Diabetes decreases rabbit bladder smooth muscle contraction while increasing levels of myosin light chain phosphorylation

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Submitted 29 January 2004; accepted in final form 10 June 2004

Su, Xiaoling, Arun Changolkar, Samuel Chacko, and Robert S. Moreland. Diabetes decreases rabbit bladder smooth muscle contraction while increasing levels of myosin light chain phosphorylation. Am J Physiol Renal Physiol 287:F690–F699, 2004. First published June 15, 2004; 10.1152/ajprenal.00027.2004.—The effect of diabetes mellitus on the regulation of urinary bladder smooth muscle contraction was studied. Diabetes was induced in the rabbit by alloxan injection followed by 16 wk of housing. The bladder was harvested and strips of wall devoid of both mucosa and serosa were examined. Intact strips of bladder smooth muscle from diabetic animals produced less stress in response to membrane depolarization than muscle from control animals; sensitivity to KCl was not changed. Carbachol responses were similar in muscle strips from the two animal groups. Basal myosin light chain (MLC) phosphorylation levels were significantly elevated in response to most stimuli in muscle strips from diabetic animals, although levels of stress were either unchanged or lower. α-Toxin-permeabilized strips that allow for control of the intracellular environment while maintaining excitation-contraction coupling showed increased levels of MLC phosphorylation but decreased sensitivity to activator Ca2+ in smooth muscle from diabetic animals. MLC phosphatase contents were similar in smooth muscle from the two animal groups; however, MLC phosphatase activity was greater in muscle from control compared with diabetic animals. These results suggest that diabetes mellitus uncouples basal MLC phosphorylation from force in the bladder smooth muscle cell.

myosin light chain phosphatase; α-toxin; myosin light chain kinase; alloxan

DIABETES MELLITUS HAS BEEN clearly shown to be associated with several functional alterations in smooth muscle, both vascular and nonvascular (4, 33, 38). Nonvascular smooth muscles such as those in the urinary bladder wall undergo some clear and significant changes as a complication of diabetes. Diabetic-induced dysfunction of the urinary bladder leads to urinary retention and associated kidney damage. Thus the pathophysiological changes in the bladder have important clinical ramifications as they present a major long-term problem in the treatment of the disease. A primary alteration that is noted both clinically and experimentally is remodeling of the bladder wall including a change in matrix composition and a prominent hypertrophy of the muscle (6, 11, 29, 39). Diabetes-induced changes in bladder muscle function have also been shown in response to changes in neuronal input (14, 27). This is primarily a change in the parasympathetic input responsible for contraction (38), but changes in the relaxant control of the bladder by sympathetic nerves have also been shown in response to diabetes (19, 24).

In addition to neural and hypertrophic effects of diabetes on the urinary bladder, direct actions on functional aspects of bladder smooth muscle have been demonstrated. Many investigators have shown diabetes-induced changes in contractile force and/or sensitivity (1, 10, 18, 37, 42). One interesting aspect of these studies is that the changes appear to be agonist specific rather than a generalized alteration in muscle contractility (15, 26, 28). This would suggest that diabetes may alter specific receptors or specific intracellular pathways that mediate the coupling between receptor binding and the contractile response.

It has been shown that the bladder smooth muscle cell has selective alterations in receptor-mediated responses (15, 26, 28), membrane ion handling (10, 12, 17), but not changes in cytosolic calcium concentrations (30, 42) and, in many cases, contractility (6, 20, 39). Therefore, diabetes induces a complex mix of effects on the bladder. One area that has not received a large amount of attention is the effect of diabetes on regulation at the level of the contractile proteins. It is possible that the apparent contradictions in the literature showing changes in receptor function without a change in contractile force may be due to specific changes at the level of the contractile apparatus. Thus the goal of this study was to determine the effect of diabetes mellitus on urinary bladder smooth muscle of the rabbit. Specifically, we were interested in determining whether the changes noted in the contractility of bladder smooth muscle can be accounted for by changes at the level of regulation of the contractile proteins. Our results suggest that diabetes alters not only the contractile response to cellular stimulation but also specific intracellular steps important in excitation-contraction coupling. Surprisingly, diabetes uncouples one of the primary steps in the activation of a smooth muscle contraction, myosin light chain (MLC) phosphorylation, from the increase in force.

MATERIALS AND METHODS

Animal model. All protocols in this study were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania School of Veterinary Medicine and Drexel University College of Medicine. Six-month-old male New Zealand White rabbits (~3 kg) were housed in a temperature-controlled room (25°C) with a 12:12-h light-dark cycle. Diabetes was induced by intravenous injection of alloxan (100 mg/kg body wt). To avoid acute hypoglycemia following alloxan injection, rabbits received 10% d-glucose in the drinking water for 48 h following the alloxan injection. The rabbits were maintained for 6 mo. Rabbits injected with normal saline served

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as controls. Rabbits receiving 5% sucrose in drinking water for 4 mo were used as a control for nondiabetic diuresis. Blood glucose values were measured using a glucometer (LIFESCAN) 7–10 days after the alloxan injection, and the final measurement was made before euthanasia. The blood glucose level of normal rabbits fell between 150 and 190 mg/dl compared with sucrose-fed rabbits ranging from 180 to 240 mg/dl, whereas the animals injected with alloxan showed increased blood glucose ranging from 200 to 500 mg/dl. In the present study, diabetic rabbits that showed a blood glucose level of 400 mg/dl or higher 4 mo after the induction of diabetes were used to evaluate the long-term effects of diabetes in the detrusor smooth muscle.

Animals were weighed and euthanized by an overdose of pentobarbital sodium followed by exsanguination. The bladders were removed, emptied, rinsed, and then weighed before usage. Six to 19 animals from each group were used for this study; the number of bladders used are indicated in the figure legends.

**Intact smooth muscle preparation.** The bladder neck, trigone, and base region were removed, leaving only the midbody for experimentation. The mucosa and serosa were carefully removed from the detrusor smooth muscle layer under a dissecting microscope. Muscle strips (~1.5 × 6 mm) were cut along the central axis of the bladder in the longitudinal orientation. At least four to eight strips were obtained from each bladder. The intact bladder strips used for isometric tension recording were mounted between two plastic clips, one attached to a micrometer for length adjustment and the other to a Grass FT.03 force transducer and a Grass model 7D polygraph, in water-jacketed muscle chambers. The tissues were bathed in a MOPS-buffered physiological salt solution (PSS; see Ref. 22 for composition) at 37°C and aerated with 100% O2. The strips were equilibrated for at least 90 min. After the equilibration period, a partial length-tension curve was performed to determine the optimal length for active force development (L0). This was performed as previously described (13). Briefly, the tissues were stretched, allowed to stress-relax, and then subjected to a quick release to a shorter length. This quick release is required to avoid including any active tone in the determination of passive force. The tissues were then subjected to a maximal stimulus, relaxed, and the procedure was repeated. This stretch, stress-relax, release, and contract cycle was repeated until at least two or three identical contractions were obtained with increasing length. Due to the rather broad plateau of the nonvascular length-tension curves, this approximates the optimal length for active force development. We did not exceed the optimal length for active force development to avoid tissue damage. Using this protocol, we were able to, with reasonable precision, set each individual muscle strip at its own L0. Unlike previous studies using whole bladder wall from diabetic animals (21), we found no significant difference in the length-tension relationship between the smooth muscle layers of bladder from control compared with diabetic animals. This is most likely due to the fact that our dissection removes both the serosal and mucosal layers that are predominantly connective tissue and therefore would have a greater influence on the passive portion of the length-tension relationship. Moreover, Longhurst et al. (21) showed that bladder responses from sucrose-fed animals were similar to those from diabetic animals and both were significantly different from control. In our study, we found no differences between sucrose-fed and control animals. Force data obtained were expressed as active stress (stress = force/cross-sectional area) or normalized as a percent-age of the maximal response to 110 mM KCl. Cross-sectional area was determined using tissue length and wet weight as previously described (22).

**Permeabilized smooth muscle preparation.** For experiments involving permeabilized tissues, muscle strips devoid of mucosal and serosal layers (200 × 800 μm) were mounted in a Muscle Research System force/length apparatus ( Axelrod Instruments, Montgomery, NY) and placed over a thermostatically controlled water bath, temperature and allowed to equilibrate for 90 min under a passive tension of 50 mg. The tissues were contracted with 10 μM ATP to desensitize purinergic receptors, followed by stimulation with 30 μM carbacol, 1 mM ATP in a Ca-free PSS containing 1 mM EGTA to deplete intracellular stores of calcium. The tissues were then permeabilized by exposure to 2,500 U/ml Staphylococcus aureus α-toxin (List Biological Laboratory, Campbell, CA) for 60 min in a solution containing 5 mM creatine phosphate, 4 mM ATP, 5 mM EGTA, 1 mM MgCl2, 20 mM imidazole (pH 6.8), and 31 mM K-acetate. Solutions for the permeabilized tissue studies contained 20 mM imidazole (pH 6.8), 1 mM Mg2+, 4 mM ATP, 5 mM EGTA, sufficient K-acetate to maintain ionic strength at 120 mM, 5 mM creatine phosphate, 1 μM ionomycin, and levels of free Ca2+ appropriate for the particular experimental design.

**MLC phosphorylation determination.** For measurement of MLC phosphorylation levels, muscle strips were mounted (permeabilized fibers were not mounted), equilibrated, and then rapidly frozen, at appropriate time points during a contractile event, in a dry ice/acetone slurry containing 6% trichloroacetic acid and 10 mM EDTA. The strips were then slowly thawed at room temperature. The tissues were rinsed in acetone, air-dried, and then dry weights were recorded. The aceto-dead tissues were homogenized in a solution containing 1% SDS, 10% glycerol, and 1 mM EDTA using glass/glass homogenizers. The homogenates were clarified by centrifugation and then subjected to two-dimensional gel electrophoresis followed by transfer to nitrocellulose membranes as previously described (23). Proteins were visualized using AuroDye fortess colloidal gold protein stain and quantified using laser-scaning densitometry (Bio-Rad Laboratories, Richmond, CA). MLC phosphorylation levels were calculated as a percentage of the sum of the densitometric analysis of both the phosphorylated and unphosphorylated forms of the MLC.

**MLC dephosphorylation rate.** MLC dephosphorylation rates, used as an index of MLC phosphatase activity, were measured in the α-toxin-permeabilized preparations as described by Somlyo and colleagues (9, 17). α-Toxin-permeabilized tissues from control and diabetic animals were contracted by the addition of 10 μM Ca2+ for 10 min. The tissues were rapidly transferred to a solution containing 1 mM MgCl2, 30 mM K-acetate, 20 mM imidazole (pH 6.8), 0 CaCl2, 10 mM EGTA to chelate any cellular calcium, 0 ATP, 10 mM glucose, and 100 U/ml hexokinase to deplete cellular ATP, and 200 μM ML-9 to inhibit MLC kinase activity. Tissues were frozen after 10 min of Ca2+-dependent stimulation and then at various times after immersion in the dephosphorylation solution. Frozen tissues were then processed for quantitation of MLC phosphorylation levels.

**Western blot analysis of MLC phosphatase content.** Intact strips of bladder smooth muscle from control and diabetic animals were homogenized, total protein content was determined, and then subjected to SDS-PAGE (7.5% acrylamide concentration), followed by transfer to nitrocellulose membranes. The membranes were immuno-blotted using antibodies against the large noncatalytic subunit of myosin phosphatase (1:10,000, clone no. ASC M130, Covance, Princeton, NJ) and actin (1:20,000, clone no. 1A4, Sigma, St. Louis, MO). The immunoblots were visualized by enhanced chemiluminescence. Actin and myosin phosphatase contents were quantified by scanning densitometry.

**Materials, statistics, and data presentation.** All standard reagents were of analytic grade or better and were obtained from Fisher Scientific (Pittsburgh, PA). All electrophoretic and blotting supplies were obtained from Bio-Rad Laboratories. ATP, glucose, hexokinase, ML-9, and creatine phosphate were obtained from Sigma. Ionomycin was obtained from Calbiochem (San Diego, CA). All results are expressed as means ± SE, with n representing the number of bladders. EC50 values were calculated using a linearization of the concentration vs. force relationship. The concentration of Ca2+ corresponding to the 50% force level was obtained from this linearization for each individual muscle strip. Data were compared for statistical significance using Student’s t-test (unpaired) and ANOVA when appropriate. A probability level of <0.05 was taken as statistically significant. All values of p provided represent the number of bladders studied.
 though diabetes significantly increases the absolute values of basal and stimulated levels of MLC phosphorylation, the relationship between suprabasal values of MLC phosphorylation and stress appears to be similar in bladder smooth muscle from the two animal groups.

Receptor-mediated contractile responses were examined using carbachol as the stimulus. Figure 3 shows the noncumulative concentration-response relationship for carbachol in bladder smooth muscle from control and diabetic animals. Carbachol-induced stresses in tissues from diabetic animals tended to be lower than those in tissues from control animals, but the differences did not achieve statistical significance. Similar to the results obtained with KCl, the sensitivity of the tissues from the two animal models to carbachol was not different (0.40 ± 0.07 μM control; 0.35 ± 0.01 μM diabetic). The time course of force and MLC phosphorylation to a single maximal concentration of carbachol (10 μM) is shown in Fig. 4. Carbachol induced the typical phasic contraction in bladder smooth muscle from both control and diabetic animals. Steady-state forces in the tissues from the diabetic animal, however, were significantly lower than those from control animals (Fig. 4A). Consistent with the results using KCl as the stimulus shown in Fig. 3, A and B, basal and carbachol-stimulated levels of MLC phosphorylation are significantly elevated in smooth muscles from the diabetic animals compared with control animals (Fig. 4B). Also consistent with the results using KCl as the stimulus, the relationship between suprabasal MLC phosphorylation and stress is unchanged by diabetes (Fig. 4C).

Because both membrane depolarization and agonist-induced contractions were altered during the diabetic state, we were interested in determining whether the changes were in the signaling to the contractile proteins or at the level of the contractile proteins. To examine this question, we used the α-toxin-permeabilized preparation that allows control of the intracellular environment while maintaining physiologically relevant signaling cascades (17, 25). The results of these studies are shown in Fig. 5. Figure 5A shows the expected increase in Ca²⁺ sensitivity by the addition of an agonist (10 μM carbachol) plus 10 μM GTP in tissues from control animals. In addition, as is typical in nonvascular smooth muscles, agonist plus GTP stimulation produced an increase in total force development in addition to the increase in Ca²⁺ sensitivity. Figure 5B shows the results of similar experiments
performed on tissues from the bladder of diabetic animals. Qualitatively similar results were obtained, an increase in total force and an increase in Ca\(^{2+}\) sensitivity, in the presence of Ca\(^{2+}\) plus carbachol plus GTP compared with Ca\(^{2+}\) alone.

To directly compare the results from control and diabetic animals, we graphed the Ca\(^{2+}\) alone curves from the two animal models together (Fig. 5C) and the Ca\(^{2+}\) plus carbachol plus GTP curves from the two animal models together (Fig. 5D). Maximal force development was not significantly different in either stimulation condition (Ca\(^{2+}\) alone or Ca\(^{2+}\) plus carbachol and GTP) between the two animal models using the α-toxin-permeabilized preparation in which [Ca\(^{2+}\)] is held constant. This would suggest that the significant decrease in maximal force developed in the intact tissue in response to membrane depolarization where cellular calcium levels are changing, from diabetic animals compared with tissue from control animals (Fig. 1A), may be related to calcium handling rather than the contractile proteins. In contrast, there were significant differences in the Ca\(^{2+}\) sensitivity of the two animal models during both stimulation regimens. The tissues from the diabetic animals were significantly less sensitive to Ca\(^{2+}\) than tissues from control animals (Ca\(^{2+}\) alone: 2.13 ± 0.12 μM control vs. 2.80 ± 0.19 μM diabetic; Ca\(^{2+}\) plus carbachol plus GTP: 0.88 ± 0.11 μM control vs. 1.31 ± 0.16 μM diabetic).

Please note that the force values in Fig. 5, C and D, exceed 100% of 10 μM Ca\(^{2+}\) and as such calculated values of the EC\(_{50}\) for Ca\(^{2+}\) do not equate to the 50% force level in the figure.

Changes in the Ca\(^{2+}\) sensitivity of MLC phosphorylation have been proposed as the primary mechanism underlying changes in the Ca\(^{2+}\) sensitivity of force in smooth muscle. Therefore, the next logical experiment was to determine the levels of MLC phosphorylation in the α-toxin-permeabilized bladder preparations. We measured steady-state MLC phosphorylation levels at 0, 0.5, 1.0, and 10.0 μM Ca\(^{2+}\) in the absence and presence of 10 μM carbachol plus 10 μM GTP. The results of these experiments are shown in Fig. 6. Steady-state MLC phosphorylation levels were slightly but not significantly elevated (with the exception of 1.0 μM Ca\(^{2+}\)) by the addition of carbachol plus GTP in tissues from the control animals. Similarly, carbachol plus GTP did not increase steady-state MLC phosphorylation levels in α-toxin-permeabilized tissues from diabetic animals. In contrast, MLC phosphorylation levels in almost all of the conditions tested were significantly higher in permeabilized tissues from the diabetic animals compared with those from the controls, regardless of stimulation conditions. The results in Fig. 5 showing a significant increase in force development in response to Ca\(^{2+}\) and carbachol plus GTP compared with Ca\(^{2+}\) alone in tissue from either animal group do not correlate with the lack of increase in MLC phosphorylation levels (Fig. 6). This would suggest that MLC phosphorylation levels may not be the only determinant of the Ca\(^{2+}\) sensitivity of smooth muscle. It should be pointed out, however, that it is technically difficult to quantitatively measure very small changes in MLC phosphorylation.

Fig. 2. Time course of force and myosin light chain (MLC) phosphorylation in response to KCl in bladder smooth muscle from control and diabetic animals. Strips of bladder smooth muscle from control (○) and diabetic animals (●) were contracted in response to 110 mM KCl. A: time course of force in response to KCl. Values were normalized in response to the maximal response in each tissue. Tissues from the diabetic animals exhibited a slight but significant decrease in the phasic profile of the contraction compared with control. B: time course of MLC phosphorylation in response to KCl. Basal and peak MLC phosphorylation levels in tissues from diabetic animals were significantly elevated compared with those from control. Steady-state MLC phosphorylation values were elevated but not to a level of significance in tissues from diabetic compared with control animals. C: stress values from A and B are shown as a function of stimulation-induced increases in MLC phosphorylation levels (actual levels — basal). The dependence of stress on MLC phosphorylation appears unchanged in tissues from diabetic animals (●) compared with tissues from control animals (○). Values are means ± SE for 14–16 force determinations and 3–5 MLC phosphorylation determinations each from a different bladder. *P < 0.05 compared with control.
This is demonstrated, for example, if one compares the MLC phosphorylation levels at 0.5 and 1.0 μM Ca²⁺ in the first set of bars in Fig. 6.

To more precisely examine the apparent diabetes-induced change in the MLC phosphorylation/force relation, we graphed force as a function of MLC phosphorylation (Fig. 7). The slopes of the MLC phosphorylation-force relationship are similar between control and diabetic animals; however, the diabetic curve is significantly shifted to the right. The data in this figure clearly demonstrate that in bladder smooth muscle from diabetic animals, any given level of force development is associated with significantly greater levels of MLC phosphorylation than in smooth muscle from control animals. Conversely, a slightly different way of looking at the same scenario is that for any given level of MLC phosphorylation, less force is developed.

Higher levels of MLC phosphorylation as demonstrated in intact tissue from the diabetic animals in Figs. 2 and 4 and in the permeabilized fibers shown in Fig. 6 could result from either a decrease in the activity of the MLC phosphatase or a decrease in the content of the MLC phosphatase. We first addressed the question of whether diabetes resulted in a decrease in the activity of the MLC phosphatase. As detailed in MATERIALS AND METHODS, we measured MLC dephosphorylation during conditions that removed all Ca²⁺ and ATP and blocked all MLC kinase activity as an index of MLC phosphatase activity. The results are shown in Fig. 8. Surprisingly, the MLC dephosphorylation rate in α-toxin-permeabilized tissues from control animals was not significantly decreased by the presence of carbachol and GTP. The MLC phosphorylation rates in α-toxin-permeabilized tissues from diabetic animals were significantly slower in the presence of either Ca²⁺ alone or in the presence of carbachol and GTP compared with tissues from control animals. Although not a significant change, the addition of carbachol and GTP to the permeabilized tissues from diabetic animals tended to slow MLC dephosphorylation rates compared with the rate in the presence of Ca²⁺ alone.

To determine whether a decrease in phosphatase content could also account for the increase in basal and stimulated levels of MLC phosphorylation, we measured MLC phosphatase levels in bladder smooth muscle from the two animal models. As can be seen in Fig. 9, there were no significant differences in actin, MLC phosphatase, or the ratio of actin to MLC phosphatase contents in bladder smooth muscle control compared with diabetic animals.

DISCUSSION

Urinary bladder dysfunction has been shown to be a complicating factor in patients with diabetes mellitus (33, 38). Although the genesis of many of the diabetic complications is thought to be due to changes in the neuronal input to the bladder, it is not known whether diabetes has a direct effect at the level of bladder smooth muscle. In this study, we examined the functional response of urinary bladder smooth muscle as well as the regulation of the response in animals subjected to chronic (4 mo) diabetes. Smooth muscle from the bladder of diabetic animals produced almost 50% less stress in response to KCl compared with bladders from control animals. In contrast, the response to carbachol tended to be less in bladders from diabetic animals, although the differences did not attain statistical significance. There were no differences in the sensitivity to either KCl or carbachol in smooth muscle from diabetic compared with control rabbits. The most striking diabetes-induced change in the bladder smooth muscle was the significant increase in levels of MLC phosphorylation without any increase in resting tone. The increase in levels of MLC phosphorylation was not due to a decrease in MLC phosphatase content but instead an apparent decrease in MLC phosphatase activity.

Diabetes appears to have differential effects on the magnitude of a smooth muscle contraction. Whether a diabetic state increases, decreases, or does not change the magnitude of a contraction depends on the species, the agonist, and the technique used to report the response. The simplest answer to explain a diabetic-induced change in magnitude of contraction is if only grams force was measured and the diabetes-dependent hypertrophy was not taken into account. For example, in streptozotocin-induced diabetes in the rat, grams force contraction of the bladder smooth muscle in response to serotonin was increased but because of a significant hypertrophy, actual values of stress were unchanged (18). Similarly, Longhurst (20) showed that in the spontaneously diabetic Brattleboro rat, grams force of contraction in response to several agonists was unchanged, but if normalized to the increase in muscle mass, there was actually a tendency for decreased contractility.
Therefore, these studies as well as data in our present report and a previous study from our laboratory (36) highlight the necessity to normalize all contractile data to bladder mass, or if possible, muscle mass. Because we use a preparation that is devoid of most if not all of the serosal and mucosal layers, our tissue mass is a close approximation of muscle mass. Moreover, we previously showed that in another model of bladder hypertrophy partial bladder outlet obstruction, that although the weight of the bladder and wall thickness increase, the percent muscle mass in a strip devoid of serosal and mucosal layer is unchanged (36). This supports our proposal that tissue mass in our tissue strips approximates muscle mass or is at least constant between the two animal models.

As stated above, we believe the most dramatic finding in our study is the diabetes-dependent increase in basal and stimulated levels of MLC phosphorylation while concomitant levels of stress are either decreased or at best unchanged. The data shown in Figs. 2, A and B, and 4, A and B, clearly demonstrate this separation in stress from basal MLC phosphorylation. However, the data shown in Figs. 2C and 4C suggest that the relationship between stress and MLC phosphorylation per se is not changed by diabetes. The data compiled in Fig. 7 show that diabetes does however produce a parallel shift to the right in the MLC phosphorylation-force relationship. Given that Ca\(^{2+}\)-dependent increases in MLC phosphorylation are believed to be the primary initiating factor for contraction (16, 35), our results are both surprising and contradictory to the common dogma.

The increase in basal MLC phosphorylation values may be the result of a difference in preload on strips from control compared with diabetic animals. Ratz and Miner (31) suggested that levels of MLC phosphorylation decrease with a change in preload from less than \(L_o\) to \(L_o\). Thus the higher levels of MLC phosphorylation in the bladder strips from diabetic animals could potentially be due to those strips being maintained at a lower preload. However, as outlined in MATERIALS AND METHODS, we equilibrated our tissues for at least 90 min followed by a partial length-tension curve performed on each and every muscle strip. Therefore, all muscle strips used in this study were at or near \(L_o\). Moreover, Ratz and Minor (31) used a bladder preparation with only the urothelium removed, producing a much thicker preparation with a greater matrix-to-muscle ratio than our dissection, which removed both the serosal and mucosal layers. One, therefore, would expect that any influence of the nonmuscle mass of the bladder wall would have a significantly lower effect in our preparation.

It is also possible that the higher basal levels of MLC phosphorylation are due to phosphatase incorporation into so-called nonfunctional sites. Protein kinase C has been shown to phosphorylate serine\(^1\), serine\(^2\), and threonine\(^9\) residues on the regulatory light chain (reviewed in Ref. 34). More importantly, phosphorylation of these sites does not affect smooth muscle contractility. Because our method for quantitation of MLC phosphorylation levels does not provide information on

![Fig. 4. Time course of force and MLC phosphorylation in response to carbachol in bladder smooth muscle from control and diabetic animals. Strips of bladder smooth muscle from control (●) and diabetic animals (○) were contracted in response to 10 μM carbachol. A: time course of force in response to carbachol. Values were normalized in response to the maximal response in each tissue. Tissues from the diabetic animals exhibited a slight but significant increase in the phasic profile of the contraction compared with control, resulting in lower levels of steady-state force. B: time course of MLC phosphorylation in response to carbachol. Basal and steady-state MLC phosphorylation levels in tissues from diabetic animals were significantly elevated compared with those from control. Peak MLC phosphorylation values were also significantly elevated in tissues from diabetic animals compared with those from control. C: stress values from A and B are shown as a function of stimulation-induced increases in MLC phosphorylation levels (actual levels – basal). The dependence of stress on MLC phosphorylation appears unchanged in tissues from diabetic animals (○) compared with tissues from control animals (●). Values are means ± SE for 18–24 force determinations and 2–5 MLC phosphorylation determinations each from a different bladder. *P < 0.05 compared with control.](http://ajprenal.physiology.org/)}
the residues phosphorylated, we do not know whether this is the case. However, we and others showed that even during maximal activation of protein kinase C, only very small increases in MLC phosphorylation occur (7, 34). This would tend to support the hypothesis that the basal values of MLC phosphorylation noted in the present study are not due to protein kinase C.

Conversely, the increase in basal MLC phosphorylation values may be the result of a diabetes-induced decrease in Na⁺-K⁺ pump activity and concomitant decrease in Na⁺-Ca²⁺ exchange activity, producing a higher resting cellular [Ca²⁺] (10, 12). The higher resting [Ca²⁺] could then produce higher resting MLC phosphorylation levels. Why then are not resting values of stress greater in diabetic bladder compared with control and/or the muscle from diabetic animals show an increased responsiveness to stimulation? It is possible that because we are using a chronic diabetic model, more closely representative of the long-term disease state compared with an acute model, the muscle has accommodated to the increased basal MLC phosphorylation levels. This could be due to an uncoupling of stress and MLC phosphorylation or more likely the expression of another regulatory system that can act as a brake for contraction during low levels of activation. Such a role has been proposed for the thin-filament proteins, caldesmon and calponin (5, 40, 41). The fact that the slope of the MLC phosphorylation-force relationship is similar in bladder smooth muscle from control compared with diabetic animals would then suggest that muscle from diabetic animals simply has a higher threshold of activation to overcome before contraction can be elicited. This could account for the decrease in force developed in response to KCI as well as the lack of change in sensitivity to stimulation between the two tissue sources. On the other hand, no change in resting or peak levels of [Ca²⁺] was seen in bladder smooth muscle from a rat model of diabetes (30, 42). Whether this is also the case in the chronic rabbit model of diabetes is not known.

Our results using the α-toxin preparation would suggest that changes in cellular [Ca²⁺] are not necessary for a change in either stress or MLC phosphorylation in smooth muscle from diabetic compared with control animals. At constant and similar levels of Ca²⁺, bladder smooth muscle from diabetic animals showed a lower sensitivity to Ca²⁺ but a consistent trend toward greater levels of MLC phosphorylation compared with permeabilized muscles from control animals. This clearly demonstrates that a fundamental change has occurred in the smooth muscle from diabetic animals that does not rely on a change in stimulation-induced levels of activator calcium.

An increase in cytosolic [Ca²⁺] would increase MLC kinase activity and therefore MLC phosphorylation levels. However, another mechanism that results in elevated MLC phosphorylation levels is a decrease in MLC phosphatase activity. This has been clearly shown to be important in the receptor and G

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Fig. 5. α-Toxin-permeabilized bladder smooth muscle from control and diabetic animals. Strips of bladder smooth muscle were α-toxin-permeabilized and then subjected to the cumulative addition of Ca²⁺ in the absence and presence of 10 μM carbachol plus 10 μM GTP. A: permeabilized strips from control animals were contracted with Ca²⁺ alone (○) or with Ca²⁺ plus carbachol plus GTP (●). The addition of carbachol plus GTP significantly increased the myofilament Ca²⁺ sensitivity as well as increased maximal force development. B: permeabilized strips from diabetic animals were contracted with Ca²⁺ alone (○) or with Ca²⁺ plus carbachol plus GTP (●). The addition of carbachol plus GTP significantly increased the myofilament Ca²⁺ sensitivity as well as increased maximal force development. C: Ca²⁺-dependent contractions of strips from control animals (○) compared with the Ca²⁺-dependent contractions of strips from diabetic animals (●). The Ca²⁺ sensitivity of strips from diabetic animals were significantly lower than those from control animals. D: Ca²⁺ plus carbachol plus GTP-dependent contractions of strips from control animals (○) compared with the Ca²⁺ plus carbachol plus GTP-dependent contractions of strips from diabetic animals (●). The Ca²⁺ sensitivity of strips from diabetic animals in the presence of carbachol plus GTP was significantly lower than those from control animals. Please note that the force values in D exceed 100% of 10 μM Ca²⁺ and as such half-maximal values of force are not 50%. Values are means ± SE for 4–7 determinations each from a different bladder. *P < 0.05 compared with control.
phosphorylation does not equate to an increase in contraction. The problem still remains as to why an increase in MLC phosphorylation levels in smooth muscle from diabetic animals was on average significantly elevated compared with tissues from control animals at each differing condition. Values are means ± SE for 3–5 determinations each from a different bladder. *P < 0.05 compared with similar conditions in tissues from control animals. #P < 0.05 compared with 0 Ca^{2+} alone in the control.

protein-mediated increase in myofilament Ca^{2+} sensitivity (35). We directly demonstrated that MLC phosphatase activity was decreased in bladder smooth muscle from diabetic animals compared with control animals (Fig. 8). Because there was no change in MLC phosphatase content (Fig. 9), we assume the decrease in activity is related to a change in phosphatase regulation.

If the hypothesis put forth above, suggesting a thin-filament regulatory protein acts as a brake against the increased number of phosphorylated myosin molecules, is correct, then can the diabetes-induced increase in MLC phosphorylation have any
function? The latch state hypothesis, presented in the early 1980s (3), stated that high levels of force could be maintained by slowly cycling dephosphorylated cross bridges, termed latch bridges. A short time later it was demonstrated that relaxation of a contraction supported by phosphorylated cross bridges was significantly faster than a contraction supported by latch bridges (8). In fact, it was later hypothesized that one mechanism to increase relaxation time may be to rephosphorylate latch bridges and therefore increase the number of phosphorylated myosin molecules (32). The transient phase of a contraction of bladder smooth muscle from diabetic animals relaxes faster than that from control animals (Figs. 2 and 4). This may result from the increased level of MLC phosphorylation.

In summary, bladder smooth muscle from diabetic animals produces less stress than bladder smooth muscle from control animals; however, the sensitivity to either KCl or carbachol is unaltered. The sensitivity to either KCl or carbachol is unaltered. The sensitivity to either KCl or carbachol is unaltered. The sensitivity to either KCl or carbachol is unaltered.

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