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ORIGINAL RESEARCH

Alcaftadine, a new antihistamine with combined antagonist activity at histamine H_1, H_2, and H_4 receptors

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Abstract: Current therapy for ocular allergy includes H_1 antihistamines, mast cell stabilizers, dual action antihistamines (H_1 antihistamines + mast cell stabilizers), and steroids. In this report, we describe the in vivo and in vitro characterization of alcaftadine, a recently approved antihistamine that exhibits a distinct set of therapeutic properties. When tested in a guinea pig model of conjunctivitis, alcaftadine prevented immediate allergic responses with an efficacy comparable with that of ketotifen, and was also able to attenuate delayed eosinophil influx with a potency similar to that of dexamethasone. Given recent reports suggesting a possible role for histamine H_2 or H_4 receptors in the etiology of ocular allergy, we examined the binding properties of alcaftadine at all histamine receptor types. Alcaftadine is a high affinity ligand for the H_1 receptor, with a pK_i (8.5) that is comparable with that of other H_1 antihistamines. It also shows an higher affinity for the H_2 receptor than ketotifen. Alcaftadine exhibited modest binding affinity for the H_4 receptor ($pK_i = 5.8$) with no affinity for the H_3 receptor. The affinity for the H_4 is higher than the value for ketotifen ($pK_i < 5$). Using a cellular assay of H_4 receptor activity, alcaftadine was shown to act as a functional antagonist of H_4 receptor signaling. Overall, the studies suggest that alcaftadine is a histamine receptor antagonist with a broad spectrum of antihistamine activity and a unique combination of therapeutic effects. As such, it represents a new therapeutic option for the treatment of allergic conditions.

Keywords: ocular, allergic conjunctivitis, antihistamine, ketotifen, pheniramine

Introduction

Histamine (2-(4-imidazolyl) ethylamine) is both an autocoid and a neurotransmitter, and exerts its biological effects through interaction with one of four distinct types of histamine receptors, termed H_1, H_2, H_3, and H_4.[1,2] Each of these receptors is a member of the G-protein coupled receptor superfamily, a class of proteins that regulate physiological activity through positive or negative modulation of canonical cell signaling molecules, such as cyclic AMP, inositol phosphates, and intracellular free calcium.[1,2] The antihistamines act by antagonizing these endogenous signaling pathways and in so doing can attenuate the pathophysiological consequences of excess histamine release associated with conditions such as allergic rhinitis or conjunctivitis.

Ocular allergy, like most allergic conditions, includes both an early phase and a late phase.[3] The early phase represents an acute response to allergen exposure, and is typically characterized by pruritus and conjunctival erythema. Both of these manifestations result from antigen-induced release of histamine and subsequent activation of histamine H_1 (and perhaps H_2) receptors.[1,3–5] Most drugs commonly used for treatment of ocular allergy are H_1 receptor antagonists/inverse agonists, and so are well suited

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to treat early phase symptoms. In contrast, the late phase is a delayed response that often occurs without sustained allergen exposure. Because the late phase involves inflammatory responses that are not strictly a result of histamine release, they tend to be less responsive to antihistamine therapy alone.

The late or delayed phase of an allergic response can occur hours, days, or months after the initial allergen challenge. During this phase, immune cells (eg, dendrocytes, basophils, and eosinophils) are recruited to the site of the allergic response, and allergic symptoms including edema, pruritus, erythema, and excess tearing persist. While dual action H_1 antihistamines (H_1 antagonism and mast cell stabilization) are more effective against the late phase than H_1 antagonists alone, the best available therapy for late phase symptoms are steroids such as loteprednol.[6] However, steroids do not effectively treat the early phase, and unlike antihistamines, they have significant ocular side effects and so are not considered "first-line" therapy. Thus, an improvement over current treatment would be a compound with a combination of early and late phase efficacy without steroid side effects.

Preliminary studies of alcaftadine (6, 11-dihydro-11-(1-methyl-4-piperidinylidene)-5*H*-imidazo [2, 1-b] [3] benzazepine-3-carboxaldehyde (CAS# 147084-10-4); Figure 1) established that the drug had antihistamine activity in several well established in vivo models. Several clinical studies have demonstrated the efficacy of alcaftadine as a treatment for allergic conjunctivitis[7,8] and a topical formulation (alcaftadine 0.25% ophthalmic solution; Lastacaft®, Allergan Inc, Irvine, CA, USA) was approved for use by the US Food and Drug Administration in 2010. In addition, Bohets et al reported on the pharmacokinetic and safety aspects of oral and topical formulations.[9]

Alcaftadine is structurally similar to the H_1 antagonist, ketotifen, but exhibits a spectrum of therapeutic activities that distinguish it from ketotifen and other similar drugs.

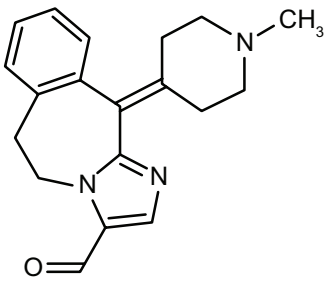

Figure 1 Structure of alcaftadine.

In the studies described here, alcaftadine was tested in an in vivo paradigm, the guinea pig conjunctival allergy challenge model. In this model, alcaftadine was effective in prevention of both early and late phase conjunctival symptoms. To understand better the role of histamine receptor antagonism underlying the action of alcaftadine, receptor binding studies were conducted. Alcaftadine was shown to be a high affinity ligand for both the histamine H_1 and H_2 receptors, and also showed H_4 receptor antagonism in a functional assay.

Materials and methods

Materials

Alcaftadine (R89674, lot #06P0149) was synthesized and analyzed as described previously.[10] For in vivo experiments, the vehicle used for the preparation of 10 mM stock solution was phosphate buffer pH 7.2; for binding experiments, the vehicle was dimethylsulfoxide. [^{3}H]-pyrilamine (30 Ci/mmol), [^{125}I]-aminopotentidine (2200 Ci/mmol), and [^{3}H]-histamine (18.1 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA, USA). Chlorophenol red β-D-galactopyranoside was from Roche Molecular Biochemicals (Indianapolis, IN, USA). All other compounds and reagents, including ketotifen fumarate, pheniramine maleate, histamine dihydrochloride, and forskolin, were purchased from Sigma-Aldrich (St Louis, MO, USA).

Dog Ascaris allergy test

The assay was performed as previously indicated.[11] Briefly, Ascaris allergens were injected at three skin sites close to the chest in Beagle dogs. Fifteen minutes later, the wheal diameters and skin thickness increases were measured. After these time zero measurements, alcaftadine or vehicle was administered orally to the dogs, with five dogs per group. Four hours later, three injections of Ascaris allergens were given and the measurements were repeated.

Guinea pig conjunctival challenge studies

These studies employed male albino Dunkin-Hartley guinea pigs weighing 200–250 g. Sensitizing antigen was Al $(OH)_3$-adsorbed rabbit squames, washed by centrifugation with sterile saline to remove the phenol preservative. The animals were anesthetized with ketamine 50 mg/kg, and sensitized with a 50 µL intramuscular injection of antigen. Sixteen days later (one day prior to conjunctival challenge) they were given oral doses of vehicle, alcaftadine, or the control drug (ketotifen or dexamethasone) at the doses indicated in Figure 2. Twenty-four hours later (day 17),

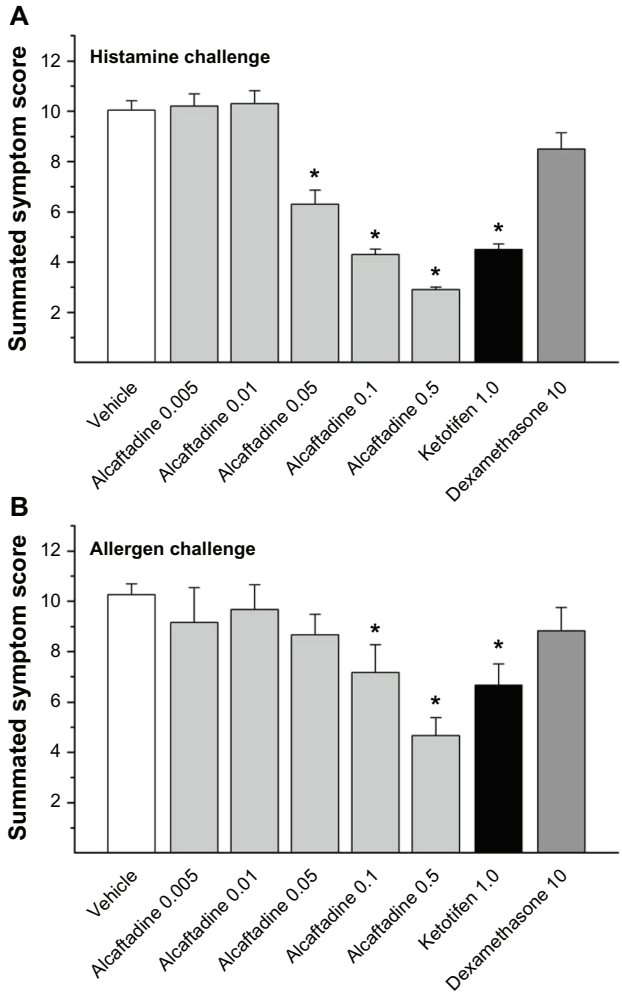

Figure 2 Alcaftadine prevents histamine-evoked and allergen-evoked symptoms in a guinea pig conjunctival allergy challenge model.

Notes: The top graph shows a dose-dependent decrease of summated symptom scores (gray bars) as a function of increasing alcaftadine doses (doses in mg/kg are shown under the bars) in response to instillation of histamine hydrochloride. The results for ketotifen and dexamethasone are also shown. The bottom graph shows a similar dose-dependent decrease of summated symptom scores in response to allergen instillation. Statistically significant changes in symptom scores relative to vehicle using the Wilcoxon–Mann–Whitney rank sum test are indicated (*$P < 0.01$).

another dose of the test drugs was administered. One hour later, the left eye was challenged by instillation of 25 µL of normal rabbit serum, while the right eye was challenged with 25 µL of 1.5 mg/mL histamine dihydrochloride. Acute phase reactions were assessed 30 minutes after allergen or histamine challenge by scoring edema and erythema in both the tarsal and bulbar conjunctiva. Blinded scoring employed a 0–4 symptom scale where 0 = absent, 1 = weak, 2 = moderate, 3 = severe, and 4 = very severe. The animals were examined before challenge, and those with pre-existing edema or erythema were excluded from subsequent analysis. Summation of scores yields an overall possible scale of 0–16.

For late phase assessments, the animals were sacrificed 24 hours after challenge, and the tarsal conjunctiva were excised and stored for subsequent quantification. Eosinophil infiltration into the conjunctiva was estimated by colorimetric assay of eosinophil-specific peroxidase.[12] The tissue was homogenized in 10% sucrose in 0.1 M phosphate buffer, pH 7.4, and pelleted by centrifugation at 10,000 × g. The pellets were resuspended in 2 mL of 0.1 M Na_2SO_4, 0.1 M $C_2H_3NaO_2$, and 0.1% cetyl-trimethyl ammonium bromide pH 7.4, and incubated for 2 hours at 4°C to allow for eosinophilic granule lysis. The samples were then frozen in liquid N_2 and stored at −20°C. Thawed samples were assayed for peroxidase by addition of 0.27 mM 3, 3′, 5, 5′-tetra methyl benzidine plus 2.8 mM H_2O_2, followed by incubation for 9 minutes at 37°C. The reactions were stopped by addition of 2 N H_2SO_4, and then measured spectrophotometrically at 450 nM (with background subtraction at 650 nM). Each sample was assayed in serial dilution to insure that the signal was in a linear range; the values plotted represent optical density measures "per eyelid". Tissues from several animals that were neither sensitized nor challenged were included as a measure of baseline conjunctival eosinophil peroxidase activity. Treated animals were compared with controls using the Wilcoxon–Mann–Whitney rank sum test. Two-sided *P* values ≤0.01 were considered to be statistically significant.

Transfected cell membrane preparations

Transfection of cloned cDNAs for the human H_1, H_2, H_3, and H_4 receptors were performed using established methods for expression of cloned genes in cultured cell lines. H_1 receptors were expressed in human kidney fibroblasts (HEK-293) cells as previously described,[13] and H_2 receptors were expressed in Chinese hamster ovary fibroblasts (CHO) cells also as described elsewhere.[14] Preparation of the H_3 and H_4 receptor cell lines and membranes was as described previously.[15] For H_1 or H_2 receptor binding studies, transfected cells were collected by scraping and homogenized in Tris-HCl 50 mM pH 7.4 using an Ultra Turrax homogenizer. The homogenate was centrifuged for 10 minutes at 23,500 × g, and the membrane pellets were washed once by rehomogenization and recentrifugation. Washed membranes were suspended in Tris-HCl 50 mM (pH 7.4), aliquoted, and stored at −80°C. For H_3 and H_4 receptor binding studies, transfected cells were collected in 20 mM Tris-HCl/0.5 mM ethylenediamine tetra-acetic acid (pH 8, TE buffer). Cell homogenates were cleared by centrifugation for 10 minutes at 800 × g, and the supernatants were collected and recentrifuged at 30,000 g for 30 minutes. The pellets were rehomogenized

in TE buffer, aliquoted, and stored at −80°C. The membrane protein content was determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Radioligand binding experiments

For H_1 receptor binding studies, membrane samples were incubated for 60 minutes with 3 nM [^{3}H]-pyrilamine (K_D for human H_1, 1.7 nM); nonspecific binding was measured by addition of 1 μM unlabeled pyrilamine as previously described.[13] For the H_2 receptor studies, membrane samples were incubated for 120 minutes at 22°C with 0.2 nM [^{125}I]-aminopotentidine (2200 Ci/mmol; K_D for human H_2, 2.9 nM); nonspecific binding was measured by addition of 100 μM unlabeled tiotidine as previously described.[14] H_3 and H_4 receptor binding assays were conducted as described elsewhere.[15] Following incubation, bound ligand was isolated by filtration and counted by scintillation. Specific binding was defined as the difference between the total binding and nonspecific binding determined in the presence of an excess of unlabelled ligand. Displacement of radioligand by pheniramine, ketotifen, olopatadine, desloratadine, or alcaftadine was expressed as a percent of control binding in the presence of increasing concentrations of the test compound. The IC_{50} values (concentration of competitor yielding 50% of maximal specific binding), were calculated from logistic curve fits of plotted binding versus concentration data. The inhibition constants (K_i) for each compound were calculated using the method of Cheng and Prusoff, and converted to pK_i values (–log of K_i) for comparison.[16] For each competition, seven concentrations of compound were tested in triplicate. Unless noted otherwise, triplicate binding assays were repeated to confirm derived constants at least once. Additional competition binding experiments were conducted to assess the ability of alcaftadine to interact with other cellular targets. These methods are described in the Supplementary Materials section.

Functional activity at the human H_4 receptor

The cAMP assay for the human H_4 receptor was carried out in SK-N-MC cell lines that express β-galactosidase under the control of cyclic AMP-responsive elements, and were carried out as previously reported.[15] The values for the duplicates were averaged and used to calculate the 50% effective concentration (EC_{50}) for inhibition of cyclic AMP production. For Schild analysis, a titration of histamine from 10^{-10} to 10^{-3} M was run in duplicate in the presence of alcaftadine 1.2, 3.7, 11, 33, and 100 μM. Duplicates were averaged, and the EC_{50} values for histamine at each concentration were used to generate the Schild plot.

Results

Alcaftadine activity in a model of allergic conjunctivitis

Preliminary studies of alcaftadine demonstrated that it exhibited properties of a classic H_1 antihistamine, including suppression of wheal and flare responses to intracutaneous antigen challenge in dogs with an ED_{50} of 7.1 μg/kg when given orally. To assess its potential efficacy in a more comprehensive way, we examined alcaftadine activity in a combined acute/late phase allergic model (guinea pig conjunctival challenge) and compared it with two agents (ketotifen and dexamethasone) with demonstrated efficacy for these two phases of allergic conjunctivitis. The results of these experiments suggest that alcaftadine has properties beyond that of a simple H_1 antagonist.

Figure 2 shows the results of the acute phase study for both histamine-induced and allergen-induced conjunctivitis. Alcaftadine shows a dose-dependent ability to reduce the overall edema and erythema response to both agents. By comparison, a 0.1 mg/kg dose of alcaftadine elicited an effect of the same approximate magnitude as ketotifen1.0 mg/kg, the positive control. Note that the steroid, dexamethasone, is ineffective in the acute phase test. To assess late phase effectiveness, eosinophil infiltration was measured by quantifying the levels of eosinophil-specific peroxidase, and the results are shown in Figure 3. Alcaftadine displays a dose-dependent ability to reduce eosinophil levels in the conjunctiva, and the reduction is statistically significant at both 0.1 and 0.5 mg/kg doses. In contrast, ketotifen reduces eosinophil peroxidase, but not to a level of statistical significance. At the highest doses tested, the reduction in eosinophil peroxidase by alcaftadine is equal to that shown by the steroid, the positive control for this assay of delayed allergic conjunctivitis. It is unknown if the effect is specific to eosinophils or a more generalized effect on overall extravasation during the late phase.

Binding to human histamine receptors

Experiments were performed to determine the affinity of alcaftadine for histamine as compared with the ocular antihistamines, ketotifen and pheniramine. As shown in Figure 4A and B, binding displacement curves were calculated for alcaftadine, ketotifen, and pheniramine at both H_1 and H_2 receptors. For each competition curve, an IC_{50} value was determined, and inhibition constants (K_i) were calculated

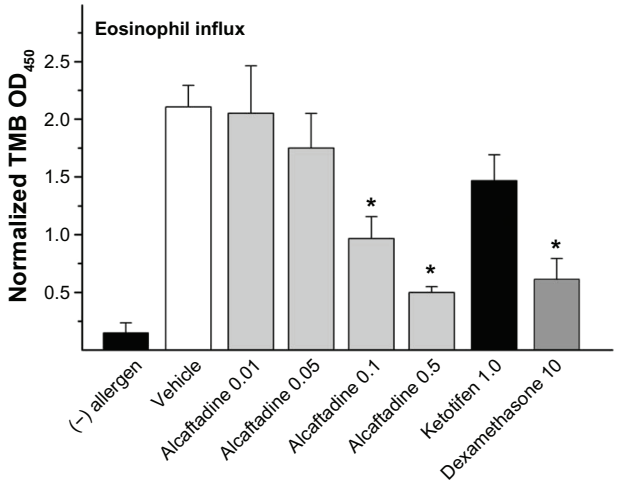

Figure 3 Alcaftadine prevents delayed conjunctival eosinophil influx in a guinea pig conjunctival allergy challenge model.
Notes: Eosinophil peroxidase levels in conjunctival tissue samples reveal a dose-dependent attenuation of influx by alcaftadine (doses in mg/kg are shown under the bars). The graph also shows the degree of influx resulting from allergen sensitization (allergen versus vehicle) as well as the effect of control drugs, ie, ketotifen and dexamethasone. Statistically significant changes in symptom scores relative to vehicle using the Wilcoxon–Mann–Whitney rank sum test are indicated (*$P < 0.01$).
Abbreviations: OD, optical density; TMB, 3,3',5,5'-tetramethylbenzidine.

using the Cheng-Prusoff equation and given as pK_i. As summarized in Table 1, the pK_i values for alcaftadine, ketotifen, and pheniramine at H_1 receptors were 8.5, 9.2, and 6.7, respectively. At the H_2 receptors, the pK_i values were 7.2 for alcaftadine, 6 for ketotifen, and <5 for pheniramine. These results show that alcaftadine binds to H_1 receptors with slightly lower affinity than ketotifen, but with much higher affinity than pheniramine. Alcaftadine showed a higher affinity for H_2 receptors than H_1 antihistamines. Alcaftadine showed no affinity for the H_3 receptor (Table 1). Binding data for olopatadine and desloratadine at the various histamine receptors are also given for comparison (Table 1). Previous data have shown that olopatadine has high affinity for the H_1 receptor ($pK_i = 7.5$), but little if any affinity for H_2 and H_3 receptors (pK_i about 4).[17]

Activity at the human H_4 receptor

The histamine H_4 receptor is expressed primarily on cells of hematopoietic origin, including basophils and eosinophils.[2] Previously, H_4 activation has been shown to mediate eosinophil chemotaxis in mouse models of asthma and dermatitis.[18–20] This, combined with the reduction of eosinophils in the guinea pig conjunctival studies, suggests a possible interaction between alcaftadine and the H_4 receptor. Therefore, alcaftadine was tested for the ability to bind to the histamine H_4 receptor. Results shown in Figure 4C indicate that alcaftadine binds to the H_4 receptor with an IC_{50} value of 4.4 ± 0.1 µM. This yields a pK_i of 5.8. Ketotifen and

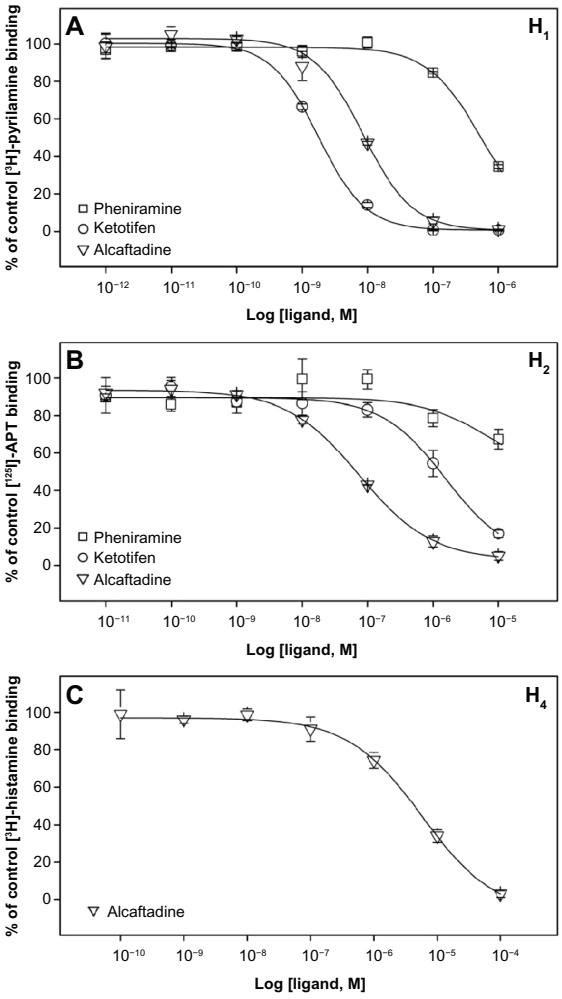

Figure 4 Alcaftadine binds specifically to human H_1, H_2, and H_4 receptors.
Notes: Competition binding studies demonstrate that alcaftadine is a high-affinity ligand for both H_1 (top panel), H_2 (middle panel), and H_4 (lower panel) receptors. For the H_1 and H_2 receptor experiments, alcaftadine was compared with two other H_1 antagonists, ie, ketotifen and pheniramine. Ketotifen shows a higher affinity at H_1 than alcaftadine (IC_{50} 1.9 nM and 8.6 nM, respectively) while alcaftadine has a higher affinity for the H_2 receptor (IC_{50} 1100 nM and 62 nM). The lower panel shows binding of alcaftadine to the H_4 receptor, with an IC_{50} of 4.4 µM.

oloaptadine have no affinity for the H_4 receptor, whereas the affinity of desloratadine is weak. The functional activity of alcaftadine was then characterized in a cell-based assay. In this assay, histamine acting via H_4 receptors causes a dose-dependent inhibition of a forskolin-induced increase in cAMP. Alcaftadine was first tested in the absence of histamine to confirm that it possesses no agonist activity at the H_4 receptor (data not shown). To test whether alcaftadine could act as antagonist at the H_4 receptor, the ability of the compound to shift the EC_{50} of histamine inhibition of the forskolin-induced increase in cAMP was assessed. The results are shown in Figure 5, and indicate that increasing concentrations of alcaftadine caused parallel and rightward shifts in the histamine dose response curves, leading to

Table 1 pK_i and IC_{50} (nM) values for human histamine receptors expressed in vitro[a]

	H_1		H_2		H_3		H_4	
	pK_i	IC_{50}	pK_i	IC_{50}	pK_i	IC_{50}	pK_i	IC_{50}
Alcaftadine	8.5 ± 0.1	8.6 ± 1.2	7.2 ± 0.1 (nM)	62 ± 3.8	<5	>10,000	5.8 ± 0.1	4400 ± 100
Ketotifen	9.2 ± 0.1	1.9 ± 0.2	6.0 ± 0.2	1100 ± 280		–	<5	>10,000
Pheniramine	6.7 ± 0.1	540 ± 22	<5	>10,000		–	<5	>10,000
Olopatadine		–		–		–	<4	>100,000
Desloratadine	8.8 ± 0.2	4.5 ± 0.1			<5	>10,000	5.1 ± 0.1	22,000 ± 300

Notes: [a]Values from this study represent mean value ± standard deviation of triplicate measures, with a minimum of two determinations; "–"represents not determined.

an increase in the apparent EC_{50} for histamine activation. This result demonstrates that alcaftadine is a competitive antagonist of the H_4 receptor. A transform of the data (Schild plot) gave a pA_2 value of 5.6, representing the negative log of the concentration of antagonist needed to induce a two-fold shift in the histamine EC_{50}. The Schild analysis allows for comparison of binding displacement with the functional assay. Theoretically, the pA_2 value should be equal to the pK_i if the inhibition is due to competition at the H_4 histamine binding site. This is consistent with what was observed, with a pA_2 of 5.6 and a pK_i of 5.8. While this represents a relatively low affinity, it remains possible that an alcaftadine-H_4 receptor interaction may underlie some aspect of its overall therapeutic effect.

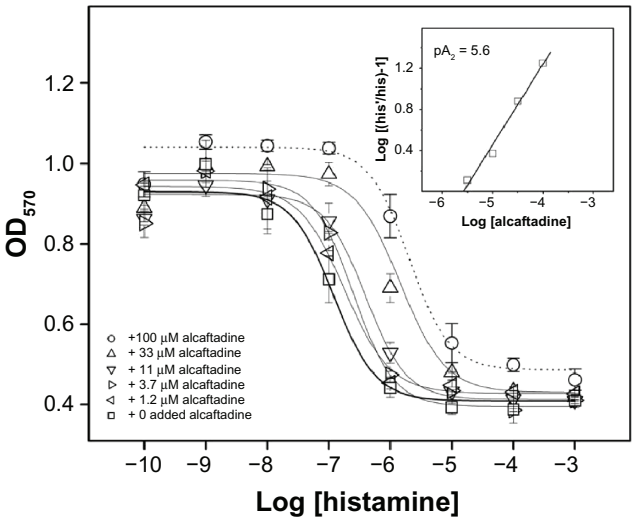

Figure 5 Alcaftadine is a competitive antagonist of histamine activation at the human H_4 receptor.

Notes: The main panel shows histamine dose-response relationships in the presence of increasing concentrations of alcaftadine. The dose response curve in the absence of alcaftadine (thick line) shifts rightward as the alcaftadine concentration is increased; the dotted curve represents the histamine dose response at the highest alcaftadine concentration. These data can be transformed using a Schild plot (inset) to derive a pA_2, ie, the concentration of antagonist that results in a two-fold decrease in agonist efficacy. That value, 5.6, is close to the pK_i of 5.8 obtained from the binding study in Figure 4.

Additional binding activity of alcaftadine

The evidence suggests that ocular antihistamines may interact with other receptors or ligand binding sites, resulting in any number of unwanted side effects.[21] Additional competitive binding experiments were conducted to assess the ability of alcaftadine to interact with other cellular targets. These are summarized in Table 2. The only significant binding activities detected in these studies were for the $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors, with pK_i values of 5.6 and 5.7, respectively. Weak affinity was noted for α1 adrenergic receptors, $5\text{-}HT_{1A}$ receptors, melanocortin MC4 receptors, and muscarinic cholinergic receptors.

Discussion

Allergic conjunctivitis is a prevalent condition affecting many people who suffer from seasonal and perennial allergies. From a pharmacological perspective, it is well established that activation of histamine H_1 receptors underlies many aspects of the allergic response.[3,22] Likewise, there is evidence to suggest that histamine H_2 receptors play a role in ocular signs and symptoms, such as conjunctival redness.[5,23,24] More recent identification of a fourth isoform of the histamine receptor, ie, the H_4 receptor, led to studies suggesting that this receptor may also play a role in the pathogenesis of ocular allergies.[25,26] Despite these observations, most of the drugs currently used to treat allergic conjunctivitis are either H_1 antagonists/inverse agonists or dual action H_1 antagonist/mast cell stabilizers.[6] Thus, it would seem that a drug with a broad spectrum (ie, one with potential for physiological effects at multiple receptor isoforms) of histamine receptor antagonism could have the potential to represent an advance over current therapeutic approaches. In this report, we have characterized the pharmacology of a newly approved antihistamine, alcaftadine, which displays just such properties.

In vivo studies have demonstrated that alcaftadine is comparable with ketotifen in prevention of conjunctival

Table 2 Binding of alcaftadine to various receptors, ion channels, and transporter proteins

Target	Species	Tissue or cell	Ligand	pK_i[a]
Adrenergic α_1	Rat	Cortex	[^{3}H]-prazosin	<5
Adrenergic α_{2a}	Human	CHO	[^{3}H]-rauwolscine	5.1
Adrenergic α_{2b}	Human	CHO	[^{3}H]-rauwolscine	<5
Adrenergic α_{2c}	Human	CHO	[^{3}H]-rauwolscine	<5
Adrenergic β_1	Human	*E. coli*	[^{125}I]-ICP	<5
Adrenergic β_2	Human	*E. coli*	[^{125}I]-ICP	<5
Dopamine D_1	Rat	Striatum	[^{3}H]-SCH23390	<5
Dopamine D_{2L}	Human	CHO	[^{3}H]-Spiperone	<5
Dopamine D_3	Human	CHO	[^{125}I]-iodosulpride	<5
Dopamine D_4	Human	L929	[^{3}H]-Spiperone	<5
5-HT_{1a}	Human	L929	[^{3}H]-8-OH-DPAT	5.4
5-HT_{1b}	Human	HEK293	[^{3}H]-5-HT	<5
5-HT_{1d}	Human	C6 glioma	[^{3}H]-alniditan	<5
5-HT_{1e}	Human	L929	[^{3}H]-5-HT	<5
5-HT_{2a}	Human	L929	[^{125}I]-RO91150	5.6
5-HT_{2c}	Pig	Choroid plexus	[^{3}H]-mesulergine	5.7
5-HT_3	Human	NxG 108CC15	[^{3}H]-GR65630	<5
5-HT_4	Guinea pig	Striatum	[^{3}H]-GR113808	<5
Muscarinic Ach	Rat	Striatum	[^{3}H]-dexetimide	5.2
NMDA	Rat	Forebrain	[^{3}H]-MK-801	<5
AMPA	Rat	Forebrain	[^{3}H]-Ro488587	<5
Opiate M	Rat	Forebrain	[^{3}H]-sufentanil	<5
Opiate K	Guinea pig	Cerebellum	[^{3}H]-U69593	<5
Opiate Δ	Human	C6 glioma	[^{3}H]-DPDPE	<5
Sigma σ_1	Guinea pig	Medulla	[^{3}H]-haloperidol	<5
Bradykinin-B_2	Human	CHO	[^{3}H]-bradykinin	<5
CCK-A	Rat	Pancreas	[^{3}H]-CCK-8	<5
CCK-B	Guinea pig	C6 glioma	[^{3}H]-CCK-8	<5
NK_1	Human	CHO	[^{3}H]-Substance P	<5
NK_2	Human	Sf9	[^{3}H]-SR48968	<5
NPY_1	Human	Sf9	[^{125}I]-PYY	<5
MC4	Human	CHO	[^{125}I]-AGRP	5.3
Dopamine transporter	Rat	Striatum	[^{3}H]-WIN35428	<5
Serotonin transporter	Human	Platelet	[^{3}H]-paroxetine	<5
Norepinephrine transporter	Rat	Cortex	[^{3}H]-nisoxetine	<5
Ca^{2+} channel	Human	Cortex	[^{3}H]-nitrendipine	<5
Na^+ channel	Human	Cortex	[^{3}H]-batrachotoxin	<5
HERG channel	Human	HEK293	[^{3}H]-astemizole	<5

Notes: [a]Values represent a single determination (when $pK_i < 5$) or triplicate determination except for compounds where $pK_i > 5$; for these compounds, n = 3.
Abbreviations: Ach, acetylcholine; CHO, Chinese hamster ovary fibroblasts; *E. coli*, *Escherichia coli*.

symptoms in a guinea pig allergen challenge model. The oral route of administration used in these studies makes it difficult to make quantitative comparisons, but it is clear from these studies that alcaftadine has the potential to be a useful new therapy for allergic conjunctivitis. In addition, this same study demonstrated a unique aspect of alcaftadine action, ie, in the same guinea pig conjunctival allergen challenge model, the drug was able to reduce the level of eosinophil infiltration to an extent similar to that of the steroid, dexamethasone. Ketotifen did not exhibit a similar ability, although there are reports that ketotifen has immunosuppressant activity.[27]

Other H_1 antagonists are also reported to exhibit immunomodulatory or anti-inflammatory activity,[27] but none of these have included the apparent inhibition of eosinophil infiltration observed with alcaftadine. Activation of the H_2 receptor has been shown to inhibit chemotaxis of eosinophils in vitro[28,29] and the H_2 receptor antagonist, cimetidine, has been shown to have no effect on eosinophils in a guinea pig conjunctival challenge study.[30] However, the combination of an H_1 antagonist and an H_2 antagonist did reduce eosinophils in the same study,[30] and may explain the effect of alcaftadine on this parameter because it has affinity at both receptors.

The effects on eosinophil recruitment may also suggest the involvement of the H_4 receptor in the mechanism of alcaftadine action, and results of H_4 receptor binding and functional studies confirm that alcaftadine is an H_4 receptor antagonist in vitro. The H_4 receptor is expressed on eosinophils and has been shown to mediate eosinophil chemotaxis.[20] The H_4 receptor is present in conjunctiva and increases in inflammatory conditions, whereas there is no evidence that the H_3 receptor is expressed.[31] In mouse models of asthma and dermatitis, treatment with an H_4 receptor antagonist leads to a reduction in infiltration of eosinophils, similar to what was seen in the model presented here.[18,19] Evidence for H_4 receptor involvement in conjunctivitis and rhinitis has been reported in mouse models of these allergic conditions.[25,32] In both of these reports, the H_4 receptor antagonist, JNJ7777120, significantly reduced allergic symptoms, and the effect could be ascribed to blockade of H_4 receptors. Combined treatment with JNJ7777120 and the H_1 antagonist levocabastine reduced signs and symptoms of allergic conjunctivitis to a greater extent than either agent alone.[25] This additive effect of the H_1 + H_4 receptors suggests that both receptors are involved in the etiology of the disease. In light of the potential role of the H_4 receptor in conjunctivitis, the H_4 receptor antagonist activity, and the effects on eosinophils observed in the preclinical model with alcaftadine, it is intriguing to speculate that antagonism of the H_4 receptor may contribute to its in vivo activity. However, the affinity for the H_4 receptor is modest, and so based upon this evidence alone, the role of H_4 receptors in the therapeutic effects of alcaftadine in allergic conjunctivitis is uncertain. Further studies will be required to address this issue unequivocally.

Another study of the action of alcaftadine in a model of allergic conjunctivitis was published recently.[33] This study showed an effect of the drug on eosinophil recruitment in mice similar to the one we report here; this effect was not seen for the H_1 antagonist, olopatadine. To test for a possible effect of alcaftadine and olopatadine on conjunctival epithelial stability, these authors also examined staining patterns for two conjunctival tight junction proteins, ZO-1 and E-cadherin. Alcaftadine was able to attenuate changes in protein expression observed in a chronic allergy paradigm, while olopatadine did not. Because these results are not readily attributable to an H_1 antagonist, they suggest that alcaftadine exerts actions on conjunctival stability via another transduction pathway.

Given the number of recent studies implicating signaling pathways beyond the H_1 receptor in allergic conjunctivitis,[34] we examined the receptor binding properties of alcaftadine at both H_1 and H_2 receptors using ketotifen as the primary comparator. Alcaftadine exhibited high affinity for both H_1 and H_2 receptors, while ketotifen appeared to have relative selectivity (about 1000-fold higher) for the H_1 receptor. Alcaftadine demonstrated a nanomolar affinity for H_2 receptors that was higher than that of ketotifen or pheniramine. The higher affinity of alcaftadine for H_2 receptors (relative to other H_1 antihistamines) may improve efficacy in the treatment of redness associated with allergic conjunctivitis.[23] With the exception of affinity for 5-HT_{2A} and 5-HT_{2C} receptors, alcaftadine did not demonstrate significant affinity for other receptors or potential "off-target" drug binding sites, so there is little likelihood of untoward side effects associated with adrenergic and muscarinic receptors in the eye. The affinity of alcaftadine at 5-HT_2 receptors appears to be similar to that reported for both ketotifen and olopatadine.[17]

Alcaftadine is an antihistamine with a unique broad spectrum of affinities for the histamine H_1, H_2 and, to a lesser extent, H_4 receptor subtypes, with no affinity for the H_3 receptor. No other antihistamine compound characterized to date exhibits this combination of affinities, suggesting that it may act as a physiological antagonist at all three receptors. Recent studies of seasonal allergic conditions such as allergic conjunctivitis suggest that H_1, H_2, and H_4 histamine receptor subtypes all participate in one or more phases of the immediate or delayed immune response that underlies ocular allergy.[1,3,34] Thus, alcaftadine may represent a significant advance over the agents currently used in the treatment and prevention of conditions such as allergic conjunctivitis. The extent to which the overall mechanism of action of alcaftadine depends upon interaction at each of these receptors remains to be determined.

Disclosure

Vistakon Pharmaceuticals, a division of Johnson & Johnson, is the developer of alcaftadine, which is marketed as Lastacaft®. Both authors are employees of divisions of Johnson & Johnson. The authors would like to acknowledge J McLaughlin from Ora for assistance with writing, and AAHP Megens, FHL Awouters, J Vermeire, G Smets, H Bruwiere, L Wouters, F Janssens, G Van Den Kieboom, G Daneels, T Jansen, and M Borgers of Janssen Research Foundation for work on the in vivo models.

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Supplementary material

Methods

Tissue or membrane suspensions were prepared from brain areas, organs, blood of animal or human origin, or cell lines derived from tumors or from cells expressing cloned human genes. The protein content was determined using a Bradford protein assay. Membrane fractions of cells or tissue homogenates were incubated with a radioactively labeled substance [^{3}H]- or [^{125}I] ligand to label a particular receptor. Experimental conditions of the binding assays for the various receptors and ion channel and monoamine transporter binding sites are summarized in Table S1. Specific receptor binding of the radioligand was distinguished from nonspecific membrane labeling by selectively inhibiting the receptor labeling with an unlabeled drug known to compete with the radioligand for binding to the receptor sites. The remaining nonspecific labeling was subtracted from all assays. Alcaftadine was dissolved in dimethylsulfoxide, and various dilutions ranging from 10^{-9} to 10^{-5} M were prepared. Details for each individual assay are given in Table S1.

Table S1

Receptor	Species	Source	Assay conditions	Labeled ligand	Nonspecific
		Cell line or tissue	Buffer, volume, temperature, time	Name, concentration, K_d (nM)	
α_1-adrenergic	Rat	Cortex	C, pH 7.7, 0.5 mL, 25°C, 60 minutes	[^{3}H]Prazosin, 0.25 nM, 0.15	Aceperone, 1 μM
α_{2A}-adrenergic	Human	CHO	J, pH 7.6, 0.5 mL, 25°C, 30 minutes	[^{3}H]Rauwolscine, 1 nM, 0.23	Oxymetazoline, 1 μM
α_{2B}-adrenergic	Human	CHO	J, pH 7.6, 0.5 mL, 25°C, 30 minutes	[^{3}H]Rauwolscine, 1 nM, 0.41	Spiroxatrine, 1 μM
α_{2C}-adrenergic	Human	CHO	J, pH 7.6, 0.5 mL, 25°C, 30 minutes	[^{3}H]Rauwolscine, 1 nM, 0.07	Spiroxatrine, 1 μM
α_1-adrenergic	Human	*E. coli*	C, pH 7.4, 0.5 mL, 37°C, 60 minutes	[^{125}I]Iodocyanopindolol, 0.025 nM, 0.029	Propranolol, 1 μM
α_2-adrenergic	Human	*E. coli*	C, pH 7.4, 0.5 mL, 37°C, 60 minutes	[^{125}I]Iodocyanopindolol, 0.025 nM, 0.017	Propranolol, 1 μM
D_1	Rat	Striatum	C, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]SCH23390, 0.25 nM, 0.39	Piflutixol, 1 μM
D_{2L}	Human	CHO	N, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]Spiperone, 0.2 nM, 0.05	Butaclamol, 1 μM
D_3	Human	CHO	F, pH 7.7, 0.25 mL, 37°C, 60 minutes	[^{125}I]Iodosulpride, 0.2 nM, 0.56	TL99, 1 μM
D_4	Human	L929	C, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]Spiperone, 0.5 nM, 0.146	Haloperidol, 1 μM
5-HT_{1A}	Human	L929	B, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]8-OH-DPAT, 0.5 nM, 1.62	Spiroxatrine, 1 μM
5-HT_{1B}	Human	HEK293	O, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]5-HT + 8OH-DPAT 30 nM + mesulergine 30 nM, 4 nM, 2.3	5-HT, 10 μM
5-HT_{1D}	Human	C6 glioma	C, pH 7.4, 0.5 mL, 37°C, 30 minutes	[^{3}H]Alniditan, 2 nM, 0.89	Sumatriptan, 10 μM
5-HT_{1E}	Human	L929	B, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]5-HT, 4 nM, 4.27	5-HT, 1 μM
5-HT_{2A}	Human	L929	C, pH 7.4, 0.25 mL, 37°C, 60 minutes	[^{125}I]R091150, 0.1 nM, 0.074	BW501, 1 μM
5-HT_{2C}	Pig	Choroid plexus	B, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]Mesulergine, 1 nM, 0.72	Ritanserin, 1 μM
5-HT_3	Mouse	NXG 108CC15	L, pH 7.4, 0.5 mL, 37°C, 30 minutes	[^{3}H]GR65630, 2 nM, 2.12	ICS-205930, 1 μM
5-HT_4	Guinea pig	Striatum	P, pH 7.4, 0.5 mL, 37°C, 20 minutes	[^{3}H]GR113808, 0.1 nM, 0.25	5-HT, 100 μM
Cholinergic muscarinic	Rat	Striatum	D, pH 7.5, 0.5 mL, 37°C, 30 minutes	[^{3}H]Dexetimide, 2 nM, 0.50	Dexetimide, 1 μM
Dopamine transporter	Rat	Striatum	U, pH 7.4, 0.5 mL, 0°C, 60 minutes	[^{3}H]WIN35428, 2 nM, 35	Mazindol, 1 μM
Serotonin transporter	Human	Platelets	V, pH 7.4, 0.5 mL, 25°C, 60 minutes	[^{3}H]Paroxetine, 0.5 nM, 0.15	Imipramine, 1 μM
Norepinephrine transporter	Rat	Cortex	V, pH 7.4, 0.5 mL, 25°C, 60 minutes	[^{3}H]Nisoxetine, 2 nM, 1.86	Mazindol, 1 μM
NMDA-MK801 site	Rat	Forebrain	M, pH 7.4, 0.5 mL, 37°C, 60 minutes	[^{3}H]MK801, + glycine 1 μM, + glutamate 1 μM, 3 nM, 12	Phencyclidine, 10 μM
NMDA glycine site	Rat	Forebrain	M, pH 7.4, 0.5 mL, 0°C, 30 minutes	[^{3}H]Glycine + strychnine 100 μM, 20 nM, 137	D-serine, 100 μM
AMPA receptor	Rat	Forebrain	M, pH 7.4, 0.5 mL, 0°C, 60 minutes	[^{3}H]Ro488,587, + KSCN 1 mM, 2 nM, 3.05	L-glutamate, 1 mM
μ-Opiate	Rat	Forebrain	A, pH 7.4, 0.5 mL, 37°C, 30 minutes	[^{3}H]sufentanil, 0.5 nM, 0.13	Dextromoramide, 1 μM
κ-Opiate	Human	C6 glioma	E, pH 7.4, 0.5 mL, 25°C, 60 minutes, P	[^{3}H]DPDPE, 2 nM, 1.38	Naltrindole, 1 μM

(*Continued*)

Table S1 (*Continued*)

Receptor	Species	Source	Assay conditions	Labeled ligand	Nonspecific
		Cell line or tissue	Buffer, volume, temperature, time	Name, concentration, K_d (nM)	
δ-Opiate	Guinea pig	Cerebellum	A, pH 7.4, 0.5 mL, 25°C, 60 minutes, P	[^{3}H]U69593, 2 nM, 1.53	Spiradoline, 1 μM
σ_1	Guinea pig	Medulla oblongata	A, pH 7.7, 0.5 mL, 25°C, 60 minutes	[^{3}H]Haloperidol, 1 nM, 0.58	+-3PPP, 10 μM
Bradykinin B_2	Human	CHO	Q, pH 7.4, 0.5 mL, 25°C, 30 minutes	[^{3}H]Bradykinin, 0.5 nM, 0.24	Bradykinin, 1 μM
CCK-A	Rat	Pancreas	I, pH 7.4, 0.5 mL, 25°C, 30 minutes	[^{3}H]CCK8, 2 nM, 1.62	Devazepide, 1 μM
CCK-B	Guinea pig	Cortex	I, pH 7.4, 0.5 mL, 37°C, 30 minutes	[^{3}H]CCK8, 1 nM, 0.57	CCK8 1 μM
Neurokinin NK_1	Human	CHO	E, pH 7.4, 0.5 mL, 25°C, 30 minutes	[^{3}H]Substance P, 0.5 nM, 0.23	Substance P, 0.1 μM
Neurokinin NK_2	Human	Sf9	E, pH 7.4, 0.5 mL, 25°C, 60 minutes	[^{3}H]SR48968, 0.3 nM, 0.172	SR48968, 1 μM
Neuropeptide Y_1	Human	Sf9	H, pH 7.4, 0.2 mL, RT, 20 hours	[^{125}I]PYY, 0.05 nM, 0.079	NPY, 1 μM
MC4	Human	CHO	S, pH 7.4, 0.2 mL, RT, 4 hours	[^{125}I]AGRP, 0.25 nM, 0.85	AGRP[83–132], 1 μM
Ca^{2+} channel	Rat	Cortex	A, pH 7.7, 0.5 mL, 37°C, 30 minutes	[^{3}H]Nitrendipine, 0.1 nM, 0.23	Nifedipine, 1 μM
Na^+ channel	Rat	Cortex	G, pH 7.4, 0.5 mL, 37°C, 30 minutes	[^{3}H]Batrachotoxin B + scorpion venom 3 μg/mL, 1 nM, 21.5	Penfluridol, 1 μM
ERG channel	Human	HEK293	R, pH 7.4, 0.25 mL, 25°C, 60 minutes	[^{3}H]Astemizole, 2 nM, 3.24	JNJ-6823076, 10 μM

Notes: Assay buffer codes: A, Tris-HCl 50 mM; B, Tris-HCl 50 mM, CaCl2 4 mM, pargyline 1 μM; C, Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, MgCl2 1 mM, CaCl2 2 mM; D, Na-K phosphate 50 mM; E, Tris-HCl 50 mM, EGTA 1 mM, $MgCl_2$ 2 mM, BSA 0.1%; F, Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, BSA 0.1%; G, HEPES-Tris 50 mM, choline chloride 130 mM, KCl 5.4 mM, $MgSO_4$ 0.8 mM, glucose 5.5 mM; H, Hepes-NaOH 50 mM pH 7.4, $MgCl_2$ 1 mM, $CaCl_2$ 2.5 mM, bacitracin 0.05%, PMSF 0.1 mM, BSA 0.1%; I, Tris HCl 10 mM, NaCl 120 mM, $MgCl_2$ 10 mM, EGTA 1 mM, soybean trypsin inhibitor, 50 μg/mL, bacitracin 0.2 mM, PMSF 10 μM, BSA 0.1%; J, glycyl glycine NaOH 25 mM; K, Tris-HCl 50 mM, NaCl 300 mM, KCl 5 mM; L, Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM; M, Tris-acetate 50 mM; N, Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, pargyline 10 μM; P, Hepes-NaOH 50 mM; Q, Tris-HCl 50 mM, $MgCl_2$ 2 mM, DTT 1 mM, o-phenantroline 1 mM, bacitracin 0.1 mM, BSA 0.1%; R, Hepes-KOH 10 mM, KCl 40 mM, KH_2PO_4 20 mM, $MgCl_2$ 5 mM, $KHCO_3$ 0.5 mM, glucose 10 mM, glutamate 50 mM, aspartate 20 mM, heptanoic acid 14 mM, EGTA 1 mM, BSA 0.1%, cyclodextrin 0.1%; S, Tris-HCl 50 mM, $MnCl_2$ 3 mM, bacitracin 40 μg/mL, leupeptin 4 μg/mL, chymostatin 2 μg/mL, BSA 0.01%.

Abbreviations: Ach, acetylcholine; CHO, Chinese hamster ovary fibroblasts; *E. coli*, *Escherichia coli*; EGTA, ethylene glycol tetraacetic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride.