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Identification of the L-menthol binding site in guinea-pig lung membranes

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1 L-Menthol inhibits both neurokinin A and capsaicin-induced bronchoconstriction in the guinea-pig and relaxes pre-constricted guinea-pig isolated bronchi. Structure-activity relationships have been defined for the action of (−)-menthol and related compounds on cold receptors, suggesting an action of L-menthol at a pharmacological receptor. We have performed radioligand binding studies to characterize the binding sites for [^{3}H]-L-menthol in whole cell membranes prepared from guinea-pig lung tissue.

2 In kinetic studies, [^{3}H]-L-menthol was found to bind rapidly and reversibly. Binding of [^{3}H]-L-menthol to lung membranes was found to be time-dependant becoming fully associated to its site within 40 min, and half-maximum association occurred within 8 min ($t_{1/2}=8$ min). [^{3}H]-L-menthol was fully dissociated from its binding site within 8 min, ($t_{1/2}=2$ min).

3 Inhibition studies presented a pharmacological profile of the 'L-menthol site'. Capsaicin, capsazepine, D-menthol, eugenol, SCH23390 and camphor were all found to displace [^{3}H]-L-menthol binding. In contrast WS3, noradrenaline, 5-hydroxytryptamine, spiperone, flunarazine, bepridil and nicardipine were without effect.

4 We have identified a L-menthol binding site in the guinea-pig, which may represent a site common to a variety of compounds.

Keywords: L-Menthol; binding site; lung membranes

Introduction

L-Menthol is a cyclic terpene alcohol, naturally occurring in the volatile oils of various species of mentha. L-Menthol has been used for many years in a wide variety of pharmaceutical preparations, generally utilizing its familiar fragrance and flavour, with little knowledge as to its pharmacological properties or medicinal benefit. Recent research into this compound has shown L-menthol to be of great interest with many specific effects. However, the mechanisms of action of L-menthol are yet to be elucidated.

The most commonly known and exploited property of L-menthol is its apparent cooling activity, which is thought to be mediated by the specific stimulation of peripheral cold fibres (Hensel & Zotterman, 1951; Hellekant, 1969). This effect is not limited to L-menthol as over 1200 compounds have been found to possess the same property. These compounds fall into a particular category which has four molecular requirements including, a hydrogen bonding group, hydrocarbon skeleton, the correct hydrophilic/hydrophobic balance and a molecular weight range of 150–350 g (Watson *et al*., 1978). These were prerequisites for intrinsic activity which suggests a specific drug-receptor interaction on peripheral cold receptors. Eight stereoisomers of L-menthol were tested by the Watson group, and L-menthol was found to have the greatest cooling activity with D-menthol being 45 times less active. Further studies on the epithelial and trigeminal responses of cold fibres of rat and dog lingual nerves have also shown L-menthol to behave very differently in comparison with a range of other related alcohols tested, indicating that the effects of menthol are through a specific receptor or channel (Simon & Sostman, 1991).

Evidence of a possible drug-receptor interaction of menthol comes from studies on Ca^{2+} channels. Menthol has been found to reduce specifically Ca^{2+}-stimulated outward current in cat lingual fibres (Shafer *et al*., 1986).

A similar action of menthol on calcium channels has also been observed in other tissues. In molluscan neurones and vertebrate dorsal root ganglion cells Swandulla *et al*. (1986), observed that L-menthol application caused a reversible reduction in Ca^{2+} current when applied extracellulary, but had no effect on Ca^{2+} current when instilled intracellularly, indicating that the site for menthol may be situated on the cell membrane. Closely related compounds cyclohexanol (cyclic alcohol from which menthol derived), thymol, and menthone showed little or no activity, D-menthol was half as active as its stereoisomer indicating a stereochemically selective effect of L-menthol on Ca^{2+} current. Chiyotani *et al*. (1994) also demonstrated a specific action of menthol in cultured epithelium cells. The effect of L-menthol on Ca^{2+} channels has not only been found to be specific, but reversible and stereochemically selective as well.

The most significant evidence of a specific action of L-menthol at the site of a pharmacological receptor comes from the work of Hawthorn *et al*. (1988). Using a receptor binding assay, this group demonstrated competitive antagonism with L-menthol of [^{3}H]-nitrendipine and the dihydropyridine radioligand [^{3}H]-(+)-PN200-110 binding to cardiac, neuronal and intestinal smooth muscle. L-Menthol inhibition of [^{3}H]-nitrendipine and [^{3}H]-(+)-PN200-110 binding was more potent in intestinal smooth muscle compared with cardiac muscle and neuronal preparations.

That the effects of L-menthol are fully reversible, and stereochemically selective, together with the fact that L-menthol has been demonstrated to displace [^{3}H]-nitrendipine and [^{3}H]-(+)-PN200-110 binding, indicate that its effects could

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result from an interaction with a specific pharmacological receptor.

Cough and bronchial smooth muscle studies in the guinea-pig indicate a pharmacological action of menthol on the respiratory tract (Laude *et al*., 1994; Wright *et al*., 1997), this may be the result of a drug-receptor interaction. The aim of the present study was to identify if a possible specific binding site for L-menthol exists in guinea-pig lung membranes and to characterize the biochemical properties of this 'L-menthol site' with ligand binding studies.

Methods

Preparation of membranes for binding experiments

Male Dunkin Hartley guinea-pigs ($n=18$, Body weight 355–750 g) were killed by dislocation of the neck, and lung tissue was collected, weighed and placed into 50 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 1 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). The tissues were coarsely chopped and homogenized in 300 ml, 50 mM Tris (pH 7.4) buffer containing, 1 mM EDTA, 0.1 mM PMSF by use of a Polytron PT3000 (Kinematica) set at maximum for 20 s.

Homogenization was repeated 3 or 4 times until a uniform consistency was reached. The resultant homogenate was centrifuged at $1000\times g$ for 5 min at 4°C (Denley Br401 refrigerated centrifuge) to allow the sedimentation of intracellular debris. The supernatant was ultracentrifuged at $40{,}000\times g$ for 30 min at 4°C (Beckman model J2-21) to pellet the cell membranes. The resultant pellet was washed twice with ice-cold 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA. The final pellets were re-suspended in 20 ml of 50 mM Tris-HCl (pH 7.4). The protein content was measured by use of the Bio Rad Bradford reagent with bovine serum albumin (BSA) as the standard (Bradford, 1976). The protein concentration was adjusted to 2 mg ml^{-1} and membranes were stored at −80°C until required.

Binding of [^{3}H]-L-menthol

Association studies Radioligand binding assays were performed in 50 mM Tris-HCl (pH 7.4) buffer containing 20 nM [^{3}H]-L-menthol in a final volume of 500 μl placed into 96 well plates. D.p.m.s added per well was 158999. The incubations were initiated by addition of cell membranes (500 μg) and samples were incubated at a range of incubation times (1–120 min) at 37°C in duplicate or triplicate. Non-specific binding was defined with 100 μM L-menthol. Incubations were terminated by filtration through GF/C filters pre-soaked with 0.1% BSA by use of a cell harvester (Brandel, SEMAT model M-48T) and washed three times with ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The filter papers were air dried in a fume cupboard and placed individually into vials containing 5 ml scintillant (Packard Ultima Gold MV). The amount of radioactivity bound to the filters was quantified by liquid scintillation spectrophotometry (Packard 2500 TR liquid scintillation analyser).

Dissociation assay Radioligand binding assays were performed in 50 mM Tris-HCl (pH 7.4) buffer containing 20 nM [^{3}H]-L-menthol in a final volume of 500 μl added to 96 well plates. Total d.p.m./assay was 165960. The incubations were initiated by addition of cell membranes (500 μg) and samples were incubated for 40 min (this was the previously calculated time taken for [^{3}H]-L-menthol to become fully associated with its binding site) at 37°C in duplicate or triplicate. Dissociation was initiated by the addition 100 μM L-menthol, samples were incubated with L-menthol at a range of incubation times (0.5–30 min). Incubations were terminated by filtration through GF/C filters pre-soaked with 0.1% BSA by use of a cell harvester and washed three times with ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The amount of radioactivity bound to the filters was quantified by liquid scintillation spectrophotometry (Packard 2500 TR liquid scintillation analyser).

Competition studies A second series of binding experiments was performed to study the displacement of specific [^{3}H]-L-menthol binding by a range of other drugs. Membranes (500 μg) were incubated with 20 nM [^{3}H]-L-menthol in the presence or absence of inhibitor over a concentration range of 0.3 nM to 100 μM for 40 min at 37°C, total d.p.m./assay was 170000. Incubations were terminated by filtration through GF/C filters pre-soaked with 0.1% BSA using a cell harvester and washed three times with ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The amount of radioactivity bound to the filters was quantified.

Further experiments examined the effects of the vehicles ethanol and acetic acid alone. At the highest concentration used (0.25%) ethanol and acetic acid (0.05%) had no effect upon specific [^{3}H]-L-menthol binding.

In all the experiments detailed, specific binding was taken as the difference between the total binding and the binding in the presence of unlabelled drug.

Materials

[^{3}H]-L-menthol (specific activity 13.6 Ci $mmol^{-1}$) was synthesized by Alchem. [^{3}H]-L-menthol was tested for purity at the beginning of this study and was found to be 95% pure as assessed by high performance liquid chromatography (h.p.l.c.). L-Menthol, D-menthol, camphor, capsaicin, capsazepine, 5-hydroxytryptamine (5-HT), noradrenaline and the calcium antagonists flunarazine, bepridil, nicardipine, were purchased from Sigma Chemical Co. (Poole, U.K.). The dopamine antagonists spiperone and SCH 23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepene hydrochloride) were purchased from RBI. N-ethyl-p-menthane-3-carboximide (WS3) was a gift from Smithkline Beecham (Weybridge, U.K.). [^{3}H]-L-menthol was diluted in the incubation buffer. Stock solutions of L-menthol and capsaicin were made up in 20% ethanol in buffer and diluted accordingly. 5-HT, noradrenaline, SCH23390 and spiperone were made up in buffer and 0.5% acetic acid.

Calcium antagonists were dissolved in the incubation buffer and where necessary 20% ethanol was used.

Result analyses

Binding parameters for specific binding were determined by use of the program SIGFIT (W.P. Bowen, Smithkline Beecham, Harlow, Essex, U.K.). The concentration of competing ligand which inhibits specific [^{3}H]-L-menthol binding by 50% (IC_{50}) was determined by use of SIGFIT which fits data to a four-parameter logistic equation:-

$$Y = \text{minimum} + \frac{\text{Max} - \text{Min}}{1 + [10^{\log IC_{50}}/10^{x}]^{\text{slope}}}$$

Results

Association studies

Specific binding [^{3}H]-L-menthol to guinea-pig lung membranes reached equilibrium after 40 min and was stable for at least 1.5 h at 37°C (Figure 1).

The association data were best fitted by a model assuming association to a single population of binding sites. ($t_{1/2} \sim 8$ min, $n = 6$). At equilibrium, specific binding represented 66% of total binding when 100 μM L-menthol was used to define non-specific binding sites present in the assay. Pre-soaking of GF/C filter with 0.1% BSA before filtration markedly increased the level of specific binding (data not shown) and therefore the treatment was included in all subsequent experiments.

Dissociation studies

Specific binding of [^{3}H]-L-menthol to guinea-pig lung membranes was rapidly displaced by the addition of 100 μM L-menthol to the assay (Figure 2). As found in association experiments, the dissociation data were best analysed with a single site dissociation model ($t_{1/2} = 2$ min, $n = 6$). Together with the results from the association experiments, it appears that guinea-pig lung membranes contain a single population of binding sites for [^{3}H]-L-menthol which possess reversible, saturable kinetics.

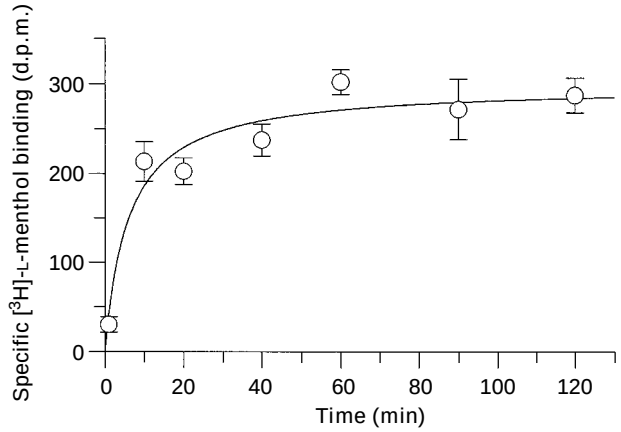

Figure 1 Association of 20 nM [^{3}H]-L-menthol with guinea-pig lung membranes. Points represent the mean of three observations; vertical lines show s.e.mean.

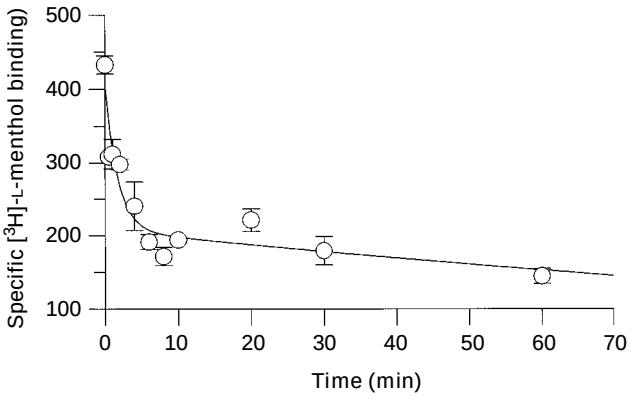

Figure 2 Dissociation of 20 nM [^{3}H]-L-menthol from the guinea-pig lung membranes. Points represent the mean of three observations; vertical lines show s.e.mean.

Inhibition studies

To characterize the specific binding of [^{3}H]-L-menthol pharmacologically, a series of displacement experiments were performed with several classes of compounds. For these studies, 20 nM [^{3}H]-L-menthol was used since this gave optimal specific binding under the assay conditions described. Higher concentrations of radioligand resulted in greater specific binding, but these could not be used due to limited availability of the radioligand. Results from the association study were used as a basis for the standard incubation period of 40 min in this assay.

To determine the selectivity of the binding of [^{3}H]-L-menthol to guinea-pig lungs a series of displacement experiments were carried out.

The most potent displacement occurred with L-menthol, SCH23390 (dopamine 1 and 5 antagonist) and capsazepine (vanilloid receptor antagonist), with mean pIC_{50} ($\pm$s.e.mean) of 5.5 ± 0.1, 5.3 ± 0.2 and 5.02 ± 0.1, respectively (Table 1).

[^{3}H]-L-menthol specific binding was displaced by related aromatic camphor but not cineole (Figure 3), or related cooling compound WS3, stereoisomer D-menthol and related alcohol eugenol also inhibited specific binding of menthol, but D-menthol was 10 times less potent than L-menthol inhibition (Figure 4). Capsaicin a vanilloid receptor agonist and counter irritant like menthol displaced L-menthol binding in the guinea-pig lung along with the capsaicin antagonist capsezepine (Figure 5). The D_1 receptor antagonist SCH23390 also displaced [^{3}H]-L-menthol binding, this was selective for the D_1 antagonist since spiperone (D_2, D_3 and D_4 antagonist) had no effect (Figure 6).

Table 1 Displacement of 20 nM [^{3}H]-L-menthol binding to guinea-pig lung membranes with a variety of compounds

Compound	n	*Slope*	*pIC_{50}*
L-Menthol	8	1.21 ± 0.1	5.55 ± 0.1
D-Menthol	6	1.00 ± 0.3	4.74 ± 0.1
Eugenol	4	0.18 ± 0.3	4.29 ± 0.2
Camphor	6	1.10 ± 0.7	4.30 ± 0.1
Capsaicin	6	1.2 ± 0.3	4.26 ± 0.1
Capsezepine	6	1.00 ± 0.02	5.02 ± 0.1
SCH23390	6	0.81 ± 0.4	5.3 ± 0.2

Data are presented as the slope and pIC_{50}

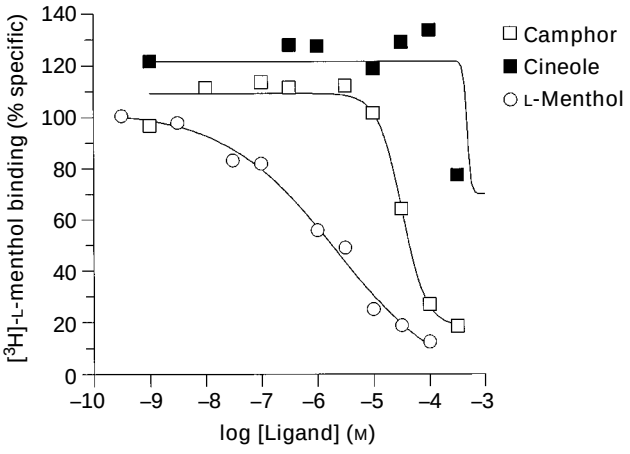

Figure 3 Inhibition of specific 20 nM [^{3}H]-L-menthol binding in the guinea-pig lung with D-menthol and eugenol. Points represent the mean of three observations.

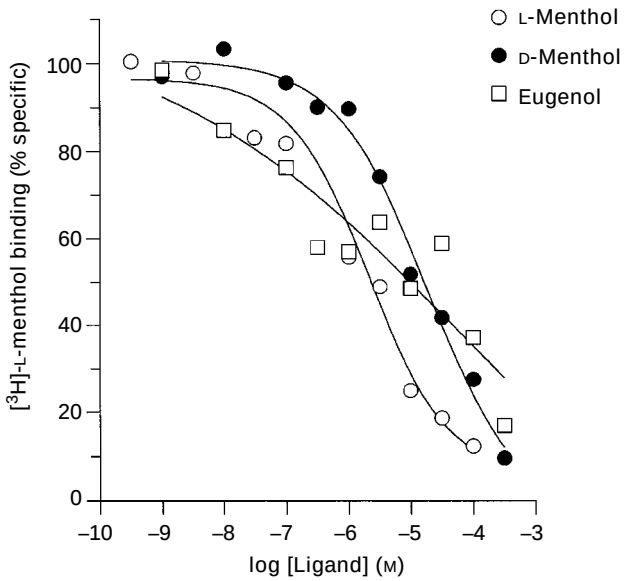

Figure 4 Inhibition of specific 20 nL [^{3}H]-L-menthol binding in the guinea-pig lung with related aromatics, camphor and cineole. Points represent the mean of three observations.

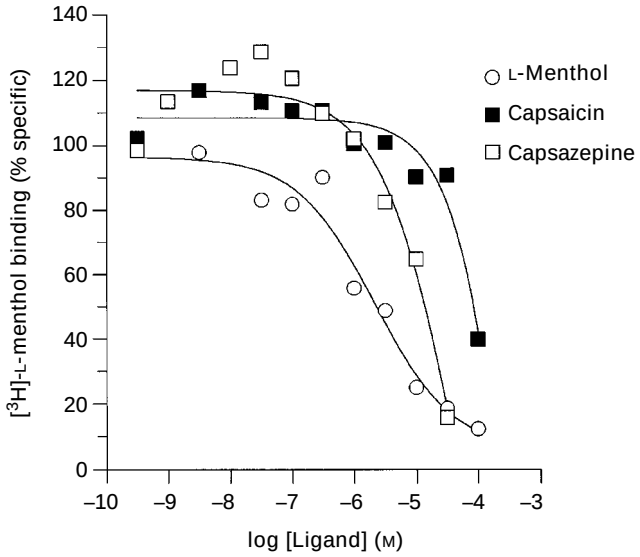

Figure 5 Inhibition of specific 20 nM [^{3}H]-L-menthol binding in guinea-pig lungs with capsazepine and capsaicin. Points represent the mean of three observations.

The majority of displacement curves possessed a slope close to unity suggesting displacement from a homogeneous population of binding sites. No evidence of heterogeneity was observed in any of the experiments.

Other compounds tested were those with known identified receptors common in the airways such as noradrenaline (adrenoceptor antagonist), 5-Hydroxytryptamine a 5-HT$_1$ receptor agonist, and calcium channel blockers nicardipine, flunarazine and bepridil. All of these compounds had no effect upon [^{3}H]-L-menthol specific binding, at concentrations up to 100 μM in guinea-pig lung membranes.

Table 1 lists compounds tested for inhibition of [^{3}H]-L-menthol specific binding which were found to displace successfully L-menthol binding, data are presented as slope and pIC$_{50}$.

Table 2 lists compounds tested for inhibition of [^{3}H]-L-menthol binding, which were without effect, data presented as pIC$_{50}$.

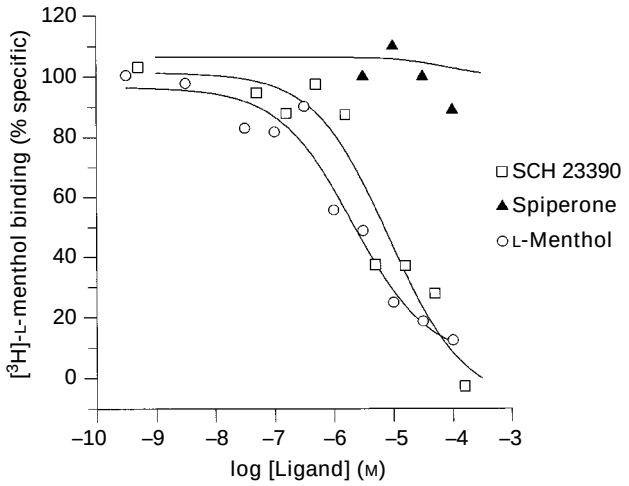

Figure 6 Inhibition of specific [^{3}H]-L-menthol binding to guinea pig airways, with spiperone and SCH23390. Points represent the mean of three observations.

Table 2 Those compounds found not to displace [^{3}H]-L-menthol binding in guinea-pig lung membranes

Compound	n	*pIC$_{50}$*
WS3	6	>4
Cineole	6	>4
Noradrenaline	6	>4
5-HT	4	>4
Spiperone	6	>4
Nicardipine	6	>4
Bepridil	4	>4
Flunarazine	4	>4

Discussion

We have demonstrated the binding of [^{3}H]-L-menthol to a specific site in guinea-pig lung tissue. The binding of [^{3}H]-L-menthol to guinea-pig lung membranes demonstrates association and dissociation kinetics, with binding being both reversible and stereochemically selective, indicating that [^{3}H]-L-menthol exhibits the binding characteristics of a compound active at a pharmacological receptor presented in guinea-pig lung tissue.

In this study we were unable to demonstrate any saturable specific [^{3}H]-L-menthol binding. This was due to the limitations encountered working with this particular radioligand. We found [^{3}H]-L-menthol to have a low affinity for its binding site and consequently extremely large volumes of the pure radioactive ligand would have been required to saturate what appears to be abundant L-menthol binding sites. Therefore, homologous association and dissociation studies were performed to demonstrate the binding kinetics of [^{3}H]-L-menthol.

In our inhibition studies we found the order of potency of compounds displacing [^{3}H]-L-menthol binding to guinea-pig lung membranes to be L-menthol = SCH23390 = capsazepine > D-menthol > eugenol = camphor = capsaicin > cineole. This is in agreement with other physiological studies in which the aromatics L-menthol, D-menthol, camphor and cineole were used. Eccles *et al.* (1988) found L-menthol to be far more potent than its isomer D-menthol in increasing nasal sensation of airflow in healthy subjects. Burrow *et al.* (1983) also demonstrated an order of potency in the production of increased nasal patency between L-menthol and the related

aromatics camphor and eucalyptus in human volunteers. The order of effectiveness was L-menthol > camphor > eucalyptus. The same order of potency of these aromatics has also been observed in their ability to reduce cough in man (Packman & London, 1980) and the reduction of citric acid-induced cough in the guinea-pig (Laude *et al*., 1994).

We have also demonstrated displacement of [^{3}H]-L-menthol binding with both capsaicin and capsazepine. In many respects both L-menthol and capsaicin have opposite effects. Capsaicin causes a warming sensation when applied to the skin, L-menthol has cooling properties, capsaicin promotes cough, L-menthol inhibits cough, capsaicin promotes bronchoconstriction, menthol bronchodilates. A recent psychophysical study by Cliff & Green (1996) of cross desensitization to capsaicin and L-menthol demonstrated that desensitizing the tongue of volunteers to L-menthol caused a sensitization to capsaicin irritation. It was concluded both L-menthol and capsaicin may act on the same neuronal pathways through different excitatory mechanisms.

The data from our inhibition studies indicate that L-menthol and capsaicin may have a common site of action, this site may be associated with facilitation of Ca^{2+} current inactivation, since both L-menthol and capsaicin have been shown to inactivate Ca^{2+} current (Swandulla *et al*., 1987; Petersen *et al*., 1989) at concentrations similar to the pIC_{50} found in our inhibition studies, 5.55 and 4.26, respectively.

In this study capsazepine (capsaicin antagonist) also displaced menthol binding with a higher potency than capsaicin. In our study, the IC_{50} of both capsaicin and capsazepine displacement of [^{3}H]-L-menthol binding is much lower than the calculated potency of these compounds for the vanilloid receptor in rat airways (Szallasi *et al*., 1993). It appears that capsazepine has dissimilar potencies for inhibiting capsaicin-induced responses in different preparations, ranging from 10 nM in guinea-pig ileum preparations to 1 μM in rat isolated saphanous nerve and hind paw (Dray *et al*., 1991). Thus capsazepine may act on more than one target. We hypothesize that both capsaicin and capsazepine displace [^{3}H]-L-menthol from a site other than the vanilloid receptor. To determine whether menthol was binding to a receptor site already characterized in guinea-pig airways, we looked at the inhibition of [^{3}H]-L-menthol binding with spiperone, SCH23390; 5-HT and noradrenaline. None of these compounds, except SCH23390, showed any displacement of [^{3}H]-L-menthol binding. The IC_{50} of SCH23390 was 5 μM, indicating binding to a low affinity site, in contrast the affinity of SCH23390 for the D_1-receptor in rabbit lung membranes is 2.05 nM (Kobayashi *et al*., 1994). Our data suggest that SCH23390 is not displacing [^{3}H]-L-menthol from D_1 receptors in guinea-pig lung membranes, but from some other site with a low affinity for SCH23390.

Like capsaicin and L-menthol, SCH23390 at 50 μM reversibly reduces HVA Ca^{2+} current in ganglion cells. This was thought to be through a receptor-mediated facilitation of the gating of a calcium channel, thus there may be a specific action of SCH23390 which is not mediated by dopamine receptors (Guenther *et al*., 1994).

Further evidence that [^{3}H]-L-menthol is binding to a site associated with a specific calcium channel comes from the work of Hawthorn *et al*. (1988) and Zygmunt *et al*. (1993). In our study we looked at the possible inhibition of [^{3}H]-L-menthol binding by a limited range of calcium channel antagonists nicardipine, flunarazine and bepridil, but we were unable to demonstrate any displacement.

In conclusion, we have shown reversible and specific binding of [^{3}H]-L-menthol to guinea-pig lung membranes. Specific [^{3}H]-L-menthol binding was also inhibited stereochemically by L- and D-menthol. The binding of [^{3}H]-L-menthol could be displaced by capsaicin, capsazepine, the related aromatics cineole, camphor and the dopamine antagonist SCH 23390.

We hypothesize that the binding site for [^{3}H]-L-menthol may involve receptor-mediated facilitation of a calcium channel which is not antagonized by either nicardipine flunarazine or bepridil. Although we have found [^{3}H]-L-menthol to have a low affinity for this site, other related compounds could bind to this site with a greater affinity than L-menthol. Such compounds could include octonol, nanonol and decanol, as like L-menthol these compounds have been shown to exert a selective action on LVA calcium currents in inferior olivary neurones at concentrations <1 μM, supporting the existence of a high affinity binding site (Llinas & Yarom, 1986).

Further research into the 'L-menthol binding site' could help in the development of more potent analogues of L-menthol with a greater therapeutic action on the respiratory tract. The specificity of L-menthol for a membrane bound receptor with diverse activity offers the possibility for pharmacological targeting of these responses.

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