Mechanism Generating Endocochlear Potential: Role Played by Intermediate Cells in Stria Vascularis

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ABSTRACT The endocochlear DC potential (*EP*) is generated by the stria vascularis, and essential for the normal function of hair cells. Intermediate cells are melanocytes in the stria vascularis. To examine the contribution of the membrane potential of intermediate cells (E_m) to the *EP*, a comparison was made between the effects of K^+ channel blockers on the E_m and those on the *EP*. The E_m of dissociated guinea pig intermediate cells was measured in the zero-current clamp mode of the whole-cell patch clamp configuration. The E_m changed by 55.1 mV per 10-fold changes in extracellular K^+ concentration. Ba^{2+}, Cs^+, and quinine depressed the E_m in a dose-dependent manner, whereas tetraethylammonium at 30 mM and 4-aminopyridine at 10 mM had no effect. The reduction of the E_m by Ba^{2+} and Cs^+ was enhanced by lowering the extracellular K^+ concentration from 3.6 mM to 1.2 mM. To examine the effect of the K^+ channel blockers on the *EP*, the *EP* of guinea pigs was maintained by vascular perfusion, and K^+ channel blockers were administered to the artificial blood. Ba^{2+}, Cs^+ and quinine depressed the *EP* in a dose-dependent manner, whereas tetraethylammonium at 30 mM and 4-aminopyridine at 10 mM did not change the *EP*. A 10-fold increase in the K^+ concentration in the artificial blood caused a minor decrease in the *EP* of only 10.6 mV. The changes in the *EP* were similar to those seen in the E_m obtained at the lower extracellular K^+ concentration of 1.2 mM. On the basis of these results, we propose that the *EP* is critically dependent on the voltage jump across the plasma membrane of intermediate cells, and that K^+ concentration in the intercellular space in the stria vascularis may be actively controlled at a concentration lower than the plasma level.

INTRODUCTION

The endolymph in the cochlea is unique as an extracellular fluid because its predominant cation is K^+ and its electrical potential (endocochlear DC potential, *EP*) is positive by 80 to 90 mV relative to the perilymph. Both the *EP* and the high K^+ concentration in the endolymph are essential for the transduction of sound by hair cells. Transduction of sound begins at the cilia of the hair cells, which bear mechano-electrical transducer channels that are in contact with the endolymph. When the transducer channels open, K^+ ions flow into hair cells. The main driving force for the K^+ influx through the transducer channels is the electrical gradient across the ciliary membrane produced by the sum of the *EP* and the resting membrane potential of the hair cells (Dallos, 1996).

It has been accepted that the stria vascularis in the cochlea produces the endolymph and the *EP*, but a general agreement on the mechanism involved in *EP* production has not been reached. Within the stria vascularis are intermediate cells, which are melanocytes and migrate from the neural crest during ontogeny (Hilding and Ginzberg, 1977). It is known that a congenital deficiency in these cells causes low *EP* and an increase in the threshold of sound pressure levels required to elicit compound action potentials (Cable et al., 1994; Steel and Barkway, 1989). Recent physiological models for the mechanism of *EP* generation suggest that K^+ channels may play an important role (Salt et al., 1987; Wangemann and Schacht, 1996), and we have proposed that the K^+ channels in intermediate cells may in particular be critical in the generation of *EP* and K^+ transport (Takeuchi and Ando, 1998b; Takeuchi and Ando, 1999). Our proposal is based on the anatomical structure of the stria vascularis and these electrophysiological observations: (i) the *EP* is reduced by some K^+ channel blockers (Marcus et al., 1985; Takeuchi et al., 1996), and (ii) intermediate cells have a relatively large K^+ conductance (Takeuchi and Ando, 1998b, 1999).

In this study, we examined the possibility that the membrane potential of intermediate cells (E_m) plays a critical role in the *EP* generation. This hypothesis can be tested by determining if agents that alter the E_m also alter the *EP* in similar measure. To accomplish this, the effect of K^+ channel blockers on the E_m were compared with their effect on the *EP*. We studied the effect of the blockers on the E_m rather than on currents because (i) we hypothesize that the E_m of the intermediate cell generates the *EP* directly, and (ii) drug effects on the E_m cannot be determined precisely from those on currents. The E_m and the *EP* were studied using the same animal species (guinea pigs) to exclude species-dependent differences in drug sensitivity.

MATERIALS AND METHODS

Measurement of membrane potentials of dissociated intermediate cells

Dissociated cells were prepared in essentially the same manner as reported previously (Takeuchi and Ando, 1998b). Cochleae of albino guinea pigs were obtained under deep anesthesia with pentobarbital sodium (75–100

Received for publication 27 March 2000 and in final form 4 August 2000.
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0006-3495/00/11/2572/11 $2.00

mg/kg, i.p.). Tissue strips of the stria vascularis were incubated for 30 min at 24–26°C in a control bath solution containing 0.2% trypsin and then kept at 4°C for up to 4 h in the same solution until use. The strips were dissected with fine needles under visual control. Though intermediate cells of albino guinea pigs do not contain pigmented inclusions, single intermediate cells can be separated from other cells on the basis of their morphological characteristics (i.e., octopus-like shape and dendrite-like projections; Fig. 1), which are similar to those seen in gerbil intermediate cells (Takeuchi and Ando, 1998b). The E_m of these cells was measured in the zero-current clamp mode of the whole cell patch clamp technique with an amplifier (3900A with 3911A, Dagan, Minneapolis, MN) at 35 ± 1°C. The above technique allowed stable measurements of the E_m for up to 30 min. Pipette resistance was 2.2–3.6 MΩ when filled with the pipette solution. Data storage and analyses were performed using pCLAMP (6.0.3, Axon, Foster City, CA). Liquid junction potentials were measured against a 3-M KCl electrode and corrected.

Two control bath solutions with different K^+ concentrations ($[K^+]_{bath}$) were prepared for the testing of the effect of K^+ channel blockers on the E_m because: (i) effects of K^+ channel blockers on the E_m might be dependent on $[K^+]_{bath}$ (see Results), and (ii) the K^+ concentration in the intercellular space in the stria vascularis, to which the intermediate cell is exposed in vivo, might differ from that in the blood (see Discussion). The bath solution with 3.6 mM K^+ contained (in mM): NaCl 110, Na-aspartate 40, KCl 3.6, HEPES 6, Tris 2.6, $CaCl_2$ 0.7, $MgCl_2$ 1, and glucose 5 (pH 7.4). The bath solution containing 1.2 mM K^+ was prepared by replacing K^+ with Na^+. The pipette solution contained (in mM): KCl 110, K-aspartate 15, KOH 10, EGTA 5, HEPES 6, Tris 6, $MgCl_2$ 1.1, and Mg-ATP 2 (pH 7.2). E_m values were recorded for 2 min in each experimental condition, and steady state values were pooled for further analysis. When E_m values flickered even after a 2-min trial (Fig. 3, *E* and *F*), means for the last 30 s were used for analysis.

Vascular perfusion and measurement of the endocochlear potential

EP levels were maintained by vascular perfusion and K^+ channel blockers were administered to the artificial blood in essentially the same manner as reported previously (Takeuchi et al., 1996), which is a modification of the technique described by others (Wada et al., 1979b; Kobayashi et al., 1984). A schematic drawing of this method is presented in Fig. 5 *G*. The vascular perfusion technique has at least two advantages for the study of the effect of K^+-channel blockers on the *EP*, namely, blockers are quickly delivered to the stria vascularis, which has a dense capillary network, and the artificial blood flows constantly and is independent of the blood circulation system of animals. Albino guinea pigs weighing 260 to 450 g were anesthetized with ketamine (70 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) and artificially ventilated with room air. A pharyngo-laryngectomy was then performed and the bony wall of the skull base was removed. The basilar artery at both ends and all of the identifiable branches from the basilar artery except for the right anterior inferior cerebellar artery were occluded and a polyethylene catheter with a tip diameter of approximately 250 μm was inserted into the basilar artery. Perfusion was performed at a constant flow rate (1.2–1.6 ml/min) using a syringe pump. The temperature of the artificial blood at the catheter tip was 35 ± 1°C. Immediately after starting vascular perfusion, the animal was severed at the neck to secure venous routes for drainage. The artificial blood contained (in mM): NaCl 126, KCl 3.6, $NaHCO_3$ 24, $CaCl_2$ 1.3, $MgCl_2$ 1.2, KH_2PO_4 0.5 and glucose 5. The artificial blood was bubbled with a mixture of 95% O_2 and 5% CO_2 for 60 min at 35 ± 1°C (pH, 7.4 ± 0.1) and stored in syringes.

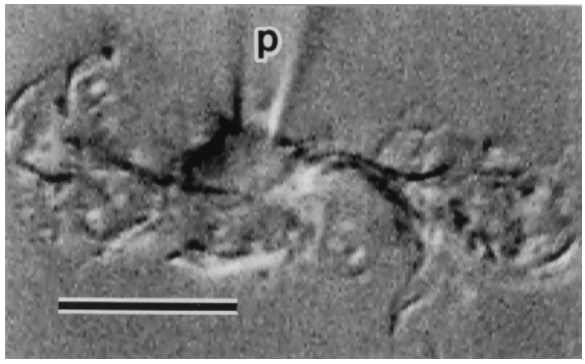

FIGURE 1 Light micrograph of an intermediate cell dissociated from albino guinea pig. *p*, patch pipette. Bar, 10 μm.

EP levels were recorded from the scala media of the basal turn using a glass microelectrode (tip diameter approximately 1 μm) filled with 150 mM KCl and an electrometer (FD223, WPI, Sarasota, FL). The electrode was inserted into the scala media of the basal cochlear turn through the lateral wall of the cochlear duct. An Ag-AgCl wire placed in the subcutaneous tissue of the head served as reference. The recording system was zeroed when the pipette tip was placed on the spiral ligament before insertion into the scala media. Voltage drifts were within ±3 mV when measured with the pipette tip pulled back to the spiral ligament after experiment. Data from animals whose *EP* (>76 mV) was stable for 6 min before the administration of K^+ channel blockers were pooled for further analysis.

The care and use of animals used in this study were approved by the Kochi Medical School Animal Care and Use Committee.

K^+ channel blockers

We examined the effect of five K^+ channel blockers on both the E_m of the intermediate cell and the *EP*. The blockers used were $BaCl_2$, CsCl, quinine-HCl, tetraethylammonium (TEA), and 4-aminopyridine (4-AP). These blockers were directly dissolved in the control bath solution and the artificial blood. As 4-AP is strongly basic, it was neutralized by equimolar HCl. When the concentration of blockers exceeded 5 mM, equimolar NaCl was removed.

Data presentation and statistics

Data were presented as mean ± SE. *N* indicates number of either cells (E_m) or animals (*EP*). Data obtained in the presence of blockers were compared with those under control conditions by paired Student's *t*-test, and changes were regarded as significant when $P < 0.05$.

RESULTS

Effect of K^+ channel blockers on the membrane potential of intermediate cells

Preliminary experiments revealed that currents in guinea pig intermediate cells were composed of two distinct K^+ currents similar to those found in gerbil intermediate cells (Takeuchi and Ando 1999). These currents were (i) inwardly rectifying K^+ currents, which were blocked almost completely by 0.5 mM Ba^{2+}, and (ii) depolarization-activated outward K^+ currents, which were resistant to 0.5 mM Ba^{2+} (Fig. 2 *A*). The chord conductances determined at the end of 500-ms voltage pulses were 152.1 ± 31.4 nS between −160mV and the reversal potential (E_{rev}, −88 mV) and 104.9 ± 9.3 nS between E_{rev} and 0 mV ($N = 9$). The

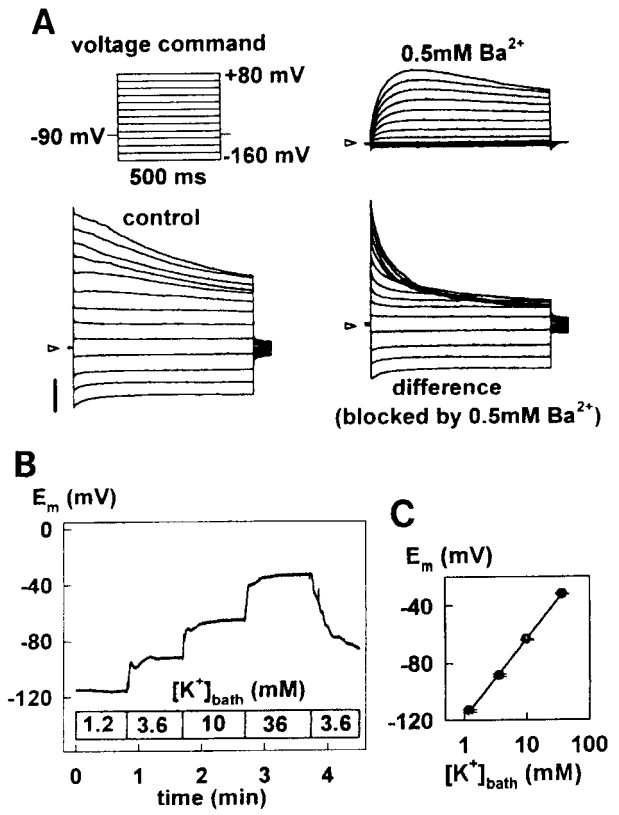

FIGURE 2 K^+ conductance in the intermediate cell. (*A*) K^+ currents in the intermediate cell. Depolarization-activated currents could be separated from inward rectifier currents by 0.5 mM Ba^{2+}. Bath K^+ concentration ($[K^+]_{bath}$) was 3.6 mM. Membrane capacitance, 30 pF. Series resistance (6 MΩ) was compensated by 80%. *Arrowheads*, zero-current level. *Bar*, 5 nA. (*B*) Trace of membrane potential (E_m) recorded in zero-current clamp mode showing effect of changes in $[K^+]_{bath}$. (*C*) E_m recorded in zero-current clamp mode plotted against $[K^+]_{bath}$ ($N = 6$). The slope of the straight line obtained after applying linear regression was 55.1 mV/10-fold change in $[K^+]_{bath}$.

membrane potential (E_m) recorded in the zero-current clamp mode was dependent on $[K^+]_{bath}$ (Fig. 2 *B*). The E_m changed by 55.1 mV per 10-fold change in $[K^+]_{bath}$ (Fig. 2 *C*), indicating that the major determinant of E_m was a K^+ conductance.

Representative E_m traces showing the effects of the K^+ channel blockers are presented in Fig. 3, and dose-response relations are summarized in Fig. 4. As the E_m in control conditions was dependent on $[K^+]_{bath}$, we calculated changes in E_m (ΔE_m) to evaluate the effect of $[K^+]_{bath}$ on drug sensitivity (Fig. 4, *B* and *D*). When extracellular K^+ concentration was set at the level of blood plasma ($[K^+]_{bath} = 3.6$ mM), the effect of Ba^{2+} on the E_m began to appear at 0.15 mM, and ΔE_m increased in a dose-dependent manner. Ba^{2+} showed stronger effects when $[K^+]_{bath}$ was reduced to 1.2 mM; the ΔE_m was statistically significant even at 0.05 mM, and increased in a dose-dependent manner. The dose-response curve of ΔE_m was shifted to the left at 10-fold less Ba^{2+} concentration when $[K^+]_{bath}$ was reduced from 3.6 mM to 1.2 mM (Fig. 4 *B*). Thus, when 5 mM Ba^{2+} is used, the resulting ΔE_m was 47.2 ± 2.6 mV when $[K^+]_{bath}$ was 3.6 mM, and 77.8 ± 2.3 mV when $[K^+]_{bath}$ was 1.2 mM. The E_m recovered almost completely upon washout of 5 or 15 mM Ba^{2+} to −85.3 ± 2.7 mV when $[K^+]_{bath}$ was 3.6 mM, and to −105.8 ± 2.7 mV when $[K^+]_{bath}$ was 1.2 mM (Fig. 3, *A* and *B*). When Cs^+ was present, the concentrations needed to obtain the same responses observed with Ba^{2+} were higher, and, as observed for Ba^{2+}, its effects were dependent on $[K^+]_{bath}$ (i.e., larger ΔE_m at lower $[K^+]_{bath}$). When 30 mM Cs^+ was present, the ΔE_m was 7.0 ± 2.6 mV when $[K^+]_{bath}$ was 3.6 mM, and 23.0 ± 2.1 mV when $[K^+]_{bath}$ was 1.2 mM. Upon washout of 50 mM Cs^+, the E_m recovered almost completely to −89.0 ± 1.1 mV when $[K^+]_{bath}$ was 3.6 mM and to −106.4 ± 2.0 mV when $[K^+]_{bath}$ was 1.2 mM (Fig. 3, *C* and *D*).

Quinine at 0.1 mM did not cause significant changes in the E_m. When 0.3 to 1 mM quinine was used, flickering of the E_m, due most likely to interactions between quinine and K^+ channels, was observed (Fig. 3, *E* and *F*). When the concentration of quinine was raised from 0.3 mM to 1 mM, a large change in the E_m appeared (Fig. 4 *C*). The ΔE_m caused by 1 mM quinine was 82.2 ± 1.9 mV when $[K^+]_{bath}$ was 3.6 mM and 85.4 ± 6.8 mV when $[K^+]_{bath}$ was 1.2 mM. $[K^+]_{bath}$-dependent differences in ΔE_m like those observed for Ba^{2+} and Cs^+, were not apparent at concentrations up to 1 mM (Fig. 4 *D*). Upon washout of 3 mM quinine, the E_m settled down to approximately 0 mV after a small transient recovery (Fig. 3, *E* and *F*). Neither 10 mM 4-AP nor 30 mM TEA resulted in apparent changes in the E_m regardless of $[K^+]_{bath}$ (Figs. 3, *G-J*, and 4, *C* and *D*).

Effect of blockers and $[K^+]_{blood}$ on the EP

The *EP* was depressed by Ba^{2+} in a dose-dependent manner, and it took 3 to 8 min for the *EP* to reach its minimum (Figs. 5 *A*, 6 *A*, and 7 *A*). When 5 mM Ba^{2+} was administered, the *EP* decreased from the control value of 83.5 ± 4.7 mV to a minimum value of 5.1 ± 5.4 mV, thereafter recovering over 20–30 min to a maximum of 13.3 ± 4.2 mV. Upon washout of 5 mM Ba^{2+}, the *EP* recovered completely to 83.6 ± 4.9 mV in 5 to 8 min. These effects of Ba^{2+} confirm those reported previously (Marcus et al., 1985).

Cs^+ had less intense effects on the *EP* than Ba^{2+} (Figs. 5 *B*, 6 *A*, and 7 *C*). When 10 mM Cs^+ was administered, the *EP* decreased from a control value of 88.9 ± 1.9 mV to 81.1 ± 2.3 mV within 5 min, and remained at this level for up to 10 min. At 30 mM Cs^+, the *EP* decreased to 72.1 ± 1.2 mV in 3 min and remained at this level for 3 to 5 min. Subsequently, the *EP* decreased to 40.0 ± 6.5 mV in the

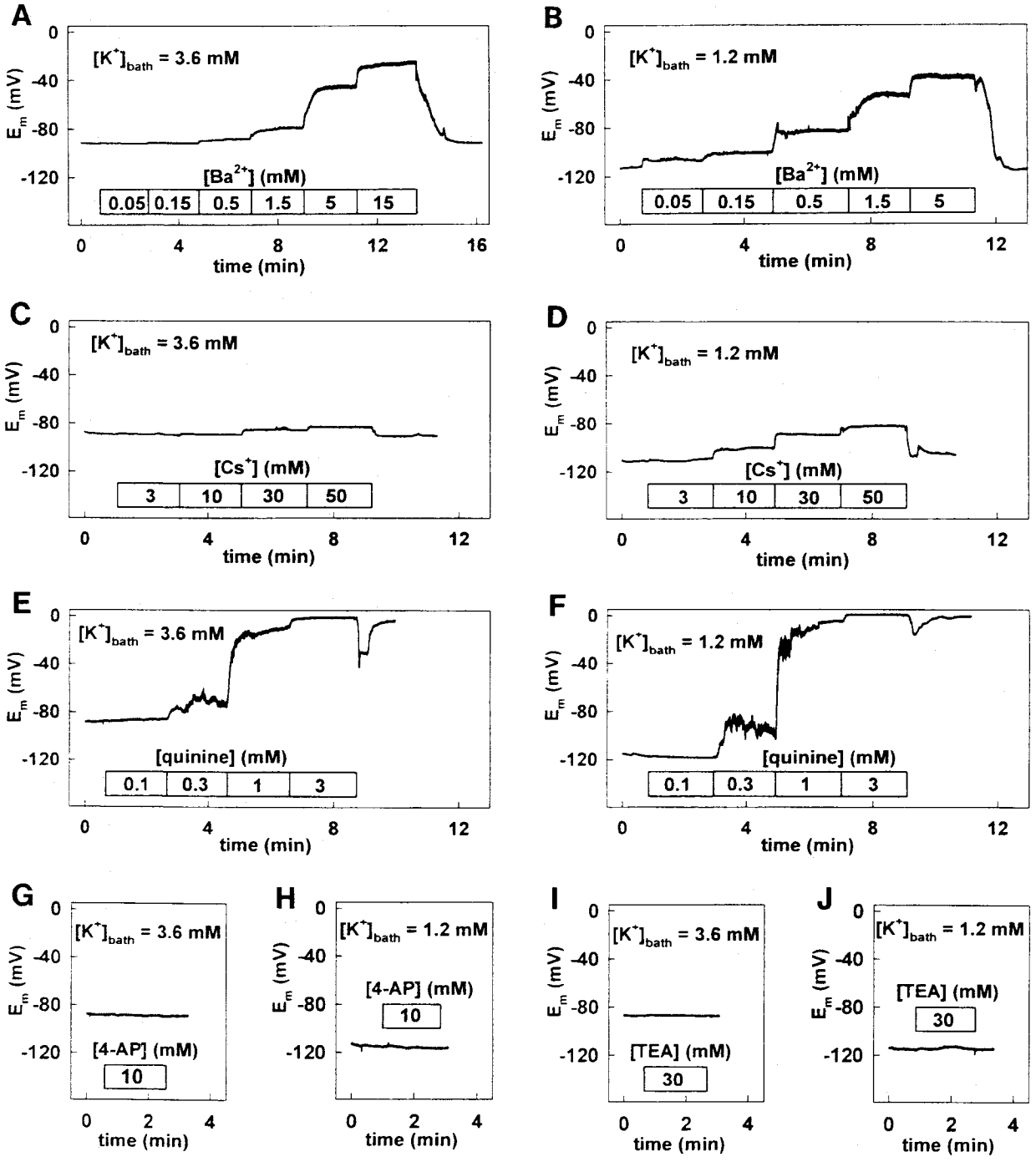

FIGURE 3 Traces of membrane potential (E_m) recorded in zero-current clamp mode showing effects of K^+ channel blockers under two $[K^+]_{bath}$ conditions. (*A* and *B*) Ba^{2+}, (*C* and *D*) Cs^+, (*E* and *F*) quinine, (*G* and *H*) 4-aminopyridine, (*I* and *J*) tetraethylammonium. In *A, C, E, G,* and *I*, $[K^+]_{bath}$ = 3.6 mM. In *B, D, F, H,* and *J*, $[K^+]_{bath}$ = 1.2 mM.

next 30 min. The *EP* recovered incompletely to 66.7 ± 6.0 mV in 10 min upon washout of 30 mM Cs^+.

The *EP* was also depressed by quinine in a dose-dependent manner (Figs. 5 *C*, 6 *A*, and 7 *B*). Quinine at 0.3 mM caused a decrease in the *EP* from the control value of 85.7 ± 2.6 mV to 81.5 ± 3.0 mV in 3 to 6 min, whereas 1 mM quinine elicited a large biphasic change in the *EP* (Fig. 5 *C*). During the first phase, which continued for 8 to 10 min, the *EP* declined to 12.8 ± 4.3 mV. During the second phase, which began before the first phase reached a steady

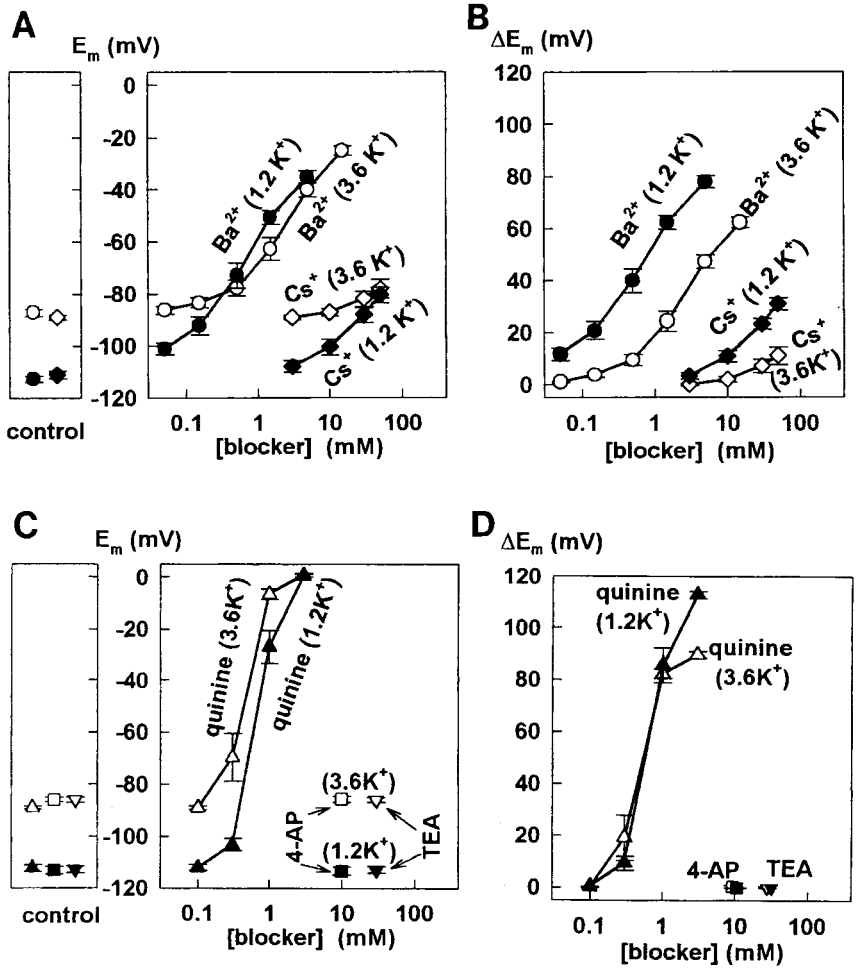

FIGURE 4 Dose-response relations of membrane potential (E_m). (*A* and *C*) Effect of blockers on E_m ($N = 8$ for each data set). E_m values under control conditions are also presented (*control*). $3.6K^+$ and $1.2K^+$ indicate $[K^+]_{bath}$ in mM. (*B* and *D*) Dose-response relations of changes in E_m from control values (ΔE_m) calculated from data presented in *A* and *C*.

level, the *EP* declined to -6.9 ± 2.1 mV. Upon washout of 1 mM quinine, the *EP* recovered only partially to 17.9 ± 4.9 mV. Neither TEA at 30 mM nor 4-AP at 10 mM caused statistically significant changes in the *EP* (Figs. 5, *D* and *E*, and 6 *B*).

Manipulations of the K^+ concentration in the artificial blood ($[K^+]_{blood}$) altered the *EP* only relatively weakly (Figs. 5 *F* and 7 *D*). Upon elevation of $[K^+]_{blood}$ from 3.6 mM to 36 mM, the *EP* declined from 83.0 ± 2.1 mV to 72.0 ± 2.2 mV in 3 min and remained at a quasi-steady level for 10 min. A reduction in $[K^+]_{blood}$ from 3.6 mM to 1.2 mM did not cause significant changes in the *EP* over 10 min of observation. These observations confirm previous reports (Wada et al., 1979a; Marcus et al., 1985).

DISCUSSION

E_m measurement and effect of K^+ channel blockers on E_m

E_m was measured in the zero-current clamp mode of the whole-cell patch clamp configuration. Though it cannot be excluded that the dialysis of cytosol by the pipette solution might suppress ion channels that are dependent on native cytosolic substances, it is unlikely that the observed K^+ conductance is silent in native cells and is artificially activated under the experimental condition. At least two distinct K^+ channels (i.e., the inward rectifier K^+ and the depolarization-activated K^+ channels) contribute to the E_m of the intermediate cell and the sensitivities of these ion channels to blockers vary (Takeuchi and Ando, 1998b, 1999).

The reduction of the E_m by Ba^{2+} and Cs^+ was enhanced by lowering the extracellular K^+ concentration from 3.6 mM to 1.2 mM (Fig. 4 *B*). Two mechanisms may be responsible for the enhanced effect of blockers at lower $[K^+]_{bath}$: (i) reduced competition between K^+ ions and blockers, and (ii) larger E_m values under control conditions. The first mechanism may apply to competitive blockers such as Ba^{2+} and Cs^+, as these blockers are known to compete with K^+ ions, most probably in the selective filter of K^+ channels (Armstrong and Taylor, 1980; Hagiwara et al., 1976). The second mechanism may apply to all the effective blockers.

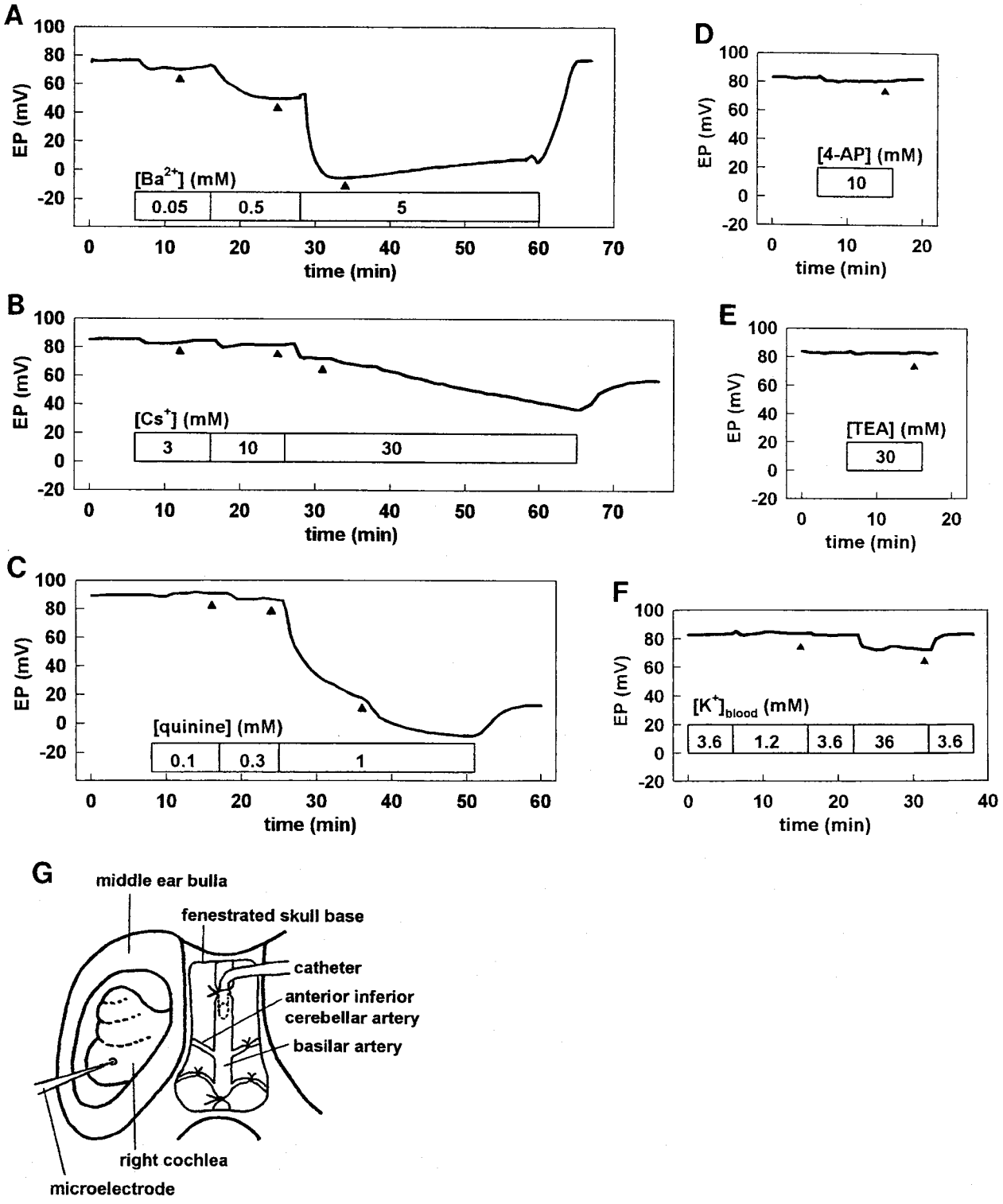

FIGURE 5 Recordings of *EP* showing the effect of blockers and the K^+ concentration in artificial blood ($[K^+]_{blood}$). (*A*) Ba^{2+}, (*B*) Cs^+, (*C*) quinine, (*D*) 4-aminopyridine, (*E*) tetraethylammonium, (*F*) $[K^+]_{blood}$. *Arrowheads*, data points for comparison with $-\Delta E_m$ (*G*) Method for vascular perfusion and recording of *EP*.

Mechanisms underlying EP inhibition by K^+ channel blockers

Ba^{2+}, Cs^+, and quinine depressed the *EP* in a dose-dependent manner, whereas TEA at 30 mM and 4-AP at 10 mM did not change the *EP* (Fig. 6). It is likely that the blockers in the artificial blood exerted most of their effects on the *EP* by affecting cells facing the intercellular space of the stria vascularis (intrastrial space) because: (i) a dense capillary network is present in the stria vascularis, and (ii) it is known that when Ba^{2+} and quinine are administered to the perilymph, these blockers cause an increase in the *EP* (Marcus, 1984; Wang et al., 1993), which is opposite to what we observed when they were administered to the artificial blood. Because the stria vascularis is one of the most densely vascularized tissues in the inner ear, it is expected that the effects of blockers will appear quickly.

With regard to the effect of Ba^{2+} on the *EP*, the relatively fast onset of *EP* decline, and the rapid and complete recovery upon washout suggest first, that the decline in *EP* was due to blockage of K^+ channels, and second, that Ba^{2+} exerts its effect extracellularly. The recovery of the *EP* observed during prolonged administration of 5 mM Ba^{2+} might be due to blockage of pathways which mediate K^+-diffusion from the endolymph to the perilymph. It is likely that such pathways reside in the basolateral membrane of hair cells, which are not well vascularized. Because administration of Ba^{2+} to the perilymph in the scala tympani causes an increase in the *EP* (Marcus, 1984), Ba^{2+} might be able to reach the perilymph and induce a slow increase in the *EP* when administered to the vascular perfusate at high concentrations.

Prolonged administration of 30 mM Cs^+ and 1 mM quinine results in a late and gradual decrease in the *EP* (Fig. 5, *B* and *C*). It is known that Cs^+ is taken up via Na^+-K^+-ATPase (Baker et al., 1969) and that Cs^+ blocks K^+ channels from within the cell. The late onset of the effect of 30 mM Cs^+ on the *EP* might be due to the gradual accumulation of Cs^+ in the cell. That the recovery of the *EP* upon washout of 30 mM Cs^+ is incomplete might be due to remaining Cs^+ within the cell. With regard to quinine, this molecule is relatively lipophilic and is thus expected to permeate through the plasma membrane to some extent. The second phase of the *EP* decline caused by 1 mM quinine might be caused by the effects of quinine on intracellular structures, such as the intracellular domains of K^+ channels and K^+ transporters in mitochondria (Diwan, 1986). Another possibility might be such that quinine inhibited a certain type of Ca^{2+} channels, whose block disturbed the normal operation of other mechanisms that were responsible for the generation of the *EP*. The limited recovery of the *EP* upon washout of 1 mM quinine (Fig. 5 *C*) may be due to the irreversible binding of quinine to K^+ channels since the effect of 3 mM quinine on the E_m of the intermediate cell was irreversible (Fig. 3, *E* and *F*). Not only intermediate cells, but also other cells in the stria vascularis, including marginal cells, might be affected by prolonged administration of 30 mM Cs^+ and 1 mM quinine.

The basilar artery and the anterior inferior cerebellar artery were expanded throughout perfusion and did not show apparent changes in their widths upon administration of blockers (data not shown). Thus, it is likely that the vascular perfusion at a constant flow rate was able to overcome any vasoconstriction caused by blockers, although possible effects of blockers on perfused vessels peripheral to the anterior inferior cerebellar artery cannot be excluded.

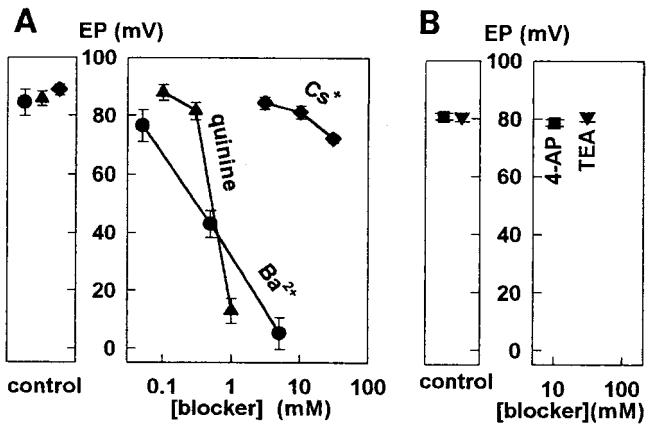

FIGURE 6 Dose-response relationships of *EP*. *EP*s under control conditions are also presented. (*A*) Ba^{2+}, Cs^+, and quinine. $N = 4$ for each data set. (*B*) 4-aminopyridine (4-AP) and tetraethylammonium (TEA). $N = 3$ for each data set.

Comparison between the effect of K^+ channel blockers on E_m and *EP*

With regard to the method used to measure *EP*, it is possible for the blockers to accumulate in native cells and thus cause effects not observed in patch-clamped cells. To enable us to compare the changes in the *EP* (ΔEP) and ΔE_m values directly, we used those *EP* values that were most likely to be affected only minimally by the intracellular presence of the blockers. Thus, because Ba^{2+} is likely to cause the decline in the *EP* by predominantly inhibiting K^+ channels from the extracellular side (see previous subsection) and the maximal effects of Ba^{2+} appeared in 3 to 8 min, we used *EP*s at the quasi steady-states observed within 3 to 8 min of blocker administration to compare ΔEP with ΔE_m (Fig. 5). With regard to diffusion of blockers, we assume that the time course of diffusion from capillaries to effective sites in the stria vascularis for Ba^{2+}, Cs^+, and quinine is similar. As 1 mM quinine caused biphasic changes in the *EP* and the second phase began before the first phase reached a steady state, we decided to use the *EP* value obtained just before

the onset of the second phase for comparison with ΔE_m (Fig. 5 *C*).

ΔE_m values were compared with ΔEPs to test our hypothesis that the E_m of the intermediate cell directly influences the *EP* (Fig. 7). The polarity of ΔE_m is reversed in Fig. 7 to compare with ΔEP because the voltage jump across the plasma membrane of intermediate cells and/or basal cells in the direction from the perilymph to the endolymph (i.e., the extracellular potential in the intrastrial space relative to the interior of intermediate cells) is likely to be the major source of the *EP* (Salt et al., 1987; Wangemann and Schacht, 1996; Takeuchi and Ando, 1999). The relationship between the Ba^{2+} concentration ($[Ba^{2+}]$) and ΔEP is similar to the relationship between $[Ba^{2+}]$ and $-\Delta E_m$ obtained at the lower $[K^+]_{bath}$, whereas this relationship is not as clear at the higher $[K^+]_{bath}$ (Fig. 7 *A*). The relationship between $[Cs^+]$ and ΔEP is also closer to the relationship between $[Cs^+]$ and $-\Delta E_m$ obtained at the lower $[K^+]_{bath}$ (Fig. 7 *C*). With regard to the effect of quinine, $[K^+]_{bath}$-dependent differences in ΔE_m were not observed at quinine concentrations up to 1 mM (Fig. 4 *D*), and the relationship between [quinine] and ΔEP is similar to the relationship between [quinine] and $-\Delta E_m$ (Fig. 7 *B*).

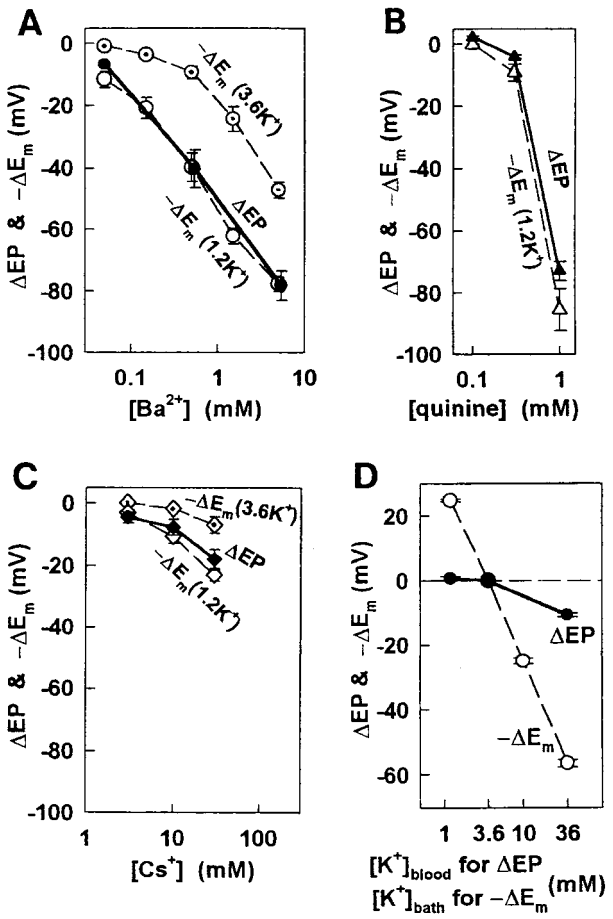

FIGURE 7 Comparison between changes in *EP* (ΔEP) and E_m ($-\Delta E_m$). (*A*–*C*) Effect of blockers. $3.6K^+$ and $1.2K^+$ indicate $[K^+]_{bath}$ in mM. Data presented in Figs. 4, *C* and *D*, and 6 *A* are rearranged. (*D*) Effect of K^+ concentration. Control values were obtained when $[K^+]_{blood}$ and $[K^+]_{bath}$ were 3.6 mM. Data presented in Fig. 2 *C* are rearranged to obtain $-\Delta E_m$. $N = 3$ for ΔEP.

Comparison of the effect of $[K^+]_{bath}$ on ΔE_m and the effect of $[K^+]_{blood}$ on ΔEP

The E_m responses to changes in $[K^+]_{bath}$ were close to those expected from the Nernst equation for a K^+ selective membrane, whereas the *EP* responses to changes in $[K^+]_{blood}$ were relatively small (Fig. 7 *D*). If, as we hypothesize, the *EP* is directly dependent on the membrane potential of intermediate cells determined by K^+ channels, then the resistance of *EP* to changes in $[K^+]_{blood}$ should be explained. In view of the close relationships between $-\Delta E_m$ obtained at $[K^+]_{bath}$ of 1.2 mM and ΔEP (Fig. 7, *A* and *C*), we suggest that the K^+ concentration in the intrastrial space ($[K^+]_{IS}$) is actively controlled at a concentration lower than $[K^+]_{blood}$, and that because of this $[K^+]_{IS}$ will not rise to $[K^+]_{blood}$ levels even if $[K^+]_{blood}$ is raised substantially. That$[K^+]_{IS}$ is actively controlled is likely when the following well known facts are considered: (i) the intrastrial space is quite small as plasma membranes facing the intrastrial space are associated very closely, and (ii) the basolateral membrane of marginal cells has strong immunoreactivity for Na^+-K^+-ATPase and Na^+-K^+-Cl^- cotransporter (Iwano et al., 1989; Crouch et al., 1997). Both transporters act to take up K^+ from the intrastrial space (Wangemann and Schacht, 1996). The enzyme activity of Na^+-K^+-ATPase in the stria vascularis is dependent on K^+ concentration (K_m, 0.87 mM) and responds sensitively to changes in K^+ concentration in the range between 0 and 5 mM (Kuijpers and Bonting, 1969). Similarly, the transport activity of Na^+-K^+-Cl^- cotransporter measured by furosemide-sensitive Rb^+ uptake is also dependent on K^+ (Rb^+) concentration (K_m, 1.80 mM), and responds sensitively to changes in K^+ (Rb^+) concentration in the range between 0 and 5 mM (Duhm, 1987). The above properties of Na^+-K^+-ATPase and Na^+-K^+-Cl^- cotransporter, in association with their abundant expression, seem to be favorable to the regulation of $[K^+]_{IS}$ at relatively low revels. In addition, it has been reported that radioactive K^+ administered to the perilymph accumulates in the endolymph and that within 40 min the concentration of radioactive K^+ in the endolymph is 12 times that in the perilymph (Konishi et al., 1978). The latter suggests that the K^+ taken up by the marginal cell is mainly from the perilymph, not the blood plasma. Though the $[K^+]_{IS}$ has been examined using double-barreled ion selective microelectrodes (Salt et al., 1987; Ikeda and Morizono, 1989), the reported values (3.6–22.1 mM) should be considered preliminary because of technical difficulties related to the small volume of this space (Wangemann and Schacht, 1996). It is thus possible that

changing the $[K^+]_{blood}$ has only limited effects on the $[K^+]_{IS}$, explaining the relatively small effect of $[K^+]_{blood}$ on the *EP*.

If the $[K^+]_{IS}$ is maintained at approximately 4 mM, there should be a mechanism other than K^+ channels in intermediate cells because the E_m of the intermediate cells is less sensitive to Ba^{2+} and Cs^+ than the *EP* when the $[K^+]_{bath}$ is 3.6 mM (Fig. 7, *A* and *C*). This putative mechanism, if any, should be insensitive to TEA and 4-AP, as sensitive to quinine as K^+ channels in intermediate cells, and more sensitive to Ba^{2+} and Cs^+ than K^+ channels in intermediate cells. Such a mechanism has not been found in the stria vascularis until now, and cannot be mediated by the large conductance K^+ channel in basal cells, as this channel is completely blocked by extracellular TEA at 1 mM (Takeuchi and Irimajiri, 1996b).

Five-compartment (two-cell) model of the stria vascularis

On the basis of the results presented in this and previous studies (Salt et al., 1987; Wangemann and Schacht, 1996; Takeuchi and Ando, 1999), we propose an extended version of the electrophysiological model of the stria vascularis (Fig. 8). The interior of the stria vascularis is isolated by two distinct cell sheets connected by tight junctions; one sheet is the marginal cell layer, and the other is the basal cell layer. Capillary endothelial cells are also connected by tight junctions. The junctions are expected to be electrically tight so as to minimize electrical shunting between the endolymph and the perilymph. The intermediate cells and the capillary network are located between the two cell sheets mentioned above. The marginal cell layer serves as a barrier between the endolymph and the intrastrial space. The basal cell layer functions as a barrier between the intrastrial space and the perilymph, and the capillary endothelial cell layer functions as a barrier between the intrastrial space and the blood in the capillary. Intermediate cells, basal cells, pericytes, endothelial cells, and fibrocytes in the spiral ligament constitute an electrical syncytium, because gap junctions connect these cells (Kikuchi et al., 1995; Takeuchi and Ando, 1998a). Accordingly, the stria vascularis, including the perilymph and the endolymph, can be regarded to be composed of five compartments separated by four distinctive membranes (*m1-m4* in Fig. 8). In this model, the electrical potential of the blood in capillaries is assumed to be the same as that of the perilymph. When E_{m1} through E_{m4} are defined as potential differences across each membrane relative to the extracellular side in vivo, the *EP* would be the sum of E_{m1}, $-E_{m2}$, E_{m3}, and $-E_{m4}$.

Measurements in vivo using intracellular microelectrodes indicate that the intracellular potential of marginal cells relative to the endolymph, which corresponds to E_{m4}, is 0 to 10 mV (Melichar and Syka, 1987; Offner et al. 1987; Salt et al., 1987; Ikeda and Morizono, 1989). As the basolateral

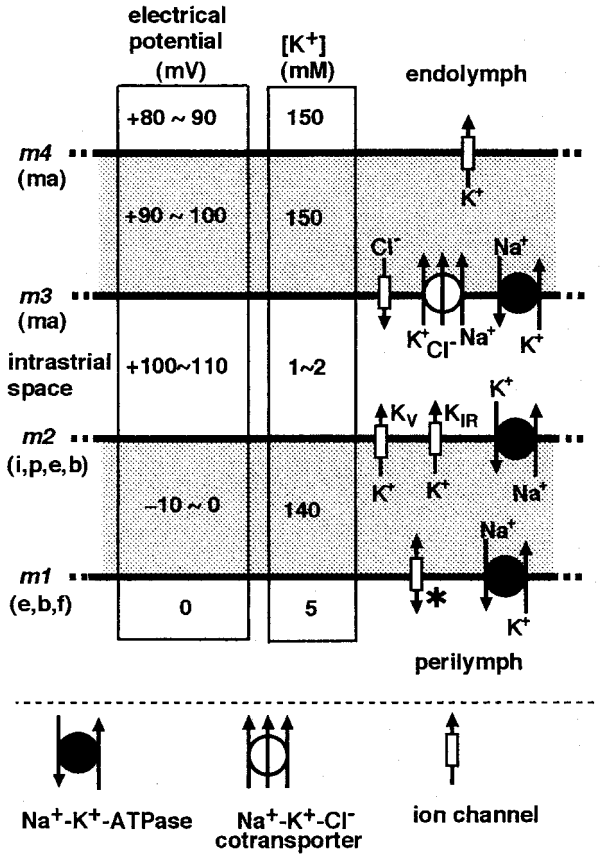

FIGURE 8 Five-compartment (two-cell) model of the stria vascularis. *m1-m4* denote membranes bordering adjacent compartments. Cells constituting each membrane are indicated in parentheses. *ma*, marginal cell; *i*, intermediate cell; *p*, pericyte; *e*, endothelial cell; *b*, basal cell; *f*, fibrocyte in the spiral ligament. Electrical potentials are referred to the perilymph. K_{IR}, inward rectifier K^+ channel. K_V, depolarization-activated K^+ channel. *Asterisk*, unspecified ion channel(s) that determine the membrane potential of *m1*. In intrastrial space, both electrical potential and $[K^+]$ are hypothetical values based on this study.

membrane of marginal cells (*m3*) has a large Cl^- conductance (Takeuchi et al., 1995; Takeuchi and Irimajiri, 1996a) and the transepithelial voltage recorded from cell sheets of the stria vascularis shows a large change when the Cl^- concentration on the basolateral side is reduced (Wangemann et al., 1995), E_{m3} is most likely to be determined predominantly by the Cl^- conductance. The E_{m3} may be estimated to be approximately -10 mV from the intracellular potential of dissociated marginal cells, which depends mostly on the Cl^- conductance in the basolateral membrane (Takeuchi et al., 1997). The intracellular potential of cells connected by gap junctions relative to the perilymph (E_{m1}) is estimated to be in the range between -10 and 0 mV, as potentials in this range have been recorded in vivo (Melichar and Syka, 1987; Salt et al., 1987; Ikeda and Morizono, 1989). The above discussion suggests that neither *m1*, *m3*, nor *m4* are likely to be the location of the positive voltage jump, which is directly related to the *EP*.

We consider here the possibility that *m2* is the location of the positive voltage jump directly related to the *EP*. The *m2* is composed of intermediate cells, pericytes, inner membranes (i.e., membranes facing the intrastrial space) of basal cells, and inner membranes of endothelial cells. With regard to the surface area of cells constituting *m2*, intermediate cells are likely to be a major component of *m2* in view of the fine network composed of these cells in situ (Ando and Takeuchi, 1999). The relatively large K^+ conductance in these cells also suggests that intermediate cells may be major determinants of the conductive properties of *m2* (Takeuchi and Ando 1998b, 1999). Assuming that (i) *m2* is a K^+-selective membrane, (ii) intracellular K^+ concentration is 140 mM, and (iii) $[K^+]_{IS}$ is actively controlled at 1–2 mM, then E_{m2} could be approximately −110 mV (the potential in the cell relative to the intrastrial space), and the electrical potential in the intrastrial space referred to the perilymph could be approximately 100 to 110 mV when E_{m1} is −10 mV. The presence of a space with both a positive potential and a relatively low K^+ concentration in the stria vascularis has been proposed previously (Salt et al., 1987; Ikeda and Morizono, 1989). This space may correspond to the intrastrial space. Using the estimated E_{m3} of −10 mV, the intracellular potential of marginal cells in vivo (relative to the perilymph) may be 90 to 100 mV. This value is in conformity with the intracellular potential of marginal cells measured in vivo (Melichar and Syka, 1987; Offner et al., 1987; Salt et al., 1987; Ikeda and Morizono, 1989). In the above discussion, we have assumed that the transepithelial voltage across the marginal cell layer (i.e., the voltage jump from the intrastrial space to the endolymph) is negative (Fig. 8). With regard to this transepithelial voltage, it has been reported that the transepithelial voltage across vestibular dark cells, which are analogous to marginal cells, changes from positive to negative when the K^+ concentration on the apical side is maintained at 145 mM and the basolateral K^+ concentration is reduced from 3.5 to 2 mM (Wangemann et al., 1996). The above report supports the model (Fig. 8), as it is well known that marginal cells and vestibular dark cells have very similar electrophysiological properties and functions (Wangemann, 1995).

Thus, in summary, it is the most likely that *m2* is the location of the voltage jump that is directly related to the *EP* generation, as suggested previously (Salt et al. 1987; Wangemann and Schacht, 1996; Takeuchi and Ando, 1999), and that the conductive property of *m2* depends largely on K^+ channels in the intermediate cell. Further studies are needed to clarify the electrophysiological properties of *m1* (*asterisk* in Fig. 8) and the non-intermediate cells constituting *m2*.

Finally, we would like to stress the important role of marginal cells in the five-compartment model. Although the plasma membranes of marginal cells (*m3* and *m4*) are not likely to be the location of the positive voltage jump as discussed above, marginal cells may contribute to *EP* generation by taking up K^+ from the intrastrial space, thus maintaining the low $[K^+]_{IS}$, which is essential for a large K^+ diffusion potential across *m2*. Marginal cells also play a major role in the K^+ secretion into the endolymph (Wangemann et al., 1995). It is known that K^+ is secreted via the KVLQT1/I_{sK} channel in the apical membrane of marginal cells, and that the K^+ secretion is impaired by a null mutation of the I_{sK} gene (Vetter et al. 1996).

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