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Temporal Relationship of Cyclic Nucleotide Levels and Calcium Exchange to Histamine-Induced Tension Development (41921)

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Abstract. The purpose of these experiments was to study the temporal relationship between tension development in incubated guinea pig tracheal smooth muscle and changes in tissue levels of cAMP and cGMP, and isotopic Ca. Dose-response studies were performed with increasing concentrations of histamine both in the absence and presence of H_1 receptor blockade using 10^{-5} *M* diphenhydramine. The time course of tension development was subsequently determined in the presence of three concentrations of histamine shown to cause 50% (3×10^{-6} *M*), 85% (9×10^{-6} *M*), and 100% (5×10^{-5} *M*) of maximal contraction. Tissue cyclic nucleotide and ^{45}Ca levels were measured 20 sec, 1 min, and 6 min after the onset of contraction. For comparison, the influence of carbachol was also studied. Our findings demonstrate that there were no detectable alterations in tissue cAMP or cGMP levels during the initial phases of contractile change. In contrast, tissue isotopic Ca uptake increased early in histamine-induced contraction and was blocked by the H_1 antagonist.

It is widely believed that changes in tissue levels of cAMP and cGMP are involved in the regulation of smooth muscle tension (1–3). A widely accepted theory describing this involvement is that increases in tissue levels of cGMP and/or decreases in cAMP promote smooth muscle contraction, and that both cAMP and cGMP are mediators of smooth muscle relaxation. A growing body of literature suggests, however, that these relationships may not be correct (4–15). As an example, a number of investigators (4, 5, 7, 11) have noted discrepancies in the relationship between drug-induced increases in smooth muscle tension and cAMP and cGMP metabolism. The general findings of these studies were that cyclic nucleotide levels did not always change in response to contractile events, or if they did change, their response lagged behind the changes in tension or occurred only at higher drug levels. It has also been reported that pharmacologic agents which cause smooth muscle relaxation, such as isoproterenol, papaverine, and nitroglycerin, are not invariably related to increases in cAMP levels (4).

In previous studies we found that low concentrations of theophylline and isoproterenol, capable of producing significant negative inotropic effects *in vivo* and *in vitro,* did not alter cAMP or cGMP levels in incubated guinea pig tracheal smooth muscle (13, 14, 16). The hypothesis of the present study was that drug-induced smooth muscle contraction is, also, not related to alterations in cAMP or cGMP levels. To test this hypothesis, as well as to extend the earlier work, the present experiments were designed to examine the relationship between histamine-induced tension development and tissue levels of cAMP, cGMP, and ^{45}Ca. The experiments were designed to examine temporal relationships and to use concentrations of histamine which produced only physiological responses. As a comparison, the positive inotropic influence of carbachol was also studied.

Methods. Male Hartley guinea pigs weighing 350–500 g were sacrificed by a sharp blow to the head. After midsternal thoracotomy, the tracheae were excised and immersed in cold oxygenated physiological medium containing (m*M*): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 8.9; $CaCl_2$, 2.5; and glucose, 5.6. After excess tissue was removed, the tracheae were divided into 4-mm segments which were then mounted on opposing L-shaped 30-gauge needles, according to the method of Hooker *et al.* (17). Each tracheal preparation was suspended in an individual incubation chamber containing 20 ml physiological medium which was aerated continuously with a mixture of O_2 and CO_2 so as to maintain the pH at 7.35 at 37°C. The tracheal rings were

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allowed to equilibrate for 90 min under a resting tension of 2 g, conditions which were found to achieve "optimal" tissue stability and responsiveness. During this time, the medium in the bath was changed every 15 min. Isometric force measurements were made using Statham UC-3 force-displacement transducers interfaced with UL-5 microscale attachments. The measurements, calibrated as grams of force, were recorded on a Beckman Dynagraph.

Tension studies. After the initial 90-min equilibration period, the contractile responses of the tracheal rings to increasing concentrations of histamine were determined. In these experiments, the administration of histamine to the tissue baths was on a cumulative basis with appropriate time lags imposed between successive administrations to allow contractile equilibration. A number of histamine dose–response studies were also performed in the presence of 10^{-5} *M* of diphenhydramine hydrochloride, an H_1 blocker. When used, diphenhydramine was added to the bath 60 min prior to the addition of histamine. All contractile data are reported as grams of tension normalized per gram of wet tissue weight.

Another series of experiments were performed which determined the time course of tension development as a function of histamine concentration. For these experiments, histamine levels were selected which caused 50% (3×10^{-6} *M*), 85% (9×10^{-6} *M*), and 100% (5×10^{-5} *M*) of maximal smooth muscle contraction.

Nucleotide and isotope studies. Determinations of tissue levels of cAMP, cGMP, and ^{45}Ca were made in tracheal rings which had been incubated under a variety of conditions. A number of tracheal rings were incubated in the presence of 3×10^{-6} *M*, 9×10^{-6} *M*, or 5×10^{-5} *M* histamine, for 20 sec, 1 min, or 6 min. As a comparison, additional timed incubation studies were performed in the presence of 10^{-6} *M* carbachol, a concentration of the parasympathomimetic airway constricting agent known to cause a maximal contractile response of the tracheal smooth muscle. In isotopic studies, tracer amounts of $^{45}CaCl_2$ (New England Nuclear Corp., 10 mCi/mg) were added to the incubation medium. Appropriate controls were obtained for each experimental condition. All incubations were terminated by rapidly immersing the tissue in liquid nitrogen.

Tissue analysis. Tissue samples to be analyzed for cAMP and cGMP were homogenized in 6% trichloroacetic acid at 4°C for 15 min. After the supernatant was decanted from the sedimented protein, extracted with water-saturated ether, and concentrated to dryness, the residue was dissolved in an acetate buffer. Radioimmunoassays of cAMP and cGMP were carried out according to the method of Steiner *et al.* (18) using reagents obtained from the New England Nuclear Corporation, Boston, Massachusetts. Protein determinations were made using the method of Lowry *et al.* (19). All cyclic nucleotide data are expressed as picomoles per milligram protein.

Tissue samples to be analyzed for ^{45}Ca content were rinsed to remove surface contamination, dried, weighed, and dissolved in a tissue solubilizer (Soluene-100, Packard Instrument Co.). The dissolved samples were mixed with 10 ml toluene, PPO, POPOP, scintillation cocktail. Isotope activity was determined by liquid scintillation counting at an average counting efficiency of 50%. The ^{45}Ca data are expressed as a ratio of the isotopic activity found in a gram of wet tissue to that found in a gram of incubation medium (abbreviated as a "T/M" Ratio).

The results of all experiments were analyzed statistically. Means and standard deviations (SD) were determined and one-way analyses of variances were used to test the significance of differences between the means of several populations of data. The unpaired *t* test was used to determine the statistical significance of differences between control values and specific experimental values.

Results. *Effective levels of histamine.* Figure 1 indicates the reactivity (grams of tension developed) and sensitivity (histamine level at which a particular level of tension develops) of guinea pig tracheal smooth muscle to increasing concentrations of histamine, both in the presence and absence of 10^{-5} *M* of diphenhydramine hydrochloride. Histamine caused a concentration-dependent increase in the isometric tension of the tracheal smooth muscle. Under control conditions, 3×10^{-6} *M*, 9×10^{-6} *M*, and 5×10^{-5} *M*

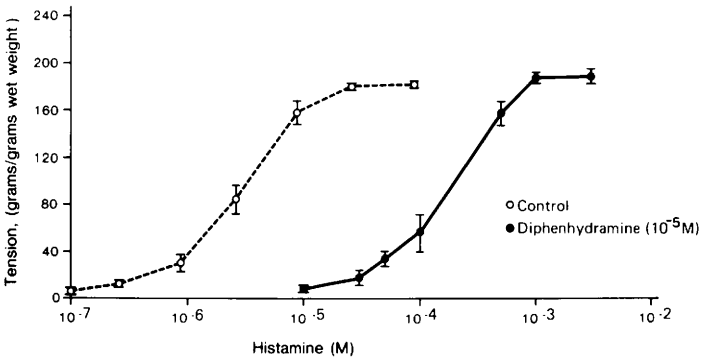

FIG. 1. The dose-response relationships of histamine-induced constriction in incubated guinea pig tracheal rings in the absence and presence of 10^{-5} *M* diphenhydramine. Each point represents the grams of tension developed per gram of wet tissue weight (*Y*-axis) and is a mean of 28 samples with the SEM indicated.

histamine corresponded to EC_{50}, EC_{85}, and $\sim EC_{100}$ concentrations, respectively. In the presence of diphenhydramine hydrochloride, the histamine dose-response curve was shifted to the right and the EC_{50} value was increased to 2.2×10^{-4} *M*. The absolute increase in smooth muscle tension caused by histamine, however, was relatively unaffected by diphenhydramine. In the absence or presence of the H_1 blocker, the tracheal smooth muscle developed 182 ± 16 and 186 ± 11 g/g wet wt, respectively.

Temporal response to histamine. Using time as a dependent variable, Fig. 2 shows the time course of tension development as a function of histamine concentration. There were no statistical differences noted between the rates of smooth muscle tension development under the influence of any one of the three levels of histamine, until 75% of the maximal potential tension has been developed. Thereafter, the final tension equilibration occurred more rapidly in the presence of lower concentrations of histamine than in the presence of higher concentrations. The tracheal rings required 2.3 min to reach contractile equilibration in the presence of 3×10^{-6} *M* histamine, and 7.5 min in the presence of 5×10^{-5} *M* histamine. These final differences were statistically significant with $P < 0.05$.

Cyclic nucleotide tissue levels. Table I records the influence of various concentrations of histamine on tissue levels of cAMP and cGMP in tracheal rings incubated for 20 sec, 1 min, or 6 min, and compares these values to appropriately timed controls. Although 6 min of incubation at all three concentrations of histamine was found to cause a substantial decrease in tissue levels of cAMP without any significant change in cGMP levels, there was no detectable alteration in tissue levels of either nucleotide at 20 sec or 1 min of incubation.

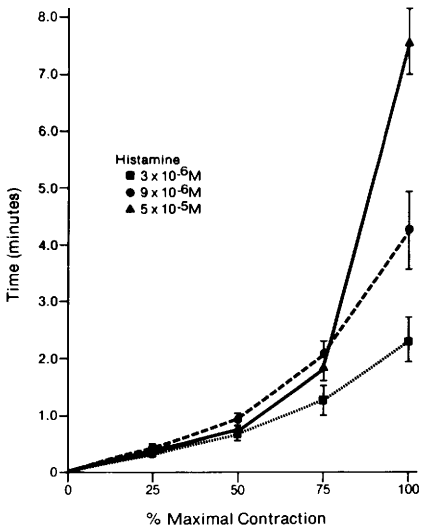

FIG. 2. The time required for incubated guinea pig tracheal rings to achieve contractile equilibrium as a function of histamine concentration. The time course of tension development is indicated with time as the dependent variable. Each point represents a mean of 14 samples with the SEM indicated.

TABLE I. TISSUE CYCLIC NUCLEOTIDE AND ^{45}Ca LEVELS

	cAMP (pmole/ mg prot)	cGMP (pmole/ mg prot)	^{45}Ca (T/M ratio)
Histamine (*M*)			
20-sec			
Control	25.4 ± 2.0	0.25 ± 0.03	0.60 ± 0.08
3×10^{-6}	21.4 ± 8.4	0.17 ± 0.02	0.53 ± 0.01
9×10^{-6}	25.3 ± 6.5	0.31 ± 0.02	0.51 ± 0.04
5×10^{-5}	28.2 ± 6.8	0.28 ± 0.08	0.80 ± 0.12*
1-min			
Control	29.6 ± 1.5	0.24 ± 0.04	1.01 ± 0.04
3×10^{-6}	28.8 ± 4.3	0.21 ± 0.04	1.10 ± 0.06
9×10^{-6}	29.0 ± 5.4	0.19 ± 0.05	1.35 ± 0.18*
5×10^{-5}	30.8 ± 1.9	0.31 ± 0.07	1.34 ± 0.09*
6-min			
Control	17.6 ± 1.9	0.26 ± 0.02	6.85 ± 0.42
3×10^{-6}	8.2 ± 2.3*	0.28 ± 0.03	7.38 ± 0.58
9×10^{-6}	2.5 ± 0.3*	0.20 ± 0.02	9.69 ± 0.68*
5×10^{-5}	3.0 ± 0.6*	0.25 ± 0.04	10.72 ± 0.44*
Carbachol (*M*)			
1-min			
Control	23.0 ± 1.4	0.20 ± 0.10	1.78 ± 0.46
1×10^{-6}	16.2 ± 4.9	0.21 ± 0.10	1.68 ± 0.74
6-min			
Control	17.5 ± 1.8	0.26 ± 0.04	6.50 ± 0.42
1×10^{-6}	12.8 ± 3.1	0.06 ± 0.02*	5.63 ± 2.13

Note. Cyclic nucleotide values are expressed as means ± SEM with $N = 5$–20. Isotopic values are expressed as dpm ^{45}Ca/g wet tissue/dpm ^{45}Ca/ml incubation fluid with $N = 10$–20.

* Values are statistically different from control, $P < 0.05$.

Table I also shows the cyclic nucleotide content of tracheal tissue incubated for 1 min and 6 min in the presence of 10^{-6} *M* carbachol, an $\sim EC_{100}$ level of the agonist. The results were quite different from those noted for tissues incubated with histamine. After 6 min of incubation, carbachol caused a 77% drop in cGMP levels without significant alterations in cAMP levels. Carbachol did not, however, affect levels of either of the cyclic nucleotides after 1 min of incubation.

Calcium tissue/medium ratios. Table I shows the influence of time and histamine on the uptake of ^{45}Ca by the incubated tracheal tissue. As would be expected, the ^{45}Ca tissue/medium ratio increased with time under control conditions as a reflection of isotope loading into the various compartments of the tracheal tissue. The tissue/medium ratio was also enhanced in the presence of histamine, an increase particularly evident at all time intervals in the presence of 5×10^{-5} *M* histamine, but also noted at 1 min and at 6 min in the presence of 9×10^{-6} *M* of histamine. When the contractile influence of histamine was blocked with 10^{-5} *M* diphenhydramine (Table II), however, the agonist no longer altered isotopic calcium levels. In contrast to histamine, 10^{-6} *M* carbachol did not alter the tissue/medium distribution of ^{45}Ca during any of the incubation times studied.

Discussion. As described in the introductory section, controversy appears to surround the current theories which link changes in smooth muscle tension to changes in tissue levels of cAMP or cGMP. Previous reports from our laboratory have presented evidence which appears to indicate a disjunction in the relationship of cyclic nucleotides to the relaxation of tracheal smooth muscle (13, 14, 16). The results of the present study tend to confirm this finding for agents which contract tracheal smooth muscle. The conclusions of this study may be stated as follows: First, increases in smooth muscle tension may not be temporally related to changes in tissue cyclic nucleotide levels; second, smooth muscle tension changes induced by pharmacological agents may not occur at the same drug dosage as do cyclic nucleotide changes; and, third, some drugs which alter smooth muscle tension do so without altering cyclic nucleotide metabolism.

The results of our study have shown that histamine-induced contraction of incubated guinea pig tracheal rings is not related temporally to alterations in tissue levels of cAMP or cGMP (Table I). Only after the tracheal smooth muscle had been exposed to histamine for 6 min, with tension development

TABLE II. EFFECTS OF DIPHENHYDRAMINE ON HISTAMINE-STIMULATED ^{45}Ca TISSUE UPTAKE

Histamine (*M*)	^{45}Ca (T/M ratio)
Control	2.01 ± 0.01
3×10^{-6}	2.10 ± 0.21
9×10^{-6}	2.17 ± 0.14
5×10^{-5}	2.13 ± 0.11

Note. Values as described in Table I. All tissues were exposed to histamine for 6 min in the presence of 10^{-5} *M* diphenhydramine.

between 50 and 100% completed (Fig. 2), did cAMP levels fall significantly. Creese and Denborough (7), using guinea pig tracheal smooth muscle strips, also reported a temporal discrepancy in the relationship between cAMP levels and tension development. Their results, however, differed from our own in that they indicated a substantial increase in cAMP levels after less than one minute of incubation with 10^{-4} *M* histamine. This variance is probably due to differences in experimental technique, as, for example, their reported rise in cAMP levels was measured in tracheal strips incubated without any imposed tension, while our measurements were made on guinea pig rings which were developing substantial levels of tension.

Our study has also shown that drug-induced smooth muscle tension changes apparently occur at dose levels different from those which alter cyclic nucleotide levels. When the tracheal smooth muscle rings were contracted in the presence of 3×10^{-6} *M* histamine there were no detectable alterations in tissue concentrations of cAMP or cGMP (Table I) until after the contractile event was almost completed (between 1 and 6 min, see Fig. 2). The same finding occurred in the presence of the $\sim EC_{100}$ concentration of carbachol for incubations of 1 min or less (Table I). A 6-min exposure to carbachol, however, caused a significant reduction in cGMP levels. Earlier work by Kolbeck *et al.* showed similar dose discrepancies for agents which caused tracheal smooth muscle relaxation. They reported that concentrations of isoproterenol (14, 16) and theophylline (13) which caused significant smooth muscle relaxation failed to alter tissue cyclic nucleotide levels. Such evidence lends credence to the hypothesis that many pharmacological agents which alter smooth muscle tension, do so at concentrations lower than those necessary to alter enzyme activities and nucleotide levels.

Our work further suggests that unrelated drugs which cause similar smooth muscle contractile responses may affect cyclic nucleotide metabolism differently. Both histamine and carbachol stimulate the tracheal smooth muscle to develop tension. Their respective influences on cyclic nucleotide metabolism, however, appear to be different (Table I). Measured tissue levels of cAMP were not significantly altered by a 6-min exposure to 10^{-6} *M* carbachol, whereas, the same length of exposure to histamine concentrations between 3×10^{-6} *M* and 5×10^{-5} *M* caused a considerable reduction in cAMP. An opposite response was noted for cGMP, with tissue levels of the nucleotide being depressed by carbachol but not by histamine.

The data in Table I indicate that the effects of the higher concentrations of histamine on tracheal smooth muscle are associated with increases in tissue calcium exchange rates, as measured by the tissue to medium ratio of ^{45}Ca. Such noted increases were temporally related to increases in muscle tone and were absent when the contractile event was blocked by the H_1 blocking agent diphenhydramine (Table II). The considerably smaller increases in muscle tone associated with carbachol (10^{-6} *M* carbachol, the ED_{100} level, developed only 26 g tension/g wet wt, while the ED_{50} level of histamine, 3×10^{-6} *M*, developed 85 g tension/g wet wt) on the other hand, occurred without altering ^{45}Ca tissue levels. While a logical explanation for the absence of a measureable carbachol-induced calcium effect may be related to the lesser level of tension production, Takayanagi *et al.* (20) and Uchida (21) recently suggested an alternative idea. They proposed that the stimulation of cholinergic receptor sites initiates smooth muscle contraction primarily by a release of calcium from intracellular sites of storage, whereas stimulation of histamine receptor sites appears to be dependent on external supplies of calcium.

It should be emphasized that the results of this study are applicable to isolated guinea pig tracheal cylinders and may not apply directly to the smooth muscle of other species. In the present study, 1×10^{-6} *M* carbachol caused a 77% decrease in cGMP levels within 6 min (Table I). Using bovine tracheal smooth muscle, Katsuki and Murad (11) reported that the same dose of carbachol markedly increased cGMP levels for at least 3 min. Similarly, in the present study, histamine had no effect on cGMP levels at any time of incubation, whereas, in the study of Katsuki and Murad, it markedly increased cGMP levels.

We acknowledge that an inability to detect changes in cyclic nucleotide levels may be

due to limitations in our methodology. It is possible that cAMP and cGMP changes caused by histamine or carbachol occur in compartments or pools which are not detected by our techniques. Alternatively, cyclic nucleotide changes may occur prior to our first 20-sec measurement. Until such findings are made, however, we feel that our original hypothesis has been adequately tested for histamine in the guinea pig tracheal smooth muscle. It appears that, under our experimental conditions, cAMP and cGMP are not involved in the initiation of tracheal smooth muscle tension.

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1. Andersson R, Nilsson K, Wikberg J, Johansson S, Mohme-Lundholm E, Lundholm L. Cyclic nucleotides and the contraction of smooth muscle. Adv Cyclic Nucleotide Res **5**:491–518, 1975.
2. Goldberg ND, Haddox MK, Nicol SE, Glass DB, Sanford CH, Kuehl FA, Estensen R. Biologic regulation through opposing influences of cyclic GMP and cyclic AMP: The yin yang hypothesis. Adv Cyclic Nucleotide Res **5**:307–330, 1975.
3. Kukovetz WR, Poch G, Holzmann S. Cyclic nucleotides and relaxation of vascular smooth muscle. In: Vasodilation. New York, Raven Press, p339, 1981.
4. Diamond J. Evidence for dissociation between cyclic nucleotide levels and tension in smooth muscle. In: Stephens NL, ed., The Biochemistry of Smooth Muscle. Baltimore, Univ Park Press, p343, 1977.
5. Diamond J. Role of cyclic nucleotides in control of smooth muscle contraction. Adv Cyclic Nucleotide Res **9**:327–340, 1979.
6. Diamond J. Lack of correlation between cyclic GMP elevation and relaxation of nonvascular smooth muscle by nitroglycerin, nitroprusside, hydroxylamine and sodium azide. J Pharmacol Exp Ther **225**:422–426, 1983.
7. Creese BR, Denborough MA. The effect of histamine on cyclic AMP levels in guinea-pig tracheal smooth muscle. Eur J Pharmacol **66**:95–101, 1980.
8. Lau YS, Lum BKB. Role of cyclic AMP in adrenergically induced tracheal smooth muscle relaxation. Proc West Pharmacol Soc **20**:33–39, 1977.
9. Janis RA, Diamond J. Relationship between cyclic nucleotide levels and drug-induced relaxation of smooth muscle. J Pharmacol Exp Ther **211**:480–484, 1979.
10. Katsuki S, Arnold WP, Murad F. Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. J Cyclic Nucleotide Res **3**:239–247, 1977.
11. Katsuki S, Murad F. Regulation of adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate levels of contractility in bovine tracheal smooth muscle. Mol Pharmacol **13**:330–341, 1977.
12. Bransome ED. Application of the Sousa (John Phillip) principles to medical research: The role of cyclic nucleotides in hormone and drug action. Trans Amer Clinic Climatol Assoc **89**:172–183, 1977.
13. Kolbeck RC, Speir WA, Carrier GO, Bransome ED. Apparent irrelevance of cyclic nucleotides to the relaxation of tracheal smooth muscle induced by theophylline. Lung **156**:173–183, 1979.
14. Kolbeck RC, Speir WA, Carrier GO, Bransome ED. Theophylline and isoproterenol: Their combined effects on calcium and cyclic nucleotide metabolism in tracheal smooth muscle. Amer Rev Resp Dis **117**:358, 1978.
15. Weinryb I. Cyclic AMP as an intracellular mediator of hormone action: Sutherland's criteria revisited. Perspect Biol Med Spring:415–420, 1979.
16. Kolbeck RC, Speir WA, Carrier GO, Bransome ED. The negative inotropic effect of beta-agonists on respiratory smooth muscle: Evidence that cyclic nucleotides are not involved and the calcium metabolism may be. Chest **72**:402–403, 1977.
17. Hooker CS, Calkins PH, Fleisch JH. On the measurement of vascular and respiratory smooth muscle response *in vitro*. Blood Ves **14**:1–11, 1977.
18. Steiner AL, Kipnis DM, Utiger R, Parker C. Radioimmunoassay for measurement of adenosine 3′,5′-cyclic phosphate. Proc Natl Acad Sci USA **64**:367–373, 1969.
19. Lowry OH, Rosebrough NJ, Fan AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
20. Takayanagi T, Hongo T, Hisayma T, Kasuya Y. Effects of acetylcholine and histamine on mechanical activity of rabbit taenia coli. Ca-incorporation and Ca-release in its microsomal fraction. J Pharm Dyn **2**:212–219, 1979.
21. Uchida M. Histamine-induced decrease of membrane-bound calcium ions in the membrane fraction of rabbit taenia coli. Eur J Pharmacol **64**:357–360, 1980.

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