

Investigation into the mechanisms by which nedocromil sodium, frusemide and bumetanide inhibit the histamine-induced itch and flare response in human skin *in vivo*

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Summary

Background In a previous study, iontophoresis of nedocromil sodium into human skin *in vivo* was shown to reduce histamine-induced itch and flare. In asthma, the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter inhibitors, frusemide and bumetanide, have been reported to have many similar actions to nedocromil sodium.

Objective To compare the effects of these drugs in the histamine-induced itch, flare and weal response in human skin *in vivo* and elucidate their site of action.

Methods Nedocromil sodium, frusemide bumetanide and reversed osmosis water (control), were introduced by iontophoresis into the forearm skin of 10 volunteers in each of two single-blind studies. In study 1, histamine (20 μL of 100 μM) or vehicle was injected into the area of iontophoresis 10 min later. In study 2, histamine or vehicle was injected 5 mm outside the area of iontophoresis so the flare developed over the area of iontophoresis. Itch was scored on a visual analogue scale every 20 s for 5 min, flare areas were assessed using scanning laser Doppler imaging up to 10 min and weal was assessed by planimetry at 10 min.

Results In study 1, nedocromil sodium, frusemide and bumetanide reduced itch scores by 36%, 48% and 34%, respectively, and flare areas by 17%, 26% and 15% respectively (all $P < 0.05$). Weal areas and blood flux in the flare were unaffected. In study 2, itch scores, flare areas and weal areas were not inhibited. Also, blood flux values in areas of drug and water iontophoresis were not different.

Conclusion This study has provided evidence to support the hypothesis that nedocromil sodium, frusemide and bumetanide inhibit sensory nerve activation to reduce the itch and flare responses induced by histamine in human skin *in vivo*. It is likely that inhibition of a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter in the sensory nerve membrane is a possible mechanism of action.

Keywords bumetanide, chloride channels, flare, frusemide, histamine, human skin, iontophoresis, itch, nedocromil sodium

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Introduction

Itch and neurogenic erythema (flare) play a major role in the morbidity of many skin disorders, including urticaria and atopic dermatitis. In a previous study, the anti-asthma drug nedocromil sodium was shown to reduce histamine-induced itch and flare, provoking discussion about its mechanism of action [1]. In asthma, the beneficial effects of cromoglycate-like drugs have been considered to be primarily a consequence of mast cell stabilization and anti-inflammatory actions [2–4]. However, following the demonstration of their ability to modulate sensory nerve function in experimental animals [5, 6], the inhibitory effects of cromoglycate-like drugs against

bronchoconstriction induced by bradykinin, sulphur dioxide, metabisulphite and ultrasonically nebulized water was suggested to have resulted from an effect on sensory nerves [7].

While the ability of cromoglycate-like drugs to inhibit sensory nerve activation by an effect on chloride channels [8] is the most likely mechanism of action, reduction of neuropeptide release from nerves [9] and tachykinin receptor antagonism [10, 11] have been offered as alternatives. Cromoglycate-like drugs have been shown to inhibit a variety of chloride channels in a wide range of cell preparations. These include a calcium-independent chloride channel on cultured mast cells [12], a volume-activated chloride channel in endothelial cells [13], calcium-dependent chloride fluxes in tracheal smooth muscle [14] and a voltage- and calcium-dependent chloride channel on airway epithelium [15]. In the rabbit vagus nerve, which is comprised mainly of C-fibres, nedocromil sodium first activates and then suppresses

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chloride ion flux, inducing a slow long-lasting nerve depolarization and thereby reducing the sensitivity of the nerve to subsequent action potentials [8].

Inhalation of frusemide and bumetanide, loop diuretics, which inhibit the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, have also been repeatedly shown to inhibit allergen-, exercise-, adenosine- and sodium metabisulphite-induced bronchoconstrictions in humans [16–19]. The original hypothesis for their mechanism of action was that they altered ion and water movement across airway epithelium. However, similarities between the inhibitory profiles and relative potencies of these drugs and those of nedocromil sodium [20] suggested other possible mechanisms. In guinea-pig airways, frusemide and bumetanide inhibit both cholinergic and excitatory non-adrenergic non-cholinergic neurotransmission, an effect that was suggested to be possibly related to their inhibitory effects on the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter [21].

From these data we hypothesize that nedocromil sodium, frusemide and bumetanide share a common mechanism in inhibiting sensory nerve function. To test this hypothesis, we used the histamine-induced weal, flare and itch response in human skin *in vivo*. Briefly, intradermal injection of histamine acts on vascular H_1 receptors to cause an initial vasodilatation at the site of injection and then leakage of plasma proteins from post-capillary venules to form a weal [22]. Histamine also activates sensory nerves. Two consequences result. First, an axon reflex induces the release of vasodilator neuropeptides to cause the widespread flare response [23, 24]. Second, histamine-activated sensory nerves carry the itch response to the central nervous system [25].

Methods

Study population

Healthy volunteers, aged 20–45 years, recruited from the staff and students of Southampton University gave written informed consent to participate in this study. Ethical approval was obtained from the Southampton and South West Hampshire Joint Research Ethical Committee (submission no: 106/01).

Iontophoresis

Nedocromil sodium (2%, 53 μM), frusemide (0.01%, 0.3 μM), bumetanide (0.01%, 0.29 μM) dissolved in reversed osmosis water (water, control) or water were introduced into the skin of the volar surface of the forearm using an iontophoresis chamber of 1 cm diameter (MICI-e, Moor Instruments Ltd, Axminster, Devon, UK). The chamber was fixed to the skin with an adhesive sticker, filled with a solution under test and a total anodal charge of 8 mC was applied (200 μA for 40 s). It was calculated that this charge would introduce theoretical doses of 30.8, 27.5 and 30.3 μg of nedocromil sodium, frusemide and bumetanide, respectively, into the skin over an area of approximately 0.8 cm^2 (a circle of 1 cm diameter).

Histamine challenge

An intradermal injection of 20 μL of 100 μM histamine (UCB, Brussels, Belgium) made up in Ringer's solution (Steriflex,

Fresenius Kabi Ltd, Warrington, UK) or Ringer's solution alone was given into the volar surface of the forearm using a sterile, disposable 0.5 mL syringe (Myjector, Terumo, Belgium).

Scoring of itch

The intensity of the itch was scored by subjects on a 100 mm visual analogue scale (VAS) at 20 s intervals for 5 min after the injection of histamine. Data are expressed as mean score for each individual.

Scanning laser Doppler imaging (SDLI)

Flare areas and blood flux within the flare, indicative of dermal perfusion up to a depth of 1 mm, were measured using an SDLI (Moor SLDI, Moor instruments Ltd) [26]. Laser Doppler images were taken over an area of skin 5 cm square. For estimations of the time course of flare development, low-resolution scans were taken every 30 s for 9 min following histamine injection. To compare the effects of drugs on flare areas and to assess blood flux, high-resolution scans were taken before, immediately prior to intradermal injection and at 10 min after injection.

Images were analysed using the accompanying image analysis software (Moor LDI version 3.0, Moor Instruments Ltd). Each scan was calibrated using a 2 cm scale drawn onto the subject's arm.

Planimetry

Weal areas were calculated from traces of their outlines on acetate sheets pressed onto the skin at 10 min after histamine injection.

Statistics

All results are shown as means $\pm$ SEM for observations in 10 subjects per group. Results in drug- and water-treated sites were compared using Student's *t*-test for paired data and $P < 0.05$ taken as statistically significant.

Protocols

This single-blind study was performed in the temperature-controlled room ($22 \pm 2^\circ\text{C}$) in the Wellcome Trust Clinical Research Facility, Southampton General Hospital. Ten subjects were recruited for each of the two studies. In each study, subjects visited the laboratory on four separate occasions at least 1 week apart, the first for enrolment and the other three for the experiment. Nedocromil sodium, 2% (Aventis, Paris, France), 0.01% frusemide (Sigma, Poole, Dorset, UK) and 0.01% bumetanide (Sigma) were iontophoresed on each of the 3 experimental days, respectively, and compared to water (control) iontophoresed in the same subject.

Study 1

On each visit, four sites, two on the volar surface of each forearm, were selected and studied sequentially, half an hour apart. At each site either drug or water was introduced into

Fig. 1. Diagrammatic representation of the experimental protocols. In study 1, drugs or water were introduced into the skin using a 1 cm diameter iontophoresis chamber. Ten minutes later, histamine was injected intradermally at point x at the centre of the area of iontophoresis. The weal and flare then developed an area indicated in the diagram. In study 2, the same protocol was followed with the exception that histamine was injected 5 mm outside the site of iontophoresis. When the response to histamine developed, the flare response spread over the area of drug iontophoresis. To examine drug effects on blood flux, flux within the area of iontophoresis was compared with that in a similar untreated area.

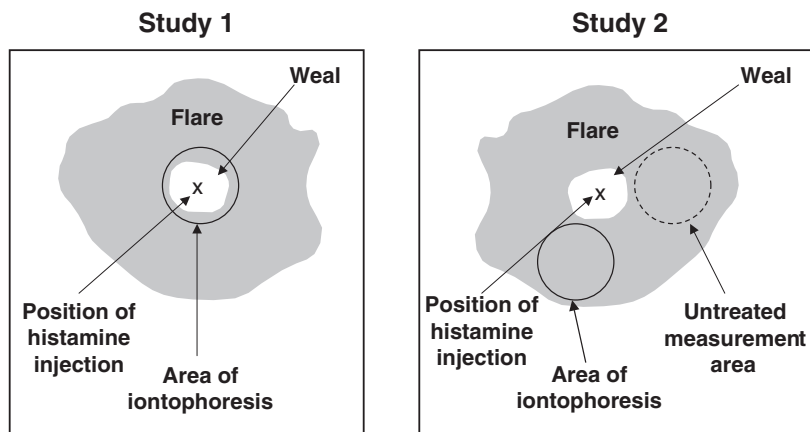
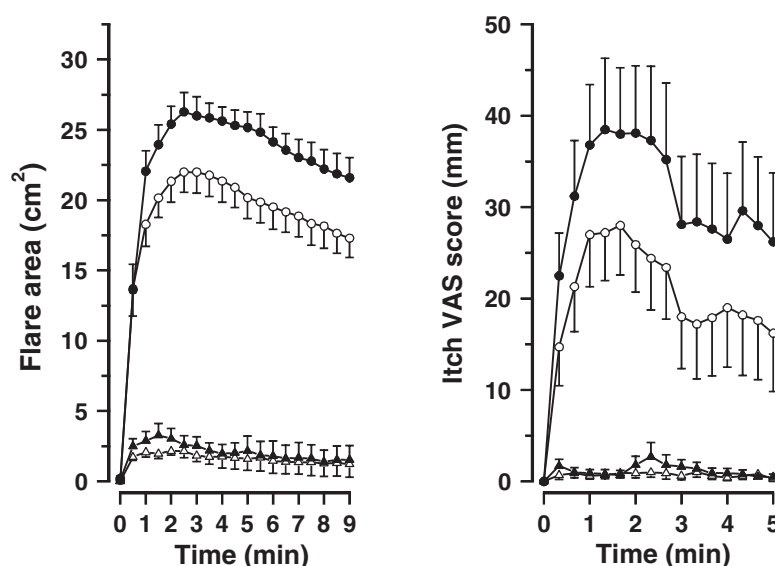


Fig. 2. Histamine-induced itch and flare in the presence and absence of nedocromil sodium. Water (control, closed circles) or nedocromil sodium (open circles) were introduced into the skin by iontophoresis 10 min before histamine injection. Itch was recorded on a 100 mm visual analogue scale (VAS) every 20 s for 5 min and flare area assessed by scanning laser Doppler imaging. As a negative control, Ringer's solution was injected into water (closed triangles) and nedocromil sodium (open triangles) treated areas. All data are mean $\pm$ SEM of observations in 10 subjects.



the skin using iontophoresis and 10 min later by an injection of histamine or Ringer's solution into the middle of the iontophoresis site (Fig. 1). Weal, flare, itch and blood flux were then assessed as described above.

Study 2

In study 2, the same protocol as study 1 was followed with the exception that the histamine injections were given 5 mm outside the site of iontophoresis (Fig. 1).

Results

Effect of iontophoresis on blood flux

To assess whether iontophoresis of either water or drugs caused a local inflammatory response, high-resolution scanning laser Doppler images were recorded before and following removal of the chamber. Blood flux values in perfusion units (PU) measured within the area of the chamber were, before and after iontophoresis, respectively, 108 ± 4

and 108 ± 6 PU for water, 105 ± 8 and 105 ± 7 PU for nedocromil sodium, 93 ± 4 and 101 ± 6 PU for frusemide and 95 ± 3 and 134 ± 26 PU for bumetanide. None of the differences were statistically significant.

Study 1

Injections of histamine (20 μ L of 100 μ M) were made central to the site of iontophoresis in order to look at the effect of drugs on histamine-induced nerve activation.

Injection of histamine into the control sites iontophoresed with water caused an itch response, which reached a maximum at $\sim 1\frac{1}{2}$ min after which it waned slowly (Fig. 2). The mean response for the total recording period was 29.8 ± 5.4 mm on the 100 mm VAS. Injection of Ringer's solution caused minimal itching (Fig. 2). Histamine-induced itch was reduced in the presence of all three drugs in comparison with control. Nedocromil sodium reduced the mean itch over the 5 min assessment period from 29.8 ± 5.4 to 19.9 ± 4.6 mm (33% reduction, $P = 0.004$), frusemide from 27.3 ± 5.5 to 17.3 ± 7.4 mm (37% reduction, $P = 0.016$) and

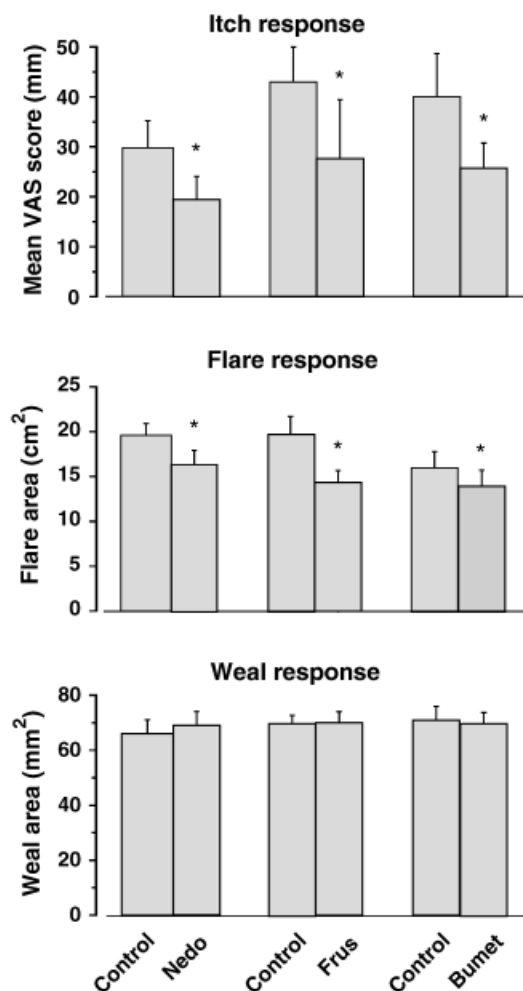


Fig. 3. Histamine-induced itch, flare and weal in the presence and absence of nedocromil sodium (nedo), frusemide (frus) and bumetanide (bumet). Itch is presented as the mean $\pm$ SEM visual analogue scale (VAS) score over 5 min, the flare area as the mean $\pm$ SEM assessed by scanning laser Doppler imaging at 10 min and weal measured by planimetry at 10 min in 10 subjects. Water (control) and drugs were introduced into the skin by iontophoresis 10 min before the injection of histamine. Asterisks indicate a statistically significant ($P < 0.05$) difference in the responses after water and drug.

bumetanide from 25.1 ± 5.3 to 16.1 ± 3.2 mm (36% reduction, $P = 0.026$) (Fig. 3).

Intradermal injection of histamine into the control sites caused a rapidly developing neurogenic flare, which reached a maximum at $2\frac{1}{2}$ min (Fig. 2). Injection of Ringer's solution caused minimal response (Fig. 2). All three drugs significantly reduced the areas of the flare responses. Iontophoresis of nedocromil sodium reduced the mean flare area at 10 min from 19.6 ± 1.3 to 16.3 ± 1.6 cm² (17% reduction, $P = 0.009$), frusemide from 19.8 ± 1.9 to 14.4 ± 1.5 cm² (27% reduction, $P = 0.002$) and bumetanide from 16.1 ± 1.8 to 14.0 ± 1.9 cm² (13% reduction, $P = 0.005$) (Fig. 3).

Blood flux measurements were also assessed within histamine-induced flare responses. The mean flux values in untreated and treated skin, respectively, were 500 ± 70 and 511 ± 73 for nedocromil sodium, 589 ± 41 and 597 ± 30 for frusemide and 510 ± 41 and 525 ± 35 for bumetanide. None of the differences were statistically significant.

Weal areas, measured 10 min after histamine injection, were not significantly different in the presence of any drug compared with water. Control and drug-treated weal areas, respectively, were 69 ± 5 and 66 ± 5 mm² for nedocromil sodium, 70 ± 4 and 69 ± 3 mm² for frusemide and 70 ± 4 and 71 ± 5 mm² for bumetanide (Fig. 3). Weal areas in sites receiving an injection of Ringer's solution were all < 3 mm².

Study 2

Injections of histamine were made 5 mm outside the sites of iontophoresis in order to look at the effect of nedocromil sodium, frusemide and bumetanide when present away from the site of initial nerve activation by histamine.

Itch responses did not significantly differ between drug-treated and control sites. Mean itch scores for control and drug-treated sites, respectively, were 21.7 ± 7.1 and 18.9 ± 5.6 mm for nedocromil sodium, 17.3 ± 5.0 and 18.2 ± 5.3 mm for frusemide and 20.7 ± 4.3 and 20.7 ± 4.7 mm for bumetanide. Itch scores in sites receiving an injection of Ringer's solution were all < 4 mm.

The mean flare areas over the total recording period were unaffected by iontophoresis of the drugs. Flare areas for control (water) and drug sites, respectively, were 20.6 ± 1.6 and 20.0 ± 1.6 cm² for nedocromil sodium, 17.3 ± 0.8 and 18.1 ± 0.9 cm² for frusemide and 17.7 ± 1.1 and 17.7 ± 1.24 cm² for bumetanide. None of the differences were statistically significant. Flare areas in sites receiving an injection of Ringer's solution were < 2 cm².

Blood flux measurements were assessed in two places within histamine-induced flare responses, one being the area of iontophoresis and the other an equal area of untreated skin (Fig. 1). Blood flux values recorded in untreated and treated areas, respectively, were 731 ± 75 and 814 ± 66 PU for water, 684 ± 78 and 720 ± 42 PU for nedocromil sodium, 696 ± 73 and 626 ± 61 PU for frusemide and 776 ± 75 and 679 ± 57 PU for bumetanide. None of the differences were statistically significant.

Like flare areas, weal areas did not significantly differ between control and drug-treated sites in this study. Weal areas for control and drug-treated sites, respectively, were 70 ± 6 and 69 ± 5 mm² for nedocromil sodium, 63 ± 03 and 66 ± 2 mm² for frusemide and 58 ± 2 and 59 ± 3 mm² for bumetanide. None of the differences were statistically significant. Weal areas in sites receiving an injection of Ringer's solution were all < 3 mm².

Discussion

This study showed that iontophoresis of nedocromil sodium into human skin *in vivo* inhibited itch and flare, but not weal, responses induced by the intradermal injection of histamine. The loop diuretics frusemide and bumetanide had similar effects. The data suggest that all three drugs inhibit sensory nerve activation.

Nedocromil sodium is not an H₁-receptor antagonist [3]. This was confirmed by the observation that it had no significant effect on the weal response to histamine injection. Frusemide and bumetanide did not inhibit the weal response, indicating that these drugs also were not acting as H₁

antihistamines. Further, it is highly unlikely that nedocromil sodium is acting as a mast cell stabilizer in this study as; first, histamine rather than a mast cell activating agent was used to provoke the response, and, second, nedocromil sodium has no effect on degranulation of human skin mast cells [27].

When considering the possible mechanisms by which nedocromil sodium, frusemide and bumetanide exert their inhibitory effects on the neurogenic responses, it is convenient to divide the responses into those components involved in both itch and flare, viz sensory nerve activation and axonal conduction, and those involved solely in the flare, viz neuropeptide release, activation of neuropeptide receptors on blood vessels and the ability of the vasculature to respond to stimulation.

Discussing the second group of possibilities first, it is unlikely that nedocromil sodium, frusemide or bumetanide modulated neuropeptide release, activation of neuropeptide receptors or had direct effects on the vasculature. If they had, then a reduced blood flux would have been expected in the area of the flare that had been treated with drug when compared to an untreated area in study 2 in which histamine was injected outside the area of drug iontophoresis. There were no statistically significant differences between untreated sites and treated sites. Also in study 2, some reduction in flare area may have been observed with drug treatment. It was not. Furthermore, although the release and receptor actions of neuropeptides are of crucial importance in the flare response [28, 29] they are suggested not to be involved in the itch response [30, 31], which was inhibited in tandem with the flare in study 1.

It also unlikely that the drugs inhibited axonal conduction. If they had, then a reduced blood flux would have been expected in the area of the flare that had been treated with drug when compared to an untreated area in study 2. As stated above, there were no statistically significant differences between untreated sites and treated sites. Furthermore, none of the drugs are local anaesthetics.

Thus, the most likely mechanism is an inhibitory effect on sensory nerve activation. This is supported by the observations that reductions in flare and itch were seen only when histamine was injected into the area of drug iontophoresis. Such an effect has been suggested for cromoglycate-like drugs from a clinical study in which sodium cromoglycate caused a feeling of warmth when infused intravenously [32]. Furthermore, nedocromil sodium has been reported to inhibit citric acid-induced cough, believed to be due to the stimulation of C-fibre endings [6] and to decrease the frequency of action potentials in C-fibres in response to capsaicin by more than 50% [33]. This is consistent with our observation that reduction of the itch sensation, which is mainly governed by the peak frequency, is greater than that of the flare, which is more dependent on the number of action potentials.

The observations that nedocromil sodium, frusemide and bumetanide have similar profiles of activity against bronchoconstriction [20, 34] opens the possibility that they may have a common mechanism of action. Nedocromil sodium has been shown to inhibit a variety of chloride channels in a wide range of cell preparations [12], loop diuretics cause diuresis by inhibiting a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter. Recently, a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter has been reported to be expressed on sensory neurones [35–37]. Frusemide and bumetanide, which

block this type of cotransporter, prevent the intracellular accumulation of Cl^- ions in dorsal root ganglion cells [38]. There is evidence to suggest that dorsal root ganglion cells, having accumulated Cl^- through the action of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, depolarize upon activation of calcium-activated chloride channels as Cl^- exits the cell [39]. They therefore provide a possible route through which Cl^- ions might leave sensory nerves in response to an increase in intracellular calcium by histamine to cause nerve depolarization. In GABAergic sensory neurones chloride efflux stimulates potassium efflux, which in turn activates the cotransporter to return the membrane potential to baseline [40]. Therefore, drugs that inhibit the cotransporter and reduce intracellular chloride would shift the membrane potential to a more hyperpolarized state. Also, the inhalation of solutions with a low chloride concentration cause coughing in man suggesting that the efflux of chloride ions from sensory nerves produces depolarization and hence the generation of action potentials [41]. The ability of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter inhibitors frusemide and bumetanide to successfully inhibit itch and flare in this model, an effect comparable to that of nedocromil sodium, indicates chloride channel blockade to be a likely common mechanism of action of these drugs.

This study has provided evidence to support the hypothesis that nedocromil sodium inhibits sensory nerve activation to inhibit the itch and flare responses induced by histamine in human skin *in vivo*. Frusemide and bumetanide produced comparable inhibitory effects to nedocromil sodium suggesting inhibition of a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter in the sensory nerve membrane to be a possible mechanism of action. At the clinical level, nedocromil sodium is effective in reducing neurogenic itch in the skin when its penetration through the stratum corneum is facilitated by iontophoresis. At mucosal membranes, including the bronchi, nose, eye and intestine, it is likely that inhibition of sensory nerve activation by nedocromil sodium contributes to its beneficial effects in allergic disease.

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