Control of the mode of excitation-contraction coupling by Ca\(^{2+}\) stores in bovine trachealis muscle

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Tao, Liang, Yu Huang, and Jean-Pierre Bourreau. Control of the mode of excitation-contraction coupling by Ca\(^{2+}\) stores in bovine trachealis muscle. Am J Physiol Lung Cell Mol Physiol 279: L722–L732, 2000.—Full muscarinic stimulation in bovine tracheal smooth muscle caused a sustained contraction and increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) that was largely resistant to inhibition by nifedipine. Depletion of internal Ca\(^{2+}\) stores with cyclopiazonic acid resulted in an increased efficacy of nifedipine to inhibit this contraction and the associated increase in [Ca\(^{2+}\)]. Thus internal Ca\(^{2+}\) store depletion promoted electromechanical coupling between full muscarinic stimulation and muscle contraction to the detriment of pharmacomechanical coupling. A similar change in coupling mode was induced byryanodine even when it did not significantly modify the initial transient increase in [Ca\(^{2+}\)], induced by this stimulation, indicating that depletion of internal stores was not necessary to induce the change in excitation-contraction coupling mode. Blockade of the Ca\(^{2+}\)-activated K\(^+\) channel by tetraethylammonium, charybotoxin, and iberiotoxin all induced the change in excitation-contraction coupling mode. These results suggest that in this preparation, Ca\(^{2+}\) released from the ryanodine-sensitive Ca\(^{2+}\) store, by activating Ca\(^{2+}\)-activated K\(^+\) channels, plays a central role in determining the expression of the pharmacomechanical coupling mode between muscarinic excitation and the Ca\(^{2+}\) influx necessary for the maintenance of tone.

calcium release; calcium influx; potassium channels; tracheal smooth muscle

AIRWAY SMOOTH MUSCLES contract tonically in response to muscarinic stimulation. This contraction is associated with a biphasic change in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and a graded membrane depolarization that often oscillates (4, 11, 17). Activation of muscarinic receptors stimulates phospholipase C, resulting in the formation of inositol trisphosphate [Ins(1,4,5)P\(_3\)] that acts as second messenger to release Ca\(^{2+}\) from internal stores (7, 8). Release of Ca\(^{2+}\) from Ins(1,4,5)P\(_3\)-sensitive internal stores is transient and occurs immediately after muscle stimulation, leading to a transient contraction. An influx of extracellular Ca\(^{2+}\) is required to maintain the sustained tension observed during prolonged muscarinic stimulation (5, 6).

The route of Ca\(^{2+}\) entry into the cytosol in airway smooth muscle cells during muscarinic stimulation is multiple. Two types (L and T) of voltage-operated Ca\(^{2+}\) channels (VOCCs) have been identified in airway muscles (13). The L-type VOCC is activated at −30 to −15 mV, and maximum current is seen at positive potentials in tracheal smooth muscle (20). Because ACh depolarizes the plasma membrane from about −60 to −40 or −30 mV (4, 9, 11), the channel is little activated, and voltage-dependent Ca\(^{2+}\) entry into cells through the VOCCs is not the main contributor for maintaining the tone induced by muscarinic stimulation in nearly all animal airway preparations (3). The Ca\(^{2+}\) influx that is the main contributor to the maintenance of tone does not occur through voltage-dependent channels and is not sensitive to inhibition by Ca\(^{2+}\) channel blockers (4–6, 23). Thus airway smooth muscle uses a mode of coupling between muscarinic stimulation and Ca\(^{2+}\) entry that is independent of membrane potential changes and was labeled pharmacomechanical coupling by Somlyo and Somlyo (30). This fact may explain the lack of beneficial clinical effects of Ca\(^{2+}\) channel blockers in the management of most obstructive lung diseases (3).

Amaoko et al. (1), Baba et al. (2), and Bourreau (4) have reported previously that the coupling between the stimulation of muscarinic receptors and airway muscle contraction can switch from the pharmacomechanical mode to a mode dependent on membrane potential, labeled electromechanical coupling (30). This switch in excitation-contraction (E-C) coupling occurs after inhibition of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) pumps with cyclopiazonic acid (CPA), and this renders Ca\(^{2+}\) entry blockers and K\(^+\) channel openers very efficient in inhibiting contractions induced by muscarinic agonists (1, 4). Because CPA eventually depletes internal Ca\(^{2+}\) stores and eliminates the Ca\(^{2+}\) release from these stores induced by muscarinic stimulation (4, 6, 10, 16), this observation suggests that the coupling mode between muscarinic receptor stimulation and muscle contraction in airway muscle may be under the influence of Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores. However, the exact mechanism(s) responsible for this switch are not known.

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The membrane of airway smooth muscle strongly rectifies due to the activity of K⁺ channels (12, 18, 19, 22). This profound rectification limits depolarization during muscarinic stimulation, which contributes to the observation that Ca²⁺ influx through the VOCCs is not the main direct contributor to the maintenance of tone. The activation of a large-conductance Ca²⁺-activated (KCa) channel by Ca²⁺ released from the SR (24, 25, 28) could be an essential element in controlling the amount of depolarization during muscarinic stimulation and, perhaps, Ca²⁺ entry through the VOCCs. It has been shown in other smooth muscles that the local increase in [Ca²⁺]i (Ca²⁺ spark) resulting from activation of ryanodine-sensitive channels in the SR limited muscle contraction by activating the KCa channel (24, 38). A similar mechanism could explain why the E-C coupling mode in bovine tracheal smooth muscle switches from a pharmacomechanical to an electromechanical mode after depletion of internal Ca²⁺ stores by CPA. Indeed, if muscarinic stimulation is no longer able to release Ca²⁺ from internal stores, one could expect that the stimulation of Ca²⁺-activated conductances would decrease (12, 14, 15, 19). Ca²⁺ entry through the VOCCs could thus be promoted, whereas Ca²⁺ entry through the non-VOCCs (23) would be decreased because the driving force for Ca²⁺ decreases when the membrane depolarizes. If this is true, compounds that block the KCa channel should have similar effects as compounds, such as CPA or ryanodine, that affect Ca²⁺ release from the SR on the E-C coupling mode in our preparation (10, 37). The aim of this study was to test this hypothesis, and we report in this manuscript supporting data from contraction experiments on bovine tracheal smooth muscle strips and studies with the fura 2 fluorescence technique in single bovine tracheal myocytes.

MATERIALS AND METHODS

Tissue Preparation and Organ Bath Experiment

Fresh bovine tracheae were obtained from a local abattoir and transported to the laboratory in ice-cold, HEPES-buffered solution containing (in mM) 154 NaCl, 4.6 KCl, 1.25 CaCl₂, 1.5 MgCl₂, 5.95 NaHCO₃, 10 HEPES, and 10 glucose as well as 3.5 mg/l of indomethacin, pH 7.4. The smooth muscle layer of the trachealis was dissected free of cartilage, mucosa, connective tissue, and fat and cut into thin strips (1 mm wide and 1 cm long) with scissors. The strips were mounted for isometric tension recording (Grass FT.03) under a load of 2 g in organ baths containing Krebs solution at 37°C and gassed with 5% CO₂:5% O₂ (pH 7.4). Preparations were equilibrated in the solution containing 154 mM NaCl, 4.6 KCl, 1.25 CaCl₂, 1.5 MgCl₂, 5.95 NaHCO₃, 10 HEPES, and 10 glucose and 3.5 mg/l of indomethacin, pH 7.4. After dissection as described in Tissue Preparation and Organ Bath Experiment, the smooth muscle strips (0.5 mm wide and 1 mm long) were transferred to a digestion solution (in mM: 136.9 NaCl, 6.2 KCl, 0.2 CaCl₂, 0.5 MgCl₂, 10 HEPES, and 10 glucose and 1.0 g/l of bovine serum albumin, pH 7.4) containing 200 U/ml of collagenase (type I), 10 U/ml of elastase (porcine pancreas), and 0.2 mg/ml of trypsin inhibitor (type 1-S). The tissues were incubated with gentle shaking for 40 min at 37°C. After this incubation, the digestion solution containing the enzymes was removed, and the preparations were washed three times in digestion solution without enzyme. Digested tissue was then gently pushed and sucked through a pipette for six steps of 5 min each to liberate individual myocytes. At the end of each step, isolated myocytes in suspension were collected (1-ml samples). The samples were pooled together, and Ca²⁺ concentration in the solution was increased gradually from 0.2 to 1.5 mM in 20 min. The solution was then centrifuged at 1,000 rpm for 5 min, and the cells were resuspended at a density of ~1 × 10⁶ cells/ml in medium 199 containing 10% fetal bovine serum. The cells were then incubated for 24 h on glass coverslips coated with poly-I-lysine in 24-well tissue culture clusters in a 95% air-5% CO₂ atmosphere at 37°C.

Measurement of [Ca²⁺]i

After incubation, the coverslips were removed from the culture well and washed in an incubation well with a physiological salt solution (PSS) containing (in mM) 130 NaCl, 5.6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 11.1 glucose and 1 mg/ml of bovine serum albumin, pH 7.4. The cells attached to the coverslips were then incubated with fura 2-AM at a concentration of 3 μM in PSS for 40 min. The unincorporated dye was then washed out twice, and the cells loaded on the coverslips were stored at room temperature (20–22°C) in the dark until used 30–240 min after the end of the loading period. The coverslips were then bathed at the bottom of an incubation chamber mounted on the stage of an inverted microscope (Nikon). The PSS solution containing 1% bovine serum albumin in the incubation chamber (1 ml) could be drained by vacuum suction and replaced with test solution within 5 s. The inverted microscope was coupled to a dual-excitation spectrofluorometer system (PIT), and the emitted light was filtered at 510 nm before being recorded. Fluorescent signals induced by excitation at 340 and 380 nm were stored for subsequent data processing and analysis. The changes in the ratio of the signals obtained at 340 and 380 nm were used as an index of the fluctuations in [Ca²⁺]i induced by drugs.

Drugs and Chemicals

Bethanechol chloride, ryanodine, nifedipine, CPA, charybdotoxin (ChTX), and iberiotoxin (IbTX) were purchased from RBI. Type I collagenase was from Worthington Biochemical; elastase was from Boehringer Mannheim; and fura 2-AM was from Molecular Probes. Trypsin inhibitor, tetracylammonium (TEA), medium 199, and bovine serum albumin were obtained from Sigma. All the chemicals were dissolved in distilled water except fura 2-AM, CPA, and ryanodine, which were dissolved in dimethyl sulfoxide, and nifedipine, which was dissolved in 95% ethyl alcohol. The final concentration of the solvents was <0.1%.
Statistical Analysis

For contractility data, the number of experiments stated refers to the number of tracheae obtained from different animals. Tests were performed in triplicate on strips dissected from each trachea. For Ca\(^{2+}\) measurements in isolated cells, sample cells in each batch prepared from tissue from one animal were tested for their ability to respond to bethanechol stimulation, and the batch was used for experimentation if bethanechol induced a biphasic change in [Ca\(^{2+}\)], in these cells similar to the biphasic change in [Ca\(^{2+}\)] induced in freshly isolated cells and muscle strips from bovine trachealis muscle. Experiments were carried out in separate batches, and the number of cells tested from these batches is given.

All data were analyzed with GraphPad PRISM software (GraphPad, San Diego, CA.) and are presented as means ± SE. Unpaired Student’s t-test was used to determine the effects of drugs. Probability values < 0.05 were considered to be significant.

RESULTS

Contractile Responses

Effect of nifedipine and removal of extracellular Ca\(^{2+}\) on atropine-sensitive tonic contractions induced by bethanechol. In Ca\(^{2+}\)-containing medium, 100 μM bethanechol induced sustained contractions of bovine tracheal smooth muscle strips (Fig. 1A), whereas in a Ca\(^{2+}\)-free solution (plus 100 μM EGTA), the contractile response to bethanechol stimulation was transient (Fig. 1B).

When the tension induced by 100 μM bethanechol in Ca\(^{2+}\)-containing medium had stabilized, the addition of 1 μM nifedipine to the medium did not abolish this tone but decreased it on average by 26 ± 5% (n = 9 experiments; Fig. 1, A and C).

Effect of nifedipine on atropine-sensitive tonic contractions induced by bethanechol in the presence of compounds that affect intracellular Ca\(^{2+}\) stores. CPA. After equilibration, the addition of 30 μM CPA to the medium induced a biphasic increase in tone, which often oscillated (Fig. 2A). The addition of 100 μM bethanechol further increased the tone, and there was no difference between the maximum amplitudes of the tonic contraction induced by bethanechol in control and CPA-containing media (P > 0.05; n = 9 experiments; Fig. 2B). In the presence of CPA, when the tension induced by bethanechol had stabilized, 1 μM nifedipine almost completely relaxed the preparations (Fig. 2, A and B). The steady-state contraction induced by bethanechol was similar in a Ca\(^{2+}\)-containing medium containing CPA and nifedipine and in a Ca\(^{2+}\)-free medium (Figs. 1C and 2B). The increase in the ability of nifedipine to relax tonic contractions induced by bethanechol was significant (P < 0.05; n = 4–9 experiments) when CPA was present at concentrations between 1 and 30 μM, and the effect of CPA was apparently concentration dependent, reaching a maximum at 10 μM (Fig. 3A).

RYANODINE. Thirty micromolar ryanodine induced an increase in tone (18 ± 3% with 60 mM KCl; n = 6 experiments) that was smaller than the increase in tone induced by CPA (73 ± 5% with 60 mM KCl; n = 12 experiments; Fig. 2C). The addition of 100 μM bethanechol further increased the tone, and there was no difference between the maximum amplitudes of the tonic contractions induced by bethanechol in control and ryanodine-containing media (P > 0.05; n = 6 experiments; Fig. 2D). In the presence of ryanodine, when the tension induced by bethanechol had stabilized, 1 μM nifedipine almost completely relaxed the preparations (Fig. 2, C and D). The increase in the ability of nifedipine to relax tonic contractions induced by bethanechol was significant when ryanodine was present at concentrations between 1 and 100 μM (P <
and the effect of ryanodine was maximum at 10 μM (Fig. 3B).

**Caffeine.** The addition of 10 mM caffeine to the solution induced a biphasic increase in muscle tone, with a maximum increase of 25 ± 8% of the tension induced by 60 mM KCl (n = 8 experiments; Figs. 2E and 4D). When the tension induced by caffeine had stabilized, the addition of 100 μM bethanechol to the solution further increased the muscle tension (Fig. 2E). In the presence of 10 mM caffeine, the maximum amplitude of the atropine-sensitive contraction induced by bethanechol was lower than in the absence of caffeine (P < 0.05; n = 8 experiments; Fig. 2F). When the tension induced by bethanechol in the presence of 10 mM caffeine had stabilized, the addition of 1 μM nifedipine to the bath slightly relaxed the preparations (Fig. 2, E and F). There was no difference in the ability of nifedipine to relax tonic contractions induced by bethanechol in the control medium or in the presence of caffeine (P > 0.05; Figs. 1C, 2F, and 3D).

**Effect of nifedipine on atropine-sensitive tonic contractions induced by bethanechol in the presence of TEA.** Ten millimolar TEA itself induced a large increase in tone. Bethanechol (100 μM) could further increase the muscle tension, and under these experimental conditions, nifedipine almost completely relaxed the tone induced by TEA and bethanechol (Fig. 5, A and B). In the presence of TEA, the maximum amplitude of the tonic contraction induced by bethanechol was smaller than that in the control medium (P < 0.05; Fig. 5B). The inhibition by nifedipine of the atropine-sensitive contraction induced by bethanechol was larger (P < 0.05) in the presence of TEA at concentrations between 0.5 and 10 mM (Fig. 3C).

Because TEA caused substantial contractions on its own, presumably by depolarization, making interpretation of the data ambiguous, we used a different approach to test the effect of K⁺ channel blockade on the ability of nifedipine to inhibit tonic contractions induced by bethanechol. The protocol is illustrated in Fig. 5C. TEA induced a large contraction, which was inhibited by nifedipine. In the presence of nifedipine and TEA, bethanechol was only able to induce a small tonic contraction, which was similar to the tonic con
tractions obtained in Ca\(^{2+}\)-free medium (compare Figs. 1C and 5D).

Depolarizing effect of CPA and ryanodine. Figure 4 shows that the contractions of bovine tracheal smooth muscle strips induced by 30 \(\mu\)M CPA and 30 \(\mu\)M ryanodine were sensitive to inhibition by the K\(^+\) channel opener cromakalim and by the L-type channel blocker nifedipine. Although contractions induced by CPA and ryanodine shared a similar sensitivity to inhibition by cromakalim and nifedipine, the amplitude of the contraction induced by CPA was larger than the contraction induced by ryanodine in this preparation (\(P < 0.05\); Fig. 4D).

Caffeine (10 mM) induced a contraction similar in amplitude to the contraction induced by ryanodine (Fig. 4D). However, this contraction was not sensitive to inhibition by cromakalim or nifedipine (Fig. 4, C and D).

Ca\(^{2+}\) Signals

Nifedipine sensitivity of the Ca\(^{2+}\) influx evoked by full muscarinic stimulation: effect of CPA and ryanodine...
dine. In Ca\(^{2+}\)-containing medium, single cells responded to bethanechol stimulation with a rapid increase in [Ca\(^{2+}\)]\(_i\), which decayed and stabilized above the resting level. This response was not affected by 1 μM nifedipine, whereas in a Ca\(^{2+}\)-free solution, the steady-state increase in [Ca\(^{2+}\)]\(_i\) induced by bethanechol was absent (Fig. 6).

CPA is a potent inhibitor of Ca\(^{2+}\)- or Mg\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake in smooth muscle SR (10) and is efficient in depleting agonist-recruitable internal Ca\(^{2+}\) stores by preventing their loading (5, 6). In our experiment, CPA (30 μM) induced an increase in [Ca\(^{2+}\)]\(_i\) in isolated myocytes as seen in Fig. 7A comparing [Ca\(^{2+}\)]\(_i\) before bethanechol stimulation in control medium and in the presence of CPA. This increase in [Ca\(^{2+}\)]\(_i\), induced by CPA was significantly decreased by 1 μM nifedipine (compare Fig. 7A, left and right) and 3 μM cromakalim (data not shown). After a 1-h incubation with 30 μM CPA, the initial increase in [Ca\(^{2+}\)]\(_i\) induced by bethanechol was absent (Fig. 7A, left). The sustained increase in [Ca\(^{2+}\)]\(_i\), was increased compared with that in control medium (Fig. 7A, left, and B) [Ca\(^{2+}\)]\(_i\), and sensitive to inhibition by nifedipine (Fig. 7A, right, and C).

Ryanodine-sensitive Ca\(^{2+}\)-release channels located in the SR membrane can be blocked by ryanodine at micromolar concentrations (37). During muscarinic activation, the transient peak and the sustained increase in [Ca\(^{2+}\)]\(_i\), were not changed by the presence of 10 μM ryanodine added to the medium 10 min before bethanechol challenge (Fig. 8). However, in the presence of ryanodine, unlike under control conditions, nifedipine (1 μM) significantly reduced the sustained increase in [Ca\(^{2+}\)]\(_i\), induced by bethanechol (Fig. 8C).

Effects of selective blockade of K\(_{Ca}\) channels on the efficacy of nifedipine to inhibit Ca\(^{2+}\) influx induced by muscarinic stimulation. Two K\(^+\) channels have been described in bovine tracheal smooth muscle cells: the delayed rectifier K\(^+\) channel, which is blocked preferentially by 4-aminopyridine, and a large-conductance K\(_{Ca}\) channel, which is inhibited preferentially by TEA at millimolar concentrations and more potently and more selectively by ChTX and IbTX (19, 21, 22). In single bovine tracheal smooth muscle cells, in the presence of ChTX (100 nM) or IbTX (100 nM), full muscarinic stimulation with 100 μM bethanechol still induced a biphasic increase in [Ca\(^{2+}\)]\(_i\) (Fig. 9, A and C), which was not significantly different from the control value (Fig. 9E). However, in the presence of ChTX (100 nM) or IbTX (100 nM), nifedipine inhibited the sustained increase in [Ca\(^{2+}\)]\(_i\), induced by bethanechol stimulation (Fig. 9, B, D, and F).

DISCUSSION

In this study, we observed that full activation of muscarinic receptors in bovine tracheal smooth muscle cells by large concentrations of bethanechol in PSS induced sustained contractions of muscle strips and a biphasic change in [Ca\(^{2+}\)]\(_i\), in isolated myocytes. This biphasic change in [Ca\(^{2+}\)]\(_i\), resulted from the recruitment of two Ca\(^{2+}\) pools, i.e., the intracellular CPA-sensitive Ca\(^{2+}\) stores and the extracellular space. The initial transient increase in [Ca\(^{2+}\)]\(_i\), was completely abolished after prolonged incubation of cells with CPA, which results in depletion of internal agonist-recruitable Ca\(^{2+}\) stores as previously described (5, 6, 10, 16). Thus this component of the Ca\(^{2+}\) signal was due to Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. The sustained increase in [Ca\(^{2+}\)]\(_i\), was absent in Ca\(^{2+}\)-free medium, indicating the requirement of the extracellular Ca\(^{2+}\) pool for this component of the Ca\(^{2+}\) signal, but was not very sensi-
tive to nifedipine, suggesting that the Ca$^{2+}$ influx induced by muscarinic stimulation and contributing to the maintenance of tone and the elevation in \([\text{Ca}^{2+}]_i\) occurs mainly via non-VOCCs. These data are in agreement with previous reports (5, 9, 11, 23) showing that airway smooth muscle uses a coupling mode between full muscarinic stimulation and contraction that is independent of membrane potential under normal conditions, labeled pharmacomechanical coupling by Somlyo and Somlyo (30).

The efficacy of nifedipine to inhibit the contractile response of strips to bethanechol stimulation was significant.

Fig. 6. Effect of nifedipine and Ca$^{2+}$-free solution on changes in intracellular Ca$^{2+}$ concentration (\([\text{Ca}^{2+}]_i\)) induced by bethanechol stimulation in isolated bovine tracheal smooth muscle cells. A: response of isolated cells to bethanechol stimulation in control medium (left), in the presence of nifedipine (middle), and in Ca$^{2+}$-free medium (right). Bethanechol induced a biphasic increase in \([\text{Ca}^{2+}]_i\), with a rapid and transient increase, referred to as initial increase in \([\text{Ca}^{2+}]_i\), and a sustained increase referred to as secondary increase in \([\text{Ca}^{2+}]_i\). 340/380, Ratio of 340-to-380-nm excitation signals used as an index of fluctuation in \([\text{Ca}^{2+}]_i\). B and C: summary of the effect of bethanechol in various conditions on the initial and secondary increases, respectively, in \([\text{Ca}^{2+}]_i\). The initial increase in \([\text{Ca}^{2+}]_i\), is expressed as the peak of 340/380 as a percentage of resting 340/380 recorded before bethanechol stimulation. The secondary increase in \([\text{Ca}^{2+}]_i\), is expressed as a percentage of resting 340/380 recorded before bethanechol stimulation. Data are means ± SE from 4–9 cells from 4 separate experiments. *Significantly different from Ca$^{2+}$-containing medium, \(P < 0.05\).

Fig. 7. Effect of CPA on the change in \([\text{Ca}^{2+}]_i\), induced by bethanechol stimulation. A: changes in \([\text{Ca}^{2+}]_i\), induced by bethanechol alone and in the presence of CPA in control medium (left) and in medium containing nifedipine (right). In the presence of CPA, the initial increase in \([\text{Ca}^{2+}]_i\), induced by bethanechol stimulation is absent, but the secondary increase in \([\text{Ca}^{2+}]_i\), is larger. Note that the resting \([\text{Ca}^{2+}]_i\), is larger in the presence of CPA (left), and this increase in resting \([\text{Ca}^{2+}]_i\), is decreased in the presence of nifedipine (right). In the presence of CPA and nifedipine, bethanechol induced little change in \([\text{Ca}^{2+}]_i\). B and C: summary of the effect of CPA and nifedipine on the initial and secondary increases, respectively, in \([\text{Ca}^{2+}]_i\), induced by 100 μM bethanechol. The initial increase in \([\text{Ca}^{2+}]_i\), is expressed as the peak 340/380 as a percentage of the resting 340/380 recorded before bethanechol stimulation. The secondary increase in \([\text{Ca}^{2+}]_i\), is expressed as a percentage of resting 340/380 recorded before bethanechol stimulation. Data are means ± SE from 6–9 cells from 4 separate experiments. # Significantly different from vehicle, \(P < 0.05\). *Significantly different from control, \(P < 0.05\).
nificantly increased after depletion of intracellular Ca\(^{2+}\) stores with CPA as previously reported (1, 4). In single cells, pretreatment with CPA abolished the initial transient increase in \([\text{Ca}^{2+}]_i\) but largely increased the sustained increase in \([\text{Ca}^{2+}]_i\) induced by bethanecol stimulation (see also Ref. 16). This larger influx of \([\text{Ca}^{2+}]_i\) induced by bethanecol stimulation in the presence of CPA was also very sensitive to inhibition by nifedipine. These results agree with previous data by Bourreau (4) suggesting that depletion of \([\text{Ca}^{2+}]_i\) stores can switch the coupling mode between muscarinic stimulation and contraction from pharmacomechanical to electromechanical and that the coupling mode between muscarinic stimulation and \([\text{Ca}^{2+}]_i\) influx is regulated by intracellular \([\text{Ca}^{2+}]_i\) stores. A simplified model to explain these observations would be that \([\text{Ca}^{2+}]_i\) released from internal stores immediately after muscarinic stimulation activates \([\text{Ca}^{2+}]_i\)-dependent \(\text{Cl}^-\) and \(\text{K}^+\) conductances (15, 34). The balance between activation of these conductances, together with activation of nonspecific cation conductances (15), would then dictate the amount of depolarization induced by agonist stimulation and the rate of \([\text{Ca}^{2+}]_i\) influx through VOCCs. Because the rate of \([\text{Ca}^{2+}]_i\) influx determines the amount of \([\text{Ca}^{2+}]_i\) that can escape the superficial buffer barrier and reach the contractile machinery (14, 32, 33), the amount of depolarization induced by agonist stimulation should affect the sensitivity of the tonic contraction to inhibition by VOCC blockers. Inhibition of \([\text{Ca}^{2+}]_i\) release from internal stores would reduce activation of \(\text{K}_{\text{Ca}}\) channels (27, 38), which might result in an increased depolarization induced by muscarinic stimulation, increased activation of VOCCs, and an increased sensitivity of the tonic contraction to inhibition by VOCC blockers (20). In our preparations, CPA could, by itself, induce a large contraction that was associated with an increase in \([\text{Ca}^{2+}]_i\). Most of the response to CPA can be inhibited if preparations are hyperpolarized with the \(\text{K}^+\) channel opener cromakalim or if L-type \([\text{Ca}^{2+}]_i\) channels are blocked with nifedipine, suggesting that the response of bovine trachealis muscle to CPA stimulation resulted from activation of L-type \([\text{Ca}^{2+}]_i\) channels after membrane depolarization, perhaps due to activation of depolarizing currents by CPA (36). In the presence of CPA, i.e., when internal \([\text{Ca}^{2+}]_i\) stores are depleted and the membrane is depolarized, both the tonic contraction and \([\text{Ca}^{2+}]_i\) influx induced by bethanecol become sensitive to inhibition by nifedipine. These data support the model described above. The model predicts that blockade of \(\text{K}^+\) channels (especially \(\text{K}_{\text{Ca}}\) channels) should promote depolarization in our preparations and should

![Fig. 8. Effect of ryanodine on the change in \([\text{Ca}^{2+}]_i\), induced by bethanecol stimulation. A: changes in \([\text{Ca}^{2+}]_i\), induced by bethanecol in the presence of 30 \(\mu\)M ryanodine in control medium (left) and in a medium containing 1 \(\mu\)M nifedipine (right). In the presence of ryanodine, the initial increase in \([\text{Ca}^{2+}]_i\), induced by bethanecol stimulation is present (left) and is not affected by nifedipine (right). Unlike in the presence of CPA, in the presence of ryanodine, the secondary increase in \([\text{Ca}^{2+}]_i\), is not larger than control value (left). However, unlike under control conditions, the secondary increase in \([\text{Ca}^{2+}]_i\), induced by bethanecol in the presence of ryanodine is inhibited by nifedipine (right). B and C: summary of the effect of ryanodine and nifedipine on the initial and secondary increases, respectively, in \([\text{Ca}^{2+}]_i\), induced by 100 \(\mu\)M bethanecol. The initial increase in \([\text{Ca}^{2+}]_i\), is expressed as the peak 340/380 as a percentage of the resting 340/380 recorded before bethanecol stimulation. The secondary increase in \([\text{Ca}^{2+}]_i\), is expressed as a percentage of resting 340/380 recorded before bethanecol stimulation. Data are means ± SE from 4–9 cells from 4 separate experiments. *Significantly different from vehicle, \(P < 0.05\).](http://ajplung.physiology.org/issue/S1062889697891583/C0663355)
result in an increased sensitivity of the tonic contraction induced by muscarinic stimulation to inhibition by nifedipine. Data obtained in the presence of TEA, ChTX, or IbTX fit this prediction and support the model.

The data obtained in the presence of ryanodine suggest that the Ins\((1,4,5)P_3\)-mediated Ca\(^{2+}\) released from agonist-recruitable stores did not control the mode of E-C coupling in our preparations. Indeed, a 10-min incubation with ryanodine was sufficient to induce a switch in coupling mode without inducing depletion of internal stores that significantly modified Ins\((1,4,5)P_3\)-mediated Ca\(^{2+}\) release induced by bethanechol stimulation. This is suggested in these experiments by the ability of bethanechol to still induce a CPA-sensitive transient increase in [Ca\(^{2+}\)]\(_i\) (see Fig. 8). On average, prolonged stimulation with ryanodine was sufficient to induce a contraction that was nifedipine sensitive. This contraction induced by ryanodine was significantly smaller than the contraction induced by CPA under similar experimental conditions. This probably reflects the ability of CPA, but not of ryanodine, to inhibit SR Ca\(^{2+}\) pumps and to disrupt the superficial buffer barrier (10, 14). Consequently, in the presence of CPA, influx via VOCCs is directly available for contraction (33). Ryanodine, on the other hand, does not inhibit SR Ca\(^{2+}\) pumps but stabilizes Ca\(^{2+}\) release channels in a subconductance state, which prevents their full opening (37). Thus in the presence of ryanodine, the superficial SR should still buffer part of the increased Ca\(^{2+}\) influx via VOCCs. This is supported by data in Figs. 7 and 8 showing that the increase in [Ca\(^{2+}\)]\(_i\) induced by ryanodine alone or by bethanechol in the presence of ryanodine is significantly smaller than the increase in [Ca\(^{2+}\)]\(_i\) induced by CPA alone or by bethanechol in the presence of CPA.

In smooth muscle cells, it has been shown that two distinct Ca\(^{2+}\)-release channels exist in the membrane of the SR, Ins\((1,4,5)P_3\)-sensitive channels and ryanodine-sensitive channels (8, 25, 26). Stimulation of muscarinic receptors induces Ca\(^{2+}\) release from both channels, and Ca\(^{2+}\) release from ryanodine-sensitive channels has been implicated in the generation of Ca\(^{2+}\) waves and sparks in smooth muscle preparations (25, 26, 28, 29). Recently, Janssen et al. (14) have shown that in airway smooth muscle, Ca\(^{2+}\) release from ryanodine-sensitive channels was targeted toward the plasma membrane and that Ca\(^{2+}\) released from these channels was not a major contributor to the average [Ca\(^{2+}\)]\(_i\) but was to activated Ca\(^{2+}\)-dependent conduc-
stances. Our data are compatible with these findings and suggest a mechanism that could be responsible for the dominant pharmacomechanical mode of muscarinic E-C coupling in airway smooth muscle.

Muscarinic stimulation causes only a limited, graded membrane depolarization from a resting value of about −60 to −30 mV (4, 11), and the strong rectification of the plasmalemma due to the activity of K⁺ channels (12, 18, 19, 22, 34) is probably responsible for limiting depolarization. Although there is little activation of L-type VOCCs within this voltage window (20), the channels are not fully inactivated, thereby allowing a substantial amount of Ca²⁺ to enter the cells through VOCCs to reload internal Ca²⁺ stores (5). Two K⁺ channels described in airway smooth muscle cells confer on the plasma membrane its outward rectifying property, the delayed rectifier and KCa channels (19, 21); and muscarinic stimulation of tracheal smooth muscle cells by acetylcholine causes activation of KCa channels (34; see, however, Ref. 35). Activation of KCa channels by ryanodine-sensitive Ca²⁺ release has been well described in vascular smooth muscle cells, where local transient increases in [Ca²⁺]i (Ca²⁺ sparks) resulting from Ca²⁺ release via ryanodine-sensitive Ca²⁺ channels in the SR just under the cell membrane can stimulate KCa channels while having little contribution to a global increase in [Ca²⁺]i (24). In airway smooth muscle, ryanodine-sensitive Ca²⁺ release (Ca²⁺ sparks) can also activate K⁺ channels (27, 29, 39), and our data with caffeine support this. In our experiment, caffeine had an effect compatible with its action on ryanodine-sensitive Ca²⁺ release channels (i.e., release of internal Ca²⁺ via these channels). However, contractions induced by caffeine were not due to membrane depolarization because they were not blocked by cromakalim or nifedipine. This suggests that unlike ryanodine, caffeine did not inhibit KCa channels but rather, perhaps, activated these channels as already proposed (34). Thus unlike ryanodine, caffeine should not modify the coupling between muscarinic stimulation and muscle contraction from pharmacomechanical to electromechanical because it should not suppress Ca²⁺ release through ryanodine-sensitive channels. Data in Fig. 3F, showing that at all concentrations tested caffeine did not promote electromechanical coupling, support this assumption.

Based on these findings, we propose that in our experimental model, blockade or removal of activation of KCa channels with TEA, ChTX, IbTX, or CPA [which also blocks KCa channels (31)] rendered bethanecholin-induced contraction very sensitive to inhibition by nifedipine, probably by increasing the rate of Ca²⁺ influx via VOCCs. The increase in the rate of Ca²⁺ influx via VOCCs was probably due to a decrease in membrane rectification (22) allowing greater membrane depolarization during muscarinic stimulation. This increased rate of Ca²⁺ influx would allow Ca²⁺ to reach the inner cytosol, bypassing the superficial buffer barrier (33), to contribute to contraction.

Thus in bovine tracheal smooth muscle cells, activation of KCa channels by ryanodine-sensitive Ca²⁺ release may play a central role in limiting the Ca²⁺ influx through VOCCs after full muscarinic stimulation. Because the Ca²⁺ influx through VOCCs is limited, the superficial SR can efficiently prevent its access to the inner cytosol (32, 33), limiting its contribution to tonic contraction (4, 5, 9) but making it a significant component of internal stores reloading (5).

What triggers ryanodine-sensitive Ca²⁺ release during muscarinic stimulation in our preparation remains to be established. Ca²⁺-induced Ca²⁺ release and cyclic ADP-ribose-induced Ca²⁺ release have both been described in airway smooth muscle cells (26, 35), and Ca²⁺-induced Ca²⁺ release can activate KCa channels (35). Also, our data show that in the presence of ryanodine or the selective KCa channel inhibitors ChTX and IbTX, the Ca²⁺ influx during muscarinic stimulation is not different from the Ca²⁺ influx observed in the absence of these compounds. This implies that only the route of Ca²⁺ entry to the inner cytosol has changed. Also, when the membrane is depolarized in the presence of TEA and Ca²⁺ entry via VOCCs is blocked by nifedipine, the tonic contraction induced by full muscarinic stimulation is not different from the Ca²⁺ influx observed in the absence of these compounds. This suggests that Ca²⁺ entry is absent in these experimental conditions. Whether Ca²⁺ entry through non-VOCCs is decreased, perhaps due to a decrease in the driving force for Ca²⁺ entry, or whether this influx is buffered remains to be established.

In conclusion, our study suggests that ryanodine-sensitive Ca²⁺ release plays a central role in determining the expression of pharmacomechanical coupling mode between stimulation of muscarinic receptors and Ca²⁺ influx by activating KCa channels under physiological conditions.

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