SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Effect of the phosphodiesterase inhibitor drotaverine on the intracellular Ca^{2+} refill mechanism in smooth muscle preparation

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The Examination takes place at the Library of the Department of Physiology, Faculty of Medicine, University of Debrecen, 10:00 am, 17th December, 2018.

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1. Introduction

Drotaverine has been present on the Hungarian pharmaceutical market as an anti-spasmodic agent, since 1962. Its molecular structure is similar to papaverine. At that time drug candidate molecules were characterized by functional tests only, which did not explain the molecular mechanism. After the discovery of the inhibitory effect of papaverine on the cyclic 3', 5'-nucleotide phosphodiesterase (PDE) enzyme, it was soon revealed that drotaverine also inhibits phosphodiesterase enzymes and its relaxant effect of smooth muscle was also associated with this intracellular mechanism. Subsequent experiments have confirmed that drotaverine can act by inhibition on the PDE IV enzyme, which has a primary role in the pathomechanism of asthma and other chronic respiratory diseases.

However, it had to wait until 2002 to find out that drotaverine can also inhibit the binding of dihydropyridine Ca^{2+} channel blockers in a micromolecular concentration. Tömösközi showed that drotaverine connects to the binding site of the L-type voltage-dependent Ca^{2+} channel (L-VOCC) blocker (3) H] nitrendipine and [(3) H] diltiazem in the pregnant rat uterus, which leads to preventing the binding of L-VOCC blockers. Consequently, it is presumable that drotaverine may have an L-VOCC blocking effects. Although, it was no information in the literature that someone could have demonstrated that the binding of drotaverine to L-VOCC would be associated with a functional change of L-VOCC, for example blocking effect. Furthermore, it is known that two other isoquinoline derivatives, papaverine and etaverine, can also bind to L-VOCC besides its PDE inhibitory effect, which suggested that these structurally similar substances exert their effect through a common molecular mechanism.

It is known that the mechanisms, which increases cAMP concentration, and the saturation of intracellular Ca^{2+} stores significantly regulating the tone of smooth muscles. Thus, such a compound as drotaverine, which inhibits the PDE IV enzymes and may have regulates the Ca^{2+} level of the intracellular Ca^{2+} stores may have a higher respiratory smooth muscle suppressing effect than PDE inhibitors. It needs special circumstances to separate the L-LVOCC blocking and PDE inhibitory effect because the result of both mechanisms is the relaxation of smooth muscle.

2. Aims and motivation

The work aim was to develop appropriate experimental conditions, which can clearly demonstrate that in addition to the PDE IV blocking effect of drotaverine it also has a functional inhibitory role on L-VOCC. Also, the Ca^{2+} is essential for respiratory smooth muscle, so it seemed relevant to examine the calcium antagonist effect of drotaverine on a respiratory smooth muscle preparation. To achieve this goal, the following experimental design was used:

1) KCl depolarization induced smooth muscle contraction. It is known from the literature that high extracellular KCl concentration can depolarize the smooth muscle cells of the airways, which cause contraction of the extracellular space through Ca^{2+} influx. Therefore, it is assumed that if the drotaverine is capable to block the L-VOCC it could also inhibit KCl-induced contraction.
2) The phenomenon of refilling the Ca^{2+} stores of smooth muscle via L-VOCC
   because the calcium depleted preparation capable to refill its calcium stores from calcium-containing solutions without agonist administration. The degree of the contraction after Ca^{2+} refill allows to measure the inhibitory effect of drotaverine on L-VOCC.
3) The movement of Ca^{2+} refilling of smooth muscle stores via ROC and L-VOCC (receptor mediated Ca^{2+} refill). The calcium depleted preparation is capable to contract in agonist containing solution after Ca^{2+} administration. The development of contraction force will be the specific parameter, which show the blockage of L-VOCC.
4) The relaxation of smooth muscle after depolarization or receptor activation. Substances capable to block the L-VOCC show a different efficacy during receptor activation or depolarization induced contraction. Comparison of the efficacy of drotaverine in receptor activation and depolarization induced contracture, the inhibitory effect on L-VOCC becomes detectable.
5) Mechanistic differences between inhibition of smooth muscle preparations by receptor activation and depolarization-based contraction. The relaxation effectiveness on the calcium release from the intracellular stores or calcium influx from the extracellular space induced smooth muscle contraction will be able to show the L-VOCC blocking of drotaverine.

3. Materials and methods

3.1 Chemicals

The ingredients of the Krebs-Henseleit solution: KCl, $CaCl_2$, $2H_2O$, KH_2PO_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, histamine dihydrochloride, D-(+)-glucose were purchased from Sigma (St Luis, MO).

3.2 Isolation of the Trachea and Organ Bath Preparation.

Dunkin-Hartley guinea pigs (body weight, 300-350 g) were used for the experiments. The animals were euthanized by pentobarbital and its trachea was rapidly removed into Krebs-Henseleit solution. The 3-4 cartilage wide rings were opened longitudinally and made a knot at the end of the tracheal strips and connected to the amplifier. The strength of the isometric contractile responses was measured by the use of force displacement transducer and preamplifier. Eight tracheal strip preparations were used from the same guinea pigs on the same day. Four preparations were used for the control groups, and the other four preparations were used for the test compounds.

3.3. KCl-induced contraction protocol

Contractions of the tracheal preparations in normal KH solution were conducted by consecutive applications of 20, 30, and 50 mM KCl, separated by washout processes. The cumulative contractions were repeated twice on every tracheal preparation before treated with the tests molecules (drotaverine, papaverine, nifedipine, diltiazem, theophylline) or the vehicle of the test molecules (control). After a washout another KCl concentration-response curve was constructed for all eight preparations and administered 10^{-7} M from test compund to 4 preparations and vehicle to another 4 control organs. The experiment was repeated twice, using 10^{-6} M (or vehicle) and 10^{-5} M (or vehicle) test molecule, with a washout between increasing doses of test molecule. The percentage of inhibition was calculated for each dose of the test molecule at every KCl concentration.

3.4. Resting refill protocol

The $3x10^{-6}$ M histamine and $5x10^{-7}$ M methacholine were used to contract the tracheal preparations to testing the intracellular Ca^{2+} dependent contraction. After the equilibration period, two consecutive contractions were evoked with either histamine or methacholine separated by a washout. After washing out the agonist, 2.5 mM Ca^{2+} containing Krebs-Henseleit solution was changed to a Ca^{2+}-free medium. Next, three consecutive contractions were evoked by the constrictor agents, each separated by a washout to deplete the sarcoplasmic Ca^{2+} stores. The third agonist stimulation usually evoked a minimal, if any, contraction, indicating depletion of the intracellular calcium stores. After the last washout 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 15-minute incubation, the solution in the bath was changed to normal Krebs-Henseleit solution, which containing 10^{-5} M of test molecule or vehicle (control). The preparations were then incubated for 30 minutes without any agonist stimulation. Next, the normal Krebs-Henseleit solution was changed to the Ca^{2+}-free Krebs-Henseleit solution in every organ baths, after which three consecutive contractions were induced by constrictor agonists, each separated by a washout period until the force of the contraction went back to baseline.

3.5. Receptor-operated refill protocol ($CaCl_2$-induced contraction)

The experimental protocol was identical to the "resting refill" protocol, up to the intracellular Ca^{2+} depletion step in Ca^{2+}-free medium. As before, the third agonist stimulation only limited, if any, contraction denoting the depleted Ca^{2+} content of the intracellular calcium stores. At this point, 10^{-5} M test molecule was added to 4 of the organ baths and the other 4 preparations were treated with the vehicle as control preparations. After 15-minute incubation, 2.75 mM $CaCl_2$ was added to all 8 organ baths. In this experimental protocol, the slope of the contraction force was the feature of the L-VOCC blocking rather than the maximum values of the contractions.

3.6. Relaxation of pre-contracted tracheal preparations protocol

In this method the aim was to examine the relaxation effect of drotaverine on precontracted trachea preparations against referent compounds. These experiments can be considered as the analog of the in vivo broncholytic effect. After the incubations, tracheal

rings were pre-contracted with the use of contractile mediators ($3x10^{-6}$ M histamine, $5x10^{-7}$ M methacholine or $2x10^{-2}$ M KCl). After the tracheal tone was stabilized, cummulative increasing concentrations of the test compounds were added to the organ bath. Finally, the preparations were fully relaxed by the administration of 2.5 mM EGTA to achieve maximal response.

3.7. Inhibition of the development of contraction protocol

The protocol was applied to determine the inhibitory effect of drotaverine on the development of smooth muscle contraction. At first, the tracheal rings were contracted with the constrictor mediators ($3x10^{-6}$ M histamine, $5x10^{-7}$ M methacholine or $2x10^{-2}$ M KCl). When the tracheal contraction reached maximum, the mediator was washed out and the process was repeated. When the preparation returned to baseline after the second contraction, the lowest concentration of the test compound was added to the organ bath. After 15 min incubation the constrictor mediators ($3x10^{-6}$ M histamine, $5x10^{-7}$ M methacholine or $2x10^{-2}$ M KCl) were administered. Once the maximal contraction was reached, the constrictor was washed out, and the process was repeated several times with increasing concentrations of test compounds, in order to obtain a concentration-response relationship.

4. Results and Discussion

4.1. The effect of drotaverine on KCl induced contraction

At the first experiment, the effect of drotaverine was compared with well-known L-VOCC blockers and PDE inhibitors. The L-VOCC blocker nifedipine or diltiazem in 10^{-5} M dose, practically abolished the KCl-induced contractions, which was predicted from the literature data. The non-selective PDE inhibitor theophylline, which is responsible for cAMP inhibition leads to activating the K_{ca} across the protein kinase A (PKA). The PKA enhances Ca^{2+} uptake of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which process finaly decreased the level of intracellular Ca^{2+}. The results clearly showed that increasing concentration of theophylline did not modify the KCl-induced contractions, suggesting that the cAMP/PDE system did not play a significant role in L-VOCC Ca^{2+} influx. Drotaverine and papaverine decreased KCl-induced contractions in a concentration dependent fashion supporting the proposed functional L-VOCC blocking activity of both moleculses rather than PDE inhibition. It is also reinforcing that hypothesis that the mixture of the equimolar concentrations of nifedipine and theophylline behaved more like a L-VOCC blockers alone in this experimental model. The inhibitory efficacy of the nifedipine and theophylline combination was significantly higher than that of nifedipine alone.

4.2. The effect of drotaverine on refilling the intracellular Ca^{2+} stores

The force of the smooth muscle contraction depends on the level of intracellular Ca^{2+}. For this reason, histamine and methacholine was applied as a constrictor because both were able to release Ca^{2+} from the internal storages. The binding of the M3 agonist methacholine and H1 agonist histamine leads to contraction evoked by receptor activation Ca^{2+} release from SR. During this phenomenon, the role of the L-VOCC was only limited as determined by the extracellular Ca^{2+} influx. It was supported that binding to L-VOCC had a limited effect on agonist induced airway contraction. The results indicated that consecutive administration of histamine and methacholine, the contraction force decreased gradually in calcium-free Krebs-Henseleit medium. After agonist-provoked calcium depletion, the preparations were put into normal Krebs-Henseleit solution (reload solution) and incubated for 30 minutes to allow the depleted calcium storages to refill (resting refill). The organ bath solution was replaced with a calcium-free medium (post reload), then the preparations were able to contract again. These results also supported that the Ca^{2+} release mediated contraction was non-agonist dependent,

as both histamine, released from granulates of mast cells and M3 receptor agonist methacholine produced the same effect in my experiments. L-VOCC blockers like nifedipine or diltiazem added to the calcium reload medium were able to reduce the agonist-induced contractions in the post reload Ca^{2+} -free medium. The nonspecific phosphodiesterase inhibitor theophylline was also tested on the resting calcium refill model. However, 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. Both drotaverine and papaverine blocked the resting Ca^{2+} refill–associated contractions at 10^{-5} M concentration, making these two is quinoline derivatives more similar to the L-VOCC blockers than to the PDE inhibitors.

4.3. Examination of $CaCl_2$ induced contraction

The use of this model enabled to detect the kinetics of Ca^{2+} influx in the presence of agonists. Our hypothesis was that the administration of extracellular Ca^{2+} should be able to induce Ca^{2+} influx across the L-VOCC. The results showed that after calcium-depleted tracheal preparation incubated with histamine ($3x10^{-6}$M) or methacholine ($5x10^{-7}$M) in Ca^{2+}-free buffer, $CaCl_2$ was unable to induce a contraction as strong as produced by the same concentration of agonists in normal Krebs-Henseleit solution at the start of the experimental protocol. Neither the maximal contraction force nor the slope of the contraction of the control preparations differed markedly between the two experimental conditions as revealed that the histamine and methacholine were able to penetrate into the cell in across the ROC and L-VOCC in the presence of histamine. The role of L-VOCC was supported by the L-VOCC blockers nifedipine or diltiazem (10^{-5} M) decreased the slope of the $CaCl_2$-induced contraction force. In the same model, both drotaverine and papaverine decreased the slope of the $CaCl_2$-induced contraction, the two tested isoquinoline derivatives behaved like the L-VOCC blockers rather than the PDE inhibitor theophylline. It had no effect on the contraction slope by none of the agonist in the organ bath. The combination of theophylline and nifedipine produced the same result as nifedipine alone.

4.4. Relaxation of the pre-contracted tracheal preparations

The most common features of respiratory diseases are difficulties in breathing and drowning, caused by bronchoconstriction induced air flow reduction. As the Ca^{2+} influx through the ion channels plays a key role in the activation of the tracheal contractions, number

of *in vivo* and *in vitro* experiments have been carried out with L-VOCC blockers on this model. For this reason, it seemed logical to test the suggested L-VOCC-PDE inhibitor drotaverine on this model with the use of three mediators, KCl, histamine and methacholine. The results showed similarities with literature data because nifedipine was able to relax the KCl evoked contractions more effectively as histamine and methacholine. Drotaverine also relaxed precontracted tracheal preparations in a concentration and mediator dependent fashion. The non-selective PDE inhibitor, theophylline, showed equipotent relaxant effects on histamine, methacholine and KCl contracted preparations with no statistically significant difference either in respect of potency or efficacy.

4.5. Inhibition of the development of contraction

The prevention of airway contraction could be potential treatment of airway obstructive diseases besides other treatments, which relax the already established airway contraction. Based on the literature and our previous results, drotaverine seemed potent to examine the preventive effect of airway contraction. The L-VOCC blocker nifedipine was able to diminish all agonist induced contraction by a concentration dependent manner but showed a greater efficiency during depolarization induced high K^+ contraction. Drotaverine rather showed an L-VOCC blocker specific effect than PDE inhibition induced contraction. It was supported by the EC_{50} values of drotaverine on KCl induced contraction because the EC_{50} value was significantly lower as histamine and methacholine contraction. The non-selective PDE inhibitor theophylline was not able to prevent any of agonist induced contraction. Moreover, an unexpected tracheal contracting effect of theophylline was observed at higher than 10^{-4} M concentrations that further limited the testing of theophylline. I tried to find an explanation for this phenomenon in the literature. Theophylline belongs to methylxanthine type drugs, therefore a structurally related to the methylxanthine derivatives, caffeine, which was described as being capable of releasing Ca^{2+} from intracellular storages. Another possible explanation is that theophylline may cause Ca^{2+} influx, which likely hyperpolarizes the membrane through the activation of K^+ channels. This hypothesis was also supported by the fact that verapamil can also block the effect of caffeine, which releases Ca^{2+} from SR but does not affect to theophylline.

5. Summary

PDE inhibitors have particular importance in the pathomechanism of asthma and other chronic respiratory diseases, and can be used to increase the concentration of cAMP. The activation of protein kinase A by cAMP leads to relaxation of the respiratory smooth muscle across different signaling pathways. On the other hand, the intracellular Ca^{2+} concentration is directly responsible for regulating the airway smooth muscle tone. Depolarization causes Ca^{2+} influx through the voltage-dependent Ca^{2+} channels by Ca^{2+} signal and further Ca^{2+} induced Ca^{2+} release which eventually leads to muscular contraction. Among the voltage-dependent Ca^{2+} channels, L-VOCC plays an important role in this process.

During the experiments, the L-VOCC blocking effect of drotaverin was tested on guinea pig trachea preparations. The aim was to create such an experimental conditions where the L-VOCC inhibitory effect of drotaverine can be measured selectively on airway smooth muscle. The difficulty of the implementation was that both the PDE inhibitor and the L-VOCC blocking effect point to the smooth muscle relaxation. To distinguish these effects the drotverin was compared to well-known PDE inhibitory and/or L-VOCC blocking substances on functional models.

It was demonstrated that selected references were able to induce smooth muscle relaxation and concentration-dependent suppression of contraction by blocking the PDE enzyme and / or blocking the Ca^{2+} influx through L-VOCC in accordance with the literature. Therefore, the selected functional models considered to be appropriate for testing the L-VOCC blocking effect of drotaverin.

The results highlighted that drotaverine was not only capable to reduce the maximum values of depolarization-induced smooth muscle relaxation, but also had an effect of the rechargement of Ca^{2+} depleted Ca^{2+} storages during in resting and receptor-activated circumstances. It was also demonstrated that its effect was not limited to reducing the maximum of the contraction, but had an impact on the kinetics of Ca^{2+} refill. There was another important observation made that L-VOCC played a key role in refilling of Ca^{2+} depleted airway smooth muscle with Ca^{2+}.

By all means, our results showed, that drotaverine that acts as PDE inhibitor and L-VOCC blocker in one compound, may have a relevant role in the treatment of obstructive airway diseases which cause the release of inflammatory mediators and increased smooth muscle contraction. Concerning our results, drotaverin probably enables to treat airway

diseases which caused by the release of inflammatory mediators and increased smooth muscle contraction due to its PDE IV and L-VOCC inhibitory properties.

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List of publications related to the dissertation

1. **Patai, Z.**, Guttman, A., Mikus, E. G.: Assessment of the Airway Smooth Muscle Relaxant Effect of Drotaverine.
   Pharmacology. 101 (3-4), 163-169, 2018.
   DOI: http://dx.doi.org/10.1159/000485921
   IF: 1.442 (2016)
2. **Patai, Z.**, Guttman, A., Mikus, E. G.: Potential L-Type Voltage-Operated Calcium Channel Blocking Effect of Drotaverine on Functional Models.
   J. Pharmacol. Exp. Ther. 359 (3), 442-451, 2016.
   DOI: http://dx.doi.org/10.1124/jpet.116.237271
   IF: 3.867

List of other publications

3. Kovács, A. L., **Patai, Z.**, Guttman, A., Kádas, J., Takács, L., Kurucz, I.: Fractionation of the human plasma proteome for monoclonal antibody proteomics-based biomarker discovery 2: antigen identification by dot blot array screening.
   Electrophoresis. 34 (20-21), 3064-3071, 2013.
   DOI: http://dx.doi.org/10.1002/elps.201200677
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