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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

（副題：ATP受容体を介する新しいインシュリン放出制御機構）

平成17年度　総括研究報告書

主任研究者　井上　和秀

平成18（2006）年　3月

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厚生労働科学研究費補助金研究報告書

平成18年３月３１日

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平成１７年度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）に係る研究事業を完了したので次のとおり報告する。

研究課題名（課題番号）：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（H17・医薬・032）
（副題：ATP受容体を介する新しいインシュリン放出制御機構）

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１．厚生労働科学研究費補助金研究報告書表紙　（別添１のとおり）

２．厚生労働科学研究費補助金研究報告書目次　（別添２のとおり）

３．厚生労働科学研究費補助金総括研究報告書　（別添３のとおり）

４．厚生労働科学研究費補助金分担研究報告書　（別添４のとおり）

５．研究成果の刊行に関する一覧表　　（別添５のとおり）

別添１

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別添２

目　　次

別添3

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（総括研究報告書）

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（副題：ATP受容体を介する新しいインシュリン放出制御機構）

（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
前年度に於いては、臨床上使用されているカルシウムチャネル拮抗薬が明らかにβ細胞からのインスリン分泌を抑制することを示し、更にATP受容体を介したインスリン分泌を促進する経路が存在する可能性を提示した。本年度はATPのラ氏島培養液中への添加、更にはATPγSやATP受容体アゴニストを用いた種々の実験を詳細に行い、その結果、ATPを介したインスリン分泌促進経路があることは明らかとなったが、ATPの作用メカニズムはかなり複雑であり、作用を及ぼす周囲環境・諸条件を明確にした上で検討をしなければならないと考えられる。

分担研究者　永松　信哉
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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。

初年度には、臨床上すでに用いられている、カルシウム拮抗降圧薬ニフェジピンの膵β細胞における作用を検討し、10μMニフェジピンはグルコース刺激による[Ca2+]iの上昇とインスリン分泌を抑制することを明らかにした。本年度は、さらに新しいインスリン分泌制御メカニズムの検討を行った。

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incu

bation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

汎用糖尿病治療薬・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、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ではないと考えられる。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受容体刺激により、インスリン分泌にどのような影響がでるかを検討した。100・M ATP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。

ついで、種々の濃度のブドウ糖にATPを加えたラ氏島batch実験を行った。その結果、低濃度グルコース存在下にてはATPのインスリン分泌促進反応は見られなかったが、200mg/dlグルコース存在下においてのみATPはインスリン分泌をATP非存在下の約30％増強した。

次に、ヒト２型糖尿病のモデル実験動物であるGKラット膵β細胞を用いてATPの効果を検討した。GKラット膵ラ氏島においては200mg/dlグルコース存在下でのATPの効果はみられなかった。更に、定量的PCR法を用いてATP受容体の遺伝子発現をGKラット膵ラ氏島を用いて検討したところ生後5週齢、6週齢どちらに於いても、P2X1、P2Y1のmRNAの発現は対照群ラットラ氏島での約半分以下にまで低下していた。他のATP受容体mRNAについては今後検討する予定である。

D.考察

前記のごとく、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ではないと考えられる。L型カルシウムチャネルが関与しない機序でインシュリン放出を制御するメカニズムが明らかになれば、新規治療薬の開発につながる。このような背景から、ATP受容体によるインスリン分泌制御の可能性をもとめて研究がスタートし、まずはATP受容体刺激によるインスリン分泌にどのような影響がでるかを検討したが、インスリン分泌に対するATPの効果は条件次第で多様に変化してしまう。その理由の一つに、ATPが培養液中にて分解されている可能性を考えては非水解型ATPγSを用いて検討し、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。また、血液中ブドウ糖濃度がATPのインシュリン放出に対する効果を変化させている可能性も考えられたので、各種ブドウ糖濃度にATPを加えたラ氏島batch実験を行った結果、低濃度グルコース存在下にてはATPのインスリン分泌促進反応は見られなかったが、200mg/dlグルコース存在下においてのみATPはインスリン分泌をATP非存在下の約30％増強した。これで決着を見たかに思えたが、実はモデル細胞Min6では逆の効果が予備試験ながら認められている。まだまだ隠されている事実が示唆され、容易に結論が出ない。一方、II型糖尿病モデルと考えられているGKラット膵β細胞では、また異なったATPの効果が認められた。定量的PCR法を用いてATP受容体の遺伝子発現をGKラット膵ラ氏島を用いて検討したところ生後5週齢、6週齢どちらに於いても、P2X1、P2Y1のmRNAの発現は対照群ラットラ氏島での約半分以下にまで低下していた。しかし、他のATP受容体mRNAについては不明であるし、mRNA発現量での議論は危険であるために、実際に受容体タンパク発現量を測定する必要がある。これは次年度の検討課題としたい。このように、ATP受容体を介するインシュリン放出メカニズムは存在するが、そのメカニズムの詳細は未だ明らかにされていないので、臨床応用にはより明確な研究進展が必要である。

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、インスリン放出に対するATP受容体の影響を、分子レベルから解析することが必要であるが、ATP受容体によるインシュリン放出制御のメカニズムは当初考えていたものよりも遙に複雑で、容易に結論出来ないものである。更なる研究の深まりが望まれる。

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

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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
前年度に於いては、臨床上使用されているカルシウムチャネル拮抗薬が明らかにβ細胞からのインスリン分泌を抑制することを示し、更にATP受容体を介したインスリン分泌を促進する経路が存在する可能性を提示した。本年度はATPのラ氏島培養液中への添加、更にはATPγSやATP受容体アゴニストを用いた種々の実験を詳細に行い、その結果、ATPを介したインスリン分泌促進経路があることは明らかとなったが、ATPの効果は限局的であるという結論に達した。一方、前年度に開発した新しいインスリン分泌測定装置であるTIRFシステムを用いてATP受容体を介する以外の種々のインスリン分泌経路についても検討した。その結果、新規糖尿病治療薬であるグリニド製剤は、少なくともSU受容体を介さない別ルートでインスリン分泌を促進するため、Caチャネル拮抗薬と併用しても有用な血糖降下剤として作用することが明らかとなった

A.研究目的

潜在患者数を含めると既に1620万人を超すと言われている糖尿病は、動脈硬化症の基礎疾患であり、更に、高血圧症が多くの患者に併発していることから、心脳血管障害を引き起こす最大の危険因子である。血糖のコントロールが、そのリスクを引き下げる最大の治療であるが、現時点では糖尿病治療薬の種類は限られている。新規糖尿病薬の開発のためにも新しいインスリン分泌経路の発見は必須の課題であり、本年度は、ATP受容体活性薬の糖尿病治療薬としての可能性、ならびに、新しい画像解析法であるTIRFシステムを用いて新しいインスリン分泌経路の解明を行う。

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、TIRF、分泌実験に用いた。
2.新たなインスリン分泌測定法であるTIRF法によるインスリン分泌経路の解明 ； 従来のRIA法を用いたインスリン分泌の測定解析では限られた情報が得られるのみなので、単一インスリン顆粒の放出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細胞に導入し、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.TATシステムによる形質膜分子とインスリン顆粒の分子間相互作用解析 ； TAT融合抗体を作製し、これをCy3標識し、インスリン顆粒との相互作用をTIRF解析した。
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) ATP受容体を介したインスリン分泌 ： 100μM ATPを用いた膵β細胞perifusionシステムによる実験を繰り返したが、その方法ではATPのインスリン分泌能に与える影響についてはっきりしなかったため、種々の濃度のブドウ糖にATPを加えたラ氏島batch実験を行った。その結果、低濃度グルコース存在下にてはATPのインスリン分泌促進反応は見られなかったが、200mg/dlグルコース存在下においてのみATPはインスリン分泌をATP非存在下り約30％増強した。次に、ヒト２型糖尿病のモデル実験動物であるGKラット膵β細胞を用いてATPの効果を検討した。GKラット膵ラ氏島においては200mg/dlグルコース存在下でのATPの効果はみられなかった。更に、定量的PCR法を用いてATP受容体の遺伝子発現をGKラット膵ラ氏島を用いて検討したところ生後5週齢、6週齢どちらに於いても、P2X1、P2Y1のmRNAの発現はcontrolウィスターラットラ氏島での約半分以下にまで

低下していた。

前年度の報告書にも記した如く、臨床的に高血圧治療薬として使用されているL 型-電位依存性Caチャネルブロッカーは、グルコース刺激インスリン分泌を著明に抑制する。一方、ATP受容体を介したインスリン分泌経路は、L型Ca2+チャネルを介さない可能性があり、この解決策になると考えられたが、GK糖尿病ラットを用いたRT-PCRの結果から、糖尿病においてATP受容体も減少していているのならば、必ずしもこの経路が有力な解決策とも言い難い。そこで昨年度に開発した新たなインスリン分泌測定法であるTIRFシステムを用いて、SNARE蛋白質を介したインスリン放出経路についての詳細な解析を行うことにより、L型Caチャネルを介さない可能性のあるインスリン分泌経路の模索を行った。

(2) TIRFシステムを用いたインスリン分泌機構の研究 ：単一インスリン顆粒の時間的空間的動態を解析するために、前年度までに開発したinsulin-GFP発現アデノウィルスベクターを用いて、初代培養膵β細胞のインスリン顆粒を標識し、TIRFシステムによりインスリン顆粒の蛍光変化を msオーダーにて追跡した。一方、SNARE蛋白質の代表格であるsyntaxin 1Aは、開口放出分子として細胞膜に局在する必須エレメントとして知られている。そこでsyntaxin 1Aとインスリン顆粒との分子間相互作用を検討するために、生きたβ細胞に於いて、syntaxin 1Aを標識し、同時にインスリン-GFPにてインスリン顆粒を標識することにより、これらの空間的時間的動態並びに相互作用を観察した。すなわち、抗syntaxin 1A抗体をCy3で標識した後にTATペプチドを融合したCy3-TAT融合抗体を作製し、これを用いて生細胞におけるsyntaxin 1を標識した。その結果、インスリン顆粒はsyntaxin 1クラスター上に局在しており、ブドウ糖刺激では、syntaxin 1クラスターは変化せず、インスリン顆粒のみがfusion、そして消失する像がみられた。一方、GKラット糖尿病β細胞ではsyntaxin 1クラスターが激減するため、膜に結合しているインスリン顆粒数が減少し、その結果としてインスリンのfusionが減少していた。ところがアデノウィルスを用いてsyntaxin 1クラスターを正常状態に回復させると、膜に結合しているインスリン顆粒数が増加、その結果としてインスリンのfusionも増えることから、SNARE蛋白質を標的とした新しい糖尿病治療薬の開発が、新規糖尿病治療としての候補と考えられる。

K_{ATP}チャネルにcouplingしているSUR1受容体を標的とするスルホニルウレア剤は、最終時にL型Caチャネルを活性化させることによりインスリン分泌を促進する作用機構を有しているため、高血圧治療薬として臨床的に用いられているCa拮抗剤であるニフェジピンが存在していると、細胞内Ca2+上昇を引き起こすことが出来ずに、インスリン分泌を促進させることか出来ない。最近、スルホニルウレア剤とは構造が違う新しいタイプのインスリン分泌促進剤(グリニド剤)が臨床上においても使用され始めた。この薬剤の作用機構の一部はL型Ca2+チャネルを介しているが、一方、Caチャネルを介さない、インスリン分泌促進経路が存在すると考えられる。そこでTIRFシステムを用いてグリニド剤のβ細胞内での作用機構について検討した。グリニド剤は、ダイアゾキサイド前処理して、L型Caチャネルの活性を完全にブロックした状態にても、細胞内由来のインスリン顆粒(newcomer)からのインスリン放出を引き起こした。すなわち、グリニド剤によるインスリン分泌促進は、高血圧治療に用いられるニフェジピンが存在している場合でも、非常に有効にインスリン分泌を引き起こすことができる新しいタイプの糖尿病治療薬であることが、TIRFを用いた薬剤の分子作用機構解明の研究結果から明らかにすることが出来た。

D.考察

一般的糖尿病治療薬であるスルホニルウレア剤にとって代わることが出来る新しい血糖降下剤の可能性としてATP受容体の活性薬を考えたが、ATP受容体を介したインスリン分泌促進作用は限局的であったため必ずしも有効な薬剤とはなり得ない様である。一方、新しいインスリン分泌経路を見出すために必要とされた新しいインスリン分泌測定法であるTIRFイメージング法を確立し、これを用いたインスリン分泌機構の研究からは、新知見を得ることが出来た。すなわち、２型糖尿病モデルラットであるGKラットβ細胞におけるインスリン分泌第一相が低下する主因に、SNARE蛋白質が大きく関与ししていること、又、新規糖尿病治療薬であるグリニド剤は、SUレセプターを介さず、newcomerインスリン顆粒からのインスリン放出を引き起こすことが明らかとなった。

E.結論

新しいインスリン分泌経路としてATP受容体を介する経路につき詳細な検討を行ったところATPはインスリン分泌を促進するものの、効果は限定的であるため、現時点においては、ATP受容体活性薬は、糖尿病治療薬の候補としては最適ではない。一方、新しい画像解析システムによるインスリン分泌測定法であるTIRF解析法を確立し、この方法を用いることにより、新規糖尿病治療薬の開発に必須であるインスリン分泌及び糖尿病におけるインスリン分泌及び糖尿病におけるインスリン分泌障害機構の解明を行うことが可能となった。

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。

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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

雑誌

氏 名	タイトル	発表雑誌名	巻 号	ページ	出版年
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Involvement of β1 Integrin in Microglial Chemotaxis and Proliferation on Fibronectin: Different Regulations by ADP Through PKA

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KEY WORDS
PKA; ATP; ADP; purinergic; glia; extracellular matrix; migration

ABSTRACT
Microglia are immune cells in the brain; their activation, migration, and proliferation have pivotal roles in brain injuries and diseases. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated. Extracellular nucleotides can serve as potent signaling molecules. Recently, ATP and ADP were revealed to possess chemoattractive properties to microglia via Gi-coupled P2Y receptors. In the present study, we report that the ADP-induced chemotaxis of microglia is mediated by P2Y12/13 receptors and is β1 integrin-dependent in the presence of fibronectin. Signals from P2Y12/13 receptors also cause β1 integrin translocation to the membrane ruffle regions, but this redistribution was lost when the intracellular cyclic AMP (cAMP) was increased by forskolin or dibutyryl cAMP. This inhibitory effect of cAMP-elevating agents did not appear when microglia were co-incubated with a protein kinase A (PKA) inhibitor, KT-5720, suggesting that PKA is a negative regulator of the β1 integrin translocation. We also show that the engagement of β1 integrin enhanced microglial proliferation. Signals from P2Y12/13 receptors attenuated the proliferation, whereas ADP itself had no effect on microglial growth. Furthermore, β1 integrin-induced proliferation is positively regulated by the cAMP-dependent PKA. Together, these results indicate the involvement of β1 integrin in microglial proliferation and chemotaxis, both of which have clinical importance. The data also suggest that PKA is inversely involved in these two cellular functions.

INTRODUCTION

Microglia are considered to act as brain macrophages. They participate in brain injuries and diseases (Nakajima and Kohsaka, 1993), in which their motility, aberrant activation, and proliferation are known to play crucial roles. Microglia quickly respond to numerous inflammatory mediators by migrating to the source of the mediators, where they become activated and exert their neuroprotective effects (Hanisch, 2002; Streit, 2002). Unfortunately, their hyperactivation often leads to neurotoxicity instead, and several pathological conditions in the CNS are, in fact, accompanied by an excess proliferation of microglia (Gehrmann et al., 1995). Thus, better understanding of the regulation of microglial chemotaxis and proliferation may have important therapeutic implications.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits; they mediate cell–cell and cell–extracellular matrix (ECM) interactions. At present, 16 α and 8 β chains have been identified, and at least 22 different complexes are known. β1 integrin, the most ubiquitous β subunit, pairs with at least 10 different α chains to comprise receptors for a wide variety of ECM proteins. Within the CNS, β1 integrin is expressed on many different cell types, including neurons, glial cells, and endothelial cells (Pinkstaff et al., 1999). As shown previously by other investigators, microglia express several integrin receptors of β1 and β2 families that are upregulated following microglial activation in vitro (Hailer et al., 1996; Yu et al., 1998; Kloss et al., 2001; Milner and Campbell, 2003). In vivo, integrin expression is found to be increased on activated microglia in Alzheimer's disease (Akiyama and McGeer, 1990), after nerve injuries (Coyle, 1998; Kloss et al., 1999; Tsuda et al., 2003) and in multiple sclerosis lesions (Bo et al., 1996). Integrins serve not only as adhesive molecules but also as signaling receptors, and they regulate numerous cellular functions (Hemler, 1998) including cell migration and proliferation. β1 integrin is closely associated with the regulation of cell motility and growth in many cell types (Hynes, 1992; Jones and Watt, 1993; Howlett et al., 1995), but its role in microglial chemotaxis and proliferation remains unclear.

Fibronectin is one of the ECM molecules; it is a large, multidomain glycoprotein that exists both as a cell surface protein and in plasma. The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the

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normal CNS. Some ECM molecules, including fibronectin, however, are upregulated after adult CNS injury. Fibronectin also exists at high concentrations in the blood plasma and a breakdown of the blood-brain barrier should result in an increase in its local concentration in the CNS. Fibronectin induces firm adhesion and activation of microglia (Milner and Campbell, 2002, 2003). It is a major ligand of the β1 integrin family, but recently Mac-1 was also reported to play a role in the adhesion of leukocytes to fibronectin (Lishko et al., 2003).

Previously, Honda et al. (2001) demonstrated that extracellular ATP and ADP could induce the chemotactic migration of microglia. Several lines of evidence so far have indicated that extracellular nucleotides serve as signaling molecules (Bodin and Burnstock, 2001). ATP, and possibly other nucleotides, are released from damaged cells or secreted via nonlytic mechanisms and activate microglia. In the work by Honda et al. (2001), extracellular ATP and ADP induced chemotaxis as well as membrane ruffling, which was possibly mediated by Gi-coupled P2Y receptors. The P2Y12 receptor is a recently cloned Gi-coupled P2 receptor (Hollopeter et al., 2001), and expressed on platelets and exclusively in microglia in the brain (Sasaki et al., 2003). The P2Y13 receptor is another Gi-coupled P2 receptor that was recently identified (Communi et al., 2001; Zhang et al., 2002). Its messenger RNA is expressed at highest levels in the brain and immune tissues, particularly the spleen (Zhang et al., 2002), suggesting its roles in neuron and in immune systems. P2Y12 and P2Y13 receptors present a very similar pharmacological profile. Both receptors show high affinities for ADP and 2MeSADP (Hollopeter et al., 2001; Zhang et al., 2002) and are selectively blocked by ARC-67085 and ARC-69931 (Ingall et al., 1999). No specific agonists/antagonists are known currently to distinguish these two receptors pharmacologically.

The involvement of β1 integrin in chemotaxis and proliferation is already well characterized. Its role in microglial chemotaxis and proliferation, however, has not been well studied, and its correlation with purinoceptors such as P2Y12/13 receptor is still unclear. We provide new evidence that (1) ADP-induced chemotaxis through P2Y12/13 receptors involves β1 integrin in the presence of fibronectin, (2) ADP induces β1 integrin redistribution which colocalizes with membrane ruffling on microglia, and PKA functions as a negative regulator of this translocation, (3) β1 integrin mediates microglial proliferation through positive regulation of PKA, and (4) signals from P2Y12/13 receptors abrogate the proliferative effect of β1 integrin. Taken together, these results indicate that β1 integrin is crucially involved in both the proliferation and chemotaxis of microglia, which are under the inverse regulation of PKA.

Abbreviations

ATP	adenosine 5′-triphosphate
ADP	adenosine 5′-diphosphate
cAMP	adenosine 2′:3′-cyclic monophosphate
CNS	central nervous system
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
CX_3CR	CX_3C chemokine receptor
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GPCR	G-protein-coupled receptor
IL-8	interleukin-8
MAP	mitogen-activated protein
2MeSADP	dimethylthioadenosine 5′-diphosphate
PKA	protein kinase A
VLA	very late antigen

MATERIALS AND METHODS

Isolation of Microglia

Rat primary cultures were derived from the cerebral cortex of neonatal Wistar rats. In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and DNase, and then centrifuged to remove dead cells. The pellet was resuspended in DMEM, filtrated and cultured in medium with 10% fetal bovine serum for 12–23 days. Microglia were isolated on day 10 and day 15 by gently shaking of the flasks for 2 min and were attached to appropriate dishes or coverslips. One flask (75 cm^2) yielded 1–2 × 10^6 microglial cells by this preparation, and the cultures were of >98% purity. The purity of microglial culture was determined by immunostaining for Ox-42 and Eva-1.

Immunofluorescence Staining of Cell Surfaces

Microglia were washed once with ice-cold staining buffer [phosphate-buffered saline (PBS) 1% fetal calf serum (FCS), 0.1% NaN_3] and then Fc blocked for 15 min on ice. After washing, they were incubated with Ha2/5 (PharMingen) for 30 min on ice. They were washed once, incubated with mouse anti-hamster IgM (PharMingen) for 30 min on ice, and then washed again. Finally, cells were incubated with Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) for 30 min on ice in the dark, washed twice, resuspended in the staining buffer, and the fluorescence intensity of the labeled microglia was analyzed with FACScan (Becton Dickinson).

Immunocytochemistry

Coverslips were briefly treated with hydrochloric acid and extensively washed with PBS. They were then coated with fibronectin (Sigma, St. Louis, MO) at 10 μg/ml overnight and washed with PBS immediately before use. Microglia were plated on coverslips and kept at 37°C for 1 h, and unattached cells were washed off gently with warm DMEM. After 1-h serum starvation, the cells were stimulated with ADP (50 μM) for 5 min at 37°C. The attached cells were then fixed in 3.7% formaldehyde in PBS for 5 min and then washed with PBS. The cells were permeabilized with 0.1% Triton-X in PBS for 5 min, washed again with PBS, and then blocked for 30 min with ACE blockase (Yukijirushi, Ltd.) with 3% goat serum at room temperature. To visualize β1 integ-

rin and membrane ruffling, the cells were stained with Ha2/5 mAb, mouse anti-hamster IgM Ab, Alexa-Fluor 488-conjugated goat anti-mouse IgG, or Texas Red-X phalloidin (Molecular Probes) and observed under a fluorescent microscope.

Defining Membrane Rufflings

To the best of our knowledge, there are no methods to quantify the degree of membrane ruffling. Therefore, we tried to define membrane ruffling by analyzing the pictures from immunocytochemistry. First we pictured cells that were stained with Texas Red-X phalloidin, and then a line was drawn across the cell and the intensity of the Texas Red-X phalloidin labeling along the line was analyzed using computer software. Membrane ruffling is characterized by the sharp, strong labeling of Texas Red-X phalloidin, which reaches >200 a.u. (out of 256) of intensity, whereas cells with no ruffling show less intense, blurred, and dispersed staining. We defined membrane ruffling as a wave-like structure that is stained with phalloidin and the labeling intensity of which reaches >200 a.u.. Cells satisfying these criteria were construed as bearing membrane ruffling morphology.

Chemotaxis Assay

Chemotaxis assays were performed using a direct-viewing Dunn chemotaxis chamber (Weber Scientific International, Teddington, UK). The details concerning this apparatus and its use are given in Webb et al. (1996) and Zicha et al. (1991). In brief, microglia were attached to coverslips that had been coated with fibronectin. After 1-h incubation, the cells were washed with warm DMEM and cultured in the absence of FCS for an additional 1 h. The coverslips were then inverted onto the slide, the inner and the outer wells of which were filled with DMEM. The edges of the coverslip were tightly sealed with adhesive tape except for one on which a thin filling slit was left. Using a needle and a syringe, the medium in the outer well was gently replaced with DMEM containing 100 μM ADP. The filling slit was quickly sealed with adhesive tape and the chamber was carefully set on the stage of a microscope equipped with a 10× phase-contrast objective. Microglia adhered to the coverslip were exposed to the ADP gradient and then monitored in the Dunn chemotaxis chamber for a period of 1 h. One region of the bridge was viewed directly via a CCD video camera and the data were recorded every 30 s during the 1-h observation using image software. After recording, cells were randomly selected from a set area of the field and the straight distance they migrated was plotted against x,y coordinates on scatter diagrams. The x-axis was parallel to the outer ring while the y-axis was vertical. Recording of the cell migration usually started within 30 min of assembling the chamber, by which time a linear diffusion gradient had been established (Webb et al., 1996).

Proliferation Assay

In this study, 96-well plates (Corning) were incubated with fibronectin (10 μg/ml), anti-β1 integrin Ab (10 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum (Sigma) overnight at 4°C and washed with PBS before use. For anti-β1 integrin Ab and normal rabbit serum coatings, the wells were pre-coated with goat anti-rabbit IgG (Sigma) for 2 h at 37°C and washed with PBS. Microglia were attached to the wells and cultured in DMEM 4% FCS for 24 h at 37°C. After the incubation, cell survival and proliferation were assayed using an MTT cell growth kit (Chemicon) according to the manufacturer's instructions and also by direct cell counting.

RESULTS

ADP-Induced Chemotaxis of Microglia Is Mediated by P2Y12/13 Receptor and Is Blocked by an Elevation of cAMP

In the previous study by Honda et al. (2001), extracellular ADP induced microglial chemotaxis through Gi-coupled P2Y receptors and the ADP-induced membrane ruffling was inhibited by a P2Y12- and P2Y13-selective antagonist, ARC-69931. First, to make the system closer to pathological conditions in the CNS, we performed the chemotaxis assay on fibronectin substrates. To examine whether microglial chemotaxis toward ADP on fibronectin substrate is also mediated by the P2Y12/13 receptor, ARC-69931 was used in the chemotaxis experiment (Fig. 1). Cultured microglia were adhered to the fibronectin-coated coverslips and their chemotaxis against the ADP concentration gradient was studied with the Dunn chemotaxis chamber (see materials and methods for details). In the absence of the ADP gradient, microglia did not move on the fibronectin-coated coverslips (Fig. 1b). When ADP 100 μM was applied to the system, however, the cells migrated toward the stimulant (Fig. 1a,c). Pretreatment of microglia with ARC-69931 at 10 μM totally blocked the ADP-induced microglial chemotaxis (Fig. 1d), suggesting that the P2Y12/13 receptor is responsible for the microglial chemotaxis toward ADP. Since the activation of P2Y12/13 receptors inhibits adenylate cyclase, the effect of changes in the intracellular cAMP level on microglial chemotaxis was tested. Forskolin is a potent adenylate cyclase activating agent and dibutyryl cAMP is a membrane permeable AMP analogue that activates cAMP protein kinases. When microglia were pretreated with forskolin (Fig. 1e) or dibutyryl cAMP (Fig. 1f), their chemotaxis toward ADP was greatly attenuated.

β1 Integrin Is Involved in the Microglial Migration Toward ADP on Fibronectin Substrate

To study the function of β1 integrin in the P2Y12/13-receptor mediated migration of microglia, we first examined its expression on cultured microglia. Flow cyto-

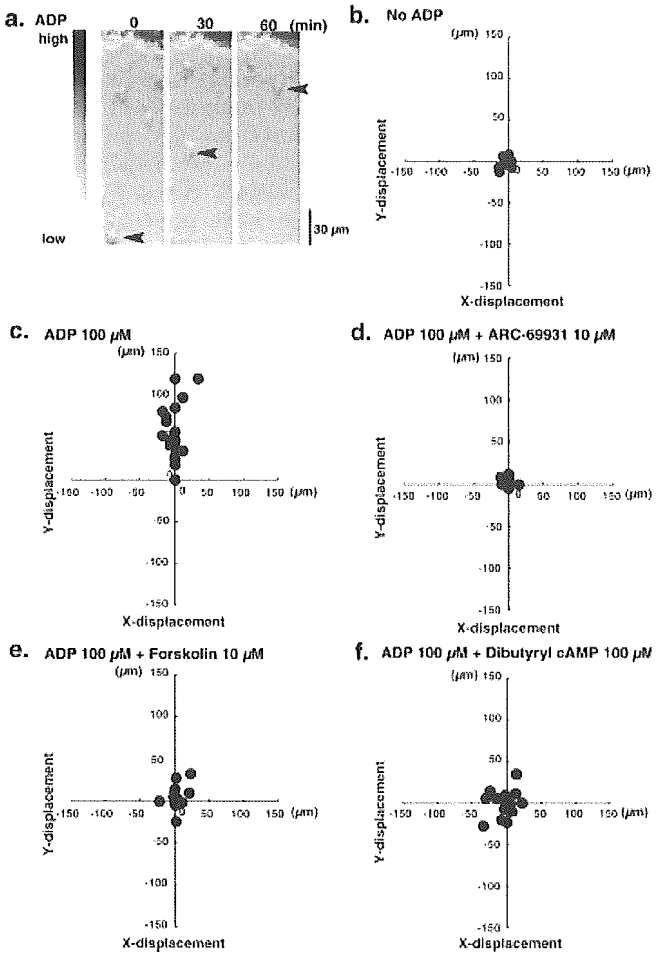

Fig. 1. ADP-induced chemotaxis of microglia was mediated through P2Y12/13 receptors and blocked by an elevation of intracellular cAMP. Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. **a:** Typical chemotactic responses of microglia toward ADP. Arrowheads depict the position of a single microglial cell at the indicated time, showing the kinetics of chemotaxis. Microglia were almost static on fibronectin in the absence of the stimulant (**b**). Microglia, however, showed chemotactic responsiveness to ADP (100 μM) (**c**), which was completely blocked by the P2Y12/13 receptor antagonist ARC-69931 (**d**), confirming that the chemotaxis to ADP is mediated by P2Y12/13 receptor. Forskolin pretreatment (**e**) and dibutyryl cAMP pretreatment (**f**) attenuated the ADP-induced chemotaxis of microglia. The data represent three independent experiments.

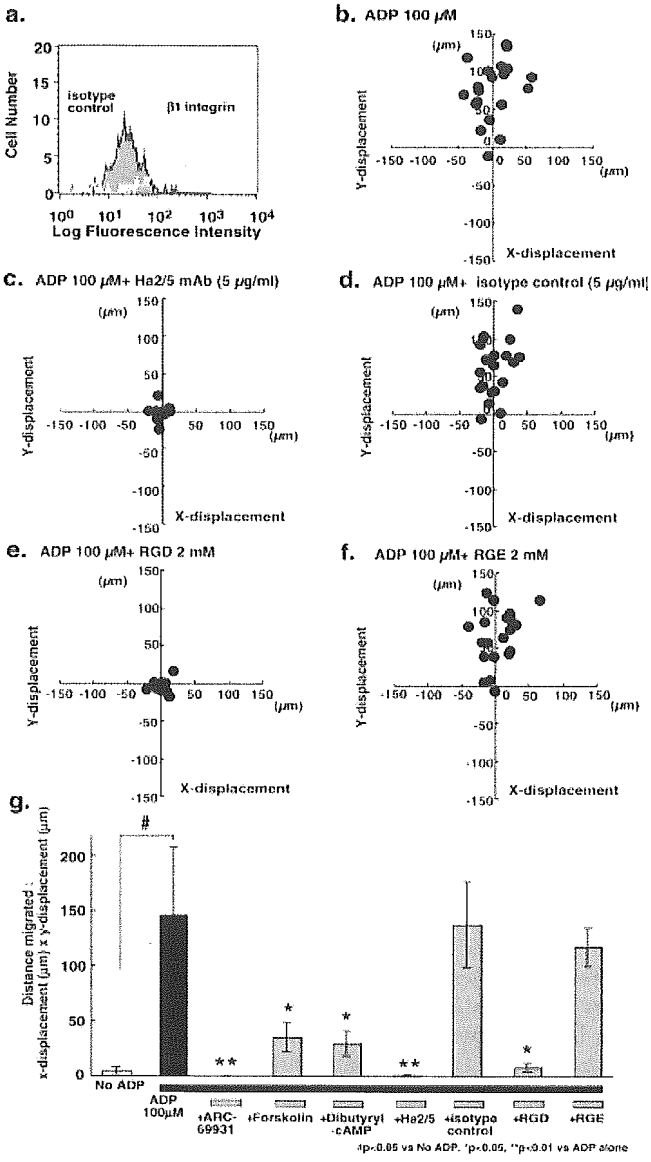

Fig. 2. β1 integrin is highly expressed on cultured microglia, and it is involved in the ADP-induced chemotaxis of microglia on the fibronectin substrate. Cultured microglia were labeled with anti-β1 integrin antibody (Ha2/5) (gray) or with isotype-matched control antibody (black). The labeling was detected by Alexa-Fluor 488-conjugated antibody and the cells were subjected to flow cytometry analysis (**a**). **b–f:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. The microglia migration toward ADP (100 μM) (b) was totally inhibited by Ha2/5 (c) and RGD peptide (e). In contrast, the migration was not affected by isotype-matched control (d) and RGE peptide (f). Data (a–f) represent three independent experiments. Each chemotaxis was quantified by calculating the x displacement (μm) multiplied by the y-displacement (μm) (**g**). Data (g) are mean ± SE of three separate experiments. #Greater than No ADP ($P < 0.05$, Student's *t*-test); *Smaller than ADP 100 μM ($P < 0.05$, Student's *t*-test); **Smaller than ADP 100 μM ($P < 0.01$, Student's *t*-test).

metric analysis confirmed that the cultured microglia expressed significant amounts of the β1 integrin subunit (Fig. 2a). Next, to assess the role of β1 integrin in the cell migration toward ADP, a chemotaxis assay was performed with the Dunn chemotaxis chamber in the presence of a monoclonal antibody specific for the β1 integrin subunit (Ha2/5) and RGD peptide (Fig. 2b–f). Treatment of microglia with Ha2/5 antibody at 5 μg/ml suppressed the ADP-induced chemotaxis (Fig. 2c), indicating that β1 integrin is required for this process. An isotype-matched control antibody did not interfere with the microglial chemotaxis toward ADP (Fig. 2d). Treatment with RGD peptide at 2 mM also perturbed the microglial migration toward ADP (Fig. 2e), whereas control RGE peptide did not inhibit the migration (Fig. 2f), suggesting that the RGD sequence is important. The RGD (Arg-Gly-Asp) sequence is present in several extra-

cellular matrix components including fibronectin, and many integrins show RGD sequence-dependent binding to their ligands. Therefore, these results demonstrate that β1 integrin was responsible for the motility of microglia on the fibronectin substrate and its interaction with fibronectin involves the RGD sequence. Each chemotaxis was quantified by calculating the x displacement multiplied by the y displacement (Fig. 2g), and thus the dot-plotted data in Figures 1 and 2 were all evaluated for statistical significance.

Stimulation of P2Y12/13 Receptor Causes β1 Integrin Redistribution That Colocalizes With Membrane Ruffling

The involvement of β1 integrin in the ADP-induced chemotaxis was further studied using immunofluorescence (Fig. 3). Extracellular ATP and ADP have been reported to induce a membrane structure called membrane ruffling (Honda et al., 2001). Membrane ruffling is a unique, wave-like structure of the plasma membrane. It is the actin polymerization seen as a projection from the cell membrane, and the most conventional way of visualizing this unique structure is to stain the cells with phalloidin. In our experiments, membrane ruffling was construed as labeling intensity that reached >200 a.u. (see Materials and Methods). Microglia were attached to fibronectin-coated coverslips, stimulated with ADP at 50 μM for 5 min, fixed and permeabilized, and then the appearance of membrane ruffling and the cellular localization of β1 integrin were studied using Texas Red-X phalloidin and Ha2/5 antibody, respectively. Without ADP stimulation, microglia showed no membrane ruffling and the β1 integrin subunit was dispersed over the plasma membrane of the microglia (Fig. 3a–c). In response to ADP stimulation at 50 μM, membrane ruffling appeared within 5 min after stimulation (Fig. 3e) and β1 integrin was redistributed to the cell edge (Fig. 3d). A merged image revealed that β1 integrin colocalized with the membrane ruffling (Fig. 3f). Treatment of microglia with ARC-69931 abrogated the membrane ruffling formation (Fig. 3h) (Honda et al., 2001), β1 integrin redistribution (Fig. 3g) and thus the colocalization of these two (Fig. 3i), suggesting that these responses were mediated by P2Y12/13 receptors. The numbers of cells with membrane ruffling were directly counted among control, ADP, and ADP with ARC-69931 populations (Fig. 3B). The result again indicated that ADP induces membrane ruffling which is inhibited by ARC-69931.

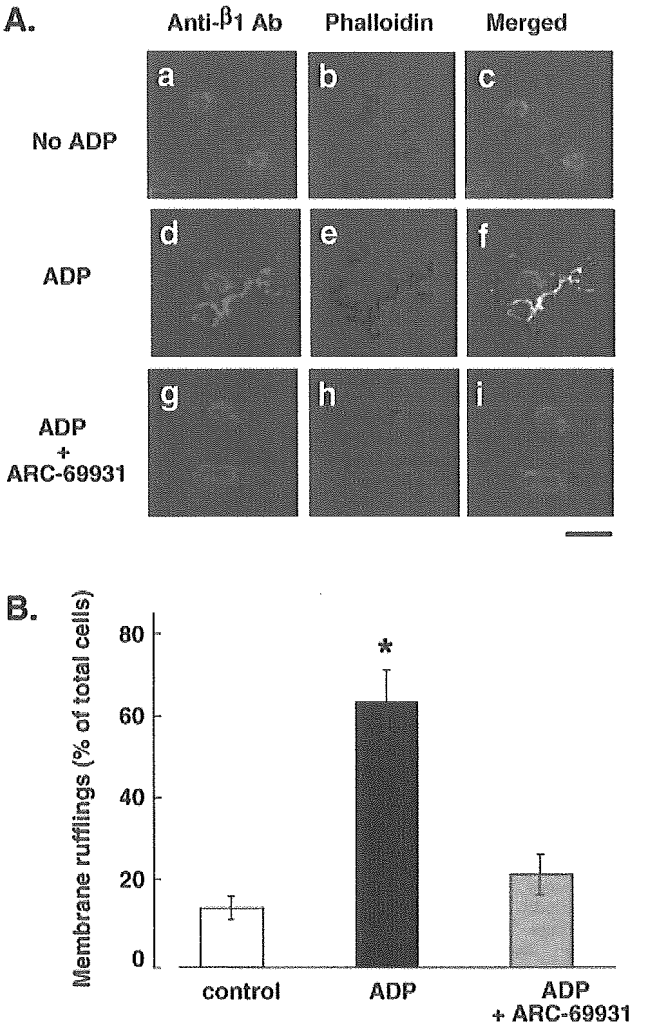

Fig. 3. P2Y12/13 receptor-mediated microglial β1 integrin redistribution that colocalized with membrane ruffling in response to ADP stimulation. **A:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. Then, for the immunofluorescence studies, the cells were incubated with anti-β1 antibody Ha2/5 (a,d,g) and phalloidin (b,e,h). a–c: Controls, i.e., PBS alone. β1 integrin redistribution and membrane ruffling occur in response to ADP stimulation (d,e), but they disappear in the presence of ARC-69931 (g,h). Merged image (f) shows that β1 integrin redistributes and colocalizes with membrane ruffling in response to ADP (50 μM). **B:** Cultured microglia on fibronectin-coated coverslips were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test). Scale bar = 10 μm in A.

An Increase in the Intracellular cAMP Level Abrogated Colocalization of Membrane Ruffling and β1 Integrin, and the PKA Inhibitor KT-5720 Restored the β1 Integrin Redistribution and Membrane Ruffling

We next investigated whether an increase in the intracellular concentration of cAMP in microglia would have any effect on the appearance of the membrane ruffling and redistribution of β1 integrin. Microglia were adhered to fibronectin-coated coverslips and pretreated either with forskolin or with dibutyryl cAMP. The cells were then stimulated with ADP at 50 μM for 5 min and

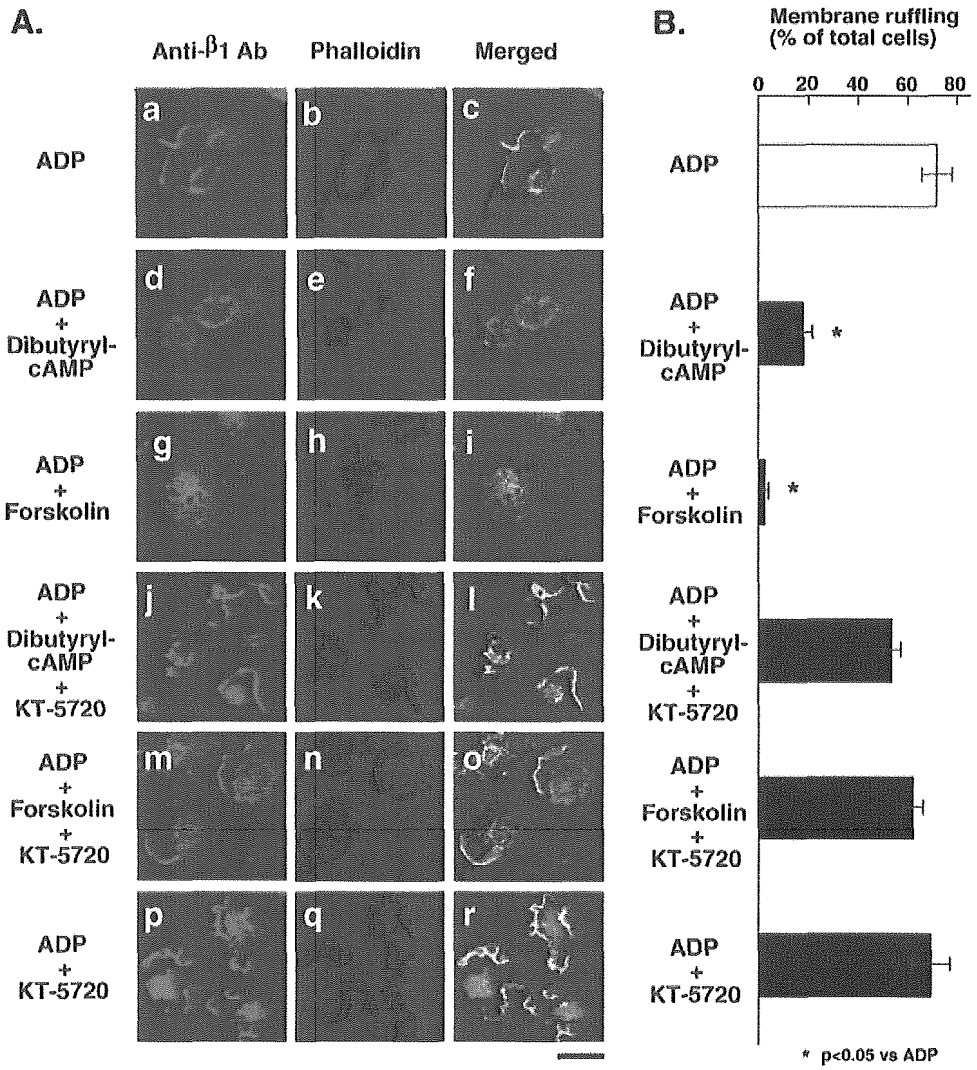

Fig. 4. Elevation of intracellular cAMP abrogated the β1 integrin redistribution and membrane ruffling in response to ADP stimulation. The PKA inhibitor KT-5720 restored the ADP-induced β1 integrin redistribution and membrane ruffling on microglia. **A:** Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min in the presence of dibutyryl cAMP (d–f) or forskolin (g–i) to study the involvement of the intracellular cAMP concentration. The elevation of intracellular cAMP abrogated the β1 integrin redistribution (d,g) and membrane ruffling (e,h) in response to ADP (50 μM). PKA involvement was examined by pretreating the cells with KT-5720 at 5 μM (j–o). KT-5720 treatment itself did not affect the morphology of the cells (data not shown) and KT-5720 pretreated cells responded normally to ADP at 50 μM (p–r). Blocking PKA by pretreatment with KT-5720 restored β1 integrin clustering and increased membrane ruffling even in the presence of dibutyryl cAMP (j–l) or forskolin (m–o). **B:** Cultured microglia on fibronectin-coated coverslips were stimulated as in A for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

studied by immunofluorescence. As seen in Figure 4Ad–i, neither the β1 integrin redistribution nor the membrane ruffling appeared on the surface of microglia when the intracellular cAMP level was elevated. These results indicate that an elevation of intracellular cAMP by dibutyryl cAMP or by forskolin inhibited the β1 integrin accumulation and membrane ruffling in response to ADP. PKA is located downstream of the cAMP elevation, and its negative regulation of the β2 integrin avidity and the integrin-mediated adhesion of lymphocytes has been described in other studies (Laudanna et al., 1997; Jones, 2002). Thus, since the PKA activity appears to regulate the integrin function, we next investigated whether PKA activation is responsible for the loss of the β1 integrin redistribution and the attenuation of the ADP-induced chemotaxis of microglia. Microglia were pretreated with forskolin or with dibutyryl cAMP, both of which were in the presence of the PKA inhibitor KT-5720. When these cells were stimulated with ADP at 50 μM, the integrin redistribution was restored (Fig. 4Aj–o). The number of cells with membrane ruffling was directly counted for each population (Fig. 4B), and the result confirmed the effect of dibutyryl cAMP, forskolin, and KT-5720 on membrane ruffling.

β1 Integrin Mediates Microglial Proliferation

β1 integrin regulates cellular proliferation, migration, survival, and differentiation via outside-in signaling. β1 integrin is involved in the proliferation of many cell types (Jones and Watt, 1993; Howlett et al., 1995), but its effect on microglial proliferation has not been clarified. To study the function of β1 integrin in microglial proliferation, an MTT assay and direct cell counting were used in this study. Figure 5 shows that microglia cultured on fibronectin substrate proliferate more than 1.5-fold after 24-h incubation as compared to the control. Microglia plated on anti-β1 integrin antibody showed similar results, suggesting that cross-linking of β1 integ-

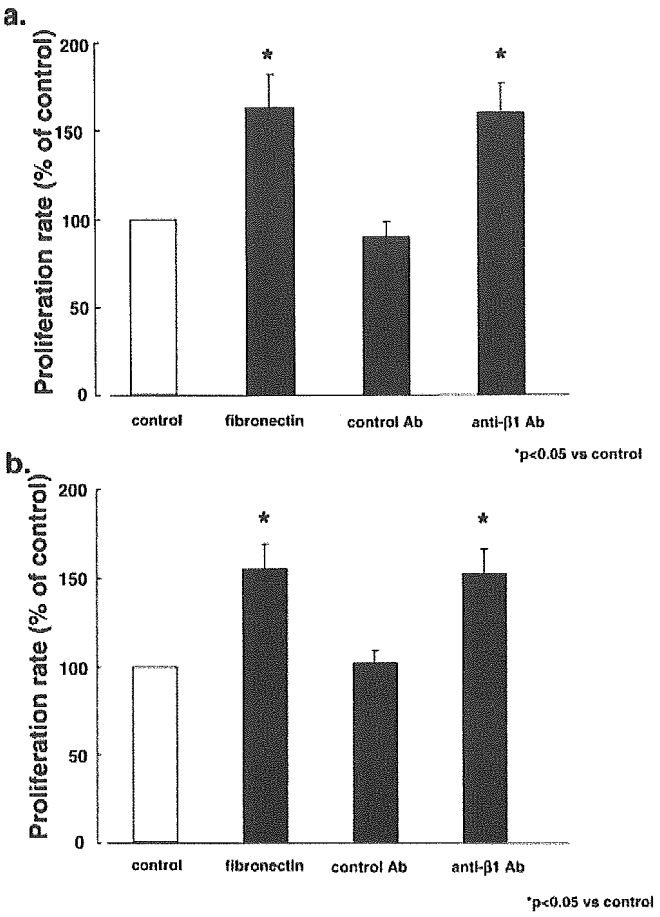

Fig. 5. β1 integrin mediates microglial proliferation. Cultured microglia were adhered to uncoated, fibronectin-coated, anti-β1 antibody-coated or control antibody-coated plates and cultured for 24 h at 37°C. After the incubation, the cell's viability was measured by (**a**) MTT assay or (**b**) direct cell counting. The data are shown as percentage of proliferation of microglia on uncoated culture plates after 24-h incubation. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

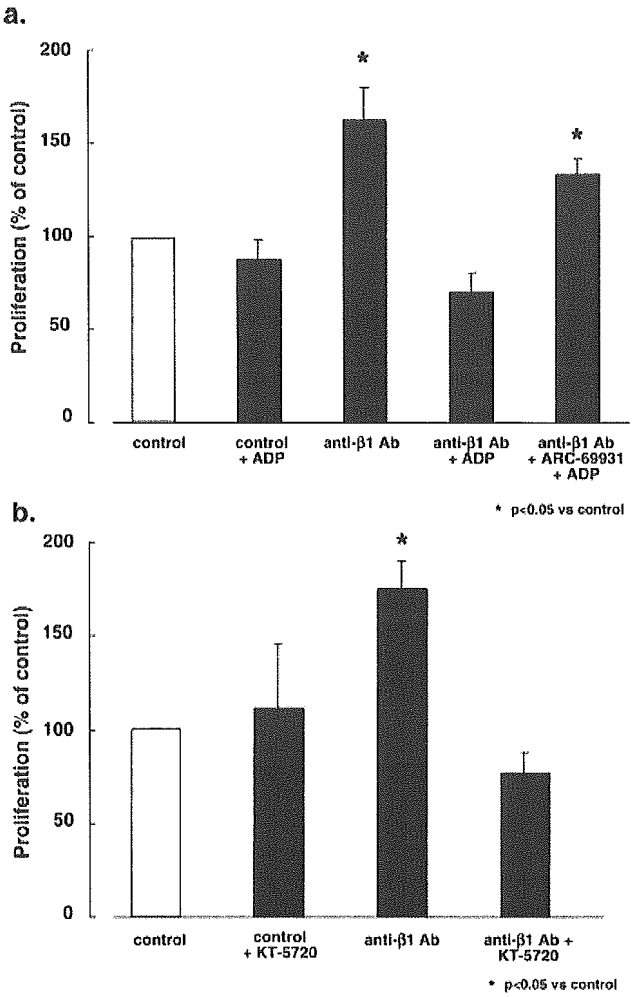

Fig. 6. β1 integrin-mediated proliferation of microglia is inhibited by ADP. PKA is a positive regulator of β1 integrin-mediated proliferation. Cultured microglia were adhered to uncoated or anti-β1 integrin antibody-coated plate in the presence of ADP (100 μM) and ARC-69931 (10 μM) (**a**) or KT-5720 (10 μM) (**b**) and incubated for 24 h at 37°C. The cells' viability was then tested by MTT assay. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

rins leads to microglial proliferation. Recent reports have shown that integrin engagements, either with ligands or with antibodies, are capable of transducing signals (Miyamoto et al., 1995). An isotype-matched control antibody did not induce microglial proliferation. In this way, it is clear from these results that microglial β1 integrin mediates the outside-in signal, which promotes the proliferation of the cell.

ADP Inhibits β1 Integrin-Mediated Proliferation of Microglia and the Mechanism Involves PKA Activation

Chemokines act through Gi-coupled GPCRs to attract the target cells (Neptune and Bourne, 1997; Rollins, 1997), and several of them are known to induce the proliferation of the cells. Several chemokine receptors including IL-8R, CXCR1-4, CCR2, CCR3, CCR5, and CX_3CR1 are detected on microglia (Hanisch, 2002; Abbadie et al., 2003) and most of them are reported to induce microglial proliferation as well. Since P2Y12/13 is also a Gi-coupled receptor, we studied its effect on cell growth. When microglia were stimulated with ADP alone at 100 μM, they did not increase in number (Fig. 6a), suggesting that ADP itself does not cause microglial proliferation. As mentioned earlier, microglia with β1 integrin cross-linking showed marked proliferation, but the integrin-induced high proliferation rate was no longer observed in the presence of ADP (Fig. 6a). The addition of ARC-69931 restored the high proliferation rate, suggesting that the ADP signal from the P2Y12/13 receptor did not synergize the proliferative signal from β1 integrin, but rather counteracted it.

We next investigated whether PKA, a major intracellular target for cAMP in mammalian cells, is involved in this process. Microglial proliferation was studied in the presence of the PKA inhibitor KT-5720 (Fig. 6b). As seen in the control, the addition of KT-5720 at 10 μM did not affect the cell's viability after 24-h incubation. When KT-5720 was added to microglia with anti-β1 integrin antibody, however, the proliferation rate was significantly reduced. These results indicate that the activation of PKA positively regulates the microglial proliferation signal from β1 integrin, in sharp contrast to its effect on P2Y12/13-induced β1 integrin translocation.

DISCUSSION

Microglia are resident tissue macrophages in the brain, exhibiting ramified morphologies in the quiescent state. Once activated by numerous soluble molecules, including cytokines, growth mediators, and nucleotides, however, they quickly respond by transforming into an amoeboid phenotype, migrating to the source of mediators, proliferating and upregulating the expression of various inflammatory cytokines (Nakajima and Kohsaka, 1993; Hanisch, 2002; Inoue, 2002). In the study described herein, we investigated the role of β1 integrin in microglial chemotaxis and proliferation, and characterized its regulation by signals delivered from P2Y12/13 receptors.

Our observation that P2Y12/13 receptors mediate the chemotaxis of microglia on the fibronectin substrate was compatible with the earlier finding by Honda et al. that membrane ruffling is cause by ADP stimulation (Honda et al., 2001). ADP is a potent agonist of P2Y1, P2Y12, and P2Y13. The purine and pyrimidine receptors known to be expressed in microglia include P2X4, P2X7, P2Y2, and P2Y12 (Inoue, 2002; Sasaki et al., 2003; Tsuda et al., 2003), and possibly P2Y13, due to its abundant mRNA in the brain and the immune system (Zhang et al., 2002). P2Y12 and P2Y13 are recently identified Gi-coupled receptors that share the same agonists and antagonists, making it very difficult to pharmacologically differentiate between these two receptors. Which of the two receptors, or both perhaps, was/were truly responsible for the chemotaxis and/or inhibition of proliferation awaits the development of a selective agonist or antagonist.

To mimic damages in the CNS, we performed chemotaxis assays on coverslips coated with an ECM molecule that is known to be expressed in the real pathological situation and thus chose fibronectin, which is upregulated following adult CNS injury (Jones, 1996). In the present study, we demonstrated that β1 integrin is highly expressed in microglia and is crucially involved in the ADP-induced chemotaxis of microglia on fibronectin, and that signals from P2Y12/13 receptors recruit β1 integrin to the membrane ruffle regions. When non-coated coverslips that were pretreated with hydrochloric acid were used in the assays, microglia neither adhered well to the coverslips nor migrated against the ADP gradient (data not shown). These results also indicate that β1 integrin is crucially involved in the adhesion to fibronectin and ADP-induced chemotaxis in the presence of fibronectin in the CNS. β1 integrin couples with α1 integrin through α6 integrin to form VLA-1 through VLA-6, respectively, and with αv integrin to form αvβ1. Among these pairs, VLA-3, VLA-4, VLA-5, and αvβ1 have fibronectin as their ligand, and microglia are known to express α4, α5, and αv chains (Kloss et al., 2001; Milner and Campbell, 2003). The ADP-induced translocation of β1 integrin may contribute to the subsequent formation of focal complexes and then focal adhesions, both of which are important for cell migration.

We have shown that ADP induced β1 integrin translocation to the membrane ruffles by lowering the intracellular cAMP via the P2Y12/13 receptor, and that β1 integrin is important for microglial chemotaxis mediated by the same receptor. Chemotaxis is an integrated process consisting of multiple steps (Lauffenburger and Horwitz, 1996). Integrins are essential for cell migration not only because they mediate adhesion directly, but also because they regulate intracellular signaling pathways required for the cell locomotion (Hood and Cheresh, 2002). When fibroblasts migrate, integrins and other molecules form focal complexes and assemble at the leading edge of the cell. These complexes evolve into highly organized focal adhesions (Laukaitis et al., 2001), which in turn generate a signal to cause actin cytoskeletal reorganization resulting in cell motility (Lauffenburger and Horwitz, 1996). However, details of the signaling pathway(s) generated by β1 integrin translocation at the membrane ruffles and the effect of this outside-in signaling on microglial chemotaxis remain unclear. Although the P2Y12/13 receptor-mediated decrease in cAMP is an important step for microglial migration, it is interesting to note that excess inhibition as well as hyperactivation of cAMP/PKA pathways also inhibits cell migration (Edin et al., 2001; O'Connor and Mercurio, 2001). Thus, an integrin-mediated outside-in signaling may function as a fine and local tuning device to control such signaling cascades for more efficient cell migration.

In the present study, we showed that cross-linking of β1 integrin resulted in a dramatic increase of microglia. CNS injuries and diseases are often accompanied by microglial proliferation (Gehrmann et al., 1995) and our results indicate that this may be due to the β1 integrin engagement by newly upregulated ECM molecules such as fibronectin. The MAP kinase cascade is an essential component of pathways that regulate cell proliferation (Widmann, 1999). In microglia, ERKs are involved in the proliferative response to granulocyte-macrophage colony-stimulating factor (Liva et al., 1999) or corticotropin-releasing hormone (Wang et al., 2003), and the importance of Jak/STAT (Liva et al., 1999) and p38 (Tikka et al., 2001) cascades in proliferation has also been suggested. Integrin engagement also causes the activation of MAP kinases (Chen et al., 1994; Zhu and Assoian, 1995; Schlaepfer and Hunter, 1998) and, therefore, it is likely that the cross-linking of β1 integrin trig-