Smooth muscle length-dependent PI(4,5)P₂ synthesis and paxillin tyrosine phosphorylation

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Sul, Donggeun, Carl B. Baron, Raymond Broome, and Ronald F. Coburn. Smooth muscle length-dependent PI(4,5)P₂ synthesis and paxillin tyrosine phosphorylation. Am J Physiol Cell Physiol 281: C300–C310, 2001.—We studied effects of increasing the length of porcine trachealis muscle on 5.5 μM carbachol (CCh)-evoked phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] synthesis and other parameters of phosphatidylinositol (PI) turnover. PI(4,5)P₂ synthesis rates in muscle held at 1.0 optimal length (Lₒ), measured over the first 6 min of CCh stimulation, were 140 ± 12 and 227 ± 14% of values found in muscle held at 0.5 Lₒ and in free-floating muscle, respectively. Time-dependent changes in cellular masses of PI(4,5)P₂, PI, and phosphatidic acid, and PI resynthesis rates, were also altered by the muscle length at which contraction occurred. In free-floating muscle, CCh did not evoke increases in tyrosine-phosphorylated paxillin (PTyr-paxillin), an index of β₁-integrin signaling; however, there were progressive increases in PTyr-paxillin in muscle held at 0.5 and 1.0 Lₒ during contraction, which correlated with increases in PI(4,5)P₂ synthesis and other parameters of CCh-stimulated inositol phospholipid turnover are muscle length-dependent and provide evidence that supports the hypothesis that length-dependent β₁-integrin signals may exert control on CCh-activated PI(4,5)P₂ synthesis.

IN BOVINE TRACHEALIS smooth muscle, carbachol (CCh)-activated inositol phosphate formation was greater when muscles were contracted at 1.0 Lₒ (1.0 Lₒ is the length at which agonists evoke maximal increases in stress) than when muscles were contracted at 0.1 or 0.2 Lₒ (1, 41). This finding implies that, in this muscle, signal transduction cascades depend on inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] are controlled in part by signal transduction cascades triggered by muscle length or stress.

It is unknown whether agonist-evoked formation of other lipid second messengers provided by inositol phospholipid metabolism (20), including phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], are length or stress sensitive and, if so, the extent of this sensitivity. Because PI(4,5)P₂ synthesis may be directed to multiple membrane domains where this lipid has different functions, it is not certain that PI(4,5)P₂ synthesis rates are equal to PI(4,5)P₂ hydrolysis and Ins(1,4,5)P₃ formation rates. Effects of changing smooth muscle length on CCh-evoked changes in inositol phospholipid metabolism have not been previously studied.

The importance of understanding how PI(4,5)P₂ pools are controlled has been emphasized with the finding of multiple cellular functions of PI(4,5)P₂. In addition to providing substrate for phospholipase C (PLC), PI(4,5)P₂ regulates fundamental cell processes, such as actin cytoskeleton remodeling, vesicular trafficking, and multiple signal transduction cascades, by anchoring proteins to membranes via pleckstrin homology and other PI(4,5)P₂-binding motifs (19, 20, 29–32, 34). In airway smooth muscle, a major function of PI(4,5)P₂ is to provide PLC substrate (3, 13). Another major role of PI(4,5)P₂ during CCh stimulation of airway smooth muscle is likely to be in involvement in remodeling of actin filaments that connect the contractile apparatus to sarcolemma (23, 35) and remodeling of actin stress fibers (16, 33, 36).

The mechanism whereby changes in smooth muscle length can modulate CCh-evoked Ins(1,4,5)P₃ formation is not established. Here we consider the following two processes: how length-stress is sensed and how length-stress signals can modulate inositol phospholipid metabolism. Increasing the muscle length of bovine trachealis smooth muscle from 0.5 to 1.0 Lₒ resulted in increases in CCh-evoked tyrosine phosphorylation of paxillin and focal adhesion kinase (35), hallmarks of β₁-integrin inward signaling that evokes Rho A-dependent actin cytoskeleton reorganization (10, 12, 28). Thus, in airway smooth muscle, β₁-integrins and associated structures may function as length-stress sensors and are candidates for a length sensor that can control CCh-evoked phosphatidylinositol (PI) turnover. There already is considerable evidence that β₁-integrin signaling can modulate PI turnover. Agonist-evoked PI turnover in some nonmuscle cells is augmented in the presence of fibronectin or by cross-linking β₁-integrin receptors, which activate β₁-
integrin inward signaling (14, 22, 24). The finding that integrins, including \( \beta_1 \)-integrins, can co-cluster with receptors that bind agonists and other signaling molecules and form functional membrane complexes offers an explanation for interactions between integrin signals and receptor-evoked signals (26).

An and Hai (1) considered that CCh-evoked increases in PLC-\( \beta \)-activity were modulated by the muscle length at which contraction occurred, but if PI(4,5)P\(_2\) concentrations are partially rate limiting for PLC-\( \beta \)-mediated PI(4,5)P\(_2\) hydrolysis, there are multiple enzymes involved in the PI cycle that could influence CCh-evoked Ins(1,4,5)P\(_3\) formation by changing rates of PI(4,5)P\(_2\) synthesis. These include the kinase-phosphatase reactions that are involved in resynthesis of PI(4,5)P\(_2\) from PI and transport events and reactions involved in PI resynthesis in the endoplasmic reticulum and membrane reinsertion of PI. Ins(1,4,5)P\(_3\) formation is known to be triggered via receptor-heterotrigem G protein activation of PLC. Agonists also activate PI 4-kinase and phosphatidylinositol 4-phosphate (PI[4]P) 5-kinase (9, 20); although activation of these enzymes should exert control on PI(4)P and PI(4,5)P\(_2\) masses, it is not established that this results in augmentation of PLC-evoked Ins(1,4,5)P\(_3\) formation.

The physiological significance of length- and stress-sensitive agonist-evoked phosphoinositide metabolism is likely to depend on whether it occurs during stress development and/or the tonic phase of contraction. Previous studies (1, 41) measured mean increases in inositol phosphate radioactivity in the presence of 10 or 20 mM Li\(^+\) over a 30-min time period, and it is unknown if muscle length effects occur during different phases of CCh-evoked contractions.

We modeled approaches in the present study after those developed in previous studies of muscarinic agonist-stimulated canine or porcine trachealis muscle (5–8). ACh or CCh evoked rapid time-dependent decreases in cellular PI and phosphatidylinositol bisphosphate (PIP\(_2\)) masses without a change in phosphatidylinositol phosphate (PIP) mass and rapid increases in phosphatidic acid (PA) and diacylglyceride masses and Ins(1,4,5)P\(_3\) formation. In muscles that had been non-steady-state-labeled with myo-[\(^3\)H]inositol, ACh stimulation evoked time-dependent incorporation in \(^3\)H into PI, PIP, and PIP\(_2\), data that were used to quantify PI resynthesis rates (8). Calculations using rates of incorporation of \(^3\)H into inositol phospholipids and rates of decreases in inositol phospholipid contents suggested that ACh stimulation resulted in a 20-fold increase in the rate of inositol phospholipid metabolism compared with that in unstimulated muscle (8). The approach of measuring multiple steps in the PI cycle has a potential of being able to determine which reactions are modulated by muscle length or stress.

Major goals in the present study were 1) to more completely characterize effects of muscle length or stress on CCh-evoked increases in Ins(1,4,5)P\(_3\) formation by determining effects on inositol phospholipid metabolism; 2) to determine if CCh-evoked PI(4,5)P\(_2\) synthesis and PI(4,5)P\(_2\) mass are altered by changing \( L_o \) and to quantitate any changes so we could evaluate whether length effects on the formation of this second messenger could potentially be important in modulating its functions; 3) to determine if changes in muscle length alter CCh-stimulated inositol phospholipid metabolism during different phases of contraction, i.e., during stress development and stress maintenance phases; and 4) to test the postulate that \( \beta_1 \)-integrin signaling is involved in length-dependent CCh-evoked PI(4,5)P\(_2\) synthesis.

**METHODS**

*Preparation of muscle sheets.* Porcine tracheas were obtained from a local meat-packing company. Porcine trachealis muscle was dissected to remove fascia (2) and was cut into 6 \( \times \) 10 × 1- to 1.5-mm sheets. We used wide sheets to obtain enough muscle for biochemical analyses; thin sheets were used to minimize O\(_2\) and CCh diffusion distances. Each sheet contained 20–30 mg muscle; we usually used 20–30 sheets/experiment. Porcine trachealis smooth muscle (PTSM) contains >98% muscle cells by histological examination. Muscle sheets were placed in cuvettes containing modified Krebs buffer (in mM: 114 NaCl, 5.9 KCl, 11.5 glucose, 1.2 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1.2 MgCl\(_2\), and 22 NaHCO\(_3\)) and were bubbled with O\(_2\)-N\(_2\)-CO\(_2\) (85-10-5%) at 37°C, pH 7.32–7.37, for 60 min to allow recovery. The general protocol was to non-steady-state label muscle with myo-[\(^3\)H]inositol, set muscle lengths at 0.5 or 1.0 \( L_o \) (isometric contractions), or use free-floating muscle. Although the free-floating muscle preparation is not physiological, it was used because CCh did not evoke increases in tyrosine-phosphorylated paxillin (P\(\text{Tyr}\)-paxillin), suggesting an absence of stress- or length-evoked signals that cause paxillin tyrosine phosphorylation. Thus free-floating muscle served as a control. After lengths were set (15–20 min), multiple free-floating and 0.5 and 1.0 \( L_o \) muscles were placed in cuvettes containing Krebs buffer, with or without 5.5 \( \mu \)M CCh. Muscles were removed from the CCh-containing solution at different times and plunged into liquid N\(_2\). In a typical experiment, we froze unstimulated muscles and muscles stimulated by CCh for different times up to 25 min. One muscle sheet was usually sufficient for all analyses. We studied only one CCh concentration, 5.5 \( \mu \)M. It was reported previously (1) that the length sensitivity of CCh-evoked inositol phosphate formation was detected over large CCh concentration ranges (1–100 \( \mu \)M).

*Non-steady-state labeling with myo-[\(^3\)H]inositol.* Unstimulated muscle sheets were transferred to a cuvette containing 1.2–1.7 ml of Krebs buffer and 100 \( \mu \)Ci (19.1 Ci/mmol) of myo-[\(^3\)H]inositol and incubated for 30–40 min at 37°C with constant aeration. Glucose was replenished at 15 min. After incubation with myo-[\(^3\)H]inositol, sheets were washed 10 times with 5 ml Krebs buffer and kept in Krebs buffer for 60 min. This protocol was shown to label muscle myo-inositol without much \(^3\)H becoming incorporated in inositol phospholipids or phosphates (8). Rates of appearance of \(^3\)H in PI, PIP, and PI(4,5)P\(_2\) during CCh stimulation allowed calculation of PI resynthesis rates.

*Setting muscle lengths.* We set the lengths of muscle strips used for biochemical analyses in a superfusion apparatus. This technique was used because we required multiple strips for analyses, and this method was rapid. There were two steps in setting muscle lengths. First, we calibrated single muscle strips in an organ bath that allowed measurement of isometric length and stress to determine the relationship of
passive length and 1.0 \( L_0 \). Muscles were transiently stretched and allowed to shorten to their passive length, which was measured. Multiple 60 mM KCl pulses were administered to the muscle held at different lengths, and stress increases were measured. This allowed us to determine the length relative to the passive length that provided maximal increases in stress. KCl was used because it is easily reversible, but data were the same with CCh pulses. This method gave reproducible data ±10%. Second, we used these calibration data to set muscle lengths in the superfusion apparatus, which allowed constant superfusion with the same solution that was present in the cuvette. Muscles were oriented parallel to muscle fibers and were placed on a 10 × 20 × 2-mm Sylgard block held in the superfusion apparatus. One end of each muscle was pinned to the Sylgard block using six butterfly pins, the muscle was transiently stretched, and the passive length was measured. Muscles were slowly stretched to ~1.0 \( L_0 \) and allowed to shorten to their passive length. Muscle lengths were then evenly increased 0.4 to the desired length, either 0.5 \( L_0 \) or 1.0 \( L_0 \), pinned to the Sylgard block, and returned to cuvettes containing bathing solution.

**Extraction of phosphoinositides and other lipids.** Tissues were ground to a fine powder under liquid N2. The extraction procedure followed previously published methods (7) using CHCl\(_3\)-CH\(_2\)OH (2:1 vol/vol) to extract major phospholipids and neutral lipids followed by CHCl\(_3\)-CH\(_2\)OH-concentrated HCl (267:133:1 vol/vol/vol) to extract polyphosphoinositides. Both extracts were dried under N\(_2\), under vacuum for 1 h, and redissolved in CHCl\(_3\)-CH\(_2\)OH.

**Analysis of polyphosphoinositides.** Samples and standards were spotted on a 1.2% oxalate-impregnated silica gel TLC plate. Plates were developed in CHCl\(_3\)-CH\(_2\)OH-concentrated NH\(_4\)OH-H\(_2\)O (45:35:2.5:7.5 vol/vol/vol/vol) and dried in a vacuum oven for 20 min at 110°C. Radioactivities in the lipids species were determined using an automatic Berthold TLC linear analyzer. We then determined lipid masses by charring TLC plates (4, 7) by spraying plates with 0.2% KMnO\(_4\) in 0.4 N NaOH, heating at 60°C for 4 min, and scanning at 553 nm using a Shimadzu densitometer. In these analyses, and in other analyses described below, measurements made using different muscle sheets were normalized in terms of myo-inositol radioactivity. Lipids were expressed as nanomoles per 100 nmol lipid Pi.

Boric acid (5%-impregnated plates were used to separate PI(4,5)P\(_2\), phosphatidylinositol 3,4-bisphosphate [PI(3,4)P\(_2\)], phosphatidylinositol 3,5-bisphosphate [PI(3,5)P\(_2\)], and PI(4)P (15).

**Analysis of PI and PA.** The major phospholipid extract and PI standards were spotted on 1.2% H\(_2\)BO\(_3\)-impregnated Whatman LK5 TLC plates. Plates were developed with CHCl\(_3\)-C\(_2\)H\(_5\)OH-(C\(_2\)H\(_5\)OH)\(_3\)-concentrated NH\(_4\)OH-H\(_2\)O (30:50:9:6 vol/vol/vol/vol). Radioactivity and lipid mass measurements were made using different muscle sheets were normalized in terms of myo-inositol radioactivity. Lipids were expressed as nanomoles per 100 nmol lipid Pi.

**Myo-inositol contents and specific activities.** This method was described previously (8). Free myo-inositol was separated by TLC on boric acid-impregnated plates using CH\(_2\)OH-H\(_2\)O (3:1 vol/vol) until the front reached 15 mm above the bottom of the plate. Plates were then dried in vacuo at 60°C for 30 min and then further developed using n-butanol-ethylicacetate-2-propanol-acetic acid-water (7:20:12:7:6 vol/vol/vol/vol/vol). Radioactivities and masses were measured as outlined above.

**Calculation of rates of PI resynthesis.** This method was described in detail previously (8). Measurements of the specific radioactivity of myo-inositol and the rate of incorporation of \(^3\)H into PI plus PI(4)P plus PI(4,5)P\(_2\) are used in this calculation. Because 1 mole of myo-inositol is used to form 1 mole of PI, moles of resynthesized PI can be computed from measurements of incorporation of \(^3\)H into inositol phospholipids as follows:

\[
\text{PI resynthesis rate (nmol · 100 nmol lipid P}_i^{-1} · \text{min}^{-1}) = \text{incorporation rate of } ^3\text{H into inositol phospholipids (cpm · 100 nmol lipid P}_i^{-1} · \text{min}^{-1})/\text{myo-inositol-specific radioactivity (cpm/nmol)}
\]

where cpm is counts per minute.

Rates of increase in radioactivity in total inositol phospholipids were used because resynthesized PI molecules are converted to PI(4)P and to PI(4,5)P\(_2\). We ignored a small correction made for movement of \(^3\)H into inositol phosphates, computed on the basis of previous studies (7, 8) to result in ~10% underestimation of the measured rate of PI resynthesis. Quantitation of PI resynthesis rates assumes that myo-[\(^3\)H]inositol is present in a homogenous pool so that PI synthesized in the ER has the same specific activity as that measured in myo-inositol. We assume that PI resynthesis occurred only via the myo-inositol pathway. That resynthesized PI replaced PI used to replenish PI(4)P and PI(4,5)P\(_2\) is supported by evidence obtained previously that PI resynthesis is precisely integrated with PI(4,5)P\(_2\) hydrolysis (8, 9, 18, 39).

**Calculation of the rate of PI(4,5)P\(_2\) synthesis.** This was determined as equal to the rate of decrease in PI plus PI(4)P contents plus the rate of PI resynthesis (8). This calculation assumes that PI is converted stoichiometrically to PI(4)P and to PI(4,5)P\(_2\) during CCh stimulation. Support for this assumption is given in the DISCUSSION.

**Measurement of tyrosine phosphorylation of particulate fraction \(\paxillin\).** This method was modified from that used by Pavalko et al. (25). All steps of this analysis were performed at 0°C. Suspensions of finely ground powder prepared from frozen muscle were homogenized in a Tenbroeck homogenizer in 6 ml of homogenization buffer [20 mM HEPES-Na (pH 7.3), 250 mM sucrose, 2 mM EGTA, 0.5 mM MgCl\(_2\) (free 4.45 mM), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μM calpain inhibitor peptide, 5 μg/ml each of aprotinin, leupeptin, and pepstatin A, 2 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 2 mM ammonium molybdate]. Homogenates were centrifuged at 15,000 g for 5 min, producing the particulate fraction. The particulate fraction was washed two times with a solution identical to that above except that sucrose and EGTA were omitted. Samples were solubilized with 1% SDS, 1 mM sodium orthovanadate was added, and aliquots were taken for a protein assay. Dithiothreitol was added, samples were placed in a boiling water bath for 5 min and centrifuged for 15 min at 15,000 g at room temperature to remove insoluble material, and SDS-PAGE and transfer were performed. Nitrocellulose membranes were blocked and then incubated with RC20 (Santa Cruz Biochemicals), an anti-phosphotyrosine antibody, followed by incubation with a secondary antibody. Immunoblots were developed as described above. Immunoblots were then stripped and reincubated with a paxillin antibody (monoclonal antibody; Transduction Laboratories) and processed as above. Bands on X-ray film were quantitated using densitometry at 448 nm. PTyr bands that contained \(\paxillin\) antibody. Immunoblots were developed as described above. Immunoblots were then stripped and reincubated with a paxillin antibody (monoclonal antibody; Transduction Laboratories) and processed as above. Bands on X-ray film were quantitated using densitometry at 448 nm. PTyr bands that matched exactly superimposed with bands obtained using the Paxillin antibody were used to measure PTyr-paxillin.

Paxillin was immunoprecipitated by incubating 400 μg of solubilized particulate fraction with 4 μg of antibody over-
night in immunoprecipitation buffer, followed by a 4-h incubation with protein G-agarose, washes, addition of SDS electrophoresis solution, electrophoresis, and transfer. We immunoblotted with RC20 and paxillin antibodies as described above.

**Statistical analyses.** We used the Microstat “Hypothesis Test” using either difference between means; paired observations, or difference between two group means; or pooled estimate of variance. Means were considered to be statistically different at $P < 0.05$.

**RESULTS**

**Measurement of molecular compositions of PIP and PIP$_2$.** Only PI(4,5)P$_2$ was detected in PIP$_2$ extracted in these experiments. PI(3,4)P$_2$ and PI(3,5)P$_2$ were not present in detectable amounts. PI(4)P was found in extracted PIP. These data are shown in Fig. 1.

**Myo-inositol content and specific radioactivity.** The myo-inositol pool size averaged 40 ± 4.3 nmol/100 nmol lipid P$_i$. PI resynthesis was computed to use ~0.25% of the total cellular myo-inositol pool per minute of CCh stimulation, and myo-[3H]inositol specific radioactivities did not decrease significantly during 25 min of CCh stimulation. Myo-inositol specific radioactivities in different preparations varied from 1,250 to 2,470 cpm/nmol.

**Comparison of 5.5 mM CCh-evoked inositol phospholipid metabolism in muscles held at 0.5 and at 1.0 $L_o$ and in free-floating muscle.** In both 0.5 and 1.0 $L_o$ muscle, 5.5 mM CCh stimulation resulted in time-dependent decreases in PI and PI(4,5)P$_2$ masses and increases in PA masses (Figs. 2 and 3). PI(4)P contents did not change significantly. In both 0.5 and 1.0 $L_o$ muscle, CCh stimulation resulted in time-dependent incorporation of $^3$H into inositol phospholipids because of PI resynthesis and conversion to PI(4)P and PI(4,5)P$_2$. These data are similar to data published previously using canine trachealis muscle (8).
Fig. 3. Mean data obtained in experiments performed at 0.5 and 1.0 L.<n>Experiments were performed as in Fig. 2. A: PA mass; B: PI mass; C: PI(4,5)P_2 mass. Muscles were prelabeled with myo-[^3]H]inositol so that rates of incorporation of [^3]H into PI (D) and PI(4,5)P_2 (E) could be determined. Means were obtained using data obtained in at least 4 experiments. *Statistically significant at P < 0.05, comparing data points obtained at the same time of CCh stimulation. The statistical significance of differences in PA formation and in evoked increases in [^3]H]PI and [^3]H]PIP_2 in 0.5 and 1.0 L. muscle are given in the text.

Figures 2 and 3 indicate that there were qualitative differences in CCh-evoked changes in PI, PI(4,5)P_2, and PA masses and incorporation of [^3]H in inositol phospholipids in muscles held at 0.5 and 1.0 L. In the best experiment (Fig. 2), differences in increases in PA mass and decreases in PI and PI(4,5)P_2 masses were seen early during CCh stimulation and persisted for up to 25 min of CCh stimulation. Means of data obtained from at least four experiments (Fig. 3) indicated that we could detect significant differences in CCh-evoked decreases in PI and PI(4,5)P_2 masses, comparing 0.5 and 1.0 L. data, only at the 15- and 25-min time points. There were no consistent changes in PI(4)P mass during CCh stimulation of 0.5 and 1.0 L. muscle (data not shown). There were larger rates of increases in PA mass determined over the first 6 min of CCh in 1.0 L. muscle than in 0.5 L. muscle, i.e., 0.082 ± 0.005 vs. 0.055 ± 0.010 nmol·100 nmol lipid P_i·min^−1, respectively (P < 0.05; n = 5).

Rates of increases of [^3]H]PI were significantly larger in CCh-stimulated muscles held at 1.0 L. compared with muscles held at 0.5 L. (Fig. 3). This was quantified by determining the rate of increase in PI specific radioactivities measured over the first 6 min of CCh stimulation, giving values of 7.43 ± 0.17 and 3.99 ± 0.54 (SD) cpm·nmol^−1·min^−1 for 1.0 and 0.5 L. muscle, respectively (P < 0.05; n = 5). Figure 3 also shows that CCh-evoked increases in [^3]H]PI(4)P_2 were 200–250% larger at the 6-, 15-, and 25-min time points in 1.0 L. muscle than in 0.5 L. muscle (P < 0.01; n = 5). Rates of increases in [^3]H]PI(4)P were larger in 1.0 L. muscle than in 0.5 L. muscle (data not shown), but this difference was not statistically significant.

Mean rates of PI resynthesis determined over the first 6 min of CCh stimulation were 0.097 ± 0.010 and 0.057 ± 0.010 nmol·100 nmol lipid P_i·min^−1 in 1.0 and 0.5 L. muscles, respectively (P < 0.05; n = 5). Rates of PI(4,5)P_2 synthesis were 0.281 ± 0.040 and 0.202 ± 0.020 nmol·100 nmol lipid P_i·min^−1 in muscles held at 1.0 and 0.5 L. muscle, respectively (P < 0.05; n = 5). PI resynthesis rate and PI(4,5)P_2 synthesis rate data are also listed in Table 1.

Mean rates of PI resynthesis and PI(4,5)P_2 synthesis computed during the 6- to 15-min measurement period are also listed in Table 1. Considering 1.0 and 0.5 L. data, 6- to 15-min values were smaller than those calculated from data obtained during the 0- to 6-min measurement period, but there was still a length dependence in that PI(4,5)P_2 synthesis rates and PI resynthesis rates were 260 and 178% larger, respectively, with 1.0 L. than with 0.5 L. muscle (P < 0.05 for both comparisons).

CCh-evoked decreases in PI content were delayed and blunted in free-floating muscle compared with data obtained either with 0.5 or 1.0 L. muscle (Fig. 4). In addition, in free-floating muscle, there were large CCh-evoked decreases in PI(4)P content that were not seen using 0.5 and 1.0 L. muscle. PI(4,5)P_2 mass decreases were similar to those measured using 0.5 and 1.0 L. muscle. Rates of incorporation of [^3]H in inositol phospholipids were smaller in free-floating muscle than in muscle held at 1.0 L. (Fig. 4) but were similar to data measured at 0.5 L. (Figs. 3 and 4). The mean PI resynthesis rate in free-floating muscles during 0–6 min of CCh stimulation was 0.053 ± 0.010 nmol·100 nmol lipid P_i·min^−1, a value statistically smaller.
than values obtained using 0.5 and 1.0 \( L_o \) muscle \((P < 0.05\) for each comparison; \( n = 4; \) Table 1). Rates of PI(4,5)P\(_2\) synthesis averaged 0.124 ± 0.020 nmol·100 nmol lipid \( P_i \) min\(^{-1}\), a value markedly smaller than that found in 0.5 and 1.0 \( L_o \) muscle \((P < 0.01; \ n = 4; \) Table 1). The mean rate of PA increases in free-floating muscle was 0.101 ± 0.020 nmol·100 nmol lipid \( P_i \) min\(^{-1}\), a value greater than that found in either 0.5 or 1.0 \( L_o \) muscle. With free-floating muscle, the decrease in PI resynthesis and PI(4,5)P\(_2\) synthesis observed in 0.5 and 1.0 \( L_o \) muscle during prolonged CCh stimulation (6–15 min of stimulation) did not occur.

**Effects of changing muscle length in unstimulated muscle.** Muscle length effects on CCh-evoked PI(4,5)P\(_2\) synthesis and PI resynthesis were not secondary to changes that occurred in unstimulated muscle. This was indicated by a lack of differences in PI, PI(4)P, and PI(4,5)P\(_2\) contents and \(^3\)H levels in these phosphoinositides in unstimulated muscle held at 0.5 and 1.0 \( L_o \), and there were no differences in PI(4)P and PI(4,5)P\(_2\) contents or phosphoinositide \(^3\)H levels in unstimulated free-floating muscle compared with unstimulated 0.5 and 1.0 \( L_o \) muscle. These findings are consistent with the previous finding (1), cited above, that inositol phosphate formation in unstimulated muscle was not length dependent.

**Tyrosine phosphorylation of paxillin in CCh-stimulated free-floating muscle and muscle held at 0.5 and 1.0 \( L_o \).** Experimental protocols were similar as above except that labeled muscle was not required. Muscle held at different lengths or free-floating muscles were frozen after 5 min of CCh. Figure 5 shows typical immunoblots using RC20 and paxillin antibody. Figure 5, A and B, shows data obtained using the solubilized particulate fraction, and Fig. 5C shows data obtained using paxillin immunoprecipitates recovered from solubilized particulate fractions. There were multiple proteins in the particulate fraction obtained from un-

### Table 1. PI resynthesis and PI(4,5)P\(_2\) synthesis rates

<table>
<thead>
<tr>
<th></th>
<th>Free Floating</th>
<th>0.5 ( L_o )</th>
<th>1.0 ( L_o )</th>
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<tr>
<td><strong>PI resynthesis rates</strong></td>
<td></td>
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<tr>
<td>0–6 min</td>
<td>0.053 ± 0.010(4)</td>
<td>0.057 ± 0.010(5)</td>
<td>0.097 ± 0.010(5)</td>
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<tr>
<td>6–15 min</td>
<td>0.114 ± 0.026(4)</td>
<td>0.037 ± 0.009(5)</td>
<td>0.066 ± 0.020(5)</td>
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<tr>
<td><strong>PI(4,5)P(_2) synthesis rates</strong></td>
<td></td>
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<tr>
<td>0–6 min</td>
<td>0.124 ± 0.020(4)</td>
<td>0.202 ± 0.024(5)</td>
<td>0.281 ± 0.040(5)</td>
</tr>
<tr>
<td>6–15 min</td>
<td>0.125 ± 0.030(4)</td>
<td>0.077 ± 0.010(5)</td>
<td>0.201 ± 0.060(5)</td>
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Data are means ± SE and were computed over 0- to 6-min and 6- to 15-min measurement periods during carbachol (CCh) stimulation; nos. in parentheses indicate no. of experiments. Units are nmol·100 nmol phospholipid \( P_i \) min\(^{-1}\). Phosphatidylinositol (PI) resynthesis rates were determined using measurements of rates of incorporation of \(^3\)H into inositol phospholipids. Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P\(_2\)] synthesis rates were computed as the sum of (rate of decreases in PI + phosphatidylinositol 4-phosphate masses) + PI resynthesis rates. PI(4,5)P\(_2\) synthesis rates and PI resynthesis rates in free-floating muscle, 0.5 optimal length (\( L_o \)) muscle and 1.0 \( L_o \) muscle were statistically significant from each other at \( P < 0.05 \) during both the 0- to 6-min and 6- to 15-min measurement periods. PI resynthesis rates in 1.0 \( L_o \) muscle were significantly greater than PI resynthesis rates in 0.5 \( L_o \) muscle and free-floating muscle \((P < 0.05)\).
stimulated muscle that were tyrosine phosphorylated. Of these, several proteins showed increases in PTyr during CCh stimulation, including 68- to 70- and 73- to 75-kDa proteins. The 68-kDa PTyr protein RC20 band was identified as paxillin on the basis of its molecular mass and immunoprecipitation analysis. This band was shown using densitometry to be separate from a 73- to 75-kDa band (data not shown). There was no consistent change in paxillin band density (per mg protein) in control or CCh-stimulated free-floating 0.5 or 1.0 Lₒ muscle.

We quantified CCh-evoked increases in PTyr-paxillin by computing the ratio of PTyr to paxillin band densities. Mean values from four experiments, given as a percentage of ratios found in CCh-stimulated 1.0 Lₒ muscle, are plotted in Fig. 6. Differences in mean PTyr-paxillin values between unstimulated muscle and CCh-stimulated free-floating muscle were not statistically significant. Mean PTyr-paxillin values during CCh stimulation of 0.5 and 1.0 Lₒ muscle were significantly greater than mean PTyr-paxillin values measured in unstimulated and CCh-stimulated free-floating muscle (P < 0.01), and the large CCh-evoked increase in PTyr-paxillin in 1.0 Lₒ muscle was statistically different from values seen in 0.5 Lₒ muscle. (P < 0.05). The finding that CCh-stimulated increases in PTyr-paxillin were small or absent in free-floating muscles and progressively increased in 0.5 and 1.0 Lₒ muscle confirms the muscle length dependence of this phenomenon (35).

**DISCUSSION**

PI(4,5)P₂ synthesis rates were computed from rates of decreases in PI plus PI(4)P masses and rates of PI resynthesis. The assumption in this calculation that PI was stoichiometrically converted to PI(4,5)P₂ (without a branch point) was supported by the finding that only PI(4,5)P₂ was detected in PIP₂ in extracted lipid. This finding excludes a possibility that, during CCh stimulation, PI 3-kinase-evoked by-products (37) were formed in significant amounts that could be detected in our extraction-plating system. The possibility that PI(5)P was phosphorylated to PI(4,5)P₂ (27) was not evaluated. Because PI(5)P likely would originate in part from PI (11), its presence should not cause error in the calculation of PI(4,5)P₂ synthesis rates.

In the studies from the laboratory of Hai et al. (1, 41), it was not determined if myo-[³H]inositol labeling was steady state or nonsteady state; therefore, it was not certain if their results reflected increases in inositol phosphate pool sizes or formation rate. (However, this does not detract from their major conclusion.) In our studies, the calculation of PI(4,5)P₂ synthesis rates and PI resynthesis rates was possible because isolation of individual inositol phospholipids allowed ³H labeling to be well defined.

We could not determine the fraction of total cellular PI, PI(4)P, and PI(4,5)P₂ masses that were involved in CCh-evoked inositol phospholipid metabolism. Our emphasis is on changes in total masses of these compounds as a measurement of changes in masses of lipid compartments involved in CCh-evoked inositol phospholipid metabolism. The cellular locations of PI, PI(4)P, and PI(4,5)P₂ that participated in length-dependent inositol phospholipid metabolism were not established. Because most cellular PI(4,5)P₂ has been found in plasma membranes (reviewed in Ref. 34), it is likely that we were primarily measuring changes in synthesis and masses of sarcolemmal-associated PI(4,5)P₂ and sarcolemmal PI and PI(4)P involved in PI(4,5)P₂ synthesis.

Muscle length determines PI(4,5)P₂ synthesis and mass changes during CCh-evoked contractions. Experiments using muscle held at 0.5 and 1.0 Lₒ produced data that were all consistent with length or stress
effects on CCh-evoked activation of inositol phospholipid metabolism and PI(4,5)P\textsubscript{2} synthesis. CCh-evoked time-dependent decreases in PI mass, increases in [\textsuperscript{3}H]PI and [\textsuperscript{3}H]PI(4,5)P\textsubscript{2}, increases in calculated PI(4,5)P\textsubscript{2} mass and PI resynthesis rates, decreases in PI(4,5)P\textsubscript{2} mass (at 15 and 25 min CCh), and increases in PA mass were all larger in muscle that contracted at 1.0 \textit{L}_o compared with muscle that contracted at 0.5 \textit{L}_o. The 140% larger rate of PI(4,5)P\textsubscript{2} synthesis found in muscle held at 1.0 \textit{L}_o compared with 0.5 \textit{L}_o muscle suggests physiological importance in terms of cellular functions of PI(4,5)P\textsubscript{2} discussed in the Introduction.

The finding, when comparing 0.5 and 1.0 \textit{L}_o muscle, that length effects were present both in the 0- to 6-min and 6- to 15-min measurement periods suggests that muscle length-stress can modulate PI(4,5)P\textsubscript{2} synthesis early during CCh-evoked stimulation and during the tonic phase of contraction.

In our experiments, the mean increase in PI(4,5)P\textsubscript{2} synthesis rate from free-floating to 1.0 \textit{L}_o muscle over the first 6 min of CCh stimulation (a 227% increase) was similar to increases in CCh-evoked inositol phosphate formation determined by Yoo et al. (41) over a 30-min time period comparing muscle held at 1.0 \textit{L}_o with muscle held at 0.2 \textit{L}_o. However, because of different conditions, species, and measurement times, this comparison does not address a question whether or not all of the PI(4,5)P\textsubscript{2} synthesized in our experiments was hydrolyzed to Ins(1,4,5)P\textsubscript{3}.

PI(4,5)P\textsubscript{2} synthesis rates and PI resynthesis rates during the 0- to 6-min measurement period were smaller in free-floating muscle than in 0.5 and 1.0 \textit{L}_o muscle, consistent with a concept that the muscle length during contraction exerted control on inositol phospholipid metabolism. However, the large increase in PA mass found in free-floating muscle during the 0- to 6- and 6- to 15-min measurement periods and high values in free-floating muscle of both PI resynthesis rates and PI(4,5)P\textsubscript{2} synthesis rates during the 6- to 15-min measurement period suggest that there may be some differences in mechanisms that control CCh-evoked inositol phospholipid metabolism in free-floating muscle compared with 0.5 and 1.0 \textit{L}_o muscle.

**Muscle length-dependent \textit{\beta}_{1}-integrin signals and CCh-evoked PI(4,5)P\textsubscript{2} synthesis.** The postulate that \textit{\beta}_{1}-integrins could provide signals that control PI(4,5)P\textsubscript{2} synthesis is attractive because integrin signals, known to evoke remodeling of actin filaments, could control PI(4,5)P\textsubscript{2} synthesis in a domain where this lipid is involved in controlling actin polymerization. In Fig. 6, it is shown that length-dependent increases in PTyr-paxillin and in PI(4,5)P\textsubscript{2} synthesis rate were identical. Thus, if \textit{\beta}_{1}-integrins are the only length-stress sensor in PTSM, our finding of identical length effects on PI(4,5)P\textsubscript{2} synthesis and increases in PTyr-paxillin implicates \textit{\beta}_{1}-integrins as providing signals that modulate CCh-evoked PI turnover. Stretch-activated ion channels that couple to PI(4,5)P\textsubscript{2} hydrolysis and produce short bursts of Ins(1,4,5)P\textsubscript{3} formation in smooth muscle (21) are unlikely to be involved in length-sensitive PI(4,5)P\textsubscript{2} synthesis studied here. At the present time, the postulate that \textit{\beta}_{1}-integrin length-stress sensors produce signals that modulate airway smooth muscle CCh-evoked PI turnover is supported by the following: 1) \textit{\beta}_{1}-integrins function as length-stress sensors in airway smooth muscle, as indicated by increases in PTyr-paxillin (35); 2) agonist-evoked Ins(1,4,5)P\textsubscript{3} formation is modulated in nonmuscle cells by perturbations that alter or stimulate \textit{\beta}_{1}-integrin interactions with extracellular matrix (14, 22, 24); 3) integrin receptors can form complexes with heterotrimeric G protein-coupled receptors and other signal transduction proteins (26); and 4) there was an identical length dependence in PTSM of CCh-evoked increases in PTyr-paxillin and PI(4,5)P\textsubscript{2} synthesis rate (the present study).

**Muscle length-stress effects on reactions in the PI cycle.** This study has characterized muscle length effects on CCh-evoked PI(4,5)P\textsubscript{2} formation in terms of...
length-dependent events that occurred in the PI cycle. With increases in muscle length from 0.5 to 1.0 L_o, there was augmented flux in reactions that involved PI 4-kinase-mediated phosphorylation of PI to PI(4)P and PI(4)P 5-kinase-mediated phosphorylation of PI(4)P to PI(4,5)P_2. This was quantitated in terms of increases in PI(4,5)P_2 synthesis rates. There also was an increase in the rate of PI resynthesis. Effects of increasing muscle length on CCh-evoked inositol phospholipid metabolism were identical to those seen previously in an airway smooth muscle when a second type of heterotrimERIC G protein-linked receptor was activated during muscarinic receptor stimulation (6).

An and Hai (1) reported that the length sensitivity of CCh-evoked inositol phosphate formation in bovine trachealis muscle occurred over a large range of CCh concentrations and that fluoroaluminate-evoked inositol phosphate formation had a similar length sensitivity to that measured during CCh stimulation. These findings indicate that signals evoked by increasing L_o modulated inositol phospholipid metabolism at a site or sites downstream of the muscarinic receptor. It is still an open question whether length- and stress-operated signal transduction cascades could recruit PLC-β, increase the activity of this enzyme, and/or control reactions that result in synthesis of PI(4,5)P_2. In our experiments, we expected that if length effects on CCh-evoked inositol phospholipid metabolism were driven by changes in PLC activity where flux in the PI cycle was secondary to a decrease in the mass of PI(4,5)P_2, thereby increasing the driving force for conversion of PI to PI(4)P and to PI(4,5)P_2, the initial rates of decreases in PI(4,5)P_2 mass should be largest for 1.0 L_o muscles > 0.5 L_o muscles > free-floating muscles. This effect was not seen (Figs. 2 and 3). Thus our experiments do not support PLC activity being under control of muscle length-stress signals.

Evidence was obtained that length-stress signals exerted control on the PI 4-kinase, PI(4)P 4-phosphatase reaction. Evidence was derived by comparison of 0.5 and 1.0 L_o data. Flux from PI to PI(4)P [measured as PI(4,5)P_2 synthesis rate] was increased in 1.0 L_o muscle compared with 0.5 L_o muscle under conditions where the PI mass was smaller and PI(4)P mass remained constant, a condition where the driving gradient was decreased. This suggests that increased flux in 1.0 L_o muscle may not have been a result of an increased chemical gradient and was due to activation of PI 4-kinase or inactivation of PI(4)P 4-phosphatase. [This assumes changes in PI and PI(4)P masses were sensed by PI 4-kinase.] Further evidence was obtained by comparing 0.5 and 1.0 L_o data with data obtained from free-floating muscle. In 0.5 and 1.0 L_o muscle, CCh-evoked decreases in PI content plus increases in PI resynthesis provide for PI(4,5)P_2 resynthesis. In contrast, free-floating muscle CCh-evoked PI(4,5)P_2 synthesis was largely effected by decreases in PI(4)P mass and PI and PI(4)P resynthesis, and decreases in PI masses were blunted. These findings indicate that, in free-floating muscle, there was a partial block at the PI 4-kinase, PI(4)P 4-phosphatase reaction or that there was decreased delivery of PI from its site of synthesis to sarcolemmal PI 4-kinase. Together, these data are consistent with graded increases in PI 4-kinase activity going from free-floating muscle to 0.5 and to 1.0 L_o muscle.

It is pertinent to this discussion that a type II PI 4-kinase extracted from a nonmuscle cell was complexed with α_3β_1-integrin (40), a finding that suggests that α_3β_1-integrins could function as direct activators of PI 4-kinase. Further studies are necessary to determine if α_3β_1-integrins are involved in length-stress activation of PI 4-kinase and inositol phospholipid metabolism.

Is muscle length-dependent PI(4,5)P_2 synthesis involved in remodeling of actin filaments? It has not yet been proven that PI(4,5)P_2 plays a role in remodeling of actin filaments or fibers during CCh stimulation of airway smooth muscle. However, this postulate is supported by the following: 1) length increases during CCh stimulation evoked increases in PTP-tyr-paxillin and PTP-tyr-focal adhesion kinase in airway smooth muscle (23, 35), events that are associated with remodeling of the actin cytoskeleton in nonmuscle cells (10, 12, 28), and this was associated with increases in PI(4,5)P_2 synthesis in the present study; 2) CCh stimulation resulted in remodeling of stress fibers in cultured airway myocytes (16, 36). (It is unknown if this is muscle.
length dependent.) In vascular smooth muscle, angiotensin stimulation produced a rapid increase in PTyr-paxillin that correlated with formation of focal adhesions (38); and 3) established roles of PI(4,5)P₂ in driving actin polymerization discussed in the Introduction.

Figure 7 shows our current working hypothesis to explain muscarinic and integrin receptor inputs that control PI(4,5)P₂ synthesis in a domain where PI(4,5)P₂ evokes actin polymerization.

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REFERENCES


following phospholipase CB activation: transport of the intermediate, phosphatidic acid, from the plasma membrane to the endoplasmic reticulum for phosphatidylinositol resynthesis is not dependent on soluble lipid carriers or vesicular transport. Biochem J 341: 435–444, 1999.
