Calcium Hypersensitivity in Airways Smooth Muscle

Isometric Tension Responses following Anaphylaxis[1]

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Abstract. The isometric tension of anaphylactic guinea pig trachealis muscle preparation was examined at subphysiologic extracellular calcium concentrations, *in vitro*. Paired observations of control to passively sensitized (egg albumin antiserum) and antigen-challenged muscles (anaphylaxis) were made by exposure to trace Ca^{++} followed by cumulative Ca^{++} replacement. Following anaphylaxis, a leftward shift of the Ca^{++} concentration-tension responses was found at 0.25–0.5 mM Ca^{++} ($p < 0.001$); EC_{50} was $1.5\times$ greater than control. A greater maximal tension was also noted at 2.52 mM Ca^{++}. Passively sensitized muscles did not exhibit this heightened response. Subthreshold tissue chemical mediators are tentatively excluded as causative. An increased sensitivity to extracellular Ca^{++} exists in resting smooth muscle following anaphylaxis.

Aside from the postulated β-adrenergic blockade mechanism and observations of smooth muscle hypertrophy, little or no evidence exists for primary alterations of airways smooth muscle in models of bronchoreactivity or asthma [1, 2]. Some studies focusing upon agonist activity in experimental anaphylaxis conclude a general lack of muscle hyperresponsiveness [3–5]. However, since passive or resting forces may be involved in the threshold for muscle contraction, the isometric tension of the normal, sensitized and anaphylactic guinea pig trachealis muscle preparation was examined at subphysiologic extracellular calcium concentrations.

Methods and Materials

Randomly bred, male, adult (Hartley) guinea pigs weighing 450–600 g were sacrificed by stunning and exsanguination. The trachea was removed, transferred to Krebs-Henseleit (KH) buffer at 37 °C, gassed with 95% O_2 and 5% CO_2, dissected free of extraneous tissue, and cut into six rings. For trachealis isometric tension measure-

ments, the cartilage was cut and one end fastened with No. 50 cotton thread to a fixed clamp, and the other via a thin piece of platinum wire (No. 25 gauge) fastened to a Grass FT03C Force Displacement Transducer amplified by a Hewlett-Packard 8805 Amplifier to record isometric tension changes, in milligrams, on a precalibrated Hewlett-Packard 7754A Thermal Tip Polygraph; full scale was 5 g. The rings were suspended and maintained throughout under 2 g tension in a 30-ml muscle chamber (Harvard) at 37 °C containing 10 ml KH buffer continuously aerated with 95% O_2 and 5% CO_2 [6]. All tracheal preparations were initially equilibrated for 60 min during which the bath fluid was exchanged three times. Oxygen and CO_2 tensions and pH of the bathing fluid were monitored during each experiment by assay in an Instrumentation Laboratories, Inc. 713 Blood Gas Analyzer. Partial pressure for CO_2 and O_2 ranged from 35 to 42 and 350 to 550 Torr, respectively, with an average pH of 7.40 ± 0.02.

Calcium responses in the normal, sensitized or anaphylactic state were examined in a paired fashion with each muscle serving as its own control. Following equilibration, a 0.18-ml aliquot of distilled, deionized water was added to 10 ml KH in the organ bath containing the control muscle and tension recorded; this volume equals the maximum volume added in calcium replacement studies. Thereafter, with three 10.0-ml KH washes (5 min/wash) and reequilibration, the muscle was exposed to trace calcium KH buffer (calcium free or zero calcium) by two organ bath exchanges of 10 ml over 45 min. Under these conditions a significant fall in tension occurs. The new zero calcium baseline was recorded to a stable position, generally over a 45-min period. This was followed by cumulative replacement of calcium (prepared in distilled, deionized water) in concentrations of 0.25, 0.375, 0.5, 1.0 and 2.52 mM by the addition of appropriate aliquots of a stock solution; volume of replaced calcium was 0.18 ml (Gilson micropipette). Tension development for each calcium concentration was measured to a constant plateau.

Thereafter, three approaches were employed.

Control Muscles. The normal control muscle was *reimmersed* into zero calcium-KH and calcium restored. The second control immersion was performed after an interval of 150 min to correspond with the total elapsed time of the postanaphylaxis study (see below) of 450 min.

Sensitized Muscles. In another group of muscles, following exposure to zero calcium, calcium replacement (to 2.52 mM) and then reequilibration in physiologic KH, passive *in vitro* sensitization was accomplished by exposure to a 1:10 physiologic saline dilution of reconstituted rabbit antichicken egg albumin antiserum (ICN Pharmaceuticals, Irvine, Calif.) in KH for 30 min at 37 °C. Excess antiserum was eluted with the KH and the muscle exposed to zero calcium.

Anaphylaxis. In another set of muscles following sensitization, specific antigen challenge was conducted with 5× recrystallized egg albumin, 100 μg/ml final bath concentration (ICN). Once the anaphylactic contraction terminated (60 min elapsed time), the bath was exchanged four times (10 ml/wash) with physiologic KH buffer and baseline tension allowed to stabilize. These bath exchanges result in elution of observable tension-inducing mediators as preanaphylaxis baselines tension are restored within ± 10% of the preanaphylactic level. Then the anaphylactic or sensitized muscle was immersed in zero calcium followed by calcium replacement identical to that cited for normal control muscle. Immunological specificity was shown by the failure of similarly sensitized muscles to react to normal saline or ragweed pollen antigen (500 μg/ml; Hollister). Neither antigen albumin nor antibody alone caused tension development in any muscle. The coefficient of variation for each calcium concentration, as measured by an autoanalyzer was ± 2% [7].

At the termination of all studies, the muscles were removed, dissected free of cartilage and dried at 50 °C to a constant weight on a five-place Mettler Balance. 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, $MgSO_4 \cdot 7 H_2O$ 2.4 mM, KH_2PO4 1.10 mM, glucose 10 mM, in distilled, deionized water. Calcium-free KH omitted the $CaCl_2$. Histamine dihydrochloride (Sigma) as the free base was prepared in distilled, deionized water and is expressed as final organ bath concentration.

Statistical Analysis. Calculations are expressed as a percentage of maximal force for each paired muscle of each experiment. In addition, milligram isometric tension per milligram muscle dry weight were analyzed. Computations of relaxation tensions include correction for baseline variation. All differences were analyzed by a two-tailed paired

sample t test, with all data expressed as mean ± SE. Nonsignificant differences by the Student's t test ($p > 0.05$) were subject to one-way analysis of variance (F test) [8, 9].

Results

Normal Muscles

Table I summarizes the effect of immersion in zero calcium for normal control muscles followed by incremental calcium restoration and then a reimmersion in zero calcium and repeat calcium replacement. Repeat immersion in zero calcium showed no significant difference in tension expressed as percent responses of maximum tension for each calcium concentration. Isometric baseline relaxation in zero calcium of

$6,022.2 \pm 507.7$ mg tension/mg dry weight muscle was found not to be statistically different from the maximal rise (calculated from the zero calcium baseline) of $5,658.9 \pm 456.1$ mg tension/mg dry weight at a Ca^{++} bath concentration of 2.52 mM ($p > 0.7$, paired t, F = 0.28). Similar findings were observed for the second immersion.

Sensitized Muscles

Passively sensitized muscles exhibited equivalent restored percentage of maximum tension following cumulative calcium replacement when paired comparison to control conditions was made (table I). Relaxation in zero calcium was not statistically different in control vs. sensitized states ($p > 0.8$, F = 0.58). Similarly restored ten-

Table I. Percentage of maximal tension response in normal, sensitized and anaphylactic states

	Extracellular calcium concentration: % of maximal response (mean ± SE)					
	0.25 mM	0.375 mM	0.50 mM	1.0 mM	2.52 mM	N°
Control muscles						
First zero calcium immersion	24.2 ± 2.6	45.1 ± 2.3	57.1 ± 2.3	87.8 ± 1.1	100	19
Second zero calcium immersion	20.2 ± 1.6	42.4 ± 2.2	58.9 ± 1.8	86.3 ± 1.5	100	19
p^x	> 0.1	> 0.2	> 0.4	> 0.3	–	
F	1.22	0.55	0.39	0.66	–	
Sensitized muscles						
Normal control	10.1 ± 1.7	24.1 ± 2.5	38.8 ± 3.0	73.8 ± 2.0	100	34
Sensitized state	8.6 ± 1.3	23.4 ± 2.4	37.7 ± 2.9	75.3 ± 1.9	100	34
p^x	> 0.2	> 0.7	> 0.5	> 0.3	–	
F	0.48	0.03	0.06	0.09	–	
Anaphylaxis						
Normal control	16.1 ± 2.0	32.1 ± 3.2	44.0 ± 2.5	80.2 ± 1.5	100	
Anaphylactic state	34.4 ± 2.8	50.5 ± 2.3	55.3 ± 1.7	82.6 ± 1.1	100	
p^x	< 0.001	< 0.001	< 0.001	> 0.2	–	
N°	32	32	48	64	64	

p^x = Significance value, paired t; F = F test; N° = number of paired experiments.

sions at 2.52 mM Ca^{++} were not different (p > 0.7, F = 0.05).

Anaphylaxis

In the third group of trachealis muscles subject to anaphylaxis, cumulative increases in bath calcium yielded a significant leftward displaced tension response, expressed as a percentage of maximum tension in the postanaphylactic period compared to control conditions (table I) by paired t test analysis up to 0.5 mM Ca^{++}. Approximately 450 min elapsed for the paired control and anaphylactic experiments; of this time, 120 min were required for full calcium replacement upon termination of anaphylaxis. From analysis of the log dose-response curve (fig. 1), the mean log EC$_{50}$ for the control state was 0.56 mM Ca^{++} vs. 0.38 mM Ca^{++} following anaphylaxis, or a ratio of 1.47. Maximal restored tension at 2.52 mM Ca^{++} was statistically different than control conditions (p < 0.05), being approximately 355 mg tension/mg dry weight greater for anaphylaxis, compared to control conditions. Relaxation was equivalent in both conditions (p > 0.5).

Evaluation of Subthreshold Tissue Mediators

Two additional studies were designed to clarify if subthreshold concentrations of chemical mediators were present in the smooth muscle preparation despite elution to baseline tensions following anaphylaxis.

Exogenous Administration of Histamine. In a normal muscle a histamine concentration of 0.025 μg/ml was determined to produce a minimal but reproducible active isometric tension of 50–100 mg at 2.52 mM Ca^{++}. Histamine was then fully eluted. Then following zero calcium immersion and
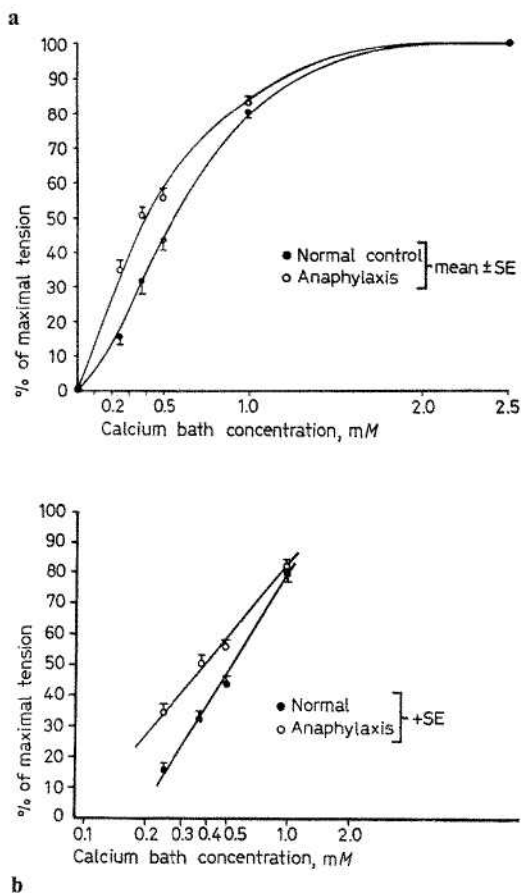

Fig. 1. Effect of extracellular calcium concentration upon isometric tension. **a** Mean (± SE) responses at cited calcium concentrations under control and anaphylactic conditions expressed as percentage of maximum at 2.52 mM Ca^{++}. **b** Semilog concentration response. The method of least squares determined the linear responses with observed mean (± SE) values superimposed; slopes were significantly different (p < 0.001) [9]: number of experimental pairs is given in table I. EC$_{50}$ is 0.56 mM for control and 0.38 mM for anaphylactic conditions. See text.

0.375 and 2.52 mM replaced calcium, the same control (nonanaphylactic) muscle was reimmersed in zero calcium and allowed to stabilize. Histamin, 0.025 μg/ml, was added, 2 min allowed for equilibration, and cal-

cium (0.375 and 2.52 mM) replaced with a 10-min response for each concentration. The mean results of 17 paired muscle strips at 0.375 mM Ca^{++} (percentage of maximal response at 2.52 mM Ca^{++}) were: control 37.2 ± 3.8% vs. trace histamine 36.6 ± 3.9% (p > 0.8).

Multiple Elutions. Following anaphylaxis, the organ bath was exchanged (10 ml) every 15 min for 2 h with physiologic KH buffer. Thereafter, calcium at 0.375 and 2.52 mM was replaced following zero calcium immersion as described previously. These multiple elutions did not abolish the enhanced response to Ca^{++} at 0.375 mM following anaphylaxis, expressed as mean percentage of maximal response at 2.52 mM Ca^{++}: control 50 ± 4.1% vs. anaphylactic 62 ± 1.7% (p < 0.05 paired t; n = 11).

Muscle Weights

Mean muscle dry weights for the three experimental groups were not found to be statistically different: control 0.28 ± 0.02 mg, sensitized 0.33 ± 0.02 mg, and anaphylaxis 0.295 ± 0.02 mg; p > 0.2 for all 3 groups.

Discussion

The present data demonstrate, as previously reported, an influence by extracellular calcium ion concentration upon the resting or passive properties of trachealis smooth muscle *in vitro* [10, 11]. The restored tension at cited Ca^{++} concentrations, expressed as a percentage of maximal response in a paired experimental sequence with each muscle serving as its own control was significantly increased following anaphylaxis only (table I). For the anaphylactic trachealis, the EC$_{50}$ derived from a log

concentration response was 1.5× greater than the normal control; anaphylaxis also exhibited a nonparallel leftward shift in calcium-tension relationships (fig. 1). In addition, the absolute maximal restored tension to 2.52 mM Ca^{++} was statistically different following anaphylaxis compared to control conditions. Although Ca^{++}-free solutions may affect the cell membrane [12], in our studies, sequential repeat immersion of the control muscle into a calcium-free buffer (table I) was not responsible *per se* for inducing a hyperresponse in tension. Similarly, passive sensitization alone had no effect.

To further evaluate other influences upon our findings, the dry weights of control, sensitized and anaphylactic muscles were compared and no differences (p > 0.2) found. By experimental design, the time interval for calcium replacement following initiation of anaphylaxis was matched to the paired reimmersion of normal control or sensitized muscles. Reported effects of osmolarity are not a consideration as the milliosmolar differences in zero calcium amount to only 1.3% of the total, a change too small to appreciably influence muscle tension [13]. Furthermore, the paired control and anaphylactic muscles were exposed to similar osmolarity conditions. Variability of the cumulatively replaced calcium concentrations is low with a coefficient of variation of calcium bath concentration measured to be ± 2% at each concentration level for the paired control and anaphylactic states. Finally, because of experimental conditions, influences upon calcium flux by changes in muscle length are not a factor [14].

Studies analyzing the effects of chemical agonists upon active tension development in animal models of experimental asthma *in vitro* and *in vivo*, conclude essentially little

or no role of airways smooth muscle in airways hyperreactivity [3, 4]. Conversely, *Popa et al.* [5] did observe a transient, exaggerated airways response *in vivo* to histamine, acetylcholine and propranolol following anaphylaxis in the guinea pig. However, the gradual restoration to normal responses was interpreted to be possibly due to the presence of smooth muscle chemical mediators in subthreshold concentrations. Hence, the possible role of subthreshold muscle concentrations of chemical mediators despite elution to baseline isometric tension as contributing to anaphylactic calcium hyperresponsiveness was evaluated in our system. One approach was to directly add exogenous histamine to normal muscles (at 2.52 mM) in a concentration determined to yield a minimal but observable active force. Then in another set of normal muscles, following zero calcium exposure, this concentration of histamine was added. However, calcium replacement at 0.375 mM was not found to be augmented. In addition, multiple elutions of the postanaphylactic tracheal muscle preparation over a period of 2 h did not abolish its tension hyperresponse to replaced calcium.

Exposure of cells to calcium-free media produces a variety of metabolic, electrochemical and physiologic changes which may or may not be related to calcium-associated cellular activities in response to stimulation [15–17]. In the present studies of anaphylaxis in this trachealis preparation, some effect, or effects, reflected at subphysiologic extracellular calcium concentrations on resting cell permeability, excitability and/or muscle tension can be alluded to. The precise mechanisms for this increased passive myosensitivity to calcium following anaphylaxis are, however, not clear. This anaphylactic disturbance to extracellular calcium may be of significance in a disease such as asthma as a factor leading to a hyperresponse in muscle contractility.

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