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Gz- and not Gi-proteins are coupled to pre-junctional μ-opioid receptors in bovine airways

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ABSTRACT

We investigated the signal transmission pathway by which activation of μ-opioid receptors attenuates acetylcholine (ACh) release in bovine trachealis. Electrical stimulation (ES)-induced [^{3}H]-ACh release was determined in bovine tracheal smooth muscle strips pre-incubated with either the Gi-protein inhibitor pertussis toxin (PTX, 500 ng/ml and 1 μg/ml) or the Gz-protein specific inhibitor arachidonic acid (AA, 10^{-6} M and 10^{-5} M) and then treated with DAMGO (D-Ala^2,N-MePhe^4,Gly-ol^5-enkephalin) 10^{-5} M. Indomethacin 10^{-5} M was used to block AA cascade. The inhibitory effect of DAMGO on ES-induced [^{3}H]-ACh release was PTX-insensitive, but, by contrast, ablated by AA in a concentration-dependent manner. AA 10^{-5} M alone reduced [^{3}H]-ACh release, an effect that was prevented by iberiotoxin 10^{-7} M, suggesting an involvement of Ca^{2+}-activated K^+-channels. Western blot analysis consistently showed immunoreactive bands against a specific antibody anti-Gz-α subunit at ~40 kDa, consistent with the presence of Gz-protein. The present findings suggest that in isolated bovine trachealis, activation of μ-opioid receptors inhibits ACh-release through a signal transmission pathway involving Gz-protein rather than Gi-protein.

1. Introduction

Acetylcholine (ACh) released by parasympathetic nerve endings is the primary neurotransmitter responsible for airway smooth muscle contraction (Baroffio et al., 2012; Belvisi et al., 1992; D'Agostino et al., 1990; Kilbinger et al., 1991; Patel et al., 1997; Wessler et al., 1990; Zappi et al., 1995). The force of airway contraction is finely tuned by ACh through the stimulation of pre-junctional M_2, M_2-like, and M_4 autoreceptors (Baroffio et al., 2012; Kilbinger et al., 1991). The latter receptors, when stimulated, activate Gi-proteins which in turn reduce the activity of adenylyl cyclase and thereby intracellular cAMP concentration (Sankary et al., 1988). Other pre-junctional receptors including μ- and κ-opioid receptors modulate airway contractility. Stimulation of pre-junctional opioid receptors reduce both electrically stimulated (ES) ACh-release and muscle contraction (Baroffio et al., 2013). Opioid receptors are localized on the vagus nerve endings but not on airway smooth muscle cells (Atweh et al., 1978; Cabot et al., 1994).

Studies on opioid pharmacology using different neural tissues or cell lines from different species have convincingly shown that the major pathways for intracellular signal transmission from the receptors to the effectors are through coupling to the inhibitory Gi (Garzón et al., 1997; Sánchez-Blázquez et al., 1993) and/or Gz (Garzón et al., 1997, 2004; Lai et al., 1995; Sánchez-Blázquez et al., 1993) proteins. In murine sympathetic nerves Gz protein may couple neurotransmitter receptors to N-type Ca^{2+} channels via a pertussis toxin (PTX)-insensitive pathway (Jeong and Ikeda, 1998).

In airways, the intracellular pathway for signal transmission from pre-junctional opioid receptors to release of ACh has not been elucidated. In preliminary studies we found that the inhibitory effect of the μ-opioid receptor agonist DAMGO (D-Ala^2,N-MePhe^{4}Gly-ol^5-enkephalin) on contraction of bovine trachealis induced by electrical field stimulation was not affected by pertussis-toxin (PTX), suggesting that Gi-proteins are not involved in the coupling to μ-opioid receptors in this tissue.

The present study was designed to test the hypothesis that the intracellular signal transmission pathway from pre-junctional μ-opioid receptors to the release of ACh involves Gz protein in bovine trachealis, a tissue widely available and used as a model for studies on airway responsiveness. The effect of DAMGO on ES-induced [^{3}H]-ACh release in muscles pre-incubated with either the Gi-protein inhibitor PTX or the Gz-protein specific inhibitor

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arachidonic acid (AA) were compared with ES-induced [^{3}H]-ACh release by DAMGO alone (Baroffio et al., 2013). The effects of AA alone or in the presence of iberiotoxin on ES-induced [^{3}H]-ACh release were also determined. Tracheal tissues were assayed for the presence of Gz-α subunit by western blot analysis.

2. Methods

2.1. Tissue preparation

Thirty-nine bovine tracheas were obtained from the local abbatoir. The tracheas were removed immediately after death and immersed in chilled (4 °C) physiologic salt solution (PSS) of the following composition (mM): $MgSO_4$, 0.8; KH_2PO_4, 1.2; KCl, 3.4; $CaCl_2$, 2.4; NaCl, 110.5; $NaHCO_3$, 25.7; and dextrose. 5.6. The animals were slaughtered according to Italian Law. The mucosa was carefully removed and muscle strips were prepared.

2.2. Assessment of ES-induced [^{3}H]-ACh release

One pair of trachealis strips was prepared from each animal; one muscle from the pair served as control and the other as test muscle. The muscles were tied at one end to a platinum electrode attached to a micromanipulator and at the other to a platinum electrode connected to a force transducer (LC 4001, G0120, Litra Co., Japan).

Both muscles were superfused (1 ml/min; calibrated roller pump, Gilson® Miniplus 3, Villiers Le Bel, France) simultaneously with aerated PSS (95% O_2 and 5% CO_2) at 37 °C. Propranolol 10^{-6} M was added to PSS to block β-adrenoceptors, indomethacin 10^{-5} M to prevent prostaglandin synthesis and AA metabolic degradation, and choline chloride 10^{-6} M to coat the surfaces of the circuits limiting loss of [^{3}H]-choline. Both muscles were contracted simultaneously every 5 min for 30 s by ES (25 Hz, 25 V, 0.5 ms stimuli duration, 0.01 ms delay) from a direct current amplifier triggered by a stimulator (S44, Grass Medical Instruments, Quincy, MA). The electric stimulations waves were monitored using an oscilloscope (GOS-622G, Melchioni Elettronica, Milan, Italy) throughout the study. The muscles were stretched after each ES until constant and maximal contractile responses resulted. This reference length (Bai et al., 2004) was not altered during the experiment. Isometric forces of the muscles were recorded continuously throughout the study (Linseis L 250 E recorder, Selb, Germany).

Utilizing a different superfusion circuit, both muscles were loaded with 15 μCi of [^{3}H]-choline (specific activity 75 Ci/mM), dissolved in 12-ml PSS, at a flow of 2 ml/min (37 °C). During loading, both muscles were continuously stimulated for 30 min (25 Hz, 25 V, 0.5 ms stimuli duration, 0.01 ms delay) to enhance neuronal uptake of [^{3}H]-choline. Then muscles were washed for 90 min with PSS (20 ml/min) containing, in addition to other compounds, hemicholinium-3 10^{-5} M to inhibit neuronal uptake of choline. Simultaneous collections of superfusates from both muscles were started at a flow of 1 ml/min. This time is defined as time zero of the study (t_0); all times (t_n) are referred to it and expressed in minutes.

Superfusates from both muscles were collected separately and simultaneously for 3 min each in scintillation vials containing 14 ml of Ready Safe™ (Beckman Coulter, Fullerton, CA) which provided accurate measurements of a known amount of [^{3}H]-choline. Collection of superfusates was interrupted for 10 min to allow incubation of test muscles with drugs (see below).

Superfusates collected from control and test muscles were assayed for radioactivity by liquid scintillation counting (LS 6500 Multi-purpose scintillation counter, Beckman Instruments, Inc. 2500 Harbor Blvd, Fullerton, CA, USA). Each sample was counted 3 times for 5 min and mean values were used.

2.2.1. Effects of DAMGO in PTX-incubated muscles

Eleven pairs of muscles from 11 animals were incubated with PTX, 500 ng/ml ($n = 5$) or 1 μg/ml ($n = 6$) for 3 h. Thereafter, strips were mounted in the tissue baths, and the experimental procedure started. After loading with [^{3}H]-choline, both muscles of each pair were electrically stimulated for 3 min at t_{18} (ES_1). At t_{39} test muscles were incubated with DAMGO 10^{-5} M for 30 min and ES was repeated at t_{67} (ES_2). At t_{88} the collections of superfusate were stopped.

2.2.2. Effects of DAMGO in AA-incubated muscles

Thirteen pairs of muscles from another 13 animals were stimulated for 3 min at t_{18} (ES_1). At t_{39}, test muscles were incubated with either AA 10^{-6} M ($n = 6$) or 10^{-5} M ($n = 7$) for 30 min and ES was repeated at t_{67} (ES_2). At t_{88}, test muscles were incubated with DAMGO 10^{-5} M for 30 min. At t_{116} both muscles were stimulated again (ES_3). At t_{137} the collections of superfusate were stopped.

2.2.3. Effects of AA in iberiotoxin-incubated muscles

Six pairs of muscles from another 6 animals were stimulated for 3 min at t_{18} (ES_1). At t_{39} test muscles were incubated first with iberiotoxin 10^{-7} M for 30 min and then with AA 10^{-5} M at t_{67} and ES was repeated at t_{94} (ES_2). At t_{115} the collections of superfusate were stopped.

2.3. Expression of Gz-α subunit

The expression of Gz-α subunit was assayed by western blot analysis performed on muscles from another 9 animals. Tissues were cut into small pieces and suspended in 1 ml of PSS containing protease inhibitors: 10 μg/ml leupeptin; 10 μg/ml aprotinin; 20 μg/ml pepstatin and 10 μM aminoethyl-benzene sulfonyl fluoride hydrochloride. Samples were then homogenized in ice for 3 min by gentle grinding (Polytron, Kinematica, Lucerne, Switzerland) and by subsequent sonication in ice for 3 min (Microson, Misonix, Farmingdale, New York, USA). Protein concentration of samples was measured according to Bradford (1976). 100 μg aliquots of each sample were diluted in Laemmli sample buffer (Laemmli, 1970), heated to 100 °C for 5 min, and subjected to SDS-PAGE on a 10% acrilamide gel followed by western blot according to Towbin et al. (1979). Saturation of 0.45 μm nitrocellulose membrane (Hybond ECL, Amersham, Italy) with milk powder, incubation with rabbit polyclonal antibody anti-Gz-α subunit (Calbiochem, VWR International, Milan, Italy) and immunoenzymatic detection were performed following instructions of the Amersham ECL kit. The specificity for bovine Gz-α subunit of the above antibody had been tested by Casey et al. (1990) in brain tissue of this species.

2.4. Data analysis

Counts per minutes were converted in disintegrations per minutes dividing them by instrument counting efficiency. Areas bound by spontaneous [^{3}H]-ACh release and [^{3}H]-ACh release evoked by ES were calculated and expressed as disintegrations per stimulus. Each area was defined as A_n where n is the number of stimulus. [^{3}H]-ACh release percent changes by each n stimulation were calculated as

$$\left(\frac{A_{n\,drug}/A_{1\,drug}}{A_{n\,contral}/A_{1\,contral}} - 1\right) \times 100$$

Data from a previous study in our laboratory carried out with the same apparatus and the same experimental procedure (Baroffio et al., 2013) were used to compare between the effects of DAMGO alone and the effects of DAMGO in muscles pre-incubated with PTX or AA.