理，聴能言語ハビリテーションなどを行っており，患児の通う聾学校（特別支援学校）や通常学校とも連携して成長を支える環境づくりをしている信州大学医学部付属人工内耳センターを訪ね，お話を伺った．

実際に人工内耳を装用している幼児との活動を見学したところ，人工内耳センターのような環境の整ったところで訓練をしていたため，話すことも聞き取りも上手く，活動の様子は普通の幼児教室のようで大変驚いた．早期の人工内耳の装用は効果的なものであると感じた．

支援員の方のお話では，人工内耳センターに積極的に通う保護者の方の願いは，通常学校に子どもを通わせる事ではなく，その時の発達状況に応じて，子に合った環境や学校で学ばせたいという想いがあるとのことであった．しかし，その反面，幼稚園児の保護者で，聾学校（特別支援学校）の小学部に見学に行ったが，手話を使っている様子を見て，通常学校に在籍させて今ある言語能力を高めていきたいという考えを持つ家庭もあった．

また，難聴児の学校選択については，通常学校に通うか特別支援学校に通うかという決断の要素には学校の設備的な環境や教師の難聴への知識だけではなく，子どもどうしの学びあいの面も考慮すべきである．

通常学校に通うメリットは言語能力を高める面である．聾学校のメリットは設備や教師の知識が整っている面である．しかし，それ以外に難聴児にかかわらず成長の過程で大切なことがある．それは，子どもが同世代のモデルを見ることである．特別支援学校に通えば同じような障害を抱えるモデルを見ることによって成長面で良い面もあるかもしれないが，通常学校に通った方がモデル数はたくさん見ることができる．

これらのことから，この人工内耳センターに通っている子どもたちは週の何日かは特別支援学校に通い，残りの日は通常学校・幼稚園に通っているということであった．

C.9.3. 難聴を疑似体験できる授業

HearLoss という難聴を疑似体験できるソフトを用いて授業案を作成し，養護教諭養成課程 2，3 年生に対して，4 年生がミニ講義「聴覚障害ってなんだろう？」を行った．

聴覚障害を持った方々がどのような聞こえ方をしているかを認識することができ，今後の支援について考えることができた．

C.9.4. 難聴者へのインタビューと健聴者への難聴に対するアンケート

アンケート結果から，過去の経験の中で難聴者と出会ったことのある人は，ない人に比べてそのイメージは大きく変わり，難聴者との出会いの経験がある人の方が難聴の症状に対して認識が高いと考えられる．

C.9.5. 小学生および大学生のヘッドホン使用状況

質問紙調査の結果から，小学生のヘッドホン使用経験のある児童は 80.1%，大学生のヘッドホン使用率は 90.8%で，ヘッドホンは社会全体に広く普及し，生活に深く根付いていると考えられる．

しかし，ヘッドホン使用に関する意識は高いとは言えないことが明らかになった．本研究では，ヘッドホンの使用により耳が聞こえにくくなると思う者の割合は小学生で 40.1%，大学生で 53.8%という結果となった．また，過去に高校生を対象とした調査では，ヘッドホン使用に関する意識はほとんど変わっていない．これらのことから，ヘッドホンによる聴覚への影響を意識せず，ヘッドホンを使用している者が増加しているということが言える．

ヘッドホン難聴の原因は，大音量で聴くこと，長時間聴くこと，周波数の高い音を聴くことなどである．しかし，ヘッドホン難聴の発症には，その時の体調や精神面，耳の強さ等，個人差があり，一概に，大音量で長時間の音を聴くと絶

対にヘッドホン難聴になるとは言えない．本調査では，ヘッドホン使用時間と自覚症状の有無に関連性が認められ（データ未掲載），ヘッドホン使用時間が長くなるほど，自覚症状のある者の割合が増える傾向がみられた．そのため，ヘッドホン使用に関する指導の際には長時間の音を聴かないことでヘッドホン難聴のリスクを軽減できるが，普通の大きさの音を聴いていても，必ずしもヘッドホン難聴を防ぐことができるとは言えないということを伝えていく必要があると思われる．

D. 考察

我々のこれまでの研究から，Pendrin 変異体の細胞内への蓄積が，感音性難聴の症状を伴う Pendred 症候群の発症の主な原因であると考えられる．日本人の難聴患者では 10 種類のミスセンス Pendrin 変異体 P123S, M147V, K369E, A372V, N392Y, C565Y, S657N, S666F, T721M 及び H723R が報告されている．このうち 2 種類（M147V と H723R）については，細胞内に蓄積することが既に報告されている．しかし，残りの 8 種類の変異体の細胞内局在は明らかにされていなかった．これまでに，本研究では，日本人の難聴患者で発見されている 10 種類の Pendrin 変異体の局在解析を行った．そして，我々は，既に報告のあった M147V と H723R の 2 種類の変異体の細胞内への蓄積を確認し，新たに 6 種類の変異体 P123S, A372V, N392Y, S657N, S666F, T721M が細胞内に蓄積することを明らかにした．従って，日本人患者で見られる 10 種類のミスセンス変異体のうち 8 種類は細胞内に蓄積され，2 種類は WT と同様に細胞膜へ局在することが明らかとなった．

また，我々は，サリチル酸による Pendirn 変異体の局在変化を調べた．その結果，細胞内に蓄積する 8 種類の変異体のうち 4 種類の変異体 P123S, M147V, S657N 及び H723R がサリチル酸の投与によって細胞膜へ移行することが明らかとなった．また，サリチル酸は 1 mM では変異体を細胞膜へ移行させる効果がなく，10 mM でその効果が見られるのに対し，サリチル酸の誘導体の 2 種類 2-Hydroxybenzyl alcohol 及び 2,3-Dihydroxybenzoic acid は，サリチル酸よりも低濃度の 1 mM で，P123S 変異体を細胞膜へ移行させる作用があることが分かった．サリチル酸は耳毒性があることが知られている．サリチル酸誘導体の耳毒性については，解析を進めた結果，現在のマウスへの手術方法においては，2 M を脱脂綿に浸み込ませて，蝸牛正円窓に設置したとき，最も効果が期待できると判断した．しかしながら，細胞実験において，サリチル酸よりも低濃度で効果があることから，サリチル酸誘導体が，副作用が少ない治療薬となる可能性があることから今後調査を進める．

薬剤の治療効果を評価する為には，Pendrin 遺伝子の変異により難聴となった動物に薬剤を投与して，その効果を確かめる必要がある．そこで，日本人の難聴患者において，最も出現頻度が高い変異 H723R（ヒト型，マウス型）の遺伝子変異をもつ 2 種類のノックインマウスの作成を委託して進めた．平成 22 年度，マウス型の Pendrin H723R 変異体を発現するノックインマウスが完成し，聴性脳幹反応（ABR）による，聴覚障害の評価を行った．その結果，20 週齢未満の若い段階で聴力が悪くなる個体群と 20 週齢を越えても聴力が衰えない個体群の 2 種類の表現型が現れた．遺伝子型が同一であるにもかかわらず，表現型に違いが出ているため，違う部分の遺伝子が影響を及ぼしたり，補償のようなメカニズムが働いている可能性が考えられる．また，マウス型ノックインマウスと通常マウスの蝸牛の形態において大きな差は見られなかった．

続いて，20 週齢未満の若い段階で聴力が悪くなっている個体にサリチル酸投与手術を行った

ところ，ドラスティックに回復する個体は見られなかった．蝸牛内への浸透が十分ではなかった可能性などが考えられる．

ヒト型ノックインマウスにおいては，予定の完成時期が大幅に遅れたことから，十分な個体数の計測ができなったが，6 週齢の非常に若い段階で，計測した 3 匹ともに 90 dB 以上の閾値と考えられるスケールアウトとなる結果を得た．現在，引き続き ABR 計測を行っているが，表現型としては，ほぼ全ての個体で聴力が悪くなっており，表現型が一致している．また，蝸牛の形態を観察したところ，内リンパ水腫とそれに伴うものと思われる Corti，Limbus，Spiral ligament において圧迫変性が見られた．また，ラセン神経節細胞の減少も見られ，難聴の原因として内リンパ水腫の関与が示唆される．さらに，前庭でも水腫を認めており，ペンドレッド症候群および前庭水管拡大を伴う難聴患者の臨床像との類似が見られ，モデル動物として有用であると考えられる．

難聴患者の治療においては，恒常的に内耳に薬剤を投与することが求められる．そのためには埋め込み型ドラッグデリバリーシステムが必要となる．初年度より試作しているドラッグデリバリーシステムを基に，生体への適用を視野に入れた形状および材料等を検討し，様々な改良を行った．最終年度には，マウスに薬剤投与する際に，目標投与量（サリチル酸の耳毒性評価実験より得られた最適と考えられる量0.5μL）を投与できるようチューブや流量を設計し，ヒト，マウスに対して薬剤注入が可能であることが分かった．

変異 Pendrin の機能を回復させる薬物の探索を目的としたスクリーニング系の確立においては，安定発現株の樹立や I⁻放出作用を指標としたスクリーニング系の構築，細胞内 pH の変化などの解析を行ったが，いずれも感度よく変異 Pendrin の機能回復を明らかにする系の確立にはいたらなかった．今後は，さらに条件検討を続け，簡易に変異 Pendrin の機能回復が観察できる系の確立を目指す．

変異 Pendrin の機能回復にサリチル酸が有効であることが確認できたが，さらに条件の良い薬剤の探索のため，サリチル酸誘導体の設計と合成について研究を行った結果，変異体 P123S においては，1,3-プロパンジオール骨格を有する環状化合物が有効である可能性が示唆されたが，変異体 H723R においては，一部，矛盾する結果も得られ，変異体毎に効果のある薬剤の構造は，異なる可能性も示唆された．今後，変異体の違いによる有効性の有無を確認する必要がある．また，機能を失った変異 Pendrin に対するシャペロン活性作用の機序解明のために作製したサリチル酸ケミカルプローブは，細胞内におけるサリチル酸の挙動をイメージングするのに有効であると考えられる．さらに，候補薬剤のスクリーニングを簡便にする目的で，変異体 P123S の定常発現株を作製し，サリチル酸の効果を調べた結果では，一時的にトランスフェクションした細胞における反応と同様であったことから，短時間で薬剤投与の効果を評価できる系の確立ができたと考えられる．

社会・教育学的取り組みとして行われた難聴に対する現状理解のアンケート結果では，社会・教育という面から難聴者と健聴者の認識や現状を把握し，難聴者の QOL を向上させるためには例えば学校教育でどのような働きかけができるか，学校という場を難聴者と健聴者の認識の差を埋める場としてどのように活用していけるのかを調査したが，難聴者の抱える悩みや想いと，健聴者の認識の差はどこにあるのか，差が出来てしまう原因はどこにあるのか，どうしたらその差を埋める事が出来るのか．難聴者を取り巻く様々な要素について調査研究を進めると，難聴者の QOL を高める視点には様々なもの （医療，教育，福祉・移行支援）があり，

それぞれがそれぞれの視点から難聴者の QOL を高めているが，最終的に QOL を高める根本的な要素は「人」であると考えられる．人々の認識が医療を高めたり，教育方法を考えたり，福祉・支援を充実させようとすることが重要であり，難聴者に関わる健聴者（学校等での友人や，社会生活上での接点を持つ全ての人）が難聴者や難聴への理解があると難聴者にとって対人関係の面でも充実し得る．難聴者にとってよりよい社会を作り出すためには，難聴者の悩みと健聴者の認識の差を埋めていく事が必須である．教育の面で考えると，学校を難聴者の悩みと健聴者の認識の差を埋める場としてもっと活用できるであろうと考えられる．小・中・高等学校のような学校において，人格形成期である就学世代に難聴を抱える子どもたちと健聴児を一緒に学ばせる事が重要であると思われる．子どもたちは人格形成期にいろんなモデルを見たり接したりする事で様々な事に理解をしており，最初は「知らなかった人・事」「身近でなかった人・事」であっても，人格形成期にある子どもたちはだんだんと日常化していくと思われる．この日常化とは，単なる「慣れ」というものではなく，遊びや生活での経験を通して障害自体に対する認識やそれを抱える人に対する認識が自然に高まることによって身近な事とされるという意味であり，人格形成期に様々な出会いをすることは，大人になってから同じような出会いがあった時にすんなりと関係を築く事が可能であると考えられる．学校とはそのような「出会い」を経験させる場であり，その「出会い」を有意義なものにさせるのは教職員をはじめとする学校組織であると考えられる．特に，学校組織の中で養護教諭はすべての児童生徒の心と身体の健康を守る任務を負っており，「人」の融合・調和を図る点で「出会い」を難聴児と健聴児双方にとって有意義なものとする為のサポートができると考えられる．そのためには難聴を抱える人々の背景にある様々な要素を理解し，その様々な要素（機関）の環に入り連携していく事も必要である．それと同時に，学校の機関の中で難聴児と健聴児が共に楽しく活躍でき，学びあえるような学校生活や行事等をコーディネートする役割も担う必要がある．今回，健聴者は難聴という症状をあまり理解できていないということが明らかになり，また，理解しづらいのではないかと考えられる．これは，自分自身のヘッドホン使用実態の質問紙調査結果にも現れた．このような点から，やはり，難聴ということを理解する前に聴覚の仕組みについての理解を深める必要があると考えられる．正常な状態の聴覚，そしてその病態，難聴という順で理解をすすめ，さらに難聴を補う補聴器や人工内耳の仕組みなどを理解してもらうような教育を行えば，補聴器や人工内耳が依然完璧なものではなく健聴者の理解や配慮も必要であるということにもつながるのではないかと思われる．また，近年，イヤホン，ヘッドホンを装着して大音量で長時間音楽を聴く風景をよく目にする．今後ヘッドホン難聴になる者が増えていく可能性は十分に考えられる．従って，ヘッドホン使用による聴覚への影響が示唆されている現代においては，学校でのヘッドホン難聴についての指導や啓蒙は，児童生徒の聴覚を守るために必要なことであると考える．様々な機械や場所において，聴覚の仕組みを理解しておけば，このようなことによる後天的な難聴の予防にもつながるのではないかと考えられる．

現在，学校現場では難聴児の受け入れ態勢，周囲の子どもたちへの教育，教職員の知識の向上等，支援態勢を整えている最中であり，課題はたくさん残っているものの「モノ」および「ヒト」による QOL 向上にむけた取り組みが必要であると思われる．

E. 結論

標的となり得る変異 pendrin の特定, 変異体の機能を回復させる化合物の抽出, 変異マウスの作製および変異マウスへの薬剤投与による回復試験の実施, 新型マイクロポンプの開発という課題を全て実施することができたが, 聴力が低下した変異マウスの聴力を薬剤によって明らかに回復させるまでには至っていない. 変異マウスの完成が計画より 8 ヶ月程遅れた点を除いて, ほぼ当初の計画通りに研究が実行されたと考えている. 今後は, 細胞系における薬剤探索, スクリーニングを行い, 効果的な候補薬剤を絞り込む. さらに, 高等な動物へのこれら候補薬剤の投与試験の実施とヒトに搭載可能なマイクロポンプシステムの更なる開発に取り組んでいく. これにより遺伝性難聴の革新的治療法の創生に向けた発展的研究へと結び付ける.

F. 研究発表

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Ⅱ．研究成果の刊行に関する一覧表

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Hetero-oligomerization between adenosine A_1 and thromboxane A_2 receptors and cellular signal transduction on stimulation with high and low concentrations of agonists for both receptors

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ABSTRACT

Growing evidence indicates that G protein-coupled receptors can form homo- and hetero-oligomers to diversify signal transduction. However, the molecular mechanisms and physiological significance of G protein-coupled receptor-oligomers are not fully understood. Both ADOR1 (adenosine A_1 receptor) and TBXA2R (thromboxane A_2 receptor α; TPα receptor), members of the G protein-coupled receptor family, act on astrocytes and renal mesangial cells, suggesting certain functional correlations. In this study, we explored the possibility that adenosine A_1 and TPα receptors form hetero-oligomers with novel pharmacological profiles. We showed that these receptors hetero-oligomerize by conducting coimmunoprecipitation and bioluminescence resonance energy transfer ($BRET^2$) assays in adenosine A_1 receptor and TPα receptor-cotransfected HEK293T cells. Furthermore, coexpression of the receptors affected signal transduction including the accumulation of cyclic AMP and phosphorylation of extracellular signal-regulated kinase-1 and -2 was significantly increased by high and low concentrations of adenosine A_1 receptor agonist and TPα agonists, respectively. Our study provides evidence of hetero-oligomerization between adenosine A_1 and TPα receptors for the first time, and suggests that this oligomerization affects signal transduction responding to different concentrations of receptor agonists.

1. Introduction

The classical concept that G protein-coupled receptors function as monomers has been changing due to the increasing evidence of G protein-coupled receptor oligomerization (Dalrymple et al., 2008; Rozenfeld and Devi, 2009). Oligomerization can increase the diversity of G protein-coupled receptor phenotypes (Panetta and Greenwood, 2008). Therefore, it is important to clarify the combinations of G protein-coupled receptors which form hetero-oligomers.

Adenosine has various physiological effects, causing reductions in sympathetic and parasympathetic activities, pre-synaptic inhibition, ischemic pre-conditioning and renal mesangial cell proliferation via adenosine receptors (Fredholm et al., 2001; Martínez-Salgado et al., 2007). Adenosine receptors are subclassified into A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm et al., 2001), all of which couple to G proteins. G proteins that are activated by G protein-coupled receptors and made up of alpha (α), beta (β), and gamma (γ) subunits. Gα subunits have many classes and behave differently in the recognition of the effectors. The adenosine A_1 receptor, the official gene name is ADOR1, which is coupled to members of the pertussis toxin (PTX)-sensitive family of G proteins, $G_{i/o}\alpha$ (G_i) proteins, which inhibits cyclic AMP production (Ralevic and Burnstock, 1998). Hetero-oligomerization of adenosine A_1 receptors with various G protein-coupled receptors was additionally reported. For example, adenosine A_1 and $P2Y_2$ receptors formed a hetero-oligomer with novel pharmacological properties, including ligand-binding pharmacology and receptor signal responses, in human embryonic kidney 293T (HEK293T) cells cotransfected with these receptors (Suzuki et al., 2009).

The thromboxane A_2 (TXA_2) is an unstable arachidonic acid metabolite. The TXA_2 receptor (TP receptor), the official gene name is TBXA2R, also belongs to the G protein-coupled receptor family, and communicates mainly with $G_{q/11}\alpha$ (G_q) (Johnston et al., 2001; Shenker et al., 1991), resulting in phospholipase C (PLC) activation. In addition, the TP receptor couples to other G proteins, including $G_{12}\alpha$ (G_{12}), $G_{13}\alpha$ (G_{13}), G_i and $G_s\alpha$ (Cordeaux et al., 2000; Djellas et al., 1999; Hirata et al., 1996; Nakahata, 2008; Offermanns et al., 1994). TXA_2 elicits diverse physiological/pathophysiological functions including the proliferation of glial cells and proliferation and contraction of renal mesangial cells upon binding to TP receptors (Nakahata,

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2008). There are two alternatively spliced isoforms, TXA_2 receptor α (TPα receptor) and TXA_2 receptor β (Hirata et al., 1991; Raychowdhury et al., 1994), which differ only at the C-terminus. Both adenosine A_1 and TP receptors are expressed in astrocytes and renal mesangial cells (Darè et al., 2007; Martínez-Salgado et al., 2007; Nakahata, 2008). In addition, both receptors are involved in decreasing the glomerular filtration rate via enhancement of renal mesangial cell proliferation and contraction (Martínez-Salgado et al., 2007; Nakahata, 2008). The contraction of mesangial cells was induced by incubation with platelet-supernatants, and abolished by a TP receptor blocker (Arribas et al., 1993). TXA_2 and adenosine are released from platelets. TXA_2 is involved in platelet activation, leading to platelet shape changes, aggregation and secretion (Nakahata, 2008). On the other hand, adenosine is a potent inhibitor of platelet activation (Cooper et al., 1995). From this evidence, it is possible that adenosine A_1 and TP receptors interact physically and functionally. In this study, we examined the hetero-oligomerization of the adenosine A_1 and TP receptors using coimmunoprecipitation and bioluminescence resonance energy transfer ($BRET^2$) techniques, and the effects on their signal transduction in HEK293T cells cotransfected with plasmids for these receptors.

2. Materials and methods

2.1. Materials

Human embryonic kidney 293T (HEK293T) were provided by Hiroyasu Nakata (Department of Molecular Cell Signalling, Tokyo, Japan). DeepBlueC was purchased from Perkin Elmer Life Sciences (Boston, MA). Forskolin was purchased from Wako Pure Chemicals (Osaka, Japan). Pertussis toxin and 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro20-1724) were from Calbiochem (San Diego, CA). 1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazine] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548) and 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_2α$ (U46619) were from Cayman Chemical (Ann Arbor, MI). N^6-cyclopentyladenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was from Sigma Aldrich (St. Louis, MO). Fura 2-AM was purchased from Dojindo (Kumamoto, Japan). Anti-HA antibody and FuGENE HD Transfection Reagent were purchased from Roche Applied Science (Manheim, Germany). HRP-conjugated anti-mouse IgG, Protein G-Sepharose™ and ECL™ Western blotting detection reagent were purchased from GE Healthcare (Piscataway, NJ). Anti-myc 9E10 antibody was purchased from Covance (Berkeley, CA). Anti-adenosine A_1 receptor antibody was from Acris Antibodies GmbH (Hiddenhausen, Germany). Anti-ERK 1/2 antibody, anti-phospho-ERK 1/2 antibody and HRP-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA). HRP-conjugated anti-rat IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Triple hemagglutinin-tagged adenosine A_1 receptor (3HA-adenosine A_1 receptor), hemagglutinin-tagged lysophosphatidic acid 1 receptor (HA-lysophosphatidic acid 1 receptor) and G-protein alpha q (G_q) plasmids were purchased from UMR cDNA Resource Center (Rolla, MO). Other chemicals used were of reagent grade or the highest quality available.

2.2. Construction of plasmids, cell culture and transfection

The HA-tagged TPα receptor (HA-TPα receptor), HA-tagged adenosine A_1 receptor (HA-adenosine A_1 receptor), myc-tagged adenosine A_1 receptor (myc-adenosine A_1 receptor), HA-adenosine A_1 receptor-Renilla luciferase (HA-adenosine A_1 receptor-Rluc) and HA-adenosine A_1 receptor-modified green fluorescent protein (HA-adenosine A_1 receptor-GFP^2) were constructed as described previously (Suzuki et al., 2006; Suzuki et al., 2009). HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO) containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. Transfections were done with FuGENE HD Transfection Reagent as described before (Suzuki et al., 2006).

2.3. Immunoprecipitation

HEK293T cells transfected with the myc-adenosine A_1 receptor, HA-TPα receptor and HA-lysophosphatidic acid 1 receptor were cultured for 48 h. Approximately 10^7 cells were collected by centrifugation at 1900×*g* and washed twice with Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4). Cells were disrupted by sonication using a Handy Sonic UR-20P (Tomy Seiko, Tokyo, Japan) in 300μl of lysis buffer (20 mM Tris–HCl pH7.4, 1 mM EDTA, 150 mM NaCl, 1% TritonX-100, and 1 mM Na_3VO_4). After incubation for 3 h at 4 °C, the solution was centrifuged at 17,400×*g* for 20 min at 4 °C, and the supernatant was pre-cleared with 30 μl/ml of 50% (w/v) Protein G-Sepharose in lysis buffer, followed by centrifugation at 17,400×*g* for 10 s to remove nonspecifically bound proteins. The supernatant was incubated with anti-myc 9E10 antibody (10 μg/ml) for 1 h, followed by Protein G-Sepharose™ (50 μl/ml) for 2 h. The mixture was centrifuged, the resulting immune complex was washed twice with 500 μl of lysis buffer, and bound proteins were eluted with 30 μl of Laemmli sample buffer (75 mM Tris–HCl, 2% SDS, 10% glycerol, 3% 2-mercaptoethanol, and 0.003% bromophenol blue).

2.4. Extracellular signal-regulated kinase1/2 (ERK1/2) assay

HEK293T cells were seeded onto 12-well plates at a density of 10^5 cells/well. At 24 h after seeding, the cells were transfected with the 3HA-adenosine A_1 receptor and HA-TPα receptor. For pertussis toxin treatment, cells were incubated with 100 ng/ml of pertussis toxin for 16 h. They were washed with a Tyrode-HEPES solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl, 1.8 mM $CaCl_2$, 10 mM HEPES, and 5.6 mM glucose, pH 7.4) and preincubated for 20 min at 37 °C. The cells were incubated with DPCPX, SQ29548 or YM-254890 (Astellas, Tokyo, Japan) for 10 min prior to stimulation with CPA and/or U46619 for 10 min at 37 °C. The reaction was terminated by aspiration of the medium and cells were lysed in 150 μl of ice-cold Laemmli sample buffer.

2.5. Western blot analysis

The samples were loaded on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis (PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in TBST (10 mM Tris–HCl, 100 mM NaCl, and 0.05% Tween 20, pH 7.4), incubated with anti-HA antibody (1:2000), anti-adenosine A_1 receptor antibody (1:2000), anti-ERK1/2 antibody (1:2000) or anti-phospho ERK1/2 antibody (1:2000) for 90 min followed by HRP-conjugated anti-rat IgG (1:2000) or anti-rabbit IgG (1:5000) for 90 min at room temperature, and detected with ECL™ Western blotting detection reagents.

2.6. $BRET^2$ assay

HEK293T cells ($5×10^5$ per 35 mm dish) were cotransfected with a fixed amount (1 μg) of HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2 plasmids and increasing concentrations of unfused receptor plasmids (0, 0.4, 0.8, 1.2, 1.6, 2.0 μg of HA-adenosine A_1 receptor or HA-TPα receptor plasmids, and 0, 0.6, 1.2, 1.8, 2.7, 3.6 μg of HA-lysophosphatidic acid 1 receptor) using the FuGENE HD Transfection reagent. For a control, non-transfected cells were used. At 48 h after transfection, the cells were harvested and suspended in assay buffer (Dulbecco's phosphate-buffered saline containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$, and 1 mg/ml D-

glucose). Suspended cells were distributed in a white-walled 96-well Plate (OptiPlate, Perkin Elmer Life Sciences) at a density of 1×10^6 cells/well, and incubated for 20 min at 37 °C. DeepBlueC was then added at a final concentration of 5 μM. Assays were conducted immediately using a Fusion α universal microplate analyzer (Perkin Elmer Life Sciences) for the detection of Rluc at 410 nm and GFP^2 at 515 nm. The BRET ratio was calculated as the ratio between GFP^2 and Rluc emission, corrected with the background emission from non-transfected cells.

2.7. Measurement of intracellular Ca^{2+} concentrations

The measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was carried out as described previously (Sasaki et al., 2006). HEK293T cells were cotransfected with 3HA-adenosine A_1 receptor/G_q, HA-TPα receptor/G_q or 3HA-adenosine A_1 receptor/HA-TPα receptor/G_q and cultured for 48 h. Transfected cells were harvested and suspended in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4), incubated with 5 mM fura2-AM for 15 min at 37 °C. Subsequently the cells were washed twice with Tyrode's solution and modified Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.18 mM $CaCl_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4), and resuspended in modified Tyrode's solution at 10^6 cells/ml. Fura2-AM fluorescence was recorded (1.5 ml aliquots) at 37 °C with gentle stirring using a FP-6500 (JASCO Corporation, Tokyo) with excitation at 340 nm and 380 nm and emission at 510 nm. Calibration of the signal was performed in each sample by adding 0.15% Triton X-100 to obtain maximal fluorescence (F_{max}) and then 2.5 mM EGTA to obtain minimal fluorescence (F_{min}). The ratio of fluorescence at 340 nm to that at 380 nm is a measure of $[Ca^{2+}]_i$, assuming a K_d of 244 nM Ca^{2+} for fura 2-AM.

2.8. Cyclic AMP assay

HEK293T cells were seeded onto 48-well plates at a density of 2×10^4/well. At 24 h after seeding, the cells were transfected with the 3HA-adenosine A_1 receptor and/or HA-TPα receptor. After 48 h, the medium was changed to Eagle's minimum essential medium—20 mM HEPES, and preincubated for 20 min at 37 °C. The cells were incubated with 100 μM of Ro 20-1724 as a phosphodiesterase inhibitor for 15 min with or without receptor antagonists. The cells were then stimulated with a receptor agonist and 100 μM forskolin, and incubated for another 10 min. Reactions were terminated by adding 2.5% perchloric acid. Acid-extracts were mixed with a 1/10 volume of 4.2 N KOH to neutralize the acid, forming potassium perchlorate as a precipitate. The cyclic AMP in the supernatant was succinylated and determined using a radioimmunoassay kit (Yamasa, Tokyo, Japan) according to the manufacturer's directions.

3. Results

3.1. Coimmunoprecipitation of myc-adenosine A_1 and HA-TPα receptors

To determine whether the adenosine A_1 and TPα receptors interact, we performed a coimmunoprecipitation analysis using HEK293T cells transiently transfected with the myc-adenosine A_1 receptor and/or HA-TPα receptor. We detected the myc-adenosine A_1 receptor in the complex precipitated with anti-myc in cells transfected with the myc-adenosine A_1 receptor and cotransfected with the myc-adenosine A_1 receptor/HA-TPα receptor (Fig. 1A, left panel, lanes 2 and 3 in 'IP', arrow), indicating the validity of this method. Importantly, we detected the band which corresponds to the HA-TPα receptor in the complex precipitated with anti-myc only in cells cotransfected with myc-adenosine A_1/HA-TPα receptors (Fig. 1B, lane 3 in 'IP', arrows). This band was not obtained from the cells transfected with

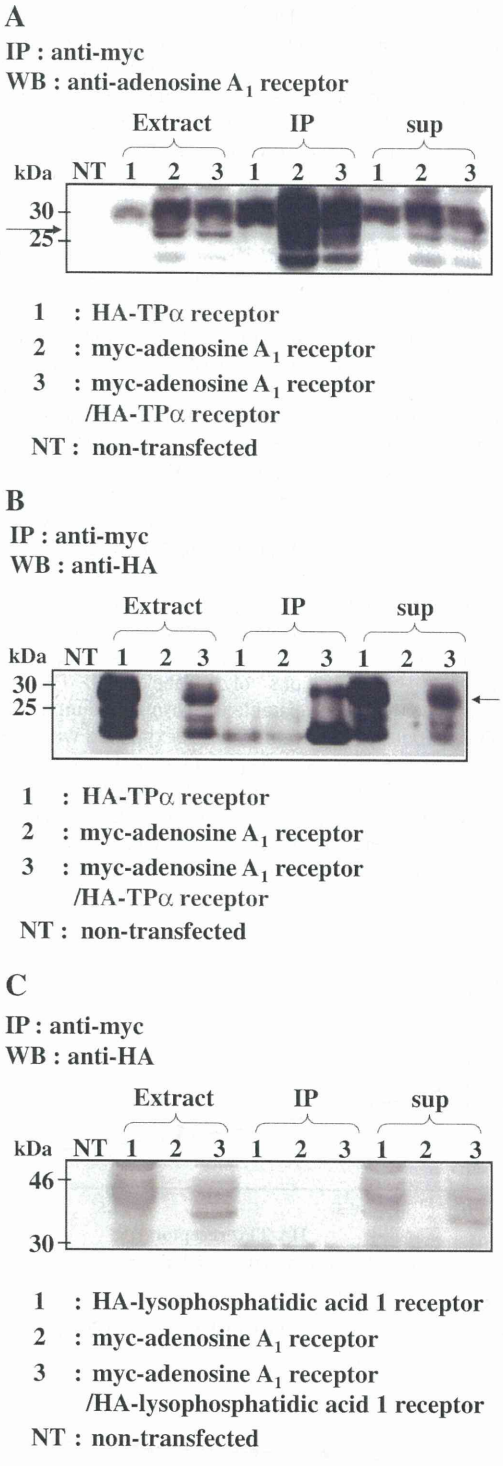

Fig. 1. Coimmunoprecipitation of the myc-adenosine A_1 receptor and HA-TPα receptor or lysophosphatidic acid 1 receptor. HEK293T cells transiently transfected with the myc-adenosine A_1 receptor and HA-prostanoid TPα receptor (A and B) or HA-lysophosphatidic acid 1 receptor (C). Cell lysates were immunoprecipitated with anti-myc antibody, and precipitates were analyzed by Western blotting with anti-HA (B and C) and anti-adenosine A_1 receptor (A) antibodies. A negative control of non-transfected cells showed no detectable bands (lane 'NT'). Arrows indicate the myc-adenosine A_1 receptor (A) and TPα receptor (B). Extract, solubilized membrane extract; IP, immunoprecipitate; sup, supernatant following immunoprecipitation; WB, Western blotting with the antibody indicated. Approximate molecular masses are shown in kDa.

the HA-TPα receptor alone (Fig. 1B, lane 1 in 'IP'). These results suggest the formation of a complex between myc-adenosine A_1 receptors and HA-TPα receptors in the cotransfected cells. We did not detect the myc-adenosine A_1 receptor in the complex precipitated with anti-myc in cells cotransfected with the myc-adenosine A_1 receptor and HA-adenosine A_1 receptor instead of HA-TPα receptor (Fig. 1C).

3.2. BRET^2 inhibition of the homo-dimerization of adenosine A_1 receptors by TPα receptors

We performed a competitive BRET^2 assay using HEK293T cells transiently transfected with Rluc- and GFP^2-fused HA-adenosine A_1 receptors and unlabeled HA-TPα receptors. With this strategy, adenosine A_1 receptor/TPα receptor interactions were assessed based on the competition of adenosine A_1 receptor homodimerization with adenosine A_1 receptor/TPα receptor heterodimerization in the living cells. The homodimerization of adenosine A_1 receptors was confirmed by BRET^2 assay using HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2 (Suzuki et al., 2009). The specificity of adenosine A_1 receptor/TPα receptor interaction was verified by the observed decrease in the BRET^2 signals of adenosine A_1 receptor homodimerization when constant amounts of HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2 were coexpressed with increasing concentrations of unlabeled HA-TPα receptor (Fig. 2A). For a control, a comparable increasing amount of unlabeled HA-lysophosphatidic acid 1 receptor was coexpressed with a constant amount of HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2, which did not cause a significant decrease in the BRET^2 signal of the HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2 pairs (Fig. 2B). These results suggested that in cotransfected living cells, the adenosine A_1 and TPα receptors formed a heterodimer whereas the adenosine A_1 receptor and lysophosphatidic acid 1 receptor did not.

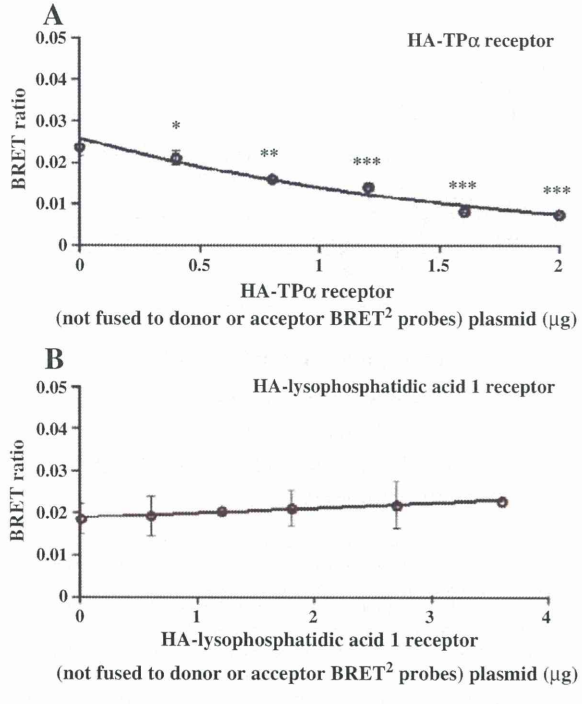

Fig. 2. Competitive BRET^2 assay. HEK293T cells were cotransfected with a fixed concentration of HA-adenosine A_1 receptor-Rluc and HA-adenosine A_1 receptor-GFP^2 plasmids and increasing amounts of (A) HA-TPα receptor, or (B) HA-lysophosphatidic acid 1 receptor plasmids. The data are the mean ± S.E.M for three experiments. $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ vs. 0 (Dunnett's test).

3.3. Effect of adenosine A_1 receptor and TPα receptor agonists on forskolin-stimulated cyclic AMP accumulation

We next examined whether the adenosine A_1 receptor/TPα receptor-coexpression affects signal transduction. We studied adenosine A_1 receptor agonist-induced inhibition of adenylyl cyclase via $G_{i/o}$. As expected, CPA (1 μM), a selective adenosine A_1 receptor agonist (Ralevic and Burnstock, 1998), decreased the forskolin-evoked increase in cyclic AMP levels in the cells expressing the 3HA-adenosine A_1 receptor alone and the cells coexpressing 3HA-adenosine A_1 and HA-TPα receptors (Fig. 3A and B, 3rd column from the left). Furthermore, DPCPX (10 μM) (Ralevic and Burnstock, 1998), a selective adenosine A_1 receptor antagonist, antagonized the CPA-induced inhibition of cyclic AMP levels in both 3HA-adenosine A_1 receptor-expressing cells and 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells (Fig. 3A and B, 6th column from the left). U46619, a selective TPα receptor agonist (Abramovitz et al., 2000), caused a small increase in forskolin-evoked cyclic AMP levels in cells

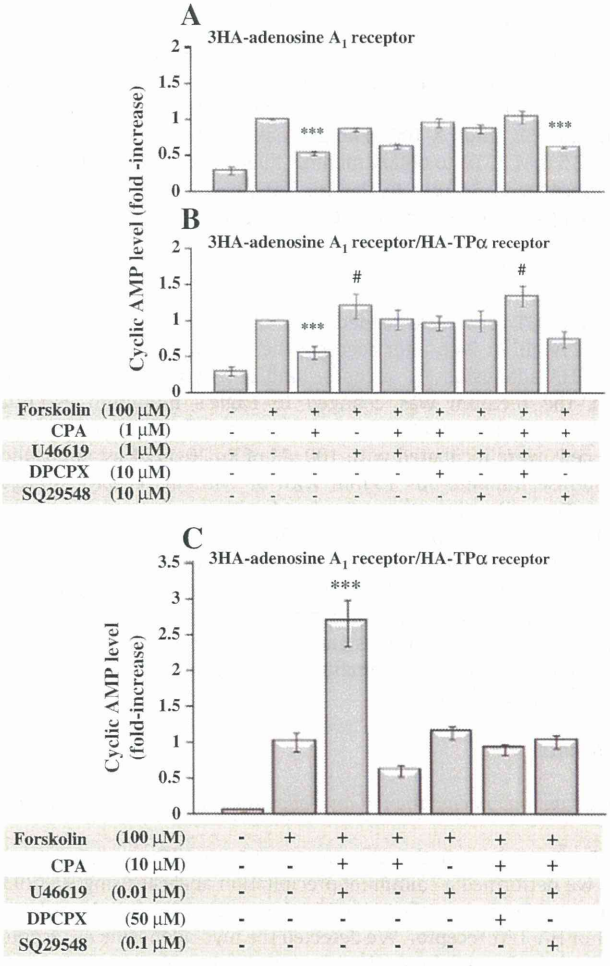

Fig. 3. Effects of adenosine A_1 receptor and TPα receptor agonists and antagonists on forskolin-stimulated cyclic AMP accumulation. Assays were performed in HEK293T cells transfected with the 3HA-adenosine A_1 receptor (A and C), HA-TPα receptor (D), and 3HA-adenosine A_1 receptor/HA-TPα receptor (B, E and F). The data were normalized to the forskolin-evoked cyclic AMP concentration in control cells (without ligands). The data are the mean ± S.E.M of triplicate determinations, and the results are representative of two similar experiments. #$P<0.05$, $^{***}P<0.001$, vs. forskolin-stimulated cyclic AMP accumulation (Tukey's test).

expressing the HA-TPα receptor alone (data not shown) and coexpressing 3HA-adenosine A_1 and HA-TPα receptors (Fig. 3B). Furthermore, the U46619-induced increase in cyclic AMP was inhibited by SQ29548 (10 μM), a TPα receptor antagonist (Nakahata, 2008), in 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells (Fig. 3B, 7th column from the left). The simultaneous addition of CPA (1 μM) and U46619 (1 μM) decreased the forskolin-evoked increase in cyclic AMP levels slightly in cells expressing the 3HA-adenosine A_1 receptor alone (Fig. 3A, 5th column from the left), though not at all in 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells (Fig. 3B, 5th column from the left). Notably, we found that the forskolin-evoked increase among 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells was markedly increased by the simultaneous addition of CPA (10 μM) and U46619 (0.01 μM) (Fig. 3C, 3rd column from the left). This characteristic increase was not induced by stimulation with CPA (10 μM) or U46619 (0.01 μM) individually (Fig. 3C, 4th and 5th columns). In addition, it was not seen in cells expressing the 3HA-adenosine A_1 receptor or HA-TPα receptor alone (data not shown). DPCPX (50 μM) and SQ29548 (0.1 μM) inhibited this synergistic increase by the simultaneous addition of CPA (10 μM) and U46619 (0.01 μM) in 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells (Fig. 3C, 6th and 7th columns from the left).

3.4. Effect of agonists for both adenosine A_1 and TPα receptors on ERK1/2 phosphorylation

It is reported that the individual stimulation of both the adenosine A_1 receptor and TPα receptor causes the activation of extracellular signal-regulated kinase-1/2 (ERK1/2), a major kinase of mitogen-activated protein kinase (MAPK) signaling pathways (Dickenson et al., 1998; Miggin and Kinsella, 2001). Then, we examined whether adenosine A_1 receptor/TPα receptor-coexpression affects ERK1/2 phosphorylation. Importantly, ERK1/2 phosphorylation in 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells was synergistically enhanced by the simultaneous addition of CPA and U46619 in all concentrations we tested, as compared with ERK1/2 phosphorylation in cells expressing the 3HA-adenosine A_1 receptor or HA-TPα receptor alone (Fig. 4A–C). This synergistic effect was not found on stimulation with CPA or U46619 alone (Fig. 4D–F). In addition, we examined the effects of receptor antagonists and pertussis toxin and YM-254890, which block signal transduction through G_i and G_q, respectively. As shown in Fig. 5, pretreatment of 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells with DPCPX significantly inhibited the synergistic effect of CPA (0.1 μM) and U46619 (0.1 μM) or CPA (1 μM) and U46619 (1 nM) (Fig. 5B and D). However, DPCPX did not block ERK1/2 phosphorylation on the addition of 1 nM CPA and 1 μM U46619 (Fig. 5C). In addition, SQ29548 did not have any inhibitory effect on ERK1/2 phosphorylation (Fig. 5A–D). Pertussis toxin and YM-254890 significantly inhibited the synergistic effect of CPA (0.1 μM) and U46619 (0.1 μM) or CPA (1 μM) and U46619 (1 nM) on ERK1/2 phosphorylation (Fig. 6A, B and D), whereas neither inhibitor had a significant effect on 1 nM CPA and 1 μM U46619-stimulated ERK1/2 phosphorylation (Fig. 6A and C).

3.5. Effect of coexpression of adenosine A_1 and TPα receptors on Ca^{2+} signaling

TPα receptor stimulation causes the activation of phospholipase C and subsequent elevation of $[Ca^{2+}]_i$ via G_q (Shenker et al., 1991). In addition, adenosine A_1 receptor stimulation also causes an elevation of $[Ca^{2+}]_i$ via Gβ and Gγ released from G_i (Dickenson and Hill, 1998; Quitterer and Lohse, 1999). We examined whether adenosine A_1 receptor/TPα receptor-coexpression affects the alteration of $[Ca^{2+}]_i$ in Fura2-AM-loaded cells. Stimulation of cells expressing the HA-TPα receptor and coexpressing the 3HA-adenosine A_1 and HA-TPα receptors with U46619 (1 μM) induced a rapid, transient elevation of $[Ca^{2+}]_i$ (Fig. 7B and C). Stimulation of 3HA-adenosine A_1 receptor-expressing cells and 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells with CPA (1 μM) induced a subtle elevation of $[Ca^{2+}]_i$ (Fig. 7D and F). Simultaneous treatment with U46619 (1 μM) and CPA (1 μM) induced elevations of $[Ca^{2+}]_i$ in 3HA-adenosine A_1 receptor-expressing cells, HA-TPα receptor-expressing cells and 3HA-adenosine A_1 receptor/HA-TPα receptor-coexpressing cells (Fig. 7G, H and I), but we could not see obvious differences from stimulation with each agonist alone. Simultaneous stimulation of U46619 and CPA-induced elevation of $[Ca^{2+}]_i$ were blocked by pretreatment with SQ29548 (3 μM, Fig. 8D–F), but not DPCPX (Fig. 8A–C).

4. Discussion

The present study proves the existence of a heteromeric complex formed by adenosine A_1 and TPα receptors in solubilized and living HEK293T cells coexpressing these receptors. Fig. 1 shows that coimmunoprecipitation revealed that the adenosine A_1 and TPα receptors associate in HEK293T cells cotransfected with these receptors. The possibility of hetero-oligomerization between adenosine A_1 and TPα receptors suggested by the coimmunoprecipitation assay using solubilized cell membranes (Fig. 1) confirmed with a competitive $BRET^2$ assay (Fig. 2). $BRET^2$ assay is using living cells, so we observed the interaction in the intact cells. The specific decrease in the $BRET^2$ signal of Rluc- and GFP^2-fused adenosine A_1 receptor pairs on transfection of an increased amount of unlabeled TPα receptor was similar to the result obtained using an unlabeled adenosine A_1 receptor substituted for the TPα receptor (data not shown). Significant $BRET^2$ signal between Rluc- and GFP^2-fused adenosine A_1 receptors indicated a homodimer as reported previously (Suzuki et al., 2009). It is likely that unlabeled TPα receptors and unlabeled adenosine A_1 receptors competitively blocked the interaction between Rluc- and GFP^2-fused adenosine A_1 receptors. In the case of nonspecific interaction, a decrease in BRET signal would not be obtained (Marullo and Bouvier, 2007), as a result using the lysophosphatidic acid 1 receptor substituted for the TPα receptor (Fig. 2B). These results confirmed that adenosine A_1 and TPα receptors form hetero-oligomers in cotransfected HEK293T cells.

G protein-coupled receptor oligomerization may increase the diversity of G protein-coupled receptor phenotypes by altering the function of receptors. It is important whether oligomerization has some specific effects on the function of the receptor. In this study, in adenosine A_1 receptor/TPα receptor-coexpressing HEK293T cells, the cyclic AMP level and ERK1/2 activation were significantly altered. However, Ca^{2+} signaling was not altered by the coexpression of adenosine A_1 and TPα receptors. In the cells which were not transfected with the TPα receptor, $[Ca^{2+}]_i$ was slightly increased by stimulation with the TPα receptor agonist, possibly because of endogenous TPα receptors in HEK293T cells (D'Angelo et al., 1996). Whereas, in the adenosine A_1 receptor/TPα receptor-coexpressing cells, costimulation with a high concentration (10 μM) of adenosine A_1 receptor agonist and low concentration (0.01 μM) of TPα receptor agonist markedly increased cyclic AMP levels (Fig. 3C). However, costimulation with equal concentrations of these agonists had no significant effect on cyclic AMP levels (Fig. 3B). This is similar to the case of adenosine A_1 receptor and $P2Y_2$ receptor-hetero-oligomer, which costimulation of high concentrations of $P2Y_2$ receptor agonist and low concentrations of adenosine A_1 receptor agonist had significantly affected cyclic AMP level (Suzuki et al., 2006). This characteristic increase of cyclic AMP levels by simultaneous treatment with a high concentration of adenosine A_1 receptor agonist and low concentration of TPα receptor agonist was inhibited by the antagonists for both receptors (Fig. 3C). Furthermore, a high concentration of

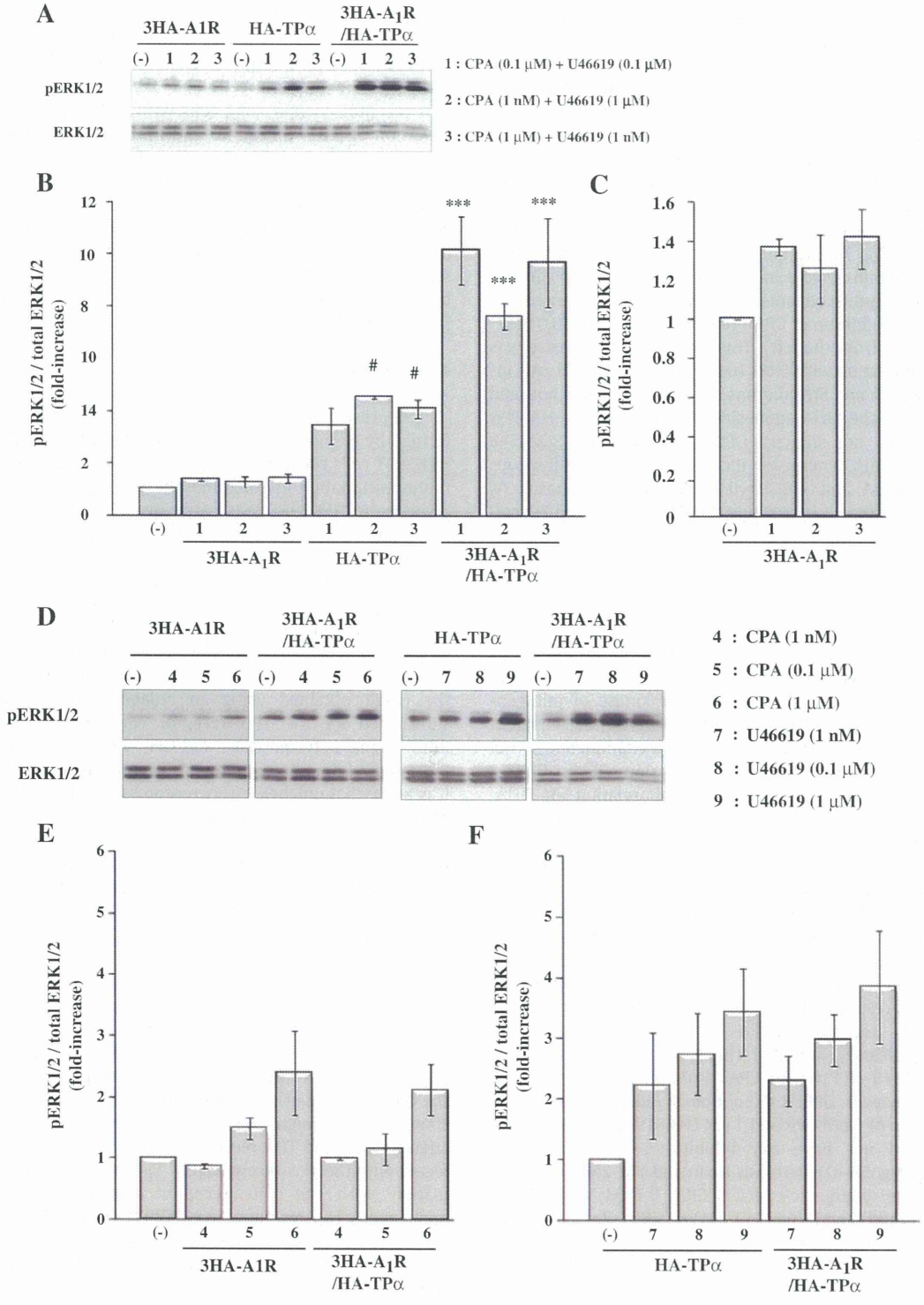

Fig. 4. Effect of CPA and U46619 on ERK1/2 phosphorylation. Assays were performed in HEK293T cells transfected with the 3HA-adenosine A_1 receptor (3HA-A_1R), and HA-TPα receptor (HA-TPα), 3HA-adenosine A_1 receptor/HA-TPα receptor (3HA-A_1R/HA-TPα) . Cells were treated with vehicle (control; (−)) or indicated concentrations of agents. The cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with anti-ERK1/2 and anti-phospho ERK1/2 antibodies (A and D). Fold increases in ERK1/2 phosphorylation in A and D, are presented as mean fold increases of control phosphorylation ± S.E.M for three experiments (B, E and F). (C) Magnified image of control and adenosine A_1 receptor in B. ***$P<0.001$, #$P<0.05$ vs. (−) group (Tukey's test).

adenosine A_1 receptor agonist or low concentration of TPα receptor agonist alone did not have a significant effect on cyclic AMP levels in cells coexpressing adenosine A_1 and TPα receptors. The TPα receptor couples with not only G_q but also G_s, and a TPα receptor-mediated increase in cyclic AMP level was reported (Nakahata, 2008). In fact, stimulation with the TPα receptor agonist induced an increase in cyclic AMP levels in cells expressing the TPα receptor alone (data not shown), though this increase was rather small compared to the

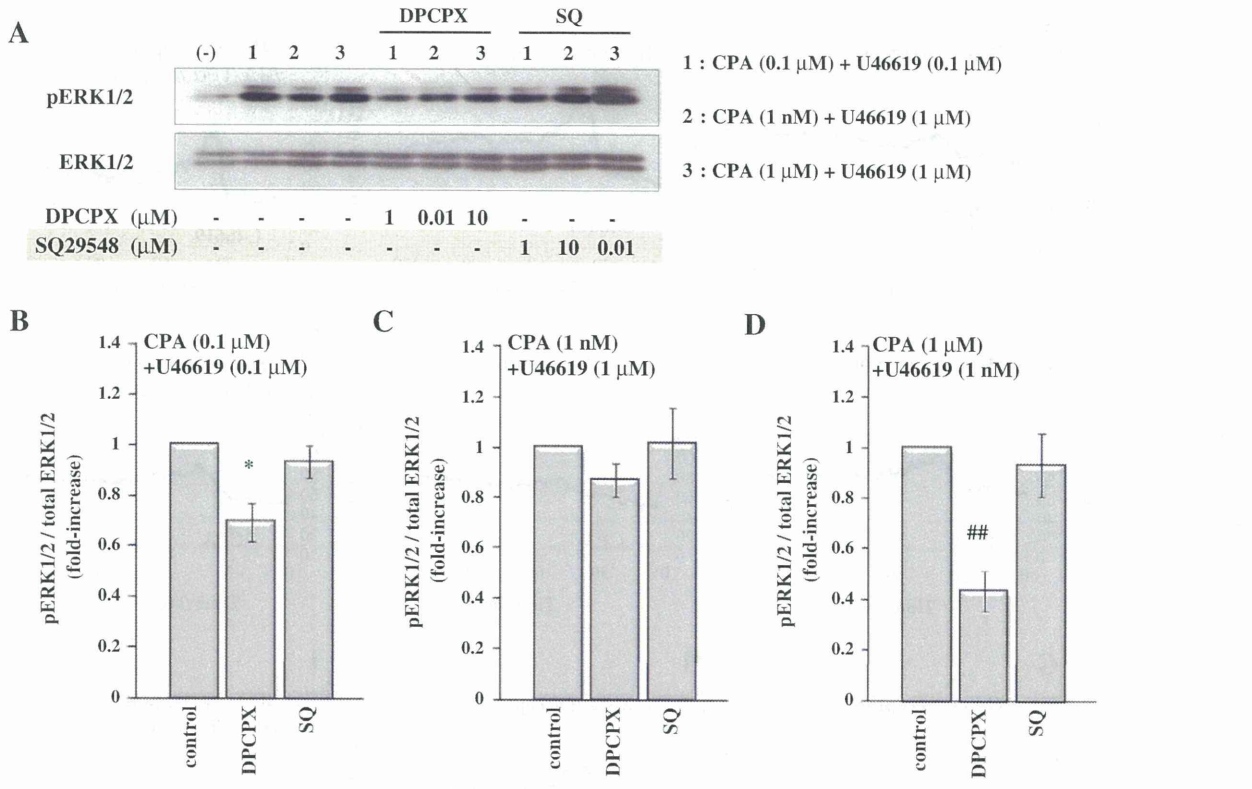

Fig. 5. Effect of DPCPX and SQ29548 on ERK1/2 phosphorylation induced by CPA and U46619. Assays were performed in HEK293T cells cotransfected with the 3HA-adenosine A_1 receptor and HA-TPα receptor. Cells were pretreated with the indicated concentration of DPCPX or SQ29548 and stimulated with vehicle (−) or indicated concentrations of agents. The cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with anti-ERK1/2 and anti-phospho ERK1/2 antibodies (A). Fold increases in ERK1/2 phosphorylation in A, are presented as the mean ± S.E.M for three experiments (B, C, D). $^{*}P<0.01$, $^{\#\#}P<0.005$ vs. control (Tukey's test).

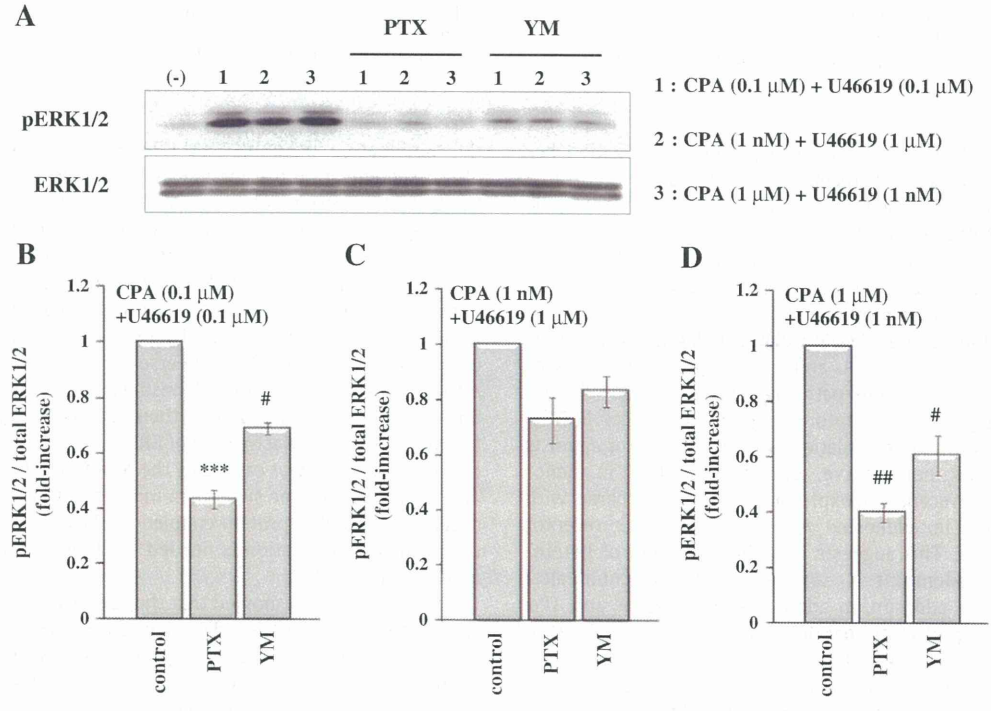

Fig. 6. Effect of pertussis toxin and YM-254890 on ERK1/2 phosphorylation induced by CPA and U46619. Assays were performed in HEK293T cells cotransfected with the 3HA-adenosine A_1 receptor and HA-TPα receptor. Cells were pretreated with 100 ng/ml pertussis toxin (PTX) for 16 h or 1 μM YM-254890 (YM) for 10 min, and stimulated with vehicle (−) or the indicated concentrations of agents. The cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with anti-ERK1/2 and anti-phospho ERK1/2 antibodies (A). Fold increases in ERK1/2 phosphorylation in A, are presented as the mean ± S.E.M for three experiments (B, C, D). $^{***}P<0.001$, $^{\#\#}P<0.005$, $^{\#}P<0.05$ vs. control (Tukey's test).