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難聴児に対する配慮のあり方

――難聴児をとりまく学校環境について――

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Careful Attention for Children with Hearing Loss
- Focusing on the School Environment -

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キーワード：難聴，学校環境，養護教諭，支援

難聴とは、聴覚障がいの中で“きこえ”に問題があるもののことを指し、そのような障がいを持った児童生徒に対する医療の発達から、近年、補聴器の性能が向上したり、人工内耳の埋め込み手術が幼少期に行われるようになったりし、聴力のレベルや本人の希望および保護者の希望等によって通常学級に在籍する難聴児が増加していると言われている。難聴児が過ごしやすく、一人ひとりの可能性を十分に発揮することの出来る学級や学校を作るためには受け入れる学校環境の整備が必要だと考えられる。そこで、本研究では、通常学級で生活する難聴児がどのようなことに困っているのかを明らかにすることによって、難聴児の過ごしやすい環境とはどのようなものであるか、また、通常学級において教員が難聴児の過ごしやすい環境作りにどのように関わっていけばよいかを考えることを目的とした。

はじめに

近年、補聴器の性能の向上や人工内耳の埋め込み手術が幼少期に行われるようになったり、新生児聴覚スクリーニング検査がよく行われるようになったりしてきたため、乳児期からの難聴児に対する早期発見・早期教育の効果が出てきている。また、特別支援教育が始まったことから、障がいのある児童生徒も一緒に通常学級で学ぶという考え方がよくされるようになってきており、メディアでも通常学級で学んだ難聴者が取り上げられて話題になっている。それらに伴い、通常学級に在

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籍する難聴児が増加していると言われている。現在、補聴器を装用している児童生徒の 63% が、人工内耳を装用している児童生徒の 70.6% が通常学級に在籍している[1]。

難聴とは、聴覚障がいの中で“きこえ”に問題があるもののことを指す。難聴以外の聴覚障がいには、聴覚鈍麻、聴覚過敏、聴野障がい、音方向感障がい、錯覚、複聴および耳鳴などがある[1,2]が、本研究では難聴を主に扱うものとする。耳の構造において、外耳と中耳は空気の振動である音を内耳の蝸牛に伝える役割を果たすため伝音系といい、蝸牛とその先の聴神経はこれを音として感じ取る役割をはたすので感音系という。これらの系のどこかに障がいが起こるときこえが障がいされ難聴になる。伝音系の障がいで起こるきこえの障がいを伝音性難聴 (伝音難聴ともいう)、感音系の障がいで起こるきこえの障がいを感音性難聴 (感音難聴ともいう) という。また、伝音性難聴と感音性難聴が同時に起こる難聴を混合性難聴という。また、発症時期による区別から、生まれつきの難聴を先天性難聴、出生後に生じた難聴を後天性難聴という。先天性あるいは生後早期の両側高度難聴の頻度は 0.1% (1000 人に 1 人) とされている[3]。このように難聴は一人一人個のなった症状を示し聞き取れる音域が異なる。

一方、近年、難聴児は、聴力のレベルや本人の希望および保護者の希望等によっていくつかの学びの場を選択することが出来るようになった[4]。聴覚特別支援学校 (旧 聾学校) だけではなく、健聴者が通う通常学校において、通常学級のみで過ごす、通常学級に籍を置き必要に応じて難聴学級で過ごす、難聴学級に籍を置き必要に応じて通常学級で過ごす、難聴学級のみで過ごす、あるいは通常学校に籍を置き通級指導教室で指導を受けるなど多様化してきている。

難聴児が通常学級でよりよい学校生活を送れるようにするためには、ともに生活する教職員や児童生徒が、難聴やその児童生徒に対して理解を深めることはもちろん必要である。ただ、難聴児は教室の雑音が大きい場合に会話の 10% をきき逃してしまう[2]と言われており周囲の理解だけでは難聴児にとって過ごしやすい環境を作ることが出来るとは言えない部分もある。そこで難聴児が過ごしやすく、一人ひとりの可能性を十分に発揮することの出来る学級や学校を作るためにはいくつかの学校環境の整備が必要だと考えられる。また、特別支援教育に関わる教員がいなかったり少なかったりする通常学校において、養護教諭が学校環境について考えコーディネーターとしての機能を果たすことが大切であると思われる。

そこで、本研究では、通常学級で生活する難聴児がどのようなことに困っているのかを明らかにすることによって、難聴児の過ごしやすい学校環境とはどのようなものであるか、また、通常学級において養護教諭を含む教員が難聴児の過ごしやすい学校環境づくりにどのように関わっていけばよいかを考察することを目的とする。

方法

[1] A 大学の学生を対象に難聴に対するイメージや難聴者との関わりの有無等について質問紙調査を行った。この質問紙調査は、「健聴者が難聴者と一緒に通常学校で過ごすことについて、どう考えているのか、どのような不安があるのか」を明らかにすることを目的として実施した。

[2] B 聴覚特別支援学校に勤務している養護教諭に対し、聴覚特別支援学校での学校環境に関する

配慮や工夫についての質問紙調査を行った。聴覚特別支援学校で行っていることを通常学校で生かすことを目的とした。

[3] 難聴者である方2名 (C さん : 22 歳, 女性: D さん : 19 歳, 男性) にインタビュー調査した。インタビュー内容は学校生活を送る上で苦労したことや、現在の生活について、また健聴者に知ってほしいことなどである。

結果

[1]　A 大学に在籍する学生を対象とした質問紙調査

A 大学に在籍する学生を対象として質問紙調査行った。質問と結果は以下の通りである。合計人数は回収数から、難聴者と無回答のものを引いたものである。

[設問 1]　健聴者と難聴者が同じ学校 (通常学校) で学ぶことについてお聞きします。健聴者および難聴者の両方の立場になったつもりで選択してください。また、その理由について回答して下さい。

(1) 健聴者として　　賛成　　反対　　わからない
(2) 難聴者として　　賛成　　反対　　わからない

[回答]

健聴者として

賛成	反対	わからない	合計人数
142　(60.4%)	19　(8.1%)	74　(31.5%)	235　(100.0%)

難聴者として

賛成	反対	わからない	合計人数
72　(31.6%)	48　(21.1%)	108　(47.4%)	228　(100.0%)

健聴者と難聴者が同じ学校 (通常学校) で学ぶことについて、健聴者という立場では、60.4% の人が「難聴児が通常学級で学ぶこと」に賛成しており、反対は 8.1% であった。一方、難聴者という立場では、賛成と答えた人が 31.6%、反対と答えた人が 21.1%、わからないと答えた人が 47.4% であった。健聴者と難聴者のどちらの立場でも賛成と答えた人の理由として「設備さえあれば賛成」という意見や、どちらの立場でも反対と答えた人の理由として「難聴者を健聴者と同様に対応できる背景が構築されていないと感じられる」や「設備が整っていないから授業以外で大変そう」という意見があった。

[設問 2] 補聴器の効果はどのようなものであると思いますか。あてはまるものを一つ選び ○ をつけて下さい。

① 音を大きくする効果　② 音を鮮明にする効果　③ 音を鮮明にし大きくする効果

[回答]

音を大きくする効果	音を鮮明にする効果	音を鮮明にし大きくする効果	合計人数
93 (39.4%)	48 (20.3%)	95 (40.3%)	236 (100.0%)

補聴器は拡声器のように「音を大きくする効果」のある機器である。しかし、「音を鮮明にする効果」や「音を鮮明にし大きくする効果」と回答し誤った知識を持つ人が約 6 割いた。

[2] 聴覚特別支援学校の養護教諭への質問紙調査

聴覚特別支援学校の養護教諭に対して質問紙調査を行った。まず、補聴器や人工内耳を装用している子どもたちがききたい音以外の音によって過ごしにくくなっている場面を目にしたことがあるかどうか質問した結果、在校生の聴力レベルはそれぞれ異なるためどれ位の大きさの音を出すのがいいのかの判断がつかずに大きな声を出してしまう児童生徒がいる。そのような友達の騒ぐ声や叫び声などを不愉快そうにしていている児童生徒がいる。また、人工内耳を装用している児童が高くて大きな声をうるさく感じて耳を塞いで嫌な顔をするという場面があったという回答が得られた。これらのことから、補償機器の使用状況や児童生徒同士のきこえの程度の違いが関係していることが想像できた。

次に、緊急時 (地震や火事など) にも連絡がより正確に伝わるような配慮について質問した結果、緊急時は、最初に音声放送が入り、幼・小学部は文字や絵を書いたカードを使用たり手話も用いたりして子どもたちを誘導する。また、保健室では、救急箱と救護の旗を持ち、非難場所に救護にあたるという回答が得られた。以上の内容より、視覚に訴えることで連絡や情報の伝達を早く正確に行うように努めていることがわかった。また、連絡の始めは音声放送であることから、教員一人ひとりの子どもたちへの伝達や誘導が安全を大きく作用することも明らかになった。

[3] インタビュー調査

C さん

C さんは先天性難聴であり、高度難聴 (聴力レベル：右・95 dB, 左・100 dB)、高校まで全て通常学級で生活してきた。口話と読話で生活している。現在は大学に通っており、ノートテイクの制度を利用している。C さんに今までの学校生活において困ったことや満足していたことについて尋ねたところ、校内放送がきき取れないことが困ったということであった。音が鳴っていることはわかるが、内容がわからなかった。「音がきこえる＝話を理解することが出来る」ということではない。また、そのような自身のきこえを例えて、水の中で話しかけられた時、外国人に話しかけられた時のようだと話していた。このように聴覚に頼った伝達の仕組みだけでは困ることが学校外でもあるようだ。それは電車が急に動かなくなった時など、なにか指示が出ているのはわかるが、内容が聞

き取れないためにどうして電車が動かないのか、どうすればいいのかがわからずに困ることがあるようである。C さんのように、授業中など話し手が見える時には読話を用いることが出来ても、音声放送など読話の出来ない状況では話の内容を理解するのに苦労することが明らかになった。

D さん

D さんは先天性難聴であり、高校までを通常学級で生活してきた。骨伝導を利用した補聴器を使用しており、これまで授業中にききたい音以外の音 (以下「雑音」とする) のない環境においては特に不自由を感じずに生活することが出来た。主に口話を用いて生活しており、聞き取りづらい環境では読話も用いている。D さんが学校生活において困ったことは、補聴器は雑音もきき取りたい音と同様に集音するため、きき取りたい音を上手くきき取ることが出来ずに苦労したようである。D さんのきこえにとって雑音がどれほど影響していたかという質問をしたところ、話し合いなどをしている時に、机をガタガタと動かす音などがすると、話の流れがわからなくなり内容をきき返すことがあったようである。単なる“うるさい場所”に行く時 (きき取りたい音は特にない時) には、補聴器のボリュームを下げればよいので大きな問題はないようであった。日常において D さんが困ることとして、会話をしている時に、きき返しが多くなってしまうことで相手をイライラさせてしまうこと、きこえなかった部分が重要な内容かどうかの判断がつかないことであるという。そのような場面は雑音の大きい時ほど多くなつようだ。以前、D さんは健聴の友人に「健聴者もきこえづらいことはあるし、そういう時はきき流すこともある」と言われ、健聴者が話をきき流すこともあるということを初めて知ったが、D さんにはその部分の話の重要性度がわからないため、きき返しも多くなってしまいそのことを今も悩んでいるそうである。D さんは雑音についての悩みは口にしなかったものの雑音が少なくなればきき返しも少なくなり過ごしやすくなると思われる。学校生活においては、机や椅子を移動させる音のような大きな音だけでなく、一斉にプリントを裏返す音や空調の音のような一見気にならないような音でも難聴児のきこえの妨げることがあるという。難聴児もこのような雑音は意識していない場合も多いそうで、無意識の内に気づかぬ雑音によってきこえの低下が見られるということである。

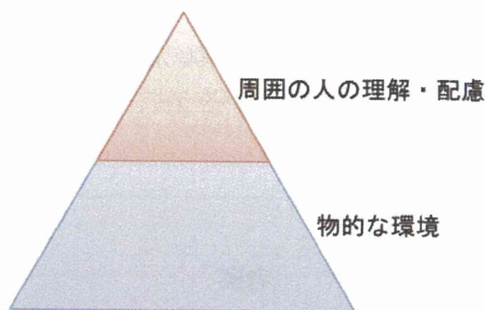
考察

本研究は、難聴の子どもたちが通常学級で生活する時に、教員や周囲の子どもたちの理解はもちろんだが、他にも何か物的な環境整備が必要なのではないか、もし必要なのであればそれはどのようなものなのであろうかという疑問から始まった。聴覚特別支援学校では、集団補聴システム (FM 補聴システム) を活用したり、授業の開始と終了や非常時を知らせるパトライトが設置されていたりする [5]。本研究において、聴覚特別支援学校の養護教諭に対して行った質問紙調査からも、緊急時は音声放送だけではなく、ライト、サイレン、絵や文字の書かれた表や旗を利用して視覚に訴えることで連絡や情報の伝達を早く正確に行うように努めていることが明らかになった。一方、健聴者と難聴者が同じ学校 (通常学校) で学ぶことについてのアンケート結果から、健聴者として考えると賛成だが、難聴者として考えるとわからないと答えた人が最も多かった。その理由として、通

常学校での設備を挙げる意見が多かった。すなわち、健聴者として受け入れようという意識はあるものの、自分が過ごしてきた学校に受け入れ設備が十分なのかと考えた場合、疑問が残るため難聴者としてはわからないという答えになるのではないだろうか。また、補聴器の効果を誤って理解している人が約 6 割もおり、補聴器をつけていればきこえていると思う人が多いことが明らかになった。従って、今後、通常学級で生活する難聴児の増加が予想されるものの、その受け入れには人的および物的な環境の整備が必要と思われる。難聴児が過ごしやすい物的環境を作るにあたっては、聴覚特別支援学校でみられるような集団補聴システムやライトなどの少し大掛かりなものだけではなく、騒音を軽減させるために机と椅子の脚に中古のテニスボール (硬式) をキャップとして取り付けて雑音を軽減させる工夫などといった取り組みやすいものもある。このような工夫は健聴児に対する騒音も軽減するため、快適に過ごせるようになることによって難聴児にとってのメリットが健聴児にとってのメリットにもなりうることを健聴児と難聴児の両方が学ぶチャンスになると考えられる。

難聴児にとって過ごしやすい環境を構築するためには、教員や友人といった周囲の人々の理解や配慮を抜きにしては不可能である。物的な環境と周囲の人々の理解や配慮には繋がりがあり、その周囲の理解と過ごしやすい環境の両方の配慮や工夫が高まったときに初めて難聴児が通常学級で学ぶメリットが発揮される。現在、通常学校での受け入れが始まった場会であることや健聴者の難聴者に対する理解が不十分であることを考慮すると、通常学校においてそこに在籍する児童生徒、教員、あるいは父兄のコンセンサスを得ながら、一歩ずつ進めることも重要であり、何かしらの問題に直面してから、難聴児、健聴児、教員がともにどうすればよいのかを考えていくことも悪いことではないのではないだろうか。

以下に筆者の考える通常学級で難聴児が過ごすにあたり必要な事柄を図に示す。



物的な環境の整備が土台となり、そこに周囲の人の理解や配慮が深まることで、難聴児は通常学級においてより過ごしやすい環境を作り出すことが出来ると思われる。しかしながら、現在、学校健診でも見つかるようになってきている心因性難聴のように、あからさまな環境への配慮を行うことが難聴児本人への負担となり、配慮がよい方向へと向かわないこともあると思われる。例えば、本人に話もせずに「この子は難聴だから席を前の方にしよう」という配慮は、本人が難聴を意識しすぎたりクラスメイトの反応を気にしてしまったりすることもある。心因性難聴児を含め様々な要因を持つ難聴児に接する際には、本人の意見や気持ちを十分にくみ取り、一人ひとりに応じた配慮を行っていく必要がある。特に、周囲に難聴について話していない場合には特に周囲に気がつかれな

いような配慮をする必要がある。難聴児はきこえにくいということでコンプレックスをもっていて、配慮や工夫に対して敏感になることも容易に想像できる。難聴児が困るであろうことを予測して配慮や工夫をすることも時には大切であるが、難聴児だから ○○ に困るというのではなく、ひとりの子が ○○ に困っているという見方も大切にする必要があると考えられる。難聴児のきこえの程度、どのような教育を受けてきてどのようなコミュニケーション手段を身につけているのか、そして何より本人が自分の障がいをどのようにとらえ、周囲にどのように理解し受け入れてもらいたいと思っているかを大切にし、一人ひとりに合った配慮や工夫をしていく必要がある。また、そのような学校環境への配慮や工夫を通して、難聴児が自信を持ったり、自己肯定感を高めていけるきっかけ作りにも繋げていったりすることが可能だと思われる。

今後、ますます通常学級で生活する難聴児の増加が予想され、その中で難聴児を含むすべての子どもたちにとって過ごしやすい学校環境となるような配慮や工夫が実践されることが求められる。難聴についてよく“耳がきこえない”という表現をされることがあるが、きこえないのは耳ではなく“音”である。バリア (障がい) は音であることを理解して人と物、両方を含んだ意味でのバリアフリーな環境が作られていく必要が増してくる。そして、難聴児について教育という括りだけで考えるのではなく医療や福祉、社会にも目を向けて考え、難聴児を含むすべての子どもたちの可能性を十分に発揮出来るような環境を作っていくように努めることが大切である。

謝辞

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注

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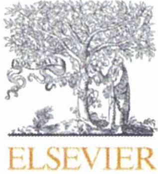

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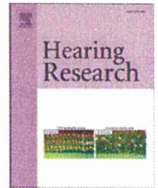

Research paper

Salicylate restores transport function and anion exchanger activity of missense pendrin mutations

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ABSTRACT

The *SLC26A4* gene encodes the transmembrane protein pendrin, which is involved in the homeostasis of the ion concentration of the endolymph of the inner ear, most likely by acting as a chloride/bicarbonate transporter. Mutations in the *SLC26A4* gene cause sensorineural hearing loss. However, the mechanisms responsible for such loss have remained unknown. Therefore, in this study, we focused on the function of ten missense pendrin mutations (p.P123S (Pendred syndrome), p.M147V (NSEVA), p.K369E (NSEVA), p.A372V (Pendred syndrome/NSEVA), p.N392Y (Pendred syndrome), p.C565Y (NSEVA), p.S657N (NSEVA), p.S666F (NSEVA), p.T721M (NSEVA) and p.H723R (Pendred syndrome/NSEVA)) reported in Japanese patients, and analyzed their cellular localization and anion exchanger activity using HEK293 cells transfected with each mutant gene. Immunofluorescent staining of the cellular localization of the pendrin mutants revealed that p.K369E and p.C565Y, as well as wild-type pendrin, were transported to the plasma membrane, while 8 other mutants were retained in the cytoplasm. Furthermore, we analyzed whether salicylate, as a pharmacological chaperone, restores normal plasma membrane localization of 8 pendrin mutants retained in the cytoplasm to the plasma membrane. Incubation with 10 mM of salicylate of the cells transfected with the mutants induced the transport of 4 pendrin mutants (p.P123S, p.M147V, p.S657Y and p.H723R) from the cytoplasm to the plasma membrane and restored the anion exchanger activity. These findings suggest that salicylate might contribute to development of a new method of medical treatment for sensorineural hearing loss caused by the mutation of the deafness-related proteins, including pendrin.

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1. Introduction

The auditory system consists of three parts: the outer ear, middle ear and inner ear. Pendrin, which is encoded by the *PDS* (*SLC26A4*) gene, is a 780 amino acid, an 85.7 kDa membrane protein having 12 putative transmembrane domains, and is mainly expressed in the inner ear, thyroid gland and kidney (Everett et al., 1997). In the inner ear, pendrin is detected in the apical membrane of the endolymphatic duct, endolymphatic sac and various cells which compose the cochlea (Yoshino et al., 2004). Pendrin belongs to the *solute carrier 26A* (*SLC26A*) family, which is a group of proteins acting as multifunctional anion exchangers, and transports anions of Cl^-, I^-, $HCO3^-$ and formate (Scott et al., 1999; Mount and Romero, 2004; Wangemann et al., 2007; Kopp et al., 2008). Pendrin is thought to play an important role in the inner ear as a $Cl^-/HCO3^-$

Abbreviations: 7-AAD, 7-amino-actinomycin D; NASEVA, non-syndromic hearing loss with enlarged vestibular aqueduct; SLC26A, solute carrier 26A.

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transporter involved in the conditioning of ion concentration of the endolymphatic fluid (Mount and Romero, 2004; Wangemann et al., 2007). The loss of pendrin in *Slc26A4*$^{-/-}$ mice, which is a model for Pendred syndrome, results in failure of $HCO3^-$ secretion into endolymph, leading to hearing loss via acidification of endolymph and inhibition of Ca^{2+} reabsorption (Royaux et al., 2001; Wangemann et al., 2007).

More than 150 different mutations of the *PDS* gene have been found in humans so far. These mutations are responsible for Pendred syndrome, which is an autosomal recessive disorder characterized by sensorineuronal hearing loss, goiter and a partial defect in iodide organification, and for non-syndromic hearing loss with an enlarged vestibular aqueduct (NSEVA) (Everett et al., 1997; Kopp, 1999; Dossena et al., 2009). In Japanese, 18 point mutations of the *PDS* gene, i.e., 10 missense mutations, one stop mutation, four frameshift mutations, and three splice site mutations, are reported to lead to these disorders (Tsukamoto et al., 2003). Although Pendred syndrome is thought to account for up to 10% of all cases of syndromic hearing loss, no effective therapy for this syndrome has been established.

Abnormal protein folding or trafficking is associated with various diseases (Thomas et al., 1995; Taubes, 1996; Welch and Brown, 1996). Although, in general, newly synthesized membrane-associated and secreted proteins are translocated to the endoplasmic reticulum, where folding is facilitated by interaction of various endogenous chaperons, abnormal proteins produced by missense mutations are retained in the endoplasmic reticulum by misfolding (Ulloa-Aguirre et al., 2004). It is suggested that such retention and defective plasma membrane targeting of pendrin mutants play a key role in the pathogenesis of Pendred syndrome (Rotman-Pikielny et al., 2002; Dossena et al., 2009). Pharmacological rescue is one method of salvaging these defective proteins by low molecular compounds such as IN3 as chaperones (Conn et al., 2002; Ulloa-Aguirre et al., 2004). Pharmacological chaperones correct errors in folding and restore activity by reestablishing correct protein routing (Ulloa-Aguirre et al., 2004). It has been reported that the misfolded mutants of membrane proteins such as V2 vasopressin receptor, P-glycoprotein and GnRH receptor can be rescued by this method (Loo and Clarke, 1997; Morello et al., 2000; Janovick et al., 2002). Therefore, it is possible that recovery of pendrin mutants by some compounds could improve the condition of Pendred syndrome and NSEVA.

Recently, we have reported that salicylate, which binds to prestin with high affinity (Tunstall et al., 1995; Kakehata and Santos-Sacchi, 1996), induces the translocation of prestin mutants to the plasma membrane from the cytoplasm (Kumano et al., 2010). Prestin is a member of the *SLC26A* gene family including pendrin, and there is approximately a 45% similarity between these proteins in amino acid sequences. Therefore, we hypothesized that pendrin mutants retained in the cytoplasm are induced to translocate to the plasma membrane by salicylate and the function is restored.

In this study, we focused on the 10 missense pendrin mutations (p.P123S (Pendred syndrome), p.M147V (NSEVA), p.K369E (NSEVA), p.A372V (Pendred syndrome/NSEVA), p.N392Y (Pendred syndrome), p.C565Y (NSEVA), p.S657N (NSEVA), p.S666F (NSEVA), p.T721M (NSEVA) and p.H723R (Pendred syndrome/NSEVA)) found in Japanese patients (Tsukamoto et al., 2003) and analyzed the localization and anion transporter activity of these mutant proteins *in vitro* and the effects of salicylate as a pharmacological chaperone for the rescue of the pendrin mutants.

2. Methods

2.1. Construction of expression vectors

The sequences encoding an N-terminal FLAG tag were added by PCR amplification to the cDNA for human wild-type pendrin (Accession No. NM_000441.1) and the resulting insert was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). *E. coli* JM109 was transformed with the constructed expression vector (pcDNA3.1/pendrin-FLAG). Mutational studies targeted the genes of pendrin missense mutations reported in Japanese patients (Tsukamoto et al., 2003). To express 10 pendrin mutants, the mutations were introduced into the pcDNA3.1/pendrin-FLAG using the primer sets shown in Table 1 and QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The constructs were verified by DNA sequence analysis.

2.2. Cell culture

HEK293 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2. For the experiments, HEK293 cells were seeded in each well of 6-well plate at a density of 5×10^5 cells in 2 ml of 10% FBS-RPMI-1640 medium. After 12 h, each expression vector (2 μg) was transfected into HEK293 cells using FuGENE HD Transfection

Table 1
The sequences of primer sets for site-directed mutagenesis.

Primers	Sequences	
p.P123S	Forward	5′ GGATATGGTCTCTACTCTGCTTTTTTCTCCATCCTGACAT AC 3′
	Reverse	5′ GTATGTCAGGATGGAGAAAAAAGCAGAGTAGAGACCATATCC 3′
p.M147V	Forward	5′ CTTTTCCAGTGGTGAGTTTAGTGGTGGGATCTGTTGTTC 3′
	Reverse	5′ GAACAACAGATCCCACCACTAAACTCACCACTGGAAAAG 3′
p.K369E	Forward	5′ CTATTGCAGTGTCAGTAGGAGAAGTATATGCCACCAAGTATG 3′
	Reverse	5′ CATACTTGGTGGCATATACTTCTCCTACTGACACTGCAATAG 3′
p.A372V	Forward	5′ GTGTCAGTAGGAAAAGTATATGTCACCAAGTATGATTACACC 3′
	Reverse	5′ GGTGTAATCATACTTGGTGACATATACTTTTCCTACTGACAC 3′
p.N392Y	Forward	5′ GCCTTTGGGATCAGCTACATCTTCTCAGGATTCTTC 3′
	Reverse	5′ GAAGAATCCTGAGAAGATGTAGCTGATCCCAAAGGC 3′
p.C565Y	Forward	5′ CGATGGTTTTAAAAAATATATCAAGTCCACAGTTGGATTTGATGCC 3′
	Reverse	5′ GGCATCAAATCCAACTGTGGACTTGATATATTTTTTAAAACCATCG 3′
p.S657N	Forward	5′ CAAAGTGCCAATCCATAACCTTGTGCTTGACTGTG 3′
	Reverse	5′ CACAGTCAAGCACAAGGTTATGGATTGGCACTTTG 3′
p.S666F	Forward	5′ CTTGACTGTGGAGCTATATTTTTCCTGGACGTTGTTG 3′
	Reverse	5′ CAACAACGTCCAGGAAAAATATAGCTCCACAGTCAAG 3′
p.T721M	Forward	5′ CAACATTAGAAAGGACACATTCTTTTTGATGGTCCATGATGC 3′
	Reverse	5′ GCATCATGGACCATCAAAAAGAATGTGTCCTTTCTAATGTTG 3′
p.H723R	Forward	5′ CTTTTTGACGGTCCGTGATGCTATACTCTATCTACAG 3′
	Reverse	5′ C TGTAGATAGAGTATAGCATCACGGACCGTCAAAAAG 3′

Reagent (Roche, Grenzacherstrasse, Switzerland) according to the manufacturer's protocol. After transfection for 24–48 h, the cells were used for the following experiments.

2.3. Immunofluorescence microscopy

After incubation for 24 h of HEK293 cells transfected with each vector, the cells were replaced in 24-well glass bottom plates and further incubated for 24 h. Salicylate dissolved in distilled water was added to the cells in each well and the cells were incubated for 12 h in the presence of salicylate. For vehicle control, distilled water was added to the medium. The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. To avoid non-specific binding, the cells were incubated in blocking solution (50% Block Ace, Dainippon Sumitomo Pharma, Osaka, Japan and 50% FBS) for 1 h at 37 °C. After washing with PBS, the cells were incubated with mouse anti-FLAG primary antibody (Sigma) for 1 h at 37 °C and then anti-mouse IgG secondary antibody TRITC conjugated (Sigma) for 30 min at 37 °C. These antibodies were diluted in 0.1% (w/v) saponin-PBS. After washing with PBS, the fluorescence of cells stained with TRITC was observed using a confocal laser scanning microscope (FV500, Olympus, Tokyo, Japan).

To clarify where pendrin mutants are mainly expressed in the cells, fluorescence intensities of the cells were analyzed by FLUOVIEW (Olympus). As shown in Fig. 1, a green line was firstly drawn on the cell expressing wild-type pendrin, and intensities of TRITC and differential interference contrast (DIC) on the line were analyzed. Two peaks at the ends of the line in the TRITC intensity map and two low peaks in the DIC intensity map were detected. The two low peaks in the DIC intensity map showed the border between the cell and the glass of the culture plate. The width of the plasma membrane was determined to be 0.8 μm from the border, based on the width of the TRITC-related peaks in the TRITC intensity map. Then, the fluorescence intensities were obtained from the 20 regions (region size, 0.8 μm × 0.8 μm) of the plasma membrane area, and such intensities were also obtained from the 20 regions of the cytoplasm area. Each region was not overlapped and equally distributed from the cell center. The former and the latter regions covered approximately 90% and 70% of the plasma membrane area and the cytoplasm area, respectively. The ratio of the mean fluorescence intensities of the 20 regions obtained from the plasma membrane area to such intensities of the 20 regions obtained from the cytoplasm area was calculated. Finally, the main localization of each pendrin mutant was estimated by calculating the averaged ratio from the ratio obtained from 6 to 9 cells.

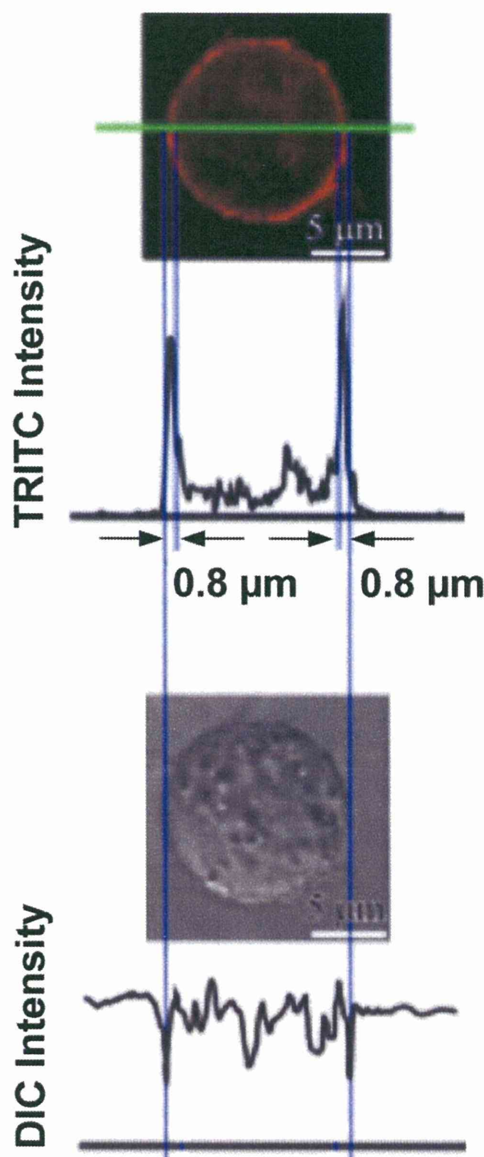

Fig. 1. Intensity analysis of intracellular localization of pendrin. A green line was drawn on the cell expressing wild-type pendrin, which mainly expresses in plasma membrane, and the intensities of TRITC and DIC on the line were analyzed. Two peaks at the ends of the line in the TRITC intensity map and the two low peaks in the DIC intensity map were detected. The two low peaks in the DIC intensity map showed the border between the cell and glass of the culture plate. The width of plasma membrane was determined to be 0.8 μm based on width of the TRITC-related peaks in the TRITC intensity map, and 0.8 μm inside from the border was defined the plasma membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Iodide efflux assay

Iodide efflux from cells was assessed by a modification of the methods described by Gillam et al. (2004) and Dossena et al. (2006). After incubation for 36 h of HEK293 cells transfected with each vector, the cells in each well of the 6-well plate were incubated for 12 h at 37 °C in the medium in the presence or absence of salicylate. The cells were then incubated for 10 min at 37 °C in 1 ml of high Cl^- buffer (2 mM KCl, 135 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 20 mM HEPES, pH7.4), and further incubated for 60 min at 37 °C in 1 ml of high I^- buffer (2 mM KCl, 135 mM NaI, 1 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 20 mM HEPES, pH7.4) containing 200 kBq/ml ^{125}I. During these processes, uptake of ^{125}I is promoted by exchange with Cl^- already taken into the cells by a mechanism independent of pendrin. After washing with high I^- buffer, the cells were incubated for 10 min at 37 °C in 1 ml of high Cl^- buffer. Replacement of high I^- buffer by high Cl^- buffer induces the export of ^{125}I from within cells to extracellular media by the activity of transfected pendrin. Finally, the cells were lysed in 200 μl of lysis buffer (0.1 M NaOH, 0.1% SDS, 2% sodium carbonate), and intracellular iodide content was determined by measuring radiolabeled-iodide in the cell lysates using a scintillation counter. The protein content in the lysates was measured by Bradford methods (Bio-Rad, Hercules, CA). Remaining radioactivity in cells was obtained by dividing intracellular iodide content in the lysates (cpm/ml) by protein content in the lysates (mg/ml).

2.5. Cytotoxicity assay

HEK293 cells cultured in each well of a 24-well glass bottom plate were incubated for 12 h at 37 °C in medium in the presence or absence (vehicle control) of 10 mM of salicylate. After incubation, nuclei in the cells were stained with 7-amino-actinomycin D (7-AAD, 200 ng/ml in medium) (Sigma) at 37 °C for 10 min. As 7-AAD penetrates plasma membranes of dying or dead cells but not viable cells, this compound is used as a marker for cell death. As a positive control, the plasma membrane of cells was permeated by incubation in methanol for 1 min and dried prior to the staining with 7-AAD. The fluorescence of the cells was observed by a confocal laser scanning microscope (Olympus).

2.6. Statistical analysis

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

3. Results

3.1. Expression of pendrin mutants in HEK293 cells

When wild-type pendrin was expressed in HEK293 cells, the fluorescence intensity of TRITC, which indicates localization of pendrin, was particularly strong on the plasma membrane compared with the fluorescence intensity of the empty vector transfected cells (Fig. 2). Two pendrin mutants, i.e., p.K369E and p.C565Y, were also localized on the plasma membrane (Fig. 2). On the other hand, other mutants, i.e., p.P123S, p.M147V, p.A372V, p.N392Y, p.S657N, p.S666F, p.T721M and p.H723R, were localized in the whole cell except for the regions of the nuclei (Fig. 2). These findings indicate that wild-type pendrin and two pendrin mutants, p.K369E and p.C565Y, were localized in the plasma membrane, whereas the other 8 pendrin mutants were retained in the cytoplasm.

3.2. Effects of salicylate on the localization of mutant pendrin

Eight pendrin mutants, i.e., p.P123S, p.M147V, p.A372V, p.N392Y, p.S657N, p.S666F, p.T721M and p.H723R, were retained in the cytoplasm of the HEK293 cells (Fig. 2). Therefore, we analyzed whether these 8 pendrin mutants are induced normal transport to the plasma membrane by salicylate. The 8 pendrin mutants were localized in cytoplasm when the cells were incubated in the absence of salicylate (Fig. 3a and c). In contrast, 10 mM of salicylate made the fluorescence intensity of TRITC of the plasma membrane stronger than that of the cytoplasm in the cells which expressed p.P123S, p.M147V, p.S657N and p.H723R (Fig. 3b and c). In this case, the fluorescence intensity of cytoplasm by TRITC becomes weaker and the nucleus and cytoplasm in the cell become unclear, because the pendrin mutants, i.e., p.P123S, p.M147V, p.S657N and p,H723R, translocate by the compound from the cytoplasm to the plasma membrane (Fig. 3b and c). As a result, the nucleus appears to be large (Fig. 3b). However, no changes were observed in the cells transfected with other mutants, i.e., p.A372V, p.N392Y, p.S666F and p.T721M (Fig. 3b and c). These findings indicate that salicylate induces the translocation of p.P123S, p.M147V, p.S657N and p.H723R pendrin mutants from the cytoplasm to the plasma membrane.

3.3. Effect of salicylate on the viability of HEK293 cells

To clarify whether 10 mM of salicylate has cytotoxicity, HEK293 cells were incubated for 12 h at 37 °C with or without 10 mM of salicylate, and the viability of cells was visualized by microscope. On treatment with salicylate, as well as vehicle control, no significant fluorescence intensity of 7-AAD binding to nuclei in the cells was observed (Fig. 4). In contrast, as a positive control, nuclei of the cells treated with methanol were clearly stained due to the increase in the permeability of the plasma membrane (Fig. 4). These findings indicate that 10 mM of salicylate has no cytotoxicity to HEK293 cells.

3.4. Effects of lower concentration of salicylate on the intracellular localization of p.P123S mutant pendrin

HEK293 cells expressing p.P123S pendrin mutant were incubated for 12 h in medium containing 1 or 10 mM of salicylate. The intracellular localization of the p.P123S pendrin mutant was detected by immunofluorescence microscopy. The p.P123S pendrin mutant was localized in plasma membrane when the cells were incubated in the presence of 10 mM of salicylate (Fig. 5). However, 1 mM of salicylate did not change the localization of p.P123S pendrin mutant when compared with vehicle control (Fig. 5). These findings indicate that salicylate at 10 mM but not at 1 mM induces the translocation of p.P123S mutant pendrin.

3.5. Effects of salicylate on the activity of pendrin

We analyzed whether the 4 pendrin mutants (p.P123S, p.M147V, p.S657N and p.H723R) translocated to the plasma membrane by salicylate act as transporters using radiolabeled-iodide. HEK293 cells transfected with the expression vector for the pendrin mutants were incubated with or without salicylate. In cells transfected with the wild-type pendrin expression vector, remaining radioactivity in cells was significantly decreased compared with that transfected with an empty vector when the cells were incubated in the absence of salicylate (Fig. 6). This finding indicates that wild-type pendrin has an activity to export iodide from cytoplasm. Remaining radioactivity in cells expressing the p.K369E or p.C565Y mutants, which were located in the plasma membrane as shown in Fig. 1, was decreased as in the case of wild-type pendrin (Fig. 6). Therefore, the mutants acted to transport iodide as do wild-type pendrin. In the expression of wild-type pendrin, the activity in the presence of 10 mM of salicylate was not significantly different from that in the absence of salicylate (Fig. 6), suggesting that the incubation with of 10 mM of salicylate does not affect the cellular condition and the wild-type pendrin activity. In case of transfection with 4 mutant pendrin genes encoding the mutants p.P123S, p.M147V, p.S657N and p.H723R, no significant change of remaining radioactivity in cells was observed compared with the empty control in the absence of salicylate treatment (Fig. 6). However, the remaining radioactivity in cells transfected with each mutant was significantly decreased by 10 mM of salicylate treatment (Fig. 6). These activities were almost the same as in the case of wild-type pendrin (Fig. 6). These findings indicate that salicylate restores pendrin activity.

4. Discussion

It is suggested that retention of some pendrin mutants in the endoplasmic reticulum is a major mechanism for Pendred syndrome (Rotman-Pikielny et al., 2002; Dossena et al., 2009). p.L236P, p.G384 and p.T416P pendrin mutants reported in Caucasians are accumulated in the endoplasmic reticulum and do not reach the plasma membrane due to their mutations (Rotman-Pikielny et al., 2002). In Japanese patients, 10 pendrin mutants, i.e., p.P123S, p.M147V, p.K369E, p.A372V, p.N392Y, p.C565Y, p.S657N, p.S666F, p.T721M and p.H723R, have been reported (Tsukamoto et al., 2003). Yoon et al. (2008) have reported that p.M147V and p.H723R pendrin mutants were accumulated in the endoplasmic reticulum due to their mutation. However,

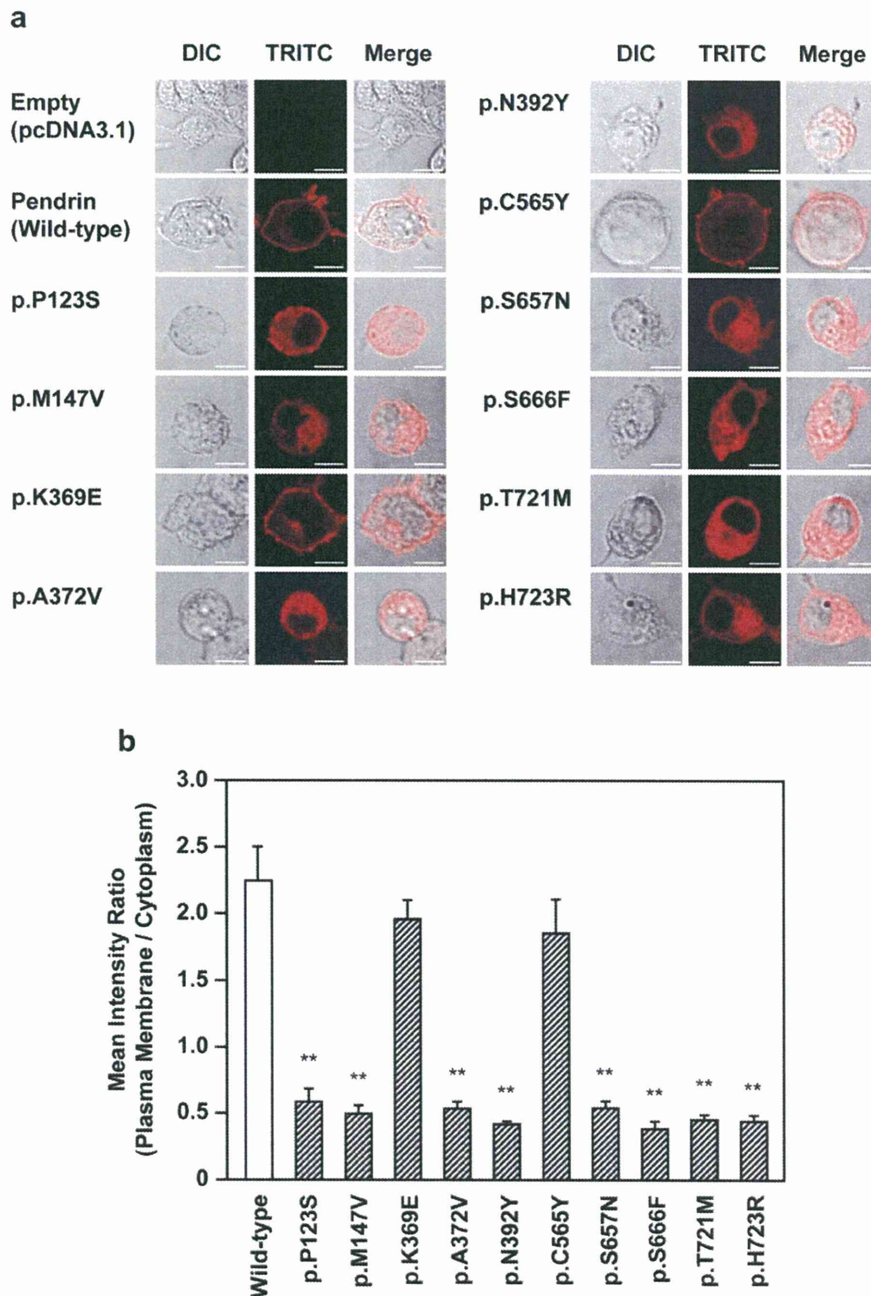

Fig. 2. Intracellular localization of pendrin and its mutants in HEK293 cells. HEK293 cells were transfected with the empty vector, the expression vector including wild-type pendrin gene or the mutant genes. (a) After 48 h, intracellular localization of pendrin was analyzed by immunofluorescent staining. The experiment shown is representative of 3 independent experiments. (b) The fluorescence intensities of the plasma membrane and cytoplasm of the cells were evaluated. The ratios were obtained by calculating the mean fluorescence intensities of 20 regions (0.8 μm × 0.8 μm) in plasma membrane and cytoplasm. Values are the means from 6 to 9 cells with SEM. $^{**}P < 0.01$ vs. wild-type. This figure shows that wild-type pendrin and two pendrin mutants p.K369E and p.C565Y were localized in the plasma membrane, whereas the other 8 pendrin mutants were retained in the cytoplasm. The bar indicates 10 μm.

intracellular localization and activity of 8 other pendrin mutants have remained to be elucidated. In this study, we demonstrated the following 4 points about the intracellular localization and activity of pendrin mutants reported in Japanese patients: (1) Among the 10 pendrin mutants reported in Japanese patients, p.K369E and p.C565Y are expressed in the plasma membrane of HEK293 cells, as is wild-type pendrin. (2) The other 8 mutants, i.e., p.P123S, p.M147V, p.A372V, p.N392Y, p.S657N, p.S666F, p.T721M and p.H723R, are accumulated in the cytoplasm of the cells. (3) In the 8 pendrin mutants retained in the cytoplasm of the cells, p.P123S, p.M147V, p.S657N and p.H723R are induced normal transport to the plasma membrane of the cells by 10 mM salicylate without cytotoxicity. (4) When induced normal transport to the plasma membrane by salicylate, these 4 pendrin mutants recovered their activity.

Pendrin is a member of the *SLC26A* gene family including prestin (Mount and Romero, 2004). In the inner ear, it is reported that the motor protein prestin plays an important role in hearing as the

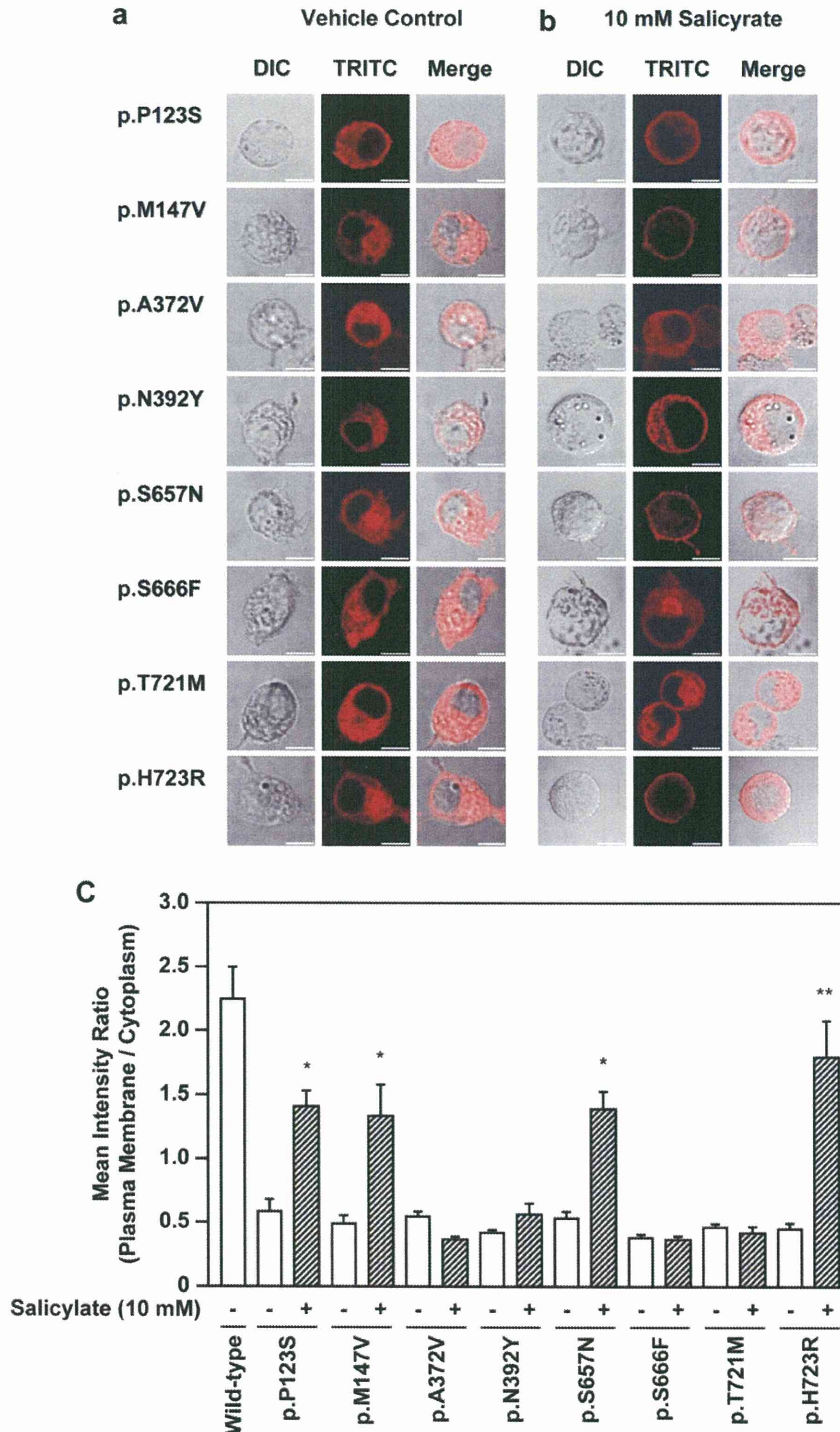

Fig. 3. Effects of salicylate on the intracellular localization of mutant pendrin. HEK293 cells were transfected with the expression vector including mutant pendrin gene. After 36 h, salicylate was added to the medium and the cells were further incubated for 12 h (a, b) Intracellular localization of pendrin was analyzed by immunofluorescent staining. The experiment shown is representative of 3 independent experiments. (c) The fluorescence intensities of the plasma membrane and cytoplasm of the cells were evaluated. The ratios were obtained by calculating the mean fluorescence intensities of 20 regions (0.8 μm × 0.8 μm) in plasma membrane and cytoplasm. Values are the means from 6 to 9 cells with SEM. *$P < 0.05$, **$P < 0.01$ vs. wild-type. This figure reveals that after incubation with salicylate, fluorescence intensity of TRITC of plasma membrane is stronger than that of the cytoplasm in the cells expressing p.P123S, p.M147V, p.S657N and p.H723R, but not in those expressing p.A372V, p.N392Y, p.S666F and p.T721M. The bar indicates 10 μm.

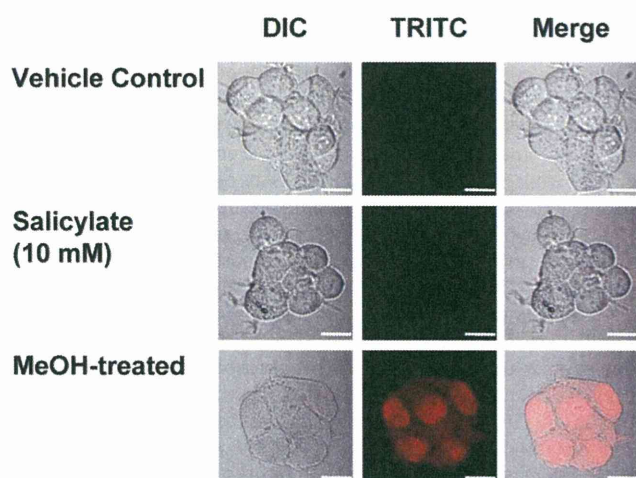

Fig. 4. Effects of salicylate on the viability of HEK293 cells. HEK293 cells were incubated for 12 h in medium containing 10% FBS in the presence or absence of 10 mM of salicylate. After incubation, the cells were stained with 7-AAD and observed by confocal microscopy. As a positive control, the membrane of cells incubated for 12 h in the absence of salicylate was permealyzed by methanol prior to the staining with 7-AAD. This figure indicates that 10 mM of salicylate has no cytotoxicity to HEK293 cells. The bar indicates 20 μm.

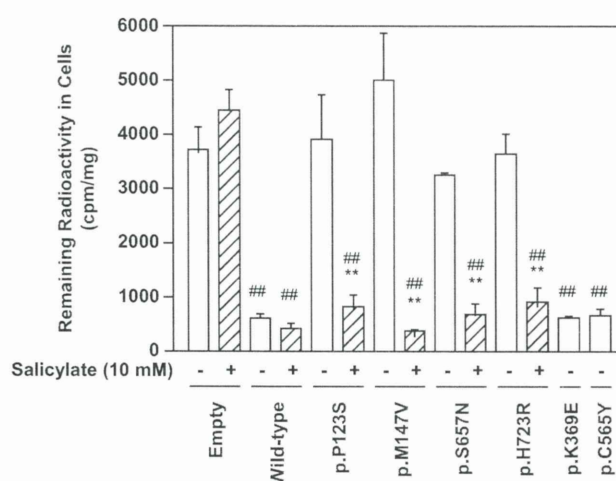

Fig. 6. Effects of salicylate on the iodide efflux from HEK293 cells. HEK293 cells were transfected with the empty vector, the expression vector including wild-type pendrin gene or the expression vector including the mutant genes. After 36 h, salicylate was added to the medium and the cells were further incubated for 12 h. The activity of pendrin was measured by radiolabeled-iodide efflux. The remaining radioactivity in cells was defined by dividing intracellular iodide content in the lysates (cpm/ml) by protein content in the lysates (mg/ml). This figure shows that the remaining radioactivity in the cells transfected with p.P123S, p.M147V, p.S657N and p.H723R was significantly decreased by salicylate. $^{**}P < 0.01$ vs. corresponding control (the cells lacking salicylate), $^{\#\#}P < 0.01$ vs. empty without salicylate.

origin of electromotility of the outer hair cells (Zheng et al., 2000). The nonlinear capacitance change involved in prestin requires some ions such as Cl^- and $HCO3^-$ and is inhibited by 10 mM of salicylate (Oliver et al., 2001). Recently, we have reported that salicylate induces the translocation of prestin mutants to the plasma membrane from the cytoplasm (Kumano et al., 2010). There is approximately a 45% similarity between pendrin and prestin in amino acid sequences and transport of anions by pendrin is similar to those transported by prestin (Mount and Romero, 2004). Therefore, we hypothesized that as is the case with prestin, salicylate modifies the function as a pharmacological chaperone for the pendrin mutants. As shown in Figs. 3 and 6, salicylate induced the transport of 4 pendrin mutants, i.e., p.P123S, p.M147V, p.S657N and p.H723R, to the plasma membrane and the recovery of their anion exchanger activity, while 4 other pendrin mutants, i.e., p.A372V, p.N392Y, p.S666F and p.T721M, were retained in the cytoplasm.

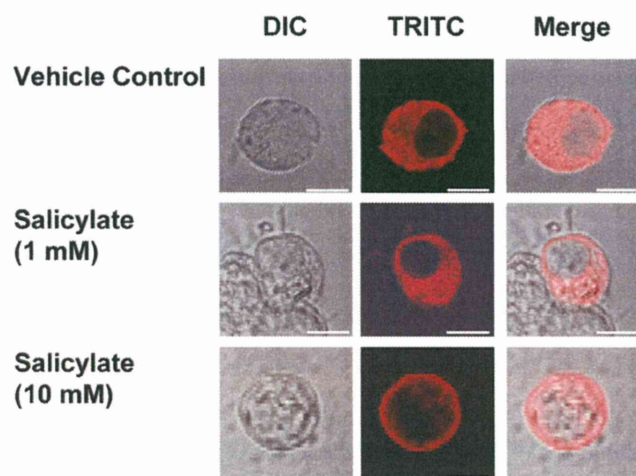

Fig. 5. Effects of lower concentration of salicylate on the intracellular localization of p.P123S mutant pendrin. HEK293 cells were transfected with the expression vector including p.P123S mutant pendrin. After 36 h, salicylate was added to the medium and the cells were further incubated for 12 h. Intracellular localization of pendrin was analyzed by immunofluorescent staining. This figure demonstrates that salicylate at 10 mM but not 1 mM induces the translocation of p.P123S mutant pendrin. The experiment shown is representative of 3 independent experiments. The bar indicates 10 μm.

Salicylate is a nonsteroidal anti-inflammatory drug (NSAID) which functions to inhibit cyclooxygenase-2 (COX-2) activity with an IC_{50} value of 14 μM (Cryer and Feldman, 1992; DuBois et al., 1996). Although salicylate at a concentration over 14 μM was expected to inhibit at least more than 50% of the COX-2 activity, in the present study, 1 mM salicylate did not induce the translocation of p.P123S pendrin mutants from the cytoplasm to the plasma membrane (Fig. 4). Therefore, it is unlikely that translocation of pendrin mutants to the plasma membrane by salicylate resulted from its COX2-inhibitory effect. Recently, it has been reported that salicylate also inhibits histone deacetylases (HDACs) at a concentration higher than that of COX-2 inhibition (DiRenzo et al., 2008). This might mean that inhibition of HDACs is one of the candidate mechanisms for the effect of salicylate on the translocation of pendrin mutants. Yoon et al. (2008) have reported that the p.H723R pendrin mutant is in the cytoplasm and is restored its location and $HCO3^-$ influx activity by 5 mM of sodium butyrate, which is an HDAC inhibitor (de Ruijter et al., 2003). HDACs are enzymes that remove the acetate from acetylated lysine residue in protein acetylated by histone acetyl transferases (HATs) (de Ruijter et al., 2003). Acetylation by HAT occurs in numerous proteins such as histone, transcription factors and heat shock proteins, and modifies their cellular functions (Ishihara et al., 2005). Therefore, hyper-acetylated-proteins by the inhibition of HDACs induced by 10 mM of salicylate might lead to the translocation of pendrin mutants to the plasma membrane and recovery of their activity. In addition, it has been reported that glycosylation of pendrin effects its folding and ultimate localization (Yoon et al., 2008; Rebeh et al., 2009). Therefore, it is necessary to analyze the involvement of pendrin acetylation and glycosylation on the translocation and recovery of the activity of the pendrin mutants by 10 mM of salicylate.

In this study, we found that 4 mutants, i.e., p.P123S, p.M147V, p.S657N and p.H723R, were induced normal transport from the intracellular region and that they were restored their activity by

10 mM of salicylate, while 4 other mutants, i.e., p.A372V, p.N392Y, p.S666F and p.T721M, were not induced normal transport and were not restored their activity (Figs. 2 and 5). We confirmed that there was no significant difference in the protein levels of expression of wild-type pendrin and 10 pendrin mutants by Western blotting. Therefore, the recovery of the activity of 4 pendrin mutants, i.e., p.P123S, p.M147V, p.S657N and p.H723R, is considered to be due to the alterations in localization rather than to the level of protein expression. It has been reported that p.H723R pendrin mutant is mostly expressed in the endoplasmic reticulum, whereas p.L236P pendrin mutant is in the centrosomal region of the cells (Yoon et al., 2008). In that report, p.H723R but not p.L236P interestingly was restored its $HCO3^-$ influx activity by sodium butyrate. Therefore, it is possible that intracellular localization of pendrin mutants (p.P123S, p.M147V, p.S657N and p.H723R) targeted to the plasma membrane by salicylate differs from that of 4 other pendrin mutants (p.A372V, p.N392Y, p.S666F and p.T721M). Clarification of the mechanism by which salicylate induces the translocation of pendrin mutants is important and requires further study.

In this study, we showed that p.K369E and p.C565Y were expressed in the plasma membrane (Fig. 1) and acted to export iodide (Fig. 5), although pendrin genes with mutations in p.K369E and p.C565Y have been recognized as candidate genes in Japanese patients with NSEVA (Tsukamoto et al., 2003). It has been shown that p.S166N found in Korean patients was expressed in the plasma membrane with Cl^-/HCO_3^- exchange activity (Yoon et al., 2008). Recently, p.C565Y pendrin mutant has also been reported to be expressed on the plasma membrane, and the exchange activities for ions such as chloride and bicarbonate were reduced in comparison to wild-type pendrin but greater than the functional null control (Choi et al., 2009). Thus, the relationship between those three mutations and NSEVA is unclear. As those three mutations were identified as compound heterozygous with other mutations in the pendrin gene (Tsukamoto et al., 2003; Park et al., 2004), it may be possible that NSEVA occurred when p.K369E, p.C565Y and p.S166N pendrin mutants were expressed with other pendrin mutants in the same cells. Further investigation is necessary to clarify how p.K369E and p.C565Y pendrin mutants are involved in NSEVA.

Pendred syndrome is characterized by sensorineural hearing impairment, presence of goiter, and a partial defect in iodide organification (Kopp et al., 2008). In the thyroid gland, pendrin releases iodide at the apical membrane of thyroid follicular cells into the follicular lumen to synthesize thyroid hormones, T3 and T4 (Kopp et al., 2008). Impairment of this function by pendrin mutants causes goiter and a partial defect in iodide organification. We have shown, in this study, that salicylate restores the iodide efflux activity of 4 pendrin mutants, i.e., p.P123S, p.M147V, p.S657N and p.H723R (Fig. 6), suggesting that salicylate restores the function in the thyroid. Although it is necessary to analyze the exchange activity for chloride and bicarbonate in the inner ear, salicylate could improve the condition of people suffering from Pendred syndrome caused by the mutation of p.P123S, p.M147V, p.S657N and p.H723R.

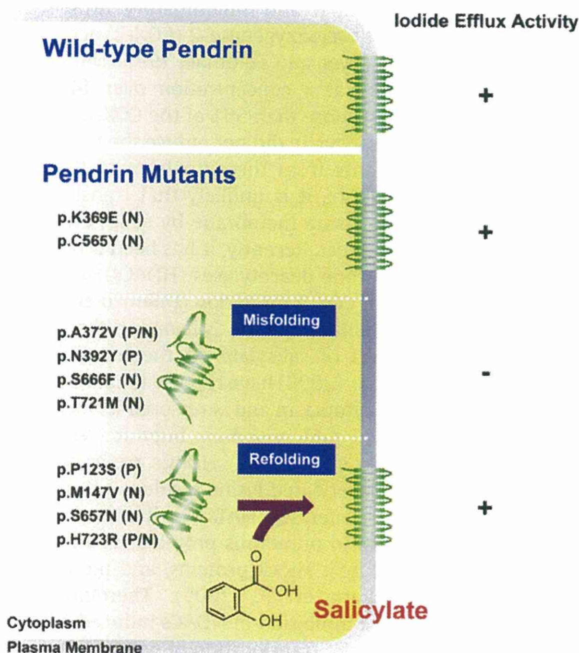

Fig. 7. Summary of pendrin mutants and *in vitro* effects of salicylate. Wild-type pendrin and two pendrin mutants, i.e., p.K369E and p.C565Y, are correctly folded, transported to the plasma membrane from the cytoplasm, and act to export iodide. In contrast, the other 8 pendrin mutants, i.e., p.P123S, p.M147V, p.A372V, p.N392Y, p.S657N, p.S666F, p.T721M and p.H723R, are retained in the cytoplasm. In the 8 pendrin mutants retained in the cytoplasm, 4 pendrin mutants, i.e., p.P123S, p.M147V, p.S657N and p.H723R, are translocated by salicylate to the plasma membrane, where the functions of 4 pendrin mutants are restored. The phenotypes (Pendred syndrome:P, NSEVA: N) related to the mutations reported by Tsukamoto et al. (2003) are represented.

5. Conclusion

In conclusion, this is the report describing the intracellular localization of reported Japanese pendrin mutants. Of 10 pendrin mutants, 2 mutants and wild-type pendrin were found to be located in the plasma membrane, while 8 mutants were retained in the cytoplasm. Furthermore, we demonstrated that the function of 4 pendrin mutants was restored by salicylate (Fig. 7). This finding suggests that salicylate is a potentially promising compound for treatment of hearing loss that is associated with defects in protein localization, including a subset of individuals with Pendred syndrome. Our findings could contribute to further investigation for understanding disorders involving pendrin mutants.

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Salicylate-induced translocation of prestin having mutation in the GTSRH sequence to the plasma membrane

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ABSTRACT

Prestin is a key molecule for mammalian hearing. The present study investigated changes in characteristics of prestin by culturing prestin-transfected cells with salicylate, an antagonist of prestin. As a result, the plasma membrane localization of prestin bearing a mutation in the GTSRH sequence, which normally accumulates in the cytoplasm, was recovered. Moreover, the nonlinear capacitance of the majority of the mutants, which is a signature of prestin activity, was also recovered. Thus, the present study discovered a new effect of salicylate on prestin, namely, the promotion of the plasma membrane expression of prestin mutants in an active state.

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1. Introduction

The motor protein prestin in the plasma membrane of cochlear outer hair cells (OHCs) is believed to be the origin of their electromotility [1]. So far, several characteristics of prestin have been clarified by introduction of mutations into prestin [2]. Mutations in membrane proteins sometimes cause the accumulation of these proteins in the cytoplasm. It has been reported that when the cells expressing such accumulated mutants were cultured with a pharmacological chaperone, which is a cell membrane-permeable molecule with high affinity for these mutants, the chaperone bound to them and promoted their transport to the plasma membrane in an active state [3–7]. Salicylate, which is known as an antagonist of prestin, is thought to have cell membrane permeability with high affinity for prestin [8,9]. Thus, salicylate was considered to be a candidate molecule to work as a pharmacological chaperone for prestin. In the present study, the aim was to investigate whether or not salicylate has the ability to promote the plasma membrane expression of prestin mutants accumulated in the cytoplasm.

2. Materials and methods

2.1. Prestin mutants

Our previous study showed that mutations in the GTSRH sequence at positions 127–131 of prestin caused a decrease in nonlinear capacitance (NLC), which is a signature of prestin activity [10]. Such decrease may be due to the accumulation of prestin in the cytoplasm. Thus, the present study used the prestin mutants created in our previous study, namely, G127A, T128A, S129A, R130A, H131A and S129T. These mutants were engineered to be expressed in HEK293 cells by transfection. As the prestin genes were co-transfected with green fluorescent protein (GFP) gene into the cells, transfected cells were selected by GFP observation.

Abbreviations: OHC, outer hair cell; NLC, nonlinear capacitance; GFP, green fluorescent protein; WT, wild-type; WGA, wheat germ agglutinin

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2.2. Incubation of transfected cells with salicylate

Transfected cells were cultured with salicylate, which is generally known to have the ability to suppress NLC as an antagonist of prestin, to confirm another effect of salicylate as a pharmacological chaperon for prestin. It was reported that, in the patch-clamp recording, 10 mM salicylate around the cells was required for almost complete suppression of NLC, which might be realized by the binding of salicylate with prestin in the plasma membrane [9]. Thus, for the binding of salicylate with prestin, at least 10 mM salicylate was considered to be necessary. Although salicylate possibly affects the cell viability, it has been reported that more than 85% of HEK293 cells were able to survive in the presence of up to 10 mM sodium salicylate [11]. In the present study, the cells were cultured for 24-36 hours in growth medium with sodium salicylate at a concentration of 10 mM from 12 hours after transfection. After such incubation, the cells were used in experiments. The cells cultured without salicylate were employed as control samples. Samples of the cells expressing wild-type (WT) prestin and its mutants which were cultured with 10 mM salicylate were termed WT prestin+Sal, G127A+Sal, T128A+Sal, S129A+Sal, R130A+Sal, H131A+Sal and S129T+Sal.

2.3. Confirmation of the localization of prestin in transfected cells

The localization of prestin in the cells was assessed by immunofluorescence staining with anti-FLAG antibody, TRITC-conjugated anti-mouse IgG antibody and wheat germ agglutinin (WGA)-Alexa Fluor 633 conjugate as described in our previous study [12]. In the present study, several tens of transfected cells were observed for

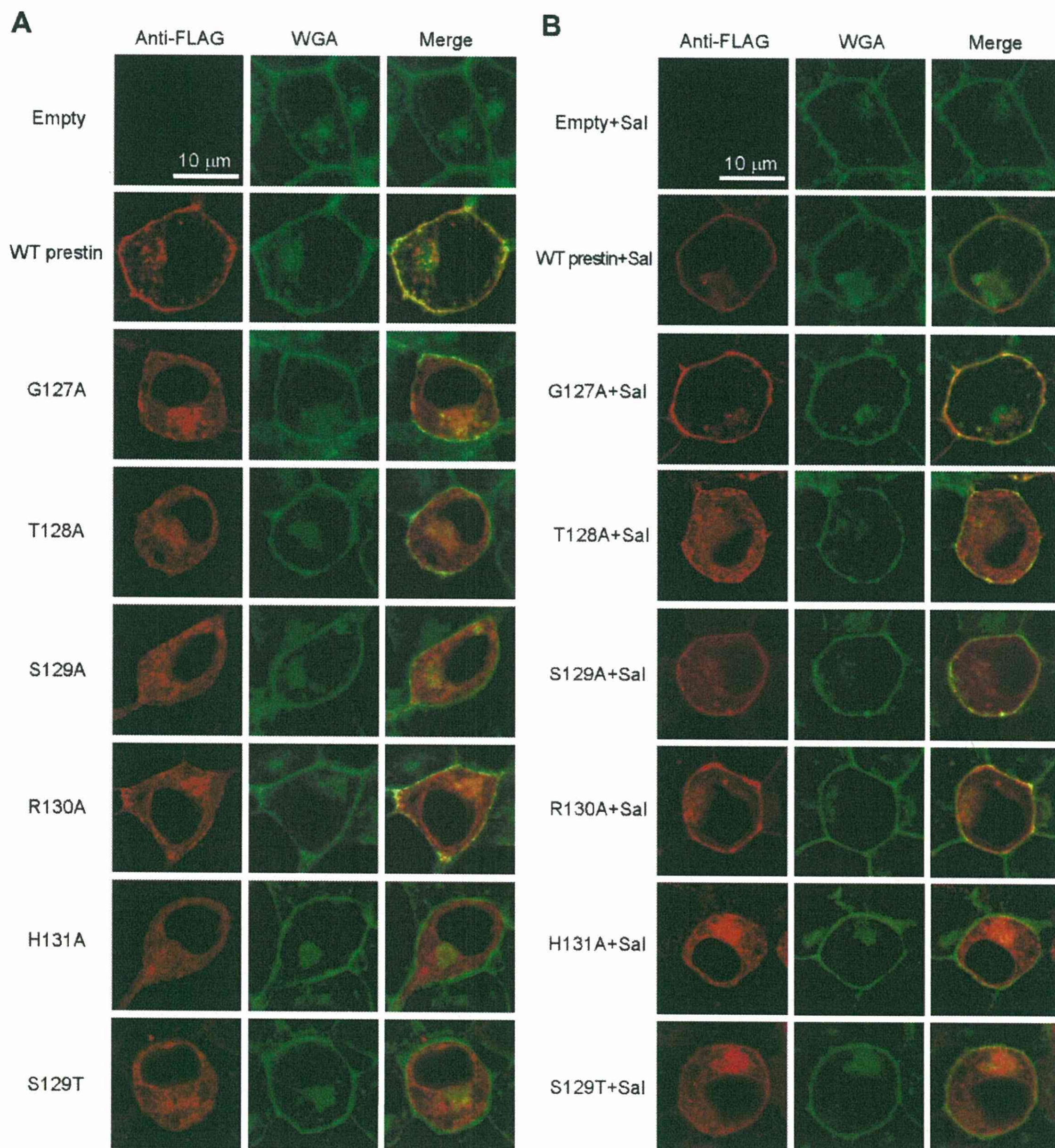

Fig. 1. Representative immunofluorescence images of transfected cells. (A) Stained cells cultured without 10 mM salicylate. (B) Stained cells cultured with 10 mM salicylate. Red and green fluorescence show prestin and both the plasma membrane and Golgi bodies, respectively. In the merged images, yellow–orange fluorescence indicates the co-localization of prestin and the plasma membrane, and that of prestin and the Golgi bodies.

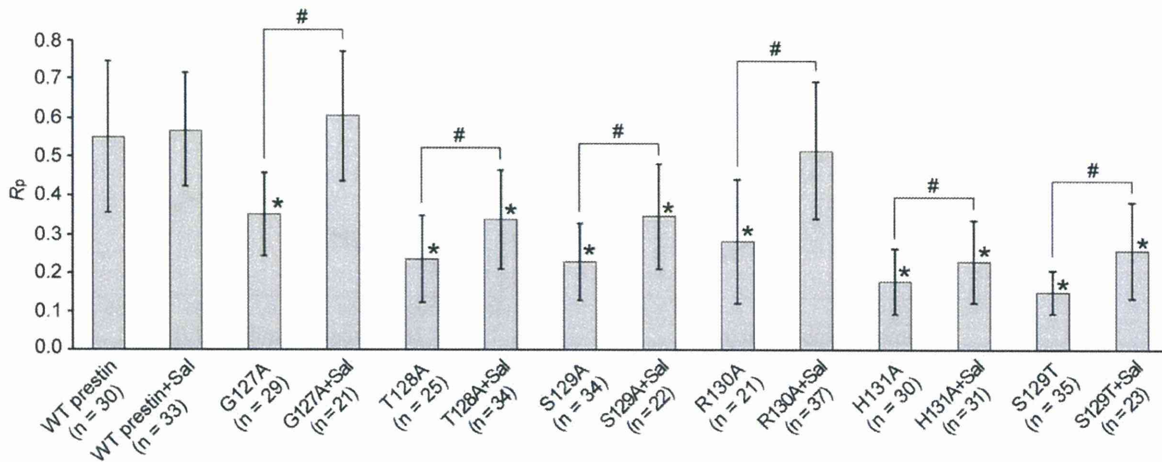

Fig. 2. R_p of WT prestin and its mutants. The R_p values of all prestin mutants were statistically lower than that of WT prestin when salicylate was not used, but they were increased by 10 mM salicylate. Asterisks show the statistical differences in the R_p values between WT prestin and the prestin mutants and between WT prestin+Sal and the prestin mutants+Sal ($p < 0.05$). Number signs indicate statistical differences between R_p values obtained from cells cultured with salicylate and those obtained from cells cultured without it in each prestin mutant ($p < 0.05$). Error bars show standard deviations.

each prestin mutant and the ratio of the amount of prestin in the plasma membrane to the total amount of prestin in the cell, R_p, was investigated. R_p was calculated by the following equation:

$$R_p = \frac{I_p}{I_w}, \quad (1)$$

where I_w is the sum of the intensity values of TRITC fluorescence of the whole area of the target cell which reflects the total amount of prestin in the cell, and I_p is the sum of the intensity values of TRITC fluorescence of only the pixels corresponding to the plasma membrane, which reflects the amount of prestin there. The I_w and I_p were calculated as described in our previous study [12].

2.4. Evaluation of electrophysiological properties of prestin

NLC, which is generally used for the analysis of prestin activity, was measured in the whole-cell patch-clamp recording as described in our previous study [13]. Transfected cells were washed just before the recording. By such washing, salicylate bound to prestin in the plasma membrane was expected to be dissociated [9]. The cells without membrane disruption which showed robust GFP fluorescence were selected for measurement. The recorded membrane capacitance was fitted with the first derivative of the Boltzmann function [14],

$$C_m(V) = C_{lin} + \frac{Q_{max}}{\alpha e^{\frac{V-V_{1/2}}{\alpha}}\left(1 + e^{-\frac{V-V_{1/2}}{\alpha}}\right)^2}, \quad (2)$$

where C_{lin} is the linear capacitance, which is proportional to the membrane area of the cells, Q_{max} is the maximum charge transfer, V is the membrane potential and $V_{1/2}$ is the voltage at half-maximal charge transfer. In Eq. (2), α is the slope factor of the voltage-dependent charge transfer and is given by

$$\alpha = kT/ze, \quad (3)$$

where k is Boltzmann's constant, T is absolute temperature, z is valence and e is electron charge. To evaluate the maximum charge transfer of prestin in the unit plasma membrane, Q_{max}, which means the maximum charge transfer of prestin in whole plasma membrane, was divided by C_{lin} and designated as charge density.

For the comparison of NLC curve, NLC had to be normalized by the area of the plasma membrane. The normalized NLC $C_{nonlin/lin}$ was defined as

$$C_{nonlin/lin}(V) = \frac{C_{nonlin}}{C_{lin}} = \frac{(C_m(V) - C_{lin})}{C_{lin}}, \quad (4)$$

where C_{nonlin} is the nonlinear component of the measured membrane capacitance.

2.5. Concentration dependence of effects of salicylate on prestin

The relationship between the concentration of salicylate and the degree of the promotion of the plasma membrane expression of prestin mutants was investigated. The cells transfected with R130A were cultured with sodium salicylate at concentrations of 1 mM and 5 mM from 12 h after transfection. By the above-mentioned method, after 24 h of incubation, the cells were subjected to immunofluorescence staining and the R_p was then calculated.

3. Results and discussion

3.1. Localization of prestin in transfected cells

Representative immunofluorescence images of stained cells which were cultured without and with 10 mM salicylate are shown in Fig. 1A and B, respectively. To statistically investigate the localization of prestin in the cells, the R_p was calculated and shown in Fig. 2. Without salicylate, the R_p values of the prestin mutants were statistically lower than that of WT prestin ($p < 0.05$), suggesting that those mutants were accumulated in the cytoplasm. To confirm whether or not salicylate has the ability to promote the plasma membrane expression of the prestin mutants, prestin-transfected cells were cultured with 10 mM salicylate. The R_p of WT prestin was unchanged by 10 mM salicylate, indicating that such amount of salicylate did not affect the process of transport of WT prestin to the plasma membrane (Fig. 2). On the other hand, the R_p values of all prestin mutants statistically increased, compared with those when salicylate was not used ($p < 0.05$). Especially, the R_p of G127A+Sal and that of R130A+Sal were similar to that of WT prestin+Sal. These results indicate that salicylate promoted the plasma membrane expression of the prestin mutants accumulated in the cytoplasm.

3.2. Electrophysiological properties of prestin

The $C_{nonlin/lin}$ (V), and charge density and α of WT prestin and its mutants are shown in Figs. 3 and 4, respectively. Without

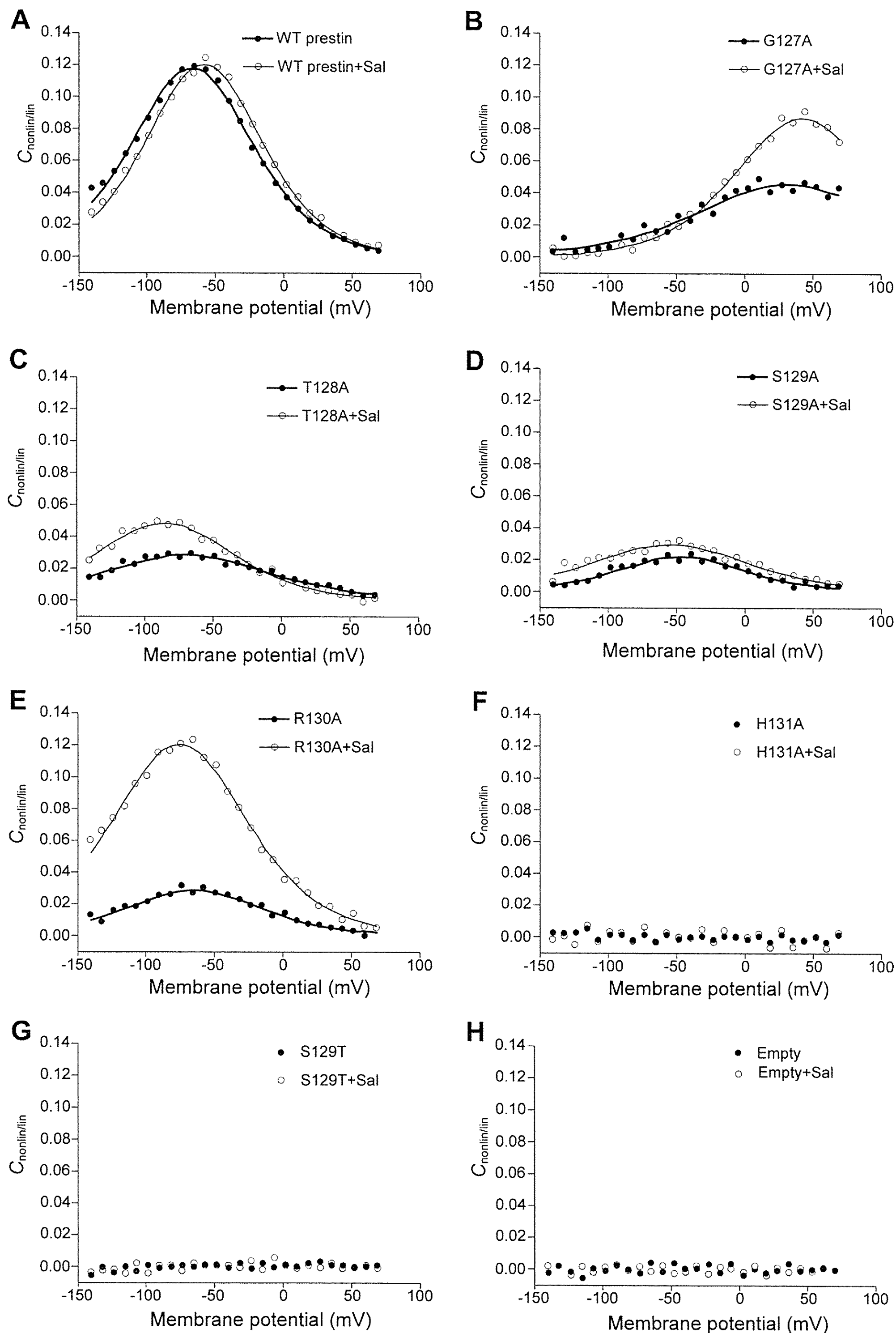

Fig. 3. Effects of salicylate on $C_{nonlin/lin}$ of prestin. Filled circles and thick lines show the results of transfected cells cultured without salicylate, while open circles and thin lines indicate the results of transfected cells cultured with 10 mM salicylate. (A) WT prestin and WT prestin+Sal. (B) G127A and G127A+Sal. (C) T128A and T128A+Sal. (D) S129A and S129A+Sal. (E) R130A and R130A+Sal. (F) H131A and H131A+Sal. (G) S129T and S129T+Sal. (H) Empty and Empty+Sal. When salicylate was used, NLC of G127A, T128A, S129A or R130A increased. On the other hand, in the case of H131A and S129T, NLC could not be detected in either type of cell, namely, cells cultured with and without 10 mM salicylate.

salicylate, G127A, T128A, S129A and R130A exhibited NLC, although their charge density was statistically smaller than that of WT prestin. On the other hand, H131A and S129T did not show NLC. The charge density of WT prestin+Sal was similar to that of WT prestin, suggesting that salicylate did not affect WT prestin itself nor the properties of the cells involved in the function of