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Hyperreactivity and ^{45}Ca movements in sensitized guinea-pig tracheal muscle

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Hyperreactivity and ^{45}Ca movements in sensitized guinea-pig tracheal muscle. M. Perpiñá, J. Cortijo, E. Fornás, M. Palau, J.L. Ortiz, E. Morcillo.
ABSTRACT: Responses to KCl and histamine and ^{45}Ca movements were studied in trachea from normal and actively sensitized guinea-pigs. Sensitized tracheas were hyperresponsive and hypersensitive to KCl and histamine. ^{45}Ca uptake experiments show that sensitized tracheal muscle behaves as normal except that the uptake of ^{45}Ca in low concentration (0.03 mM) Ca^{2+} solution was higher and the number of binding sites for the high affinity component of ^{45}Ca uptake (as estimated by Scatchard-coordinate plot) was augmented. Additionally, in sensitized tracheal muscle, incubation in low (0.03 mM) Ca^{2+} solution followed by La^{3+} wash-out resulted in a greater amount of residual ^{45}Ca than in normal tissues. KCl, but not histamine, increased the La^{3+} resistant ^{45}Ca content. This increase was greater in sensitized than in normal trachea. This demonstrates the existence of hyperreactivity and altered ^{45}Ca movements in sensitized trachealis muscle.
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The airways of asthmatic patients are hyperresponsive to a wide array of bronchoconstrictor stimuli [1]. The mechanisms responsible for this nonspecific hyperresponsiveness are unclear although several hypotheses have been considered [1, 2]. One such hypothesis is the existence of a fundamental defect in the cellular processes which regulate airway smooth muscle contraction [3]. Calcium ions have a central role in excitation-contraction coupling in airways smooth muscle cells and are under a precise homeostatic control [4]. Therefore, an alteration in any of the steps of this complex regulation which finally increases the intracellular Ca^{2+} concentration may underly airway hyperresponsiveness. This constitutes essentially the calcium hypothesis in asthma [5, 6].

The immunological sensitization of laboratory animals, in particular of the guinea-pig, has become a widely adopted model of allergic asthma. The purpose of mimicking human asthma makes the existence of hyperreactivity in the experimental model desirable. *In vitro* studies with tracheal and lung parenchymal strips obtained from actively sensitized guinea-pigs demonstrate a nonspecific hyperresponsiveness to autonomic drugs, endogenous autacoids and other exogenous spasmogens [7–12]. This enhanced responsiveness has characteristics resembling those of post-junctional supersensitivity [9, 13]. This type of supersensitivity suggests the existence of an abnormality at a step beyond the level of receptor-agonist interaction [14]. This alteration may be related to differential Ca^{2+} movements in sensitized, as compared to normal, airway smooth muscle cells.

The aim of the present work was the search for differences between normal and sensitized guinea-pig tracheas in their ^{45}Ca movements, both basal (unstimulated) and after stimulation with two selected spasmogens, KCl and histamine.

Methods

Male guinea-pigs (325–375 g) were randomly allocated into two groups, nonsensitized and sensitized. The sensitization procedure was: on day 0 the animals were injected subcutaneously 0.25 ml of Freund's complete adjuvant plus 1.25 $\mu g \cdot g^{-1}$ body weight of bovine serum albumin (BSA) dissolved in 0.25 ml saline; on day 2 and day 4 the animals received the same amount of Freund's complete adjuvant and BSA by the intramuscular route. The animals were used for experiments on days 21 to 25. The nonsensitized group was subjected to the same protocol but received only saline. The animals were killed by stunning and exsanguination and their tracheas removed and dissected free of extraneous tissues.

Tracheal strips were mounted for isometric recording of tension as outlined previously [15]. Only one cumulative concentration-response curve to KCl or histamine was constructed in each tracheal strip after 60 min equilibration under a resting tension of 1 g. Tension and effective concentration 50% (EC_{50}) were determined and expressed as $g \cdot mm^{-2}$ and pD_2 values ($-\log_{10} EC_{50}$), respectively. The tension induced by a maximally

effective concentration of KCl or histamine was considered to be a measure of the responsiveness of the tissue while the pD_2 values indicated the sensitivity of the preparations.

Tissues were prepared for ^{45}Ca studies essentially as described by WEISS *et al.* [16]. Tracheal strips were dissected free of cartilage which interferes with ^{45}Ca measurements [17, 18]. For ^{45}Ca uptake experiments, tissues were equilibrated for 120 min in Tris buffered solution (composition in mM: NaCl 154.4; KCl 5.4; $CaCl_2$ 2.5; dextrose 11.0; and Tris-(hydroxymethyl) aminomethane 6.0; pH 7.3±0.1 maintained at 37°C and gassed with 100% O_2.

After equilibration, tissues were placed for 30 min in Tris solutions containing either 2.5 mM $CaCl_2$ (normal Ca^{2+} solution) or 0.03 mM $CaCl_2$ (low Ca^{2+} solution) and then were transferred to the same respective solution with added ^{45}Ca (0.76 μM Ca^{2+}; specific activity, 0.4 μCi·ml^{-1}) for a specified period of time. At the end, tissues were blotted on absorbent paper, dipped four times (2–4 s) in non-radioactive Tris solution, blotted again and weighed. The tissues were then placed into scintillation vials and the ^{45}Ca extracted overnight with 20 mM ethylene glycol tetra-acetic acid (EGTA). The extracts and 100 μl aliquots of the loading and La^{3+}-containing solutions were prepared for scintillation counting to determine ^{45}Ca content of the tissues and media, using Triton X-toluene scintillation fluid. The radioactivity was counted on an LKB 1219 Rackbeta. Tissue/medium ratios of ^{45}Ca (ml·g^{-1}) and total ^{45}Ca uptake (μmol·g^{-1} tissue wet weight) were calculated in a manner similar to that previously reported by WEISS *et al.* [16].

In some experiments, [^{14}C]sucrose was used to determine the extracellular space. Tissues were incubated in a Tris solution (2.5 mM $CaCl_2$) containing [^{14}C]-sucrose (1 μCi·ml^{-1}). The procedure to count radioactivity was similar to that used for ^{45}Ca as indicated by WEISS *et al.* [16]. Scatchard-coordinate plots were constructed from the values of total Ca^{2+} uptake minus that Ca^{2+} present in the extracellular space ([^{14}C]sucrose space) as outlined by WEISS *et al.* [16]. The x-axis intercept estimates the number of binding sites (n) present per unit of tissue and the ratio of x-axis intercept/y-axis intercept estimates the dissociation constant (k_D).

The wash-out of ^{45}Ca into a cold (0.5°C) isosmotic lanthanum-substituted solution (composition in mM: $LaCl_3$ 80.8 mM; dextrose 11.0 mM; and Tris 6.0 mM; pH adjusted to 6.8 with maleic acid) was also studied. After equilibration, tissues were exposed for 60 min to a Tris solution containing either 2.5 mM $CaCl_2$ (normal Ca^{2+} solution) or 0.03 $CaCl_2$ (low Ca^{2+} solution) with ^{45}Ca (0.76 μM Ca^{2+}; 0.4 μCi·ml^{-1}) added. Then, tissues were blotted, dipped in non-radiactive Tris solution and placed into successive vials containing 2 ml of cold isosmotic La^{3+}-substituted solution for the desired time intervals. The content of each vial was prepared for counting radioactivity. Tissues were also processed to measure radioactivity as in the uptake experiments. Data are expressed as desaturation curves *i.e.* percentage of ^{45}Ca remaining in the tissue after each wash-out interval [16].

The number of linear components of desaturation curves were also determined by linear regression analysis. The best line fitting the points for the slowest of the components was determined for each curve. The slope of this line is the rate constant (k) from which the half-time ($t_{0.5}$) was calculated ($t_{0.5}$=0.693/k). The points on this line were then subtracted from points for the next slowest component and a new line was obtained with these points. The half-time for this component was calculated by the same procedure. A third linear component was analysed in a similar manner.

The residual ^{45}Ca uptake was also measured according to WEISS *et al.* [16] but incorporating aspects of the technique used by FOSTER *et al.* [17] and RAEBURN and RODGER [18]. In each experimental run, one strip of trachea served as control (basal uptake) and the remainder as test (drug-stimulated uptake) strips. After equilibration, tissues were incubated in Tris solution (2.5 mM Ca^{2+}) with ^{45}Ca (0.76 μM Ca^{2+}; 0.4 μCi·ml^{-1}) added for 5 min. Then, either vehicle (control strips) or a certain concentration of the agonist (test strips) was added to the loading solution and left in contact for an additional period of time. This additional time of incubation was previously determined in tension experiments as the contact time necessary, for each concentration of KCl or histamine, to develop almost all the tension rise attainable by that concentration of the agonist [17]. At the end of the incubation period, the tissues underwent a 60 min wash-out into a cold isosmotic La^{3+}-substituted solution (composition as in ^{45}Ca efflux experiments). Tissues were prepared for counting as described previously.

Data are presented as mean±standard deviation (SD) and were analysed for statistical significance by the use of Student's unpaired t-test.

Materials

Histamine and BSA were obtained from Sigma (St. Louis, M.O.) and Freund's complete adjuvant from Difco (Detroit, MI). All other chemicals used were of reagent quality. Stock solutions of drugs were prepared in twice distilled water. ^{45}Ca was supplied as an aqueous solution of $CaCl_2$ by Amersham. The specific activity of the material was 25 mCi·μg^{-1} Ca^{2+}. [^{14}C]-sucrose (specific activity, 1 μCi·ml^{-1}) was supplied by Amersham.

Results

Tension experiments

Both KCl (1 to 100 mM) and histamine (0.1 μM to 1 mM) produced concentration-related contractions of tracheal strips from normal and sensitized guinea-pig. The concentration-response curves generated in sensitized trachea exhibited a left upward shift compared to those obtained in the nonsensitized group (fig. 1). This displacement translates into the differences between groups for the maximal effect (*i.e.* responsiveness) and effective concentration 50% (*i.e.* sensitivity), as presented in table 1.

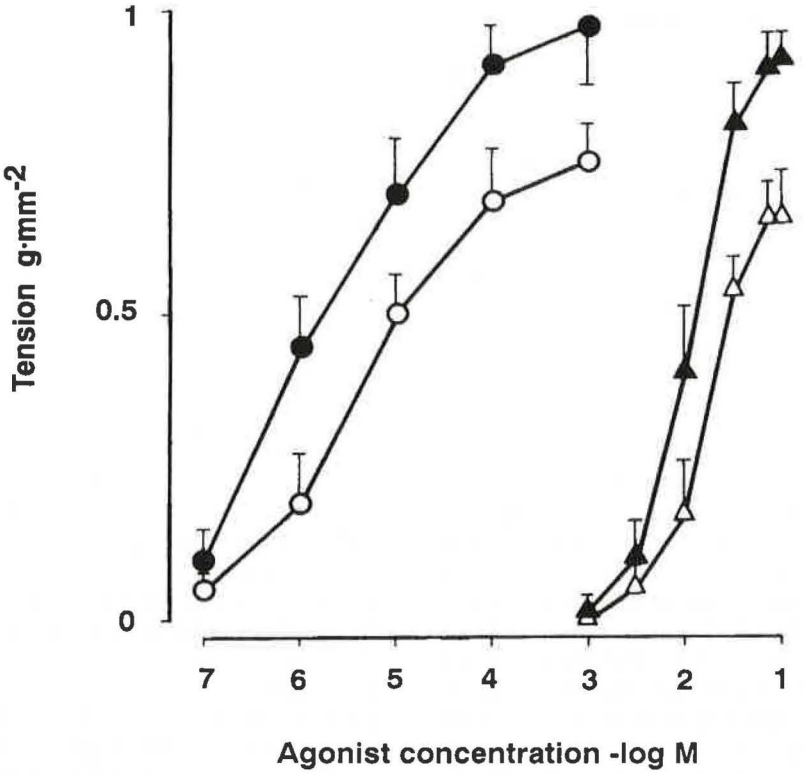

Fig. 1. – Concentration-response curves for concentration of tracheal strips by KCl (Δ, ▲) and histamine (○, ●) in normal (open symbols) and sensitized (closed symbols) guinea-pigs. Point and bar are the mean value and SD, respectively. The number of experiments for each group is shown in table 1.

Table 1. – Responsiveness (estimated by the maximal effect, E_{max}) and sensitivity (shown as the pD_2 value *i.e.* $-\log_{10} EC_{50}$) of tracheal strips to KCl and histamine in normal and sensitized guinea-pigs

	Normal			Sensitized		
	n	E_{max} g·mm^{-2}	pD_2	n	E_{max} g·mm^{-2}	pD_2
KCl	6	0.66±0.07	1.78±0.03	6	0.91±0.05*	2.02±0.02*
Histamine	8	0.75±0.06	5.04±0.05	8	0.98±0.08*	5.83±0.11*

n: number of experiments; *: $p<0.05$ compared to normal.

^{45}Ca uptake studies

Tracheal muscle from nonsensitized and sensitized guinea-pigs reached equilibrated levels of ^{45}Ca uptake within 30 to 60 min in both normal (2.5 mM) and low (0.03 mM) Ca^{2+} solutions (fig. 2). The equilibrated ^{45}Ca uptake was much greater in normal than in low Ca^{2+} solutions. There was no difference for ^{45}Ca uptake in normal Ca^{2+} solution between nonsensitized and sensitized tissues. Conversely, the uptake of ^{45}Ca in low Ca^{2+} solution was higher ($p<0.05$) in sensitized than in nonsensitized tissues (fig. 2).

Extracellular space, as determined by [^{14}C]sucrose tissue/medium ratios, was 0.420±0.029 ml·g^{-1} (n=20) in normal and 0.410±0.016 ml·g^{-1} (n=18) in sensitized tracheal strips.

Uptake of ^{45}Ca by tracheal strips was determined after 60 min incubation in solutions with Ca^{2+} concentrations ranging from 0.01 to 5.00 mM. The Scatchard-coordinate plot constructed with these data (fig. 3) showed that Ca^{2+} uptake by guinea-pig trachealis muscle may be split into two components.

This provides the basis for a distinction between high and low affinity populations of binding sites. Thus, low Ca^{2+} solutions (0.01 to 0.30 mM) favour high affinity whilst high Ca^{2+} solutions (1.00 to 5.00 mM) favour low affinity Ca^{2+} uptake.

In addition, in the sensitized group, the number (n) of binding sites for the high affinity component were augmented with respect to the nonsensitized tissues while no difference between groups was observed for the calculated dissociation constant (k_D). Conversely, n and k_D values for the low affinity component were similar in nonsensitized and sensitized tissues.

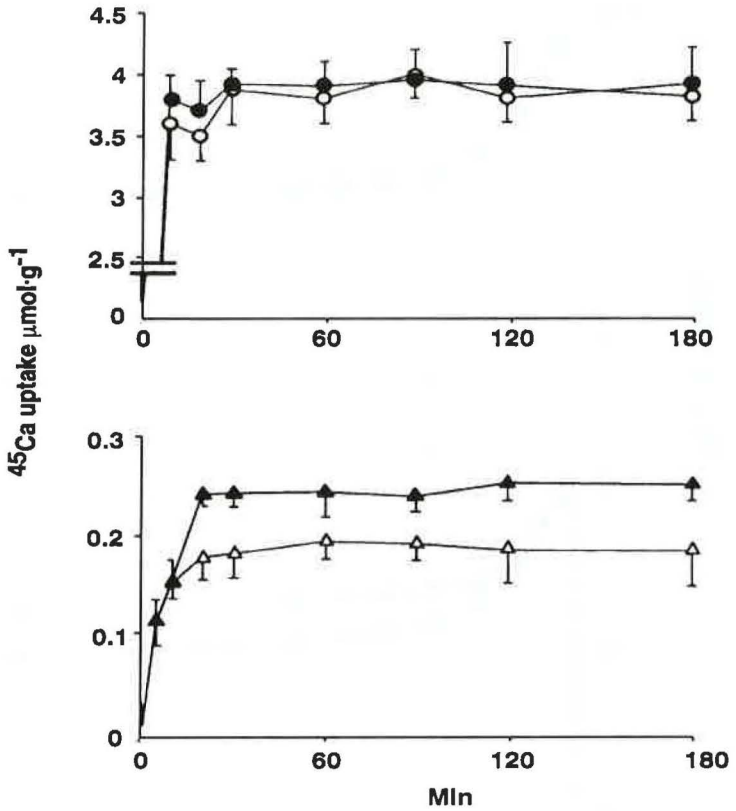

Fig. 2. – Uptake of ^{45}Ca in tracheal strips from normal (open symbols) and sensitized (closed symbols) guinea-pigs in either normal (2.5 mM) (○, ●) or low (0.03 mm), (Δ, ▲) Ca^{2+} solution (see Methods for details). Points are mean values of 8 observations and vertical lines represent SEM.

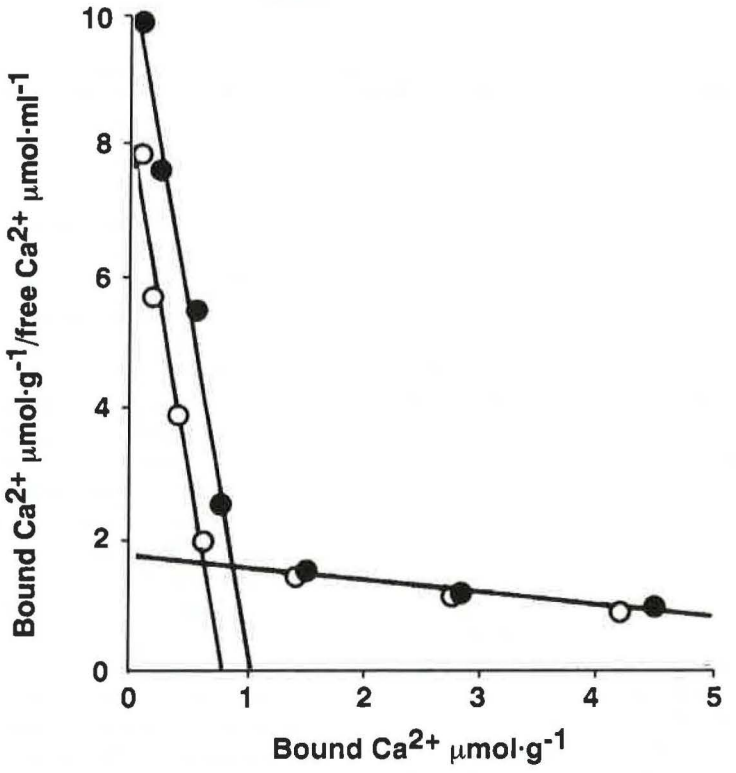

Fig. 3. – Scatchard-coordinate plot of ^{45}Ca uptake in tracheal strips from normal (○) and sensitized (●) guinea-pigs after 60 min incubation in Tris solutions containing the following extracellular Ca^{2+} concentrations: 0.01, 0.03, 0.10, 0.30, 1.00, 2.50 and 5.00 mM. Points are the means of at least 5 experiments. For the high affinity component, the number of binding sites (x-axis intercept) were 0.76 in nonsensitized *versus* 0.93 in sensitized tissues and calculated k_D values (x-axis intercept/y-axis intercept) were 0.098 in nonsensitized *versus* 0.087 in sensitized tissues.

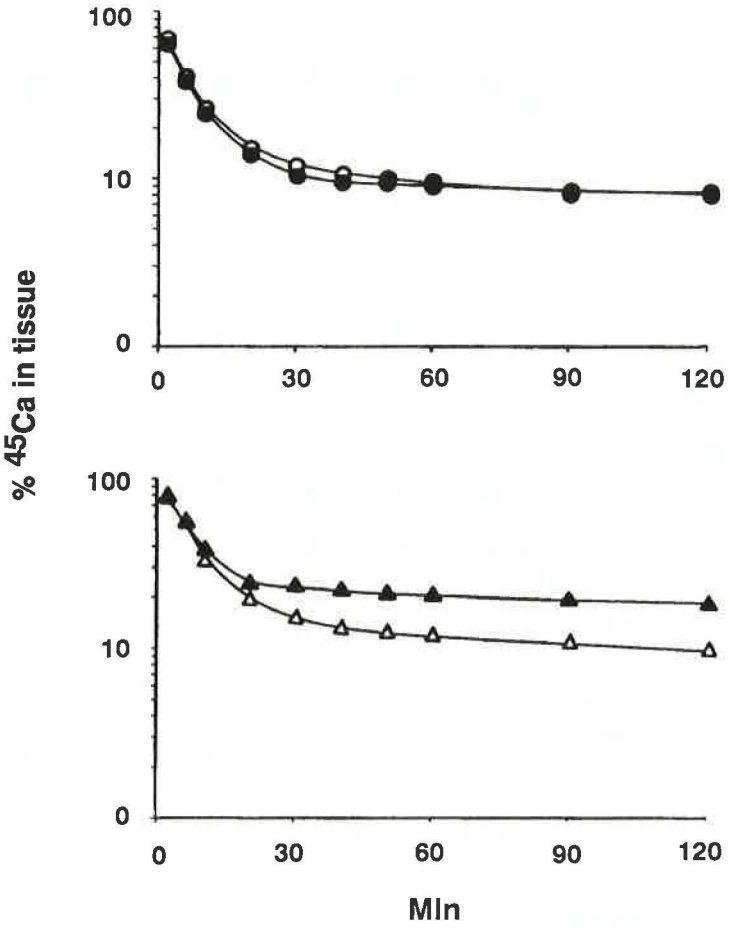

Fig. 4. – Effect of La^{3+}-substituted (80.8 mM) solution at 0.5°C on ^{45}Ca efflux from tracheal strips obtained from normal (open symbols) and sensitized (closed symbols) guinea-pigs. Tissues were incubated in 2.5 mM (○, ●) or 0.03 mM (△, ▲) Ca^{2+} Tris solutions with ^{45}Ca for 60 min before beginning wash-out. Each desaturation curve is the average of five experimental ones.

^{45}Ca washout studies

After 60 min incubation with ^{45}Ca in normal (2.5 mM) or low (0.03 mM) Ca^{2+} solution, wash-out in substituted La^{3+} solution yielded the desaturation curves shown in figure 4. As can be observed, cold La^{3+} did not greatly interfere with ^{45}Ca loss from the tissue in any of the experimental situations. However, ^{45}Ca efflux from sensitized tissues incubated in low Ca^{2+} solution reached a higher steady level (18.1±2.1%) when compared to normal tissues (9.5±1.7%, $p<0.05$) as shown in the lower panel of figure 4.

The analysis of the linear components of the ^{45}Ca desaturation curves yielded the following results expressed as the half-time in minutes for each component: 2.46, 12.30 and 230.0 in nonsensitized *versus* 2.31, 12.10 and 233.0 in sensitized tissues, both incubated in normal (2.5 mM) Ca^{2+} solutions; 3.35, 11.45 and 250.6 in nonsensitized *versus* 3.5 and 246.8 in sensitized tissues, both incubated in low (0.03 mM) Ca^{2+} solutions. All curves exhibited three linear components except that obtained in sensitized tissues incubated in low Ca^{2+} medium, which only had two components.

Residual ^{45}Ca uptake studies

The ^{45}Ca uptake, after 60 min wash-out into cold La^{3+}-substituted solution, was studied in the absence or presence of various concentrations of KCl and histamine (table 2). KCl (10 to 100 mM) caused a significant and concentration-dependent increase in the ^{45}Ca-uptake. This increase was significantly greater in sensitized, compared to normal, tissues for KCl (30 to 100 mM). Histamine (1 to 100 μM) did not cause any significant change in ^{45}Ca uptake by tracheal strips, in either normal or sensitized tissues.

Agonist-induced responses were at the same concentration, greater in sensitized than in normal tissues (fig. 1). Therefore, additional experiments were carried out to compare ^{45}Ca uptake after single equi-effective concentrations of the agonists. Concentrations of the agonists were chosen to produce around 40% (KCl 14 mM, histamine 4 μM) or 80% (KCl 27 mM, histamine 50 μM) of the maximal response in nonsensitized tracheal strips and those concentrations producing approximately the same level of contraction in the sensitized strips were found to be KCl 6 and 12 mM, and histamine

Table 2. – Effect of various concentrations of KCl and histamine (HA) on ^{45}Ca uptake, after 60 min wash-out into cold La^{3+}-substituted solution, by tracheal strips from normal (nonsensitized) and sensitized guinea-pigs

		Normal ^{45}Ca uptake nmol·g^{-1}		Sensitized ^{45}Ca uptake nmol·g^{-1}	
		Basal	Stimulated	Basal	Stimulated
KCl	10 mM	196±45	267±39*	229±42	319±37*
KCl	30 mM	187±41	305±40*	215±53	397±53*#◆
KCl	60 mM	205±49	392±62*◆	266±56	558±94*#◆
KCl	100 mM	196±41	401±52*	247±37	583±62*#
HA	1 μM	187±41	251±51	215±43	268±40
HA	10 μM	205±49	239±73	266±56	277±63
HA	100 μM	196±41	266±64	247±37	296±53

Number of experiments in each group was 6 *: $p<0.05$ with respect to basal; #: $p<0.05$ with respect to the corresponding value in the normal group; ◆: $p<0.05$ with respect to the preceding value in the same column.

Table 3. – Effect of single equi-effective concentrations of KCl and histamine (HA) on ^{45}Ca uptake, after 60 min wash-out into cold La^{3+}-substituted solution, by tracheal strips from normal (nonsensitized) and sensitized guinea-pigs

		Normal				Sensitized		
			^{45}Ca uptake nmol·g^{-1}				^{45}Ca uptake nmol·g^{-1}	
		Contractile response g·mm^{-2}	Basal	Stimulated		Contractile response g·mm^{-2}	Basal	Stimulated
KCl	14 mM	0.224±0.017	193±36	268±32*	6 mM	0.23±0.021	215±35	281±27*
KCl	27 mM	0.496±0.014	188±45	372±19*	12 mM	0.483±0.019	209±27	369±21*◆
HA	4 μM	0.280±0.041	227±33	228±21	0.4 μM	0.320±0.034	237±36	225±19
HA	50 μM	0.573±0.024	203±29	235±31	3 μM	0.581±0.037	206±30	240±22

The number of experiments was 6 for each group. *: $p<0.05$ with respect to basal value; ◆: $p<0.05$ with respect to the preceding value in the same column.

0.4 and 3 μM, respectively. The effect of these concentrations of KCl and histamine on ^{45}Ca uptake is shown in table 3. KCl enhanced concentration-dependently the ^{45}Ca uptake but no difference was found between normal and sensitized tissues. Histamine did not stimulate ^{45}Ca uptake (table 2).

Discussion

Tracheal strips from actively sensitized guinea-pigs were hyperresponsive (*i.e.* greater maximal effect) and hypersensitive (*i.e.* smaller effective concentration 50%) to KCl and histamine. These results confirm previous findings from this laboratory [8, 9, 15] and are in agreement with those from other research groups [7, 10–12, 19]. Differences with other reports [20–22] may be related to methodological differences, mainly in the immunization procedure, and have been discussed previously [9, 15].

Therefore, ^{45}Ca movements were then investigated in this model since an alteration in Ca^{2+} handling by sensitized airway smooth muscle has been suggested from previous studies, which found differences both in the response to agonists in Ca^{2+}-free medium [15, 23] and in the efficacy of calcium antagonists [24–26], between naive and sensitized tissues.

In normal (nonsensitized) tracheal muscle, the time-course of ^{45}Ca uptake, the amounts taken up in normal (2.5 mM) and low (0.03 mM) Ca^{2+} solutions and the distribution of Ca^{2+} binding into high and low affinity populations (as determined by the Scatchard-coordinate plot), are in accordance with results previously reported for tracheal muscle of the guinea-pig [27] and other animal species [16]. Sensitized guinea-pig trachea behaves in a manner similar to that of naive animals except that: i) the uptake of ^{45}Ca in low (0.03 mM) Ca^{2+} solution was higher; and ii) the number of binding sites for the high affinity component of Ca^{2+} uptake was augmented.

In sensitized guinea-pig tracheal muscle, incubation in low (0.03 mM) Ca^{2+} solution followed by La^{3+} wash-out resulted in a greater amount of residual ^{45}Ca compared to normal tissues. The analysis of the linear components of the desaturation curve under these experimental conditions showed only two components instead of the three usually observed in normal tissues in this and other studies [28].

Taken together, the results from the ^{45}Ca uptake and efflux studies indicate that immunological sensitization produces a preferential enhancement of those Ca^{2+} movements related to the high affinity component *i.e.*

more high affinity Ca^{2+} is taken up and retained by sensitized tracheal muscle compared to normal tissues. The possible relevance of this finding has to be weighed considering that the high affinity Ca^{2+} presumably represents the cellular or membrane bound Ca^{2+} store which is primarily linked to the response to agonists [29].

As pointed out by GOODMAN *et al.* [27] and as can also be derived from results of the present study, the use of cold La^{3+} wash-out to estimate residual ^{45}Ca uptake and its modification by agonists is less feasible in guinea-pig trachea than in other tissues. The values for basal (unstimulated) ^{45}Ca uptake obtained in this study are lower than those found by FOSTER *et al.* [17, 30] and RAEBURN *et al.* [31] but close to those of RAEBURN and RODGER [18]. This may be related, as suggested by RAEBURN *et al.* [31], to the presence of Tris as buffer in the incubation medium.

Potassium chloride, but not histamine, produced a concentration-dependent increase in La^{3+} resistant ^{45}Ca uptake by guinea-pig trachea. The results for normal tissues are in good agreement with those previously reported [18, 30–32]. The increase in ^{45}Ca uptake by KCl is apparently due to Ca^{2+} entry through voltage-operated Ca^{2+} channels [18, 30]. This increase was significantly greater in sensitized than in normal guinea-pig trachealis. In contrast, RAEBURN *et al.* [31] found no significant difference between normal and sensitized preparations for KCl-stimulated tissues. However, this group used a different sensitization protocol which did not produce hyperreactivity [22]. The small but significantly greater Ca^{2+} entry following KCl stimulation may contribute to the lesser efficacy of verapamil in guinea-pig sensitized airway smooth muscle [24]. This greater ^{45}Ca uptake in sensitized tissues was not found for equi-effective concentrations of KCl. On the other hand, tension development by histamine mainly involves intracellular Ca^{2+} release [32] whilst Ca^{2+} influx, if existing, lies under the level of detection by the lanthanum technique [32].

In summary, sensitized guinea-pig tracheal muscle shows hyperreactivity to histamine and KCl, and alterations in ^{45}Ca movements consisting of an augmented uptake and retention of high affinity Ca^{2+} and a greater increase of La^{3+} resistant ^{45}Ca uptake after KCl-stimulation. Although certain biochemical [33] and electrophysiological [34] abnormalities have been described in sensitized guinea-pig airways, the mechanism underlying these alterations remains to be determined. Although caution is needed in extrapolating findings from experimental models of asthma, further knowledge of Ca^{2+} handling by airway smooth muscle may prove decisive in the search for new anti-asthma drugs. This may be of importance given the lack of clinical effectiveness of calcium antagonists in asthma [35].

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Hyperréactivité et déplacement du ^{45}Ca dans le muscle trachéal de cobayes sensibilisés. M. Perpiña, J. Cortijo, E. Fornàs, M. Palau, J.L. Ortiz, E. Morcillo.

RÉSUMÉ: Nous avons étudié, dans la trachée de cobayes normaux et sensibilisés activement, les réponses au KCl, à l'histamine et les mouvements du ^{45}Ca. Les trachées sensibilisées s'avèrent hyperréactives et hypersensibles au KCl et à l'histamine. Les expériences de résorption de ^{45}Ca montrent que le muscle trachéal sensibilisé se comporte comme un muscle normal, à l'exception d'une résorption plus élevée de ^{45}Ca dans des solutions de Ca^{2+} à faible concentration (0.03 mM), et d'une augmentation du nombre de sites de liaison pour la résorption du composant de ^{45}Ca à forte affinité (estimée par le Scatchard-coordinate plot). En outre, dans le muscle trachéal sensibilisé, une incubation dans une solution à faible concentration de Ca^{2+} (0.03 mM), suivie par un lavage au La^{3+}, entraîne la persistance d'une quantité plus grande de ^{45}Ca résiduel que dans les tissus normaux. Le KCl, mais non l'histamine, augmente le contenu en calcium résistant à La^{3+}. Cette augmentation est plus importante dans les trachées sensibilisées que dans les trachées normales. Ceci démontre l'existence d'une hyperréactivité et de mouvements altérés du ^{45}Ca dans le muscle trachéal sensibilisé.

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