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Activation of potassium conductance by ophiopogonin-D in acutely dissociated rat paratracheal neurones

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1 The effect of ophiopogonin-D (OP-D), a steroidal glycoside and an active component of Bakumondo-to, a Chinese herbal antitussive, on neurones acutely dissociated from paratracheal ganglia of 2-week-old Wistar rats was investigated using the nystatin-perforated patch recording configuration.

2 Under current-clamp conditions, OP-D (10 μM) hyperpolarized the paratracheal neurones from a resting membrane potential of -65.7 to -73.5 mV.

3 At the concentration of 1 μM and above, OP-D concentration-dependently activated an outward current accompanied by an increase in the membrane conductance under voltage-clamp conditions at a holding potential of -40 mV.

4 The reversal potential of the OP-D-induced current ($I_{OP\text{-}D}$) was -79.4 mV, which is close to the K^+ equilibrium potential of -86.4 mV. The changes in the reversal potential for a 10 fold change in extracellular K^+ concentration was 53.1 mV, indicating that the current was carried by K^+.

5 The $I_{OP\text{-}D}$ was blocked by an extracellular application of 1 mM Ba^{2+} by 59.0%, but other K^+ channel blockers, including 4-aminopyridine (3 mM), apamin (1 μM), charybdotoxin (0.3 μM), glibenclamide (1 μM), tolbutamide (0.3 mM) and tetraethylammonium (10 mM), did not inhibit the $I_{OP\text{-}D}$.

6 OP-D also inhibited the ACh- and bradykinin-induced depolarizing responses which were accompanied with firing of action potentials.

7 The results suggest that OP-D may be of benefit in reducing the excitability of airway parasympathetic ganglion neurones and consequently cholinergic control of airway function and further, that the hyperpolarizing effect of OP-D on paratracheal neurones *via* an activation of K^+ channels might explain a part of mechanisms of the antitussive action of the agent.
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Abbreviations: ACh, acetylcholine; CNS, central nervous system; $E_{OP\text{-}D}$, reversal potential of the OP-D-induced current; EPSP, excitatory post synaptic potential; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; HOE-140, D-Arg-[Hyp[3],Thi[5],D-Tic[7],Oic[8]]-bradykinin; $I_{OP\text{-}D}$, ophiopogonin-D-induced current; NANC, non-adrenergic non-cholinergic; OP-D, ophiopogonin-D; TEA, tetraethylammonium; Tris-OH, tris(hydroxymethyl)amino-methane; V_H, holding potential

Introduction

Ophiopogonin-D (OP-D) (Figure 1) is a steroidal glycoside, originally isolated from Ophiopogonis tuber which is a main component of a traditional Chinese medicinal prescription, Bakumondo-to (Mai-men-dong-tang). This prescription consists of six herbs (*Ophiopogonis tuber*, *Pinelliae tuber*, *Zizyphi fructus*, *Glycyrrhizae radix*, *Ginseng radix* and *Oryzae fructus*) and has been used for the treatment of severe dry cough in patients with bronchitis and pharyngitis (Asano *et al*., 1993; Takahama & Miyata, 1995). We have previously found that Bakumondo-to has potent antitussive action in several experimental models of cough in guinea-pigs (Miyata *et al*., 1989; 1999), such as inhibition of capsaicin-, citric acid-, or substance P-induced coughs, inhibition of codeine-resistant coughs which are caused by neurokinin A-inhalation (Takahama *et al*., 1993) and by treatment with an angiotensin-converting enzyme inhibitor (Miyata *et al*., 1999). Bakumondo-to also inhibited codeine-resistant coughs in bronchitic guinea-pigs prepared by SO_2-exposure. In addition, Bakumondo-to inhibited the increased discharges of the airway vagal afferents in bronchitic animals (Miyata *et al*., 1989; 1999), suggesting that the site of the antitussive action of Bakumondo-to may be in the airway. These pharmacological characteristics of Bakumondo-to in antitussive actions fit well with those of OP-D (Miyata *et al*., 1999), suggesting that OP-D may be one of antitussive components in Bakumondo-to. Recently, we have reported that the cough induced by stimulation to the bifurcation of trachea is more resistant to an antitussive codeine than that

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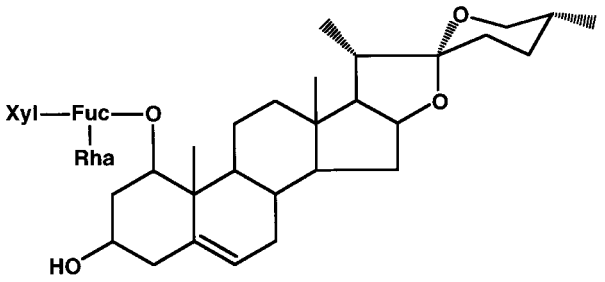

Figure 1 Chemical structure of ophiopogonin-D (OP-D). Fuc, Rha and Xyl stand for fucose, rhamnose and xylose, respectively.

caused by larynx stimulation (Takahama *et al*., 1997). In this model, OP-D also suppressed not only codeine-sensitive but also codeine-resistant cough in the guinea-pig (Takahama & Miyata 1995). However, the antitussive mechanism of OP-D remains largely unknown.

In the lower airway, there are a number of parasympathetic ganglia and the network of nerve fibre situated on the serosal surface of the dorsal tracheal wall (Baker *et al*., 1986; Baluk *et al*., 1985). From these ganglia, short postganglionic fibres innervate the effector organs including airway smooth muscle, blood vessels and submucosal glands, and the innervation plays an important role in the regulation of airway function (Barnes, 1987; 1992; Smith & Taylor, 1971). The predominant control of airway is thought to be exerted by vagal cholinergic innervation (Barnes, 1987; 1992). However, the vagal control of airway is complex. For instance, the stimulation of the vagus nerve in guinea-pigs *in vivo* induces cholinergic bronchoconstriction, and also produces bronchodilation which is neither adrenergic or cholinergic (NANC) (Barnes, 1992; Diamond & O'Donnell, 1980; Lammers *et al*., 1992). On the other hand, the NANC bronchodilator action could not be elicited by the *in vitro* vagal stimulation if connections between the esophagus and trachea were disrupted, suggesting that the NANC pathway may be associated with the esophagus (Canning & Undem, 1993; Diamond & O'Donnell, 1980). The NANC nerve cell bodies innervating the trachea are also found to be located in the esophagus, but not in the tracheal wall (Moffatt *et al*., 1998). Thus, the paratracheal ganglia are thought to be the last relay stations for the cholinergic excitatory inputs to the airway. In addition to the regulation by vagal preganglionic nerve fibres from the central nervous system, the excitability of paratracheal ganglion neurones can be modulated by a peripheral reflex mechanism (Myers & Undem, 1993; Myers *et al*., 1996), because the branches of tachykinin-immunoreactive afferent nerves project to paratracheal neurons (Day *et al*., 1996; Diamond & O'Donnell, 1980). Thus, the airway ganglia are thought to be not only integrative sites for the neuromodulation of the normal airway function, but also important sites for pathogenesis in airway inflammation associated with cough.

The aim of the present study is to study the effect of OP-D on parasympathetic neurones acutely dissociated from paratracheal ganglia of the rat trachea by using the nystatin perforated patch-clamp recording configuration (Horn & Marty, 1988).

Methods

Preparation

Experiments were performed on paratracheal neurones freshly dissociated from 2-week-old Wistar rats. The procedure for obtaining dissociated paratracheal ganglion neurones is similar to that described elsewhere (Murai *et al*., 1998). Briefly, the paratracheal ganglia were rapidly removed from the trachea of rats under pentobarbital anaesthesia, and the rats were subsequently killed by exsanguination. The isolated ganglia were treated with a normal external solution containing 0.3% collagenase and 0.3% trypsin for 40 min at 35°C. Following this treatment, single neurones were dissociated mechanically by triturating the ganglia with the fire-polished Pasteur-pipette in a culture dish. The dissociated neurones adhered to the bottom of the dish within 20 min. The neurones were then prepared for electrophysiological experiments.

Solutions and chemicals

The ionic composition of the normal external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2 N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris(hydroxymethyl)aminomethane (Tris-OH). The ionic composition of the patch pipette (internal) solution was (in mM): KCl 80, K-gluconate 70 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. Nystatin was dissolved in methanol, resulting in a 10 mg ml^{-1} stock solution, and added to the internal solution at a final concentration of 400 μg ml^{-1} just before use. A rapid application of external solution was performed with 'Y-tube' technique as described previously (Murase *et al*., 1989). Using this technique, external solution could be completely exchanged within 20 ms.

Drugs used in the present study were ophiopogonin-D (OP-D) (Tsumura, Tokyo, Japan), acetylcholine, collagenase, nystatin, pirenzepine, trypsin, glibenclamide (Sigma, St. Louis, MO, U.S.A.), apamine, bradykinin, charybdotoxin (Peptide Institute, Osaka, Japan), D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (HOE-140), tolbutamide (Research Biochemical International, Natick, MA, U.S.A.), 4-aminopyridine and tetraethylammonium chloride (Wako, Tokyo, Japan). OP-D was first dissolved in dimethyl sulphoxide (DMSO) at a concentration of 5 mM, and then diluted to the final concentration in an external solution. The final concentration of DMSO was $<0.6\%$, in which DMSO alone had no effect on membrane potential or electrical activity.

Electrophysiological recordings

Electrical measurements were performed using the nystatin perforated patch recording configuration (Horn & Marty, 1988). Patch pipettes were prepared using a vertical micropipette puller (PP-83, Narishige, Tokyo, Japan). The resistance between the patch pipette filled with the internal solution and the reference electrode in the normal external solution was 2–5 MΩ. The junction potential between the external and the patch-pipette solution was maximally +4 mV, which was subtracted from the recorded potentials. After stable perforated patch formation, series resistance

ranged from 8 to 15 MΩ and was compensated in the same manner as that explained previously (Llano *et al*., 1991).

Ionic currents were measured with a patch clamp amplifier (Axopatch-200B, Axon Instruments, Foster, CA, U.S.A.), low-pass filtered at 1 kHz (Model 3611, NF Electric Instruments) and monitored on an oscilloscope (CS-6040, Kenwood, Tokyo, Japan). Data were stored on video tapes after being digitized with a digital audio-processor (RP-880, NF Electric Instruments, Tokyo, Japan) for subsequent analysis using the pCLAMP system (Axon Instruments). All electrophysiological measurements were done at room temperature (21–24°C). The data are presented as mean ± standard error of the mean (s.e.mean, n = number of experiments).

Results

Recordings were made from more than 90 paratracheal neurones from 2-week-old Wistar rats. The average resting membrane potential after making the stable perforated patch-recording configuration was −58.9 ± 2.1 mV (range: −48 to −72 mV, n = 42). The mean input resistance was 42 ± 9 MΩ (n = 20). These values were close to those previously reported in paratracheal neurones of guinea-pigs (Myers *et al*., 1990) and rats (Allen & Burnstock, 1990).

OP-D response

In order to characterize the electrophysiological properties of the OP-D responses, the effect of OP-D was studied under current clamp conditions. In all seven neurones tested, the external application of 10 μM OP-D hyperpolarized the neurone from the resting membrane potential of −65.7 ± 3.1 to −73.5 ± 3.1 mV (Figure 2A).

Voltage-clamp studies were done in order to further analyse the hyperpolarizing effect. A typical OP-D-induced current response is shown in Figure 2B. The neurone was voltage-clamped at a holding potential (V_H) of −40 mV. Hyperpolarizing voltage steps of 20 mV and 500 ms duration were continuously applied to monitor the changes in the membrane conductance. OP-D at 10 μM evoked a slow outward current with increasing the membrane conductance. This OP-D-induced current ($I_{OP\text{-}D}$) reached the steady state within 20 s and did not show obvious inactivation during a continuous application of the agent (data not shown). This response then gradually recovered after wash-out (Figure 2B).

Concentration-response relationship

The amplitude of the $I_{OP\text{-}D}$ increased in a concentration-dependent manner. As shown in Figure 3A, OP-D at a concentration of 1 μM and above consistently evoked the outward currents in a concentration-dependent manner at a V_H of −40 mV. Figure 3B summarizes the concentration-response relationship for OP-D. OP-D was not tested at concentrations above 30 μM since OP-D at high concentrations >50 μM sometimes increased leakage currents and disturbed stable recordings (not shown), and, therefore, it was not possible to obtain the accurate estimate of the half-maximum effective concentration.

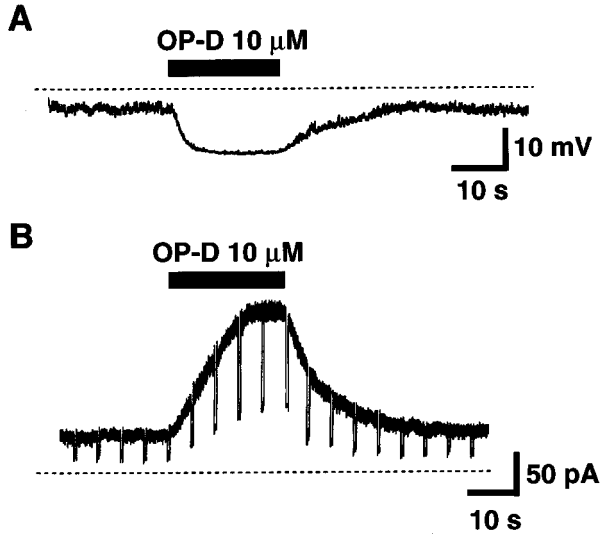

Figure 2 The response for OP-D. (A) OP-D hyperpolarized the paratracheal neuron. A dotted line shows the level of −60 mV. The figure is representative of seven reproducible observations. (B) The outward current induced by OP-D at a holding potential (V_H) of −40 mV. A brief hyperpolarizing pulse of −20 mV with 500 ms duration was applied every 5 s. A dotted line shows the zero current level. This current response was obtained from the different neuron shown in A. The figure is representative of seven reproducible experiments.

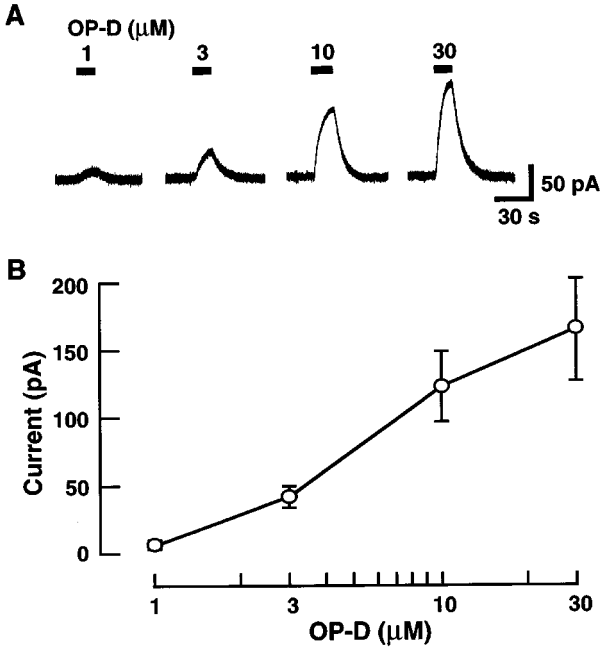

Figure 3 Concentration-response relationship for OP-D. (A) Typical current traces elicited by various concentrations of OP-D at a V_H of −40 mV. (B) Concentration-response relationship for OP-D. Each point represents a mean outward current from 4–5 neurones; vertical bars show ± s.e.mean.

Ionic mechanism for $I_{OP\text{-}D}$

Figure 4A shows the typical $I_{OP\text{-}D}$ induced by 10 μM OP-D in the normal external solution containing 5 mM K^+ at various

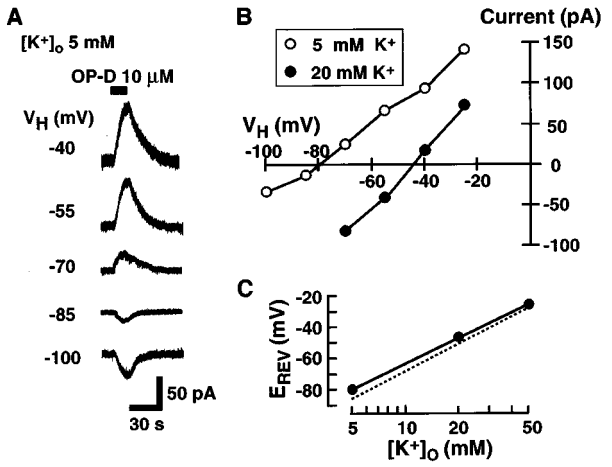

Figure 4 Current-voltage relationships for the OP-D-induced response. (A) Currents elicited by 10 μM OP-D at various V_Hs. (B) Representative current-voltage relationships of OP-D-induced currents ($I_{OP\text{-}D}$) in external solution with two different K^+ concentrations of 5 (open circle) and 20 mM (closed circle). The intracellular K^+ concentration was 150 mM throughout the experiments. The data were obtained from the same neurone as shown in A. (C) The relationship between the external K^+ concentration ($[K^+]_o$) and the reversal potential of the $I_{OP\text{-}D}$ ($E_{OP\text{-}D}$). The change in the $E_{OP\text{-}D}$ values for a 10 fold change in $[K^+]_o$ was 53.1 mV. The dotted line represents the change in 58 mV for 10 fold change of $[K^+]_o$ predicted from the Nernst equation. Each point is the mean reversal potential of 5–6 neurones. No error bar was depicted, because the size of error bars was within each closed circle.

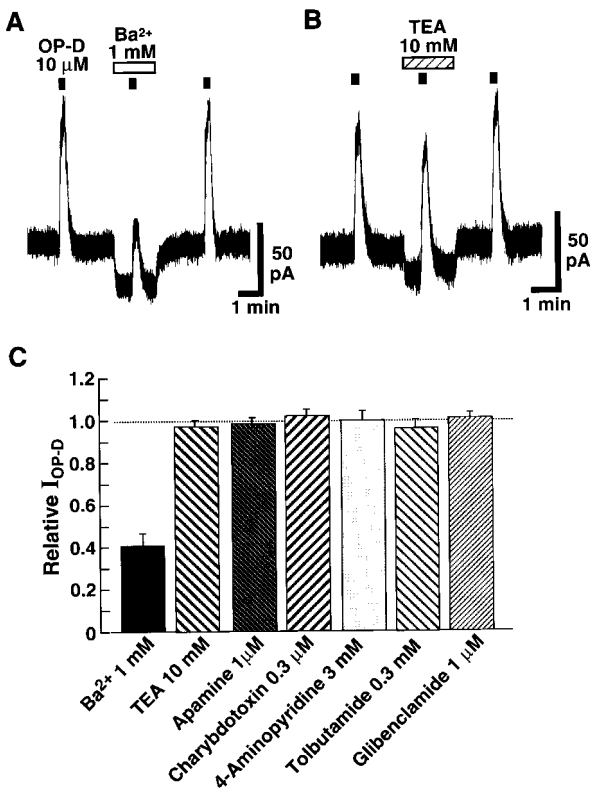

Figure 5 Effects of K^+ channel blockers. (A) Effect of Ba^{2+} on the $I_{OP\text{-}D}$. (B) Effect of TEA on the $I_{OP\text{-}D}$. (C) Effects of various K^+ channel blockers on the $I_{OP\text{-}D}$. Each column is the average of 4–7 neurones. The $I_{OP\text{-}D}$ caused by OP-D alone was taken as 1.

V_Hs. The reversal potential of the $I_{OP\text{-}D}$ ($E_{OP\text{-}D}$) estimated from the current-voltage (I–V) relationship was -79.4 ± 0.8 mV ($n=6$) in the normal external solution with 5 mM K^+ (Figure 4B). This value was almost identical to the theoretical K^+ equilibrium potential of -86.4 mV calculated from the Nernst equation, at the given K^+ concentration of the external and pipette solutions. As the extracellular K^+ concentration was increased to 20 and 50 mM, the $E_{OP\text{-}D}$ shifted to -46.0 ± 1.5 ($n=6$) and -26.5 ± 1.1 mV ($n=5$), respectively. These values were derived from the line of best fit to the plot obtained by pooling the reversal potentials obtained at various K+ concentrations in several cells, and were close to the calculated K^+ equilibrium potentials of -51.2 and -27.9 mV in solutions containing 20 and 50 mM K^+, respectively. The slope of the $E_{OP\text{-}D}$ values for a 10 fold change in K^+ concentration was 53.1 mV and was also close to the predicted value (58 mV) of the slope (Figure 4C). This finding thus indicates that the $I_{OP\text{-}D}$ is selectively carried by K^+.

Effect of K^+ channel blockers

For the pharmacological characterization, we examined the effects of several K^+ channel blockers on the $I_{OP\text{-}D}$. Figure 5A shows the effect of 1 mM Ba^{2+} on $I_{OP\text{-}D}$ at a V_H of -40 mV. The holding current shifted inwardly with the application of Ba^{2+}, and the $I_{OP\text{-}D}$ was reversibly inhibited by $59.0\pm5.9\%$ ($n=5$). Although tetraethylammonium chloride (TEA-Cl, 10 mM) also shifted the holding current inwardly, it produced no significant effect on $I_{OP\text{-}D}$ ($n=6$, Figure 5B). On the other hand, other K^+ channel blockers applied extracellularly, such as 1 μM apamine, 0.3 μM charybdotoxin (Ca^{2+}-activated K^+ channel blockers), 3 mM 4-aminopyridine (a blocker of K^+ channels including delayed rectifiers and A channel), 0.3 mM tolbutamide and 1 μM glibenclamide (ATP-sensitive K^+ channel blockers) did not affect either the holding current or $I_{OP\text{-}D}$ (Figure 5C). The outward current evoked by OP-D was not observed when all of K^+ in the pipette solution was replaced with equimolar Cs^+ ($n=5$, not shown).

Effect of OP-D on ACh- and bradykinin-induced depolarizing responses

The paratracheal neurones receive the cholinergic excitatory input from the efferent vagal nerves (Day *et al*., 1996; Kalia & Mesulam, 1980). In addition, bradykinin has recently been reported to cause the membrane depolarization of the airway parasympathetic ganglion neurones in the guinea-pig (Kajekar & Myers, 2000). In the present study, therefore, the effect of OP-D on the acetylcholine (ACh)- and bradykinin-induced depolarizing responses was also investigated under current-clamp conditions. Both 0.3 μM ACh and 0.1 μM bradykinin depolarized the membrane potential, resulting in the burst of action potentials (Figure 6). The depolarizing responses induced by ACh and bradykinin were almost fully inhibited by pirenzepine (1 μM, $n=4$, not shown) and HOE-140 (1 μM, $n=4$, not shown), respectively, thus suggesting the involvement of muscarinic receptor in the ACh response and B_2-receptor in the bradykinin response (Proud *et al*., 1993).

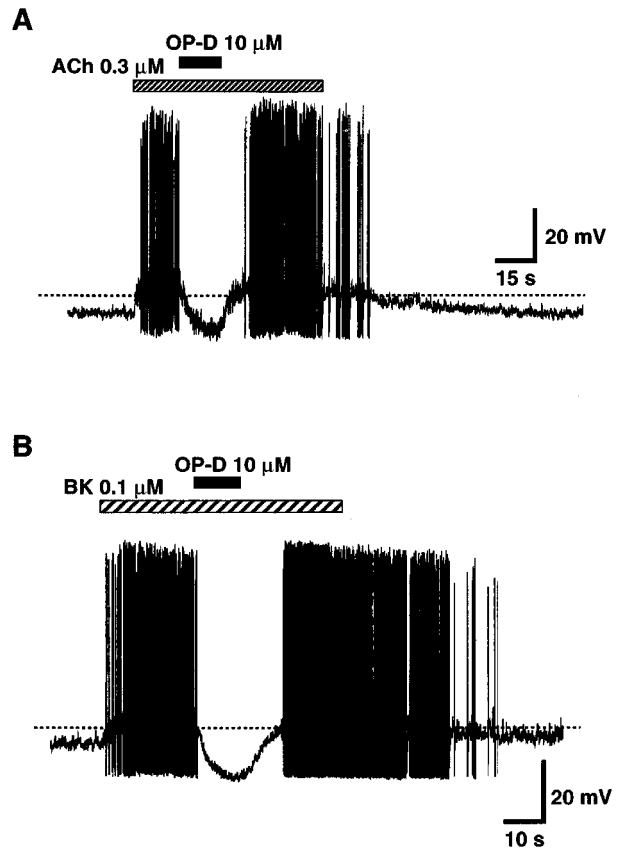

Figure 6 Effect of OP-D on ACh- and bradykinin-induced depolarizing responses. All recordings were obtained under current clamp conditions. Dashed line represents the level of −60 mV. (A) Effect on ACh-induced depolarization with the action potential firing. This recording is representative of five reproducible experiments. (B) Effect on bradykinin-induced depolarization accompanied with the action potential firing. The recording was obtained from the different neurone shown in A. The figure is representative of four reproducible experiments.

As shown in Figure 6, both the ACh- and bradykinin-induced depolarizing responses which were accompanied with firing of action potentials were markedly inhibited by the application of OP-D (10 μM). The membrane potential and action potential firing recovered after washing out of OP-D.

Discussion

The present study demonstrated that OP-D hyperpolarizes the paratracheal ganglion neurone *via* an activation of K^+ channels. The paratracheal neurone has been reported to have several types of K^+ currents including TEA-sensitive delayed rectifier currents, 4-AP-sensitive transient currents (A-currents), Ca^{2+}-activated currents and voltage-sensitive M-currents (Aibara *et al*., 1992; Allen & Burnstock, 1990). In the present study, however, neither TEA nor 4-AP blocked $I_{OP\text{-}D}$ (Figure 5), and $I_{OP\text{-}D}$ did not show marked voltage-dependency (Figure 4). In addition, the failure to block $I_{OP\text{-}D}$ with glibenclamide and tolbutamide rules out the idea of ATP-sensitive K^+ channel involvement. On the other hand, $I_{OP\text{-}D}$ was blocked by Ba^{2+}, which is known as an inhibitor of Ca^{2+}-activated K^+ channels (Miller *et al*., 1987). However, the lack of effects of apamine, charybdotoxin and TEA on $I_{OP\text{-}D}$ is incompatible with the characteristics of the Ca^{2+}-activated K^+ channels. The present pharmacological properties of the $I_{OP\text{-}D}$ is also consistent with an inwardly rectifying K^+ (Kir) channels (Hille, 1992; Katayama *et al*., 1997), although the current-voltage relationship of $I_{OP\text{-}D}$ is not in agreement with that of the Kir channel current. Nonetheless, since the Kir channels are operable *via* a whole-range of G-protein coupled receptors (Wickman & Clapham, 1995), it may be possible to assume that OP-D might act as an agonist at such an unidentified receptor. While the present result provides the first data showing that OP-D selectively activates K^+ channels in rat paratracheal ganglion neurones, the distinct type of K^+ channels regulated by OP-D was not fully explored. Further studies are needed to clarify the mechanism by which OP-D activates the K^+ channels.

The paratracheal neurones receive the cholinergic input from the vagal preganglionic nerve. Consistent with the previous report (Aibara & Akaike, 1991), ACh produced the depolarization, which is accompanied by firing of action potentials, in rat paratracheal ganglion neurones (Figure 6). At the paratracheal ganglion synapses, nicotinic cholinergic receptors are responsible for the fast excitatory postsynaptic potential (EPSP), and this is followed by a slow EPSP which is mediated by muscarinic receptors (Barnes, 1992; Barnes *et al*., 1988). ACh elicits the inward currents *via* nicotinic receptors with a threshold concentration of 3 μM in the isolated rat paratracheal neurones (Aibara & Akaike, 1991), while the muscarinic receptor mediated response is activated by nanomolar concentrations of ACh (Murai *et al*., 1998). In the present study, the depolarizing response induced by low concentration of ACh (0.3 μM) was inhibited by pirenzepine, thus suggesting that the ACh-response observed in the present study may be regulated by muscarinic receptors. Application of OP-D inhibited the depolarizing action of ACh (Figure 6), suggesting that OP-D may inhibit or reduce the parasympathetic control of airway functions.

We have previously shown that OP-D has a strong antitussive activity in guinea-pig (Takahama & Miyata, 1995). The cough reflex is often associated with inflammatory airway disease (McEwan *et al*., 1990). Bradykinin is implicated in a lot of inflammatory responses in the airway (Barnes *et al*., 1998) and is known to stimulate cough response in guinea-pigs (Takahama & Miyata, 1995; Fox *et al*., 1997; Fuller *et al*., 1987). On the other hand, the bronchoconstrictor effect of bradykinin is reduced by atropine, suggesting the involvement of cholinergic system in the bradykinin action (Ichinose & Barnes, 1990). Recently, the parasympathetic ganglion neurones in the airway of guinea-pigs have been reported to be activated by bradykinin (Kajekar & Myers, 2000). The present study also demonstrated that bradykinin directly excites the cholinergic airway ganglion neurones of rats (Figure 6). OP-D inhibited this bradykinin-induced depolarizing response (Figure 6). Therefore, the present result suggests the possibility that OP-D may be of benefit in reducing inflammatory response in the airway.

In conclusion, the hyperpolarizing effect of OP-D on paratracheal neurones *via* an activation of K^+ channels observed in the present study might explain a part of mechanisms of the antitussive action of the agent.

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