A Comparison of the Effects of Olopatadine and Ketotifen on Model Membranes

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ABSTRACT. Olopatadine is a human conjunctival mast cell stabilizer with antihistaminic activity. Ketotifen is an older molecule that possesses antihistaminic activity and is reported to have additional pharmacological properties. The interactions of these two compounds with model membranes (i.e., monolayers of 1-stearoyl-2-oleoyl-sn-glycerophosphocholine at the argon-buffer interface), and natural (i.e., erythrocyte) membranes were compared in an effort to understand the differences in their biological activities. Drug-lipid interaction with monolayers was determined by monitoring the surface pressure as a function of the drug concentration in the aqueous phase supporting the monolayer. Drug interaction with erythrocyte membranes was determined by monitoring changes in the permeability of the membranes to hemoglobin and 6-carboxyfluorescein as a function of drug concentration in the medium. Olopatadine and ketotifen are both intrinsically surface active and both interact with phospholipid monolayers. However, in both the presence and absence of lipid monolayers, the changes in surface pressure induced by olopatadine are lower than those caused by ketotifen. The effects of these two drugs on cell membranes were dramatically different. Exposure of bovine erythrocytes to increasing concentrations of ketotifen (1–10 mM) resulted in complete hemolysis of the cells, whereas olopatadine (1–10 mM) caused only minimal hemolysis (<8%). Consistent results were obtained in experiments measuring the leakage of 6-carboxyfluorescein from erythrocyte ghosts as a more sensitive marker of membrane perturbation. Olopatadine treatment (0.1–10 mM) minimally perturbed the cell membrane while ketotifen (1–10 mM) caused a concentration dependent release of the fluorescent marker. These data demonstrate fundamental differences between the two drugs in their effects on cell membranes. Moreover, the differences are consistent with the surface activities of the two compounds measured in monolayers and with reported differences in their pharmacological activities. These findings offer an explanation for the biphasic non-specific cytotoxic effect of ketotifen on histamine release from mast cells and may account for the nonlytic mast cell stabilizing activity of olopatadine.

Key words: olopatadine – ketotifen – erythrocyte membranes – phospholipid monolayers.

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Introduction

Olopatadine hydrochloride 0.1% (Patanol®) is an effective, topical ocular antiallergic drug which inhibits pro-inflammatory mediator release from human conjunctival mast cells and possesses selective and specific histamine H_1 antagonist activity (1, 2). Ketotifen is a benzocycloheptathiophene compound that has significant antihistaminic activity and has been reported to prevent the release of histamine and other chemical mediators from various mast cell preparations (3, 4). It has recently been approved in the United States as a topical anti-allergic agent. During the evaluation of these two molecules for effects upon human conjunctival mast cell mediator release, olopatadine produced concentration dependent inhibition of histamine release (100%) which was maintained while concentrations of the test drug were increased 10-fold. In contrast, ketotifen at low concentrations inhibited histamine release but actually stimulated release at slightly higher concentrations (5). A similar biphasic effect of ketotifen was reported as early as 1982 (6) in other mast cell populations.

This biphasic effect of antihistamines on mast cell mediator release (inhibition at low concentrations and precipitous increases in mediator release at slightly higher concentrations) was first studied by Mota and da Silva in 1960 (7). Numerous investigators have confirmed these initial observations over the decades (8–11). Significantly, the ability of antihistamines to liberate mast cell mediators or prevent mast cell-mediator release is not correlated with intrinsic histamine H_1-receptor antagonism (12, 13). Simons suggested that antihistamines are lipophilic, cationic drugs capable of partitioning into cell membranes. This drug-membrane interaction expands and stabilizes the membrane, making it less permeable to ion fluxes (14). However, correlation between lipophilicity and an antihistamine's ability to inhibit mast cell-mediator release or to cause release has not been convincingly demonstrated (15). Despite the lack of correlation between

lipophilicity and cytotoxic mediator-liberating effects of antihistamines, evidence of interaction between antihistaminic drugs and membrane phospholipids has been obtained using lecithin monolayers (16). The authors suggested this interaction may be attributable to the surface activity of these molecules at the air/water interface (17).

Olopatadine inhibits mast cell-mediator release without liberating pro-inflammatory mediators at higher concentrations while ketotifen produces the characteristic antihistaminic biphasic curve upon mast cell-mediator release. However, both compounds possess histamine H_1-receptor antagonist activity. Therefore, the reported studies were undertaken to elucidate differences in surface activity and membrane interactions which may explain the dissimilar effects on mast cell populations noted with these drugs.

Materials and Methods

Reagents – Olopatadine and ketotifen (solid substance) were provided by Alcon Laboratories, Inc., Fort Worth, TX. Water was purified by reverse osmosis and carbon filtration, passage through an Elix 3 deionization system (Millipore) and passage through a Milli Q UV Plus polishing system (Millipore). Buffer, comprised of 10 mM HEPES containing 0.1 M NaCl pH 7.5, was used for preparation of solutions of olopatadine and ketotifen and for control experiments. After mixing the drug with the buffer, the pH was readjusted to a value of 7.5 with 5 M NaOH. All other chemicals used were of reagent grade or highest purity available.

Measurement of surface tension/surface pressure – Surface tension was measured using an automated interfacial monitor-controller built around a Cahn 27 electrobalance (18) equipped with a 24 ga. Nichrome wire Wilhelmy probe (19). The two aqueous compartments (circular and rectangular) of the keyhole-shaped Teflon trough were disconnected in this study and only the circular compartment (area=25.5 cm^2, volume=24.4 ml) was used for monolayer formation. Temperature in both compartments was maintained at 24°C using a thermostatic base plate controlled by a precision water bath. Precise positioning of the Wilhelmy probe in the aqueous phase, correction for probe buoyancy due to immersion, subphase stirring, data collection and data display were under microprocessor control (18).

Exchange of aqueous phase contents – The circular compartment was fitted with an inlet tube (1/32" ID Teflon) and an outlet tube (18 ga. Teflon) which entered through the outer wall of the sample compartment. These were connected to 25-mL, gas-tight syringes (model 1025, Hamilton, Reno, NV) mounted in a microprocessor-controlled push-pull dual syringe pump (model sp260p, World Precision Instruments, Sarasota, FL) through three-way Teflon valves (Hamilton, Reno, NV) which were used for filling and flushing. About 42 cm of the inlet tube was coiled in the water-filled rectangular compartment of the trough in order to equilibrate the incoming solution to the temperature of the circular compartment. A custom Teflon-coated magnetic stirring bar (length=3.6 cm, diameter 2 mm) was used to mix the aqueous contents. The bar was maintained at 50 rpm by stepper motor-driven magnet mounted beneath the circular compartment and controlled by the microprocessor. The relatively slow stirring speed and small bar diameter were used to minimize disturbance of the lipid monolayer. To exchange the contents of the circular compartment with the solution in the inlet syringe while maintaining constant volume, the syringes were operated in unison, but in opposite directions, by the syringe pump. Control experiments (not shown) demonstrated that, during exchange of 25 ml of aqueous phase, the volume of liquid removed from a test container remained constant to within an average deviation of 0.023 ml (n=2), or ~0.1%. This insured that the depth of immersion of the Wilhelmy probe was constant to within ~10 μm and, hence, the contact angle of the aqueous phase with the probe, remained essentially constant during exchange experiments. Control experiments were also conducted (not shown) with solutions of sodium deoxycholate to validate the linear relationship between surface tension and the log of surfactant concentration (20, 21) under static and dynamic exchange conditions.

Measurement of erythrocyte plasma membrane leakage

Bovine blood was collected immediately after slaughter, EDTA added to a concentration of 2.7 mM, and the mixture cooled to 4°C. Erythrocytes were prepared as described elsewhere (22). Briefly, a 10 ml aliquot of blood was centrifuged at 3,000 g for 10 min at 4°C, the buffy coat was removed by aspiration and the supernatant was discarded. The erythrocyte pellet was suspended in 10 ml of 150 mM NaCl, centrifuged as before and the supernatant discarded. This procedure was twice repeated with a suspension medium containing 2.7 mM KCl, 1.5 mM KH_2PO_4, 139 mM NaCl and 16 mM Na_2HPO_4, adjusted to pH 7.4 (PBS). The ~4 ml of washed erythrocytes were resuspended in PBS to a total volume of 10 ml and stored at 4°C. For determination of lipid phosphorous, 1 ml of erythrocyte suspension was solvent extracted as described (23) and phosphorous content of the extract determined (24). The concentration of phospholipid in the cell suspensions used was 1.5–3.0 mM.

Erythrocyte lysis (hemolysis) was measured spectrophotometrically (22). The test compounds olopatadine and ketotifen were prepared in PBS at concentrations approaching maximum compound solubility. The pH was adjusted to 7.4 with concentrated NaOH. The solutions were centrifuged to sediment undissolved test compound. The supernatant fraction was collected. An aliquot of the drug-saturated supernatant solution was appropriately diluted, and test compound concentration was determined spectrophotometrically at 299 nm using corresponding calibration standard solutions of test compound. To 2 ml of test solution at 37°C was added 10 μl of erythrocyte suspension (9 nmol lipid phosphorous). Following 30 min of incubation at 37°C, each sample was centrifuged at 4,000 g for 15 min at 15°C, the cell-free supernatant was decanted and its optical density at 577 nm was determined. Control experiments were performed with PBS buffer at pH 7.4 to measure erythrocyte stability and with Triton X-100 at 0.1% (v/v) in PBS at pH 7.4 to obtain complete hemolysis. Percentage hemolysis was calculated as 100 times the optical density of the sample supernatant at 577 nm divided by that of the control treated with Triton X-100.

Erythrocyte ghosts, loaded with 6-carboxyfluorescein (CF-ghosts), were prepared by rupturing and resealing erythrocytes in the presence of the dye (22). To 0.8 ml of erythrocyte suspension was added 8 ml of 1.2 mM acetic acid, 4 mM $MgSO_4$, pH 3.2, at 4°C. After 5 min incubation at 4°C, the pH was adjusted to 7.4 using 1 M Tris base. The sample was centrifuged at 16,000 g for 10 min at 0°C

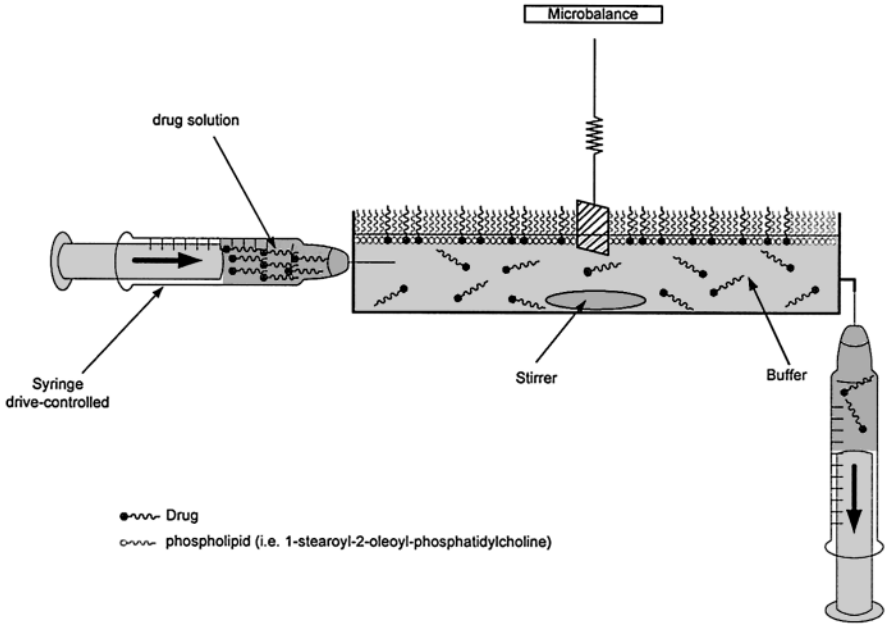

Fig. 1. Interfacial monitor/controller (Wilhelmy film balance) for the assessment of phospholipid/drug interactions.

and the pellet was re-suspended in 400 µl of 80 mM 6-carboxyfluorescein, 20 mM HEPES, 170 mM NaCl, pH 7.4, at 37°C. The ghosts were resealed by incubation for 1 hr at 37°C, after which free 6-CF was removed at 24°C by passage of the sample through a column of Sephadex G-75 equilibrated with 10 mM HEPES, 140 mM NaCl buffer adjusted to pH 7.4. The brownish CF-ghosts eluting well ahead of the 6-CF were pooled and used to measure the effects of test compounds on 6-CF leakage. CF-ghosts were maintained at 24°C and were used within 6 days of preparation. The quantity of membranes used in each experiment was not assessed, but was much less than 9 nmol of phospholipid-phosphorous that used for hemolysis experiments. Thus, CF leakage was also measured under conditions of high dilution.

For measurement of drug-induced 6-carboxyfluorescein leakage from CF-ghosts, 2 ml of a solution of the test compound in 10 mM HEPES, 140 mM NaCl, pH 7.4, were added to a 1-cm cuvette and equilibrated with stirring for 3 min at 37°C. Fluorescence was recorded continuously at 510 nm using an excitation wavelength of 485 nm. Following addition of 50 µL of CF-ghosts and recording of fluorescence emission intensity for an additional 30 min, 20 µL of a 10% Triton X-100 solution (vol/vol) were added, and the emission intensity was recorded for ~5 min. Zero CF leakage was defined as the fluorescence intensity obtained immediately after addition of CF-ghosts to the cuvette in the absence of test compound. Complete CF leakage was defined for each sample as the value obtained after the addition of Triton X-100, less the fluorescence intensity representing zero leakage. CF leakage for each experimental sample and control was calculated as the fluorescence intensity at 30 min after CF-ghost addition, less the zero CF leakage value intensity. Percent CF leakage was defined as the net sample fluorescence defined above divided by the net fluorescence intensity for complete CF leakage, multiplied by 100.

Results

Interaction with the argon-buffer interface

To determine whether olopatadine or ketotifen has the potential to interact with lipids of cell membranes, measurements were conducted to initially assess the intrinsic surface activity of each compound at the argon/buffer interface. Surface tension measurements were recorded while exchanging the content of the modified Langmuir trough by continuous infusion

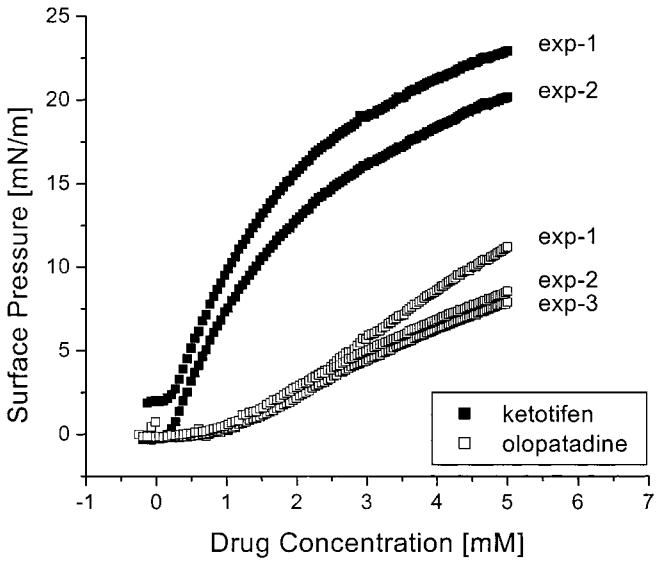

Fig. 2. Intrinsic surface activity of olopatadine and ketotifen.

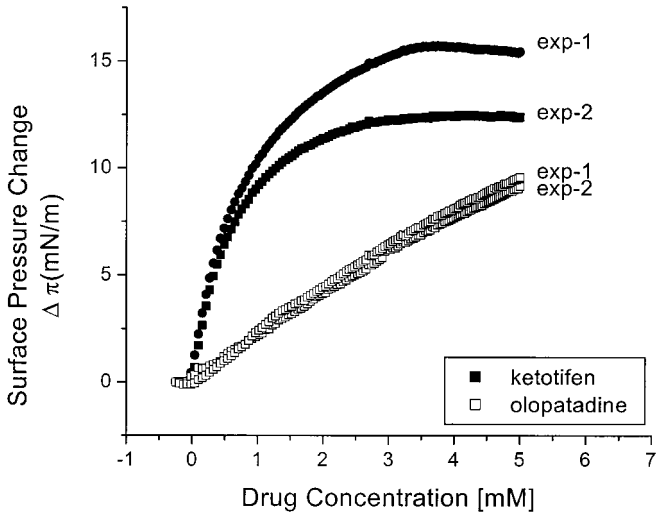

Fig. 3. Change in surface pressure of SOPC monolayers with increasing concentrations of olopatadine and ketotifen.

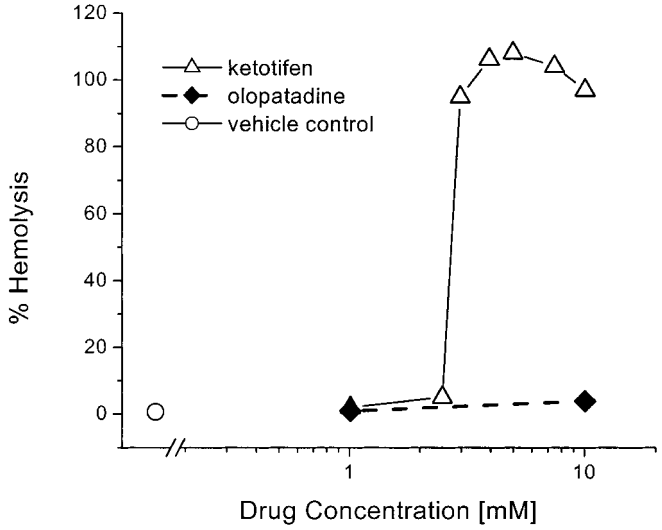

Fig. 4. Effects of olopatadine and ketotifen on the in vitro stability of bovine erythrocyte membranes.

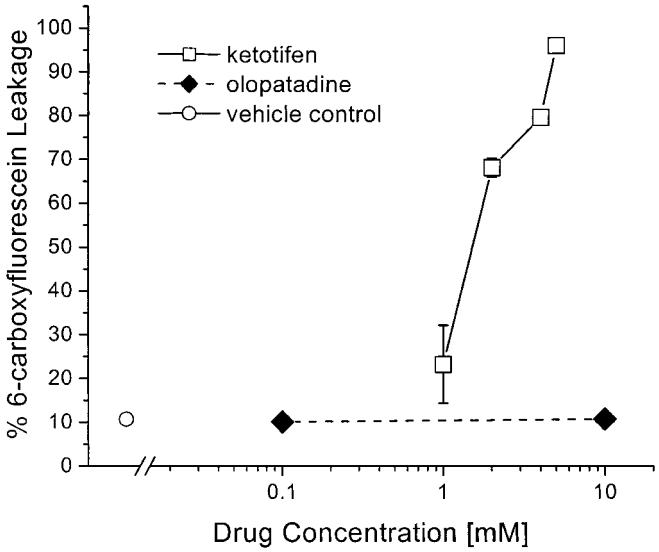

Fig. 5. In vitro effects of olopatadine and ketotifen on carboxyfluorescein release from preloaded bovine erythrocyte ghosts.

of a 10 mM drug solution and concurrent removal of an equal volume from the trough without changing the trough volume (Fig. 1). The changes in surface tension (expressed as surface pressure π; mN/m) obtained as a function of drug concentration are shown in Fig. 2. Both compounds exhibited an affinity for the argon/buffer interface. Although plateau (saturation) levels in surface pressure were not achieved with either drug, ketotifen exhibited an apparent greater affinity for the argon/buffer interface (greater intrinsic surface activity) than olopatadine. Moreover, at the maximum concentrations achieved in the trough (5 mM), ketotifen produced a >2-fold increase in surface pressure compared to that attained with the same concentration of olopatadine.

Interaction with SOPC model membranes

With an obvious differentiation between olopatadine and ketotifen in their amphipathic behavior at the argon-buffer interface it was of interest to determine whether these properties are also recognizable by compound interactions with SOPC monolayers. Surface activity was determined with the interface occupied by a monolayer of SOPC at an initial surface pressure of 28–32 mN/m. This surface pressure range is achieved at an SOPC lateral packing density believed to be close to that of cellular membranes (25, 26).

Compared to sham experiments with buffer exchange alone (not shown) olopatadine promoted a continuous change in surface pressure over the entire concentration range (0–5 mM) without ever reaching a plateau (Fig. 3). Ketotifen at low concentrations also caused a continuous increase in the surface pressure, but at a subphase concentration above 2–3 mM changes in surface pressure reached plateau levels. Olopatadine, at the highest achievable concentration (0.5 times that of the infusion stock solution; i.e., 5 mM), produced surface pressure changes that remained below those attained with ketotifen (i.e., 8 mN/m vs. 12–15 mN/m). The concentration dependent responses in surface pressure change indicate that ketotifen exhibits a greater affinity than olopatadine for the SOPC monolayer and, hence, possesses a greater potential to perturb the integrity of biological membranes.

Interaction of drugs with biological membranes

As a test of the abilities of olopatadine and ketotifen to interact with biological membranes, their effects on the permeability of erythrocytes were measured using two techniques. The first measured the ability of the compounds to cause leakage of cytoplasm, i.e., hemoglobin (MW=64.500), from intact erythrocytes, and the second measured the leakage of 6-carboxyfluorescein (MW=376.32) from CF-ghosts. In both cases the cells were incubated with a solution of the drug at 37°C for 30 min, after which the percentage of solute leakage was quantified as described in Materials and Methods.

Fig. 4 summarizes the effects of the compounds on erythrocyte hemolysis. When cells were incubated in buffer alone, hemolysis was ~1%. While olopatadine caused only minimal hemolysis (≤8%) over a concentration range of 1–10 mM, exposure of red cells to ketotifen concentrations >2.5 mM produced complete hemolysis. During the experiments using ketotifen it was noted that at concentrations just below the onset of hemolysis the cells were already 'fragile' in that the centrifugation process caused them to rupture in the bottom of the tube.

A similar drug-induced leakage was observed with ketotifen using erythrocyte ghosts loaded with CF (Fig. 5). Increasing the concentration of ketotifen from 1 to 5 mM caused a concentration dependent release of intracellularly trapped CF. At the highest concentration virtually all CF was released from the erythrocyte ghosts. Olopatadine on the other hand caused only minimal release of CF. The levels of leakage were indistinguishable from those observed in vehicle treated controls (2.3%).

Discussion

These studies demonstrate that olopatadine and ketotifen are both surface active. Compared to ketotifen, however, olopatadine produced more shallow surface pressure-concentration isotherms, indicating a lower affinity for its interactions with both the argon-water interface and the phospholipid monolayer. In addition, the maximum total surface pressure at-

tained with olopatadine in the SOPC monolayer (approximately 38 mN/m) was lower than that produced with equal molar concentrations of ketotifen (42–45 mN/m). This latter range is well above the postulated internal surface pressure (30–35 mN/m) of biological membranes (25, 26) and is near the monolayer collapse pressure of 47 mN/m exhibited by phosphatidylcholine (27). Consistent with the differences in surface activity of the compounds, ketotifen, but not olopatadine, caused lysis of erythrocytes (Fig. 4) and induced CF leakage from erythrocyte ghosts (Fig. 5). These data, considered in relationship to previously published reports showing that olopatadine completely inhibits mast cell mediator release without causing cell lysis at high concentrations (1, 5, 28), suggest that the limited changes in membrane surface pressure induced by olopatadine, as measured by the described techniques, are insufficient to cause membrane disruption. Conversely, the greater extent of ketotifen interaction with phospholipid surfaces prompts an explanation of the cell lytic effect of this molecule as previously demonstrated (5, 6). These observations suggest that concentrations of ketotifen placed onto the eye may non-specifically cause perturbations in cell membranes.

Recently, a more extensive series of classical histamine H_1-receptor antagonists were examined for intrinsic surface activity, effects upon mast cell mediator release, and the ability to interact with phospholipid monolayers. Results obtained using the methodology described in this article demonstrated that pyrilamine, clemastine, diphenhydramine and ketotifen all produced a biphasic effect upon human conjunctival mast cells and caused significant changes in surface pressure greater than those produced by olopatadine (29). Thus, olopatadine appears to be a unique topical ocular antiallergic agent that is devoid of membrane lytic activity as a consequence of its low intrinsic surface activity and limited nonspecific interaction with biological membranes.

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