muscle tissue. The data do not support the conclusion that fundus smooth muscle CaM kinase II is fully Thr\(^{305/306} \) phosphorylated. The total CaM kinase II activity is higher than the autonomous activity, indicating that Ca\(^{2+} \)/CaM is binding to and activating the holoenzyme. It should be noted that kinetic analyses of purified CaM kinase II have been used to distinguish non-Thr\(^{305/306} \)-phosphorylated from Thr\(^{305/306} \)-phosphorylated CaM kinase II (13). These studies have shown that when Ca\(^{2+} \)/CaM is bound to the CaM kinase II holoenzyme, the \( K_m \) for the
autocamtide-2 peptide substrate is 0.3 μM. However, in the absence of Ca2+/CaM, the K_m of autonomous CaM kinase II for autocamtide-2 is 3 μM (13). Because Thr305/306-phosphorylated CaM kinase II cannot bind Ca2+/CaM, its K_m for autocamtide-3 is 3 μM in the presence of Ca2+/CaM (13). However, we cannot use kinetic analyses to investigate Thr305/306 phosphorylation of fundus and proximal CaM kinase II holoenzymes. In each tissue, the CaM kinase II holoenzymes are not fully phosphorylated on Thr286 and Thr305/306, unlike in vitro studies (12, 13). In addition, the CaM kinase II protein levels in each tissue are unknown, so it is not possible to assay and compare equal amounts of CaM kinase II in kinetic assays.

ACh binding to m2 and m3 muscarinic receptors on gastrointestinal smooth muscle cells causes contraction by Ca2+ influx through L-type Ca2+ channels and inositol 1,4,5-trisphosphate-induced Ca2+ release (27). ACh treatment of fundus and proximal colon smooth muscle tissues activates CaM kinase II as indicated by our results showing increases in autonomous CaM kinase II activity levels. KN-93 inhibited the phasic contractile activity of proximal colon smooth muscles induced by ACh. In contrast, KN-93 enhanced the ACh-induced increase in tonic contractions of fundus smooth muscle. These findings suggest different functional roles for CaM kinase II in modulating the contractile activities of fundus and proximal colon smooth muscles. Our results with the CaM kinase II inhibitor KN-93 suggest that CaM kinase II activation inhibits fundus contractions but enhances proximal colon contractions. CaM kinase II sensitizes the contractile apparatus of vascular smooth muscle to Ca2+ via MAP kinase activation and LC20 phosphorylation (18, 26). In addition, KN-93 inhibited CaM kinase II activation and LC20 phosphorylation (18, 26). These findings and our results (Fig. 11) suggest that CaM kinase II activation and regulation of contraction is a common feature of smooth muscle contraction. Although ACh increased autonomous CaM kinase II activity in fundus smooth muscle tissues from 30 to 55% of total activity, our results indicate that CaM kinase II in fundus lysates is unresponsive to Ca2+/CaM-induced activation and increases in autonomous activity compared with CaM kinase II in proximal colon lysates. Additional studies are underway to elucidate the mechanism of CaM kinase II activation and its substrates during ACh-induced contractions in fundus and proximal colon smooth muscles. In summary, we have demonstrated that transcripts for two α and four β CaM kinase II isoforms are expressed in murine gastric fundus and proximal colon smooth muscle tissues. In addition, we found a pattern of expression of the γ and δ isoforms unique to these two smooth muscle tissues. Expression of the γ and δ CaM kinase II isoforms appears to be prevalent in fundus and proximal colon smooth muscle tissues by Western blot analysis. ACh activated CaM kinase II in fundus and proximal colon smooth muscle tissues. The CaM kinase II inhibitor KN-93 enhanced ACh-induced contractions of fundus but inhibited contractions of proximal colon smooth muscles induced by ACh. Although both tissues express the same α, β, γ, and δ CaM kinase II isoforms, the two tissues exhibited differences in CaM kinase II expression levels and total and autonomous CaM kinase II activity levels. Although total CaM kinase II activity levels are lower in fundus than in proximal colon smooth muscle, the percentage of autonomous CaM kinase II activity in fundus smooth muscle is higher. Proximal colon smooth muscle CaM kinase II readily generated additional autonomous activity. In contrast, gastric fundus smooth muscle CaM kinase II was largely unresponsive to Ca2+/CaM activation and required alkaline phosphatase treatment to generate additional autonomous activity. Alkaline phosphatase treatment of fundus smooth muscle lysates lowered autonomous and increased total CaM kinase II activity levels. These results suggest that CaM kinase II holoenzymes in fundus smooth muscle tissues are characterized by a high level of Thr286 and Thr305/306 autophosphorylation. The proximal colon and fundus smooth muscle tissues used in these studies to measure total and autonomous CaM kinase II activities and identify the expressed CaM kinase II isoforms contain the circular and longitudinal smooth muscle layers and are predominantly composed of myocytes. However, interstitial cells of Cajal and enteric ganglia are also present interspersed among the myocytes (16). Further investigations using in situ hybridization and immunohistochemical analyses are necessary to determine the cellular distribution of the CaM kinase II isoforms and autonomous CaM kinase II in gastric fundus and proximal colon smooth muscle tissues. In addition, future studies must investigate the basis for the differences in CaM kinase II autophosphorylation at Thr286 and Thr305/306 in gastric fundus and proximal colon smooth muscle tissues.

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