High K⁺-induced contraction requires depolarization-induced Ca²⁺ release from internal stores in rat gut smooth muscle

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Aim: Depolarization-induced contraction of smooth muscle is thought to be mediated by Ca²⁺ influx through voltage-gated L-type Ca²⁺ channels. We describe a novel contraction mechanism that is independent of Ca²⁺ entry.

Methods: Pharmacological experiments were carried out on isolated rat gut longitudinal smooth muscle preparations, measuring isometric contraction strength upon high K⁺-induced depolarization.

Results: Treatment with verapamil, which presumably leads to a conformational change in the channel, completely abolished K⁺-induced contraction, while residual contraction still occurred when Ca²⁺ entry was blocked with Cd²⁺. These results were further confirmed by measuring intracellular Ca²⁺ transients using Fura-2. Co-application of Cd²⁺ and the ryanodine receptor blocker DHBP further reduced contraction, albeit incompletely. Additional blockage of either phospholipase C (U 73122) or inositol 1,4,5-trisphosphate (IP₃) receptors (2-APB) abolished most contractions, while sole application of these blockers and Cd²⁺ (without parallel ryanodine receptor manipulation) also resulted in incomplete contraction block.

Conclusion: We conclude that there are parallel mechanisms of depolarization-induced smooth muscle contraction via (a) Ca²⁺ entry and (b) Ca²⁺ entry-independent, depolarization-induced Ca²⁺-release through ryanodine receptors and IP₃, with the latter being dependent on phospholipase C activation.

Keywords: calcium channels L-type, ryanodine receptor, inositol phosphates, phospholipase C

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Introduction

Smooth muscle contraction is widely accepted to be triggered by a rise in cytosolic free Ca²⁺ concentration ([Ca²⁺]), which is regulated by either pharmacomechanical coupling to agonists that induce contraction without membrane depolarization, or by membrane depolarization itself in a process known as electromechanical coupling[1-3]. The rise in [Ca²⁺], leads to calmodulin-dependent activation of myosin light chain kinase (MLCK) and results in the phosphorylation of the 20-kDa light chain of myosin[4, 5]. Pharmacomechanical coupling may depend on Ca²⁺ influx through receptor-operated Ca²⁺ channels[6] or on Ca²⁺ release from the sarcoplasmic reticulum following an intracellular second messenger cascade involving G protein-coupled receptor-dependent phospholipase C (PLC) activation[7]. When activated, PLC utilizes membrane-bound phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate (IP₃), which in turn activates IP₃ receptors at the sarcoplasmic reticulum, resulting in IP₃-induced Ca²⁺ release (IICR)[8]. In addition to these IP₃ receptors, [Ca²⁺], can also be increased by ryanodine receptors (RyRs) through a mechanism called Ca²⁺-induced Ca²⁺ release (CICR). In this process, cytosolic Ca²⁺ ions can trigger further Ca²⁺ release[9, 10]. Alternatively, this may also occur through the Ca²⁺-dependent activation of the cyclic ADP-ribose pathway[7, 11].

Electromechanical coupling, such as high K⁺-induced contraction, is believed to rely on cell surface voltage-gated Ca²⁺ channels (VGCCs), which are activated by cell membrane depolarization and cause an increase in [Ca²⁺][12]. However, it remains unknown whether depolarization-induced contraction in smooth muscle is entirely dependent on the influx of extracellular Ca²⁺. At the very least, extracellular Ca²⁺ ions entering the cell through VGCCs might also trigger Ca²⁺ release from internal stores via RyRs. Another possibility is that depolarization is directly coupled to sarcoplasmic Ca²⁺ release channels. Indeed, depolarization-induced Ca²⁺ release (DICR) is known to be essential for excitation-contraction coupling in the skeletal muscle, and this mechanism relies on a mechanical linkage between RyR1 and cell surface volt-
In the present study, we assessed the contributions of extracellular versus intracellular Ca\textsuperscript{2+} during high K\textsuperscript{+}-induced contraction in the smooth muscle of the small intestine. Our data suggest that depolarization-induced contraction involves substantial Ca\textsuperscript{2+} release from internal stores. Moreover, we found that depolarization without Ca\textsuperscript{2+} influx into the cell can induce the opening of Ca\textsuperscript{2+} release channels (ryanodine and IP\textsubscript{3} receptors). We conclude that DICR is a physiologically relevant mechanism of electromechanical coupling in gut smooth muscle.

**Materials and methods**

**Preparation and maintenance of gut pieces**

Adult male Wistar rats (70–90 d) were deeply anesthetized with diethyl ether and decapitated. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The abdomen was carefully opened and the full-length small intestine was quickly removed. In order to allow the discrimination between the different intestinal segments, three distinct portions were prepared. One portion included the initial 15–20 cm from the stomach and was denoted as the proximal portion. Another portion included the last 15–20 cm before the ileocecal transition and was referred to as the distal portion. The third portion included the initial 15–20 cm from the stomach and was denoted as the proximal portion. Another portion included the central 15–20 cm and was labeled as the middle portion. The proximal, middle and distal portions were subsequently cut into pieces of 1–2 cm in length. Thin nylon threads were sutured to both ends of these pieces to enable longitudinal fixation in the organ bath (Panlab ML0146/C, ADInstruments, Spechbach, Germany). The gut preparations were transferred into a HEPES-buffered storage solution (in mmol/L: 120 NaCl, 4.5 KCl, 26 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 0.02 CaCl\textsubscript{2}, 1.0 MgSO\textsubscript{4}, 0.25 Na\textsubscript{2}-EDTA, 5.5 glucose, 5 HEPES, pH=7.4) for recovery for 15–60 min at 4 °C before they were fixed into the organ bath. The organ bath (volume 25 mL) was filled with a recording solution that contained (in mmol/L) 120 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 30 NaHCO\textsubscript{3}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 0.5 Na\textsubscript{2}-EDTA, 5.5 glucose, 2 sodium-pyruvate, 0.8 µmol/L atropine, 0.8 µmol/L hexamethonium (pH=7.4, gassed with carbogen) at a flow rate of ~4 mL/min. Longitudinal smooth muscle cells were visualized using a digital video camera (cooled Retiga-2000RV, QImaging\textsuperscript{®}) and a fluorescence imaging system (ICU Photonics by Agilent Technologies) including a software package (TILLvisION 4.0.1.3, TILL Photonics by Agilent Technologies) for data acquisition and analysis. Fura-2 fluorescence (510 nm) was measured at room temperature (22–24 °C) while alternating the two excitation wavelengths (355 and 380 nm) at 2 Hz. The gut patches were challenged with a high K\textsuperscript{+}-containing solution (KCl 90 mmol/L, osmolality corrected by equimolar reduction of NaCl) to evoke depolarization-induced Ca\textsuperscript{2+} transients.

**Recording isometric contractions and relaxations**

After fixation in the organ bath, the preparations were allowed to recover for another 30 min before the experiments were started. During this time the organ bath temperature was raised from room temperature to 37 °C. The initial tension of the pieces of small intestine within the organ bath was adjusted to yield a stable baseline tension with regular phasic contractions. The gut preparations were challenged with high K\textsuperscript{+} solution to induce depolarization-induced contraction or with acetylcholine to study cholinergic effects. Isometric contractions and relaxation were measured by force transducers (MLT0201, ADInstruments) and recorded with a bridge amplifier (ML224, ADInstruments) connected to an analog-to-digital converter (Powerlab 4/30, ADInstruments) and analyzed by the Chart 5 software (ADInstruments).

**FURA-2 loading and Intracellular Ca\textsuperscript{2+} measurements**

To analyze intracellular Ca\textsuperscript{2+} transients, the rat gut was cut into small patches and transferred into HEPES-buffered storage solution containing (in mmol/L) 120 NaCl, 26 NaHCO\textsubscript{3}, 4.5 KCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 1 MgSO\textsubscript{4}, 1.6 CaCl\textsubscript{2}, 0.25 Na\textsubscript{2}-EDTA, 5.5 glucose, 5 HEPES (pH=7.4). After careful removal of the mucosa, gut patches were transferred into a small jar filled with HEPES-buffered loading solution with 10 µmol/L Fura-2 AM (Molecular Probes\textsuperscript{TM}) and incubated for 60 min at room temperature in the dark followed by a 30 min wash in HEPES-buffered solution. For the fluorescence measurement the gut patches were transferred into a perfusion chamber on an inverted fluorescence microscope (Nikon Eclipse FN1, Tokyo, Japan, equipped with an immersion objective: NIR Apo 40x/0.80W DIC N2, Nikon). The patches were perfused with a recording solution containing (in mmol/L) 120 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 30 NaHCO\textsubscript{3}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 0.5 Na\textsubscript{2}-EDTA, 5.5 glucose, 2 sodium-pyruvate, 0.8 µmol/L atropine, 0.8 µmol/L hexamethonium (pH=7.4, gassed with carbogen) at a flow rate of ~4 mL/min. Longitudinal smooth muscle cells were visualized using a digital video camera (cooled Retiga-2000RV, QImaging\textsuperscript{®}) and a fluorescence imaging system (ICU Photonics by Agilent Technologies) including a software package (TILLvisION 4.0.1.3, TILL Photonics by Agilent Technologies) for data acquisition and analysis. Fura-2 fluorescence (510 nm) was measured at room temperature (22–24 °C) while alternating the two excitation wavelengths (355 and 380 nm) at 2 Hz. The gut patches were challenged with a high K\textsuperscript{+}-containing solution (KCl 90 mmol/L, osmolality corrected by equimolar reduction of NaCl) to evoke depolarization-induced Ca\textsuperscript{2+} transients.

**Chemicals and data analysis**

The muscarinic antagonist atropine was purchased from Merck. Verapamil, 1,1’-diheptyl-4,4’-bipyridinium dibromide (DHPB), 2-aminoethoxydiphenylborane (2-APB), 1-[6-[[17b]-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrole-2,5-dione (U 73122), and thapsigargin (TG) were purchased from Tocris (Bristol, UK). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). DHPB was chosen as a ryanodine receptor blocker because ryanodine itself has both antagonistic and agonistic effects, depending on its dosage\textsuperscript{[15–17]}. In a whole muscle preparation, this dosage is difficult to control with bath application; hence the effects of ryanodine would have been less predictable than the effects of DHPB.

Smooth muscle contractions and relaxation were analyzed as the absolute isometric contraction strength values as well as the percentage of the individual high K\textsuperscript{+}-induced isometric contraction strength. We did not observe any differences between the proximal, middle and distal portions in all our experiments, so we pooled the data for our analysis. Statistical
analyses were performed using the paired two-tailed Student’s t test with a statistical significant difference referred to as P value less than 0.05. All data are presented as the mean±SEM.

Results

Depolarization-induced and acetylcholine-induced contraction

Smooth muscle contraction can be triggered by high K+-induced depolarization (electromechanical coupling) or by acetylcholine application (pharmacomechanical coupling). To investigate viability, rat gut preparations were probed with exogenous acetylcholine to demonstrate intact pharmacological contraction in the longitudinal smooth muscle. Bath application of acetylcholine (1.2 μmol/L) caused a stable tonic contraction that was highly sensitive to the muscarinic receptor antagonist atropine (0.8 μmol/L; Figure 1A, left panel) and less sensitive to the nicotinic receptor antagonist hexamethonium chloride (0.8 μmol/L; Figure 1A, right panel). As shown in Figure 1B, bath application of acetylcholine (1.2 μmol/L) caused a tonic contraction of 17.4±1.5 mN (n=30) that was inverted to a relaxation value of -1.3±1.3 mN following atropine application (n=14, P<0.001). This indicated substantial endogenous tonic acetylcholine release by parasympathetic nerves. A smaller, but significant relaxing effect was achieved with hexamethonium chloride (14.7±2.1 mN, n=16, P<0.05). In order to rule out the possibility that depolarization with high K+ could excite gut parasympathetic nerve fibers leading to acetylcholine release, both atropine and hexamethonium chloride (0.8 μmol/L each) were added to the bath in all of the subsequent experiments.

Application of high K+-containing solution to the bath (15–120 mmol/L external K+) caused a dose-dependent contraction of the longitudinal smooth muscle (n=12, Figure 1C, closed circles). As a control for osmolarity changes caused by the application of high K+ concentrations, equivalent amounts of saline solution were applied to the smooth muscle (n=6, Figure 1C, open circles). Intriguingly, increasing osmolarity with saline alone had a strong relaxing, rather than contracting effect indicating that the observed contraction by high K+ was caused by depolarization rather than by the hyperosmolar conditions. In the remaining experiments, 45 mmol/L was used as the standard concentration to study high K+-induced contraction. Repetitive depolarization with 45 mmol/L of K+ induced reproducible contractions in gut smooth muscle (Figure 1D, n=43).

Depolarization-induced contraction requires Ca2+ release from internal stores

Depolarization-induced contraction is mediated by Ca2+ influx through voltage-gated Ca2+ channels, but it may also require Ca2+ release from internal stores. Therefore, we studied high K+-induced contraction before and after depletion of internal Ca2+ stores (Figure 2). At the beginning of the experiment, high K+ application (45 mmol/L) caused a contraction of 16.2±2.4 mN (n=9; Figure 2Aa), which was entirely

Figure 1. Acetylcholine-induced and depolarization-induced contraction of gut smooth muscle. (A) Acetylcholine (1.2 μmol/L) evoked strong and long-lasting contraction of gut smooth muscle that was abolished by atropine (0.8 μmol/L), and partly antagonized by hexamethonium chloride (0.8 μmol/L). (B) Bar graph summarizing the acetylcholine (ACh)-induced contraction with and without the muscarinic blocker atropine and the nicotinic blocker hexamethonium, respectively. Note that atropine caused a relaxation (negative force) indicating endogenous ACh release. (C) Depolarization-induced contraction with increasing concentrations of KCl. Equivalent concentrations of NaCl caused relaxation indicating that contraction was not due to the increase of osmolality. (D) Repetitive depolarization with 45 mmol/L KCl induced reproducible contractions in gut smooth muscle. bP<0.05, cP<0.01.
Ca²⁺ free solution containing 4 mmol/L EGTA almost abolishes high K⁺-induced contractions, although these effects were incomplete (caffeine: 2.8±0.6 mN, P<0.01; thapsigargin: 7.4±1.2 mN, P<0.01). Looking at these relative effects, caffeine reduced the contraction strength by 78%±6%, which was significantly greater than the thapsigargin-induced reduction of 52%±5% (P<0.01). Ryanodine receptor blockade with DHPB (100 µmol/L) did not counteract the caffeine effect (data not shown). Whereas internal store depletion by caffeine might have been prevented by DHPB in these experiments, the blocking effect of caffeine on MLCK[18] probably occluded any of the ryanodine receptor blocking effects of DHPB. The internal store depletion experiments showed that Ca²⁺ release from internal stores is required for high K⁺-induced contractions. Since residual contractile activity may be due to Ca²⁺ influx through voltage-gated Ca²⁺ channels, the Ca²⁺ channel pore blocker CdCl₂ (1 mmol/L) was added to the bath containing thapsigargin. Cd²⁺ and thapsigargin further reduced the high K⁺-induced contraction significantly (2.8±0.5 mN, P<0.01; Figure 2Ae) as compared with thapsigargin alone. Altogether, thapsigargin and CdCl₂ blocked 76%±9% of the initial contraction strength, which was very similar to the effect of caffeine alone.

Depolarization-induced contraction involves depolarization-induced Ca²⁺ release through ryanodine and IP₃ receptors via PLC activation

In addition to CICR, Ca²⁺ release from internal stores may also be evoked by depolarization directly in a process referred to as depolarization-induced Ca²⁺ release (DICR). To test this, we used CdCl₂ to abolish Ca²⁺ influx through voltage-gated Ca²⁺ channels without interfering with putative depolarization-induced conformation changes in these channels. As shown in Figure 3, high K⁺-induced contraction was significantly reduced by 1 mmol/L CdCl₂ from 22.2±2.0 mN (Figure 3Aa) to 11.9±1.9 mN (n=16, P<0.001; Figure 3Ab), which was equivalent to a reduction of contraction strength by 46%±7%. In addition, CdCl₂ caused a minor but significant delay in the peak latency (9.0±2.8 s versus 6.8±0.4 s). When the ryanodine receptor blocker DHPB (100 µmol/L; Figure 3Ac) or the IP₃ receptor blocker 2-APB (100 µmol/L; Figure 3Ad) was added to the CdCl₂-containing bath, a more pronounced reduction in the high K⁺-induced contraction was observed as compared with CdCl₂ alone (DHPB: 6.4±1.8 mN, P<0.05; 2-APB: 7.8±1.9 mN, P<0.05). In contrast to their similar effects on contraction strength, the peak latencies of high K⁺-induced contraction were different between these two blockers. Whereas 2-APB did not have an effect on peak latency (9.1±2.4 s), DHPB significantly delayed the peak to 23.3±4.6 s (P<0.05 compared with the CdCl₂-containing bath). Finally, a cocktail consisting of CdCl₂, DHPB and 2-APB inhibited smooth muscle contraction even more markedly (4.1±0.9 mN, P<0.05 compared with CdCl₂ and DHPB or 2-APB alone; Figure 3Ae). These blockers reduced contraction strength by 79%±5%, which closely resembled the relative reduction observed after caffeine treatment or with co-application of thapsigargin and CdCl₂ (Figure 2). Following wash-out, high K⁺-induced contraction did not fully recover, indicating poor wash-out of DHPB and/or 2-APB (Figure 3Af). To avoid biasing effects
due to prolonged drug action, the order of DHBP and 2-APB application was randomized (2-APB after DHBP: n=10, 2-APB before DHBP: n=6). However, no significant differences were observed when comparing the different experimental protocols, and importantly, complete contraction inhibition could not be achieved. However, when the L-type Ca²⁺ channel blocker verapamil (40 µmol/L), which not only blocks Ca²⁺ influx but also inhibits conformational changes in the channel[19], was applied, virtually no contraction could be observed (0.5±0.2 mN, P<0.001). In a separate set of experiments, we tested the effect of DHBP and/or 2-APB without CdCl₂ on high K⁺-induced contraction. Interestingly, neither DHBP nor 2-APB reduced contraction when applied without CdCl₂. The initial high K⁺-induced contraction value of 17.1±2.5 mN (n=11) was slightly higher following pre-application of DHBP (20.9±3.3 mN) or 2-APB (18.2±3.0 mN). When both DHBP and 2-APB were co-applied, the high K⁺-induced contraction value was 17.8±3.2 mN (n=11). At the end of the experiment, the high K⁺-induced contraction fully recovered (18.4±3.1 mN, n=11). In addition, both DHBP and 2-APB relaxed the basal tone of the smooth muscle (-4.3±0.7 mN and -9.0±1.6 mN, respectively) indicating constitutive Ca²⁺ release under control conditions via both ryanodine receptors and IP₃ receptors. Overall, this suggested that unless transmembrane Ca²⁺ influx is blocked, neither ryanodine receptor nor IP₃ receptor blockade alone or in combination prevents contraction. In summary, Ca²⁺ release from internal stores can be independent of Ca²⁺ influx through voltage-gated Ca²⁺ channels. In addition, depolarization-induced Ca²⁺ release in gut smooth muscle involves both ryanodine and IP₃ receptors, probably due to depolarization-induced conformational changes in voltage-gated Ca²⁺ channels.

Whereas ryanodine receptors directly couple to voltage-gated Ca²⁺ channels, IP₃ receptors require IP₃ production through phospholipase C (PLC) activation. Thus, we asked if depolarization-induced conformational changes in voltage-gated Ca²⁺ channels can lead to IP₃ production. As shown in Figure 4, high K⁺-induced contraction (25.6±2.2 mN; Figure 4Aa) was again significantly reduced by 1 mmol/L CdCl₂ treatment to 8.7±1.6 mN (n=17, P<0.001; Figure 4Ab), which also increased the peak latency (from 7.8±0.5 s to 11.7±1.0 s, P<0.01). Similar to the experiments described above, additional application of either the ryanodine receptor blocker DHBP or the PLC blocker U 73122 (5 µmol/L) further reduced this contraction significantly (DHBP: 4.4±0.8 mN, P<0.01, U 73122: 4.0±0.6 mN, P<0.01; Figure 4Ac,d). With respect to peak latencies, U 73122 again had no effect (13.8±1.6 s), whereas DHBP significantly slowed peak contraction (14.7±1.3 s, P<0.05 compared with the CdCl₂-containing bath). Finally, the combined application of CdCl₂, DHBP and U 73122 further reduced the high K⁺-induced contraction (3.3±0.6 mN, P<0.05; Figure 4Ae) as compared with CdCl₂ and DHBP or U 73122 alone. Interestingly, the relative reduction of contraction strength following CdCl₂, DHBP and U 73122 application (85%±4%) was again as high as that observed for caffeine treatment. High K⁺-induced contraction did not fully recover following wash-out indicating poor removal of these lipophilic drugs from the tissue (Figure 4Af). Therefore, we again changed the order of application between DHBP and U 73122 without obtaining significant differences between these two experimental groups (U 73122 after DHBP: n=14, U 73122 before DHBP: n=3). The application of verapamil (40 µmol/L) at the end of the experiment abolished high K⁺-induced contraction (0.5±0.2 mN, P<0.001; Figure 4Ag).

Figure 3. Depolarization-induced contraction depends on depolarization-induced Ca²⁺ release through ryanodine receptors and IP₃ receptors. (A) Consecutive traces of a representative experiment. High K⁺ (45 mmol/L) evoked a strong contraction that was significantly reduced in solution containing 1 mmol/L CdCl₂. Additional application of the ryanodine receptor blocker DHBP (100 µmol/L) further reduced the contraction, albeit incompletely. Combined application of CdCl₂ and the IP₃ receptor blocker 2-APB (100 µmol/L) also incompletely reduced the contraction. Application of CdCl₂, DHBP and 2-APB caused a significantly stronger reduction of contraction than CdCl₂ with DHBP or 2-APB alone. Verapamil abolished the depolarization-induced contraction. (B) Bar graph summarizing 16 experiments (mean±SEM). *P<0.05, #P<0.01.
These results demonstrate again that Ca²⁺ release from intracellular stores can be independent of Ca²⁺ entry but can also be triggered by depolarization itself through coupling to ryanodine and IP₃ receptors. When pooling all of the experimental results described above, CdCl₂ reduced the contraction strength by approximately 56%±5% (n=33), whereas DHBP together with CdCl₂ caused a relative reduction of 75%±5%. By contrast, verapamil almost completely abolished smooth muscle contraction (inhibition by 98%±6%).

Depolarization-induced contraction is largely paralleled by intracellular Ca²⁺ transients

Our findings so far relied on measurements of isometric contraction strength. However, the correlation between the intracellular rise in Ca²⁺ concentration and contraction strength is uncertain. Thus, we employed FURA-2 recordings in longitudinal smooth muscle cells in situ in order to measure intracellular Ca²⁺ transients evoked by high K⁺ bath application. As demonstrated in Figure 5Aa, high K⁺ solution evoked an increase in intracellular Ca²⁺ concentration (114.2%±2.3%, n=9). Whereas this increase was significantly reduced in a solution containing 1 mmol/L CdCl₂ (103.9%±0.4%, P<0.01, Figure 5Ab), it was almost abolished in a solution containing 40 µmol/L verapamil (101.8%±0.4%, P<0.01 vs CdCl₂, Figure 5Ac). These results indicate that inhibition of Ca²⁺ influx through voltage-gated Ca²⁺ channels by Cd²⁺ cannot fully prevent the rise in intracellular Ca²⁺ concentration. Verapamil, however, showed significantly less Ca²⁺ transients compared with CdCl₂. Hence, these results recapitulate our observations on isometric contraction because the Ca²⁺ transients with CdCl₂ administration was roughly 30% of that obtained in the control situation. Thus, this indicates a parallel reduction of contraction strength with a rise in intracellular Ca²⁺ concentration in the presence of the Ca²⁺ channel pore blocker CdCl₂.

Discussion

Role of internal Ca²⁺ stores in smooth muscle contraction

Smooth muscle contraction is well known to be triggered by a rise in the cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) leading to Ca²⁺/calmodulin-dependent activation of MLCK, which in turn phosphorylates the 20-kDa light chains of myosin[4, 5]. This rise in [Ca²⁺]ᵢ can be attained by either Ca²⁺ entry through VGCCs following depolarization, or through receptor-operated Ca²⁺ channels following ligand binding. However, additional Ca²⁺ release from internal Ca²⁺ stores through Ca²⁺ release channels appears to be a substantial prerequisite for normal smooth muscle contraction. Thus, we found a 50% reduction in depolarization-induced contraction after the depletion of internal Ca²⁺ stores with thapsigargin. Moreover, our experiments using Cd²⁺ ions to block Ca²⁺ entry into the smooth muscle cells also showed a comparable residual contraction strength that was half the control value. Therefore we conclude that roughly half of the contraction strength in gut smooth muscle is due to Ca²⁺ entry through Ca²⁺-permeable channels in the plasma membrane and the other half is due to Ca²⁺ release from internal stores.

The residual contraction strength with caffeine treatment was only ~20% of the original amplitude. Interestingly, in addition to activating ryanodine receptors[8, 20, 21] caffeine also directly inhibits MLCK[18]. This probably explains this pronounced reduction in depolarization-induced contraction in the presence of caffeine as compared with thapsigargin alone, which selectively inhibits SERCA[22, 23]. Thus, caffeine may inhibit all MLCK-dependent and hence Ca²⁺-dependent,

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**Figure 4.** Depolarization-induced contraction depends on depolarization-induced Ca²⁺ release through ryanodine receptors and PLC activation. (A) Consecutive traces of a representative experiment. High K⁺ (45 mmol/L) evoked a strong contraction that was significantly reduced in solution containing 1 mmol/L CdCl₂. Additional application of the ryanodine receptor blocker DHBP (100 µmol/L) further reduced the contraction albeit incompletely. Combined application of CdCl₂ and the PLC blocker U 73122 (5 µmol/L) also incompletely reduced the contraction. Application of CdCl₂, DHBP and U 73122 caused a significantly stronger reduction of contraction than CdCl₂ with DHBP or U 73122 alone. Verapamil abolished the depolarization-induced contraction. (B) Bar graph summarizing 17 experiments (mean±SEM). *P<0.05, **P<0.01.
mechanisms of smooth muscle contraction. However, there is evidence that these mechanisms may coincide with Ca\(^{2+}\)-independent, but Rho kinase-dependent pathways of smooth muscle contraction, which leads to MLCK-independent phosphorylation of myosin-light chain. This is often referred to as Ca\(^{2+}\)-sensitization\(^{[24]}\). Consistent with this idea, the residual contraction strength following caffeine treatment was mirrored by the combined application of CdCl\(_2\) with thapsigargin, which abolished Ca\(^{2+}\)-dependent mechanisms of smooth muscle contraction. The same holds true for our experiments using CdCl\(_2\), DHBP, and 2-APB or U 73122. Interestingly, residual smooth muscle contraction was abolished by verapamil, which only inhibits L-type Ca\(^{2+}\) channels after a large conformational change in the channels\(^{[19]}\). Therefore, the residual contraction following Cd\(^{2+}\) treatment, which was fully sensitive to verapamil, indicates that Ca\(^{2+}\)-independent mechanisms of contraction such as the Rho kinase pathway depend on depolarization-induced conformational changes in L-type Ca\(^{2+}\) channels, but are independent of Ca\(^{2+}\) entry or Ca\(^{2+}\) release. The involvement of additional Ca\(^{2+}\) channel types is unlikely, as the inhibition of T-type, N-type and P/Q-type Ca\(^{2+}\) channels by verapamil occurs at higher concentrations\(^{[25, 26]}\). Thus, even though T-type and P/Q-type, but not N-type Ca\(^{2+}\) channels are expressed in smooth muscle\(^{[27, 28]}\), the concentration used in our study probably reflects Ca\(^{2+}\) influx through L-type channels in smooth muscle cells.

**Coupling between voltage-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) release**

It has been shown in several smooth muscle preparations that Ca\(^{2+}\) ions entering the cell can evoke CICR through direct interaction with ryanodine receptors\(^{[29, 30]}\). In general, CICR is believed to be related to the cardiac subtype RyR2, whereas direct mechanical coupling has been demonstrated in skeletal muscle between the surface voltage-gate Ca\(^{2+}\) channel Ca\(_{1.1}\) and RyR\(_1\)\(^{[13, 14, 31, 32]}\). Thus, ryanodine receptors in smooth muscle cells primarily act as intracellular Ca\(^{2+}\) sensors and promote a further increase in [Ca\(^{2+}\)]\(_i\). In line with this scenario, Ca\(^{2+}\) release through RyRs should be entirely dependent on Ca\(^{2+}\) entry. However, after blocking Ca\(^{2+}\) entry with CdCl\(_2\), we obtained a substantial residual contraction of about half of the initial strength. This residual contraction was paralleled by a Ca\(^{2+}\) transient of 30% of the control in a solution containing CdCl\(_2\). On the basis of our force measurements, this contraction was caused by Ca\(^{2+}\) release from internal stores because the ryanodine receptor blocker DHBP further reduced this contraction significantly by approximately 70% of the control. But how might voltage-gated Ca\(^{2+}\) channels couple to ryanodine receptors? Smooth muscle cells express all ryanodine receptor subtypes. Among these, the skeletal subtype Ca\(_{1.1}\) appears to have a significant role in smooth muscle cell function\(^{[33–35]}\). Moreover, in airway smooth muscle cells it has recently been shown that the skeletal subtype Ca\(_{1.1}\) may co-localize with RyR\(_1\)\(^{[36]}\). Thus, it is at least conceivable that in gut smooth muscle cells Ca\(_{1.1}\) may also be functionally present. However, the direct coupling between RyR1 and the predominant smooth muscle VGCC subtype Ca\(_{1.2}\) is still under debate\(^{[37]}\). Our data support the existence of a coupling mechanism between smooth muscle L-type VGCCs and ryanodine receptors beyond CICR. Further studies are required to elucidate the interconnection between these channels.

In the present study, CdCl\(_2\) and DHBP were not able to fully abolish gut smooth muscle contraction. We also observed a significant reduction of contraction by additional inhibition of IP\(_3\) receptors. Likewise, inhibition of PLC had the same effect.
on smooth muscle contraction. From these results we infer that voltage-gated Ca$^{2+}$ channels couple to G proteins and lead to the PLC-dependent production of IP$_3$, which then acts on IP$_3$-gated Ca$^{2+}$ release channels at the sarcoplasmic reticulum. Indeed, L-type Ca$^{2+}$ channels have recently been demonstrated to exhibit metabotropic actions following membrane depolarization. In particular, they appear to be linked to PLC-coupled G proteins enabling IP$_3$-induced Ca$^{2+}$ release (IICR) from internal stores, which has been termed Ca$^{2+}$ channel-induced Ca$^{2+}$ release[38, 39]. Thus, in addition to the Ca$^{2+}$ inward current, Ca$^{2+}$ channels may serve as voltage sensors even in the absence of any Ca$^{2+}$ influx. Such a mechanism has been found in viscera[40, 41] as well as vascular smooth muscle[42, 43]. Although we could not directly test G protein activation in our preparations, our data at least suggest a metabotropic coupling between L-type Ca$^{2+}$ channels and IP$_3$-mediated Ca$^{2+}$ release because DHBP treatment significantly slowed contraction compared with CdCl$_2$ treatment. By contrast, blocking IP$_3$ receptors with 2-APB had no effect itself on peak latencies. This may be interpreted as an induction of fast Ca$^{2+}$ rise by ryanodine receptors and a significantly slower Ca$^{2+}$ rise by IP$_3$ receptors, presumably because of the slower kinetics of G protein-dependent and PLC-mediated IP$_3$ production.

Figure 6 summarizes our current knowledge about electromechanical coupling of gut smooth muscle cells. Ca$^{2+}$ ions entering the cell through VGCC increase cytosolic Ca$^{2+}$ concentration and also activate CICR through ryanodine receptors (RyR). The cytosolic Ca$^{2+}$ concentration can be lowered by active transport into internal stores by the sarcoendoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) or by extrusion out of the cell by the plasma membrane Ca$^{2+}$ ATPase (PMCA) and the secondarily active Na$^+$/Ca$^{2+}$-exchanger (NCX), respectively. Caffeine acts as an inhibitor of phosphodiesterase[44, 45] and thereby activates SERCA through an increase in cyclic AMP. It also directly inhibits MLCK[21]. In addition to these well-known cascades, we describe two novel pathways for electromechanical coupling (see dotted arrows). On the one hand, depolarization-dependent activation of voltage-gated Ca$^{2+}$ channels can open ryanodine receptors without Ca$^{2+}$ influx and lead to depolarization-induced Ca$^{2+}$ release (DICR) from internal Ca$^{2+}$ stores. On the other hand, VGCC activation can cause PLC-dependent IP$_3$ production and Ca$^{2+}$ release through IP$_3$ receptors. Thus, we conclude that DICR from internal stores, which is independent of Ca$^{2+}$ entry, is a physiologically relevant mechanism of electromechanical coupling in gut smooth muscle.

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Author contribution
Timo KIRSCHSTEIN designed the study, performed research, analyzed data, and wrote the manuscript; Mirko REHBERG performed research, and analyzed data; Rika BAJORAT performed research, analyzed data, and wrote the manuscript; Katrin PORATH performed research, analyzed data, and wrote the manuscript; Rüdiger KOHLING designed the study, performed research, written data, and wrote the manuscript; Tursonjan TOKAY performed research, analyzed data, and wrote the manuscript; Karin PORATH performed research, and analyzed data; Rüdiger KOHLING designed the study, and wrote the manuscript.

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Figure 6. Ca$^{2+}$-induced and depolarization-induced Ca$^{2+}$ release. This synoptic overview shows the well-known pathways of Ca$^{2+}$-dependent mechanisms of smooth muscle contraction (black arrows). Thus, Ca$^{2+}$ entry through L-type voltage-gated Ca$^{2+}$ channels (VGCC) leads to a rise in cytosolic Ca$^{2+}$ concentration, which can be enhanced by Ca$^{2+}$-induced Ca$^{2+}$ release via ryanodine receptors (RyR), and causes MLCK-dependent contraction. In turn, smooth muscle cells relax when Ca$^{2+}$ is actively transported into the sarcoplasmic reticulum by SERCA, or when Ca$^{2+}$ is extruded by the plasma membrane Ca$^{2+}$ ATPase (PMCA) or by the Na$^+$/Ca$^{2+}$-exchanger (NCX). The dotted arrows indicate the new pathways demonstrated by the present study. Thus, voltage-dependent conformational changes of VGCCs can directly open RyRs and IP$_3$ receptors without any Ca$^{2+}$ influx, the latter via PLC activation. *P<0.05, **P<0.01.
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