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Cooling-Induced Contraction of Guinea Pig Tracheal Smooth Muscle[1] (41944)

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Abstract. Cooling of isolated guinea pig tracheal smooth muscle from 38 to 28°C over 2.25 min produced a transient contraction followed by sustained relaxation. The cooling-induced contraction was blocked either by pretreatment with ouabain at concentrations of 10^{-5} *M* or greater or by substitution of normal physiological salt solution with K-free solution. In contrast, the contractile response to cooling was not inhibited by pretreatment with phentolamine (10^{-5} *M*), atropine (10^{-5} *M*), tetrodotoxin (3×10^{-7} *M*), diphenhydramine (10^{-5} *M*), cromolyn sodium (10^{-3} *M*), indomethacin (3×10^{-7} *M*), nifedipine (10^{-7} *M*), or verapamil (3×10^{-6} *M*). Addition of $NaHCO_3$ to the bath during cooling, preventing a change in pH of the physiological salt solution, did not affect the cooling-induced contraction. It is concluded that cooling of isolated guinea pig trachea produces a transient ouabain-sensitive contraction, and that the data suggest the contraction is mediated by inhibition of Na–K-ATPase in the smooth muscle rather than through neuronal stimulation or chemical mediator release.

Many asthmatic patients experience exacerbation of asthmatic symptoms following exposure to allergens or following exercise, especially in cold air. The effects of exposure to allergens on pulmonary mechanics are well known and are relatively easy to control. However, the mechanism of the exercise-induced bronchoconstriction is poorly understood. Among the proposed mechanisms are hypocapnia (1, 2), stimulation of airway receptors that trigger a vagal reflex (3, 4), lactic acidosis from exercising muscles (5), stimulation of pharyngeal receptors (6), release of stored chemical mediators (3, 7, 8), and imbalance of α- and β-adrenoceptor activity (9, 10). Recent studies have suggested that heat loss from the airway and low water content of inspired air (11–16) may be critical in the production of bronchoconstriction during exercise. However, the mechanisms by which cooling produces bronchoconstriction are not known. The present study was undertaken to elucidate responses of isolated guinea pig tracheal smooth muscle to cooling and to investigate effects of various blocking agents on the responses. A preliminary result of this study has been presented elsewhere (17).

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Materials and Methods. Albino guinea pigs of either sex (Hartley strain, 600–1000 g) were sacrificed by exsanguination from common carotid arteries. The trachea was rapidly removed and immersed in a preoxygenated normal physiological salt solution with the following composition in millimoles: NaCl, 115.3; KCl, 4.7; $NaHCO_3$, 23.0; $CaCl_2$, 1.8; $MgSO_4$, 1.2; Na_2(EDTA), 0.03; KH_2PO_4, 1.2; and glucose, 7.9. Surrounding connective tissue was removed from the trachea. Tracheal smooth muscle was isolated from the posterior trachea by longitudinal cuts including 1–2 mm of tracheal cartilage on each side. The smooth muscle was then cut into strips 1.5 mm wide along the direction of the tracheal rings by the use of specially arranged razor blades to ensure equal width of all strips. Using small stainless-steel clamps, one end of the tissue was fixed to a holder and the other end was connected to a force-displacement transducer (Grass FT 03C, Grass Instrument Co., Quincy, Mass.). The tissue was vertically suspended in a 10-ml isolated tissue bath (Metro Scientific, Farmingdale, N.Y.) containing the physiological salt solution. The solution was initially maintained at 38°C and aerated with a gas mixture of 95% O_2 and 5% CO_2. Changes in isometric force were recorded with a Beckman Type R Dynograph (Beckman Instruments, Schiller Park, Ill.). The strips were placed at an

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isometric resting force of 1.0 g. This resting force gave the greatest contraction in response to 40 m*M* KCl among strips tested at 0.25, 0.5, 1.0, 1.5, and 2.0 g. Before measurements were taken, the tissue was allowed to equilibrate for 100–120 min, and the physiological salt solution was replaced with a fresh solution every 15–20 min. The resting force was readjusted to approximately 1 g after each solution change and before each cooling experiment.

Following the equilibration period, the tracheal preparations were contracted with 40 m*M* KCl to allow expression of subsequent changes in isometric force as a percentage of the initial KCl contractions. Following washout of the KCl and an appropriate recovery period, tissue baths were cooled from 38°C to 28°C in 2.25 ± 0.18 min (N = 18) by a circulator (Lauda Thermostat K-21R, Lauda, West Germany). During cooling, the temperature of the nutrient solution was monitored with a thermometer which was placed in a bath without tissue within a series of tissue-containing baths.

The pH of the solution at 38 and 28°C was 7.36 ± 0.01 and 7.30 ± 0.01, respectively (N = 4, Radiometer PHM 82, Copenhagen, Denmark). To clarify whether the observed responses with cooling were due to these pH changes alone, the following three experiments were undertaken: (a) 10 μl 1 *N* HCl was added to the 10-ml bath at 38°C to determine the effect of lowering pH on isometric tension. The fall in pH was 0.23, 0.16, and 0.07 at 1, 2, and 5 min, respectively, after bolus addition of the HCl. (b) Sufficient $NaHCO_3$ (3.5 m*M*) was infused simultaneously with cooling to maintain a near constant pH (7.36), and (c) 3.5 m*M* $NaHCO_3$ was added to the bath maintained at 28°C to raise the pH of the solution to the precooling level.

Five periods of repeated cooling to 28°C from 38°C were carried out on the tracheal strips to see whether tracheal responses developed tachyphylaxis to repeated cooling. To find whether the rate of cooling was an important factor, tracheal strips from four guinea pigs were slowly cooled at an average rate of −0.36°C/min (over 27.50 ± 0.29 min), and the responses to the cooling compared with those to cooling at a faster rate of −4.44°C/min (over 2.25 min) in the same preparation. The magnitude of relaxation was expressed as the percentage of the relaxation produced by papaverine 10^{-4} *M* added to the bath (18).

After the response to the more rapid cooling (over 2.25 min) was observed, blocking agents were added to the bath and left in the bathing solution for 20–40 min. We tested individual strips with only one blocking agent. Specific agents were examined and their concentrations are listed in Tables I through III. A second cooling was then carried out in the same manner as the first. At the end of each experiment, the contractile response to 40 m*M* KCl at 38°C was reexamined to determine the effect of the blocking agent on this contraction. A separate tracheal strip from each animal was used as a time control for each experiment to account for any time-related changes. In all experiments involving blocking agents, cooling was begun only after adjusting the resting force of the tracheal strip to 1.0 g. Studies with nifedipine were carried out with the tissue bath shielded from light. Cooling-induced changes in isometric force were also examined in K-free solution. The K-free solution was prepared by replacing KH_2PO_4 with equimolar NaH_2PO_4, and by deleting KCl from the physiological salt solution. Tissues were left in contact with the K-free solution for 40–60 min before a second cooling was carried out.

Results shown in the text, tables, and figures are expressed as mean values ± SEM. The data obtained were analyzed for statistical significance by the two-tailed Student t test with statistical significance accepted at the 5% level.

Drugs used in this study with their sources are as follows: atropine sulfate (Sigma Chemical Co., St. Louis, Mo.), phentolamine mesylate (Ciba Pharmaceutical Co., Summit, N.J.), cromolyn sodium (Fisions Corp., Bedford, Mass.), diphenhydramine HCl (Sigma), indomethacin (Sigma), nifedipine (Pfizer Pharmaceuticals, N.Y.), papaverine HCl (Sigma), verapamil HCl (Knoll Pharmaceuticals, Whippaney, N.J.), tetrodotoxin (Sigma), ouabain octahydrate (Sigma), and propranolol HCl (Sigma).

Results. Cooling of the bath solution from 38°C to 28°C over 2.25 min resulted in a transient contraction followed by a sustained

relaxation of the guinea pig tracheal smooth muscle. The time course and magnitude of contraction and relaxation are shown in Fig. 1. When the bath temperature was subsequently kept at 28°C, the contraction was followed by a relaxation to a tension below that seen at 38°C. The maximum cooling-induced contraction was 8.6 ± 1.3% of 40 m*M* KCl-induced contraction (N = 7), and the maximum relaxation was 34.5 ± 6.2% of the relaxation produced by 10^{-4} *M* papaverine (N = 7). When the bath was rewarmed to 38°C, resting force returned to baseline in most tissues.

When the tissues were cooled more slowly (over 27.5 min), the response was still a transient contraction followed by relaxation. The slower cooling, however, produced a significantly ($P < 0.02$) smaller maximum contraction than did the faster cooling (Fig. 2). Repeated cooling at the faster rate did not show significant changes in the magnitude of the contraction. When the contraction with the first cooling was taken as 100%, responses to the second, third, fourth, and fifth cooling periods were 100 ± 3%, 117 ± 8%, 125 ± 13%, and 96 ± 11%, respectively (N = 3).

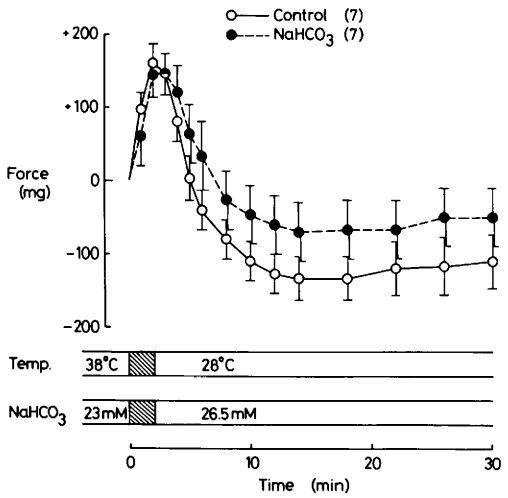

FIG. 1. Contractile and relaxing responses of guinea pig trachea to cooling from 38 to 28°C over 2.25 min (indicated by shaded area on time line). In test strips (closed circles), $NaHCO_3$ (3.5 m*M*) was continuously infused to the bath during 2 min immediately after the start of cooling. There is no significant difference between control and test groups ($P > 0.05$).

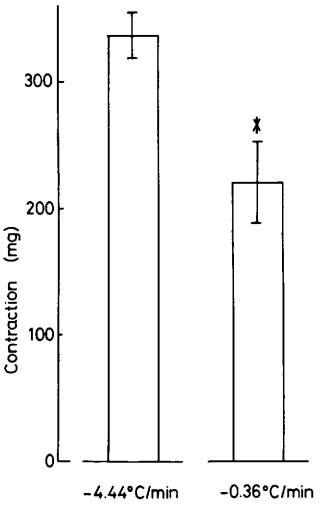

FIG. 2. Maximum contractile responses to different rates of cooling. Tracheas (N = 4) were cooled from 38 to 28°C at an average rate of −4.44°C/min or at −0.36°C/min. The faster cooling produced a significantly greater maximum contraction than the slow cooling ($P < 0.02$).

As shown in Table I, ouabain in concentrations of 10^{-5} *M* or greater blocked the cooling-induced contraction in a dose-dependent manner, while time controls did not show significant changes in contraction. Ouabain did not affect 40 m*M* KCl-induced contractions. Substitution of the physiological salt solution with K-free solution abolished the cooling-induced contraction; 1.5 ± 1.5% (N = 6) of the first cooling-induced contraction. The substitution with K-free solution or addition of ouabain (10^{-4} *M*) produced transient contractions of the tracheal smooth muscle; 300 ± 80 mg (N = 6) or 341 ± 97 mg (N = 9), respectively.

Pretreatment with phentolamine (10^{-5} *M*), atropine (10^{-5} *M*), diphenhydramine (10^{-5} *M*), indomethacin (3×10^{-7} *M*), cromolyn sodium (10^{-3} *M*), or tetrodotoxin (3×10^{-7} *M*) did not affect the contraction induced by cooling (Table II). Neither nifedipine (10^{-7} *M*) nor verapamil (3×10^{-6} *M*) blocked the cooling-induced contraction (Table II). However, the KCl-induced contraction was markedly reduced by nifedipine or verapamil to 10.6 ± 6.8% (N = 5) and 39.7 ± 8.8% (N = 5), respectively, of the initial KCl-induced contraction.

TABLE I. EFFECTS OF OUABAIN TREATMENT ON COOLING-INDUCED CONTRACTION[a]

	N	Pre-treatment[a]	Post-treatment[b]
Control	6	19.2 ± 1.9	20.6 ± 3.6
Ouabain (10^{-4} *M*)	5	23.8 ± 6.9	0.2 ± 0.2[c]
Ouabain (3×10^{-5} *M*)	5	18.1 ± 3.2	1.0 ± 0.7[d]
Ouabain (10^{-5} *M*)	6	18.0 ± 3.2	4.3 ± 1.0[c]
Ouabain (10^{-6} *M*)	4	20.9 ± 5.2	22.1 ± 2.6

Note. Values are expressed as percentages of the initial contraction by 40 m*M* KCl.

[a] Contraction before the addition of ouabain.

[b] Contraction after the addition of ouabain.

[c] Significantly different from pretreatment values ($P < 0.01$).

[d] Significantly different from the pretreatment values ($P < 0.001$).

Bolus addition of 10 μl of 1 *N* HCl to the bath at 38°C resulted in a pure relaxation; the maximum relaxation was 290 ± 10 mg ($N = 3$) at 3 min after the start of cooling. With the continuous infusion of 3.5 m*M* $NaHCO_3$ into the tissue bath during the cooling process, the response of the tissue to cooling was still an initial contraction followed by relaxation. The maximum contraction was 10.6 ± 1.7% of 40 m*M* KCl-induced contraction, which was not significantly different from that obtained with cooling alone ($N = 7$) (Fig. 1). The maximum cooling-induced relaxation was 33.4 ± 7.1% of 10^{-4} *M* papaverine-induced relaxation, again not significantly different from the relaxation produced by cooling without a bicarbonate infusion into the bath ($N = 7$) (Fig. 1). Bolus addition of 3.5 m*M* $NaHCO_3$ to the bath at 28°C, returning the pH to 7.36, produced neither contraction nor relaxation ($N = 7$).

TABLE II. EFFECTS OF VARIOUS BLOCKING AGENTS ON COOLING-INDUCED CONTRACTION

	N	Pre-treatment[a]	Post-treatment[b]
Control	6	12.7 ± 1.9	11.9 ± 2.1
Phentolamine (10^{-5} *M*)	5	11.7 ± 2.4	13.0 ± 2.8
Atropine (10^{-5} *M*)	5	11.6 ± 2.5	11.9 ± 2.6
Diphenhydramine (10^{-5} *M*)	5	15.3 ± 2.5	14.0 ± 2.1
Indomethacin (3×10^{-7} *M*)	6	11.4 ± 1.4	11.4 ± 1.9
Cromolyn Na (10^{-3} *M*)	5	9.1 ± 1.6	9.7 ± 2.3
Tetrodotoxin (3×10^{-7} *M*)	5	8.0 ± 0.5	9.1 ± 0.7
Nifedipine (10^{-7} *M*)	5	12.8 ± 2.5	11.6 ± 3.4
Verapamil (3×10^{-6} *M*)	5	12.7 ± 3.1	7.3 ± 1.4

Note. Values are expressed as percentages of the initial contraction by 40 m*M* KCl.

[a] Contraction before the addition of blocking agents.

[b] Contraction after pretreatment with blocking agents.

TABLE III. EFFECTS OF BLOCKING AGENTS ON COOLING-INDUCED RELAXATION

	N	Pre-treatment[a]	Post-treatment[b]
Control	6	12.7 ± 3.5	7.8 ± 3.8
Cromolyn Na (10^{-3} *M*)	5	10.3 ± 5.2	6.4 ± 4.8
Propranolol (10^{-6} *M*)	5	17.2 ± 2.4	12.6 ± 3.6
Tetrodotoxin (3×10^{-7} *M*)	5	16.3 ± 3.9	12.2 ± 3.5
Ouabain (10^{-4} *M*)	5	28.0 ± 4.1	5.7 ± 4.7[c]
Ouabain (10^{-5} *M*)	6	22.9 ± 4.7	27.0 ± 8.0

Note. Values are expressed as percentages of the initial contraction by 40 m*M* KCl.

[a] Relaxation before the addition of blocking agents.

[b] Relaxation after pretreatment with blocking agents.

[c] Significantly different from the pretreatment values ($P < 0.01$).

Cooling-induced relaxation was not inhibited by pretreatment with cromolyn sodium, propranolol, or tetrodotoxin (Table III). Ouabain at a concentration of 10^{-5} *M*, which significantly inhibited the cooling-induced contraction, did not inhibit the subsequent cooling-induced relaxation. Ouabain at a higher concentration (10^{-4} *M*) inhibited the cooling-induced relaxation (Table III). However, the same concentration of ouabain (10^{-4} *M*) markedly reduced the relaxation of the tracheal smooth muscle in response to 10^{-4} *M* papaverine as well; papaverine-induced relaxations in the absence and presence of ouabain were 540 ± 62 mg and 102 ± 12 mg ($N = 6$), respectively.

Discussion. Cooling of isolated guinea pig tracheal smooth muscle from 38 to 28°C over 2.25 min results in a biphasic response: a transient contraction followed by a prolonged relaxation. Ouabain, a potent inhibitor of Na–K-ATPase, in concentrations of 10^{-5} *M* or greater blocked the cooling-induced contraction in a dose-dependent manner. Moreover, a K-free solution, known to inhibit Na–K-ATPase (19), also abolished the contraction. These findings strongly suggest in-

volvement of membrane Na–K-ATPase in the cooling-induced contraction. In addition, the fact that addition of ouabain or use of K-free solution produced a transient contraction suggests that Na-pump inhibition results in a contraction in guinea pig trachea. It may be that when Na–K-ATPase is fully inhibited by ouabain or by other procedures, cooling produces no further inhibition of the enzyme, and therefore, no contraction. This hypothesis is supported by electrophysiologic evidence in which ouabain (10^{-5} *M*) at 37°C produced a significant depolarization of the resting membrane potential of tracheal smooth muscle, but at 21°C failed to further lower the membrane potential which was reduced by the lower temperature itself (20). The concentration of ouabain required to inhibit the cooling-induced contraction in this study (10^{-5} *M*) is higher than 10^{-6} *M* ouabain which blocks relaxant response to a small amount of K addition in rabbit arterial smooth muscle (21). Thus, the possibility that the inhibition of cooling-induced contraction by ouabain seen in this study may be unrelated to inhibition of Na–K-ATPase is not ruled out.

Suppression of the Na pump increases intracellular Na concentration. It is not entirely clear how such changes in Na concentration result in increased intracellular Ca which causes contraction; it may be either by a decrease in Ca efflux (22) or by increased Ca influx (23, 24). For the cooling-induced contraction of guinea pig stomach, taenia coli, and urinary bladder, translocation of intracellular Ca has been proposed (25, 26). In the present study, calcium entry blockers (nifedipine and verapamil) blocked the KCl-induced contraction but not the cooling-induced contraction (Table II). It seems likely that the cooling-induced contraction does not depend on a membrane potential-dependent influx of calcium (27). However, the mechanism for the cooling-induced contraction remains unresolved as cooling may alter the contractile machinery or biochemical process involved in the contraction of smooth muscle.

While cooling lowers the pH of the physiological salt solution, the change in pH does not explain our observation because: (a) reduction of pH of the bath at 38°C produces a pure relaxation of the tracheal smooth muscle; (b) maintenance of pH constant during cooling did not significantly alter either the contraction or relaxation responses to cooling; and (c) restoration of pH after the relaxation phase at 28°C by addition of $NaHCO_3$ did not cause a contraction. It is therefore highly unlikely that the temperature-induced change in pH can account for either the contraction or relaxation responses to cooling.

Release of chemical mediators or neural activation implicated in *in vivo* bronchoconstriction also does not appear to be involved in the cooling-induced contraction observed here. The failure of atropine, diphenhydramine, phentolamine, or tetrodotoxin to attenuate cooling-induced contraction mitigates against the involvement of acetylcholine, histamine, α-adrenergic receptors, or action potential generated release of neurotransmitters. The conclusion that mediator release is not involved is further supported by the lack of attenuation in the contractile response following repeated cooling experiments and by failure of cromolyn sodium to inhibit cooling-induced contraction. In the present study, indomethacin at a concentration of 3×10^{-7} *M*, sufficient to inhibit prostaglandin synthesis in other smooth muscle (28, 29), did not modify the cooling-induced contraction. However, indomethacin at a higher concentration of 3×10^{-6} *M* inhibited the cooling-induced contraction in a preliminary study ($92.2 \pm 7.8\%$ of inhibition, $N = 5$). This finding may suggest participation of prostaglandin biosynthesis in the cooling-induced responses. Such a suggestion has been made by Souhrada *et al.* (30) based on the potentiating effect of aspirin (50 μg/ml = 2.8×10^{-4} *M*) on the contractile response. However, the reason for opposite direction of effects of the prostaglandin synthesis inhibitors is unclear. The possibility that the inhibitory effect of indomethacin is not directly related to cyclooxygenase, e.g., Na–K-ATPase inhibition (31) and nonselective inhibitory action on smooth muscle (32), cannot be ruled out. Thus, further study is required to determine the involvement of prostaglandin synthesis in the cooling-induced responses.

Our findings of a contraction followed by relaxation are discordant with the observation

of Chaudhary *et al.* (33) that cooling induced only relaxation. The major difference between these two studies appears to be the rate of cooling. They cooled the tissue at a rate of −0.3°C/min while our rapid cooling was at a rate of −4.44°C/min which is similar to the rate reported in cooling-induced bronchoconstriction in humans (15). When we cooled tracheal smooth muscle strips at −0.36°/min, we still observed a contraction, but one which was significantly less than that seen with the more rapid cooling.

The tracheal smooth muscle exhibited a rather striking relaxation following the initial contraction with cooling of the bath. Although it was not the primary purpose of this study to elucidate the mechanism of this relaxation, a few comments can be made. The relaxation does not appear to be caused by a cooling-induced fall in pH of the solution as discussed earlier. The relaxation was not blocked by propranolol, eliminating a role for β-adrenergic receptors. In contrast to the cooling-induced contraction, the relaxation process appears to be unrelated to alterations in the Na-pump activity based on the following observations: (a) There was a differential effect of ouabain on the contraction and relaxation processes; ouabain (10^{-5} *M*) significantly blocked the contraction but not the relaxation (Tables I and III). (b) Although pretreatment with ouabain (10^{-4} *M*) resulted in a significant reduction in the cooling-induced relaxation process, it also blocked papaverine-induced relaxation, suggesting that ouabain at this concentration exerts a nonspecific influence on the relaxation process.

In conclusion, we have demonstrated that isolated guinea pig tracheal smooth muscle initially contracts and then relaxes in response to cooling. The lowering of pH of the solution associated with cooling does not appear to cause or modify the contraction–relaxation response of the tracheal smooth muscle. The contraction was not blocked by many of the drugs commonly used to treat exercise-induced asthma, but was blocked by procedures which inhibit Na-K-ATPase activity. We conclude that Na-K-ATPase inhibition in the smooth muscle is involved in the mechanism for the cooling-induced contraction.

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