ABSTRACT — Single cells were prepared from guinea pig tracheal smooth muscle and used in an experiment on the contraction mechanisms for norepinephrine and a study on the change of $\alpha_2$-adrenoceptors with age. Specific bindings of $[^3H]$p-aminoclonidine to the single cells from the tracheal smooth muscles of 6- and 40-week-old guinea pigs were saturable and analyzed by Scatchard plot. The maximum number of $[^3H]$p-aminoclonidine binding sites was larger in the preparation from 40-week-old guinea pigs than that from 6-week-old animals, while its dissociation constant did not change with age. The amount of prostaglandin F$_{2\alpha}$ released from the single cells was increased by norepinephrine, not affected by phenylephrine, and reduced by an $\alpha_2$-antagonist such as yohimbine. The amount released by norepinephrine was significantly larger in the preparation from 6-week-old guinea pigs than that from 40-week-old animals. These results suggest that the age-related decrease in the potency of norepinephrine is due to reduction in the amount of excitable prostaglandin F$_{2\alpha}$ released by the drug, but not to a change in the total amount of $\alpha_2$-adrenoceptors or the dissociation constant of the drug with respect to these adrenoceptors. Furthermore, $\alpha_2$-adrenoceptors in the tracheal smooth muscle cell play an important role in the release of prostaglandin F$_{2\alpha}$ and the production of contractile responses of these muscles.
the guinea pig tracheal muscle, we used single cells prepared from the tracheal smooth muscles of 6- and 40-week-old animals which contained no small vessels or vascular cells.

MATERIALS AND METHODS

Preparation of single cells

Single smooth muscle cells from guinea pig trachea were prepared by the method of Momose and Gomi (5), with some modifications. Male Hartley strain guinea pigs (6 weeks old: 314 ± 9.2 g, 40 weeks old: 882 ± 14.3 g; body weight was expressed as the mean ± S.E. of about 25 animals) were killed and the trachea quickly removed and suspended in an incubation medium consisting of 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl$_2$, 0.18 mM CaCl$_2$, 5.6 mM glucose, and 4.2 mM HEPES, pH 7.4, and kept at 32°C for 90 min. Slices of the tissue were incubated in a medium containing 0.2% collagenase (Amano), 0.05% elastase (Sigma Type IV) and 1.0% bovine serum albumin at 32°C for 15 min; then 6 units/ml papain (Sigma Type III) was added and incubation continued for 15 min, followed by centrifugation at 1,000 × r.p.m. for 5 min. The pellets were resuspended in the incubation medium at 35°C for 15 min. Thereafter, the single cells could be readily separated from the muscle by gently pipetting the muscle strips in the same solution through a wide-pore Pasteur pipette. The suspension was filtered through nylon mesh.

Viability of the single cells was assessed by the trypan blue exclusion test (6, 7). This involved the addition of 20 μl of 0.4% trypan blue solution into a Burker-Turk hemocytometer. The total number of stained and viable single smooth muscle cells was then determined.

Binding assay

Single cells were incubated with various concentrations of [³H]p-aminoclonidine in a total volume of 0.3 ml of incubation medium at 22°C for 60 min. The incubation mixture was rapidly filtered through a Whatman GF/B glass filter and the filter washed 3 times with 3 ml of ice-cold 50 mM Tris/HCl buffer (pH = 7.4). After the passage, the filter was dried and radioactivity was determined in a toluene base scintillator with a liquid scintillation spectrometer (Aloka LSC-900).

Nonspecific binding was determined as the radioactivity bound to the single cells that was not displaced by 10 μM phentolamine. Specific binding was determined as the difference between total binding and nonspecific binding. Results and calculations are based on the values of specific binding. The binding to the single cells was analyzed using Scatchard (1949) analysis to determine the affinity and the number of binding sites (8).

The total number of receptor binding sites per cell (sites/cell) was determined by multiplying the maximum binding capacity by Avogadro's number and dividing by the cell number per milliliter of incubation volume.

Measurement of prostaglandin F$_{2α}$

Estimation of prostaglandin F$_{2α}$ in the single cells prepared was made as follows: The suspension of single cells in the physiological solution containing desmethylimipramine (10$^{-7}$ M), pargyline (10$^{-4}$ M) and propranolol (3 × 10$^{-6}$ M) was incubated at 32°C and gassed with a mixture of 95% O$_2$ and 5% CO$_2$ and then divided into samples of 1 ml. These were individually incubated as above for 10 min. A 10-μl aliquot of norepinephrine (10$^{-4}$ or 10$^{-3}$ M), phenylephrine (10$^{-3}$ M) and yohimbine (10$^{-3}$ M) were added to each sample, and they were again incubated at 32°C for 10 min. Then each sample was centrifuged at 2,000 × r.p.m. for 5 min at 0°C. A 50-μl aliquot of flurbiprofen (10$^{-4}$ M) was added to each sample of the supernatant to inhibit breakdown of the prostaglandin released. Prostaglandin F$_{2α}$ in 100 μl of the incubation medium was then measured by radioimmunoassay using a prostaglandin F$_{2α}$ [³H]assay kit (TRK 900) obtained commercially from Amersham. Cross-reactivities of prostaglandin F$_{2α}$ antisemur using the assay kit with prostaglandin E$_2$ and 13,14-dihydro-15-keto-prostaglandin F$_{2α}$ are 0.01 and 0.03%, respectively.
Contraction of single cells

Contraction of the single cells prepared under the three conditions was determined as follows: The cells were perfused continuously with the incubation medium on a dimethyl-dichlorosilane-coated glass slide, and prostaglandin \( F_2 \alpha \) was applied with the perfusate. The incubation medium was kept at 35°C and gassed with carbogen. Contraction of cells was observed with a phase-contrast microscope and a computer-assisted light microscopic image analyzer. Five minutes after perfusion began, prostaglandin \( F_2 \alpha \) was added, and the cell size was measured on photographic paper. Cells from ten or eleven different preparations (5 cells in each preparation) were analyzed. The total length of a cell was divided into straight parts, and each straight part was measured; total cell size was then estimated to be the sum of the lengths of all straight parts (9). Contraction percent in the presence of various concentrations of prostaglandin \( F_2 \alpha \) were estimated as:

\[
\left(\frac{S_0 - S}{S_0 - S_m}\right) \times 100
\]

where \( S_0 \) is the size in the absence of prostaglandin \( F_2 \alpha \); \( S_m \) is the size in the presence of prostaglandin \( F_2 \alpha \) \((10^{-3} \text{ M})\), which was the highest concentration used; and \( S \) is the size in the presence of carbachol in the actual concentration used. Thus, a concentration-response curve was obtained.

Agonistic activity of prostaglandin \( F_2 \alpha \) was expressed as the pD\(_2\) value, which was the molar concentration producing a 50% maximum response (10).

Statistical analyses

Numerical results are expressed as means ± S.E., and statistical significance was calculated by Student’s \( t \)-test or Duncan’s new multiple range test. A P value less than 0.05 was considered to indicate a significant difference.

Drugs

\( L \)-Norepinephrine hydrochloride, desmethylinimipramine hydrochloride, pargyline, and \( d,l \)-propranolol hydrochloride (Sigma); prostaglandin \( F_2 \alpha \) (Ono); phenolamine mesylate (Ciba Geigy); and flurbiprofen (Kaken Seiyaku), all in powder form. \([3\text{H}]p\)-Amino-\( p \)-aminoclonidine (specific activity: 45.6 Ci/mmol) was obtained from New England Nuclear. Other chemicals used were of analytical grade.

RESULTS

Binding assay of \([3\text{H}]p\)-aminoclonidine to the single cells of the tracheal smooth muscles of guinea pig

Viabilities of the single cells prepared from 6- and 40-week-old guinea pigs were 82.7 ± 0.35% and 86.0 ± 0.37%, respectively. These values are in agreement with those reported by Koike et al. (1990) for the smooth muscle single cells of guinea pig (11). Yields of the single cells prepared under this condition were 5.21 ± 0.52 \( \times 10^6 \) and 2.46 ± 0.67 \( \times 10^6 \) cells/g tissue (mean with S.E. of 10 groups), respectively. A \([3\text{H}]p\)-aminoclonidine binding experiment and measurement of prostaglandin \( F_2 \alpha \) released from the single cells resulted in 4.92 ± 0.77 \( \times 10^4 \) cells/tube and 1.88 ± 0.23 \( \times 10^4 \) cells/tube, respectively.

The specific binding of \([3\text{H}]p\)-aminoclonidine to the single cells of the tracheal smooth muscles of guinea pigs was saturable (Figs. 1 and 2). Scatchard plots of these bindings yielded two straight lines which were parallel (Figs. 1 and 2). The Hill coefficients were not significantly different from unity, suggesting that the interactions between \([3\text{H}]p\)-aminoclonidine and \( \alpha_2 \)-adrenoceptor are a single bimolecular reaction (Table 1). An age-dependent increase in the maximum number of binding sites was observed, though the dissociation constant did not alter with age. The data are shown in Table 1.

Measurement of prostaglandin \( F_2 \alpha \) released by adrenoceptor

The amounts of prostaglandin \( F_2 \alpha \) released by norepinephrine \((10^{-5} \text{ M})\) were measured in the single cells from the two age groups. Norepinephrine-evoked release of prostaglandin \( F_2 \alpha \) significantly increased in the prepara-
Fig. 1. $[^3H]$p-Aminoclonidine binding to single cells from 6-week-old guinea pig tracheal muscle. Abscissa: concentration (nM) of radioligand and ordinate: radioligand bound in fmol per $10^5$ cells. Inset: Scatchard plot of $[^3H]$p-aminoclonidine binding. Abscissa: concentration of $[^3H]$p-aminoclonidine bound (fmol/ $10^5$ cells). Ordinate: the ratio of bound to free $[^3H]$p-aminoclonidine in the incubation medium. 0, Total binding; •, specific binding; A, non-specific binding.

Fig. 2. $[^3H]$p-Aminoclonidine binding to single cells from 40-week-old guinea pig tracheal muscle. Abscissa: concentration (nM) of radioligand and ordinate: radioligand bound in fmol per $10^5$ cells. Inset: Scatchard plot of $[^3H]$p-aminoclonidine binding. Abscissa: concentration of $[^3H]$p-aminoclonidine bound (fmol/ $10^5$ cells). Ordinate: the ratio of bound to free $[^3H]$p-aminoclonidine in the incubation medium. 0, Total binding; •, specific binding; A, non-specific binding.

Table 1. Maximum binding ($B_{max}$), apparent dissociation constant ($K_D$-value) and Hill coefficient obtained from specific binding of $[^3H]$p-aminoclonidine to single cells derived from the tracheal smooth muscles of 6- and 40-week-old guinea pigs

| Age     | n  | $B_{max}$ (fmol/ $10^5$ cells) | $K_D$ (nM)   | Hill coefficient |
|---------|----|--------------------------------|--------------|-----------------|
| 6 weeks | 5  | 10.28 ± 1.27                   | 1.62 ± 0.17  | 0.92 ± 0.04     |
| 40 weeks| 5  | 14.52 ± 1.33*                  | 1.83 ± 0.19  | 0.97 ± 0.03     |

n represents the number of experiments. Each value is presented as a mean ± S.E.M. of 5 experiments. *: significant difference from the corresponding value by Student's t-test (P < 0.05).
tions from 6-week-old guinea pigs but not in those from 40-week-old animals (Table 2). This enhancement of prostaglandin F$_{2\alpha}$ release induced by adrenoceptors in the single cells from 6-week-old guinea pigs was studied (Table 3). The amount of prostaglandin F$_{2\alpha}$ released was enhanced by an $\alpha_1$- and $\alpha_2$-agonist, norepinephrine (10$^{-6}$ M), but was not enhanced by an $\alpha$-agonist, phenylephrine (10$^{-5}$ M); this enhancement was blocked by an $\alpha_2$-adrenoceptor antagonist, yohimbine (10$^{-5}$ M), which did not change the amount of prostaglandin F$_{2\alpha}$ from the single cells (Table 3).

Table 2. Amounts of prostaglandin F$_{2\alpha}$ in the absence (control) and presence of norepinephrine (10$^{-5}$ M) in the tracheal preparation from 6- and 40-week-old guinea pigs

| Age       | Prostaglandin F$_{2\alpha}$ (pg/10$^5$ cells) |
|-----------|-----------------------------------------------|
|           | Control | Norepinephrine (10$^{-5}$ M) |
| 6 weeks   | 389.0 ± 16.2 (4) | 538.5 ± 26.0** (4) |
| 40 weeks  | 417.0 ± 10.9 (4) | 421.5 ± 11.5 (4) |

Each value is presented as a mean ± S.E.M. of 4 experiments. **: significant difference from the corresponding control value by Student's t-test (P < 0.01).

Table 3. Amounts of prostaglandin F$_{2\alpha}$ in the absence (control), presence of norepinephrine (10$^{-6}$ M), phenylephrine (10$^{-5}$ M) and effect of yohimbine (10$^{-5}$ M) on the prostaglandin F$_{2\alpha}$ release in the presence of norepinephrine (10$^{-6}$ M) from single cells of 6-week-old guinea pig tracheal smooth muscles

| Prostaglandin F$_{2\alpha}$ release (pg/10$^5$ cells) |
|-----------------------------------------------------|
| Control | 356 ± 10.1 (n = 4) |
| Norepinephrine 10$^{-6}$ M | 517 ± 14.1** (n = 3) |
| Phenylephrine 10$^{-5}$ M | 317 ± 13.9 (n = 4) |
| Yohimbine 10$^{-5}$ M | 370 ± 18.5 (n = 4) |
| Norepinephrine 10$^{-6}$ M | |

Each value is presented as a mean ± S.E.M. of 3 or 4 experiments. **: significant difference from the other three groups by Duncan's new multiple range test (P < 0.01).

Contractile response of single cells induced by prostaglandin F$_{2\alpha}$

The prepared tracheal single smooth muscle cell was contracted in a concentration-dependent manner by prostaglandin F$_{2\alpha}$ (Fig. 3). Eighty to eighty-five percent of the total population of cells prepared was contracted by carbachol (10$^{-4}$ M), being similar to results in the guinea pig taenia caecum (11). The total length of these cells was shortened by a perfusion of prostaglandin F$_{2\alpha}$ (10$^{-3}$ M). The maximum contraction with 6- and 40-week-old animals were 16.1 ± 2.19% (n = 11) and 9.87 ± 2.92% (n = 10), respectively. That of the single cells prepared from 6-week-old animals was greater than that in the cells prepared from the older animals. The pD$_2$ value of prostaglandin F$_{2\alpha}$ estimated in the single cells is summarized in Table 4. Sensitivity of the single cells prepared from 6-week-old animals was significantly greater than that in the cells prepared from the older animals.

DISCUSSION

The present results obtained in single cells clearly indicate the existence of $\alpha_2$-adrenoceptors in the guinea pig tracheal smooth mus-
cles, confirming the findings of Takayanagi et al. (2, 3) in the smooth muscle preparations and membrane fractions from guinea pigs. Barnes et al. (12, 13) reported that the density of \( \alpha_2 \)-adrenoceptors predominates over \( \alpha_1 \)-adrenoceptors in canine trachea, and our results are similar to their findings.

Table 4. The pD\(_2\) values and maximum responses in the single cells

| Age   | pD\(_2\) value       |
|-------|-----------------------|
| 6 weeks| 6.16 ± 0.16 (11)     |
| 40 weeks| 5.47 ± 0.24* (10)  |

Each value is presented as a mean ± S.E.M. The number of experiments is shown in parentheses. *: significant difference from the corresponding value by Student's t-test (\( P < 0.05 \)).

In the present study, using single cells prepared with a mixture of purified collagenase and papain, the dissociation constant (\( K_D \) value) of \( [^3H]p\)-aminoclonidine estimated from the Scatchard plot did not change with age, while an age-dependent increase in maximum binding sites (\( B_{max} \)) was observed (Figs. 1, 2 and Table 3). The \( K_D \) value of \( [^3H]p\)-aminoclonidine in this experiment was similar to those in previous experiments using a membrane preparation (2, 3). In the membrane preparation also, the \( K_D \) value estimated from the Scatchard plots did not change with age, while the maximum binding sites (\( B_{max} \)) increased with age from 6- to 40-weeks. These results are in good agreement with the findings of Takayanagi et al. (1991) who reported on the membrane fractions from guinea pig trachea (4). When the membrane fraction were used, about 50 guinea pigs were used in one binding experiment; the experiment was repeated several times to obtain the mean value, so that we used about 150 animals (4). The dissociation constants for \( [^3H]p\)-aminoclonidine are in good agreement with the values estimated in the membrane fractions derived from the guinea pig trachea. With this preparation, we can obtain several different types of receptors on one cell, which allows us to discuss the efficacies of various full or partial agonists. Thus the single cell method is useful for our pharmacological studies.

On the other hand, there was a significant amount of norepinephrine-released prostaglandin \( F_2\alpha \) in the preparations from 6-week-old guinea pigs, but there was no release of prostaglandin \( F_2\alpha \) after stimulation with norepinephrine in the preparations from 40-week-old animals (Table 2), suggesting the decrease in prostaglandin \( F_2\alpha \) release is age-related. An important finding in the present study was that the release of prostaglandin \( F_2\alpha \) in single cells from tracheal smooth muscles was increased by norepinephrine, an \( \alpha_1 \)- and \( \alpha_2 \)-adrenoceptor agonist, but was not changed by phentolamine, an \( \alpha_1 \)-selective adrenoceptor agonist, and that the enhancement of prostaglandin \( F_2\alpha \) release produced by norepinephrine was inhibited by yohimbine, an \( \alpha_2 \)-selective adrenoceptor antagonist. This suggests that prostaglandin \( F_2\alpha \) is released through \( \alpha_2 \)-adrenoceptors. Furthermore, as shown in Fig. 2 and Table 4, the sensitivity of contractile responses to prostaglandin \( F_2\alpha \) was significantly greater in the preparations from 6-week-old guinea pigs than in those from 40-week-old animals. In a preliminary examination using tracheal strips, we observed that the maximum contraction for prostaglandin \( F_2\alpha \) becomes smaller with age. It is connected with a reduction in the sensitivity and the maximum response for prostaglandin \( F_2\alpha \) in the single cells. The tracheal smooth muscle prepared from 6-week-old guinea pigs was slightly contracted by norepinephrine, but we could not observe any contraction in the muscle from 40-week-old animals. These results probably indicate that the elimination of prostaglandin \( F_2\alpha \) released by norepinephrine and the reduction of norepinephrine induced contractile responses with age are due to a functional disorder of physiological mechanisms from \( \alpha_2 \)-adrenoceptors to prostaglandin \( F_2\alpha \) release. From these observations, we think that the age-dependent increase in the maximum number of binding sites is caused by the diminishment of...
these physiological responses.

It is well-documented that the pD₂ value of an agonist is greater in tissues where there are many receptors than in tissues where there are few receptors (14, 15). However, this does not apply to our results. The age-related decrease in the pD₂ value of norepinephrine found in the previous study can be explained by the decrease in the amount of excitatory prostaglandin F₂α released by norepinephrine and in its contractile response to prostaglandin F₂α. It is known that prostaglandin D₂ contracts the tracheal muscles; however, no significant release of prostaglandin D₂ from the guinea pig tracheal preparation was observed (3). Therefore we did not test the action of prostaglandin D₂ on the single cells in this study.

In conclusion, an age-related decrease in the potency (pD₂ value) of norepinephrine is the result of the decrease in the amount of prostaglandin F₂α released by norepinephrine and the decrease in the potency of prostaglandin F₂α, but is not the result of a change in the total number of α₂-adrenoceptors or in the dissociation constant of the drug from α₂-adrenoceptors.

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