Effects of cromolyn and nedocromil on ion currents in canine tracheal smooth muscle

L.J. Janssen, J. Wattie, P-A. Betti

Electrophysiological studies of airway smooth muscle (ASM) have identified several ionic currents which are activated during membrane depolarization. In direct response to the change in membrane potential, voltage-dependent Ca\(^{2+}\) currents and at least two different K\(^+\) currents (delayed rectifier and Ca\(^{2+}\)-dependent) are activated [1–4]. Opening of the Ca\(^{2+}\) channels results in Ca\(^{2+}\) influx, which in turn contributes further to Ca\(^{2+}\)-dependent K\(^+\) channel activity and activates Ca\(^{2+}\)-dependent Cl\(^{-}\) currents [5]. All of these currents have unique activation, inactivation and deactivation characteristics and distinct pharmacological sensitivities which allow them to be distinguished and studied in relative isolation using patch-clamp techniques. Various physiological responses are closely linked to changes in activity of these membrane currents. For example, voltage-dependent Ca\(^{2+}\) channels are involved in excitation-contraction coupling by mediating voltage-dependent Ca\(^{2+}\)-influx (electromechanical coupling) as well as refilling of the internal Ca\(^{2+}\) pool [2–4, 6]. K\(^+\) channels mediate membrane hyperpolarization and are therefore important in setting the resting state of the cell and/or inhibiting excitation. As such, many relaxants act by opening these channels while spasmogens act in part by suppressing their activity [7]. Cl\(^{-}\) channels contribute to the membrane depolarization in response to spasmodic stimulation [7–9]. In addition to the conductance changes described above, many spasmodens also activate a nonselective cation current [8, 9], although the signalling mechanisms underlying this response are still unclear.

Asthma is characterized by airway hyperresponsiveness and bronchoconstriction. Since it is believed that the sequence of events leading to asthma begins with airway inflammation, the widely accepted prophylactic approach to the treatment of asthma is the use of anti-inflammatory agents, of which the most effective are inhaled corticosteroids [10, 11]. In addition to steroids, other drugs including the chromones nedocromil and cromolyn sodium mediate a variety of effects including suppression of the release of inflammatory mediators and cytokines from mast cells [10–12]. However, there is still considerable controversy with respect to the precise mechanism of action of these non-steroidal anti-inflammatory agents [10, 11].

Recently, a number of these nonsteroidal anti-inflammatory agents have been found to exert effects on ion channels. For example, cromolyn sodium and nedocromil block Cl\(^{-}\) channels in cultured mast cells [13, 14], colonic carcinoma cells [14], airway epithelial cells [15], mouse 3T3 fibroblasts [16] and bovine pulmonary arterial endothelial cells [17]. In rabbit vagal C-fibres, however, nedocromil first activates Cl\(^{-}\) channels, then suppresses them [18]. Cromolyn sodium also blocks the Ca\(^{2+}\) conductance, which mediates refilling of the internal Ca\(^{2+}\) pool of rat peritoneal mast cells [13].

In light of the influence that these commonly used clinical agents may have on Ca\(^{2+}\) homeostasis and ion channels, which play central roles in ASM excitability, this study sought to examine their effects on the activity of canine ASM.
Materials and methods

Preparation of tissues and cell dissociation

Adult mongrel dogs were killed with pentobarbital sodium (100 mg·kg⁻¹). Tracheae were excised, kept in Ringer's solution and dissected to remove overlying connective tissue, vasculature and epithelium. Tracheal muscle was then cut into strips parallel to the muscle fibres (~1 mm wide) and transferred to Ringer's buffer for use in muscle baths (see below) or in enzyme-containing dissociation buffer (0.5–1.0 g wet weight·mL⁻¹; composition given below). Tissues were either used immediately or stored at 4°C for use up to 48 h later; it was previously found that tissues or cells studied immediately exhibit similar functional responses to those used after refrigeration. To liberate single ASM cells, tissues in enzyme-containing solution were incubated at 37°C for 60–120 min, then gently triturated.

Patch-clamp electrophysiology

Single ASM cells were allowed to settle and adhere to the bottom of a recording chamber (1 mL bath volume, perfused at 2–3 mL·min⁻¹) and were studied within 6 h. Membrane currents were recorded using the nystatin perforated patch method to limit "run-down" of currents. Electrode tip resistances ranged from 3–5 MΩ, with access resistance of 10–20 MΩ. Data were filtered at 1 kHz and stored on magnetic tape using a digital data recorder (Instrutech, GT Neck, NY, USA) while being simultaneously digitized using pClamp 6.1 (Axon Instruments, Foster City, CA, USA; sampling rate of 2 kHz). In many cases, voltage protocols were repeated two or three times and the resultant currents averaged. Corrections were not made for liquid junction potentials. During recording of membrane currents evoked by step depolarizations, leak and capacitance currents were subtracted using the P/4 method with hyperpolarizing pulses from the holding potential.

Fura-2 fluorimetry

Single ASM cells were studied using a Deltascan system (Photon Tech. Int., 5th Brunswick, NJ, USA). After settling onto a glass coverslip mounted on a Nikon inverted microscope (Diaphot, Garden City, NY, USA), cells were loaded with the membrane-permeant form of fura-2 (fura-2/AM; 2 µM) at 37°C for 30 min, then superfused continuously with Ringer's buffer 2–3 mL·min⁻¹. Only cells which exhibited reversible contractions to acetylcholine were used. Light from a 75 W xenon lamp was monochromatically filtered to restrict excitation light to 340 or 380 nm wavelengths (10 nm bandwidth). Cells were illuminated alternately at excitation wavelengths of 340 and 380 nm and the emitted fluorescence (measured at 510 nm) in-vitro alternated at excitation wavelengths of 340 and 380 nm wavelengths (10 nm bandwidth). Cells were illuminated at 1 and 10 Hz with 5 min between each of these pulse trains, after which a carbachol dose-response curve was generated (10⁻⁶ to 10⁻⁴ M). Tissues were dried and weighed in order to express the contractile responses as grams tension developed per mg dry weight tissue; those tissues weighing <1 mg were not included in the statistical analysis.

Solutions and chemicals

Single cells were dissociated in Ringer's buffer containing the following (in mM): NaCl, 125; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 10; EDTA, 0.25; d-glucose, 10; taurine, 10; pH 7.0; collagenase (type IV, Sigma, Oakville, Ontario, Canada; 2.6 U·mL⁻¹); elastase (type IV, Sigma; 12.5 U·mL⁻¹); bovine serum albumin (BSA; 1 mg·mL⁻¹). During electrophysiological and fluorimetric studies, cells were bathed in Ringer's buffer containing (in mM): NaCl, 130; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 20; d-glucose, 10; pH 7.4. For electrophysiological studies, the electrode (i.e. intracellular) solution contained (in mM): KCl, 140; CaCl₂, 4.0; MgCl₂, 1; EGTA, 1; HEPES, 20; pH 7.2. To facilitate the study of Ca²⁺ currents, K⁺ channels were blocked using an electrode solution containing (in mM): CsCl, 130; tetraethyl ammonium chloride, 10; MgCl₂, 1; HEPES, 20; EGTA, 5; pH 7.2. Nystatin (300 units·mL⁻¹) was also added to all electrode solutions to perforate the membrane and allow electrophysiological recording.

Data analysis

Responses are reported as means±SEM; n refers to the number of cells tested. Statistical comparisons were made using a Student's t-test and analysis of variance (ANOVA), with p-values <0.05 being considered significant.
Fig. 1. – After establishing a nystatin-perforated patch preparation, canine tracheal smooth muscle was dialysed with a K+ containing electrode solution. a) Depolarizing pulses (350 ms duration; 10 mV increments) from a holding potential of -80 mV were used to evoke outward K+ currents; in this particular cell, repolarization to the holding potential evoked inward Cl- tail currents. b) Application of cromolyn (10^{-4} M in application pipette) markedly decreased the outward K+ currents and eliminated the inward Cl- tail currents. Mean current-voltage relationship for peak outward K+ current (I_K) in the absence (❍) and presence (●) of c) 10^{-4} cromolyn (n=7) or d) 10^{-4} M nedocromil (n=6); currents are expressed as a percentage of the control current evoked at +30 mV in each cell.

Table 1. – Effect of chromones on ionic mechanisms

<table>
<thead>
<tr>
<th>Chromone</th>
<th>K+ current (mV)</th>
<th>Ca2+ current (mV)</th>
<th>Cl- current (%)</th>
<th>Ca2+ transient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cromolyn</td>
<td>57.1±7.9* (7)</td>
<td>9.1±7.3* (7)</td>
<td>31±10* (8)</td>
<td>67.8±4.7 (20)</td>
</tr>
<tr>
<td>Nedocromil</td>
<td>48.4±7.6* (6)</td>
<td>3.2±8.2* (3)</td>
<td>111.0±20.4 (6)</td>
<td>48.4±7.6* (3)</td>
</tr>
<tr>
<td>Ringer’s</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
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</table>

K+ and Ca2+ currents were evoked by depolarizing pulses (to +30 and +10 mV, respectively), while Cl- currents and Ca2+ transients were evoked by acetylcholine (10^{-4} M). After a control response, cells were exposed to drugs (10^{-4} M) for 5–10 min or Ringer's buffer followed by a second test response. Data is expressed as percentage of the initial control response obtained within the same cell. For statistical analysis, Ca2+ and Cl- currents and Ca2+ transients were compared with responses during application of Ringer's (*: p<0.05), while K+ currents were compared with their own control responses. Values in parenthesis indicate number of cells or tissues tested. ND: not done.

Fig. 2. – Spontaneous outward K+ currents in a cell held continuously at 0 mV and dialysed with a K+ containing electrode solution. Neither the frequency nor the amplitude of these currents was altered by cromolyn (10^{-4} M in application pipette; 30 s application). The dotted line indicates the zero current level.
Results

Effects of chromones on K+ currents

K+ currents evoked by depolarizing pulses were greatly suppressed within ~30 s after application of cromolyn (fig. 1). In seven cells, cromolyn reduced the K+ currents recorded at +30 mV from 1736±287 pA to 917±146 pA (fig. 1c and table 1). Using the same experimental approach, outward K+ currents were suppressed to a comparable degree by nedocromil (1917±280 pA versus 876±142 pA, n=6; fig. 1d and table 1). The study also investigated the effect on K+ currents triggered by Ca2+ released spontaneously from the internal store [20]; figure 2 shows such spontaneous transient outward currents (STOC) in a cell held constantly at 0 mV. Cromolyn did not reduce either the amplitude or the frequency of the STOC, indicating that it does not antagonize Ca2+-dependent K+ channels directly.

Effects of chromones on voltage-dependent Ca2+ currents

After blocking K+ currents (by replacement of K+ with Cs+), voltage-dependent Ca2+ currents were evoked by repeated depolarizations to +10 mV (100 ms duration; from the holding potential of -80 mV) at 15 s intervals in the absence and presence of chromones (10-4 M in application pipette). The control responses to these depolarizing pulses are given in figure 3a, while the mean current-voltage relationship is given in figure 3b. Cromolyn did not alter the holding current, but greatly reduced the Ca2+ currents (fig. 3b). In seven cells studied, cromolyn reduced the peak calcium current from 46.2±7.6 pA to 5.9±3.8 pA (fig. 3b and table 1). Likewise, nedocromil did not alter the holding current but suppressed the peak calcium current from a mean value of 40.8±15.9 pA to 3.0±2.9 pA (table 1; n=3). Ca2+ currents were also reduced, though to a much lesser extent, during application of vehicle alone (Ringer's) (table 1), possibly due to run-down [21].

Effects of chromones on agonist-evoked Cl- current and Ca2+-release

Figure 4 shows the membrane current evoked by acetylcholine (ACh) (10-4 M) in a single canine tracheal ASM cell; this current was considerably smaller in the presence of cromolyn than in its absence (fig. 4). In eight cells studied in the same way, the ACh-evoked current was 161±38 pA and 958±120 pA in the presence and absence of cromolyn, respectively (table 1). While cromolyn seemed to moderately reduce ACh-evoked Ca2+-release (fig. 5), this reduction was not significantly different from the run-down seen after cells were exposed to vehicle (table 1; responses expressed as a fraction of the control response evoked prior to exposure to cromolyn or vehicle). The absolute magnitudes of the cholinergic Ca2+ transients were 552±72 nM (n=20) in the presence of cromolyn and 318±44 nM (n=15) during application of vehicle alone.

Nedocromil had no significant effect on ACh-evoked membrane currents (these were 1165±577 pA and 1398±247 pA in the absence and presence of nedocromil, respectively; table 1). Similarly, there was not a significant difference between ACh-evoked Ca2+ transients in nedocromil-treated and vehicle-treated cells when these were expressed in absolute terms (311±76 nM (n=12) and 318±
Effects of chromones on agonist-evoked contractions

Cumulative dose-response curves for carbachol were generated in the absence or presence of cromolyn or nedocromil (final bath concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M). Neither chromone induced a change in basal tension. While the maximal response to carbachol was significantly smaller in the presence of 10^{-5} and 10^{-4} M cromolyn compared with control (fig. 6a; table 2), there was no accompanying change in the carbachol medium effective concentration (EC50) values (table 2); the contractile responses to electrical stimulation were also unaffected by cromolyn (fig. 6b). Nedocromil had no significant effect on the responses to either exogenously added carbachol (neither EC50 nor maximum) or nerve-released ACh (fig. 7 and table 2).
Discussion

In this study, the aim was to investigate the effects of the anti-inflammatory agents cromolyn sodium and nedocromil on ion currents and responses evoked by cholinergic stimulation. Depolarizing pulses in single myocytes dissociated from airway smooth muscle evoke a series of ion conductance changes (exemplified in fig. 1a) which include an initial small inward voltage-dependent Ca\(^{2+}\) current [1–4] followed by large outward delayed rectifier and Ca\(^{2+}\)-dependent K\(^+\) currents [1, 4], as well as a Ca\(^{2+}\)-dependent Cl\(^-\) current [5]. Upon repolarization to the holding potential, some cells (including the one in fig. 1a) exhibit large Cl\(^-\) tail currents with time constants of hundreds of milliseconds [5].

V\(^{+}\) current

Cromolyn and nedocromil antagonized voltage-dependent Ca\(^{2+}\) currents in canine tracheal myocytes. The observation that vehicle alone did not mediate the same suppression of Ca\(^{2+}\) current indicates that a nonspecific effect (e.g., puffer pressure or trace amounts of metals in the applied solution) is not responsible for the chromone-induced suppression. These agents also suppress a Ca\(^{2+}\) conductance in mast cells, although this pathway is voltage independent and activated by depletion of the internal Ca\(^{2+}\) pool [13]. It is not surprising that these agents are capable of influencing voltage-dependent Ca\(^{2+}\) channel activity, given that their chemical structure, i.e., a series of aromatic rings with highly polar molecular groups, is similar to that of a number of other classes of Ca\(^{2+}\) channel blockers, including dihydropyridines (e.g., nifedipine), piperidines, benzothiazepines and phenylalkylamines [21]. Although able to abolish voltage-dependent Ca\(^{2+}\) currents, cromolyn and nedocromil were relatively ineffective against contractile responses to cholinergic stimulation (which are largely pharmacomechanically mediated); in like fashion, classical blockers of voltage-dependent Ca\(^{2+}\) channels are generally not useful in reversing cholinergically induced bronchoconstriction.

K\(^+\) current

Cromolyn and nedocromil also suppressed depolarization-evoked K\(^+\) currents in these cells. This was clearly not a direct effect on Ca\(^{2+}\)-dependent K\(^+\) currents, since spontaneous K\(^+\) currents (which are triggered by Ca\(^{2+}\) released from the sarcoplasmic reticulum) were unaffected. However, Ca\(^{2+}\)-dependent K\(^+\) currents are also triggered by voltage-dependent Ca\(^{2+}\) influx, and the latter were found to be nearly abolished by the chromones. Previous studies using canine tracheal ASM have shown that any method which abolishes Ca\(^{2+}\) currents also suppresses the depolarization-evoked Ca\(^{2+}\)-dependent K\(^+\) current in exactly the same manner as shown in this study during application of chromones [4, 22]. It is unlikely that the delayed rectifier K\(^+\) current was suppressed greatly by the chromones. Otherwise, the abolition of the voltage-dependent Ca\(^{2+}\) current and Ca\(^{2+}\)-dependent K\(^+\) current, together with a suppression of delayed rectifier K\(^+\) current, would result in nearly complete reduction of all depolarization-evoked current: contrary to this prediction, a sizeable fraction of K\(^+\) current remained in the presence of the chromones. Thus, the partial reduction of K\(^+\) current by chromones was interpreted as reflecting decreased Ca\(^{2+}\)-dependent K\(^+\) current secondary to inhibition of voltage-dependent Ca\(^{2+}\) current. Suppression of K\(^+\) currents is one pathway by which spasmodens depolarize a cell and mediate their mechanical effects [5, 8, 9]; however, chromones do not evoke such an electromechanically mediated contraction, since voltage-dependent Ca\(^{2+}\) channels are also blocked.

Cl\(^-\) current

Protocols have previously been developed whereby Ca\(^{2+}\)-dependent Cl\(^-\) currents are elicited by depolarization-evoked Ca\(^{2+}\) influx [5] or by agonist-evoked release of internally sequestered Ca\(^{2+}\) [8, 9]. While the former protocol might have been useful in investigating the effects of the anti-inflammatory agents on Cl\(^-\) currents, the finding that voltage-dependent Ca\(^{2+}\) influx is abolished by the clinical agents precludes this approach. Therefore, the effects
of these agents on acetylcholine-evoked Ca\(^{2+}\) release and Cl\(^{-}\) currents were investigated and it was found that the latter were also reduced by cromolyn. Others have shown cromolyn to suppress intermediate conductance, Ca\(^{2+}\)-independent Cl\(^{-}\) channels on mast cells and colonic carcinoma cells [14], a small conductance, volume-activated Cl\(^{-}\) current on endothelial cells [17], a Ca\(^{2+}\)-independent Cl\(^{-}\) channel on mouse 3T3 fibroblasts [16] and a voltage-dependent, Ca\(^{2+}\)-dependent Cl\(^{-}\) channel on airway epithelial cells [15]. The Cl\(^{-}\) channels in canine airway smooth muscle are of a small-conductance, Ca\(^{2+}\)-dependent variety [5, 20]; the agonist-evoked current remaining in the presence of cromolyn may represent the nonselective cation current described previously [8, 9]. Cromolyn seemed to be acting directly on the Cl\(^{-}\) channels, since ACh-evoked Cl\(^{-}\) transients (which trigger the Cl\(^{-}\) currents) were not significantly affected. The chemical structure of cromolyn (a large carboxylic acid with a number of aromatic rings) is similar to that of many other Cl\(^{-}\) channel blockers, including niflumic acid and 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid (SITS). Curiously, nedocromil had relatively little effect against ACh-evoked Cl\(^{-}\) currents in this study of canine airway smooth muscle, even though it has many structural similarities with cromolyn, niflumic acid and SITS. One important difference between them, however, is that the three aromatic rings in nedocromil are not free to rotate relative to one another, while those of cromolyn, niflumic acid and SITS are free to do so around the alkyl chain or amine group which joins them. In contrast to the present findings made in ASM, others found nedocromil to be more potent than cromolyn with respect to blocking Cl\(^{-}\) channels in mouse 3T3 fibroblasts [16]. This tissue-related difference may reflect a channel subtype specificity of the chromones (Ca\(^{2+}\)-dependent versus Ca\(^{2+}\)-independent, respectively) and/or differing mechanisms of actions of these drugs (cromolyn decreases the unitary conductance of Cl\(^{-}\) channels in airway epithelial cells, while nedocromil decreases the open probability without any change in unitary conductance [15].

Conclusions

Cromolyn and nedocromil markedly antagonized voltage-dependent Ca\(^{2+}\) currents in canine airway smooth muscle. While neither agent significantly altered the acetylcholine-triggered release of internal Ca\(^{2+}\), high concentrations of cromolyn seemed to also partially suppress acetylcholine-evoked Cl\(^{-}\) currents and contractions. Although it might be argued that the concentrations required in this study to achieve statistical significance greatly exceed conventional therapeutic plasma concentrations, it should be pointed out that cromolyn and nedocromil are delivered by inhalation; thus, the concentrations attained in the tissues lining the airways (including the smooth muscle layer) would be likely to reach much higher levels than those found in the plasma.

References