Suppression of the cough reflex by inhibition of ERK1/2 activation in the caudal nucleus tractus solitarii of the rabbit

Donatella Mutolo¹, Fulvia Bongianni¹, Elenia Cinelli¹, Maria Grazia Giovannini² and Tito Pantaleo¹

¹Dipartimento di Scienze Fisiologiche, Viale G.B. Morgagni 63 and ²Dipartimento di Farmacologia Preclinica e Clinica, Viale Pieraccini, 6, Università degli Studi di Firenze, 50134 Firenze, Italy.

Running Head: ERK1/2 activation within the NTS and the cough reflex

CORRESPONDING AUTHOR:

Donatella Mutolo
Dipartimento di Scienze Fisiologiche
Viale G.B. Morgagni 63
50134 Firenze - Italy

e-mail: donatella.mutolo@unifi.it
Phone: +39 055 4237319
Fax: +39 055 4379506
ABSTRACT

The caudal nucleus tractus solitarii (cNTS), the predominant site of termination of cough-related afferents, has been shown to be a site of action of some centrally acting antitussive agents. A role of Extracellular Signal Regulated Kinases-1 and -2 (ERK1/2) has been suggested in acute central processing of nociceptive inputs. Since pain and cough share similar features, we investigated whether ERK1/2 activation could also be involved in the central transduction of tussive inputs. To this purpose, we undertook the present research on pentobarbitone anesthetized, spontaneously breathing rabbits by using microinjections (30-50 nl) of an inhibitor of ERK1/2 activation (U0126) into the cNTS. Bilateral microinjections of 25 mM U0126 caused rapid and reversible reductions in the cough responses induced by both mechanical and chemical (citric acid) stimulation of the tracheobronchial tree. In particular, the cough number and peak abdominal activity decreased. Bilateral microinjections of 50 mM U0126 completely suppressed the cough reflex without affecting the Breuer-Hering inflation reflex, the pulmonary chemoreflex and the sneeze reflex. These U0126-induced effects were, to a large extent, reversible. Bilateral microinjections of 50 mM U0124, the inactive analogue of U0126, at the same cNTS sites had no effect. This is the first study that provides evidence that ERK1/2 activation within the cNTS is required for the mediation of cough reflex responses in the anesthetized rabbit. These results suggest a role for ERK1/2 in the observed effects via nontranscriptional mechanisms, given the short time involved. They also may provide hints for the development of novel antitussive strategies.

Keywords: airway defensive reflexes; cough; nucleus tractus solitarii; MAP kinase; ERK1/2
INTRODUCTION

Cough is one of the most important airway defensive reflexes (29) involving several brainstem structures (e.g., Refs. 4, 5, 30, 31, 40-43, 50). Antitussive drugs possess little clinically relevant efficacy and their use is limited by important side effects (1). A better understanding of the neural mechanisms involved in acute and chronic cough will result in more effective antitussive treatments.

While it is well known that tracheobronchial rapidly adapting receptors (RARs) are involved in cough mediation, the role of bronchopulmonary C-fibers and Aδ-nociceptive pulmonary afferent fibers in this reflex remains controversial (9, 32, 65). The role of C-fibers in the cough production has been discussed in previous studies (8, 41). Recently, we have shown in the rabbit that cough evoked by mechanical stimulation of the tracheobronchial tree and by citric acid inhalation is primarily mediated by glutamatergic neurotransmission at the level of the caudal aspect of the nucleus tractus solitarii (cNTS) and, especially, within the commissural subnucleus (comNTS) where cough-related afferents terminate (41, 43). This medullary region has been identified as the predominant site of central projections of tracheobronchial RARs (30, 31) and, possibly, of the so-called “cough receptors” responsive to mechanical stimuli and acid, recently described in the larynx and rostral trachea of guinea pigs (6, 7, 35; see Ref. 8 also for further Refs.). Both pain and cough share similar features, such as central and peripheral sensitization (e.g. Refs. 8, 10, 20-24, 26, 35, 43, 47, 66, 67). In more detail, in agreement with results obtained in the guinea pig (8, 24, 35), we have shown that substance P microinjections into the cNTS potentiate the cough reflex in the rabbit (43). We have also shown that the cNTS is a site of action of some centrally active antitussive drugs and a likely component of the complex neural system involved in cough regulation (40). In agreement with Bonham et al. (5), the NTS is the first central site where the sensory inputs related to cough can be modulated and a possible target for synaptic plasticity.
The Mitogen Activated Protein Kinase (MAPK) signal transduction pathways are ubiquitous and evolutionarily well conserved protein kinases involved in relaying extracellular signals into intracellular responses (e.g. Refs. 16, 22, 47, 55). The Extracellular Signal Regulated Kinases-1 and -2 (ERK1/2) were the first members of the MAPK family to be discovered, followed by p38/HOG, JNK/SAPK (c-jun N-terminal kinase/stress activated protein kinase), and ERK5. The classic ERK1/2 signal transduction pathway consists of a three-layered cascade of protein kinases in which MAPK kinase (MEK) is a dedicated dual-specificity kinase that phosphorylates and thereby activates only ERK1/2 (55).

Recently, it has been reported that MAPK pathways are involved in signal transduction mechanisms of primary afferent neurons, in particular of those responsible for the modulation of pain transmission. Activation of MAPKs may result in the induction and maintenance of pain hypersensitivity via nontranscriptional and transcriptional central and peripheral mechanisms (22, 47, 67). In particular, MEK inhibitors reduce acute pain within a short time, thus suggesting a role of ERK1/2 in acute processing of nociceptive inputs at many levels, including dorsal root ganglia, spinal cord and amygdale, not only through transcriptional, but possibly also through nontranscriptional mechanisms (10, 14, 20, 21, 23, 26). Interestingly, it has been suggested that a better understanding of intracellular signaling pathways involved in nociception and nerve sensitization may lead to novel antitussive drugs such as p38 MAPK inhibitors (1). Owing to the similarities between the characteristics of central processing of nociceptive and cough-related inputs, we hypothesized that ERK1/2 activation could also have a role in triggering and modulating the cough reflex at the central level. Thus, in an attempt to disclose an involvement of the ERK1/2 transduction pathway in the regulation of the cough reflex in response to mechanical or chemical stimulation of the tracheobronchial tree, we undertook the present research on pentobarbitone anesthetized, spontaneously
breathing rabbits by inhibiting ERK1/2 activation by means of microinjections of the MEK inhibitor U0126 into the cNTS.

We also investigated whether U0126 microinjections affected the cough reflex without any influence on the Breuer-Hering (B-H) inflation reflex (4, 43, 64) and the pulmonary chemoreflex (27, 32, 39, 43) due to slowly adapting stretch receptor (SAR) and C-fiber afferent stimulation, respectively. These reflexes have their second order neurons mainly located in different albeit neighbouring subnuclei of the NTS (30, 31, 43). An attempt was also made to ascertain whether the sneeze reflex was affected by U0126 microinjected into the cNTS since the results of different reports (12, 34, 49, 61) suggest that the cNTS and convergent inputs from nasal and vagal afferents may have a role in the regulation of nasotrigeminal reflex responses.
MATERIALS AND METHODS

Animal preparation. Experiments were performed on 22 male New Zealand white rabbits (2.7-3.5 kg) anesthetized with sodium pentobarbitone (40 mg kg⁻¹ i.v., supplemented by 2-4 mg kg⁻¹ every 30 min; Sigma-Aldrich, St. Louis, MO, USA). Atropine (0.15 mg kg⁻¹ i.m.) was administered to reduce mucosal secretion in the airways. The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. Additional criteria were the presence of a stable and regular pattern of phrenic bursts and the absence of fluctuations in arterial blood pressure or phrenic nerve activity, whether spontaneous or in response to somatic nociceptive stimulation. All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Community Council on the use of laboratory animals (Directive 86/609/EEC). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize both the number of animals used and their suffering. Experimental procedures and details about the methods employed have previously been described (40, 41, 43).

Recording procedures. Efferent phrenic nerve activity was recorded using bipolar platinum electrodes from the central stump of the cut and desheathed C₃ or C₅ phrenic root. The electromyographic (EMG) activity of abdominal muscles was recorded by wire electrodes (Nichrome wires, insulated except for 1 mm at the tips, diameter 0.1 mm) inserted into the external or the internal oblique abdominal muscles. Phrenic and abdominal activities were amplified, full-wave rectified, and “integrated” (low-pass RC filter, time constant 100 ms). Arterial blood pressure was recorded by a strain-gauge manometer. End-tidal CO₂ partial pressure was measured by an infrared CO₂ analyzer (Datex, CD-102; Normocap, Helsinki, Finland). Integrated phrenic and abdominal activities as well as the signals of the other variables studied were recorded on an eight-channel rectilinearly writing chart recorder.
Cardiorespiratory variables were also acquired and analyzed using a personal computer, equipped with an analog-to-digital interface (Digidata 1200, Axon Instruments, Union City, CA, USA) and appropriate software (Axoscope, Axon Instruments).

Microinjection procedures. Microinjection procedures have been fully described in previous reports (40, 41, 43). Bilateral microinjections (30-50 nl) were performed via a glass micropipette (tip diameter 10-25 μm) into the cNTS and particularly into the lateral comNTS (Fig.1). The following drugs (Tocris Cookson, Bristol, UK) were used: U0126 (25-50 mM), a potent MEK inhibitor, and U0124 (50 mM), the inactive analogue of U0126. Both drugs were dissolved in 50% dimethyl sulfoxide (DMSO, Sigma-Aldrich) /saline. A single preparation was employed to test the effects of each drug at a single concentration. Drug concentrations were in the same range as those previously used in in vivo preparations (2, 11, 13, 54). Control injections of equal volumes of the vehicle solution containing equivalent amounts of DMSO at the responsive sites were also made. In each experiment, bilateral microinjections were performed at two different sites along the rostrocaudal extent of the cNTS mainly corresponding to the comNTS (Fig. 1) following a procedure previously described (40, 41, 43). Briefly, the stereotaxic coordinates were selected according to the atlas of Meessen and Olszewski (36). They were approximately as follows: the first site at the level of the caudal-most end of the area postrema, 0.6-0.8 mm lateral to the midline and 0.7-0.8 mm below the dorsal medullary surface, the second 0.5 mm more caudal, usually 0.4-0.5 mm lateral to the midline and 0.7-0.8 mm below the dorsal medullary surface.

Stimulation procedures. Cough was induced by both mechanical or chemical stimulation of the tracheobronchial tree. Mechanical stimulation (4-5 s) was performed by means of a 0.5-mm diameter nylon fiber inserted through a lateral port of the tracheal cannula until the tip was judged to be near the carina and main bronchi (for further details see Refs. 40, 42, 43). An
interval of ~ 1 min was scheduled between cough stimulations. As a rule, 3 stimulation trials were performed in succession before drug administration. These stimulation trials were also accomplished ~ 5 min after the completion of all the microinjections and repeated at appropriate intervals (at least 4-5 min) until complete recovery was observed or for a maximum of ~ 3 h. Chemical stimulation of the tracheobronchial tree was performed by means of citric acid inhalation (41). Citric acid (1 M, Sigma-Aldrich) was freshly dissolved in saline and nebulized (particle diameter 80% from 0.5 to 8 µm; nebulization rate 0.5 ml min\(^{-1}\)) via an ultrasonic nebulizer (Projet, Artsana, Grandate, CO, Italy). The opening of the tracheal cannula, through which the rabbits were spontaneously breathing, was exposed to a steady stream of the nebulized citric acid solution for ~ 3 s (see Ref. 41). The interval between chemical challenges was > 10 min (usually ~ 15 min) since similar cough reflexes could be reliably obtained at minimal intervals of 7 min (41). Chemical stimulation was always applied 2-3 min after mechanically-induced cough. As a rule, chemical stimulation was performed both before and ~ 15 min after the completion of the injections and repeated at appropriate intervals to follow the time course of the recovery process for a maximum of ~ 3 h.

The B-H inflation reflex due to the stimulation of SARs was elicited by tracheal occlusion at end-inspiration. The reflex is characterized by a prolongation of the expiratory phase and by expiratory muscle activation (4, 43, 64). The pulmonary chemoreflex is a composite response mainly consisting of bradycardia, hypotension and tachypnea (27, 32, 39, 43). It was evoked by stimulating bronchopulmonary C-fibers by right atrial injections of phenylbiguanide (PBG, Sigma-Aldrich). To this purpose, the right femoral vein was cannulated and the tip of the polyethylene cannula was advanced to the right atrium. The position of the cannula was confirmed post mortem. In these experiments the same cannula was used to introduce PBG and other drugs. PBG was dissolved in saline and administered in dose of 80 µg kg\(^{-1}\) in 300 µl volumes over ~ 2 s (27, 39, 43). The volume of PBG utilized was less than the dead space of
catheter. The PBG was loaded into the catheter, and the drug was infused with a 0.5-ml saline flush. Sneezing was induced by using a 0.3-mm diameter nylon fiber with a smoothed tip inserted into one nostril and pushed 2 times forward 1.5 cm into the nose. This mechanical stimulation was gentle and short-lasting (~ 3 s) to avoid as much as possible traumatic effects. Before nasal stimulation, the nylon fiber was positioned into one nostril for an extent (starting point) proved in preliminary trials to be suitable for the generation of consistent reflex responses (29, 41). The B-H inflation reflex, the sneeze reflex and the pulmonary chemoreflex (single trials) were elicited in succession (with at least a 3-min interval between trials) before and ≥ 30 min after U0126 only at the higher concentration (50 mM), i.e. when the cough responses were abolished (see Results). Microinjections of U0126 were performed at least 30 min after the completion of control reflex responses. In all instances, stimulation procedures were repeated at appropriate intervals until complete recovery was observed or for a maximum of ~ 3 h after the injections.

Histology. At the end of each experiment, the brain was perfused via a carotid artery with saline and subsequently with 10 % formalin solution. After at least a 48-h immersion in 10 % formalin solution, the brain was placed in a hypertonic sucrose solution. Frozen 20-μm coronal sections stained with cresyl violet were used for the histological control of pipette tracks and injection sites. The atlas of Meessen and Olszewski (36) and the atlas of Shek et al. (56) were used for comparison (see also Ref. 43).

Data collection and analysis Respiratory variables were measured during eupneic breathing and reflex responses. The inspiratory (T_i) and expiratory (T_e) times, as well as the total duration of the respiratory or cough cycle (T_T) were measured on recordings of raw phrenic nerve activity. The respiratory frequency was subsequently calculated (breaths min⁻¹). T_T was measured from the onset of phrenic nerve activity to the onset of the next phrenic burst. T_i was defined as the period from the onset of phrenic nerve activity until its maximum, while T_e as the interval from
the maximum of phrenic nerve activity to the onset of the next phrenic burst (e.g. Ref. 50). Peak amplitude (arbitrary units) of the phrenic nerve activity and abdominal EMG activity were measured on integrated traces. Normalization of the amplitudes of phrenic and abdominal activities was performed by expressing them as a fraction (or percentage) of the highest achievable amplitude observed in each animal. The highest peak values were consistently observed during coughing. Therefore, all amplitudes have been expressed in relative units (RU; see e.g. Refs. 40-43). Breathing pattern variables were measured for an average of five consecutive breaths prior to and following drug bilateral microinjections into the cNTS. Furthermore, systolic and diastolic blood pressures were measured at 2 s intervals; mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. Average values of cardiorespiratory variables observed in control conditions and at the time when the maximum response occurred were considered for statistical analyses (Sigma Stat, Jandel Scientific Software, San Rafael, CA). Owing to the small variations in respiratory and cardiovascular variables within each measurement period, average values were taken as single measurements for the purpose of analysis. To provide an estimate of the intensity of the pulmonary chemoreflex, respiratory frequency and mean arterial blood pressure were measured and averaged both during the period immediately preceding PBG injections (10 s) and during the time interval (4 s) at which the maximum response occurred. Also in this case, average values for each period were taken as single measurements for the purpose of analysis.

The cough motor pattern in response to mechanical or chemical stimulation of the tracheobronchial tree is usually characterized by repeated coughs. Each cough consists of an augmented phrenic burst (preparatory inspiration) immediately followed by a burst of expiratory abdominal activity (40, 41, 43). In agreement with our previous results, repeated coughs usually started during stimulation and continued shortly after stimulus cessation. Respiratory variables of coughs (cough-related variables) included cough-related $T_T$, $T_1$ and
as well as peak phrenic amplitude, peak abdominal activity and cough number, i.e. the number of coughs following each stimulation. Cough-related variables were measured and averaged before and after drug microinjections (3 trials for mechanical stimulation and a single trial for citric acid inhalation). The average values of cough-related variables were taken as single measurements for subsequent statistical analysis. In some cases, the first obvious response following mechanical stimulation of the tracheobronchial tree was a small-amplitude expiratory effort without a preceding preparatory inspiration (4, 42, 43). This pattern could fit more appropriately the definition of expiration reflex that is typically evoked by mechanical stimulation of the vocal folds (29), but that can be also produced by mechanical stimulation of the tracheobronchial tree (58, 63). For further details on this topic see our previous reports (40, 42, 43). However, in our study an expiration reflex only occurred as the first motor event in a cough epoch, and its appearance was limited to a few occasions. Therefore, these expiratory responses were not considered for data analysis. Sneezing responses induced by mechanical stimulation consisted of an attack of 3-5 sneezes. Each sneeze consisted of a preparatory augmented inspiration, followed by an intense burst of expiratory activity (29, 41, 61). For simplicity, we considered only some sneeze-related variables, i.e. the number of expiratory thrusts and average peak abdominal activity before and after drug microinjections into the cNTS. Comparisons between cough-related variables recorded under control conditions and after administrations of 25 mM U0126 were performed by means of the one-way repeated-measures analysis of variance (ANOVA) followed by Student-Newman-Keuls tests. Drug-induced cardiorespiratory changes in the remaining experimental conditions were evaluated by Student’s paired t-tests. All reported values are means ± SE; \( P < 0.05 \) was taken as significant.
RESULTS

Effects of microinjections of U0126 and U0124 on the cough reflex. Bilateral microinjections \((n = 6)\) of 25 mM U0126 (30-50 nl; 0.75-1.25 nmol) at the two selected cNTS sites caused within ~30 min consistent and marked reductions in the cough response induced by both mechanical and chemical stimulation of the tracheobronchial tree (Table 1). The cough number and peak abdominal activity decreased, while the cough-related \(T_T\) increased due to a rise in \(T_E\). Bilateral microinjections \((n = 6)\) of 50 mM U0126 (30-50 nl; 1.5-2.5 nmol) into the cNTS progressively depressed cough responses up to the complete suppression of them (Fig. 2). Cough suppression occurred within 30 min after U0126 administration and persisted for ~30-40 min. Cough reflex responses displayed a progressive recovery, thus showing that these effects were reversible. With the lower concentrations of U0126 a complete recovery was seen after 60-90 min, while with the higher concentrations only a partial recovery was observed within ~3 h. Bilateral microinjections \((n = 6)\) of 50 mM U0124 (30-50 nl; 1.5-2.5 nmol), the inactive analogue of U0126, at the two selected cNTS sites did not produce any effect on cough reflex responses (Table 1) and baseline cardiorespiratory variables (not shown). Although our attention was mainly focused on cough responses, we also observed that bilateral microinjections of U0126 into the cNTS did not affect cardiorespiratory variables during eupneic control breathing (see e.g. control recordings before reflex responses in Figs 2 and 3; statistical data not shown). Mean arterial blood pressure was always between 95 and 102 mmHg. For a general evaluation of cardiorespiratory variables in the rabbit under control conditions see our previous reports (40, 41, 43).

Effects of microinjections of U0126 on the B-H inflation reflex, the pulmonary chemoreflex and the sneeze reflex. The B-H inflation reflex, the pulmonary chemoreflex and the sneeze reflex, evaluated \(\geq\) 30 min following bilateral microinjections of 50 mM U0126 into the cNTS, were unaffected. Following U0126 microinjections, tracheal occlusion at end-inspiration still
induced the characteristic increase in $T_E$ associated with abdominal muscle activation (Fig. 3). Under control conditions, $T_E$ during B-H inflation reflex was $3.21 \pm 0.23$ s, while peak abdominal activity $0.10 \pm 0.004$ RU. No significant changes in this reflex were observed following U0126 microinjections. B-H reflex-related $T_E$ and peak abdominal activity were $3.32 \pm 0.28$ s and $0.12 \pm 0.006$ RU, respectively. As for the pulmonary chemoreflex, PBG injections caused within 3-4 s bradycardia, hypotension, tachypnea accompanied by the development of tonic inspiratory activity and marked decreases in expiratory muscle discharges. The maximum cardiorespiratory effects were achieved within $\sim 15$ s. A complete recovery was achieved within 20 min. These cardiorespiratory responses to PBG were largely unaffected by U0126 microinjections (Fig. 3). Under control conditions, respiratory frequency raised from 56.6 ± 3.5 to 140.2 ± 7.5 breaths min$^{-1}$, while mean arterial blood pressure decreased from 100.5 ± 2.1 to 58.2 ± 2.8 mmHg. Similar changes in respiratory frequency (from 58.2 ± 2.9 to 145.5 ± 9.2 breaths min$^{-1}$) and in mean arterial blood pressure (from 98.8 ± 3.0 to 55.8 ± 2.3 mmHg) were seen following U0126 microinjections. Mechanical stimulation of the nasal mucosa still produced the sneeze reflex after U0126 microinjections (Fig. 3). Under control conditions, the number and intensity of expiratory thrusts were 3.3 ± 0.17 and 0.49 ± 0.03 RU, respectively. No significant changes in the number of expiratory thrusts and in peak abdominal activity were observed in response to U0126 microinjections (3.5 ± 0.22 and 0.50 ± 0.04 RU, respectively).

Controls. The localization of the injection sites was confirmed by histological control (see Fig. 1). Despite employed coordinates could display a certain degree of variability among preparations, the histological control showed that injection sites were always within the target area or in close vicinity to it. In 4 additional preparations, control injections of equal volumes of the vehicle solution (50 % DMSO/saline) at the responsive sites were ineffective. In these preparations, bilateral control microinjections of 50 mM U0126 were performed at medullary
locations sufficiently far from the responsive sites (see e.g. Refs. 38, 40, 41, 43, 45). They were made [for comparisons see the atlas of Meessen and Olszewski (36)] lateral to the responsive sites into the nucleus cuneatus or the nucleus tractus spinalis nervi trigemini (5 trials), as well as > 0.8 mm caudal to the responsive sites into the nucleus gracilis or the adjacent reticular formation (3 trials). These injections caused no appreciable change in the cough responses.
DISCUSSION

This is the first study that provides evidence that local inhibition of ERK1/2 activation by microinjections of the MEK inhibitor U0126 into the cNTS decreases or completely abolishes within 30 min cough responses induced by mechanical stimulation of the tracheobronchial tree or by citric acid inhalation in the anesthetized rabbit. These results suggest a role for the ERK1/2 pathway in the processing of tussive inputs by nontranscriptional mechanisms given the short time lapsed from ERK1/2 inhibition and cough response suppression. Present results confirm that these NTS regions are important components of the neural system involved in the central regulation of cough (7, 8, 35, 40, 41, 43 also for further Refs.). Furthermore, in agreement with our previous results (43), the investigated NTS subregions appear to be specifically involved in the mediation of the cough reflex since the B-H inflation reflex, the chemoreflex and the sneeze reflex were not affected by U0126 microinjections within these NTS locations.

General remarks. As in our previous studies (40, 41, 43), all efforts were made to minimize the number of animal used. We have already provided a detailed description of the microinjection techniques used, along with a discussion on their reliability and the spread of injectate (e.g. Refs. 40, 41, 43 also for further Refs.). Injection sites were selected by using stereotaxic coordinates according to the atlas of Meessen and Olszewski (36). The histological control confirmed their localization (Fig.1). Our previous observations on the spread of the injectate ≤ 50 nl (38, 44) are in agreement with theoretical calculations by Nicholson (45) suggesting that a volume of 50 nl should spread < 385 μm in any direction from the injection site. Accordingly, U0126 microinjections into regions sufficiently away from the responsive sites did not affect the cough reflex. Given the relatively high concentrations used in microinjection studies, it seems very likely that the injected drugs are pharmacologically active within the whole spread area, although with a decreasing trend beginning from the micropipette
tip (e.g. Refs. 3, 45, 46 also for further Refs.). The specificity of drug-induced effects is also supported by the absence of changes in the cough reflex following control bilateral microinjections of the vehicle solution or the inactive analogue U0124 into the cNTS. As to the specific involvement of the cNTS in the mediation of the cough reflex, it should be recalled in particular (see also Ref. 43) that second order neurons of SAR afferents responsible for the B-H inflation reflex are located in the medial portion of NTS rostral to the caudal margin of the area postrema (30, 31). On the other hand, bronchopulmonary C-fibers project to several NTS subnuclei (30, 31), including the dorsomedial aspect of the comNTS. As to the sneeze reflex, it should be mentioned that Fos-immunoreactive neurons have been observed in the comNTS in response to nasal mucosa stimulation (12) and that SARs and RARs may have a modulatory role (34, 61).

Effects of the MEK inhibitor U0126 on the cough reflex. Our results show that activation of ERK1/2 is involved in the mediation of the cough reflex at the level of the cNTS. Owing to the similarities between the central processing of nociceptive and cough-related inputs, it is not surprising that present findings are in agreement with previous observations on the involvement of ERK1/2 in the central effects on both acute pain behaviour and neuronal plasticity underlying pain hypersensitivity (21-23, 26, 47, 67).

It is well known that the classic ERK1/2 signal transduction pathway is involved in neuronal plasticity, such as long term potentiation, learning and memory (16, 18, 57). ERK1/2 is downstream of MEK, a dedicated dual-specificity kinase (55), phosphorylated by an upstream kinase which in turn is activated by membrane depolarization and Ca\(^{2+}\) influx (52). The rapid time-course of the U0126-induced cough suppression cannot be explained by modification of gene expression but rather by nontranscriptional mechanisms (14, 20-23, 25, 26, 28, 47) that are, at present, only speculative. Nevertheless, it has been described that ERK1/2 produces long-term adaptive changes increasing gene transcription in chronic pain
states (20, 22, 23, 47), but it is also involved in mediating the rapid acute phase of central pain sensitization via activation and downstream modulation of Kv4.2-containing K⁺ channels by direct phosphorylation (17). The duration of ERK1/2 activation seems to be limited to a restricted time window after stimulus. An inherent signal termination process seems to exist that determines the duration of ERK1/2 activation (51) which depends upon a balance between the activity of kinases and phosphatases (37). Many of these latter proteins are induced by stimuli that also activate ERK1/2 and participate in a negative feedback control of ERK1/2 activity (48) in which ERK1/2 itself determines the duration of its own activation by in turn activating phosphatases (51). The strict regulation of ERK1/2 activation appears to be very important since the downstream effectors of ERK1/2 activation are determined, in part, by the duration of ERK1/2 phosphorylation itself. Indeed, transient activation of ERK1/2 triggers cell differentiation in PC12 cells while prolonged activation results in cell proliferation (59). In neuronal cell culture only repeated depolarization, rather than a single depolarization, causes sustained ERK1/2 activation accompanied by new spine formation, which, on the contrary, is prevented by ERK1/2 inhibition (68). Thus, further studies could be devoted to investigate the role, if any, and the time-course of ERK1/2 activation in cough sensitization and chronic cough. Sensitization of the cough reflex has also been described in recent years in response to different experimental manoeuvres (5, 8, 24, 43).

It has been repeatedly demonstrated that the NMDA receptor is an upstream component of ERK1/2 activation (reviewed in Ref. 62). Activation of the NMDA receptors increases intracellular Ca²⁺ which in turn activates several Ca²⁺-dependent kinases to increase ERK1/2 phosphorylation leading ultimately to changes in neuronal plasticity. Indeed, blockade of hippocampal NMDA receptors by administration of NMDA receptor antagonists blocks ERK1/2 activation and impairs learning (53). Consistently, changes in cough-related
variables induced by 25 mM U0126 in the present study are similar to those observed following microinjections of the NMDA receptor antagonist D-AP5 into the cNTS (41, 43).

The absence of effects of the MEK inhibitor on the sneeze reflex supports the specificity of the injected area in the control of the cough reflex. However, it raises the question of which mechanisms underlie the role of convergent inputs from nasal and vagal afferents within the cNTS in the regulation of naso-trigeminal reflex responses. The lack of significant changes in the sneeze reflex in response to nasal mucosa stimulation could be mainly related to the fact the main central site of nasal projections is the trigeminal sensory complex (33, 60; 41 also for further Refs.), while NTS may represent only a site of a supplementary regulatory function (12, 34, 49, 61). Nevertheless, the mechanisms that mediate this function are at present unclear and deserve further investigation.

**Perspectives and significance.** The results of the present study contribute to improve our knowledge on the central neural mechanisms involved in the generation of the cough motor pattern and especially of those operating within the cNTS. They also confirm that the cNTS is one of the different neural structures responsive to centrally acting antitussive drugs. This study aimed at investigating the role of MAPK activation in the short-term central effects of tussive stimuli represents a first step toward more comprehensive investigations on MAPK involvement in the transduction of cough-related extracellular stimuli into intracellular posttranslational and transcriptional responses. Present findings are, in our opinion, of particular interest since they are the first to show the suppressant effects exerted by MEK inhibitors on the cough reflex. Fos-like immunoreactivity revealed that not only NTS subnuclei, but also several other medullary and pontine respiration-related regions were activated by tussive stimulation (15, 19). Studies on the role of the activation of MAPK pathways in these brainstem regions would be of interest. The involvement of MAPK pathways in central and peripheral long-term plasticity phenomena induced by tussive stimuli
and certain pathophysiological conditions (5, 24) deserves consideration. On the other hand, the role of MAPK activation in a variety of different reflex responses remains to be investigated in order to ascertain whether ERK1/2 signal transduction pathway is specific for certain reflexes, such as pain and cough. Intriguingly, studies on this matter may provide fruitful strategies for the development of novel antitussive therapies.
GRANTS

This study was supported by grants from the Ministero dell’Istruzione, dell’Università e della Ricerca of Italy and from the Compagnia di San Paolo. E.C. is supported by a Postdoctoral Fellowship from the A. Menarini Industrie Farmaceutiche Riunite Srl, Firenze, Italy.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
Fig. 1. Localization of injection sites. A, a diagrammatic representation of a dorsal view of the medulla oblongata of the rabbit showing the sites where multiple microinjections (●) have been performed into the cNTS. Abbreviations: AP, area postrema; DRG, dorsal respiratory group. B, photomicrographs of coronal sections of the medulla oblongata at the levels indicated in panel A (horizontal dashed lines) showing the location of the tracks along which bilateral microinjections were performed. Abbreviations: comNTS, commissural subnucleus of the nucleus tractus solitarii; NDV, nucleus dorsalis nervi vagi; NV, nucleus tractus spinalis nervi trigemini; NXII, nucleus nervi hypoglossi. The dotted line in B delineates the approximate edges of the comNTS. The atlas of Meessen and Olszewski (36) and the atlas of Shek et al. (56) were used for comparison.

Fig. 2. Effects of U0126 microinjections into the cNTS on cough reflex responses evoked by mechanical stimulation of the tracheobronchial tree and by the inhalation of 1 M citric acid in one anesthetized spontaneously breathing rabbit. Reduction and complete suppression of cough responses at different times after bilateral microinjections of 50 mM U0126. Recovery of cough responses was taken after ~ 3 h. Stimulation periods marked by filled bars. Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity.

Fig. 3. Persistence of the Breuer-Hering inflation reflex, the pulmonary chemoreflex and the sneeze reflex ≥ 30 min after bilateral microinjections of 50 mM U0126 into the cNTS in different preparations, i.e. when cough responses were abolished. The Breuer-Hering inflation reflex was induced by tracheal occlusion (filled bar) at end-inspiration. The pulmonary chemoreflex was evoked by right atrial injections of 250 μg PBG given at arrow. Right panels
illustrate the recovery of cardiorespiratory variables 20 min after PBG infusion. The sneeze reflex was induced by mechanical stimulation of the nasal mucosa (filled bar). Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity; BP, arterial blood pressure.
REFERENCES


Table 1. Changes in respiratory variables of coughs (cough-related variables) recorded 30 min following bilateral microinjections of 25 mM U0126 or 50 mM U0124 into the cNTS

<table>
<thead>
<tr>
<th></th>
<th>CN</th>
<th>Tₜ, s</th>
<th>Tᵢ, s</th>
<th>Tₑ, s</th>
<th>PPA, RU</th>
<th>PAA, RU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanical stimulation (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.5 ± 0.22</td>
<td>0.53 ± 0.03</td>
<td>0.35 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.66 ± 0.06</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>25 mM U0126</td>
<td>1.2 ± 0.16*</td>
<td>0.81 ± 0.11*</td>
<td>0.43 ± 0.04</td>
<td>0.37 ± 0.08*</td>
<td>0.71 ± 0.04</td>
<td>0.36 ± 0.05*</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.4 ± 0.23</td>
<td>0.52 ± 0.04</td>
<td>0.36 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.65 ± 0.06</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td><strong>Citric acid inhalation (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.7 ± 0.33</td>
<td>0.49 ± 0.03</td>
<td>0.34 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.70 ± 0.05</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>25 mM U0126</td>
<td>1.3 ± 0.17*</td>
<td>0.66 ± 0.02*</td>
<td>0.39 ± 0.03</td>
<td>0.27 ± 0.04*</td>
<td>0.67 ± 0.06</td>
<td>0.36 ± 0.05*</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.6 ± 0.27</td>
<td>0.50 ± 0.03</td>
<td>0.34 ± 0.02</td>
<td>0.16 ± 0.01</td>
<td>0.69 ± 0.05</td>
<td>0.45 ± 0.04</td>
</tr>
</tbody>
</table>

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanical stimulation (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.50 ± 0.22</td>
<td>0.52 ± 0.03</td>
<td>0.34 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.65 ± 0.06</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>50 mM U0124</td>
<td>3.33 ± 0.21</td>
<td>0.55 ± 0.02</td>
<td>0.36 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.66 ± 0.06</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td><strong>Citric acid inhalation (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.66 ± 0.33</td>
<td>0.49 ± 0.03</td>
<td>0.34 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.70 ± 0.05</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>50 mM U0124</td>
<td>3.20 ± 0.29</td>
<td>0.51 ± 0.03</td>
<td>0.35 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>0.68 ± 0.06</td>
<td>0.42 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals. Cough-related variables: CN, cough number; Tₜ, cycle duration; Tᵢ, inspiratory time; Tₑ, expiratory time; PPA, peak phrenic activity in relative units (RU); PAA, peak abdominal activity in relative units (RU). Recovery was taken ~ 90 min after 25 mM U0126 injections. *P < 0.05 compared with control cough as well as with recovery.
Mechanical stimulation

50 mM U0126

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>15 min</th>
<th>30 min</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phr IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phr N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abd IEMG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abd EMG</td>
<td></td>
<td></td>
<td></td>
<td>0.4 mV</td>
</tr>
</tbody>
</table>

Citric acid inhalation

50 mM U0126

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>15 min</th>
<th>30 min</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phr IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phr N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abd IEMG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abd EMG</td>
<td></td>
<td></td>
<td></td>
<td>0.4 mV</td>
</tr>
</tbody>
</table>

5 s