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Title Page

Potential L-type voltage operated calcium channel blocking effect of drotaverine on functional models

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Running Title Page

Running title: Functional calcium channel blocking effect of Drotaverine

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Abstract

Drotaverine is considered as an inhibitor of cyclic-3′,5′-nucleotide-phophodiesterase (PDE) enzymes. However, published receptor binding data supports the potential L-type Voltage Operated Calcium Channel (L-VOCC) blocking effect of drotaverine too. Hence, in this work we are focusing on the potential L-VOCC blocking effect of drotaverine using L-VOCC associated functional in vitro models. Accordingly, drotaverine and reference agents were tested on KCl-induced guinea pig tracheal contraction. It was found that drotaverine, like the L-VOCC blockers nifedipine or diltiazem, inhibited the KCl-induced inward Ca^{2+}- induced contraction in a concentration dependent fashion. The PDE-inhibitor theophylline had no effect on the KCl-evoked contractions indicating its lack of inhibition on inward Ca^{2+} flow. Drotaverine was also tested on the L-VOCC mediated resting Ca^{2+} refill model. In this model the extracellular Ca^{2+} enters the cells to replenish the emptied intracellular Ca^{2+} stores. Drotaverine and L-VOCC blocker reference molecules inhibited the Ca^{2+} replenishment of Ca^{2+} depleted preparations detected by agonist-induced contractions in post Ca^{2+} replenishment Ca^{2+} free medium. Theophylline didn't modify the Ca^{2+} store replenishment following contraction. It seems that drotaverine but not theophylline inhibit the inward Ca^{2+} flux. Addition of $CaCl_2$ to Ca^{2+} free medium containing the agonist, induced inward Ca^{2+} flow and subsequent contraction of Ca^{2+} depleted tracheal preparations. Drotaverine similar to the L-VOCC blockers, inhibited inward Ca^{2+} flow and blunted the slope of $CaCl_2$-induced contraction in agonist containing Ca^{2+} free medium withCa^{2+} depleted tracheal preparations. These results show that drotaverine behaves like L-VOCC blockers but unlike PDE inhibitors using L-VOCC associated in vitro experimental models.

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Introduction

Two families of drugs which inhibit PDE activity, methylxanthines and isoquinolines, are used clinically in 2 distinct therapeutic areas involving smooth muscle function. Methylxanthines, such as theophylline, are frontline asthma medications, while the isoquinolines, like papaverine, are used to ameliorate the symptoms of visceral smooth muscle spasm and associated pain. However, neither of them are active if used in the opposite field – why is that? Based on indirect experimental evidence the difference may be associated with their differing activity on the voltage operated calcium channel (L-VOCC).

The natural isoquinoline alkaloide of Papaver sumniferrum, papaverine and its synthetic derivate drotaverine have been broadly used as antispasmodic agents in human medicine for decades. It has been published that drotaverine binds to the L-VOCC on pregnant rat uterine membranes (Tömösközi et al., 2002) while Ca^{2+} activated potassium channels and L-VOCCs may be involved in papaverine-induced vascular relaxation in rat basilar artery (Han et al., 2007). Moreover, it was shown that both molecules inhibit cyclic-3′,5′-nucleotide-phophodiesterase (PDE) enzymes with concentration dependent specificity (Kukovetz and Pöch,1970; Triner et al.,1970; Pöch and Kukovetz, 1971; Kukovetz et al., 1976, Ji-Qun et al., 1995). However, the detailed molecular mechanism of action and associated function of drotaverine on airway smooth muscle has not yet been systematically investigated. Especially, the interaction of drotaverine with the Ca^{2+} flux essential for the contraction-relaxation machinery of airway smooth muscle has not been tested. Both of these cellular mechanisms play a fundamental regulatory role in smooth muscle function. In airway smooth muscle, bronchodilatory agents increase the intracellular

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cAMP level (e.g. by the inhibition of the PDEs) and cause the relaxation of precontracted (post-agonist administration) preparations (Torphy and Undem, 1991; Frossard et al., 1981). However, these agents are less effective in the inhibition of the agonist-induced contraction (pre-agonist administration) (Bilcíková et al., 1987). The airway smooth muscle contractile mechanism is considered to be triggered either by release of intracellularly sequestered Ca^{2+} or by the increased influx of extracellular Ca^{2+}. In airway smooth muscle the Ca^{2+} release from the intracellular stores predominates over inward Ca^{2+} flux through L-VOCCs after receptor activation-induced airway smooth muscle contraction (Pelaia G et al., 2008). While operation of L-VOCCs can induce some relaxation of agonist-induced pre-contracted airway smooth muscle (Fanta et al., 1982) they are not effective prior to agonist administration (Foster et al., 1984). This may explain why L-VOCC blockers have no principal therapeutic benefit for the treatment of bronchial asthma (Hirota et al., 2003). Airway smooth muscle receptor mediated activation uses intracelleluar Ca^{2+} stores, whereas vascular smooth muscle is more dependant on Ca^{2+} influx. However, the L-VOCCs may play a role in the Ca^{2+} balance necessary for the maintenance of the physiological airway smooth muscle function. In this context the principal role of L-VOCCs may be the regulation of the Ca^{2+} influx responsible for refilling of the intracellular Ca^{2+} stores that are partly depleted during agonist-induced contraction in airway smooth muscle (Bourreau et al., 1993). It has been demonstrated that L-VOCC blockers are able to inhibit the refill of the depleted sarcoplasmatic Ca^{2+} stores in resting conditions (Bourreau et al., 1991; Liu and Farley, 1996), while their impact on receptor activation associated inward Ca^{2+} currents and subsequent contractions in normal Ca^{2+} containing medium is minimal (Cheng and Townley, 1983). We have hypothesized that the L-VOCCs may also play

an important role in receptor mediated inward Ca^{2+} current associated contractions when Ca^{2+} depleted preparations in Ca^{2+} free medium were supplemented with extracellular Ca^{2+}. Our aim with this study was to generate functional data supporting the potential L-VOCC blocking effect of drotaverine using proven L-VOCC dependent tracheal models (e.g. KCl depolarisation induced contractions (Foster et al., 1983), resting Ca^{2+}-refill linked contractions and receptor mediatedinward Ca^{2+}-induced contractions).

Methods

Chemicals

The ingredients of the Krebs-Henseleit solution: KCl, $CaCl_2.2H_2O$, $KH2PO_4$, $NaHCO_3$, $MgSO_4.7H_2O$ were obtained from Merck Inc (Darmstadt, Germany). NaCl from Reanal ZRt. Budapest, Hungary.The drotaverine HCl was synthesized in Chinoin Co.Ltd., Budapest, Hungary; EGTA, indomethacin, nifedipine, theophylline, papaverine HCl, diltiazem HCl, Acetyl-β-methylcholine chloride (methacholine), histamine dihydrochloride, D-(+)-glucose were purchased from Sigma (St Louis, MO).

Animals

Male guinea pigs 300-350 g bodyweight (Harlan MD, distributed by INNOVO Ltd., Gödöllő, Hungary) were used. All experiments were performed in accordance with the Institutional Ethical Codex, Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243/1998) and the European Union guidelines (directive 2010/63/EU). The animals were housed in open cages in a temperature-controlled and ventilated environment (21-23°C) with a 12-hour light-dark cycle. Water and standard ascorbic acid containing guinea pig chow (Altromin) were provided ad libitum. The animals were tissue donors therefore, the approval of the experimental

protocol by the Hungarian Governmental Animal Ethics Committee (ÁTET) was not mandatory.

Isolation of the trachea, and organ bath preparation

On the day of the experiments the guinea-pigs were euthanized by an overdose of pentobarbital sodium. The trachea was rapidly removed and placed in Krebs-Henseleit (KH) solution of the following composition (mM): NaCl-119, $NaHCO_3$-25.0, $CaCl_2$-2.5, KCl-4.7, KH_2PO_4-1.2, $MgSO_4$-1.2, glucose-11.1, $5 \cdot 10^{-6}$ M indomethacin. The cartilaginous rings from the distal part of the trachea were opened longitudinally and mounted in a 20 ml organ bath containing KH solution, maintained at 37.4^{o}C, and continuously bubbled with 95 % O_2 and 5 % CO_2 to give a pH of 7.4 ± 0.1. The preparations were preloaded with 5 millinewton (mN; approx. 0.5 g). The tissues were then allowed to equilibrate for at least 1 hour, during which time they were washed approximately every 20 min with fresh KH solution. Eight tracheal strip preparations were used from the same guinea pigs on the same day. Four preparations were used for the control groups while the other four preparations were used for the test compounds. Earlier studies showed no difference in reactivity to histamine or methacholine with respect to tracheal ring location from this part of the trachea (data not shown). The strength of the isometric contractile responses was measured (mN) using a force displacement transducer and preamplifier (MDE Co. Ltd., Budapest, Hungary). The experimental data were collected and evaluated by a computer aided data acquisition system (SpelIso v3.2 software; MDE Co. Ltd., Budapest, Hungary).

Due to small variations in the baselines and the acclimation of the tissue preparations to the organ bath environment, the second contraction force was in many cases

higher than the first (but not as high as the third contraction force). Consequently the second contraction was used as the control in all the experimental protocols.

KCl induced contraction protocol

Contractions of the 8 mounted tracheal preparations in normal KH solution were evoked by consecutive applications of 20, 30 and 50 mM KCl, separated by a wash out Figure 1A). The contraction force was continuously registered. The contraction force derived from the second concentration-response curve was used as the control value (100% contraction). Once the control value had been established, 4 of the 8 preparations were treated with10^{-7} M of the test moleculeswhile the other 4 were treated with the vehicle of the test molecule (control preparations). Following 15 min incubation, another concentration-response curve to KCl was constructed for all 8 preparations. After a wash out, the experiment was repeated using 10^{-6} M (or vehicle) and 10^{-5} M (or vehicle) test molecule, with a wash out between increasing doses of test molecule. The experimental design is shown in Figure 1A. The percentage inhibition was calculated for each dose of the test molecule at every KCl concentration. The test molecule dose causing a 50 % inhibition of KCl contraction was calculated in each case, when feasible.

Ca^{2+} reload following agonist-induced contractions in Ca^{2+} free medium (resting refill)

The intracellular Ca^{2+} dependent contractions were tested using two mediators that induce contraction of airway smooth muscle, histamine ($3x10^{-6}$ M) and methacholine ($5x10^{-7}$ M). The concentrations chosen were the calculated EC_{50} values for these mediators from previous experiments in our laboratory (data not shown). Histamine and methacholine were used in separate experiments. Following the

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equilibration period two consecutive contractions were evoked with either histamine or methacholine separated by a wash out. The second contraction was considered the control (100%) contraction. After washing out the agonist, 2.5 mM Ca^{2+} containing KH solution was changed to a Ca^{2+} free medium. Next, 3 consecutive contractions were evoked by the constrictor agents each separated by a wash out in order to deplete the sarcoplasmatic Ca^{2+} stores. The third agonist stimulation usually evoked a minimal, if any, contraction indicating depletion of the intracellular calcium stores (Table 2A, B, 5^{th} contraction column). Following the last wash out in Ca^{2+} free medium, test compounds (10^{-5} M) were added to 4 of the 8 organ baths. The other 4 preparations served as controls with only the vehicle added to the baths. After a 15 min incubation the solution in the bath was changed to normal KH solution (2.5 mM Ca^{2+}) containing 10^{-5} M test molecule (or vehicle). The preparations were then incubated for 30 min without any agonist stimulation. Previous studies have shown this was sufficient time for the Ca^{2+} refill of intracellular calcium stores. The normal KH solution was then changed to the Ca^{2+} free KH solution in all eight organ baths, after which three consecutive contractions were evoked by constrictor agonists, each separated by a wash out period. The contraction force was measured (mN) and the percentage contraction values were calculated using the second agonist-induced contraction as 100 % contraction. The experimental design is shown in Figure 1B.

$CaCl_2$-induced contraction in agonist containing Ca^{2+} free medium (receptor operated refill)

The experimental protocol was indentical with the “resting refill” protocol up to the intracellular Ca^{2+} depletion step in Ca^{2+} free medium. As before, the third agonist stimulation evoked only limited if any contraction denoting the depleted Ca^{2+} content of the intracellular calcium stores (Table 2A, B 5^{th} contraction column). At this point,

10^{-5} M of test molecule was added to 4 of the organ baths while the other 4 preparations were treated with the vehicle as control preparations. After 15 min incubation, 2.75 mM $CaCl_2$ was added to all 8 organ baths. The experimental design is presented in Figure 1C. In this experimental protocol both the maximum contraction and the slope of the contractions were evaluated. The percentage decrease of the contraction force was also calculated.

Statistics

Statistical comparison of the treated versus non-treated (control) groups was conducted by using Student's t-test (GraphPad Prism v6.0, La Jolla, CA). Differences among groups were considered statistically significant when the P value was <0.05.

Results

KCl depolarisation

KCl caused contraction of the guinea pig tracheal preparation in a concentration dependent fashion. The contraction force generated by 20 mM, 30 mM and 50 mM was 4.0 ± 2.2, 9.5 ± 2.5 and 13.9 ± 3.1 mN (n=122), respectively. The KCl responses were reproducible in control preparations each time they were repeated for constructing drug dose-response curves (data not shown). L-VOCC blockers e.g. nifedipine and diltiazem decreased the KCl-induced contractions in a concentration dependent manner. 10^{-5} M nifedipine or diltiazem practically abolished the KCl-induced contractions (Figure 2A,B,C) proving that the applied experimental protocol was suitable to test the functional consequences of L-VOCC blockade. The two isoquinoline derivatives also decreased KCl-induced contractions in a concentration dependent fashion (Figure 2A,B,C), supporting the proposed functional L-VOCC blocking activity of both drotaverine and papaverine. However, the potency and

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efficacy of both drotaverine and papaverine were weaker than that of the reference L-VOCC blockers (Figure 2, Table 1). Unlike the L-VOCC blockers, the PDE inhibitor theophylline did not modify the KCl-induced contractions indicating that the cAMP/PDE system doesn't play a significant role. The mixture of the equimolar concentrations of nifedipine and theophylline behaved more like an L-VOCC blockers alone in this experimental mode (Figure 2, Table 1). Interestingly, the inhibitory efficacy of the nifedipine and theophylline combination was significantly higher than that of nifedipine alone. This observation will be explored in future studies.

Ca^{2+} reload followed agonist-induced contractions in Ca^{2+} free medium (resting refill)

Both $3x10^{-6}$ M histamine and $5x10^{-7}$ M methacholine in normal KH solution induced tracheal smooth muscle contractions of similar strength (11.9 ± 3.2 mN n=43 and 10.4 ± 2.8 mN n=32, respectively). With consecutive administration of histamine and methacholine, the contraction force decreased gradually in calcium free KH medium to 70.7 ± 14.8%, 22.9 ± 25.1% and 3.0 ± 6.3% (n=43) and 76.2 ± 12.4%, 13.3 ± 16.8% and 0.4 ± 1.1% (n=32), respectively of the original reference contraction (Table 2A and 2B). Following agonist-provoked calcium depletion the preparations were put into normal KH solution (reload solution) and incubated for 30 min in order to let the depleted calcium stores refill (resting refill). The organ bath solution was replaced with a calcium free medium (post-reload), after which the preparations were able to contract again with agonist stimulation, indicating refilling of their intracellular calcium stores (Table 2A and Table 2B, 6^{th} contraction column)**.** L-VOCC blockers like nifedipine (Figure 3C,4C), or diltiazem (Figure 3D,4D) added to the calcium reload medium were able to reduce the agonist-induced contractions (6th contraction) in the post-reload Ca^{2+} free medium. This observation indicates that both

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L-VOCC blockers may inhibit the resting Ca^{2+} refill of emptied intracellular calcium stores regardless of the constrictor mediator used (Table 2).

The non-specific phosphodiesterase inhibitor theophylline was also tested on the resting calcium refill model. However, as can be seen in Figure 4E, theophylline, unlike L-VOCC blockers enhanced, rather than inhibited the histamine-induced contraction. The combination of 10^{-5} M theophylline with 10^{-5} M nifedipine in the Ca^{2+} refill medium inhibited the agonist-induced contraction in the post-reload calcium free medium (Figure 3F and Figure 4F).

Drotaverine and papaverine were also tested on this model (Figure 3A, 4A and Figure 3B, 4B). Both drotaverine and papaverine blocked the resting Ca^{2+}refill associated contractions at 10^{-5} M concentration, making these two isoquinoline derivatives more similar to the L-VOCC blockers than to the PDE inhibitors.

$CaCl_2$-induced contraction in agonist containing Ca^{2+} free medium (receptor operated refill)

Administration of 2.75 mM $CaCl_2$ to the calcium depleted tracheal preparation incubated with histamine ($3x10^{-6}$M) or methacholine ($5x10^{-7}$M) in Ca^{2+} free (0.25 mM EGTA) buffer induced a contraction as strong as that produced by the same concentration of agonists in normal (2.5 mM Ca^{2+}) KH solution at the start of the experimental protocol. Neither the maximal contraction force, nor the slope (Table 3) of the contraction of the control preparations differed markedly between the two experimental conditions. So the mechanical response to the agonist-induced contraction in normal KH solution is identical with the contraction developed by adding $CaCl_2$ to the Ca^{2+} free KH solution containing the agonist.

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The L-VOCC blockers, nifedipine or diltiazem (10^{-5} M), decreased the slope of the $CaCl_2$-induced contraction force. This effect was not agonist specific (Table 3), as they blunted the force development slope in the presence of both histamine and methacholine.

In the same model, both drotaverine and papaverine decreased the slope of the $CaCl_2$-induced contraction (Table 4), and in this context the two tested isoquinoline derivatives behaved like the L-VOCC blockers. However, the PDE inhibitor theophylline had no effect on the contraction slope with either agonist in the organ bath. The combination of theophylline with nifedipine produced the same result as nifedipine alone. There were a variety of potencies among the test compounds with respect to maximum contraction. The rank order for decreasing the contraction amplitude in the presence of histamine was; diltiazem<theophylline<drotaverine=nifedipine=nifedipine+theophyilline<papaverine, while in the presence of methacholine the rank order was; theophylline=drotaverine<nifedipine=nifedipine+theophylline<diltiazem<papaverine (Figure 5A,B). Based on these results it has been suggested that in the case of diltiazem sufficient Ca^{2+} penetrated the cells to develop contractions as strong as histamine or methacholine-induced contractions in normal KH solution. This did not appear to be the case with other test molecules because as well as decreasing the contraction slope, they modified the maximum $CaCl_2$ –induced contraction force as well. Theophylline alone had no effect on either slope or contaction maximum, and the nifedipine and theophylline combination acted like nifedipine alone.

Discussion

Phosphodiesterdase inhibitors have long been used in the treatment. Methylxanthines (e.g. theophylline) are in the frontline for treatment of asthma, while isoquinolines (e.g. papaverine) ameliorate the symptoms of visceral smooth muscle spasm and associated pain. The medical use of these structurally disimilar PDE inhibitors is based on bedside experience rather than on a rational knowledge of their molecular mechanism of action.The question to be considered is what molecular mechanisms support the different clinical uses for thesetwo types of PDE inhibitor? Based on indirect experimental evidence the difference may be associated with their differing activity on the voltage operated calcium channel (L-VOCC).

It was published that drotaverine increases intracellular cAMP levels by the inhibition of PDEs and it may also have an allosteric L-VOCC regulating effect, as proved by the displacement of [H^3]-nitrendipine (Tömösközi et al., 2002) from its binding site on pregnant rat uterine membranes. Two other isoquinoline derivatives, papaverine and ethaverine also inhibit PDEs and bind to the L-VOCC (Wang and Rosenberg, 1991; Iguchi et al., 1992) suggesting a common cellular mechanism of action for these structurally similar derivatives. However, neither the interaction of drotaverine with the L-VOCC binding site(s) of guinea pig airway smooth muscle, nor the functional L-VOCC blocking effect of drotaverine has been investigated to date. Therefore, our aim was to provide functional data supporting the L-VOCC blocking effect of isoquinoline derivatives using proven L-VOCC dependent tracheal models like the KCl depolarisation induced contraction model, the resting Ca^{2+}-refill linked contraction model, and the receptor activation associated inward Ca^{2+}-induced contraction model.

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Traditional L-VOCC blockers and papaverine are able to inhibit the KCl-induced contraction through the inhibition of depolarisation triggered inward calcium current (Cheng and Townley, 1983; Cerrina et al., 1983; Foster et al., 1984; Baersch and Frölich, 1995; Ohashi M and Takayanagi, 1983). Our results showed that both papaverine and drotaverine but not theophylline inhibited the KCl-induced contractions in a dose-dependent fashion. Others (Small et al., 1989) also demonstrated the lack of inhibition by theophylline on the amplitude of KCl-induced tracheal contractions. A combination of theophylline with nifedipine produced the same effect as nifedipine alone indicating that cAMP/PDE related mechanism wasn't related to L-VOCC function on KCl-induced contraction on guinea pig isolated tracheal preparations. Despite widespread use of the KCl model for testing L-VOCC blockers it is not truly physiological, as it is not the high extracellular concentration of KCl that is responsible for membrane depolarization and the subsequent smooth muscle contraction. Instead, airway smooth muscle contraction is considered to be Ca^{2+} dependant, either by the release of intracellularly sequestered Ca^{2+} or by an increase in influx of extracellular Ca^{2+}. In airway smooth muscle Ca^{2+} release from intracellular stores favors inward Ca^{2+} flux through L-VOCCs following receptor activation-induced airway smooth muscle contraction. This mechanism is indirectly supported by the relative ineffectiveness of L-VOCC blockers on agonist-induced tracheal contractions described by others (Drazen et al., 1983; Advenier et al., 1984; Ahmed et al., 1985; Baersch and Frölich, 1995). Repeated agonist stimulation in the presence of L-VOCC blockers results in gradual but only moderately decreasing maximal contraction force (Flores-Soto et al., 2013). This indicates that the airway smooth muscle is able to contract in a condition when the inward Ca^{2+} current via L-VOCCs is blocked. So the intracellular Ca^{2+} stores are the primary Ca^{2+} sources for

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contractions (Creese and Denborough, 1981) and the partly emptied sarcoplasmic Ca^{2+} stores are able to be replenished before a new contraction even if the L-VOCCs are blocked. In this case the receptor operated and other calcium channels are responsible for the inward Ca2+ current (McFadzean and Gibson, 2002). Contrary to the results obtained in normal KH solution, consecutive constrictor mediator stimulation in a Ca^{2+} free medium resulted in gradually decreasing contractions (Creese and Denborough, 1981; Noguera et al., 1994). If the Ca^{2+} depleted preparation is then transferred to a normal Ca^{2+} containing buffer, the intracellular Ca^{2+} stores are refilled (Noguera et al., 1995). It is highly probable that inward Ca^{2+} flux through the L-VOCC is the main mechanism for the post contraction Ca^{2+} refill (Bourreau et al., 1991; Bourreau et al., 1993; Dessy and Godfraind, 1996; Hirota and Janssen, 2007; Flores-Soto et al., 2013). This is called resting Ca^{2+} refill because the agonist is not present between two stimulations when the Ca^{2+} refill takes place. In our studies, repeated histamine or methacholine administration in a Ca^{2+} free medium elicited gradually decreasing contractions indicating the possible depletion of the internal Ca^{2+} stores. Changing the Ca^{2+} free medium to a normal 2.5 mM Ca^{2+} containing KH buffer (reload medium), the agonists were again able to evoke contraction in the post-reload Ca^{2+} free medium. The observed reaction was mediator independent since both the mast cell mediator histamine, and the muscarinic M_3 receptor agonist methacholine, produced the same result. Similarly to the L-VOCC blockers (e.g.nifedipine or diltiazem), isoquinolines introduced to the Ca^{2+} reload medium inhibited the histamine or methacholine-induced contraction in the post reload Ca^{2+} free solution suggesting that the observed mechanism is linked to L-VOCC function. In contrast, using the PDE inhibitor theophylline in the Ca^{2+} reload medium, did not block, and rather increased the magnitude of the constrictor

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mediator induced contraction. These observations suggest that the Ca^{2+} refill model is L-VOCC dependent, and not inhibited by blocking the cAMP/PDE system.

Receptor activation induces the depolarisation of airway smooth muscle resulting in inward Ca^{2+} flux via opening of both ROCCs and L-VOCCs (Cuthbert et al., 1994; Flores-Soto et al., 2013) with subsequent smooth muscle contraction. Airway smooth muscle can replenish the intracellular Ca^{2+} stores through both ROCCs and L-VOCCs. The Ca^{2+} depleted tracheal preparation in an agonist containing Ca^{2+} free medium responded with as strong a contraction by the addition of $CaCl_2$ to the organ bath, as the same dose of agonist in normal KH solution. There was also no difference in the development speed of the contraction (slope) indicating that with the added $CaCl_2$, Ca^{2+} penetrates rapidly into the Ca^{2+} depleted smooth muscle and evokes a contraction in the presence of either histamine or methacholine. The receptor activated refill process was significantly decreased by L-VOCC blockers or isoquinoline derivatives (e.g. drotaverine or papaverine) as shown by the reduced slope of the $CaCl_2$ contraction curve, but they only moderately affected the amplitude of the contraction.. This indicates that the speed of the Ca^{2+} entry is reduced after L-VOCC blockade but after a while sufficient Ca^{2+} becomes accessible to the contractile apparatus to allow maximal contraction. So, unlike the resting Ca^{2+} refill model, the L-VOCCs are not the only channel type where the Ca^{2+} entry into the smooth muscle takes place. It seems that L-VOCCs are responsible for rapid Ca^{2+} entry, and other channels allow a slower entry. The PDE inhibitor, theophylline, had no effect on either the contraction slope or on the magnitude of contraction, indicating that the mechanism is independent of the cAMP/PDE system. This hypothesis is further supported by the result that mixed equimolar concentrations of nifedipine and theophylline behaved more like L-VOCC blockers.

In conclusion, the experiments we have performed demonstrate a mechanistic rationale why the 2 classes of PDE inhibitor successfully treat separate clinical indications, but are not interchangeable.

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Authorship Contributions

Participated in research design: Endre G. Mikus

Conducted experiments: Zoltán Patai

Contributed new reagents or analytic tools: Endre G. Mikus

Performed data analysis: Endre G. Mikus, Zoltán Patai

Wrote or contributed to the writing of the manuscript: Endre G. Mikus, András Guttman

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Footnotes

None

Legends for Figures

Figure 1A. The experimental design for demonstrating the effect of test molecules on KCl depolarization-induced contractions. Consecutive 20, 30 and 50 mM KCl-induced contractions were evoked twice in normal Krebs-Henseleit solution (KH). The value of the second contraction at each KCl concentration was taken as 100%. This was followed by three cumulative KCl concentration-response curves, in the presence of increasing concentration of the test molecule (or vehicle) with a wash out between each. Please note that this is an illustration of the method, not a real trace. W-wash out.

Figure 1B. The experimental design for investigating the intracellular Ca^{2+} store depletion associated decrease in contraction, and subsequent calcium reload dependent contraction recovery. Agonist-induced contractions were evoked twice in normal Krebs-Henseleit solution (KH), followed by three consecutive agonist stimulations (separated by a wash out) in Ca^{2+} free KH medium. After the third stimulation and wash out, the medium is changed to normal KH solution containing the test molecule. After a 30 min incubation, the medium was again changed to Ca^{2+} free KH solution and three agonist-induced contractions were evoked ieach separated by a wash out. Please note that this is an illustration of method, not a real trace. C_1-control contraction, C_2-contraction after test molecule; W-wash out.

Figure 1C. Experimental protocol for investigating the effect of test molecules on $CaCl_2$-induced contraction. Agonist-induced contractions are evoked twice in normal Krebs-Henseleit solution (KH) , followed by three consecutive agonist stimulations, each separated by a wash out in Ca^{2+} free KH medium. After the final wash out, the medium was changed to Ca^{2+} free KH buffer containing the agonistand 2.75 mM

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$CaCl_2$ was added to the buffer in order to evoke the Ca^{2+} infux associated contraction. Please note that this is an illustration of method, not a real trace. C_1-contraction without test molecule C_2-contraction after test molecule, W-wash out.

Figure 2. The effect of test molecules on KCl induced tracheal contractions. (20 mM Figure 2A, 30 mM Figure 2B, 50 mM Figure 2C). The experiments were carried out in normal Krebs-Henseleit solution supplemented with the appropriate concentration of KCl. Values were expressed as mean ± SD (n =4-8). Student's t-test was used to compare the test molecule treated organs to the vehicle treated control ones. * P< 0.05 **P< 0.01 or ***P<0.001.

Figure 3. Histamine-induced guinea pig isolated tracheal contractions in normal and calcium free Krebs-Henseleit (KH) solution before and after 30 min incubation in normal KH solution (Ca^{2+} reload) with or without 10^{-5} M Drotaverine (A), Papaverine (B), Nifedipine (C), Diltiazem (D), Theophylline (E) or Theophylline+Nifedipine (F). Values represent mean ± SD (n=4-12 preparations). Student's t-test was used for the comparison of control and test molecule treated groups. *P<0.05, **P<0.01 ***P<0.001.

Figure 4. Methacholine-induced guinea pig isolated tracheal contractions in normal and calcium free Krebs-Henseleit (KH) solution before and after 30 min incubation in normal KH solution (Ca reload) with or without 10^{-5} M Drotaverine (A), Papaverine (B), Nifedipine (C), Diltiazem (D) or Theophylline (E) or Theophylline+Nifedipine (F). Values represent mean ± SD (n=4-12 preparations). Student's t-test was used for the comparison of control and test molecule treated groups. *P<0.05, **P<0.01 ***P<0.001.

Figure 5. The maximum $CaCl_2$-induced contraction force of intracellular calcium pre-depleted tracheal preparations in $3x10^{-6}$ M histamine (Figure 5A) or $5x10^{-7}$ M methacholine (Figure 5B) containing Ca^{2+} free KH solution. The percentage change of the contraction force is also indicated on the figures. Values represent the mean ± SD (n=4-8) Student's t-test was used to compare the $CaCl_2$ induced contraction in non-treated to the treated organs. *P<0.05, **P<0.01 ***P<0.001.

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Tables

TABLE 1

EC_{50} (μM) values of the test molecules

The EC_{50} values of the test compounds calculated at 20, 30 and 50 mM KCl induced tracheal contractions.

Compound	20 mM KCl	30 mM KCl	50 mM KCl
Drotaverine	9.2	>10	>10
Papaverine	0.6	5.8	>10
Nifedipine	0.6	0.6	0.7
Diltiazem	0.8	4.4	5.7
Theophyline	> 10	> 10	> 10
Theophyline+ Nifedipine	< 0.1	< 0.1	< 0.1

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TABLE 2A

Tracheal smooth muscle contraction in (mN)

Histamine ($3x10^{-6}$ M) or methacholine ($5x10^{-7}$ M)-induced guinea pig isolated tracheal contractions in normal (1st contraction and 2nd contraction) and calcium free Krebs-Henseleit (KH) solution before (3rd contraction, 4th contraction and 5th contraction) and after incubating for 30 min in normal KH solution (6th contraction and 7th contraction).

	Normal KH solution (2.5mM Ca^{2+})		Ca^{2+} free KH solution			Ca^{2+} free KH solution	
Agonist	1st	2nd	3rd	4th	5th	6th	7th
Histamine	10.9 ± 2.8	11.9 ± 3.2	8.6 ± 3.3	2.6 ± 2.5	0.4 ± 0.8	6.5 ± 3.9	0.3 ± 0.9
Methacholine	9.3 ± 2.5	10.4 ± 2.8	8.0 ± 2.7	1.5 ± 2.2	0.4 ± 0.1	4.4 ± 2.6	0.11 ± 0.3

[a]Values represent mean ± SD (n=32-43).

TABLE 2B

Tracheal smooth muscle contraction in percentage (mean±SD)

	Normal KH solution (2.5mM Ca^{2+})		Ca^{2+} free KH solution			Ca^{2+} free KH solution	
Agonist	1st	2nd	3rd	4th	5th	6th	7th
Histamine	92.5 ± 8.0	100	70.7 ± 14.8	22.9 ± 25.1	3.0 ± 6.3	52.7 ± 25.4	3.1 ± 10.1
Methacholine	89.2 ± 5.8	100	76.2 ± 12.4	13.3 ± 16.8	0.4 ± 1.1	42.0 ± 21.1	1.2 ± 2.9

[a]Values represent mean ± SD (n=32-43).

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TABLE 3

Contraction force (mN) and slope values of different protocols

Comparison of the contraction force (mN) and slope values of the contractions were obtained using four experimental protocols. Histamine (3x10-6 M; Protocol 1), and methacholine (5x10-7 M; Protocol 3)-induced tracheal contraction in normal Krebs-Henseleit (KH) solution. CaCl2-induced contraction of intracellular calcium pre-depleted tracheal preparations in histamine (Protocol 2) or methacholine (Protocol 4) containing Ca2+ free KH solution.

	Protocol-1	Protocol-2	Protocol-3	Protocol-4
Contraction force	9.6 ± 2.8	10.2 ± 2.8	11.3 ± 2.5	10.9 ± 2.3
Slope (x 10^{-3})	19.9 ± 7.9	12.7 ± 6.0	15.8 ± 5.29	14.5 ± 6.20

[a]Values represent the mean ± SD.

[b]n=36 (histamine), n=24 (methacholine).

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TABLE 4

The slopes of the $CaCl_2$-induced contractions

The $CaCl_2$-induced contraction slope values for intracellular calcium pre-depleted tracheal preparations in histamine ($3x10^{-6}$ M) or methacholine ($5x10^{-7}$ M) containing Ca^{2+} free KH solution.

	$CaCl_2$ added to histamine containing Ca^{2+} free medium (x 10^{-3})	p<	$CaCl_2$ added to methacholine containing Ca^{2+} free medium (x 10^{-3})	p<
control	9.7 ± 3.2		19.6 ± 5.9	
10^{-5}M Drotaverine	1.2 ± 1.1	0.001	3.5 ± 2.5	0.01
control	19.1 ± 13		11.8 ± 4.6	
10^{-5}M Papaverine	1.1 ± 0.5	0.05	0.8 ± 0.3	0.01
control	11.4 ± 4.2		10.1 ± 3.3	
10^{-5}M Nifedipine	1.1 ± 0.4	0.001	1.0 ± 0.5	0.01
control	14.5 ± 5.0		13.7 ± 2.4	
10^{-5}M Diltiazem	2.4 ± 1.1	0.001	0.7 ± 0.3	0.001
control	10.6 ± 3.6		12.6 ± 6.4	
10^{-5}M Theophylline	13.9 ± 4.1	n.s.	8.4 ± 1.4	n.s.
Control	13.9 ± 3.3		17.9 ± 9.4	
10^{-5}M Theophylline +10^{-5}M Nifedipine	0.9 ± 0.3	0.001	0.9 ± 0.2	0.05

[a]The slope values represent the mean ± SD (n=4-8).

[b]Student's t-test was used to compare the non treated to the treated slope values.

Figure 1

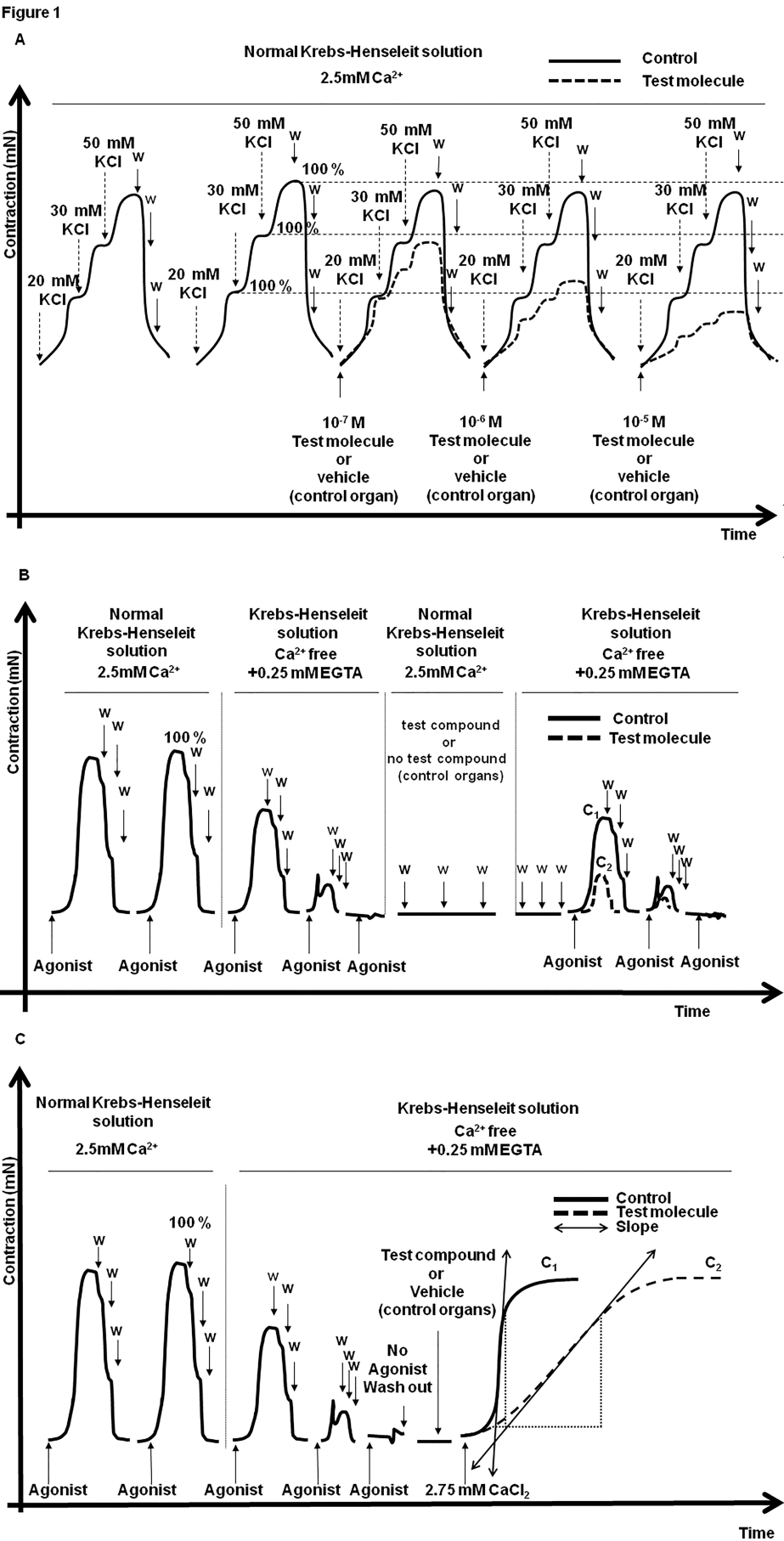
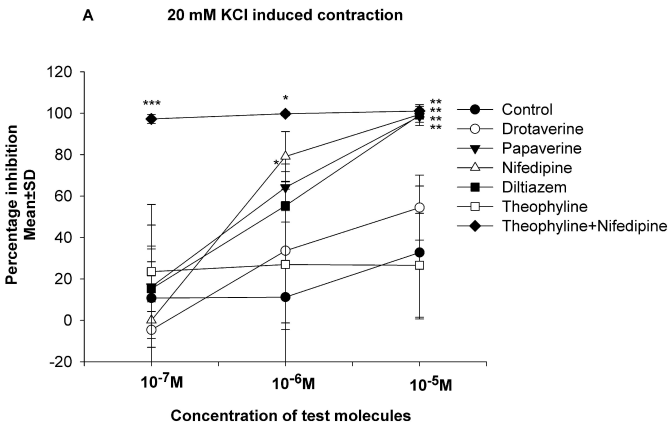
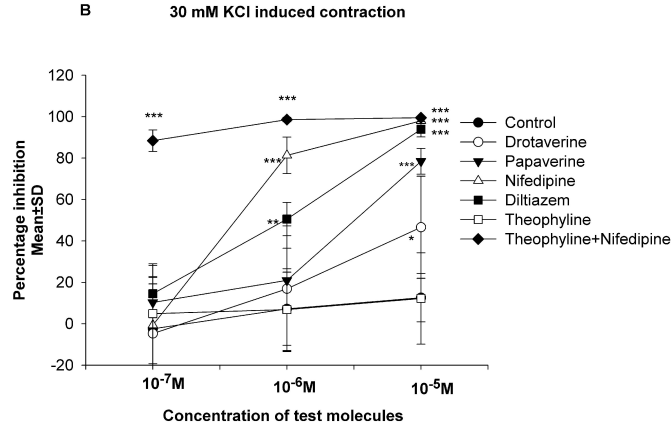

A
Normal Krebs-Henseleit solution
2.5mM Ca2+
Control
Test molecule
Contraction (mN)
50 mM KCl
30 mM KCl
20 mM KCl
W
100 %
10-7 M Test molecule or vehicle (control organ)
10-6 M Test molecule or vehicle (control organ)
10-5 M Test molecule or vehicle (control organ)
Time
B
Normal Krebs-Henseleit solution 2.5mM Ca2+
Krebs-Henseleit solution Ca2+ free +0.25 mM EGTA
Normal Krebs-Henseleit solution 2.5mM Ca2+
Krebs-Henseleit solution Ca2+ free +0.25 mM EGTA
Contraction (mN)
test compound or no test compound (control organs)
Control
Test molecule
C1
C2
Agonist
Time
C
Normal Krebs-Henseleit solution
2.5mM Ca2+
Krebs-Henseleit solution
Ca2+ free
+0.25 mM EGTA
Contraction (mN)
Control
Test molecule
Slope
Test compound or Vehicle (control organs)
No Agonist Wash out
2.75 mM CaCl2
Agonist
Time

Figure 2


A
20 mM KCl induced contraction
Percentage inhibition Mean±SD
120
100
80
60
40
20
0
-20
10-7M
10-6M
10-5M
Concentration of test molecules
Control
Drotaverine
Papaverine
Nifedipine
Diltiazem
Theophyline
Theophyline+Nifedipine


B
30 mM KCl induced contraction
Percentage inhibition Mean±SD
120
100
80
60
40
20
0
-20
10-7M
10-6M
10-5M
Concentration of test molecules
Control
Drotaverine
Papaverine
Nifedipine
Diltiazem
Theophyline
Theophyline+Nifedipine

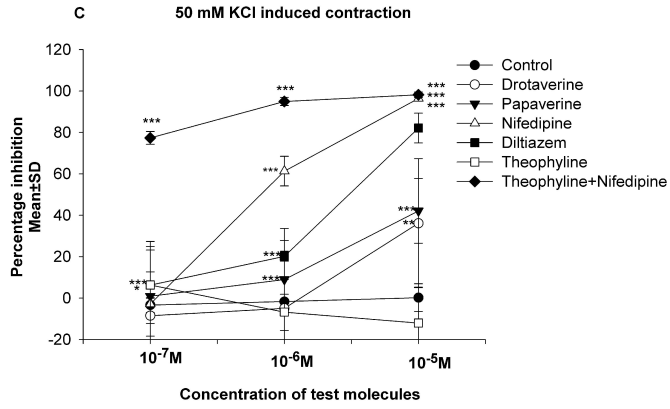

C
50 mM KCl induced contraction
Percentage inhibition Mean±SD
120
100
80
60
40
20
0
-20
10-7M
10-6M
10-5M
Concentration of test molecules
Control
Drotaverine
Papaverine
Nifedipine
Diltiazem
Theophyline
Theophyline+Nifedipine

Figure 3

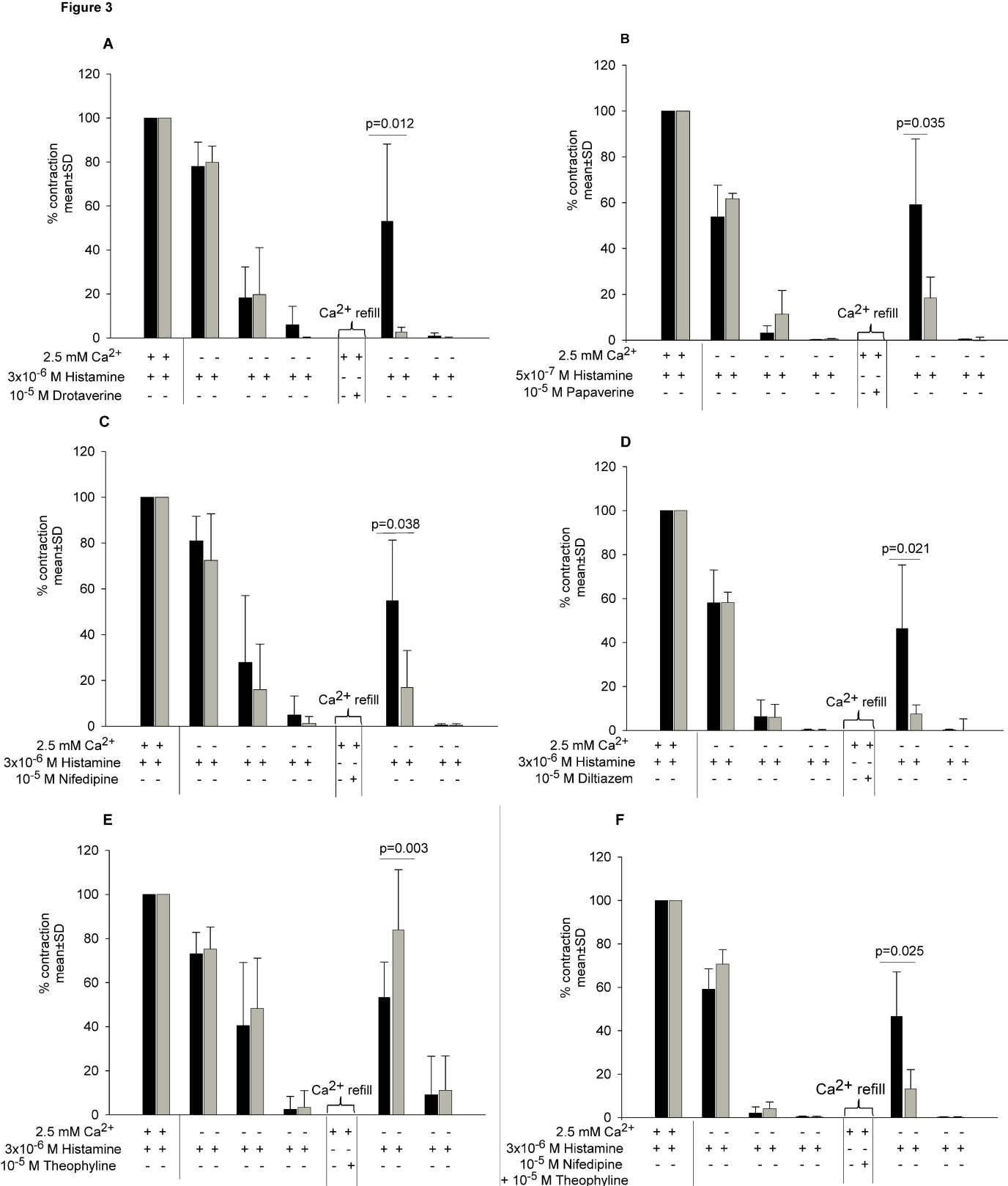

A
% contraction mean±SD
p=0.012
Ca2+ refill
2.5 mM Ca2+
3x10-6 M Histamine
10-5 M Drotaverine
B
% contraction mean±SD
p=0.035
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Histamine
10-5 M Papaverine
C
% contraction mean±SD
p=0.038
Ca2+ refill
2.5 mM Ca2+
3x10-6 M Histamine
10-5 M Nifedipine
D
% contraction mean±SD
p=0.021
Ca2+ refill
2.5 mM Ca2+
3x10-6 M Histamine
10-5 M Diltiazem
E
% contraction mean±SD
p=0.003
Ca2+ refill
2.5 mM Ca2+
3x10-6 M Histamine
10-5 M Theophyline
F
% contraction mean±SD
p=0.025
Ca2+ refill
2.5 mM Ca2+
3x10-6 M Histamine
10-5 M Nifedipine
+ 10-5 M Theophyline

Figure 4

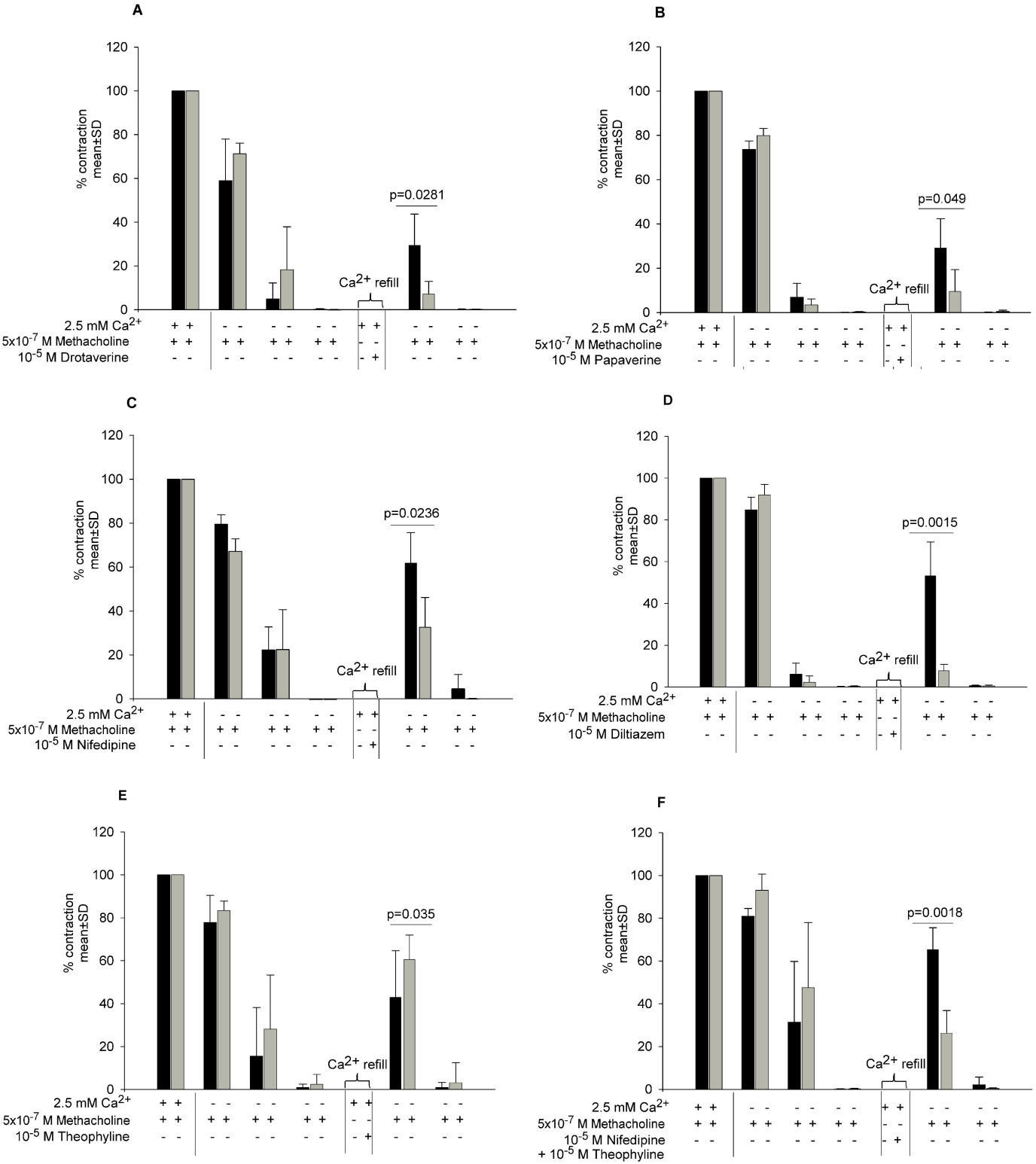
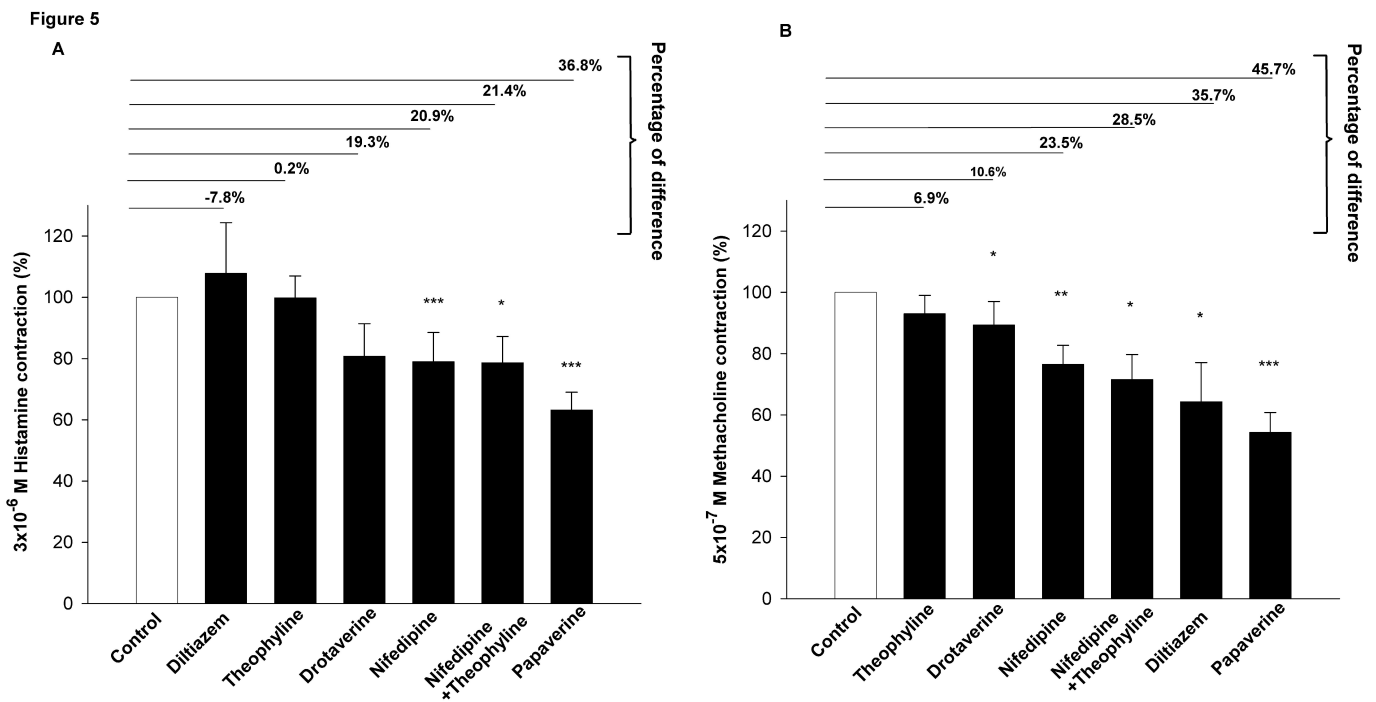

A
% contraction mean±SD
p=0.0281
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Drotaverine
B
% contraction mean±SD
p=0.049
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Papaverine
C
% contraction mean±SD
p=0.0236
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Nifedipine
D
% contraction mean±SD
p=0.0015
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Diltiazem
E
% contraction mean±SD
p=0.035
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Theophyline
F
% contraction mean±SD
p=0.0018
Ca2+ refill
2.5 mM Ca2+
5x10-7 M Methacholine
10-5 M Nifedipine
+ 10-5 M Theophyline

Figure 5


A
36.8%
21.4%
20.9%
19.3%
0.2%
-7.8%
Percentage of difference
3x10-6 M Histamine contraction (%)
120
100
80
60
40
20
0
***
*
***
Control
Diltiazem
Theophyline
Drotaverine
Nifedipine
Nifedipine +Theophyline
Papaverine
B
45.7%
35.7%
28.5%
23.5%
10.6%
6.9%
Percentage of difference
5x10-7 M Methacholine contraction (%)
120
100
80
60
40
20
0
*
**
*
*
***
Control
Theophyline
Drotaverine
Nifedipine
Nifedipine +Theophyline
Diltiazem
Papaverine