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Effect of Calcium Antagonists in Experimental Asthma

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Verapamil was found to be an effective inhibitor of isometric tension in *in vitro*, experimental anaphylaxis in guinea pig trachealis smooth muscle. The mean IC_{50} for protection studies was 2×10^{-4} M; the drug was also effective as a bronchoreversal agent. The inhibitory effect of verapamil upon the initial rate of isometric muscle tension suggests an action beyond simple calcium channel inhibition. No inhibition of tracheal mast cell histamine release was observed. Verapamil was slightly more potent than theophylline in this *in vitro* anaphylactic model.

Key words: anaphylaxis; asthma; calcium antagonists; smooth muscle.

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The cellular regulatory functions influenced by calcium (Ca^{2+}) are well described (11). Within the airways, the elements of smooth muscle and mast cells are subject to the effects of free intracellular Ca^{2+} and the binding to Ca^{2+} modulating proteins which couple respective cellular functions to membrane-initiated processes. We previously detailed the inhibitory action of lidocaine in *in vitro* anaphylaxis because the myostabilizing action and the inhibition of exocytic histamine secretion are events which local anesthetics stabilize in part by antagonism with calcium, and which may serve as a model for asthma (15). More recently, there has been greater attention directed to a group of drugs which act with greater specificity as calcium channel antagonists. Because such agents could affect anaphylaxis, this study evaluated the inhibition of airways smooth muscle isometric tension and mast cell histamine release by verapamil during the Schultz-Dale reactions following passive *in vitro* sensitization.

MATERIAL AND METHODS

Adult male Hartley guinea pigs (Elm Hill Breeding Laboratory, Chelmsford, MA) weighing 450 to 600 g were sacrificed by stunning and exsanguination. The trachea was removed, placed in a Krebs-Henseleit (KH) buffer at 37°C, gassed with 95% O_2 and 5% CO_2, and cut into rings (15). For tension measurements the cartilage was cut, and one end was fastened with No. 50 cotton thread to a fixed clamp; the other end was fastened with a thin piece of platinum wire to a Grass FTO3C force displacement transducer amplified by a Hewlett-Packard 8805B amplifier to record isometric tension changes, in milligrams on a precalibrated Hewlett-Packard 7754A thermal tip polygraph; full scale was 5 g. The muscle was oriented parallel to the direction of the force displacement. The strips were suspended at 37°C under 2 g of initial tension in a 20 ml chamber (Harvard) containing 10 ml KH solution aerated with 95% O_2 and 5% CO_2.

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Optimal length-tension relationships for these experiments were determined by exposing muscle to increasing tensions 0.25, 0.5, 1.0, 2.0 and 3.0 g against histamine, 1.0 μg/ml; verification of the 2 g resting tension was conducted periodically. Resting tension was less than 10% of the maximum active tension generated. All tracheas were analyzed for force development, and those exhibiting baseline instability (±10%), inconsistent rates or erratic responses were discarded. All preparations were initially equilibrated for 60 min with the bath fluid changed three times. Before any new experiment the bath was flushed three times, and the muscles re-equilibrated for 10 min. PO_2, PCO_2 and pH were monitored in an 813 blood gas analyzer (Instrumentation Laboratories, Inc.); PCO_2 and PO_2 ranged from 33 to 44 mmHg and 350 to 550 mmHg respectively, with a mean pH of 7.42 ± 0.02.

Passive, *in vitro* sensitization was accomplished under isometric tension by immersing the muscle in a 1:10 normal saline dilution of reconstituted rabbit antichicken egg-albumin antiserum (ICN Pharmaceuticals, Irvine, CA) in KH buffer for 30 min at 37°C. The sensitized trachealis was then eluted of excess antibody (three washes of 10.0 ml) with KH. Antigen challenge was performed with 5 × recrystallized egg albumin; final bath concentration of 100 μg/ml (ICN Pharmaceutical, Irvine, CA). Tension responses to challenge with antigen egg albumin (anaphylaxis) were recorded at the point of maximal and constant plateau. For protection studies, sensitized muscles were incubated with and without verapamil until baseline equilibration occurred (20 to 30 min) and before antigen challenge. For reversal experiments, verapamil was added at maximal anaphylactic force (usually 5 min). Inhibition of anaphylactic force was calculated as follows: a) *protection studies:* The mean maximal force during anaphylaxis was obtained from a group of non-drug-treated muscles. Inhibition for each drug dose was then expressed as a percentage of this mean maximal value (group t); b) *reversal studies:* Percentage relaxation was computed by comparing the developed force after each drug dose to the maximal anaphylactic force for that same muscle prior to drug exposure (paired t).

Histamine assay

Trachealis muscle was employed for histamine release studies. Excised trachealis rings 2 mm in width were pooled to yield approximately 30 mg wet weight and then equilibrated in 1.0 ml KH aerated with 95% O_2 and 5% CO_2 at room temperature for 45 min. Tissue aliquots were segregated as follows: 1) control: KH buffer alone; 2) control and verapamil: for 30 min at concentrations specified in the results; 3) anaphylactic challenge: passively sensitized with rabbit antichicken egg-albumin antiserum for 90 min at 37°C and then exposed to antigen 100 μg/ml; 4) anaphylaxis: preceded by verapamil incubation for 30 min. Effluents (0.8 ml) were removed for assay after 5 min of reaction time, the point of observed maximal anaphylactic tracheal force. The aliquot was added to 0.8 ml of 0.8N $HClO_4$ yielding a final concentration of 0.4N $HClO_4$. A 1.6 ml aliquot of this solution was transferred to an extraction vessel containing 2.5 ml *n*-butanol and 600 mg NaCl and then histamine assayed by the procedure of May et al. (12) as we have previously detailed (15). Fluorescence at 450 mμ was measured on an Aminco-Bowman dual monochromatic spectrophotofluorometer from activation at a wavelength of 360 mμ and was normalized to a quinine sulfate standard. The concentration of histamine was determined from a standard curve. Verapamil, at cited concentrations, had no effect on fluorescence or upon the histamine assay for the wavelengths cited.

Solutions and drugs

The Krebs-Henseleit buffer was prepared as follows: NaCl, 118.1 mM; KCl, 4.7 mM; $NaHCO_3$, 24.8 mM; $CaCl_2$, 2.52 mM; $MgSO_4.7H_2O$, 2.4 mM; KH_2PO_4, 1.10 mM; glucose, 10 mM; in distilled, deionized water. Histamine dihydrochloride, anhydrous theophylline, and acetylcholine (Sigma Pharmaceuticals) and verapamil HCl (gift of Knoll Pharmaceuticals) were prepared daily in distilled,

deionized water to produce the desired final bath concentrations; volume additions to the 10 ml bath volume were 0.25 ml or less.

Statistical analysis

Differences between paired or group observations were analyzed by Student's t test with data expressed as mean ± SE.

RESULTS

Effect of verapamil in normal trachealis

To test the action of verapamil an evaluation was conducted in normal trachealis. First, the dependency of trachealis isometric tension to histamine, acetylcholine and depolarizing potassium (80 mM) upon extracellular calcium was demonstrated (Fig. 1A). Verapamil 2.2×10^{-4} M and 5.5×10^{-4}M yielded a 53.0 ± 3.0% and 147.0 ± 13.0% inhibition respectively of a mean maximal contracture of 1580 ± 112 mg induced by 5.4×10^{-6} M histamine ($n = 6$). In addition, the initial rate or fast phasic component was analyzed. Fig. 1B reveals a dose-dependent depression by verapamil upon the initial rate of isometric force development following histamine. A relationship between inhibition of this initial rate to the maximum inhibition of isometric force by verapamil is indicated in Fig. 1C. Verapamil also inhibited acetylcholine contractures (5.5×10^{-6} M) by 69.0 ± 5.0% and 91.0 ± 3.0% at 2.2×10^{-4} M and 5.5×10^{-4} M respectively ($n = 6$). For depolarizing potassium (80 mM), phasic and tonic components were

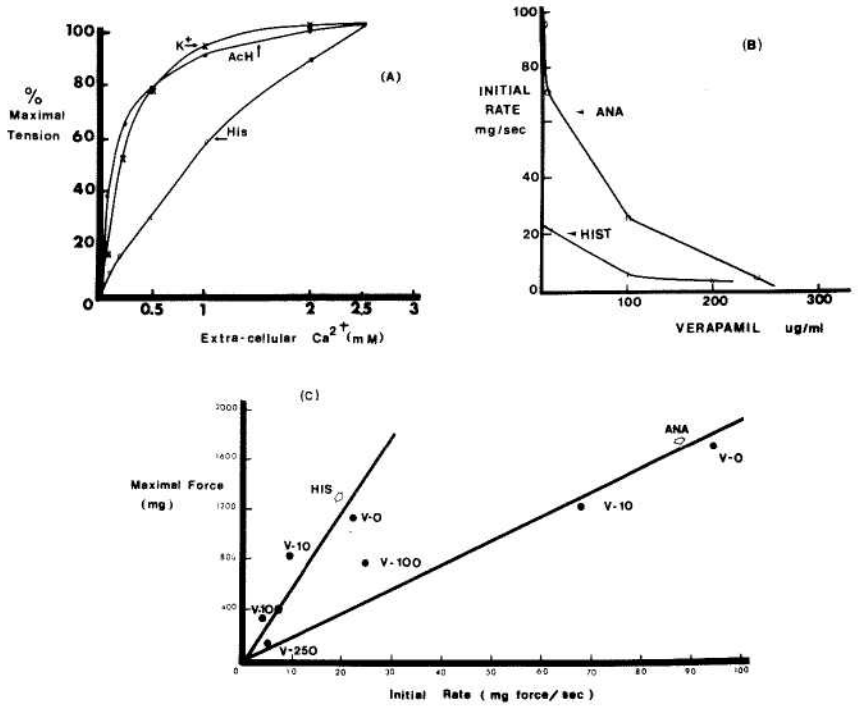

Fig. 1A. Extracellular calcium dependency of trachealis muscle tension with various agonists (80 mM K^+, histamine and acetylcholine at 5.4×10^{-6} M). Data expressed as a percentage of the maximal tension at 2.54 mM Ca^{++}.

B. Effect of verapamil upon the initial rate (mg force/sec) of isometric tension following histamine (5.4×10^{-6} M) or anaphylaxis. The initial rate was analyzed as the slope of the initial 2–3 min of developed tension. Control initial rate is shown at 0 μg/ml verapamil.

C. The effect of verapamil upon the relationship between initial rate of force (mg/sec) and the maximum force development (mg); histamine contraction (HIS) and anaphylaxis (ANA). Control (no verapamil) = V-0. V-10, 100, 250 indicates μg/ml verapamil concentrations for the ANA and HIS conditions studied (100 μg/ml = 2.2×10^{-4} M).

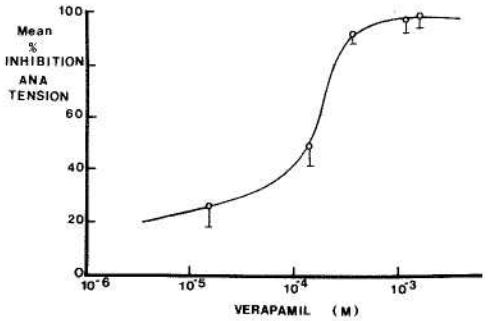

Fig. 2. Verapamil inhibition of anaphylactic isometric tension. Tension was measured at its peak development with inhibition expressed as a percentage of mean control anaphylactic contraction; $IC_{50} = 2 \times 10^{-4}$ M.

inhibited by 65% and 95% respectively with 0.2×10^{-5} M verapamil.

Immunogenic specificity, effect upon passive sensitization, and inhibition of anaphylactic (ANA) force

Neither egg albumin antigen or antibody alone caused force development in control muscles. To examine the effect of verapamil upon passive *in vitro* sensitization, antibody and verapamil (1.1×10^{-3} M) were added simultaneously for 30 min to the trachealis. Then, following multiple KH buffer elutions, specific egg albumin antigen challenge was conducted. Mean control ANA force was 900 ± 282 mg, while the mean ANA force in muscles passively sensitized in the presence of verapamil was 1050 ± 212 mg ($n = 4$, $P = 0.2$, NS).

The effect of verapamil during *in vitro* ANA was then evaluated. A dose-dependent suppression of ANA isometric force was observed with pretreatment of the sensitized muscles by verapamil (Fig. 2). The mean IC_{50} was found to be 2.2×10^{-4} M verapamil; maximal inhibition in this system was at 2.2×10^{-3} M. As with agonist histamine, verapamil also inhibited the initial rate of force development during ANA (Fig. 1B). Verapamil was also found to reverse ANA-induced force when the drug was applied at the point of maximal contraction. Verapamil 5.5×10^{-4} M ($n = 4$) yielded a 155.0 ± 36% reversal and verapamil 2.2×10^{-4}M ($n = 8$), a 90.0 ± 9.0% inhibition. Approximately 15 min elapsed for this maximal inhibition to occur.

Comparison of potency of verapamil vs. theophylline

Since the *in vitro* ANA model employed releases a variety of chemical mediators, the resulting intrinsic physiologic effects or potency upon isometric force could influence the effective inhibitory concentration of verapamil. To clarify the relative potency of verapamil under our ANA conditions, we compared the inhibition by verapamil with another standard antagonist, theophylline. Following a histamine exposure (5.4×10^{-6} M), the same control muscle was then pretreated with verapamil 2.2×10^{-4} M or theophylline 2.2×10^{-4} M and the mean percent inhibition obtained. The mean maximum histamine force was 1047 ± 112 mg; the mean percent inhibition by verapamil was 53.0 ± 5.0% ($n = 6$) and by theophylline was 29.3 ± 9.0% ($n = 8$) ($P < 0.05$, group t). The ratio of verapamil/theophylline (V/T) inhibition was 1.80.

We then compared these same inhibitors at 2.2×10^{-4} M in a similar pretreatment of ANA by adding the respective drug for 20 min following passive *in vitro* sensitization. Anaphylaxis was then induced with egg albumin antigen. The data were as follows: mean ANA force was 1658 ± 198 mg ($n = 10$); mean verapamil inhibition of anaphylaxis was 51.0 ± 10.0% ($n = 10$); mean theophylline inhibition was 26.0 ± 6.0% ($n = 6$, $P < 0.01$, group t); the V/T inhibition ratio was 1.96.

Histamine release

Effluent histamine concentration (μg/g dry weight trachealis) for control trachealis muscles was 10.7 ± 0.9 ($n = 30$). The addition of antiserum to the control muscle had no effect upon histamine concentration, 11.7 ± 3.9 μg/g ($n = 8$, $P > 0.4$). Following anaphylaxis ($n = 24$) effluent histamine was 15.4 ± 1.2 μg/g ($P < 0.01$ compared to control). Verapamil at 2.2×10^{-4} M and 1.1×10^{-3} M ($n = 10$) had no significant effect upon the control effluent histamine con-

Table 1

Net effluent histamine concentrations in anaphylaxis with verapamil

Condition	μg Histamine/ g dry weight (± SE)	n	P
A) anaphylaxis	5.4 ± 0.7	25	–
B) anaphylaxis + 2.2×10^{-4} M verapamil	5.6 ± 1.8	10	NS, $P > 0.9$ (A vs. B)
C) anaphylaxis + 1.1×10^{-3} M verapamil	6.0 ± 0.9	10	NS, $P > 0.5$ (C vs. A or B)

centrations with 9.1 ± 2.0 and 11.6 ± 0.8 observed, respectively. Net effluent histamine concentrations following anaphylaxis with verapamil are given in Table 1.

DISCUSSION

Transmembrane influx of extracellular calcium, one major factor in smooth muscle tension development, may be inhibited by specific calcium channel drugs. Verapamil is one such agent, but data on its effect in airways smooth muscle is limited and extrapolation of its activity from vascular or intestinal tissue is not necessarily valid. Furthermore, its mechanism of action as inhibition of the slow calcium channel is derived largely from studies in cardiac muscle where contraction is associated with cellular depolarization (13).

We observed that verapamil inhibited both phasic and tonic components of an 80 mM K^+ depolarization in trachealis, with a preferential effect upon the tonic component. A similar action was described in canine trachealis muscle by Coburn (4). He suggested that the major, but not the exclusive, mechanism of D600 (methoxy-verapamil) was to disrupt electromechanical coupling by inhibiting membrane potential dependent calcium influx during K^+ depolarization. A similar inhibition was described in serotonin-induced tension changes, but no inhibition to acetylcholine was observed (4). In contrast, in our study, verapamil was found to inhibit acetylcholine and histamine-induced increases in isometric force in normal guinea pig trachealis. Verapamil (2×10^{-4} M) yielded approximately a 50% inhibition of maximal force to either agonist at 5×10^{-6} M by treatment prior to agonist exposure. Our data is consistent with that reported by Farley and Mills (7) that sensitivity to verapamil inhibition of acetylcholine-induced tension in canine trachea is dependent upon the acetylcholine dose employed. These workers found that this dose dependency coincided with a shift in the dependence upon transmembrane calcium influx that could be related to the relative contribution of cellular depolarization to tension.

In our study, both slow and fast phases of histamine force change were affected. At lower verapamil concentrations (10^{-5} M and 10^{-6} M), the initial rate of force development (fast phase) was inhibited, with slight inhibition of force during the slow tonic phase. A significant myoinhibition to histamine or acetylcholine did not occur at these lower concentrations of verapamil (below 10^{-5} M) in contrast to ID_{50}'s observed in other tissues. For example, the ID_{50} for verapamil in rat uterus against angiotension II was reported at 5×10^{-7} M (6). However, inhibition to agonist norepinephrine in rat aorta ($ID_{50} > 10^{-4}$ M) or rabbit aorta and angiotension II in rabbit aorta ($ID_{50} > 10^{-5}$ M) indicates verapamil to be equipotent in guinea pig trachealis (1, 14). Differences in the above-cited inhibitions may vary with tissue sources, intrinsic potency of selected agonists, technical features, or, in fact, with whether these calcium channel inhibitors affect other aspects of calcium dynamics that are not confined to the slow inward current. Hence, effective inhibitory concentrations of verapamil might vary for a variety of causes.

During passive *in vitro* anaphylaxis (ANA) we found verapamil to be an effective inhibitor of isometric force. Both protection and drug reversal studies revealed a mean IC_{50} of 2×10^{-4} M. Analysis of the effect of pretreatment by verapamil under ANA conditions revealed a concentration-related inhibition of both initial phasic force rate and a reduction in the absolute maximal tonic isometric force. This inhibition is similar to that observed with agonist histamine or acetylcholine in normal trachealis (Figs. 1, 2). Presuming that early and late portions of ANA isometric tension are related to histamine and SRS-A activation, inhibition by verapamil suggests their calcium dependency (Fig. 1B, 1C). However, the action of verapamil in guinea pig trachealis is not necessarily limited to the slow inward current as with cardiac muscle. Also, Kass & Tsien (10) and Galper & Catterall (8) have suggested other cellular inhibitory mechanisms by verapamil in heart and liver. Our observation also indicates that ANA can be reversed by verapamil when applied at the point of maximal tension. Finally, concurrent incubation with verapamil and antiserum with subsequent elution revealed that verapamil did not inhibit the binding of antibody to mast cell as evidenced by the subsequently normal ANA response obtained.

By comparing the standard bronchorelaxant theophylline with verapamil, we found verapamil to be twice as potent in inhibiting histamine, and immunologically stimulated muscle contraction. This would suggest a potential use for a calcium entry blocker such as verapamil for bronchodilation in diseases such as asthma. Alternatively, Himori & Taira (9), comparing verapamil with isoprenaline in canine tracheal musculature and vasculature, found isoprenaline to be at least 60 times more potent than verapamil in tracheal relaxation measured via intraluminal pressure. In this system verapamil was a more potent vasodilator than an airways dilator, suggesting some limitation of the drug's usefulness in asthma therapy.

Finally, we could not document any significant inhibition of histamine release from trachealis under our ANA conditions; histamine levels were comparable to cited histamine release in ANA (5). In a measurement of plasma histamine in placebo or nifedipine-treated patients with exercise-induced asthma, venous plasma histamine levels were found to double with placebo treatment, but were at the baseline level in nifedipine-treated patients during exercise (2). Alternatively, employing an *in vitro,* sensitized human lung system, no differences in histamine release were found in nifedipine or verapamil (100 μM) treated lungs when compared to controls (3). However, *in vitro* and *in vivo* systems may differ in their sensitivity to calcium entry blocker inhibition of spasmogen release.

It would appear that the action of verapamil *in vitro* may be more than simple slow channel inhibition. As such it may share with agents such as lidocaine some non-specific effects on calcium permeability or intracellular binding in airways structures as we have previously reported (15). Overall, from the available literature, control of calcium dynamics may offer innovative therapeutic modalities in the management of bronchial asthma.

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