EFFECT OF 4-AMINOPYRIDINE ON POTASSIUM PERMEABILITY OF CANINE TRACHEAL SMOOTH MUSCLE CELL MEMBRANE

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Abstract—Effect of 4-aminopyridine (4-AP) on the potassium permeability of the cell membrane of canine tracheal smooth muscle was investigated using $^{86}$Rb-efflux. The $^{86}$Rb-efflux from the strip was remarkably increased by 20 mM 4-AP in normal physiological solution in the presence of 1 μM atropine. In high potassium and chloride deficient solution, 4-AP (0.3–20 mM) decreased the rate of $^{86}$Rb-efflux in a dose-dependent manner. This finding indicates that 4-AP interacted with a single class of site of action in depolarized tracheal smooth muscle. The maximum decrease in $^{86}$Rb-efflux and the inhibitory dissociation constant of 4-AP were 42.3% and 1.49 mM, respectively. 4-AP had an inhibitory effect on the K permeability of the cell membrane of canine tracheal smooth muscle, and it seemed to interact with the same site of action of tetraethylammonium (TEA), but was less effective than TEA.

It is well known that 4-AP decreases K-conductance (gK) of the cell membrane in many excitable tissues (squid giant axon (1), Ranvier's node (2), cardiac muscle (3), skeletal muscle (4)). In smooth muscle preparations containing peripheral autonomic nervous systems, the drug at low concentrations (<1 mM) is effective on nerve endings and potentiates the release of transmitter (5–7). At higher concentrations, however, 4-AP also acts on the smooth muscle cell membrane (8, 9). Moreover, it has been found that 4-AP is more effective on transmitter release from sympathetic nerve endings in guinea pig vas deferens than TEA (6), which is also well known as a selective blocking agent of gK in excitable cell membranes (10–12).

This work was undertaken to elucidate the effect of 4-AP on the K permeability of canine tracheal smooth muscle using $^{86}$Rb-efflux in comparison with that of TEA.

Materials and Methods
Circular muscle strips were obtained from the cervical trachea of male dogs weighing 7–15 Kg. After the mucosa and adventitial areolar tissue were carefully removed, the strip was cut into a segment, 2–3 mm in width and about 15 mm in length.

$^{86}$Rb was used as a tracer of K-ion flux because the half-decay time of $^{42}$K is very short (12.36 hr). The validity of $^{86}$Rb as a substitute for $^{42}$K was discussed in a previous paper (13). For the ion-flux experiment, a strip was mounted on a stainless steel rod isometrically and preincubated for 2 hr in Krebs' Ringer solution (112 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl$_2$, 25 mM NaHCO$_3$, 1.2 mM MgCl$_2$, 1.2 mM KH$_2$PO$_4$, 14 mM glucose, pH 7.4 when aerated with 95% O$_2$ plus 5% CO$_2$ at 37°C). After the preincubation, the strips were immersed in a loading solution containing $^{86}$Rb for 2 hr. They were then
transferred through a series of test tubes containing 1.5 ml of washing fluid bubbled with 95% O₂-5% CO₂ gas and maintained at 37°C. The efflux period was 10 min for each. At the end of an efflux sequence, the tissue was removed from the rod, blotted gently on filter paper (Toyo No. 2), and the wet weight was determined. The tissue was then dissolved in NH₄OH, and the volume of sample was adjusted with water to 1.5 ml. Radioactivities of samples were measured in an Aloka autogamma spectrometer (model JDC-755). The composition of the loading solution was the same as that of Krebs’ Ringer solution except that it contained ^86RbCl at a concentration about 10 uM (2-5 μCi/ml). High potassium solutions were made by replacing sodium chloride with an equivalent amount of potassium acetate. Low chloride solutions were prepared by replacing chloride salts with corresponding acetate salts. For example, 70 mM potassium and chloride deficient solution was of the following composition (mM): Na+, 45; Cl⁻, 20; K⁺, 70; acetate, 102.6; other compositions were the same as that of the normal solution. Solutions containing 4-AP was prepared by replacing sodium chloride with equimolar 4-AP. A stock solution of 1 M 4-AP was prepared at pH 7.6±0.2 by HCl. In a given series of experiments, isotonic solutions containing the same concentration of chloride ion were prepared by keeping the concentration of 4-AP-HCl plus sodium chloride constant. Radioactive Rb (⁸⁶Rb) was obtained from New England Nuclear Inc. Potassium ion in the loading and washing fluid was not replaced with rubidium ion. All solutions used in efflux experiments contained 1 μM atropine.

Membrane potential was measured using glass-microelectrodes filled with 3 M KCl and with a resistance between 35 and 70 M ohm. Electrical activity was displayed on an oscilloscope (Nihon Koden Ltd. model VC-9) and on an ink writing recorder (Nihon Koden Ltd., model WL-180).

Mechanical responses of the strips set in a 20 ml organ bath were measured isometrically with a force displacement transducer which was domestically made using strain gages (Shinkoh, Type B-FAE-5 50 T1). The electrical signal from the transducer was amplified by an operational amplifier (nA 725) and recorded with a pen recorder (Toa-denpa Kohgyo, model FBR-252A). For direct electrical stimulation, the muscle was passed through a pair of platinum ring electrodes. The diameter of the rings and the distance between the rings were 3 mm and 15 mm, respectively. Direct stimulation was carried out by a train of 20 square pulses of 10 msec duration and frequency of 3 Hz, instead of single pulse of long duration, because the magnitude of contraction was irregular when a single pulse at a duration of 200 msec was applied for a direct stimulation in the presence of TTX (0 5 g/ml). The stimulations were applied at intervals of 5 min. The stimulator used was domestically made and was able to send current up to 250 mA by FET (VN 8AF). The voltage difference between both ends of a resistance (1 ohm) set in series to the electrodes was always monitored on an oscilloscope (Trio model CS-1562A), and the current through the electrodes during stimulating pulses was kept constant (200±5 mA).

The following drugs were used: 4-amino-pyridine, tetraethylammonium chloride (Wako Pure Chem.), atropine sulfate (Tokyo-Kasei Co. Ltd.), tetrodotoxin (a gift from Dr. Tsuda, Tokyo University), D-600 hydrochloride (Knoll A.G.).

Data were evaluated by the Student’s t-test.

Results

Canine tracheal smooth muscle have no spontaneous electrical and mechanical
activities under normal conditions (14–16). A sustained contraction was produced by an application of 4-AP at concentrations over 10 mM, and this contraction was completely inhibited by 1 µM atropine (Fig. 1A). The amplitude of the sustained contraction induced by 1 mM 4-AP was 30–70% of the response to 1 mM acetylcholine. In the presence of atropine, 4-AP at concentrations over 10 mM induced rhythmic or repetitive phasic contractions in 16 preparations out of 21 obtained from 18 dogs. A typical recording of spontaneous mechanical activity in the presence of 4-AP (>10 mM) is shown in Fig. 1A. 4-AP, at concentrations over 3 mM, depolarized the cell membrane, and it occasionally produced oscillations of the membrane potential in the presence of 1 µM atropine. A higher concentration of 4-AP (20 mM) significantly depolarized the cell membrane from 59.1±0.2 mV (mean±S.E.M., 97 cells from 7 dogs) to 38.4±3.1 mV (22 cells from 4 dogs), and it induced spike potentials, 15–30 mV in amplitude in most preparations. As shown in Fig. 1B, transient depolarization (at a duration of a few minutes and more than 5 mV in amplitude) superimposed by action potentials occurred repetitively at intervals of 5–10 min. Similar spontaneous electrical and mechanical activities induced by TEA have been reported in tracheal smooth muscle (16–19). In some preparations, not phasic but slowly developing contractions were observed in the presence of atropine. Both contraction and spike potential were almost completely suppressed by 0.1 µM D-600 or 2 mM MnCl₂.

Figure 2 illustrates the effect of 20 mM 4-AP on ⁸⁶Rb-efflux in normal solution containing 1 µM atropine. O: control, ■: 20 mM 4-AP. Each point is the mean value of three experiments. Abscissa: time after beginning of efflux in min. Ordinate: rate coefficient of ⁸⁶Rb-efflux.
in $^{86}$Rb-efflux was observed in the presence of 4-AP at concentrations over 10 mM. The increase in the rate of $^{86}$Rb-efflux was remarkably reduced by 0.3 μM D-600, while the rate was not affected by 0.3 μM D-600 alone (not shown).

An inhibitory effect of 4-AP should be examined in high K solution to avoid the affect of membrane potential changes on $^{86}$Rb-efflux. The rate of $^{42}$K- and $^{86}$Rb-efflux from the strip in high K (70–100 mM) solution is very high and did not remain constant (20, 21). When chloride ion in the medium was replaced by a larger anion such as acetate ion, the rate of $^{42}$K and $^{86}$Rb-efflux decreased and remained constant for more than 2 hr even in high K solutions (13, 20). Thus, the effect of 4-AP on $^{86}$Rb-efflux from depolarized muscle was examined in chloride deficient, acetate rich solutions with various K concentrations. Figure 3 shows the relationship between extracellular K concentrations and the rate of $^{86}$Rb-efflux and also an inhibitory effect of 10 mM 4-AP on it. The rate coefficient became constant within 40 min after the beginning of an efflux experiment. In the absence of 4-AP, the threshold concentration of extracellular K ion necessary to increase the rate seemed to lie between 10 and 20 mM. As has been already demonstrated in Fig. 2, not the inhibition but the potentiation of $^{86}$Rb-efflux by 4-AP was found in solutions containing relatively low concentrations of potassium (≤20 mM). At higher concentrations of potassium (50 and 70 mM), the rate of efflux was significantly suppressed by 4-AP.

As the inhibitory effect of 10 mM 4-AP on $^{86}$Rb-efflux was revealed in high K solution, the relationship between the concentration of 4-AP and the extent of blockade of $^{86}$Rb-efflux was determined in this solution (Fig. 4: 70 mM K+, 102.6 mM acetate, see Methods). The effect of 4-AP was evaluated as a percentage of the rate coefficient of the control group (Fig. 4A). 4-AP reduced the efflux in a concentration dependent manner between 0.3 and 20 mM (Fig. 4B). The data in Fig. 4B were replotted according to an equation of the single site of action model in Fig. 4C. The plot was fitted by a straight line very well, suggesting either that there is a single class of site of action for 4-AP or that there are more than one class of site of action with very similar inhibitory dissociation constants. If there is a single class of site of action, the maximum decrease in rate coefficient and the dissociation constant of 4-AP calculated from the plot are 42.3% and 1.49 mM, respectively.

In order to compare 4-AP and TEA for their effects on K permeability, the interactions
between these two drugs were examined (Fig. 5). Simultaneous application of 4-AP and TEA, at the concentration of 1 mM, decreased $^{86}$Rb-efflux from depolarized muscle in an additive fashion. At the concentration of 10 mM, however, simultaneous
decrease

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**Fig. 4.** A: Effect of 4-AP on $^{86}$Rb-efflux from depolarized tracheal smooth muscle with 70 mM K solution. Each point is a mean value of 3 experiments. ○: control. ●: 10 mM 4-AP. Abscissa: time after beginning of $^{86}$Rb-efflux in min. Ordinate: rate coefficient of $^{86}$Rb-efflux. 4-AP was applied during the latter 50 min of the efflux period. Effect of 4-AP was evaluated as the percentage of the rate coefficient and was calculated by the following equation:

$$U = \left[1 - \frac{A_1 B_2}{A_2 B_1}\right] \times 100\,\%.$$

$A_1$ and $A_2$ are the rate coefficients of the control and another group at 50 min, respectively. $B_1$ is the mean of the rate coefficient of the control group at 90 and 100 min, and $B_2$ is that of the 4-AP treated group. B: Relationship between dose and the effect of 4-AP. Experiments and evaluation were performed in the same way as shown in A. Each point is the mean value±S.E.M. Figures in parentheses are numbers of dogs and preparations used, respectively. The sum of dogs and preparations used in these experiments were 12 and 58, respectively.

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**Fig. C** curve of 4-AP in accordance with a single class of site of action: $U = \frac{MC}{(C+KD)}$, where M (maximum decrease in rate coefficient caused by 4-AP) = 42.3% and KD (dissociation constant) = 1.49 mM. These values were obtained from a plot of the data as shown in C. C: The data in B were replotted in accordance with the following equation: $U/C = -\frac{C}{KD} + \frac{M}{KD}$. Abscissa: concentration of 4-AP in mM. Ordinate: $U\,\%$/concentration of 4-AP (mM). A linear regression line was fitted by the least squares method: $r=0.98$. The equation of the line is: $Y = -0.67X + 28.4$. 

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application did not produce a further decrease than the maximum reduction induced by TEA.

Figure 6 illustrates the effects of 4-AP and TEA on the contractile response to direct electrical stimulation in the presence of 1 μM atropine. The stimulation induced contraction was almost completely blocked by 2 mM MnCl₂. Although both 4-AP and TEA increased the amplitude in a dose-dependent manner, 4-AP was less effective than TEA. The effect of 4-AP and TEA at higher concentrations could not be evaluated, because spontaneous contraction was produced.

Discussion

4-AP increased the rate of ⁸⁶Rb-efflux in a physiological solution containing 3 μM atropine. This increase probably resulted from the initiation of spontaneous electrical activities because both the increase in ⁸⁶Rb-efflux and the spontaneous activities were inhibited by similar concentrations of D-600 (0.1 or 0.3 μM). The same mechanism was suggested when ⁸⁶Rb-efflux was increased by TEA (13). Although it was found that verapamil decreased ⁴²K-efflux from cardiac muscle (22), D-600 (0.3 μM) per se did not affect ⁸⁶Rb-efflux from tracheal smooth muscle.

In high K solution, the inhibitory effect of 4-AP on ⁸⁶Rb-efflux was obvious in a similar manner as that of TEA (13, 21, 23). It can be postulated that 4-AP interacts with a single class of site of action which probably regulates K permeability directly. The result obtained from the experiment where 4-AP and TEA were applied simultaneously suggests that these two drugs act on the same site (probably the K channel) at low concentrations. The maximum effect of 4-AP (42.3%) was, however, smaller than that of TEA (52.3%) (13). The possibility that the difference in maximum effects of these drugs is an artifact can not be ruled out. However, two distinct K channels on the uterine smooth muscle cell membrane have been detected pharmacologically using TEA and 4-AP in a voltage clamp study (9). Thus, there might be a site in depolarized tracheal smooth muscle which regulates K permeability and is blocked by TEA, but not blocked by 4-AP.

**Fig. 5.** A comparison of the effect of 4-AP and TEA on ⁸⁶Rb-efflux. Each point and vertical bar are the mean value from 5 experiments and S.E.M., respectively. □: 4-AP, ▼: TEA, □: 4-AP+TEA. Abscissa: concentration of 4-AP or TEA in mM. For example, at the concentration of 1 mM, 4-AP+TEA indicates that 1 mM 4-AP and 1 mM TEA were added simultaneously. Ordinate: U(%) obtained from the experiments performed in the same way as shown in Fig. 3A. *, P<0.05 vs. 4-AP, **: P<0.05 vs. TEA.

**Fig. 6.** Effect of 4-AP and TEA on contractile response to direct electrical stimulation in the presence of 1 μM atropine. Stimulation was performed by trains of 20 pulses (10 msec, 3 Hz, 200±5 mA). Each point and vertical bar are the mean value of 6 experiments and S.E.M., respectively. ○: 4-AP, ●: TEA. Abscissa: concentration of 4-AP or TEA. Ordinate: contractile response represented as % of the control response in the absence of 4-AP or TEA. *: P<0.01 vs. TEA.
If this is true, TEA may have more than two sites of action with very similar dissociation constants since only one site of action seemed to be detected in our previous study using $^{86}$Rb-efflux (13). Another possible mechanism which explains why the maximum effect of 4-AP was smaller than that of TEA may be partial recovery of the effect of 4-AP in high K solution. Although we did not try to detect it, it has been reported that the inhibitory effect of 4-AP on voltage-dependent potassium conductance is partly recovered when the membrane is kept at a depolarized level in some tissues (1, 2).

In this study, chloride ion in the medium was replaced by acetate ion in order to evaluate the quantitative effect of 4-AP on K permeability. Although it has been known that K permeability is decreased by substitution of extracellular chloride ion with low permeable anions (20), the characteristics of K permeability decreased by this procedure is not yet clear in smooth muscle. Thus, a possibility that the effect of 4-AP might be partly masked by this procedure cannot be ruled out. Moreover, $^{86}$Rb-efflux was similar to, but not the same thing as $^{42}$K-efflux in canine tracheal smooth muscle (21). The effect of 4-AP on $^{86}$Rb-efflux is probably equal in quality, but might not be equal in quantity to that on $^{42}$K-efflux.

Even considering the possibilities discussed above, the result obtained in this study indicates that 4-AP decreases K permeability in tracheal smooth muscle. Both TEA and procaine which are also known as gK inhibitors (12, 24) increased the excitability of the canine tracheal smooth muscle and induced spontaneous activity (13, 25) as 4-AP did. Therefore, it can be concluded that the suppression of gK is the most important factor to induce spontaneous excitations including action potentials in tracheal smooth muscle, which is one of the lowest excitable smooth muscles. In fact, this muscle produces neither a spontaneous nor an evoked action potential under a normal condition (15, 16, 19).

The atropine sensitive contraction induced by 4-AP is probably due to spontaneous release of endogenous acetylcholine from parasympathetic nerve endings as has been already pointed out in the canine trachea (26), and such remarkable increase in spontaneous release of acetylcholine was found in the rat superior cervical ganglion (27). At low concentrations (0.1 mM), 4-AP seemed to produce spontaneous transmitter release and potentiated contractile responses to nerve stimulation (not shown). On the other hand, the effects of the drug on smooth muscle such as the membrane depolarization, the potentiation of direct electrical stimulation, the production of spontaneous activity in the presence of atropine, and the decrease in $^{86}$Rb-efflux were observed at higher doses of 4-AP. These findings are in good agreement with the suggestion that the nerve ending is more sensitive to 4-AP than smooth muscle (6).

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