Caffeine relaxes smooth muscle through actin depolymerization

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Tazzeo T, Bates G, Roman HN, Lauzon A, Khasnis MD, Eto M, Janssen LJ. Caffeine relaxes smooth muscle through actin depolymerization. Am J Physiol Lung Cell Mol Physiol 303: L334–L342, 2012.—Caffeine relaxes smooth muscle through actin depolymerization. Am J Physiol Lung Cell Mol Physiol 303: L334–L342, 2012. First published June 8, 2012; doi:10.1152/ajplung.00103.2012.—Caffeine is sometimes used in cell physiological studies to release internally stored Ca2+. We obtained evidence that caffeine may also act through a different mechanism that has not been previously described and sought to examine this in greater detail. We ruled out a role for phosphodiesterase (PDE) inhibition, since the effect was 1) not reversed by inhibiting PKA or adenylate cyclase; 2) not exacerbated by inhibiting PDE4; and 3) not mimicked by submillimolar caffeine nor theophylline, both of which are sufficient to inhibit PDE. Although caffeine is an agonist of bitter taste receptors, which in turn mediate bronchodilation, its relaxant effect was not mimicked by quinine. After permeabilizing the membrane using β-escin and depleting the internal Ca2+ store using A23187, we found that 10 mM caffeine reversed tone evoked by direct application of Ca2+, suggesting it functionally antagonizes the contractile apparatus. Using a variety of molecular techniques, we found that caffeine did not affect phosphorylation of myosin light chain (MLC) by MLC kinase, actin-filament motility catalyzed by MLC kinase, phosphorylation of CPI-17 by either protein kinase C or RhoA kinase, nor the activity of MLC-phosphatase. However, we did obtain evidence that caffeine decreased actin filament binding to phosphorylated myosin heads and increased the ratio of globular to filamentous actin in precontracted tissues. We conclude that, in addition to its other non-RyR targets, caffeine also interferes with actin function (decreased binding by myosin, possibly with depolymerization), an effect that should be borne in mind in studies using caffeine to probe excitation-contraction coupling in smooth muscle.

Excitation-contraction coupling; Ca2+ handling; airway smooth muscle; ryanodine; asthma

Contraction of smooth muscle (SM) is ultimately dependent on phosphorylation of the regulatory light chain of myosin (MLC20) at Ser19, leading to a marked increase in myosin ATPase activity and cross-bridge cycling with actin. MLCK is widely recognized as the primary effector of this phosphorylation and is activated directly by Ca2+. Phospho-MLC20 (P-MLC20) is then dephosphorylated by myosin light chain phosphatase (MLCP). Contraction can also involve a suppression of tonic MLCP activity, via phosphorylation of MLCP and the MLCP inhibitor protein, CPI-17, in response to the activation of RhoA kinase (ROCK) and PKC pathways, in Ca2+-independent and -dependent fashions, respectively. Furthermore, accumulating evidence suggests that Ca2+-dependent activation of calcineurin causes the actin polymerization mediating agonist-induced canine airway smooth muscle (Sm) contraction (51). Thus both MLC20 phosphorylation and actin polymerization are involved in Ca2+-induced SM contraction in response to agonist stimuli.

Given this central role for Ca2+ in contraction, considerable attention has been focused on the regulation of cytosolic concentration of calcium ([Ca2+]i) in SM. [Ca2+]i, is elevated through yet another mechanism, distinct from disruption of Ca2+ handling or PDE activity. Here, we characterize that, in addition to its other non-RyR targets, caffeine also interferes with actin function (decreased binding by myosin, possibly with depolymerization), an effect that should be borne in mind in studies using caffeine to probe excitation-contraction coupling in smooth muscle.

Because of the importance of the SR in determining [Ca2+]i, many tools have been identified that modulate the activity of these Ca2+ flux pathways. One of the most commonly used among these is caffeine, which at millimolar concentrations increases the Ca2+ sensitivity of the RyR such that they become activated at basal [Ca2+]i. Moreover, caffeine is often used to study excitation-contraction coupling in SM, with the intent of inducing a massive release of internally sequestered Ca2+ or possibly even outright depletion of the SR. However, caffeine is also known to mediate other effects at even lower concentrations, including inhibition of phosphodiesterase (PDE) activity and blockade of adenosine receptors (9, 50). In the course of investigating the mechanisms underlying contractile responses to 5-HT in bovine tracheal SM (TSM), we collected evidence that it might also be acting through yet another mechanism, distinct from disruption of Ca2+ handling or PDE activity. Here, we characterize that other mechanism, finding it to comprise a direct inhibition of the contractile apparatus, in part through disruption of actin filaments. Comparisons are made with other molecules that share caffeine’s identity as a methylxanthine and PDE inhibitor [theophylline; isobutylmethylxanthine (IBMX)] and as agonist at bitter taste receptors (quarine).
METHODS

Preparation of isolated tissues. All experimental procedures were approved by the McMaster University Animal Care Committee, the McMaster University Biosafety Committee, and the St. Joseph’s Healthcare Research Ethics Board and conform to the guidelines set out by the Canadian Council on Animal Care. Tracheas were obtained from cows (200–500 kg) euthanized at the local abattoir and transported to the laboratory in ice-cold Krebs buffer (see Solutions and chemicals). Upon arrival at the laboratory, the epithelium was removed, and TSM strips (~2–3 mm wide, ~10 mm long) were excised and used immediately or stored at 4°C for use up to 48 h.

Muscle bath technique. ASM strips were mounted vertically in organ baths using silk suture (Ethicon 4-0) tied to a Grass FT.03 force transducer on one end and to a glass rod that served as an anchor on the other end. These were bathed in Krebs-Ringer buffer containing (in mM) 116 NaCl, 4.2 KCl, 2.5 CaCl₂, 1.6 NaH₂PO₄, 1.2 MgSO₄·7H₂O, 22 NaHCO₃, 11 glucose, bubbled with 95% O₂-5% CO₂ to maintain pH at 7.4 and maintained at 37°C. The Krebs-Ringer buffer was replaced with a buffer that mimics the physiological pH at 7.4 and maintained at 37°C. C₈-nitro-arginine (10⁻⁴ M) and indomethacin (10⁻³ M) were also added to prevent generation of nitric oxide and of cyclooxygenase metabolites of arachidonic acid, respectively. Tissues were passively stretched to impose a preload tension of ~1 g. Isometric changes in tension were digitized (2 samples per second) and recorded online (DigiMed System Integrator, MicroMed, Louisville, KY) for plotting on the computer. Tissues were equilibrated for 1 h before commencing the experiments, during which time they were challenged with 60 mM KCl three times to assess the functional state of each tissue.

β-Escin permeabilized muscle strips. Bovine TSM was excised in the form of strips ~50–100 μm thick and 3–4 mm long. One end of these was anchored, and the other end was tied to a force transducer (SensoNor AE801; Norway) with single fibers of silk suture, stretched to its in situ length, and bathed in standard Krebs-Ringer buffer (composition given below). After 30–60 min of equilibration, the Krebs-Ringer buffer was replaced with a buffer that mimics the cytosol (i.e., high-[Ca²⁺] and low free [Ca²⁺]) developed by Rodat-Despoix et al. (38), comprised of the following (in mM): 87 KCl, 5.1 MgCl₂, 5.2 ATP (disodium salt), 10 creatine phosphate, 2 EGTA, 30 PIPES, pH adjusted to 7.4 by use of KOH. Various C₈a were obtained by dilution of a high-Ca²⁺ form of this cytosolic buffer (one supplemented with 6.5 mM CaCl₂ and 10 mM EGTA, estimated to give pCa 5) with appropriate volumes of the nominally Ca²⁺-free form of this buffer. Tissues were initially bathed in pCa 8 cytosolic buffer, then permeabilized with β-escin (10 μM) for 40 min. Unless noted otherwise, the Ca²⁺ ionophore A23187 (10⁻⁴ M) was also added to this pCa 8 cytosolic buffer to deplete the SR. The permeabilized tissues were then challenged with cytosolic buffers having pCa ranging from 8 to 5 in the presence of various pharmacological tools.

In vitro motility assay. The in vitro motility assay was used to quantify the velocity of actin propulsion by myosin (v_max) as previously described (5, 26). Myosin was purified from pig stomach antrum (43) and was either phosphorylated (46) in the absence or presence of caffeine (1 and 10 mM), or thiophosphorylated (46) in the absence of caffeine [phosphorylation levels were assessed by phos-tag analysis (44)]. Actin was purified from chicken pectoralis acetone powder (32) and fluorescently labeled with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (P1951, Sigma-Aldrich Canada) (49). The pte- teins and buffers were introduced into the flow-through chamber as previously described with the exception that caffeine (or vehicle) was added to the thiophosphorylated myosin and motility buffer before being introduced into the chamber. Measurements were performed at 30°C. Motility was assayed by use of an inverted microscope (IX70, Olympus, Melville, NY) equipped with a high numerical aperture objective (×100 magnification Ach 1.25 numerical aperture, Olympus) and rhodamine epifluorescence. An image-intensified video camera (KP-E500 CCD Camera, Hitachi Kokusai Electric, Woodbury, NY) was used to visualize and record the actin filament movement on computer (Custom Built by Norbec Communication, Montreal, QC, Canada) by using a frame grabber (Pinnacle Studio AV/DV V.9 PCI Card) at 29.94 Hz and an image capturing software (AMCap software V9.20) at 29.94 Hz. The v_max was determined from the total path described by the filaments divided by the elapsed time via our automated version of the National Institutes of Health tracking software (NIH macro in Scion Image 4.02, Scion). The assay was also modified to quantify the number of actin filaments bound by thio- phosphorylated myosin. Four washes of actin buffer were added after the labeled actin incubation to wash away unbound actin filaments. No ATP was added to the motility buffer because only binding was assessed. Caffeine (or vehicle) was added to the myosin, to the labeled actin, and to the actin buffer washes. Actin filament binding was assessed by quantifying the number of filaments present in five spots per flow-through chamber throughout the motility surface.

Myosin light chain phosphatase assay. The recombinant MLCP complex, consisting of the regulatory subunit (MYPT1) and the catalytic subunit (PP1C₈), was prepared by using a mammalian-cell coexpression system. COS1 cells were transiently transfected for 48 h with a pair of DNA vectors FLAG-metal affinity tag (MAT)-tagged full length human MYPT1 and HA-tagged PP1C₈ using FuGeneHD reagent (Roche). After transfection, the cells expressing recombinant MYPT1 and PP1C₈ were lysed with the lysis buffer containing 20 mM Tris-HCl, pH 8.0, plus 0.5 M NaCl, 0.1 mM EDTA, 5% glycerol, 0.1% Tween-20, 4 mM Pefabloc (Roche), and 0.5 mM Tris[2-carboxyethyl]phosphine (TCEP, Pierce). The clarified cell lysates were subjected to metal affinity purification by use of Talon beads (Invitrogen) and eluted with the buffer including 0.5 M imidazole-HCl, pH 7.0. The eluant, including MYPT1 and PP1C₈, was used as the MLCP preparation. The MLCP activity was determined from the amount of inorganic phosphate liberated from the substrate, phospho- peptide mimicking MLC20 (3–26) with phospho-Ser19. The amount of released phosphate was determined by using a BIOMOL Green kit (Enzo). The conditions were 25 mM MOPS-NaOH, pH 7.0, plus 0.1 mM EDTA, 0.5 mM TCEP, 4 mM Pefabloc, 1 mM okadaic acid, and caffeine at the indicated concentration. Okadaic acid at low concentration was added to avoid the trace of the activity of PP2A in the MLCP preparation. The reaction was initiated by addition of the substrate and terminated by addition of BIOMOL Green reagent, followed by the measurement of OD 650 nm. The thiolphosphorylation of the recombinant MLCP was carried out by preincubating for 30 min at 30°C with ROCK and 1 mM ATPγS prior to the assay. Mean values ± SE were obtained from triplicate assays in three independent experiments (n = 9).

Kinase assay. Phosphorylation in the presence of caffeine was assayed by using MLCK purified from chicken gizzard, PKC purified from human red cells (7), and the recombinant human ROCK2 (Millipore). Chicken gizzard MLCK20 and recombinant CPI-17 (7) were used as substrates. MLCK, ROCK, and PKC assays were performed using phospho-specific antibodies for phospho-MLC20 Ser19 (Cell Signaling) and phospho-CPI-17 Thr38 as described previously (8).

F-actin-to-G-actin ratio assay. SM strips were homogenized in F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% anti-foul, 1 mM ATP, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, 500 μg/ml tosyl arginine methyl ester) provided within a standard commercially available assay kit (Cytokeleton, Denver, CO). The homogenates were centrifuged at 100,000 g for 60 min at 30°C, then the supernatants (containing G-actin) were collected and kept on ice. The pellets (containing F-actin), on the other hand, were resuspended in ice-cold distilled H₂O plus 1 μm cytochalasin D and incubated on ice for 1 h to dissociate the actin filaments into G-actin monomers; the resuspended pellets were gently mixed every 15 min. This cytochalasin-digested mixture was centrifuged at 2,300
Statistically significant.

by Student's number of flow-through chambers. Statistical comparisons were made ...

IP3-sensitive Ca\textsuperscript{2+} and shown complete overlap between ryanodine-sensitive and deplete the SR in ASM (13, 18, 19, 23, 24); others have acid (CPA), which we have previously shown is sufficient to to 5-HT, then this effect should be mimicked by cyclopiazonic acid (CPA) plus/minus ryanodine (10\textsuperscript{-5} M) (Fig. 1). This inhibitory effect of caffeine developed within minutes, being half-maximal within 1–2 min, and more than 90% complete within 10 min (Fig. 1B).

If depletion of the SR accounts for the loss of responsiveness to 5-HT or KCl were expressed as a percentage of the response to bolus addition of 60 mM KCl added during the equilibration period (immediately before onset of the experiment). Reversals of tone in permeabilized tissue strips are expressed as a percentage of the tone evoked by pCa 6.5 buffer. Values are reported as means \pm SE; n refers to the number of animals. For the in vitro motility assay, n refers to the number of flow-through chambers. Statistical comparisons were made by Student’s t-test or one-way ANOVA; P < 0.05 was considered statistically significant.

RESULTS

Caffeine inhibits excitatory responses in intact bovine TSM strips. Serotonin evoked contractions in a concentration-dependent fashion (Fig. 1A); these were abolished by pretreatment with 10 mM caffeine, both in the presence and absence of ryanodine (10\textsuperscript{-5} M) (Fig. 1). This inhibitory effect of caffeine developed within minutes, being half-maximal within 1–2 min, and more than 90% complete within 10 min (Fig. 1B).

If depletion of the SR accounts for the loss of responsiveness to 5-HT, then this effect should be mimicked by cyclopiazonic acid (CPA), which we have previously shown is sufficient to deplete the SR in ASM (13, 18, 19, 23, 24); others have confirmed this property of CPA specifically in bovine TSM (6) and shown complete overlap between ryanodine-sensitive and IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores (2). However, we show here (Fig. 1A) that 5-HT-evoked contractions are relatively unaffected by pretreatment with CPA plus/minus ryanodine (10\textsuperscript{-5} M; the latter added to promote Ca\textsuperscript{2+} release through RyR).

Similarly, if the mechanism of action underlying this inhibitory effect of caffeine is simply through SR depletion, then 1 mM caffeine should also exert some inhibitory effect, since that concentration is also sufficient to sensitize RyR (34). However, this too was not the case (Fig. 1C).

Finally, caffeine-induced depletion of the SR should have little effect on contractions evoked by high millimolar KCl, which in ASM are mediated by promotion of voltage-dependent Ca\textsuperscript{2+} influx and/or stimulation of RhoA kinase activity (22, 28). However, we show here that caffeine reduces by more than 50% the magnitudes of contractions evoked by all concentrations of KCl (Fig. 2), irrespective of whether the SR has previously already been emptied or not. That is, depletion of the stores using CPA and ryanodine caused a modest left-hand shift in the KCl concentration-response relationship [Fig. 2; which we have previously shown is due to abolition of the superficial buffer barrier (18, 24)], but this prior depletion had no effect on the caffeine-induced reduction of KCl-evoked contractions (Fig. 2). Our observation that caffeine antagonizes KCl-evoked contractions also argues against the possibility that caffeine’s effect on 5-HT-evoked contraction is mediated through blockade of 5-HT receptors.

Caffeine acts through inhibition of PDE? Altogether, then, the data suggest that mere depletion of the SR does not account for the inhibitory effect of millimolar caffeine on contractions in ASM. Being a methylxanthine, caffeine at concentrations 100-fold lower than those used here can also inhibit PDE activity (50), which might functionally antagonize the excitatory responses. IBMX (10\textsuperscript{-4} M), another methylxanthine with well-documented inhibitory action of PDE activity, fully reproduced the effect of caffeine (Fig. 3A). However, theophyl-
line, a methylxanthine that has been used clinically to reverse bronchoconstriction through its ability to inhibit PDE activity (35, 37), when applied at a concentration of 100 μM [sufficient to fully inhibit PDE (35)], had no effect against KCl-evoked contractions (Fig. 3A). Similarly, 1 mM caffeine [which is also sufficient to inhibit PDE activity (50)] applied for 30 or 120 min did not reproduce this inhibitory effect (Fig. 3B), and 10 mM caffeine applied for 120 min did not have any greater effect than that at 10 min (Fig. 3C). Also not consistent with the interpretation that caffeine inhibits contraction by causing accumulation of cyclic nucleotides was our finding that its inhibitory effect was not augmented by rolipram [10⁻⁵ M; selective inhibitor of PDE4, the cAMP-dependent subtype that is prevalent in bovine ASM; (39), nor reduced by the adenylate cyclase inhibitors SQ22536 or MDL 12330A (10⁻⁵ M) (Fig. 3D). Finally, we tested the effect of the protein kinase A inhibitor H89 (3 × 10⁻⁵ M) (Fig. 3D) on this effect of caffeine. Given that H89 is also a powerful inhibitor of ROCK (3), which in turn modulates the contractile response to KCl (22, 28, 40), we compared the effect of H89 in tissues that had concurrently been pretreated with or without the ROCK-selective inhibitor Y27632 (3); H89 did not remove the caffeine-induced suppression (Fig. 3D). Altogether, these various lines of evidence rule out the interpretation that this caffeine-induced suppression that we describe here is owing to inhibition of PDE, accumulation of cAMP and activation of PKA.

Caffeine acts as a direct functional antagonist? Next, we explored the possibility that caffeine was acting as a direct functional antagonist by using the permeabilized muscle strip approach. First, to confirm that our preparation allowed direct access to the contractile apparatus, we compared responses to pCa 6.5 conditions before and after membrane permeabilization. TSM strips did not exhibit a contractile response (Fig. 4, A and B) that was unaffected by subsequent application of nifedipine to block voltage-dependent Ca²⁺ channels (Fig. 4B). Altogether, these observations confirm that the tone evoked in β-escin-permeabilized tissues by pCa 6.5 cytosolic buffer is due to direct stimulation of the contractile apparatus by free Ca²⁺.

In bovine TSM strips permeabilized and then constricted using pCa 6.5 cytosolic buffer as described above, subsequent challenge with 10 mM caffeine led to a transient spike-like increase in tone, followed by a precipitous abolition of all tone (Fig. 4, A–C). The initial small contraction appears to be mediated by release of internal Ca²⁺, since it was occluded by pretreatment of the tissues using A23187 (10⁻⁵ M; e.g., compare Fig. 4, A and B). One millimolar caffeine evoked only a small drop in pCa 6.5 tone (Fig. 4C). The relaxant effect of caffeine is not mediated by inhibition of PDE, since the nonselective PDE inhibitor IBMX (10⁻³ M) exerted the opposite effect on pCa 6.5 tone (Fig. 4C). Theophylline at a concentration of 100 μM [sufficient to fully inhibit PDE (35)] caused no statistically significant reversal of pCa 6.5 tone (Fig. 4C); 1 and 10 mM theophylline; however, completely reversed pCa 6.5 tone. Finally, caffeine is also an agonist of bitter taste receptors (27), which have been shown to be bronchodilators (4): here, we found quinine, another bitter taster, to be considerably and statistically significantly less effective than caffeine in reversing the contractile response to pCa 6.5 when applied at 1 mM or at 10 mM (Fig. 4C).

Caffeine disrupts the contractile apparatus. Altogether, the data presented above indicate that the inhibitory effect of caffeine and theophylline are downstream of [Ca²⁺]i elevation. We therefore used a variety of assays to probe for any potential effect of caffeine on the various molecular events that determine the interactions between actin and myosin.

First, we used a standard commercially available ELISA kit to compare levels of filamentous and monomeric actin, to determine whether caffeine promoted the depolymerization of actin filaments. Tissues were challenged with carbachol for 20 min, then challenged with either 10 mM caffeine or vehicle (Krebs buffer) for another 20 min before flash freezing and processing for F-actin vs. G-actin. In this way, we found the F-actin-to-G-actin ratio in caffeine-treated tissues to be 66 ± 22% of that in control tissues (4 assays using tissues from 8 animals; P < 0.05).

A standard in vitro motility assay was used to quantify actin-myosin interaction directly. We found that the vmax was not significantly altered when the myosin had been phosphorylated in the presence of caffeine vs. control (Fig. 5A; ○). When caffeine was added to the thio phosphorylated myosin and the motility buffer, vmax was not significantly different from control either (Fig. 5A, △; P = 0.09), suggesting that caffeine does not alter the cross-bridge cycling. On the other hand, we found that the number of actin filaments bound by thio phosphorylated myosin in the assay (in which there is no cell membrane to act as a barrier to caffeine) was statistically significantly reduced by 1 and 10 mM caffeine compared with control (Fig. 5B; P < 0.05).

Using the phospho-immunoblotting approach, we compared phosphorylation of MLC20 by MLCK (Fig. 6A, top; in the presence of Ca²⁺/calmodulin), of CPI-17 by ROCK (Fig. 6A, middle), or of CPI-17 by PKC (Fig. 6A, bottom) in the presence vs. absence of caffeine (0, 0.1 or 1.0 mM). We found phos-
phorylation of MLC20 or of CPI-17 to be unaffected by caffeine, suggesting that the latter does not inhibit MLCK, and further suggesting that it does not affect MLCP through inhibition of ROCK or PKC.

Finally, we further tested whether caffeine directly activates MLCP using the recombinant MLCP complex. Unphosphorylated MLCP (Fig. 6B) was slightly activated in the presence of 0.1 mM caffeine (*P < 0.05), but not affected at the higher concentrations. MLCP is inactivated when the regulatory subunit MYPT1 is phosphorylated at Thr696 or Thr853. Nonetheless, the inactivation of MLCP by ROCK-mediated thiphosphorylation (Fig. 6C) was not affected by the addition of caffeine (0.1 to 10 mM). We found no effect of caffeine on P-MLC20 dephosphorylation in either case, suggesting caffeine does not suppress contractions by enhancing MLCP activity.

**DISCUSSION**

In this study, we examined a wide variety of possible mechanisms by which 10 mM caffeine might inhibit contractions in ASM. Our group and others have elsewhere shown that it can release internally sequestered Ca²⁺ from the SR when applied at millimolar concentrations (18, 19, 23) and can inhibit PDE activity as well as block adenosine receptors at 10- to 100-fold lower concentrations (9, 50). Here, we identified another mechanism by which it can interfere with excitation-contraction coupling when applied at millimolar concentrations. This suppression was not due to suppression of PDE activity (with consequent accumulation of cyclic nucleotides and activation of PKA), since it was not prevented by inhibiting adenylate cyclase (SQ22536 or MDL12330A) (10⁻⁵), the PDE4-selective inhibitor rolipram (10⁻⁵), the ROCK-inhibitor Y27632 (10⁻⁵), Y27632 plus the ROCK/PKA-inhibitor H89 (both 10⁻⁵), or vehicle, as indicated; *n = 5 for all. There was no statistically significant reversal of the caffeine-induced effect in response to SQ22536, MDL12330A, H89 (+/- Y27632), nor any significant augmentation of this caffeine-induced inhibition in response to rolipram.

**Fig. 3.** A: cumulative concentration-response relationships for KCl after pretreating tissues for 30 min with vehicle, caffeine (10 mM), theophylline (10⁻⁴), or IBMX (10⁻⁴), as indicated; *n = 4. Significantly different from control. Cumulative concentration-response relationships for KCl after incubations for various durations (as indicated) with 1 mM caffeine (B) or 10 mM caffeine (C); *n = 5 for both. Significantly different from control. D: cumulative concentration-response relationships for KCl after pretreating the tissues for 20 min with caffeine plus/minus either one of the adenylate cyclase inhibitors SQ22536 (10⁻⁵) or MDL12330A (10⁻⁵), the PDE4-selective inhibitor rolipram (10⁻⁵), the ROCK-inhibitor Y27632 (10⁻⁵), Y27632 plus the ROCK/PKA-inhibitor H89 (both 10⁻⁵), or vehicle, as indicated; *n = 5 for all. There was no statistically significant reversal of the caffeine-induced effect in response to SQ22536, MDL12330A, H89 (+/- Y27632), nor any significant augmentation of this caffeine-induced inhibition in response to rolipram.
downstream of \( \text{Ca}^{2+} \)-mediated activation of the contractile apparatus. We dissected the latter using a variety of molecular techniques, finding caffeine to have no effect on phosphorylation of \( \text{MLC20} \) by \( \text{MLCK} \), actin filament motility when using myosin phosphorylated in the presence of caffeine, actin filament motility catalyzed by \( \text{MLCK} \), phosphorylation of \( \text{CPI-17} \) by either \( \text{PKC} \) or \( \text{ROCK} \), nor the activity of \( \text{MLCP} \). However, two observations suggest to us that caffeine exerted its effect through actin. First, it increased the ratio of globular to filamentous actin in TSM tissues that had been stimulated with the cholinergic agonist carbachol. Second, it dramatically reduced the binding of actin filaments by thio-phosphorylated myosin in the in vitro motility assay. We acknowledge that the latter assay used purified skeletal actin with smooth muscle myosin, as our group (5, 26) and others have done many times in the past. This is done to simplify the assay: actin is otherwise accompanied by many regulatory proteins (calponin, caldesmon, tropomyosin, SM22), each exhibiting a variety of isoforms and phosphorylation states. Nonetheless, this approach provides an invaluable starting point from which to begin to understand the function and regulation of the contractile apparatus. Thus, from the data so obtained, it seems that caffeine dramatically reduces actin binding by myosin and/or promotes thin filament depolymerization (i.e., decreases the F- to G-actin ratio). Recent studies suggest that ASM contraction is also regulated through actin polymerization. Several groups have already established that disruption of actin filaments using latrunculin can severely impair contraction of ASM (1, 29). Zhao et al. (51) reported that ACh-triggered \( \text{Ca}^{2+} \) transients activate the \( \text{Ca}^{2+} \)-calmodulin-dependent phosphatase calcineurin, which dephosphorylates ADF/cofilin and promotes actin polymerization in parallel with TSM contraction. Actin polymerization also occurs in arterial SM in response to \( \alpha \)-adrenergic stimulation and leads to vasoconstriction (25). We presume that caffeine-induced actin depolymerization disrupts cross-bridge formation in the intact and permeabilized SM tissues, leading to a profound relaxation, without affecting myosin motor function per se.

Others have examined the effect of caffeine on the contractile apparatus in other muscle preparations and found this agent to inhibit MLCK itself in chicken gizzard SM (31) or to interfere with actin-myosin cross-bridge cycling in dog cardiac

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**Fig. 4.** A: original recordings from tissue challenged with pCa 6.5 buffer before and after permeabilization, evoking a contractile response only in the latter case. Subsequent application of IBMX (10\(^{-3}\)) evoked a further increase in tone, whereas caffeine (10 mM) evoked a small and brief contraction but then completely reversed all tone. B: in a second tissue permeabilized by \( \beta \)-escin and treated with A23187 to release internal \( \text{Ca}^{2+} \) (as evidenced by the increase in tone) before constricting with pCa 6.5 buffer, nifedipine (10\(^{-6}\) M) had no effect but caffeine evoked only a reversal of tone. C: mean reversals of tone evoked by caffeine, IBMX, quinine, or thio-phylline applied at concentrations indicated. *Significantly different from the mean response evoked by 10 mM caffeine. Italicized numerals in parentheses indicate value of \( n \) in each case.

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**Fig. 5.** A: velocity of actin propulsion by myosin \( (v_{\text{max}}) \) when \( I \) myosin is phosphorylated in the presence of caffeine (or vehicle) (i.e., prior to introduction onto the motility surface; \( I \) and 2) caffeine (or vehicle) is added to the already thio-phosphorylated myosin as well as the final assay buffer containing ATP, to ensure that the caffeine is not flushed out of the motility assay during velocity measurements \( (a) \); \( n = 3 \) for both assays. B: number of actin filaments bound to the motility surface when caffeine (or vehicle) was added to the thio-phosphorylated myosin, the labeled actin, and the actin buffer used to wash away the unattached actin filaments (no ATP was present in these conditions); \( n = 5 \).
Neither of those two studies assessed actin filament polymerization state nor its binding to myosin filaments, although the second study did find actomyosin Mg\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{-ATPase activity to be decreased by 20 mM caffeine (36). Theophylline (another methylxanthine, like caffeine) inhibited actin polymerization in guinea pig peritoneal macrophages, although that study linked the change to elevation of cAMP levels (11). Yet another group found caffeine inhibits the activation of JNK and thereby decreases the phosphorylation of c-Jun in cultured cerebellar granule neurons, but apparently through a mechanism that does not involve RyR (48). All of these effects of caffeine on non-RyR targets should be kept in mind in studies that use millimolar concentrations of caffeine to probe excitation-contraction coupling in SM.

Our finding that caffeine reversed KCl-evoked contractions led us to conclude that it is acting through some mechanism other than depletion of the internal Ca\textsuperscript{2+} pool, since our previous studies have shown that KCl contractions are augmented under conditions in which the internal Ca\textsuperscript{2+} store is depleted by cyclopiazonic acid plus/minus ryanodine, a phenomenon involving disruption of the “superficial buffer barrier” (18, 24). In contrast, Perez and Sanderson (33) have reported that KCl-evoked contractions are actually dependent on Ca\textsuperscript{2+} release from the SR and, as such, whether a caffeine sensitivity of KCl contractions indicates a mechanism involving depletion of the store (their view) or some non-SR-dependent mechanism (our view). Our group (22, 28) and several others (10, 40, 41, 47) have shown that depolarizing stimuli like KCl can activate the RhoA/ROCK signaling cascade and thereby sensitize the contractile apparatus to even small bursts of Ca\textsuperscript{2+} from an overloaded and decanting SR (which would be abolished by depletion of the store), but this does not mean that the KCl contraction is actually dependent on store-derived Ca\textsuperscript{2+}.

These findings may be useful in developing therapeutic agents for the treatment of asthma. Although it is unrealistic to propose applying or delivering millimolar concentrations of caffeine to the airways, a better understanding of how this agent exerts its inhibitory effect(s) may set the stage for a more potent and clinically safe therapeutic tool. Theophylline (a methylxanthine like caffeine) and PDE inhibitors (of which IBMX is a nonselective congener) have been used in the treatment of asthma, and caffeine can act through that same mechanism that they share in common (50), but that pathway does not explain the phenomenon we describe here. It is interesting that caffeine is also an agonist for bitter taste receptors, which have recently been shown to elicit bronchodilatory actions through a mechanism that does not involve the cyclic nucleotide signaling cascade (4). We wondered whether caffeine might also be acting through those receptors but did

Fig. 6. A: kinase assays were performed with regulatory light chain of myosin (MLC20; LC20) plus Ca\textsuperscript{2+}/calmodulin-MLCK (MLCK) (top), or recombinant CPI-17 plus ROCK (middle) or PKC (bottom), and the reaction products were subjected to immunoblotting using the antibodies indicated. Myosin light chain phosphatase (MLCP) assay was performed as described in MATERIALS AND METHODS. The specific activity (nmol released phosphate-min\textsuperscript{-1}mg\textsuperscript{-1} of MLCP) was determined by using untreated (B) or thiophosphorylated MLCP (C) incubated with phospho-MLC20 peptide in the presence of caffeine indicated. Of note, thiophosphorylation of MLCP reduced the specific activity 16-fold. Mean value ± SE was obtained from triplicate assays in 3 independent experiments (n = 9).
not find quinine, a potent bitter tastant, to fully mimic the inhibition of KCl-evoked contractions.

In conclusion, we find that millimolar concentrations of caffeine can reverse ASM contraction in part through disruption of actin filaments. This mechanism joins several other ones through which caffeine can exert its actions, including inhibition of adenosine receptors (9), PDE activity (50), JNK (48), MLCK (31), and actin-myosin cross-bridge cycling (36), as well as sensitization of RyR (30, 34), and future studies should keep these in mind when using millimolar caffeine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Janssen, Lauzon, Eto. Conducted experiments: Tazzeo, Bates, Roman, Khasnis. Contributed new reagents or analytic tools: not applicable. Performed data analysis: Tazzeo, Bates, Lauzon, Khasnis, Eto, Janssen. Wrote or contributed to the writing of the manuscript: Tazzeo, Bates, Lauzon, Eto, Janssen.

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