

*Full Paper***Verapamil Induces Calcium Influx in the Trachea**Seham Mustafa<sup>1,\*</sup>, Abdulkareem Abdulraheem<sup>1</sup>, and Abdulhakeem Alsughayer<sup>1</sup><sup>1</sup>Department of Pharmaceutical Sciences, College of Health Sciences, The Public Authority for Applied Education & Training, Safat 13092, Kuwait

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**Abstract.** Verapamil, a Ca<sup>2+</sup> entry blocker, can induce bronchorelaxation and bronchoconstriction. The mechanism of verapamil-induced bronchoconstriction is poorly understood. The present study determines the direct effect of verapamil on smooth muscle of isolated ovine airways and analyzes the mechanisms involved. Isolated tracheal strips were suspended in organ baths containing Krebs solution for isometric tension recording. Tissue responses to verapamil as assessed by basal tone were examined in the presence or absence of epithelium. The effects of verapamil on carbachol and cooling-induced contraction were also recorded. Measurement of unidirectional fluxes was carried out using <sup>45</sup>Ca<sup>2+</sup> in the absence or presence of verapamil. Verapamil induced contractions of basal tracheal smooth muscle that were proportional to its concentrations. Removal of epithelium did not affect the verapamil contractile effect. Verapamil-induced contractions were abolished in Ca<sup>2+</sup>-free Krebs solution containing 2 mM EGTA. Verapamil increased the <sup>45</sup>Ca<sup>2+</sup> influx into the tracheal smooth muscle. It caused relaxation of the muscle tone induced by carbachol or KCl, but it potentiated the effect of cooling-induced contraction. Verapamil induced Ca<sup>2+</sup> influx that may lead to bronchoconstriction. These results proved that verapamil may worsen bronchoconstriction; therefore verapamil should be used with caution in asthmatic individuals.

**Keywords:** airway smooth muscle, trachea, calcium antagonist, asthma, <sup>45</sup>Ca<sup>2+</sup>

**Introduction**

Calcium (Ca<sup>2+</sup>) plays an important role in airway hyperactivity and in the pathophysiology of asthma (1). It is essential for the synthesis of bronchoconstrictor mediators and release from mast cells and neuronal conductions. Ca<sup>2+</sup> can enter cells across the plasma membrane through voltage-operated calcium channels (VOC), ligand-gated calcium channels, Na<sup>2+</sup>/Ca<sup>2+</sup> exchange, and store-operated calcium channels. The voltage-operated calcium channels are highly selective for Ca<sup>2+</sup>. There are five distinct subtypes of these channels: L, T, N, P, and R (2–5). L-channels (L-VOC) are particularly important in regulating contraction of smooth muscle. It has been reported that L-VOC is involved in the sarcoplasmic reticulum calcium refilling (6–8). The term “calcium antagonists” is used for drugs

that block cellular entry of Ca<sup>2+</sup> through calcium channels. L-type antagonists comprise three chemical distinct classes: phenylalkylamine (e.g., verapamil), dihydropyridines (e.g., nifedipine), and benzothiazepines (e.g., diltiazem) (4). The main effects of calcium antagonists, as used therapeutically, are on cardiac and vascular smooth muscle (9). Calcium antagonists led to speculation that it may be beneficial in the treatment of bronchial asthma. However, most clinical investigations suggest that calcium antagonists may produce variable effects on airway tone. It was reported that verapamil inhalation produces bronchoconstriction (10). Therefore these drugs no longer considered for asthma management. However, calcium antagonists are often used in the treatment of tachycardia and hypertension in asthmatic patients even if these agents are not used as bronchodilators (11–13). The aim of this study was to determine the direct effect of verapamil on smooth muscle of ovine trachea and analyzes the mechanisms involved.

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## Materials and Methods

### Preparation of ovine isolated tracheal strips

The trachea of Merino sheep were obtained from the slaughter house, placed in chilled Krebs solution (118 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 11.1 mM glucose, pH 7.4), and transported to the laboratory within 30 min. A piece of the trachea was cleaned of adhering adipose and connective tissue and opened longitudinally through the cartilage rings diametrically opposite the trachealis muscle. Thereafter it was pinned flat on a cork board and strips of smooth muscle, 10 mm in length and 5 mm in width, were dissected free from the underlying cartilage. In some experiments, the epithelium and sub-mucosa were carefully removed from the strips leaving smooth muscle only. Preparations were suspended in 20-ml organ baths containing Krebs solution, maintained at 37°C, and gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Tension was recorded using computerized, fully automated isometric transducers (Schuler organ bath type 809; Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a Gould recorder. Tracheal strips were suspended at a preset-tension of 2 g. The tracheal preparation was allowed to equilibrate for 60 min, during which time they were washed twice before adding agonists. At the end of each experiment, the muscle was weighed and the contractions were corrected for the weight of muscle. Responses were calculated as mg/mg tissue weight and in some cases, expressed as % of maximum response.

### Cooling protocol

Lowering the temperature of the Krebs solution in the chambers was achieved by changing the circulating water jacket fluid to a second circulator (Haake F3 Fisons, Germany), which had been set to 20°C. This allowed rapid temperature change in Krebs solution (2–3 min).

### Measurement of Ca<sup>2+</sup> influx

Measurement of unidirectional fluxes was carried out. The tracheal strips were incubated in Krebs' solution for up to 60 min at 37°C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Two strips were then exposed to Krebs' solution containing 1 μCi/ml <sup>45</sup>Ca<sup>2+</sup> for 5 min, one as the control and the second one with 10 μM verapamil. The amount of <sup>45</sup>Ca<sup>2+</sup> entering the tissue during such a short period can be assumed to be primarily due to Ca<sup>2+</sup> influx. At the end of a 5-min exposure, the tissues were bathed in 100 ml ice-cold Ca<sup>2+</sup> free-Krebs' solution containing 2 mM EGTA for 2 min and then immersed in 500 ml of this solution for 3 min to remove extracellular <sup>45</sup>Ca<sup>2+</sup>. At

the end of the experiment the tissues were blotted dry, weighed, and solubilized in 1 ml of 1 M benzethonium hydroxide in methanol by incubation at 50°C for 10–12 h. A 10-ml aliquot of ready gel liquid scintillation cocktail was added. The radioactivity was measured as dpm (disintegrations per min) in a Wallac Liquid Scintillation Counter (Model 1411) (USA). The <sup>45</sup>Ca<sup>2+</sup> remaining in the muscles was then calculated (14).

### Drugs

Carbachol hydrochloride, verapamil hydrochloride, and ethylene glycol bis ( $\beta$ -aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA) were all obtained from Sigma Chemicals, (St. Louis, MO, USA). <sup>45</sup>Ca<sup>2+</sup> was obtained from Amersham (UK), and ready gel liquid scintillation cocktail was obtained from Beckman (USA). Carbachol and verapamil were dissolved in distilled water. EGTA was dissolved in 0.1 N sodium hydroxide.

### Statistical analyses

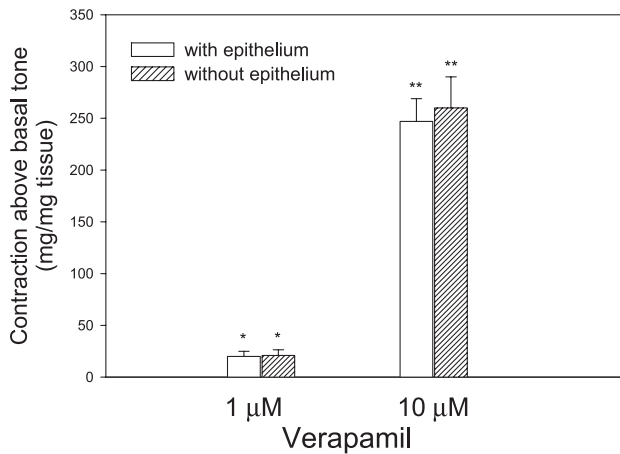
Data are each presented as the mean  $\pm$  S.E.M of the number of sheep used in the experiments (n). Where necessary, differences between two mean values were compared by using Students *t*-test, paired or unpaired as appropriate. Where multiple comparisons were necessary, one way analysis of variance (ANOVA) was used followed by the Student-Newman-Keuls test. The difference was assumed to be significant at  $P < 0.05$ .

## Results

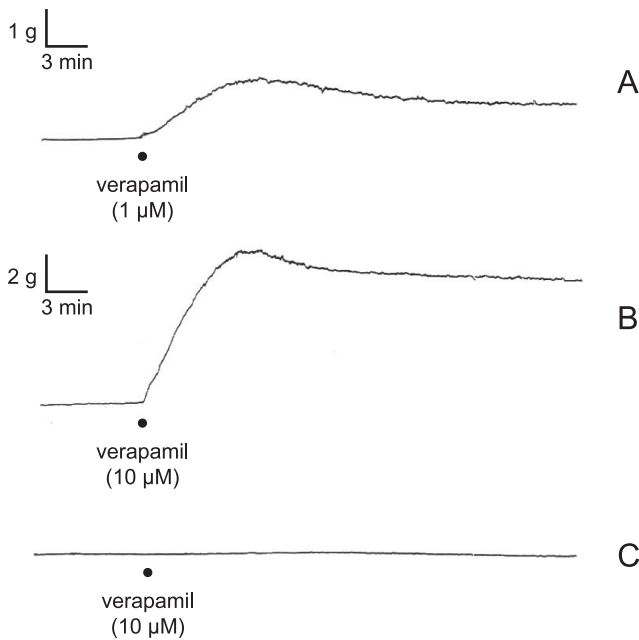
All the tracheal strip preparations maintained a consistent steady baseline. Verapamil, a calcium channel-blocking agent at 1 or 10 μM raised the tone of the tracheal smooth muscle preparations by  $22.19 \pm 12.0$  and  $85.46 \pm 43.0$  (mg/mg tissue wt), respectively, ( $P < 0.05$ ,  $n = 5$ ). Removal of epithelium did not affect the contractile responses to verapamil as shown in Fig. 1. Verapamil-induced contractions peaked after approximately 8 min. Incubation of the tracheal strips for 30 min in Ca<sup>2+</sup>-free Krebs solution containing 2 mM EGTA had no effect on the basal tension, but verapamil-induced contraction was abolished (Fig. 2).

### Calcium influx

To determine the role of Ca<sup>2+</sup>-influx in verapamil-induced contractions, the uptake of <sup>45</sup>Ca<sup>2+</sup> into the tracheal smooth muscle was measured in the absence (control) and presence of verapamil. <sup>45</sup>Ca<sup>2+</sup> influx in tracheal strips that were incubated with verapamil (10 μM) is more than that in the strips under the control condition. The uptake of <sup>45</sup>Ca<sup>2+</sup> increased significantly from  $37.07 \pm 1.39$  to  $46.25 \pm 2.4$  μmol/kg. Verapamil



**Fig. 1.** Effect of verapamil (1 and 10  $\mu\text{M}$ ) on basal tone of ovine tracheal smooth muscle with or without epithelium. Each point represents the mean  $\pm$  S.E.M. of 4 experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

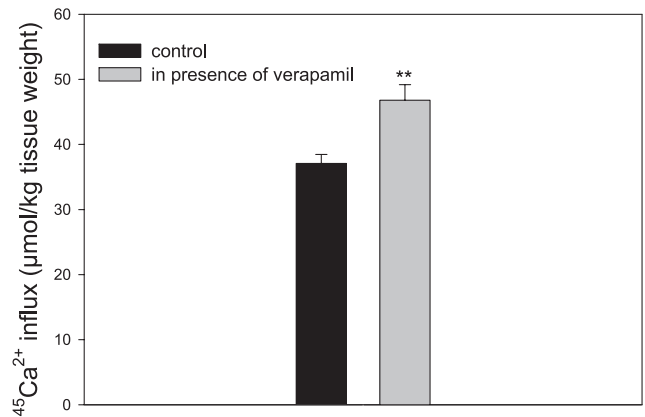


**Fig. 2.** Typical tracing of the effect of verapamil at 1  $\mu\text{M}$  (A) and 10  $\mu\text{M}$  (B) in normal Krebs solution and 10  $\mu\text{M}$  in presence of  $\text{Ca}^{2+}$ -free Krebs solution (C) on tracheal smooth muscle.

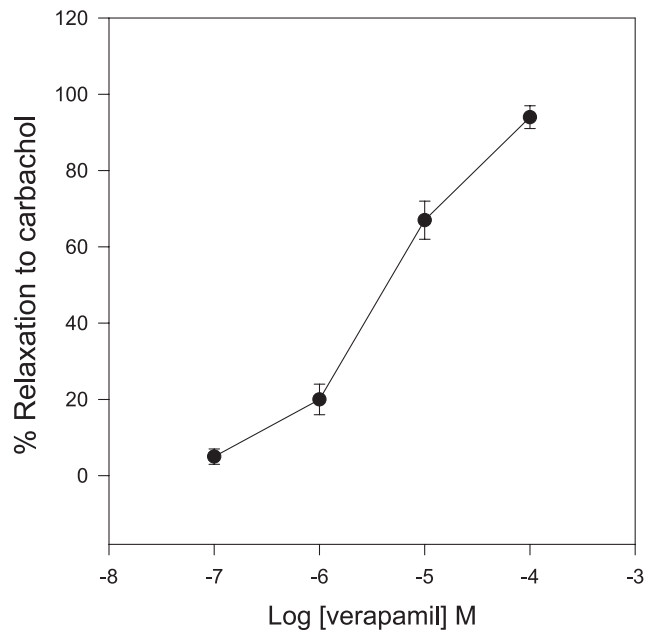
increased the influx of  $^{45}\text{Ca}^{2+}$  by 26% ( $P < 0.01$ ,  $n = 4$ ) as shown in Fig. 3.

#### Verapamil and bronchoconstrictors

In order to determine whether verapamil-induced contractions were a receptor-specific phenomenon or related to altered signal transductions mechanisms, or affecting intracellular calcium, the effect of verapamil on carbachol, KCl, and cooling-induced contractions was examined.



**Fig. 3.** Influx of  $^{45}\text{Ca}^{2+}$  in the control condition and in the presence of verapamil (10  $\mu\text{M}$ ) of ovine tracheal smooth muscle. Each point represents the mean  $\pm$  S.E.M. of 4 experiments. \*\* $P < 0.01$ .



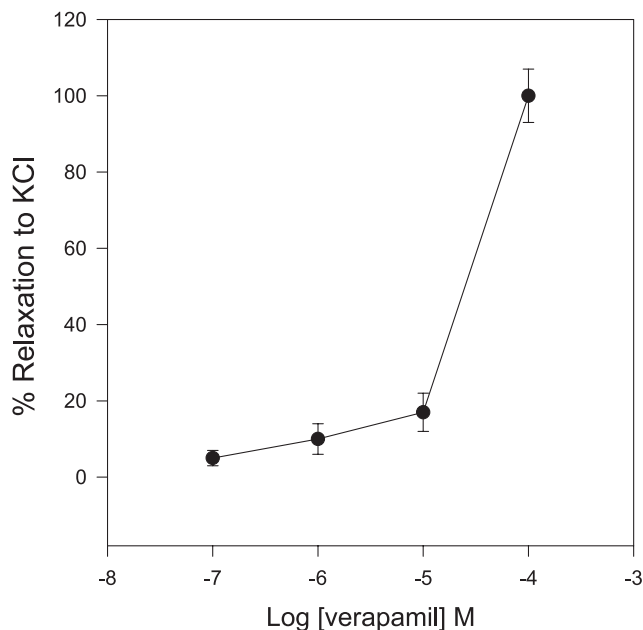
**Fig. 4.** Verapamil-relaxation curve to carbachol (10  $\mu\text{M}$ ) of ovine tracheal smooth muscle. Each point represents the mean  $\pm$  S.E.M. of 4 experiments.

#### Verapamil and carbachol

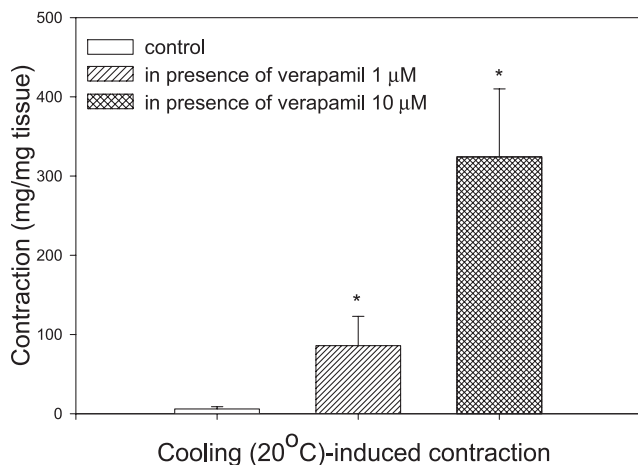
The effect of verapamil on carbachol-induced contraction was examined. Carbachol (10 nM) increased tracheal strip tension. Verapamil reversed carbachol-induced contraction in a concentration-dependent manner. The relaxation reached  $94 \pm 3\%$ , ( $P < 0.05$ ,  $n = 4$ ) (Fig. 4).

#### Verapamil and KCl

KCl (100 mM) increased tracheal strip tension.



**Fig. 5.** Verapamil-relaxation curve to KCl (100 mM) of ovine tracheal smooth muscle. Each point represents the mean  $\pm$  S.E.M. of 4 experiments.



**Fig. 6.** Effect of verapamil (1, 10  $\mu$ M) on cooling (20°C)-induced contractions in ovine tracheal smooth muscle. Each point represents the mean  $\pm$  S.E.M. of 4 experiments. \* $P < 0.05$ .

Verapamil reversed KCl-induced contraction in a concentration-dependent manner. The relaxation reached  $100 \pm 5\%$  ( $P < 0.05$ ,  $n = 4$ ) (Fig. 5).

#### Verapamil and cooling

Cooling (20°C) induced contraction in tracheal smooth muscle. Verapamil (1, 10  $\mu$ M) significantly potentiated the effect of cooling-induced contraction. The potentiation was proportional to the concentration of verapamil (Fig. 6).

## Discussion

The main effects of calcium blockers, as used therapeutically, are on cardiac and smooth muscle. Selectivity between heart and smooth muscle varies. Verapamil preferentially affects the heart compared to smooth muscle. Its clinical uses include antidysrhythmic therapy, angina (by reducing cardiac work), and hypertension (9).

Several reports suggested that verapamil may have bronchodilating effects. The relaxant effects of calcium entry blockers on bronchial smooth muscle have been demonstrated both clinically in exercise-induced bronchospasm (15) and experimentally against the broncho-constriction induced by histamine, acetylcholine, and  $\text{PGF}_{2\alpha}$  or by inhalation of antigen in sensitized animals, using dogs (16) and guinea pigs (17), and in humans (18). Inversely, other studies showed that VOC-blocking drugs, such as nifedipine, have proved disappointing as a therapy for asthma (19) and have a limited effect in alleviating bronchial constriction (20–22). It was proven that blockade of L-type  $\text{Ca}^{2+}$  channels at the extracellular (dihydropyridine and benzothiazepine group) rather than intracellular (phenylalkylamine group; e.g., verapamil) surface of the smooth muscle cell membrane may attenuate bronchoconstriction (23). It is also shown that extracellular-site  $\text{Ca}^{2+}$ -entry blockers inhibited histamine- and allergen-induced bronchoconstriction, whereas the intracellular-site  $\text{Ca}^{2+}$ -entry blockers did not (24). It was proven that verapamil significantly worsened the bronchoconstriction induced by methacholine in vivo (23). Inhaled verapamil has been reported to produce bronchoconstriction (10, 24, 25). It was also reported that acute asthma attack was associated with sustained-release verapamil in humans (26). However, these studies showed the responses but did not explain the mechanism of action of the bronchoconstriction effects.

In this study, our results showed that verapamil raised the basal tone of the tracheal smooth muscle leading to bronchoconstriction. This contraction was completely abolished in the absence of extracellular calcium. These results confirm that verapamil action is through influx of calcium. The results obtained with  $^{45}\text{Ca}^{2+}$  reinforce this theory since it increased the influx of  $^{45}\text{Ca}^{2+}$  to the tracheal smooth muscle strip. This would result in an increase of intracellular  $\text{Ca}^{2+}$  and consequently in an enhancement of the contractile response.

Our study also showed the effect of verapamil on carbachol-, KCl-, and cooling-induced contraction. The relaxant effect of verapamil on carbachol- and KCl-induced contraction of tracheal smooth muscle strip were clear. This indicates that verapamil (VOC) has a

potent direct action on the cell membrane after the addition of bronchoconstrictors acting on carbachol acting through ligand-gated calcium channels and KCl acting through VOC. The exposure of the airways to low temperature is a well-known trigger of bronchoconstriction and asthma (27). Mustafa et al. showed that cooling induced tracheal smooth muscle contractions. The contraction was inversely proportional to the cooling temperature. Cooling-induced contraction is due to an increase in intracellular calcium.  $\text{Ca}^{2+}$  antagonists did not inhibit cooling-induced contractions, but instead, it potentiated the cooling effects (28, 29). Verapamil can induce calcium influx, which explains all the unexpected results of verapamil and also can explain the discrepancies between all the results including ours. In addition, the possibility that verapamil has other unknown mechanisms (30) that lead to the influx of extracellular calcium under specific circumstances and act as an agonist might be true. The cooling effects may suggest that verapamil could induce  $\text{Ca}^{2+}$  influx through nonselective channels activated by the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum.

The pH of the physiological Krebs solution was lowered about 0.05 unit during cooling. This change in pH had no effect on the experimental results. This finding is similar to other reports, indicating that the temperature-induced change in pH cannot account for either the contraction or relaxation responses (31).

In conclusion, our results proved that verapamil induces calcium influx; therefore, verapamil should be used for cardiovascular conditions with caution in asthmatic subjects since it may worsen their bronchoconstriction states.

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