別添１

厚生労働科学研究研究費補助金

医薬品・医療機器等レギュラトリーサイエンス総合研究事業

# 副作用の発現メカニズムを考慮した対応方策に関する研究

平成１６年度　総括研究報告書

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# 副作用の発現メカニズムを考慮した対応方策に関する研究

（主任研究者　井上　和秀　九州大学大学院薬学研究院）

研究要旨
臨床的に用いられるCaチャネル拮抗降圧剤は、膵β細胞機能を抑制し、糖尿病治療薬であるスルフォニルウレア剤の効果を減弱させる可能性がある。従ってL型Caチャネルを介さないインスリン分泌機構の検索が必要であり、ATP受容体を介した経路はその候補のひとつである。一方、この様な研究推進のためには、新たなるインスリン分泌・測定法の開発が必須であり、本研究ではTIRFMシステムを用いた新しいインスリン開口放出測定系を確立した。

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## A. 研究目的

我が国の高血圧症の患者802万人（1999年国民栄養調査）の95%は本態性高血圧であり、一般に中年以降に発症することが多い。糖尿病患者（現在潜在患者数を含めると約1620万人）が高血圧を併発した場合、心脳血管障害へ至る確率は格段と跳ね上がる。糖尿病はそれ自体が動脈硬化症を引き起こす主要な疾患であり、その結果、心・循環器系の病態も増悪し、脳梗塞や心筋梗塞の合併率が極めて高い。そこで、降圧剤による治療は特に糖尿病と高血圧症を併発している患者にとって必須であり、現在臨床ではカルシウム拮抗薬が広く用いられている。その作用メカニズムはL型電位依存性カルシウムチャネルの抑制であり、結果として末梢血管拡張・降圧という治療効果をもたらす。一方、インシュリンを分泌する膵臓β細胞には同じくL型電位依存性カルシウムチャネルが発現しており、インシュリン分泌の要として機能している。従って、カルシウム拮抗薬がインシュリン分泌を抑制しても不思議ではなく、高血圧の患者が降圧剤を使用中に緩徐な慢性的インシュリン分泌低下およびマイルドな血糖上昇を来しているばかりではなく糖尿病患者の血糖コントロールに悪影響を及ぼす可能性がある。現在、副作用情報としてカルシウム拮抗薬の代表例であるニフェジピンやアムロジピンでは、0.1%未満の発生頻度で「高血糖」があると添付文書に出ている。糖尿病合併症患者ではさらに頻度は高くなる可能性もあるし、また、単に糖尿病が進行したと考えて副作用としなかったりするなど、成人病を併発しやすいという病態像に隠れて発現頻度が低く見積もられているのかもしれない。まずこの点を緊急に明らかにしなくてはならない。

そこで、本研究の目的は、上市されているカルシウム拮抗薬が膵臓β細胞からのインシュリン分泌を抑制するか否かを明らかにし（新しい副作用とそのメカニズム・予防法の提唱）、ついで、その副作用を未然に防ぐ方法を考案することである。その方法は次の２点。（１）カルシウム拮抗薬の中で、膵β細胞に発現しているL- type Ca Channel (alpha1CとalphaID)には効かないものを探す。（２）新しいインシュリン放出メカニズムを探索し、それに基づく副作用治療法を提案する。

## B. 研究方法

1. 膵β細胞の調整・培養 ； 麻酔下にWistar ratを開腹し、総胆管よりコラジネース液を注入し、膵摘出後、ラ氏島を回収し、Ca2+free溶液にて単一β細胞を採取、glass bottom dishにまき、RPMI1640溶液中にて培養した。

2. Fura-2AM負荷細胞を用いた細胞内カルシウムイメージング ； [Ca2+]iのイメージング・解析にはArugus/HiSCAを使用した。β細胞をFura-2AMにてincubation後、各種刺激物質にて15分間細胞を刺激し、励起光によりFura-2が発する510nm蛍光波長をCCDカメラにて測定し、340nm/380nmの蛍光波長強度比を測定することにより、細胞内Ca2+濃度を測定した。

3. 新たなインスリン分泌測定・解析法の開発 ； β細胞からのインスリン分泌を従来の様なRIA法を用いたmassとして測定解析するのではなく、単一インスリン顆粒の放出として捉え、これをナノスケールから解析するために、TIRFM(total internal reflection fluorescence microscopy)法を膵β細胞に導入応用した。インスリン顆粒をGFP標識するためにヒトプレプロインスリンのC末端にGFPを導入した

cDNAを作製、更にそのrecombinant adenovirusを作製。ラットβ細胞にこのウィルスを感染させ、GFP標識単一インスリン顆粒の動態をTIRFMシステムを用いてCCDカメラにて0.1秒毎に取得し、メタモルフソフトウェアにて時間的・空間的解析を行った。

4.膵β細胞のperifusionと分泌インスリン量の定量(RIA)；膵β細胞をペリスタポンプにより灌流し、1分毎の流出液を回収、回収液を125I-インスリン、及び抗インスリン抗体を用いてRadio immuno assay (RIA)を行った。

C.研究結果

1.カルシウム拮抗降圧薬の膵β細胞における影響；臨床上すでに用いられている、ニフェジピンの膵β細胞における作用を検討した。10μMニフェジピンはグルコース刺激による[Ca2+]iの上昇を完全に抑制し、1μMでも約50%の抑制がみられた。RIAにより測定したインスリン分泌も同様に抑制された。L型Caチャネルにより選択的なIsradipineは、1μMにおいて完全に[Ca2+]i上昇を抑制した。

2.新しいインスリン分泌メカニズムの検索；現在、糖尿病治療に於いて最も一般的に使用されている治療薬はスルホニルウレア剤である。この薬は、KATP-チャネルを閉じ、L型カルシウムを開くことによってインスリンの分泌を促進する。従って、前述したCaチャネルブロッカーを降圧剤として用いている高血圧を併発している糖尿病患者にとっては、スルホニルウレア剤は必ずしも最適の血糖降下剤とは言い難い。そこで、KATP-チャネル→L型カルシウムチャネル系の活性化を介さない様な血糖降下剤の開発が必要である。よって、ATP受容体を活性化することにより、インスリン分泌が活性化されるか否かについて検討した。100μMATPを用いて膵β細胞のperifusionを行い、インスリン分泌量を測定したところ、第1相、第2相のインスリン分泌を明らかに増強する場合と、全く影響のない実験データが得られた。ATPが培養液中にて分解されている可能性があるため、P2Y受容体の選択的アゴニストであるADPβS、更には非水解型ATPγSを用いて、検討したところ、ATP受容体の活性化はインスリン分泌を促進する可能性が強いdataを得ることができた。

3.ナノテクテクノロジーを用いた新しいインスリン分泌測定・解析法の開発；新たなインスリン分泌機構の検索、及び新規糖尿病治療薬の開発には、単に放出されたインスリンをmassとして測定するのみではなく、β細胞内における単一顆粒の動態を時間的空間的に解析出来るようなインスリン放出の新しい測定・解析システムを開発することが必須である。従って、本年度は単一インスリン顆粒の細胞膜へのdocking/fusionが0.1秒のオーダーで解析できるTIRFシステムを確立した。まず、β細胞内のインスリン顆粒を標識するために、ヒトインスリンcDNAの3'端にGFPcDNAを挿入、これをadenovirusへと変換した(pchi-GFP)、pchi-GFPを感染したβ細胞をTIRFMによって観察し、GFPの動きを0.1秒毎に、CCDカメラにより取得、メタモルフソフトウェアを用いて、インスリン顆粒動態を詳細に解析することに成功した。この方法を用いることにより、種々の物質のインスリン開口放出における分子機構、更には新たなインスリン分泌メカニズムを明らかにすることが可能となった。

D.考察

臨床上頻繁に用いられているカルシウム拮抗降圧薬の膵β細胞における作用を検討した結果、ニフェジピンはグルコース刺激による[Ca2+]iの上昇を完全に抑制し、インスリン分泌も同様に抑制した。L型 Caチャネルにより選択的なIsradipineは、1μMにおいて完全に[Ca2+]i上昇を抑制した。この様に、降圧剤として用いられているカルシウム拮抗薬は膵β細胞における[Ca2+]i上昇を抑制し、その結果としてインスリン分泌に抑制的に作用するが、臨床的に用いられる濃度(~100nM)では、必ずしも明らかな抑制はみられない。しかしながら、糖尿病状態における作用がいかに変化しているかについては定かではない。来年度には、糖尿病ラットを用いた検討する必要があると考えられる。

現在、糖尿病治療に於いて最も一般的に使用されている治療薬はスルホニルウレア剤は、KATP-チャネルを閉じ、L型カルシウムを開くことによってインスリンの分泌を促進する。従って、上記のCaチャネルブロッカーを降圧剤として用いている高血圧を併発している糖尿病患者にとっては、スルホニルウレア剤は必ずしも最適の血糖降下剤とは言い難い。そこで、KATP-チャネル→L型カルシウムチャネル系の活性化を介さない様な血糖降下剤の開発が必要である。よって、本年度はATP受容体を活性化することによりインスリン分泌が活性化されるか否かについて検討した。その結果、P2Y受容体の選択的アゴニストであるADPβS、更には非水解型ATPγSはインスリン分泌を促進する可能性が示唆された。来年度はこの系を介したインスリン分泌機構につき更に詳細な検討を行う予定である。

E.結論

臨床的に用いられているカルシウム拮抗薬は、ある条件下においては、膵β細胞機能を抑制し、糖尿病治療薬であるスルホニルウレア剤の効果を阻害する。従って今までとは全く違った分子を標的とした新規糖尿病治療薬の開発が必須であり、ATP受容体の活性化がその一つの方向性を示すものと思われる。その様な意味からは、インスリン放出を、分子レベ

ルから解析することが必要であり、本年度私達が確立したβ細胞におけるTIRFMシステムは、次年度からの研究を推進する上で強力にtoolとなる。

F. 健康危機情報

本研究成果からは現段階において特段の健康危機情報は得られていない。

G. 研究発表


1. Y. Shinozaki, S. Koizumi, S. Ishida, J. Sawada, Y. Ohno and K. Inoue: Cytoprotection against oxidative-stress-induced damage of astrocytes by extracellular ATP via P2Y1 receptors. Glia 49, 288 - 300, 2004

2. S. Ishida, Y. Shigemoto-Mogami, Y. Shinozaki, H. Kagechika, K. Shudo, S. Ozawa, J. Sawada, Y. Ohnol, K. Inoue. Differential modulation of PI3-kinase/Akt pathway during all-trans retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis. Biochem. Pharmacol., 68, 2177-2186, 2004

3. S. Koizumi, K. Fujishita, K. Inoue, Y. Shigemoto-Mogami, M. Tsuda and K. Inoue. Ca2+ waves in keratinocytes are transmitted to sensory neurons: the involvement of extracellular ATP and P2Y2 receptor activation. Biochem. J. 380, 329-338, 2004

4. T. Suzuki, I. Hide, K. Ido, A. Inoue, S. Kohsaka, K. Inoue and Y. Nakata. Production and release of neuroprotective TNF by P2X7 receptor-activated microglia. J. Neurosci., 24, 1-7, 2004

5. M. Ohara-Imaizumi, C. Nishiwaki, Y. Nakamichi, T. Kikuta, S. Nagai & S. Nagamatsu Corelation of syntaxin1 and SNAP-25 clusters with docking and fusion of insulin granules analyzed by total internal reflection microscopy. Diabetologia (in press)

6. M. Ohara-Imaizumi, A.K. Cardozo, T. Kikuta, D.L. Eizirik & S. Nagamatsu. The cytokine IL-1β reduces the docking and fusion of insulin granules in pancreatic β-cells, preferentially decreasting the first phase of exocytosis. J. Biol. Chem. 279: 41271-41274, 2004

7. M. Ohara-Imaizumi, C. Nishiwaki, T. Kikuta, S. Nagai, Y. Nakamichi & S. Nagamatsu TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic β-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat β-cells. Biochem. J. 381, 13-18, 2004

8. S. Torii, T. Takeuchi, S. Nagamatsu & T. Izumi. Rab27 Effector Granuphilin Promotes the Plasma Membrane Targeting of Insulin Granules via Interaction with Syntaxin 1a. J. Biol. Chem. vol. 279 p22532-22538 , 2004

9. M. Ohara-Imaizumi, C. Nishiwaki, T. Kikuta, K. Kumakura, Y. Nakamichi & S. Nagamatsu Site of Docking and Fusion of Insulin Secretory Granules in Live MIN6 Cells Analyzed by TAT-conjugated Anti-syntaxin 1 Antibody and Total Internal Reflection Fluorescence Microscopy . J. Biol. Chem. 279 :8403-8408 , 2004


H. 知的財産権の出願・登録状況

1. 特許取得

現段階ではなし。

2. 実用新案登録

現段階ではなし。

別添４

厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）
（分担研究報告書）

# 副作用の発現メカニズムを考慮した対応方策に関する研究

（分担研究者　永松　信哉　杏林大学・生化学教室・教授）

研究要旨
臨床的に用いられるCaチャネル拮抗降圧剤は、膵β細胞機能を抑制し、糖尿病治療薬であるスルフォニルウレア剤の効果を減弱させる可能性がある。従ってL型Caチャネルを介さないインスリン分泌機構の検索が必要であり、ATP受容体を介した経路はその候補のひとつである。この様な研究推進のための新たなるインスリン分泌・測定法としてTIRFMシステムを用いた新しいインスリン開口放出測定系を確立した。

## A. 研究目的

我が国の糖尿病患者数は近年著明に上昇してきており、潜在患者数を含めるとに於いては1620万人に達すると言われている。糖尿病は、動脈硬化症の基礎疾患であり、その多くが高血圧症を併発していることは臨床医の常識である。その場合、心・脳血管障害へ至る確立は格段と跳ね上がり、致命的な脳梗塞、心筋梗塞を発症する。従って糖尿病患者のQOLを高めるためには血糖のコントロールはいうでもなく、降圧剤による血圧コントロールが必須である。一般診療に於いては降圧剤としてカルシウム拮抗薬が1st チョイスではあるが、本剤は、L型電位依存型Caチャネル活性を抑制してしまうため、血糖コントロールという面では悪影響を及ぼす場合がある。そこで、本研究では、臨床的に使用されているカルシウム拮抗薬の膵β細胞からのインスリン分泌に対する影響を検討し、L型Caチャネルを介さない新しいインスリン分泌メカニズムを検索し、更にこのメカニズムを検索、新規糖尿病治療薬開発のための新たなインスリン放出測定法の開発を行う。

## B. 研究方法

1. 膵β細胞の調整・培養 ； 麻酔下にWistar ratを開腹し、総胆管よりコラジネース液を注入し、膵摘出後、ラ氏島を回収し、Ca2+free溶液にて単一β細胞を採取、glass bottom dishにまき、RPMI1640溶液中にて培養した。
2. 新たなインスリン分泌測定・解析法の開発 ； β細胞からのインスリン分泌を従来の様なRIA法を用いたmassとして測定解析するのではなく、単一インスリン顆粒の放出として捉え、これをナノスケールから解析するために、TIRFM(total internal reflection fluorescence microscopy)法を膵β細胞に導入応用した。インスリン顆粒をGFP標識するためにヒトプレプロインスリンのC末端にGFPを導入したcDNAを作製、更にそのrecombinant adenovirusを作製。ラットβ細胞にこのウィルスを感染させ、GFP標識単一インスリン顆粒の動態をTIRFMシステムを用いてCCDカメラにて0.1秒毎に取得し、メタモルフソフトウェアにて時間的・空間的解析を行った。
3. 膵β細胞のperifusionと分泌インスリン量の定量(RIA) ； 膵β細胞をペリスタポンプにより灌流し、１分毎の流出液を回収、回収液を125I-インスリン、及び抗インスリン抗体を用いてRadio immuno assay (RIA)を行った。

## C. 研究結果

1. 新しいインスリン分泌メカニズムの検索 ； 現在、糖尿病治療に於いて最も一般的に使用されている治療薬はスルホニルウレア剤である。この薬は、KATP-チャネルを閉じ、L型カルシウムを開くことによってインスリンの分泌を促進する。従って、前述したCaチャネルブロッカーを降圧剤として用いている高血圧を併発している糖尿病患者にとっては、スルホニルウレア剤は必ずしも最適の血糖降下剤とは言い難い。そこで、KATP-チャネル→L型カルシウムチャネル系の活性化を介さない様な血糖降下剤の開発が必要である。よって、ATP受容体を活性化することにより、インスリン分泌が活性化されるか否かについて検討した。100μMATPを用いて膵β細胞のperifusionを行い、インスリン分泌量を測定したところ、第１相、第２相のインスリン分泌を明らかに増強する場合と、全く影響のない実験データが得られた。ATPが培養液中にて分解されている可能性があるため、P2Y受容体の選択的アゴニストであるADPβS、更には非水解型ATPγSを用いて、検討したところ、

ATP受容体の活性化はインスリン分泌を促進する可能性が強いdataを得ることができた。

2. ナノテクテクノロジーを用いた新しいインスリン分泌測定・解析法の開発 ; 新たなインスリン分泌機構の検索、及び新規糖尿病治療薬の開発には、単に放出されたインスリンをmassとして測定するのみではなく、β細胞内における単一顆粒の動態を時間的空間的に解析出来るようなインスリン放出の新しい測定・解析システムを開発することが必須である。従って、本年度は単一インスリン顆粒の細胞膜へのdocking/fusionが0.1秒のオーダーで解析できるTIRFシステムを確立した。まず、β細胞内のインスリン顆粒を標識するために、ヒトインスリンcDNAの3'端にGFPcDNAを挿入、これをadenovirusへと変換した(pchi-GFP)、pchi-GFPを感染したβ細胞をTIRFMによって観察し、GFPの動きを0.1秒毎に、CCDカメラにより取得、メタモルフソフトウェアを用いて、インスリン顆粒動態を詳細に解析することに成功した。この方法を用いることにより、種々の物質のインスリン開口放出における分子機構、更には新たなインスリン分泌メカニズムを明らかにすることが可能となった。

D. 考察

現在、糖尿病治療に於いて最も一般的に使用されている治療薬はスルホニルウレア剤は、KATP-チャネルを閉じ、L型カルシウムを開くことによってインスリンの分泌を促進する。従って、上記のCaチャネルブロッカーを降圧剤として用いている高血圧を併発している糖尿病患者にとっては、スルホニルウレア剤は必ずしも最適の血糖降下剤とは言い難い。そこで、KATP-チャネル→L型カルシウムチャネル系の活性化を介さない様な血糖降下剤の開発が必要である。よって、本年度はATP受容体を活性化することによりインスリン分泌が活性化されるか否かについて検討した。その結果、P2Y受容体の選択的アゴニストであるADPβS、更には非水解型ATPγSはインスリン分泌を促進する可能性が示唆された。来年度はこの系を介したインスリン分泌機構につき更に詳細な検討を行う予定である。

E. 結論

臨床的に用いられているカルシウム拮抗薬は、ある条件下においては、膵β細胞機能を抑制し、糖尿病治療薬であるスルホニルウレア剤の効果を阻害する。従って今までとは全く違った分子を標的とした新規糖尿病治療薬の開発が必須であり、ATP受容体の活性化がその一つの方向性を示すものと思われる。その様な意味からは、インスリン放出を、分子レベルから解析することが必要であり、本年度私達が確立したβ細胞におけるTIRFMシステムは、次年度からの研究を推進する上で強力にtoolとなる。

F. 健康危機情報

本研究成果からは現段階において特段の健康危機情報は得られていない。

G. 研究発表

1. M. Ohara-Imaizumi, C. Nishiwaki, Y. Nakamichi, T. Kikuta, S. Nagai & S. Nagamatsu Corelation of syntaxin1 and SNAP-25 clusters with docking and fusion of insulin granules analyzed by total internal reflection microscopy. Diabetologia (in press)

2. M. Ohara-Imaizumi, A.K. Cardozo, T. Kikuta, D.L. Eizirik & S. Nagamatsu. The cytokine IL-1β reduces the docking and fusion of insulin granules in pancreatic β-cells, preferentially decreasting the first phase of exocytosis. J.Biol.Chem. 279: 41271-41274, 2004

3. M. Ohara-Imaizumi, C. Nishiwaki, T. Kikuta, S. Nagai, Y. Nakamichi & S. Nagamatsu TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic β-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat β-cells. Biochem. J. 381, 13-18, 2004

4. S. Torii, T. Takeuchi, S. Nagamatsu & T. Izumi. Rab27 Effector Granuphilin Promotes the Plasma Membrane Targeting of Insulin Granules via Interaction with Syntaxin 1a. J.Biol.Chem. vol.279 p22532-22538 , 2004

5. M. Ohara-Imaizumi, C. Nishiwaki, T. Kikuta, K. Kumakura, Y. Nakamichi & S. Nagamatsu Site of Docking and Fusion of Insulin Secretory Granules in Live MIN6 Cells Analyzed by TAT-conjugated Anti-syntaxin 1 Antibody and Total Internal Reflection Fluorescence Microscopy . J.Biol.Chem. 279 :8403-8408 ,2004

H. 知的財産権の出願・登録状況

1. 特許取得

現段階ではなし。

2. 実用新案登録

現段階ではなし。

別添５

研究成果の刊行に関する一覧表レイアウト（参考）

雑誌

| 氏 名 | タイトル | 発表雑誌名 | 巻 号 | ページ | 出版年 |
|---|---|---|---|---|---|
| Y. Shinozaki et al. | Cytoprotection against oxidative-stress-induced damage of astrocytes by extracellular ATP via P2Y1 receptors. | Glia | 49 | 288 - 300 | 2004 |
| S. Ishida et al. | Differential modulation of PI3-kinase/Akt pathway during all-trans retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis. | Biochem. Pharmacol. | 68 | 2177-2186 | 2004 |
| S. Koizumi et al. | Ca2+ waves in keratinocytes are transmitted to sensory neurons: the involvement of extracellular ATP and P2Y2 receptor activation. | Biochem. J. | 380 | 329-338 | 2004 |
| T. Suzuki et al. | Production and release of neuroprotective TNF by P2X7 receptor-activated microglia. | J. Neurosci. | 24 | 1-7 | 2004 |
| Mica Ohara-Imaizumi et. al | TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic β-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat β-cells | Biochem. J | 381 | 13-18 | 2004 |
| S. Torii et. al | Rab27 Effector Granuphilin Promotes the Plasma Membrane Targeting of Insulin Granules via Interaction with Syntaxin 1a. | J. Biol. Chem | 279 | 22532-22538 | 2004 |
| Yoko. Nakamich et. al | Functions of pancreatic beta cells and adipocytes in bombesin receptor subtype-3-deficient mice. | Biochem, Biophys. Res. Commun | 318 | 698-703 | 2004 |
| Mica Ohara-Imaizumi et. al | The cytokine IL-1β reduces the docking and fusion of insulin granules in pancreatic β-cells, preferentially decreasting the first phase of exocytosis | J. Biol. Chem | 279 | 41271-41274 | 2004 |

| | | | | | |
|---|---|---|---|---|---|
| Mica Ohara-Imaizumi et.al | Corelation of syntaxinl and SNAP-25 clusters with docking and fusion of insulin granules analyzed by total internal reflection microscopy | Diabetologia | 47 | 2200-2207 | 2004 |
| K. Kasai, et.al | Rab27a mediates the tight docking of insulin granules onto the plasma membrane during glucose stimulation. | J. Clin. Invest | 115 | 388-396 | 2005 |

GLIA 49:288–300 (2005)

# Cytoprotection Against Oxidative Stress-Induced Damage of Astrocytes by Extracellular ATP Via $P2Y_1$ Receptors

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*KEY WORDS* ATP; $P2Y_1$ receptors; astrocytes; oxidative stress

*ABSTRACT* Oxidative stress is the main cause of neuronal damage in traumatic brain injury, hypoxia/reperfusion injury, and neurodegenerative disorders. Although extracellular nucleosides, especially adenosine, are well known to protect against neuronal damage in such pathological conditions, the effects of these nucleosides or nucleotides on glial cell damage remain largely unknown. We report that ATP but not adenosine protects against the cell death of cultured astrocytes induced by hydrogen peroxide ($H_2O_2$). ATP ameliorated the $H_2O_2$-induced decrease in cell viability of astrocytes in an incubation time- and concentration-dependent fashion. Protection by ATP was inhibited by P2 receptor antagonists and was mimicked by $P2Y_1$ receptor agonists but not by adenosine. The expressions of $P2Y_1$ mRNAs and functional $P2Y_1$ receptors in astrocytes were confirmed. Thus, ATP, acting on $P2Y_1$ receptors in astrocytes, showed a protective action against $H_2O_2$. The astrocytic protection by the $P2Y_1$ receptor agonist 2-methylthio-ADP was inhibited by an intracellular $Ca^{2+}$ chelator and a blocker of phospholipase C, indicating the involvement of intracellular signals mediated by Gq/11-coupled $P2Y_1$ receptors. The ATP-induced protection was inhibited by cycloheximide, a protein synthesis inhibitor, and it took more than 12 h for the onset of the protective action. In the DNA microarray analysis, ATP induced a dramatic upregulation of various oxidoreductase genes. Taken together, ATP acts on $P2Y_1$ receptors coupled to Gq/11, resulting in the upregulation of oxidoreductase genes, leading to the protection of astrocytes against $H_2O_2$. 

## INTRODUCTION

Astrocytes are much more than merely support cells for neurons in the central nervous system (CNS). They can receive inputs, assimilate information, and send instructive chemical signals to neighboring glial cells as well as neurons (Araque et al., 1999a, b, 2001; Haydon, 2001). Thus, communication among astrocytes would play an important role in brain function. Initially, so-called gliotransmission, a glia-to-glia communication or even neuron-to-glia communication, was reported to be mediated by glutamate (Cornell-Bell et al., 1990; Charles et al., 1991; Parpura et al., 1994; Innocenti et al., 2000) because astrocytes express glutamate receptors and release glutamate. However, re-

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cent accumulating evidence has shown that extracellular ATP released from astrocytes has a central role in astrocyte-to-astrocyte (Guthrie et al., 1999), astrocyte-to-microglia (Verderio and Matteoli, 2001; Schipke et al., 2002), and even astrocyte-to-neuron communication (Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003).

ATP is an endogenous ligand for P2 receptors that are classified into ligand-gated P2X and G-protein-coupled metabotropic P2Y receptors (Abbracchio and Burnstock, 1994). Astrocytes express both types of P2 receptors (James and Butt, 2002; Fumagalli et al., 2003) and can release ATP in response to various stimuli (Guthrie et al., 1999; Queiroz et al., 1999; Koizumi et al., 2003). Astrocytic ATP acting on these P2 receptors forms intercellular $Ca^{2+}$ waves that mediate long-range communications in astrocytes (Fam et al., 2000; Gallagher and Salter, 2003). However, the physiological or pathological significance of such an ATP/P2 receptor-mediated response in astrocytes remains largely unknown.

It has been reported that ATP inhibits excess neuronal excitations by inhibiting the release of glutamate (Koizumi and Inoue, 1997; Zhang et al., 2003) or by facilitating inhibitory γ-aminobutyric acid (GABA) release in the hippocampus (Aihara et al., 2002) and is therefore presumably involved in protecting neurons against excitotoxicity. With regard to neuroprotective actions, however, adenosine, a metabolite of ATP, has received much attention as an important inhibitory molecule because it is formed by the immediate degradation of ATP by ectonucleotidases, potently inhibiting the excitability of neurons and protecting them against various neurodegenerative disorders including excitatory neuronal death (Jones et al., 1998; Behan and Stone, 2002; Hentschel et al., 2003; Schwarzschild et al., 2003). This might be why the functional role of ATP in relation to neuroprotection has received only limited attention. Interestingly, however, adenosine does not show any protective action in astrocytes, rather it induces the cell death of astrocytes (Abbracchio et al., 1995; Appel et al., 2001; Di Iorio et al., 2002). It has been reported that ATP protects astrocytes against glucose deprivation-induced cell death, although this protection appears to be independent of P2 receptors (Shin et al., 2002). ATP is released from both neurons (Wieraszko et al., 1989; Inoue et al., 1995) and astrocytes (Guthrie et al., 1999; Ahmed et al., 2000) in physiological and pathological conditions, and astrocytes could receive the ATP signal via various P2 receptors, including a high-affinity $P2Y_1$ receptor (Koizumi et al., 2002). These findings raise the possibility that, unlike neurons, astrocytes mainly use ATP/P2 receptor-mediated pathway(s) for their own survival.

We report that ATP acting on $P2Y_1$ receptors protects astrocytes from cell death induced by hydrogen peroxide ($H_2O_2$), one of the main reactive oxygen species (ROS) generated by traumatic brain injury, hypoxia/reperfusion, and various neurodegenerative disorders (Agardh et al., 1991; Lei et al., 1997; Cuajungco et al., 2000; Huang et al., 2000; Tabner et al., 2001; Tamagno et al., 2003). We further demonstrate by using differential gene expression analysis that ATP induces the upregulation of oxidoreductase genes, suggesting the involvement of these genes in the protective action.

## MATERIALS AND METHODS

### Chemicals

Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), adenosine, 2-methylthio-adenosine diphosphate (2MeSADP), adenosine 5-o-(2-thiodiphophate) (ADPβS), α,β-methylene-adenosine triphosphate (α,βmeATP), suramin, reactive blue 2 (RB2), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), MRS2179, U73122, U73343, glutamate, 1-octanol, DL-2-amino-5-phosphonopentanoic acid (AP-V), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (RS)-α-methyl-4-carboxyphenylglycine (MCPG) were purchased from Sigma Chemical Co. (St Louis, MO). The sources of other chemicals are shown in parentheses as follows; trypsin-EDTA, M-MLV reverse transcriptase, 100 mM dNTP set, recombinant ribonuclease (RNase) inhibitor and deoxyribonuclease (DNase) I (GIBCO/Invitrogen, Tokyo, Japan), RNA STAT 60 (Tel-Test, Friendswood, TX), hydrogen peroxide ($H_2O_2$) (Wako Pure Chemicals, Osaka, Japan), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Chemicon International, Temecula, CA), GeneAmp PCR Reagent Kit and AmpliTaq DNA polymerase (Perkin-Elmer, (Foster City, CA) Roche Molecular Systems, (Pleasanton, CA), O,O′-Bis (2-aminophenyl) ethyleneglycol-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) (Calbiochem Biosciences, San Diego, CA).

### Cells and Cell Culture

Astrocytes were prepared from neonatal rat forebrain. The cells were cultured in Dulbecco's modified essential medium (DMEM, GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS; GIBCO/Invitrogen). After 3 weeks with changing of the medium every 3 days, the medium was changed to DMEM with 5% horse serum (HS; GIBCO/Invitrogen) and 5% FBS; the cells were shaken for 15 h at 100 rpm. Then, the cells were washed 3 times with phosphate-buffered saline (PBS) (10 ml each) and supplemented with 0.025% trypsine-EDTA (diluted with PBS), and incubated for 2 min under 10% $CO_2$ /90% air at 37°C. After the cells were harvested, $2 \times 10^5$ cells were seeded on 60 × 15-mm dishes (Falcon/Becton Dickinson, San Jose, CA) and cultured in DMEM with 5% HS and 5% FBS. Total RNA was collected from five dishes. For the cell viability assay, cells were seeded on 96-well plates (NUNC, Roskilde, Denmark) at a density of $1.25 \times 10^4$ cells/well. At 24 h after the seeding, the medium was

changed. The cells were used for experiments 72 h after the medium exchange.

### Experimental Design of Hydrogen Peroxide ($H_2O_2$)-Evoked Cell Death

Astrocytes were exposed to various concentrations of $H_2O_2$ (75–300 μM) for 1–24 h, and then the cell viability was investigated. In the present study, we chose a $H_2O_2$ concentration of 250 μM and an incubation period of 2 h to assess the effect of ATP.

### Cell Viability Assay

For the cell viability assay, we used an MTT assay. MTT is a yellow tetrazolium salt that is reduced to purple formazan (Altman, 1976). The MTT assay assesses cell viability by measuring the mitochondrial function (Twentyman and Luscombe, 1987). After incubation with $H_2O_2$ for 2 h, a 1/10 volume of MTT solution (5 mg/ml in PBS) was added and incubated for 4 h under 10% $CO_2$/90% air at 37°C. Then an equal volume of isopropanol (with 0.04 N HCl) was added to the cells, and the MTT formazan was dissolved by pipetting. The absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (ASYS Hitech, Eugendorf, Austria) with a test and reference wavelength of 570 and 630 nm, respectively.

### Expression of $P2Y_1$ Receptors in Astrocytes

The expression of $P2Y_1$ receptor mRNA was analyzed by single reverse transcription-polymerase chain reaction (RT-PCR). For RT-PCR analysis, astrocytes were directly lysed with 0.5 ml of RNA STAT-60 (Tel-Test B) and total RNA was isolated; 1 μg of RNA was reverse-transcribed with M-MLV transcriptase. Aliquots (1 μl) of the RT product were added to the reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq* polymerase and $P2Y_1$ receptors specific primers according to the nucleotide sequences as follows; forward, 5′-ctgatcttgggctgttatgg-3′ and reverse, 5′-gctgttgagacttgctagac-3′. Amplification was performed in a Gene Amp PCR System 2400-R (Perkin-Elmer/Roche Molecular Systems) thermal cycler for 30–40 cycles, after an initial denaturation at 94°C for 2 min by utilizing sense and antisense primers specifically designed for $P2Y_1$ receptors. The PCR product was resolved on agarose gel stained by 2% ethidium bromide and visualized under ultraviolet (UV) light.

### Measurement of Intracellular $Ca^{2+}$ Concentration ($[Ca^{2+}]_i$) in Single Cells

The increase in $[Ca^{2+}]_i$ in single cells was measured by the fura-2 method as described by Grynkiewicz et al. (1985) with minor modifications (Koizumi et al., 2002). In brief, the cells were washed with a balanced salt solution (BSS) of the following composition (in mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 25, and D-glucose 10 (pH = 7.4). Cells were then loaded with 5 μM fura-2 acetoxymethylester (fura-2 AM) at room temperature in BSS for 45 min, followed by a BSS wash and a further 30-min incubation to allow de-esterification of the loaded dye. For the $Ca^{2+}$-free experiment, $Ca^{2+}$ was removed from the BSS ($Ca^{2+}$-free BSS). The coverslips were mounted on an inverted epifluorescence microscope (TE-2000-U, Nikon, Tokyo, Japan). Fluorescent images were obtained by alternate excitation at 340 nm (F340) and 380 nm (F380). The emission signal at 510 nm was collected by a charge-coupled device camera (C-6790, Hamamatsu Photonics, Hamamatsu, Japan) coupled with an image intensifier (GaAsP, C8600-03, Hamamatsu Photonics); digitized signals were stored and processed using an image processing system (Aquacosmos, Hamamatsu Photonics). Drugs were dissolved in BSS and applied by superfusion.

### Measurement of Extracellular ATP Concentration

The extracellular ATP concentration in astrocytes was detected with a luciferin-luciferase bioluminescence assay. After glutamate stimulation or exogenous ATP application, supernatants were collected at different time points and were mixed with luciferase reagents (ATP bioluminescence assay kit CLS II; Roche Diagnostics, Mannheim, Germany). ATP bioluminescence was detected by a luminometer (Lumiphotometer TD-4000, Labo Science, Tokyo, Japan). The absolute ATP concentration was estimated using a standard ATP solution (0.001–1 μM).

### Total RNA Preparation

After washing the cells twice with PBS, total RNA was prepared with RNeasy Mini total RNA Preparation Kit (Qiagen GmbH, Tokyo, Japan) according to the manufacturer's instructions.

### DNA Microarray Analysis

Converting total RNA to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to the manufacturer's instructions. The targets were hybridized to rat genome U34A Gene Chip microarray (Affymetrix) for 16–24 h at 45°C. After the hybridization, the DNA microarrays were washed and stained on Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. Then, the DNA microarrays were scanned, and the images obtained

were analyzed by Microarray Suite Expression Analysis Software (version 5.0; Affymetrix). To analyze the gene expressions in astrocytes, differences in the mean level of the gene expression index between the control group and drug-treated group were assessed using the Student's *t*-test for each probeset.

Astrocytes were incubated for 2 h with ATP at a final concentration of 100 μM. Total RNA was prepared at the end of incubation and converted to the target for GeneChip hybridization. The gene expression was analyzed in duplicate by Rat Genome U34A GeneChip using these targets. The addition of ATP and the preparation of total RNA was done four times independently.

### Selection of Differentially Expressed Genes

The first step was selecting genes whose expression levels were increased 2-fold by treatment with ATP. The second step was selecting genes whose *P*-values were $P < 0.05$ using Student's *t*-test. The last step was selecting genes whose expression levels of the drug treated group were 1,000.

### Quantitative RT-PCR of Oxidoreductase Genes

RT-PCR amplifications were performed using Taqman One-step RT-PCR Master Mix Reagents and, 200 nM oxidoreductase-specific primers. Using the computer software Primer Express (Applied Biosystems), clone-specific primers were designed to recognize rat oxidoreductase genes, i.e., rat carbonyl reductase (CBR, Taqman Probe, 5′-cctcctgaatgcctgccctg-3′; forward, 5′-tgaggagaggagagaggacaaga-3′; reverse, 5′-cctgccatgtcggttctga-3′), schlafen-4 (SHL4, Taqman probe, 5′-aggccttatcgaggccagatggtttg-3′; forward, 5′-tcttgtttttcctagaactgttgtg-3′; reverse, 5′-ggtgaggtagcctggctatagc-3′), and thioredoxin reductase (TrxR, Taqman probe, 5′-attgaagcagggacaccaggccg-3′; forward, 5′-gtgccgacgaaaattgaaca-3′; reverse, 5′-gtggatttagcggtcacctga-3′). RT-PCR was performed by 30 min reverse transcription at 48°C, 10 min Amplitaq Gold activation at 95°C, then 15-s denaturation at 95°C, 1 min annealing and elongation at 60°C for 40 cycles in a PRISM7700 (Applied Biosystems). To exclude contamination by nonspecific PCR products such as primer dimmers, melting curve analysis was applied to all final PCR protocols after the cycling protocol. Each experiment was performed in triplicate.

## RESULTS

### Protection by ATP Against Oxidative Stress-Induced Cell Death in Astrocytes

Using an MTT assay, we tested the effect of hydrogen peroxide ($H_2O_2$) on cell viability in astrocytes. We found that $H_2O_2$ caused a time- (Fig. 1A) and concentration-dependent (Fig. 1B) decrease in the cell viability of the astrocytes, i.e., cell death of the astrocytes. When incubated for 1 h at 250 μM, the cell viability was almost halved and then was gradually decreased to ~20% of the non-treated control level by a further incubation (2–24 h, Fig. 1A). When the $H_2O_2$ concentrations were varied, the cell viability was decreased in a concentration-dependent fashion and reached the minimum at 250 μM. We therefore chose an $H_2O_2$ concentration of 250 μM and an incubation period of 2 h for the following experiments.

We tested the effect of exogenously applied ATP on the $H_2O_2$-induced astrocytic cell death. ATP was applied to the cells 24 h before and during $H_2O_2$ applica-

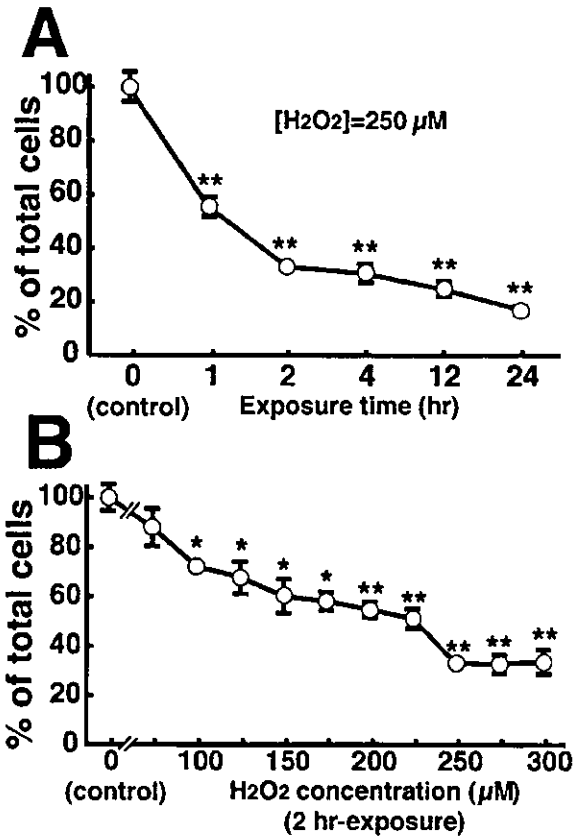


Fig. 1. Changes in cell viability of astrocytes by $H_2O_2$. **A:** Cells were incubated with 250 μM $H_2O_2$ for various periods before the cell viability test. The cell viability was evaluated by the MTT assay as described in Materials and Methods. $H_2O_2$ induced a decrease in cell viability in an exposure time-dependent fashion. **B:** Cells were stimulated with various concentrations of $H_2O_2$ for 2 h; cell viability was then examined. $H_2O_2$ evoked cell death in a concentration-dependent fashion. Sequential plots show mean ± SEM of triplicate measurements, depicting a representative experiment (n = 3). Values were normalized to total cell number (control) and the cell viability was expressed as percentage of total cell. Asterisks show significant differences from the control response (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

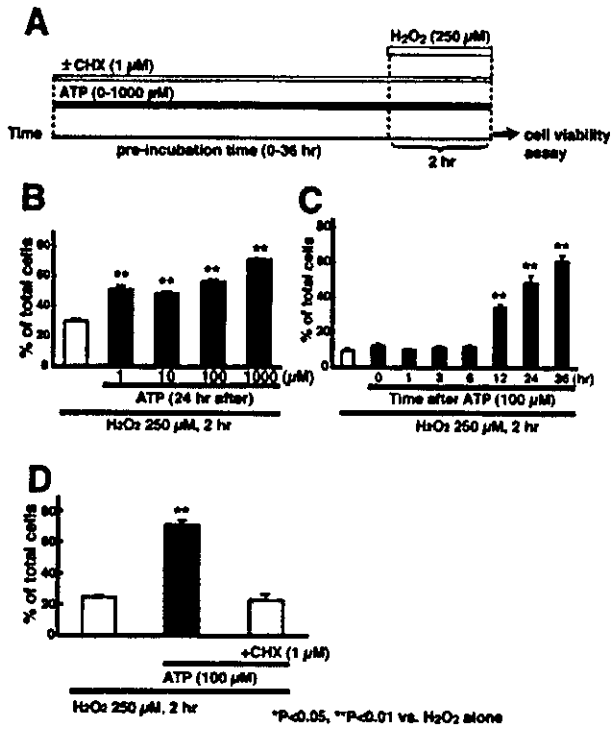


Fig. 2. Protection by ATP of $H_2O_2$-evoked cell death in astrocytes. A: Stimulus regime. B: ATP protected against $H_2O_2$-evoked cell death in a concentration-dependent fashion. Cells were incubated with ATP (1–1,000 μM) 24 h before and during $H_2O_2$ application. C: Protective effect of ATP was dependent on the duration of preincubation. Cells were incubated with ATP (100 μM) for various periods (from 0 to 36 h), and then exposed to $H_2O_2$ together with ATP. Without any preincubation periods, ATP did not show any significant protective effect. D: Inhibition by CHX of the ATP-induced protection. Incubation of cells with CHX (1 μM for 24 h) abolished the protective effect of ATP. Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number and the cell viability was expressed as percentage of total cell number. Asterisks show significant difference from the response evoked by $H_2O_2$ alone (*$P < 0.05$, **$P < 0.01$, Student's $t$-test).

tion. Pretreatment with ATP significantly inhibited the $H_2O_2$-induced cell death in a concentration-dependent manner over a concentration range of 1–1,000 μM (Fig. 2B). When pretreated for 12–36 h, the $H_2O_2$-induced cell death in astrocytes was significantly reduced to about 60% of control (Fig. 2C). However, ATP did not show any cytoprotective action when the exposure time of ATP was less than 12 h. When astrocytes were pretreated with ATP plus cycloheximide (CHX, 1 μM), a protein synthesis inhibitor, the protection by ATP (100 μM for 24 h) disappeared (Fig. 2D). CHX alone had no effect on the viability of astrocytes (control; 100 ± 6%, CHX 1 μM; 90 ± 6%, n = 6).

Protection by ATP against the $H_2O_2$-induced cell death of astrocytes was evaluated pharmacologically. As shown in Figure 3B, when the P2 receptor antagonists suramin (100 μM), PPADS (300 μM), and RB2 (10 μM) were added to the cells 15 min before and during ATP (100 μM) application, ATP protection was almost abolished, indicating the involvement of P2 receptors. UTP (100 and 1,000 μM), an agonist of $P2Y_2$ and $P2Y_4$ receptors, α,βmeATP (100 μM), an agonist of $P2X_1$ and $P2X_3$ receptors, had no effect on the $H_2O_2$-evoked cell death (Fig. 3C). Adenosine (10 μM) did not show any protection against the cell death. The $P2Y_1$ receptor agonists 2MeSADP (1 μM) and ADPβS (1 μM) provided significant protection against cell death (Fig. 3D) and the ATP-induced protection was inhibited by the $P2Y_1$ receptor antagonist MRS2179 in a concentration-dependent manner (Fig. 3E). Thus, ATP appears to show its protective action mainly via a $P2Y_1$ receptor-mediated pathway(s) in astrocytes. None of the agonists and antagonists alone had any effect on the cell viability of astrocytes (Fig. 3B–E, gray columns).

We tested whether prolonged ATP is required or a brief exposure of ATP is enough to trigger its protective action in astrocytes. Since the ATP-induced protection is mediated by $P2Y_1$ receptors (Fig. 3), we added the $P2Y_1$ receptor antagonist MRS2179 (1 μM) to the culture medium 15 min before or 30 min after ATP stimulation, and then further incubated for 24 h prior to $H_2O_2$ exposure. MRS2179 reversed the ATP-induced protection only when it was added to the cells 15 min before and during ATP stimulation (MRS2179 15 min before ATP, 33.6 ± 5.9% of total cells, n = 3, $P = 0.91$ vs. $H_2O_2$ alone; MRS2179 30 min after ATP, 62.9 ± 3.5 of total cells, n = 3, $P < 0.05$ vs. $H_2O_2$ alone; Fig. 4B). Furthermore, we analyzed the time-course of ATP degradation in astrocytes. ATP was exogenously applied to astrocytes, and the supernatants were collected at different incubation periods. Exogenously applied ATP (100 μM) was soon metabolized; the concentrations at 5, 15, 30, 60, and 120 min were 76.0 ± 17.8, 18.9 ± 23.7, 1.2 ± 1.0, 0.3 ± 0.36 and 0.02 ± 0.03 μM, respectively (Fig. 4C). Although longer periods (>12 h, see Fig. 2C) were required for the onset of the cytoprotective action, prolonged exposure of ATP was not necessarily required for the protection in astrocytes.

## Intracellular Signaling Cascades Involved in $P2Y_1$ Receptor-Mediated Protection

We investigated the involvement of $P2Y_1$ receptor-mediated intracellular signaling cascades in the protection against the $H_2O_2$-induced cell death in astrocytes. Both the PLC inhibitor U73122 (5 μM) and the rapid intracellular $Ca^{2+}$ chelator BAPTA-AM (25 μM) inhibited the protection by 1 μM 2MeSADP (Fig. 5). The much less active PLC inhibitor U73343 (5 μM) had no effect on the ATP-evoked protection. These chemicals were added to the cells 1 h before and during 2MeSADP-application and were washed away before $H_2O_2$ application. These blockers themselves had no effect on the cell viability under the normal condition (control, 100 ± 3%; U73122, 90 ± 5%; U73343, 92 ± 6%; and BAPTA-AM, 105 ± 3%, n = 6) (Fig. 5B, gray columns) nor affected the $H_2O_2$-induced cell death in

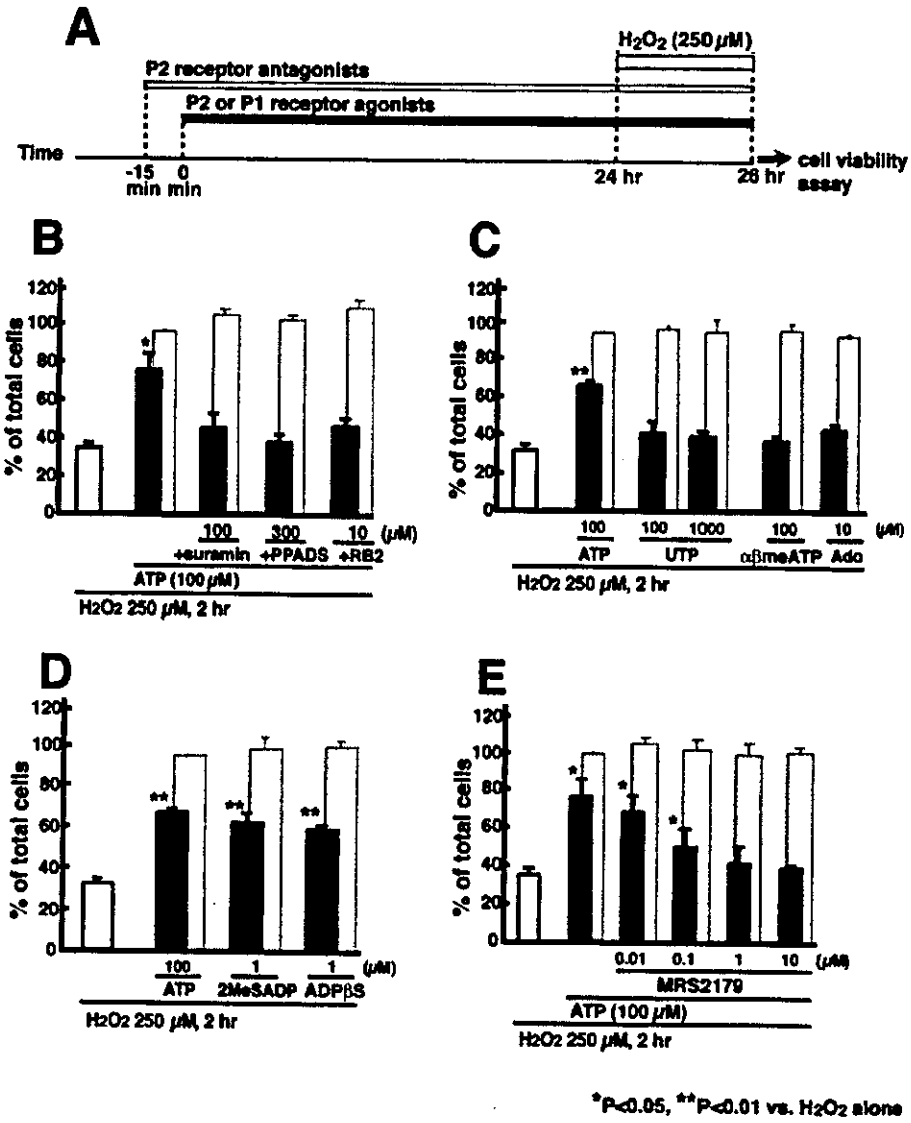


Fig. 3. Effect of P2 receptor agonists and antagonists on the $H_2O_2$-evoked cell death of astrocytes. **A:** Stimulus regime. **B:** Inhibition by P2 receptor antagonists of ATP-induced protection. Suramin (100 μM), PPADS (300 μM) and RB2 (10 μM) reversed the effect of ATP. Cells were incubated with ATP (100 μM) for 24 h before $H_2O_2$ application. Each antagonist was added to the cells 15 min before and during ATP application. **C:** Effect of P1 and P2 receptor agonists. UTP (100 and 1,000 μM), α,βmeATP (100 μM) and adenosine (Ado, 10 μM) had no significant protective effect against the $H_2O_2$-evoked death in astrocytes. **D:** Protection by $P2Y_1$ selective agonists of $H_2O_2$-evoked cell death in astrocytes. The $P2Y_1$ selective agonists 2MeSADP and ADPβS (1 μM) mimicked the cytoprotective effect of ATP. **E:** Inhibition by $P2Y_1$ selective antagonist of ATP-induced protection. The $P2Y_1$ selective antagonist MRS2179 inhibited the effect of ATP in a concentration-dependent manner. Various concentrations of MRS2179 were added to the cells 15 min before and during ATP application. Gray columns show the effects of agonists or antagonists alone on the cell viability in the normal condition. Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number. Asterisks show significant difference form the response evoked by $H_2O_2$ alone (*$P < 0.05$, **$P < 0.01$, Student's $t$-test).

astrocytes ($H_2O_2$ alone, 36 ± 2%; +U73122, 36 ± 1%; +U73343, 34 ± 4%; and BAPTA-AM, 35 ± 13%, n = 6).

We also studied the effect of these blockers on the 2MeSADP-evoked increase in $[Ca^{2+}]_i$ in astrocytes (Fig. 5C). Both BAPTA-AM (25 μM) and U73122 (5 μM) inhibited the 2MeSADP-evoked increase in $[Ca^{2+}]_i$, whereas U73343 (5 μM) did not. BAPTA-AM and U73122 also reduced 2MeSADP-responders (Fig. 5C, open circles). U73122, U73343 and BAPTA-AM were added to the cells 15 min before and during 2MeSADP application.

Glutamate is another important gliotransmitter that leads to an increase in $[Ca^{2+}]_i$ in astrocytes via PLC-linked metabotropic glutamate receptors (Pasti et al., 1997; Porter and McCarthy, 1996). We therefore tested the effect of pretreatment with glutamate on the $H_2O_2$-induced cell death in astrocytes. As shown in Figure 6A, pretreatment of glutamate (100 μM for 24 h) significantly protected the $H_2O_2$-induced cell. Glutamate alone had no effect on cell viability (Fig. 6A, gray column). Interestingly, such protection by glutamate disappeared when the $P2Y_1$ receptor antagonist MRS2179 was added to the cells 15 min before and during glutamate application (Fig. 6B). We further investigated whether exogenously applied glutamate induces the release of ATP from astrocytes and found that it evoked ATP release that lasted for 15 min (Fig. 6C).

## Expression and Function of $P2Y_1$ Receptors in Astrocytes

To elucidate whether $P2Y_1$ receptors are actually expressed and functional in astrocytes, we analyzed the expression of $P2Y_1$ receptors by RT-PCR and mea-

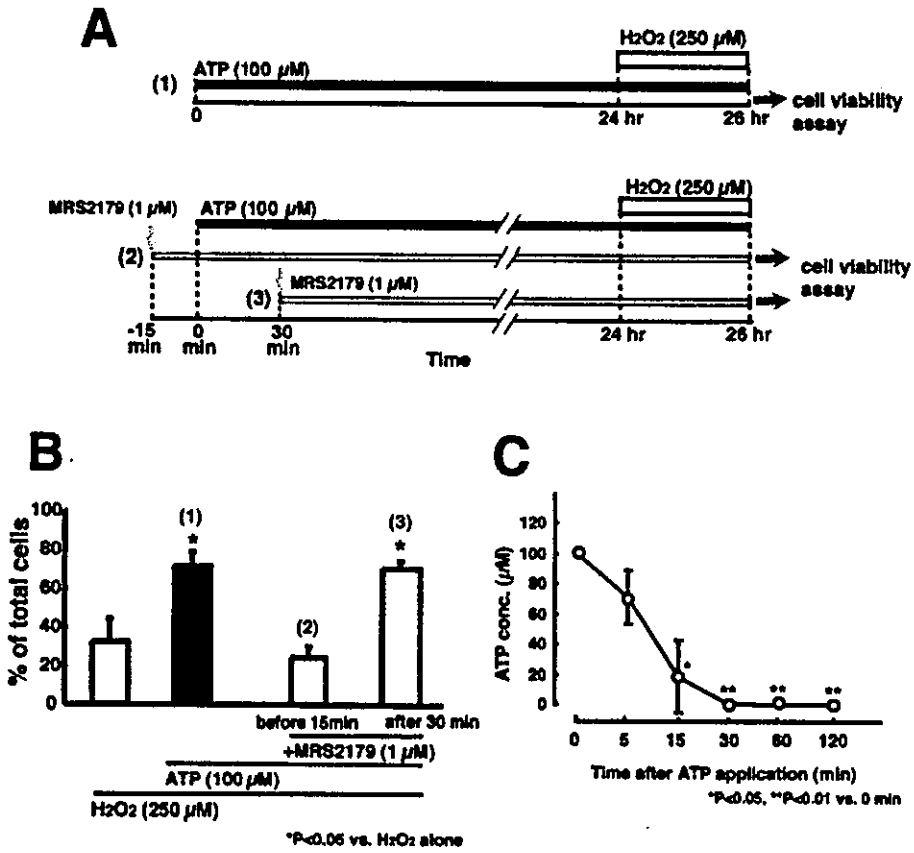


Fig. 4. ATP activates $P2Y_1$ receptors in a short time window. A: (1)–(3) depict the stimulus regime. B: MRS2179 (1 μM) was applied to the cells 15 min before and during ATP application (2), or 30 min after ATP stimulation (3). MRS2179 did not antagonize the effect of ATP when it was applied to the cells 30 min after ATP stimulation (3). Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number. Asterisks show significant differences from the response evoked by $H_2O_2$ alone ($^*P < 0.05$, Student's $t$-test). C: Exogenously applied ATP is metabolized rapidly on astrocytes. Extracellular ATP concentration was measured using a luciferin-luciferase method. ATP (100 μM) was added to the cells, and then supernatant was collected at the time indicated. Exogenously applied ATP was soon metabolized and almost disappeared 30 min after the stimulation. The extracellular ATP concentrations at 5, 15, 30, 60, and 120 min were 76.0 ± 17.8, 18.9 ± 23.7, 1.2 ± 1.0, 0.3 ± 0.36, and 0.02 ± 0.03 μM, respectively. At least three such experiments were performed. Asterisks show significant differences from the ATP concentration at 0 min after 100 μM ATP application ($^*P < 0.05$, $^{**}P < 0.01$, Student's $t$-test).

sured the increases in $[Ca^{2+}]_i$ in astrocytes (Fig. 7). Single RT-PCR analysis revealed that astrocytes express $P2Y_1$ receptor mRNA (Fig. 7A). The $[Ca^{2+}]_i$ analysis showed that ATP (100 μM) evoked an increase in $[Ca^{2+}]_i$ in about 90% of the astrocytes [Fig. 7B(1)], which was independent of the extracellular $Ca^{2+}$ ($0Ca^{2+}$), but was inhibited by the P2 receptor antagonists PPADS (300 μM), reactive blue 2 (RB2) (10 μM), suramin (100 μM), and the $P2Y_1$ receptor antagonist MRS2179 (1 μM) [Fig. 7B(1)]. Similar to ATP, the $P2Y_1$ agonists ADP (100 μM) [Fig. 7B(2)] and 2MeSADP (1 μM) [Fig. 7B(3)] evoked $[Ca^{2+}]_i$ elevations, which were again inhibited by PPADS and MRS2179. Another $P2Y_1$ receptor agonist, ADPβS (1 μM), also produced an increase in $[Ca^{2+}]_i$ (responder, 97 ± 1%; mean amplitude, 0.53 ± 0.03, n = 63). These results suggest that the metabotropic $P2Y_1$ receptor has a dominant role in the $Ca^{2+}$ responses to extracellular nucleotides in astrocytes. UTP, an agonist of UTP-preferring $P_2Y_2/4$ receptors, also evoked an increase in $[Ca^{2+}]_i$ in a concentration-dependent fashion (100–1,000 μM) (Fig. 7C, gray columns) and at 1,000 μM almost all astrocytes responded to UTP (Fig. 7C, open circles). The mean amplitude of the $[Ca^{2+}]_i$ elevation evoked by UTP, however, was less than that evoked by 100 μM ATP (ATP, 0.92 ± 0.04, n = 103 vs. UTP, 100 μM, 0.46 ± 0.03, n = 182; 1,000 μM, 0.69 ± 0.03, n = 167). Neither adenosine nor α,βmeATP, an agonist of $P_2X_1$ and $P_2X_3$ receptors, evoked the $[Ca^{2+}]_i$ elevation in astrocytes (Fig. 7C).

Since glutamate and gap junction are the most probable factors that may affect increases in $[Ca^{2+}]_i$ in astrocytes (Chen et al., 1997; Finkbeiner, 1992; Glaum et al., 1990), the effects of glutamate antagonists and a gap junction inhibitor on the $Ca^{2+}$ responses to ATP were investigated. As shown in Figure 7D, neither the amplitude of the $[Ca^{2+}]_i$ elevations evoked by 100 μM ATP (columns) or the fraction of ATP-responders (open circles) was affected by the gap junction inhibitor 1-octanol (500 μM), the NMDA receptor antagonist AP-V (100 μM), the AMPA receptor antagonist CNQX (30 μM) or the metabotropic glutamate receptor antagonist MCPG (300 μM). All inhibitors were applied to the cells 15 min before and during ATP application.

## Gene Expression Changes by ATP

To show the effect of ATP on the gene expression of astrocytes, we investigated the differential gene expression induced by ATP in astrocytes using Affymetrix GeneChip. We analyzed ATP-induced genes based on the information obtained from Genbank, Unigene, Locuslink, and PubMed at NCBI. As expected

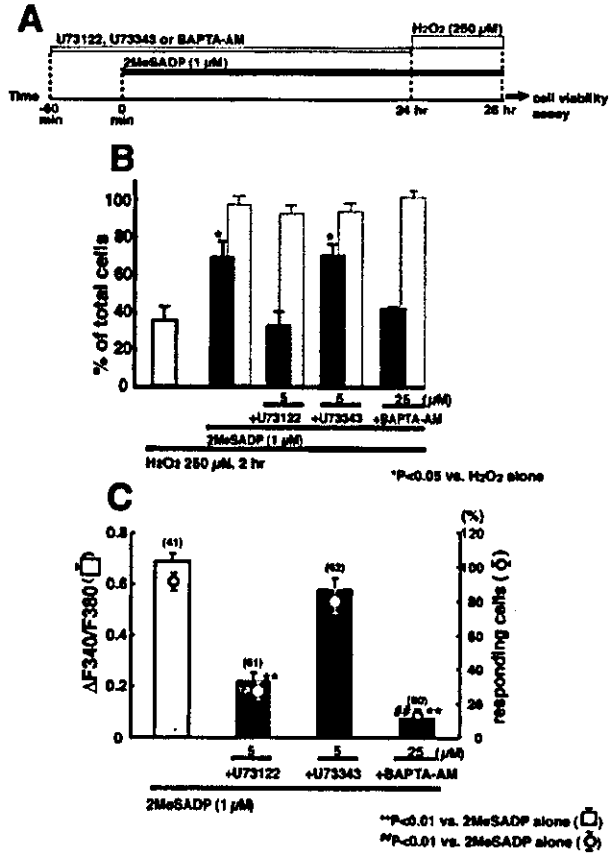


Fig. 5. Intracellular signaling cascades associated with $P2Y_1$ receptor-mediated cytoprotective action. **A:** Stimulus regime. **B:** When cells were pretreated with U73122 (5 μM) or BAPTA-AM (25 μM), the 2MeSADP (1 μM)-induced protective action against $H_2O_2$ disappeared, whereas U73343 (5 μM), a much less active PLC inhibitor, had no effect on it. 2MeSADP was added to the cells 24 h before and during $H_2O_2$ application, and each inhibitor was added 1 h before and during 2MeSADP application. These inhibitors were washed out just before $H_2O_2$ application. Gray columns show the effects of 2MeSADP alone or inhibitors alone on the cell viability in the normal condition. Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number. Asterisks show significant differences from the response evoked by $H_2O_2$ alone (*$P < 0.05$, ** $< P < 0.01$, Student's $t$-test). **C:** Increases in $[Ca^{2+}]_i$ evoked by 2MeSADP, showing the effects of the blockers listed in B. The increase in $[Ca^{2+}]_i$ evoked by 2MeSADP (1 μM) was inhibited by U73122 (5 μM) or BAPTA-AM (5 μM) but not by U73343 (5 μM). These inhibitors were added to the cells 15 min before and during 2MeSADP application. The increases in $[Ca^{2+}]_i$ (Δ340/F380) and fraction of responders are shown as columns and open circles, respectively. The number of cells tested is shown in parentheses. Asterisks show significant difference from the amplitude of $[Ca^{2+}]_i$ and the number of responders evoked by 2MeSADP alone, respectively ($[Ca^{2+}]_i$, *$P < 0.05$, **$< P < 0.01$ vs. 2MeSADP alone; numbers of responders, #$P < 0.05$, ##$P < 0.01$ vs. 2MeSADP alone; Student's $t$-test).

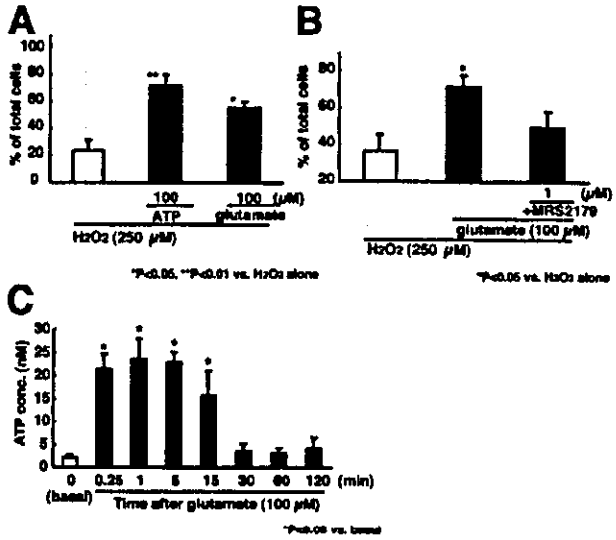


Fig. 6. Effects of glutamate on the $H_2O_2$-induced cell death in astrocytes. **A:** Preincubation with glutamate protected astrocytes against $H_2O_2$-evoked cell death. Cells were incubated with glutamate (100 μM) for 24 h, and then was exposed to $H_2O_2$. Glutamate showed significant protection against $H_2O_2$-induced cell death. Gray columns show the cell viability after a 24-h incubation with agonists alone. Glutamate itself had no effect on the cell viability. Asterisks show significant differences from the response evoked by $H_2O_2$ alone (*$P <$ 0.05, **$P < 0.01$, Student's $t$-test). **B:** Selective $P2Y_1$ receptor antagonist MRS2179 inhibited the protective effect by glutamate. MRS2179 (1 μM) was added to the cells 15 min before and during glutamate application. Asterisks show significant differences from the response induced by $H_2O_2$ alone (*$P < 0.05$, Student's $t$-test). **C:** Glutamate (100 μM) produced release of ATP from astrocytes. Cells were incubated with 100 μM glutamate for the time indicated, the supernatants were collected, and then extracellular ATP concentrations were measured using a luciferin-luciferase method. Significantly higher ATP concentration above basal was observed from 0.25 to 15 min after glutamate (100 μM) stimulation. Asterisks show significant differences from the basal extracellular ATP concentration (*$P < 0.05$, Student's $t$-test).

from the previous results, ATP induced a dramatic upregulation of oxidoreductase genes such as TrxR, CBR, and SHL4 (similar to superoxide dismutase SOD-2) (Table 1). These genes were classified on the basis of information from Gene Ontology Consortium (http://www.geneontology.org/). Using a quantitative RT-PCR method, we confirmed that these oxidoreductase genes including TrxR, CBR and SHL4 were upregulated by ATP (100 μM, 2 h). The fold increases are shown in parentheses in Table 1 [i.e., CBR (8.9), SHL4 (17.2), and TrxR (2.9)].

## DISCUSSION

The importance of dynamic communication among glial cells in the CNS has been recognized, and astrocytic ATP has a dominant role in such gliotransmission (Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003). In the present study, we demonstrated that such ATP-mediated gliotransmission is important for astrocytic survival because ATP protected astrocytes from $H_2O_2$-induced cell death. This effect was mediated by the activation of $P2Y_1$ receptors but not by adenosine receptors although adenosine, a metabolite of ATP, is well known to protect neurons from various pathological conditions. After the activation of $P2Y_1$ receptors, it took more than 12 h for the protective action to be revealed, and ATP upregulated several "oxidoreduc-

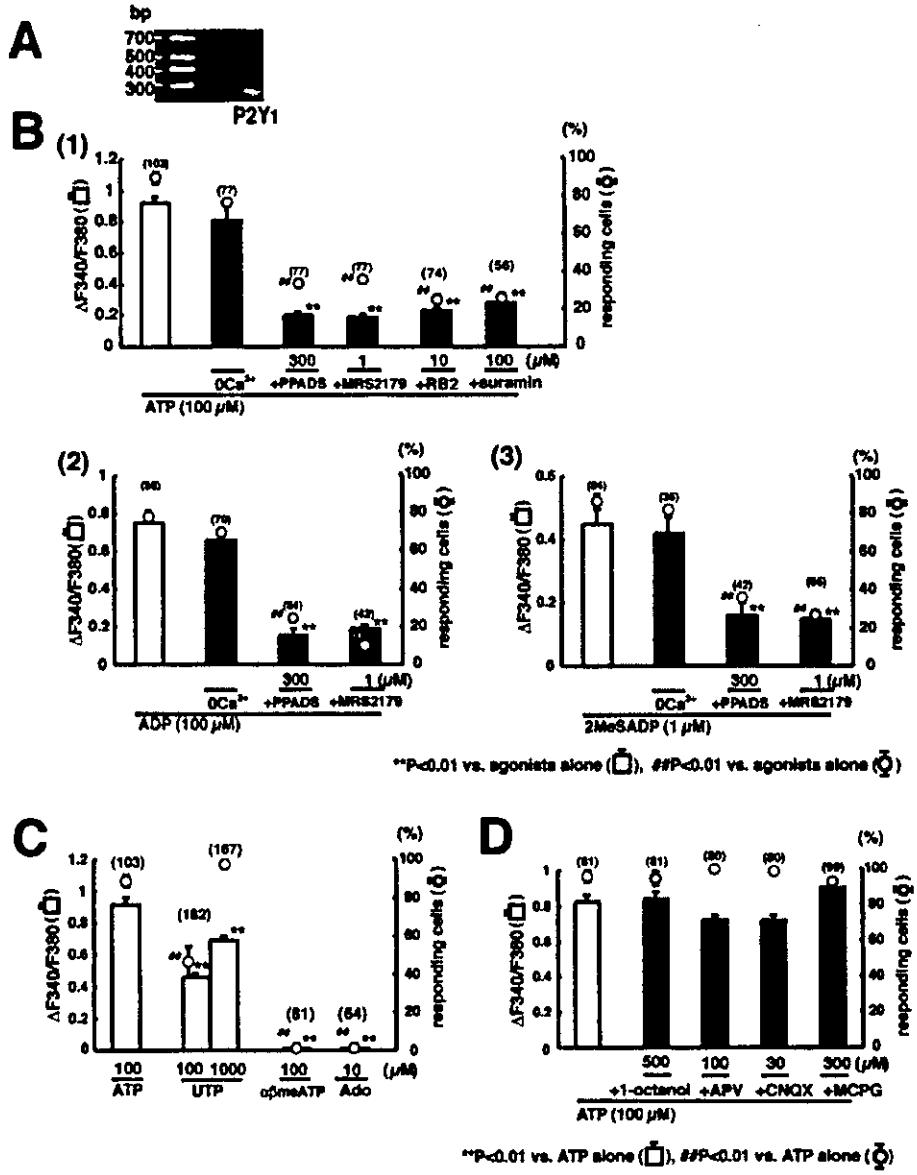


Fig. 7. Expression and function of $P2Y_1$ receptors. **A:** RT-PCR analysis showing expression of $P2Y_1$ receptor mRNA in astrocytes. Total cellular RNA was subjected to RT-PCR. The resulting cDNA was amplified with specific primers for $P2Y_1$ receptors. PCR product was resolved on agarose gel stained by 2% ethidium bromide and visualized under UV light. **B:** Functional $P2Y_1$ receptors are present in astrocytes. (1) ATP (100 μM) produced a rise in $[Ca^{2+}]_i$, which was independent of extracellular $Ca^{2+}$ ($0Ca^{2+}$), but was inhibited by PPADS (300 μM), MRS2179 (1 μM), reactive blue 2(RB2) (10 μM), or suramin (100 μM). Similar to ATP, (2) ADP (100 μM) and (3) 2MeSADP (1 μM) induced rapid $[Ca^{2+}]_i$ increases, which were independent of extracellular $Ca^{2+}$ but were sensitive to PPADS and MRS2179. **C:** $P2Y_{2/4}$ receptor agonist UTP also evoked elevations in $[Ca^{2+}]_i$ in a concentration-dependent fashion and at 1,000 μM produced $[Ca^{2+}]_i$ increases in almost all astrocytes (97 ± 2%, n = 167), although the amplitude of $[Ca^{2+}]_i$ elevation was significantly lower than that evoked by 100 μM ATP. Neither α, βmeATP (100 μM) nor adenosine (10 μM) produced any $[Ca^{2+}]_i$ elevations. **D:** Effects of glutamatergic receptors and gap junctions on the ATP-evoked increase in $[Ca^{2+}]_i$ in astrocytes. 1-Octanol (500 μM), AP-V (100 μM), CNQX (30 μM) and MCPG (300 μM) were added to the cells 15 min before and during ATP-application. All these antagonists had no effect on the amplitude of $[Ca^{2+}]_i$ or the number of responders. The number of cells examined was shown in parentheses. Columns and circles show the mean amplitude of $[Ca^{2+}]_i$ elevations and percentage of responders, respectively. * and # show significant difference from the amplitude of $[Ca^{2+}]_i$ and the number of responders evoked by agonist alone, respectively ($[Ca^{2+}]_i$, $^*P < 0.05$, $^{**}P < 0.01$ vs. agonist alone; numbers of responders, $\#\#P < 0.01$ vs. agonist alone; Student's *t*-test).

tase genes." Thus, astrocytes use $P2Y_1$ receptor- but not adenosine P1 receptor-mediated signals to upregulate self-protection genes, thereby leading to resistance to oxidative stress.

$P2Y_1$ receptors are expressed in various tissues (Tokuyama et al., 1995; Akbar et al., 1996), including the CNS (Tokuyama et al., 1995; Ayyanathan et al., 1996; Webb et al., 1998; Moore et al., 2000). Astrocytes ex-

*TABLE 1. List of Genes Upregulated by ATP in Astrocytes*

| Identifer | Title | Fold increase (RT-PCR) | Oxidoreductase activity[a] |
|---|---|---|---|
| D89069 | Inducible carbonyl reductase | 6.5 (8.9) | GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD, or NADP as acceptor |
| D89070 | Noninducible carbonyl reductase | 6.8 | GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD or NADP as acceptor |
| X95986 | Carbonyl reductase | 5.9 | GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD, or NADP as acceptor |
| AA926129 | Schlafen-4 (similar to SOD-2) | 3.1 (17.2) | GO:0016721; oxidoreductase activity, acting on superoxide radicals as acceptor |
| U63923 | Tissue type liver thioredoxin reductase | 2.3 (2.9) | GO:0016654; oxidoreductase activity, acting on NADH or NADPH, disulfide as acceptor |

SOD-2, superoxide dismutase-2; RT-PCR, reverse transcription-polymerase chain reaction.
[a]GO ontology defined by Gene Onthology Consortium (http://www.godatabase.org/htdocs.html).

press several types of metabotropic P2Y receptors such as $P2Y_1$ (Ho et al., 1995; Fam et al., 2000) and $P2Y_{2,4,6,12,14}$ (Idestrup and Salter, 1998; Lenz et al., 2000; Fumagalli et al., 2003) as well as ionotropic P2X receptors ($P2X_{1,2,3,4,5,7}$). Our present findings showed that the protective effect by ATP against $H_2O_2$-induced cell death was dependent on both PLC activation and stored $Ca^{2+}$, suggesting that the protective action of ATP works via metabotropic PLC-linked P2Y receptors in astrocytes (Fig. 5B). The pharmacological analysis revealed that the responsible receptors for the protective action were $P2Y_1$ receptor (Fig. 3). In addition to $P2Y_1$ receptors, $P2Y_2$ receptors, another type of PLC-linked P2Y receptor, are also expressed in astrocytes. UTP, however, failed to protect astrocytes from $H_2O_2$-induced cell death (Fig. 3C) in spite of the fact that UTP produced increases in $[Ca^{2+}]_i$ via a PLC-linked mechanism (Shahidullah and Wilson, 1997; Idestrup and Salter, 1998; Viana et al., 1998). Both ATP and UTP activate $P2Y_2$ receptors almost equally (Lustig et al., 1993), whereas ATP activates $P2Y_1$ receptors more potently than UTP, and the $ED_{50}$ for ATP to evoke a $[Ca^{2+}]_i$ elevation is almost 10-fold smaller than that of UTP in astrocytes (Koizumi et al., 2002). Thus, this discrepancy might be explained by the lower affinity of $P2Y_2$ receptors to ATP in astrocytes. In addition, although ATP and the selective $P2Y_1$ agonist 2MeSADP evoked increases in $[Ca^{2+}]_i$ in almost all of the astrocytes, UTP (100 μM) produced the $[Ca^{2+}]_i$ elevation in a smaller population of cells (Fig. 7C). The discrepancy may also be explained by the functional heterogeneity of $P2Y_2$ receptor expression among astrocytes. However, when the UTP concentration was raised up to 1,000 μM, it produced elevations in $[Ca^{2+}]_i$ in almost all astrocytes (Fig. 7C) but still failed to protect against cell death in astrocytes (Fig. 3C). These results suggest that the PLC-linked $Ca^{2+}$ mobilization is required for the ATP-induced cytoprotection but is not sufficient to reveal its protective action. Other than PLC-linked $Ca^{2+}$ mobilization, the $P2Y_1$ receptor might stimulate other pathways closely involved in the cytoprotective action. The finding that, although glutamate could mobilize $Ca^{2+}$ and protect against $H_2O_2$ induced cell death in astrocytes, the glutamate-induced cytoprotection also involved the activation of $P2Y_1$ receptors (Fig. 6) may support this idea.

Since Servitja et al. (2000) showed that $H_2O_2$ activates PLC in astrocytes, previous exposure of ATP might reduce the amount of PLC available during the application of $H_2O_2$, thereby leading to the decrease in $H_2O_2$-induced cell death in astrocytes. Although we cannot exclude this possibility completely, such a PLC reduction, if it occurs, does not seem to be involved in the protective action by ATP for the following reasons. Firstly, activation of $P2Y_1$ receptors by 2MeSADP results in an increase in $[Ca^{2+}]_i$ via PLC-mediated mechanisms. The 2MeSADP-evoked increases in $[Ca^{2+}]_i$ in ATP-treated (24 h) and ATP-untreated control cells were almost identical (ATP treated cells: 0.64 ± 0.05, n = 65; ATP untreated cells: 0.65 ± 0.04, n = 70), suggesting that the $P2Y_1$/PLC-mediated pathway(s) is not affected by ATP pretreatment. Secondly, $H_2O_2$-induced cell death was unaffected by the PLC blocker U73122, suggesting that PLC itself is not involved in the $H_2O_2$-induced cell death ($H_2O_2$ alone; 36 ± 2%, and $H_2O^{2+}$U73122; 36 ± 1% of control). Judging from these findings, it is unlikely that a reduction of PLC is involved in the ATP-evoked protection against $H_2O_2$ in astrocytes.

Cells in the CNS have many chances to be exposed to ATP because ATP is released or leaked from both neurons and astrocytes in physiological and pathological conditions. Extracellular ATP, however, is soon metabolized into adenosine by ectonucleotidases (Zimmermann, 1996), and some ectonucleotidases are upregulated after brain ischemia (Braun et al., 1998) especially in glial cells (Braun et al., 1997). Adenosine therefore is considered one of the major molecules that show neuroprotective effects against several types of neuronal damage in the CNS, such as ischemic/hypoxic brain damage or post-hypoxic repurfusion-evoked neuronal injury (Behan and Stone, 2002; Jones et al., 1998), and Parkinson's disease (Schwarzschild et al., 2003). The main mechanism underlying the adenosine-induced neuroprotection appears to be the inhibition of excess excitability of neurons (Fredholm and Dunwiddie, 1988). In the present study, however, adenosine showed no protective effect against $H_2O_2$-evoked cell death in astrocytes. Although some groups already reported that adenosine protected astrocytes from glucose deprivation-evoked cell death, this protection appeared to be independent of adenosine receptor

activation since the protective action was mimicked by other ATP metabolites, such as AMP, ADP, and inosine, and antagonists to adenosine receptors did not inhibit the effect of adenosine (Schubert et al., 1997; Jurkowitz et al., 1998). This nucleotide/nucleoside-induced protection seems to be due to an inhibition of the decrease in the intracellular ATP levels evoked by glucose deprivation. Instead, it has been reported that adenosine rather induce the cell death of astrocytes via adenosine receptors (Abbracchio et al., 1995; Appel et al., 2001; Di Iorio et al., 2002) without affecting the neuronal cell survival (Ceruti et al., 2000). In addition, adenosine acting on adenosine A3 receptors causes apoptosis in astrocytes (Ceruti et al., 2000; Di Iorio et al., 2002). In contrast, ATP is well known to show trophic effects in astrocytes such as proliferation/gliosis (Brambilla et al., 1999; Neary et al., 1999; Franke et al., 2001b), induce trophic factors such as leukemia inhibitory factor (Yamakuni et al., 2002) and MCP-1 (Panenka et al., 2001) and protect astrocytes against TNF-α-induced cell death (Kim et al., 2003a, b). Thus, unlike neurons, astrocytic survival appears to be mainly controlled by ATP/P2 receptor-mediated but not by adenosine/P1 receptor-mediated pathways. As described above, the responsible receptors for the ATP-induced protective action in astrocytes were $P2Y_1$ receptors. Astrocytes express P2Y receptors (Ho et al., 1995; Idestrup and Salter, 1998; Fumagalli et al., 2003), P2X receptors (Franke et al., 2001a; Fumagalli et al., 2003) and several adenosine receptors as well (Peakman and Hill, 1994; Porter and McCarthy, 1995; Ciccarelli et al., 2001). It appears that ATP and its metabolites have functionally distinct roles in astrocytes.

We demonstrated that the ATP-induced protection of astrocytes required a preincubation period (12–36 h). This may involve two possibilities, namely that prolonged activation of $P2Y_1$ receptors is needed for the protection, or short-time exposure of ATP is enough to trigger the protection but longer periods (>12 h) are required to reveal the protective action. The $P2Y_1$ agonist MRS2179 could not reverse the effect of ATP when it was applied 30 min after ATP stimulation, and exogenously applied ATP was soon metabolized and almost disappeared 30 min after ATP the application (Fig. 4). These findings suggest that exogenously applied ATP should work only for limited periods (30 min), and therefore the short-time effect of ATP should be sufficient to trigger the protective action against $H_2O_2$ in astrocytes.

After the activation of $P2Y_1$ receptors, it took more than 12 h (12–36 h) for the onset of the ATP-induced protective action in astrocytes (Fig. 2C), and the protection was inhibited by the protein synthesis inhibitor CHX (Fig. 2D). These findings suggest that the protection by ATP is mediated by the upregulation of some proteins that are involved in anti-oxidative functions. In fact, DNA microarray analysis and quantitative RT-PCR analysis demonstrated that ATP upregulated oxidoreductase genes such as TrxR, CBR, and superoxide dismutase-like gene (SHL4, SOD-2 like gene) (Table 1). TrxR reduces Trx and is known to be involved in various important antioxidant functions (Eftekharpour et al., 2000). CBR belongs to a class of oxidoreductase proteins that are part of the family of short-chain dehydrogenase reductase (Inazu et al., 1992; Wirth and Wermuth, 1992), and it detoxifies toxic carbonyl compounds. SOD-2 is the mitochondrial form of superoxide dismutase and reduces superoxide anion ($O_2$) to $H_2O_2$ (Furuta et al., 1995). All these upregulated genes are expressed in both neurons and astrocytes, are somehow involved in the protective action against oxidative stress (Rozell et al., 1985; Hansson et al., 1989; Wirth and Wermuth, 1992; Eftekharpour et al., 2000; Forrest and Gonzalez, 2000), and are also known to be increased in some pathological conditions such as Alzheimer's disease (Lovell et al., 2000; Balcz et al., 2001; Kim et al., 2001; Butterfield et al., 2003) and Down syndrome (Balcz et al., 2001; Kim et al., 2001). Interestingly, such an upregulation is observed rather in astrocytes in some pathological conditions or by chemical treatment. For example, the antioxidant response element activator t-butylhydroquinone increases TrxR in astrocytes, but not in neurons (Eftekharpour et al., 2000), and upregulation of SOD-2 in reactive astrocytes is more predominant than that in neurons in Alzheimer's disease brain (Furuta et al., 1995). Astrocytes greatly promote the survival of neurons (Desagher et al., 1996), and also affect neuronal functions (Haydon, 2001). $H_2O_2$ generation is observed in many pathological conditions and can be a trigger of some brain disorders, including ischemic brain damage (Agardh et al., 1991; Lei et al., 1997), Alzheimer's disease (Cuajungco et al., 2000; Huang et al., 2000; Tabner et al., 2001; Tamagno et al., 2003), and Parkinson's disease (Tabner et al., 2001). Thus, the ATP-induced upregulation of oxidoreductase genes and the protection against cell death in astrocytes seen in the present study might be a key event for even neuronal survival, and possibly be involved in these diseases. However, the direct interaction between the upregulation of these oxidoreductase genes and the ATP/$P2Y_1$ receptor-mediated protection of cell death in astrocytes remains to be clarified.

In conclusion, we demonstrated that ATP protected astrocytes from $H_2O_2$-induced cell death via $P2Y_1$ receptor-mediated pathways and that the ATP-induced protection of astrocytes required upregulation of oxidoreductase genes. Unlike neurons, adenosine had no such effect in astrocytes. The precise target genes or mechanisms underlying the $P2Y_1$ receptor-mediated protective actions in astrocytes remain to be clarified. Our present findings suggest that one important role of ATP-mediated gliotransmission would be such a protective effect in astrocytes since ATP is released or leaked when cells in the CNS are damaged in several pathological conditions (Dubyak and el-Moatassim, 1993; Lutz and Kabler, 1997; Ahmed et al., 2000; Zhang et al., 2000; Parkinson et al., 2002).