別添１

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

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１８年度　研究報告書

主任研究者　井上　和秀

平成１９（２００７）年　３月

別添2

目　　次

別添3

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）
（総括研究報告書）

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
（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

グルコース誘発インシュリン分泌に対するATP受容体の作用は２相性であり、低濃度（１μM）では増強し、高濃度（100μM）では逆に抑制した。受容体サブタイプの関与をアゴニストやアンタゴニストを用いて検討した結果、増強作用はP2X5およびP2X6の可能性が考えられ、抑制作用はP2Y1の可能性が高まった。この増強のかなりの部分は電位依存性Ca^{2+}チャネル依存性であったが、電位依存性Ca^{2+}チャネル以外の経路も関与すると考えられる。一方、P2Y1受容体を介するグルコース誘発インシュリン分泌の抑制は著明であり、過剰なインシュリン分泌を制御するという重要な役割をATPが担っている可能性が考えられる。このように、ATP受容体は様々な形で膵β細胞の機能維持において重要な役割を果たしていると考えられる。

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、L-type Ca Channelとは独立した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ことである。

これまでに、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細胞からのインシュ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シュリン分泌を促進する経路が存在する可能性を提示した。その後更に研究を進めた結果、ATPを介したインシュ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事が判明した。そこで本年度は、ATP受容体刺激によるインシュリン放出メカニズムについてモデル細胞MIN6を用いて詳細に検討した。

B.研究方法

MIN6 細胞を、5 × 10^5 cells / well となるよう24 well plate に播種し、DMEM medium を加えて全量 1 ml / well とし5-7 日間培養する。ほぼ90%程度に増殖が達した状態で medium を除去し、HBSS で wash 後、2.2 mM Glu / HBSS (3 ml/ well)

を加えて2 時間の pre incubation を行う。その後、medium を除き、各種刺激とする溶液を3 ml / well で添加し5 時間の incubation を行う。反応終了時にmedium 1 ml/well を回収し、その上清中のインシュリン濃度をELISA法により測定した。

C.研究結果

1．グルコース刺激によるMIN6細胞からのインシュリン放出：基礎的検討

ATP受容体の関与を検討する前に、MIN6細胞からのグルコース刺激によるインシュリン放出に関して、グルコース濃度依存性および刺激時間依存性について検討した。その結果、グルコース濃度（0.44～22 mM）に依存したインシュリン放出が認められた。ヒト血清中のグルコース濃度との対比から、グルコース22mM でのインシュリン濃度とグルコース0.44mM でのそれを差し引いたものをグルコース誘発インシュリン分泌とした。また時間依存性に関しては、検討した時間経過では刺激後５時間目がもっともインシュリン分泌量が多くて安定していた。なお、グルコース無添加でATP刺激した場合にはインシュリン放出は認められなかった。そこで以下の実験では、５時間目のグルコース誘発インシュリン分泌に対するATP受容体関連化合物の作用を検討した。

2．ATP受容体アゴニスト

ATPがグルコース誘発インシュリン分泌に影響を及ぼすとすれば、それはどのP2受容体サブタイプであるのか特定しなければならない。そこで、様々なP2受容体アゴニスト（ATP, ADP, UTP, UDP）ならびにアデノシンを用いてインシュリン分泌に対する影響を検討した。その結果、ATP、ADPは低濃度（1μM）ではグルコース誘発インシュリン分泌を増強し、高濃度（100μM）では逆に抑制するという２相性の作用を引き起こした（図１）。一方、UTPやUDPではグルコース誘発インシュリン分泌作用に対してほとんど影響が見られなかった。

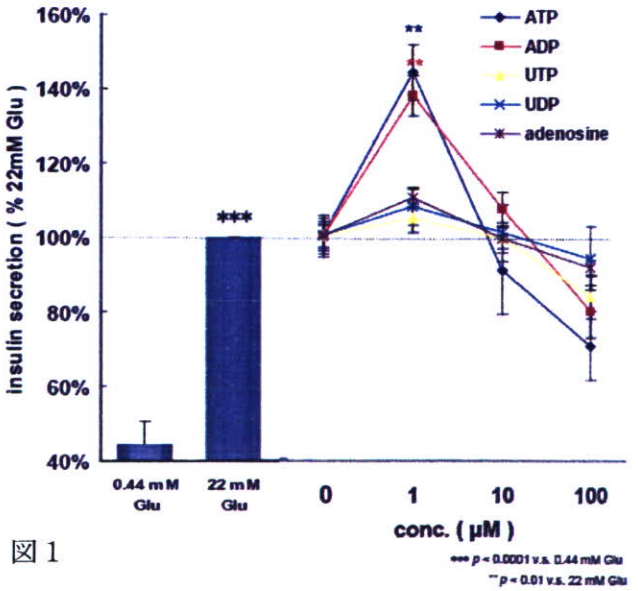

図１

アデノシンもまたほとんど効果を示さず、ATPやADPが分解されてアデノシンとして作用することはないと考えられる。

次に、ADPとATPがほぼ同様な効果を示したことから、両アゴニストに親和性の高いP2受容体サブタイプP2Y1受容体の関与が考えられる。そこで、強力なP2Y1受容体の選択的アゴニストである2-MeSADPを用いて検討した。その結果、グルコース誘発インシュリン分泌作用は2-MeSADPの10～1000 nMの範囲で有意に抑制され、ATP高用量（100μM）で認められたような増強作用はみとめられなかった（図２）。ATPの２相性の作用の内P2Y1受容体が抑制系を担っていると考えられる。

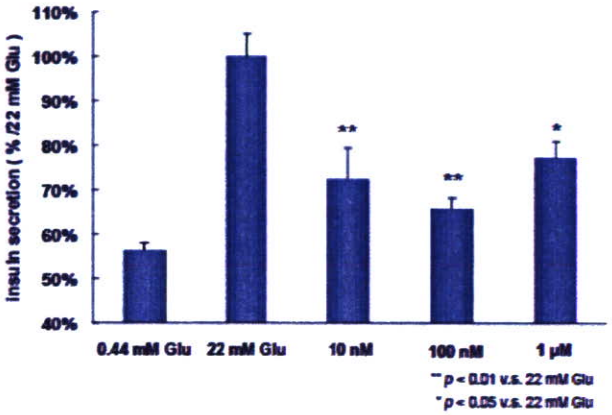

図2　22mMグルコース誘発インシュリン放出に対する2-MeSADP（10～1000 nM）の影響

3．ATP受容体アンタゴニスト

グルコース誘発インシュリン分泌に対するATPの作用がどの受容体を介したものであるのかを次にP2受容体のアンタゴニスト３種類（TNP-ATP、PPADSおよびsuramin）を用いて検討した。

TNP-ATPはP2X1, 2, 3, 4, 7 のアンタゴニストとして知られているが、今回用いた用量（0.1～5μM）ではATPの作用に影響を及ぼさなかった。ATPのインシュリン分泌促進作用にP2X1,2,3,4,7受容体が関与する可能性は低い。

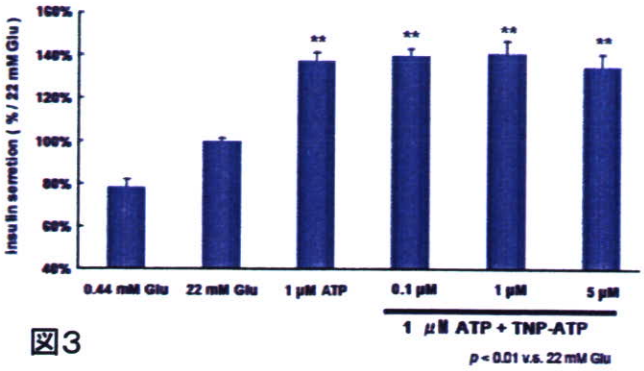

図3

次いで、P2X1, 2, 3, 5,6, 7 のアンタゴニストとして知られているPPADSを用いて検討したところ、1～10μMの濃度ではATPの作用に影響を認めなかったが、100μMの濃度ではATPのグルコース誘発インシュリン分泌増強作用を完全に抑制した（図４）。PPADS単独では、１～100μMの範囲内で22 mMグルコースのインシュリン分泌作用には何の影響もないことを確認している。

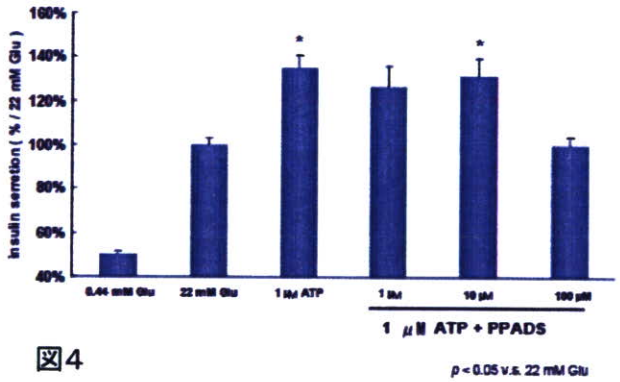

図4

なお、非選択的なATP受容体アンタゴニストsuraminについては、50μMのsuramin自体にインシュリン分泌抑制作用が見られたためにそれ以上の検討はできなかった。

４．電位依存性Ca^{2+}チャネルの関与

グルコース誘発インシュリン分泌に対するATPの２相性の作用の内、増強作用はP2X5および６の可能性が指摘された。これらイオンチャネル型P2X受容体はCa^{2+}およびNa^{+}を通し、脱分極や細胞内Ca^{2+}上昇を引き起こすことができる。脱分極を介する場合には電位依存性Ca^{2+}チャネルの関与があることになる。それを確認するために、電位依存性Ca^{2+}チャネル阻害剤nifedipine を用いてグルコース誘発インシュリン分泌に対するATPの増強作用への影響を検討した。　まず、nifedipine単独の作用を検討した。その結果、22 mMグルコースによるインシュリン分泌作用に対して0.1μM nifedipineはほとんど影響を及ぼさなかったが、１および10μMのnifedipine は完全に抑制してしまった。このことは、22 mMグルコースによるインシュリン分泌は電位依存性Ca^{2+}チャネル依存性であることを示している。

次に、グルコース誘発インシュリン分泌に対するATPの増強作用へのnifedipine の影響を検討した。その結果、nifedipine１μM存在下、1 μM ATPによるグルコース誘発インシュリン分泌増強作用は大きく減少した。しかし、nifedipine１μM単独時のインシュリン分泌に比較して、有意差を持ってインシュリン分泌は増加していた。

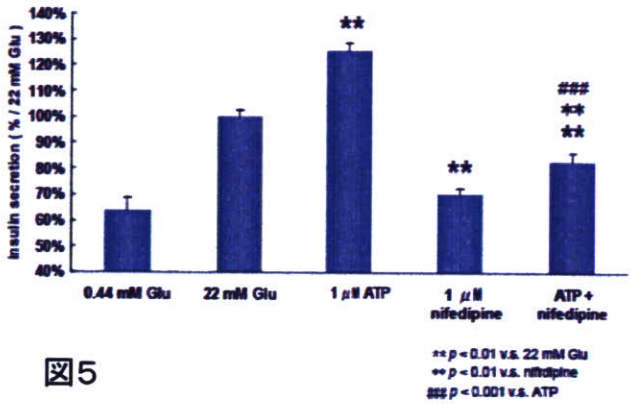

図5

D. 考察

前述してきたように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の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受容体によるインシュ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シュリン分泌にどのような影響がでるかを検討したが、インシュリン分泌に対するATPの効果は条件次第で多様に変化してしまう。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る。II型糖尿病モデルと考えられているGKラット膵β細胞で定量的PCR法を用いてATP受容体の遺伝子発現を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。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。そこで、本年度

は、グルコース誘発インシュリン分泌に対するATP受容体刺激効果についてモデル細胞MIN6を用いて詳細に検討した。

本年度の成果で最も特徴的なことは、グルコース誘発インシュリン分泌に対するATP受容体の作用は２相性であることである。ATPおよびADPは低濃度（１μM）ではグルコース誘発インシュリン分泌を増強し、高濃度（100μM）では逆に抑制するという２相性の作用を引き起こした。ATPが細胞内に入りK^+_{ATP}チャネルを不活性化する経路は考えにくいので、この作用は受容体サブタイプの機能差と考えるのが自然である。そこで、受容体サブタイプの関与を検討するためにアゴニストやアンタゴニストを用いて検討した結果、増強作用はP2X5および６の可能性が考えられ、抑制作用はP2Y1の可能性が高まった。なお、MIN6（ passage 43-53 ）においてP2Y4受容体とP2Y6受容体のmRNAの発現が報告されているが、UTPやUDPではグルコース誘発インシュリン分泌作用に対してほとんど影響が見られなかったことから、P2Y4およびP2Y6受容体がインシュリン分泌に関与するとは考え難い。これまで、様々な動物実験を用いた成績が報告されているが、ある論文ではインシュリン分泌を抑制したとされ、別の論文では逆に増強したとされ、互いに矛盾していたが、今回の報告でその矛盾は見事に解決された。

さて、本研究の最終目的は電位依存性Ca^{2+}チャネルに依存しないインシュリン分泌経路を発見することであった。しかし、ATP単独ではインシュリン分泌を引き起こさない。生理的条件ではグルコースが存在しない状況は考えられないことから、このような条件設定はあまり意味がない。そこで、我々は、グルコース存在下のインシュリン分泌に対するATPの影響を調べ、その結果、P2Xを介してインシュリン分泌を増強することを見いだしたのであるが、予想に反してこの増強のかなりの部分は電位依存性Ca^{2+}チャネル依存性であった。しかし、nifedipine では抑制しきれないインシュリン分泌部分も残るため、電位依存性Ca^{2+}チャネル以外の経路も関与すると考えられる。一方、P2Y1受容体を介するグルコース誘発インシュリン分泌の抑制は非常に著明であり、過剰なインシュリン分泌を制御するという重要な役割をATPが担っている可能性が考えられる。すなわち、ATP受容体は様々な形で膵β細胞の機能維持において重要な役割を果たしていると考えられる。

E. 結論

グルコース誘発インシュリン分泌に対するATP受容体の作用は２相性であり、低濃度（１μM）では増強し、高濃度（100μM）では逆に抑制した。受容体サブタイプの関与をアゴニストやアンタゴニストを用いて検討した結果、増強作用はP2X5およびP2X6の可能性が考えられ、抑制作用はP2Y1の可能性が高まった。これまで、様々な動物実験を用いた成績が報告されているが、ある論文ではインシュリン分泌を抑制したとされ、別の論文では逆に増強したとされ、互いに矛盾していたが、今回の報告でその矛盾は見事に解決された。この増強のかなりの部分は電位依存性Ca^{2+}チャネル依存性であったが、電位依存性Ca^{2+}チャネル以外の経路も関与すると考えられる。一方、P2Y1受容体を介するグルコース誘発インシュリン分泌の抑制は非常に著明であり、過剰なインシュリン分泌を制御するという重要な役割をATPが担っている可能性が考えられる。このように、ATP受容体は様々な形で膵β細胞の機能維持において重要な役割を果たしていると考えられる。

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

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
Nishimaki-Mogami T, Kawahara Y, Tamehiro N, Yoshida T, Inoue K, Ohno Y, Nagao T, Une M.	Alpha-bile alcohols function as farnesoid X receptor antagonists.	Biochem Biophys Res Commun.	339	386-391	2006
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5α-Bile alcohols function as farnesoid X receptor antagonists ☆

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Abstract

The farnesoid X receptor (FXR) is a bile acid/alcohol-activated nuclear receptor that regulates lipid homeostasis. Unlike other steroid receptors, FXR binds bile acids in an orientation that allows the steroid nucleus A ring to face helix 12 in the receptor, a crucial domain for coactivator-recruitment. Because most naturally occurring bile acids and alcohols contain a *cis*-oriented A ring, which is distinct from that of other steroids and cholesterol metabolites, we investigated the role of this 5β-configuration in FXR activation. The results showed that the 5β-(A/B *cis*) bile alcohols 5β-cyprinol and bufol are potent FXR agonists, whereas their 5α-(A/B *trans*) counterparts antagonize FXR transactivation and target gene expression. Both isomers bound to FXR, but their ability to induce coactivator-recruitment and thereby induce transactivation differed. These findings suggest a critical role for the A-ring orientation of bile salts in agonist/antagonist function.

Keywords: Nuclear receptor; Farnesoid X receptor; Bile acid; Bile alcohol; Agonist; Antagonist; Coactivator

The farnesoid X receptor (FXR; NR1H4) is a nuclear receptor that is activated by bile acids [1,2] and bile alcohols [3,4], and it plays an essential role in bile acid/cholesterol homeostasis [5,6]. FXR belongs to the steroid hormone receptor superfamily, however, crystal structure studies have suggested that bile acids bind FXR with their steroid backbone flipped head to tail, the reverse orientation of all other steroid hormones, when they bind to their cognate receptors. Steroid hormones, such as testosterone, glucocorticoids, and estrogen, are oriented with their D rings facing helix 12 of their respective receptors [7–9], whereas the A ring of bile acids faces helix 12 of FXR [10,11].

Helix 12, the most C-terminal helix in the ligand binding domain of nuclear receptors, plays a crucial role in ligand-dependent receptor activation. Binding of an agonist to a receptor leads to a conformational change that allows the receptor to interact with a coactivator, which mediates ligand-dependent transcription of the receptor [12]. In this activated state, helix 12, the activation function 2 (AF2), functions as a molecular switch and forms one side of the coactivator binding pocket [13]. Structural analysis studies have demonstrated that agonist and antagonist bind at the same site within the core of the ligand-binding domain, but induce different conformations [8,10,11,14]. Agonists have been shown to stabilize the agonist conformation of helix 12 via direct or indirect interactions, and partial agonists or antagonists have been shown to destabilize it.

Unlike those of other steroids and cholesterol metabolites, the A rings of most naturally occurring bile acids are *cis*-oriented (5β-configuration). Because structural studies have shown that the A ring of bile acids is in contact with several amino acid residues on helices 11 and 12

☆ *Abbreviations:* FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α-hydroxylase; SHP, the small heterodimer partner; BSEP, bile salt export pump; PXR, pregnane X receptor.

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[10,11], it seems likely that the 5β-(A/B *cis*) ring juncture of bile acids plays a critical role in stabilizing the agonist-bound conformation of helix 12.

Bile alcohols are produced as intermediates in the bile acid synthetic pathway in mammals and as end-products of cholesterol catabolism in most evolutionarily primitive vertebrates [15]. We have shown that bile alcohols possess FXR-ligand properties similar to those of the corresponding bile acids [3]. Although the majority of naturally occurring bile alcohols are 5β-bile alcohols, few species of fishes and frogs produce 5α-bile alcohols containing a *trans*-oriented A ring. The bile alcohol 5α-cyprinol was originally isolated from the bile of *Cyprinus carpi* [16], the Asiatic carp, and 5α-bufol was isolated from the bile of lungfish [17] and frogs [18] (Fig. 1). Since our preliminary experiments showed that their 5β-counterparts, 5β-cyprinol and 5β-bufol, are potent agonists of human FXR, in this study we investigated whether these 5α-bile alcohols possess the ability to bind to FXR and recruit a coactivator. The results showed that both of these 5α-bile alcohols are capable of binding to FXR but are unable to induce coactivator-association, and as a result antagonize FXR activation.

FXR activation has been shown to repress the expression of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme in the bile acid biosynthetic pathway, by inducing an orphan nuclear receptor, the small heterodimer partner (SHP) [19,20]. FXR also up-regulates expression of the bile salt export pump (BSEP) [21], which represents the major canalicular bile salt export pump of the liver. We also investigated whether the 5α-bile alcohols modulate FXR-target gene expression.

Materials and methods

Bile alcohols and chemicals. Cholic acid and chenodeoxycholic acid were commercial products. 5α- and 5β-Cholestane-3α,7α,12α,26,27-pentols (5α-cyprinol and 5β-cyprinol) were isolated from the bile of carp [16,18]. 5α- and 5β-Cholestane-3α,7α,12α,25,26-pentols (5α-bufol and 5β-bufol) were isolated from the bile of frogs and toads, respectively [18,22]. GW4064 is synthesized according to the published procedures [23].

Transient transfections and reporter gene assays. HepG2 cells were maintained in DMEM containing 10% FCS and 100 μg/ml kanamycin, and they were seeded in 24-well plates 24 h prior to transfection. Cells were transfected with 85 ng pFXRE-tk-Luc [3], 25 ng each of the pcDNA3.1 expression vectors for human FXR (NR1H4) and RXRα, and either 65 ng of *Renilla* luciferase vector (phRL-TK) or pSV-β-galactosidase vector (Promega) with Effectene (Qiagen). Three hours after transfection, cells were exposed for 24 h to bile acids or bile alcohols in the medium containing 0.5% delipidated FBS. Cells were lysed and luciferase activity was determined. Firefly luciferase activity was normalized to *Renilla* luciferase or β-galactosidase activity for each well.

Coactivator-association assay using fluorescence polarization. The assay was performed essentially according to the published procedure [4]. TAMRA-labeled peptide (100 nM, with amino acid sequence ILRKLLQE) was incubated for 1 h with 1.5 μM of purified GST-fused human FXR ligand binding domain (residues 244–472) and ligands in 100 μl of buffer (10 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT at pH 7.9) in a black polypropylene 96-well plate on a shaker. Ligand-dependent recruitment of the coactivator peptide was measured as increases in fluorescence polarization with a Mithras LB-940 multilabel reader (Berthold).

mRNA analysis by real-time quantitative RT-PCRs. Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism

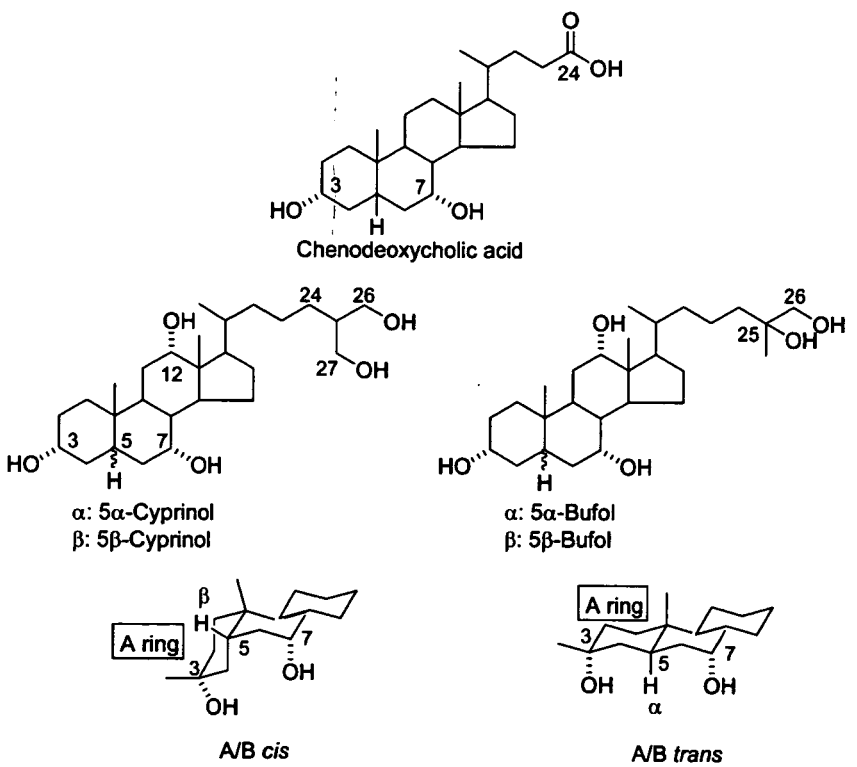

Fig. 1. Structure of bile alcohols and chenodeoxycholic acid (CDCA). 5α- and 5β-Cyprinol, 5α- and 5β-cholestane-3α,7α,12α,26,27-pentols; 5α- and 5β-bufol, 5α- and 5β-cholestane-3α,7α,12α,25,26-pentols; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.

7700 sequence detection system (Applied Biosystems). Human hepatoma-derived HepG2 cells were exposed to bile acids or bile alcohols in DMEM containing 0.5% delipidated FBS for 20 h. Total RNA extracted with the RNeasy Mini Kit (Qiagen) was treated with DNAase according to the manufacturer's instructions (Qiagen). The relative expression levels of mRNA were determined using the TaqMan one-step RT-PCR Master Mix Reagent Kit. The primer/probe sequences for human BESP, CYP7A1, and SHP have been reported previously [4,24].

Other methods. Cell viability was checked by leakage of lactate dehydrogenase into the medium. Statistical significance was determined by ANOVA followed by the Student Newman–Keuls method.

Results

The ability of bile alcohols to activate human FXR was assessed by means of a transient transfection assay. HepG2 cells were cotransfected with a FXRE-driven luciferase reporter plasmid and expression plasmids for FXR and RXRα. Exposure of the cells to 5β-cyprinol or 5β-bufol, bile alcohols containing two hydroxyl groups in their side chain (Fig. 1), led to the induction of luciferase activity at levels comparable to that of the most potent physiological FXR ligand, CDCA (Fig. 2A), whereas their 5α-counterparts, 5α-cyprinol and 5α-bufol, had little effect. However, these 5α-bile alcohols inhibited the transactivation elicited by either 50 μM CDCA (Fig. 2B) or 1 μM GW4064, a synthetic FXR agonist structurally unrelated to bile acids [23] (Fig. 2C).

In an in vitro coactivator-recruitment assay, 5β-cyprinol and 5β-bufol induced a dose-dependent interaction of SRC-1 peptide with FXR, but not with LXRα (Fig. 3A). By contrast, 5α-cyprinol induced a very weak interaction, accounting for 20% of 5β-cyprinol-induced interaction (Fig. 3B). 5α-Bufol induced no interaction at all. When assayed with 1 μM GW4064, these 5α-bile alcohols reduced (by 80%) the GW4064-elicited interaction (Fig. 3C). These findings show that 5α-cyprinol and 5α-bufol act as FXR antagonists, whereas their 5β-counterparts, 5β-cyprinol and 5β-bufol, are FXR agonists.

We used real-time quantitative RT-PCR to investigate the effect of these bile alcohols on the expression of FXR-target genes in HepG2 cells. 5β-Cyprinol and 5β-bufol increased the BSEP mRNA level, whereas 5α-cyprinol and 5α-bufol had little effect (Fig. 4A). When combined with 50 μM CDCA, these 5α-bile alcohols decreased CDCA-elicited induction of BSEP mRNA in a dose-dependent manner (Fig. 4B).

5β-Cyprinol and 5β-bufol increased the SHP mRNA level and markedly reduced the CYP7A1 mRNA level (Fig. 4A). The SHP mRNA elevation by 5α-bufol and 5α-cyprinol was small or insignificant. However, unexpectedly, these 5α-bile alcohols markedly repressed CYP7A1 expression (by 90% and 80%, respectively, at 50 μM). By contrast, 90% reduction in the CYP7A1 expression by CDCA was accompanied by a 5.2-fold elevation of the SHP level. When combined with CDCA, these 5α-bile alcohols further enhanced CDCA-elicited repression of CYP7A1, although SHP expression was unchanged or decreased instead (Fig. 4B).

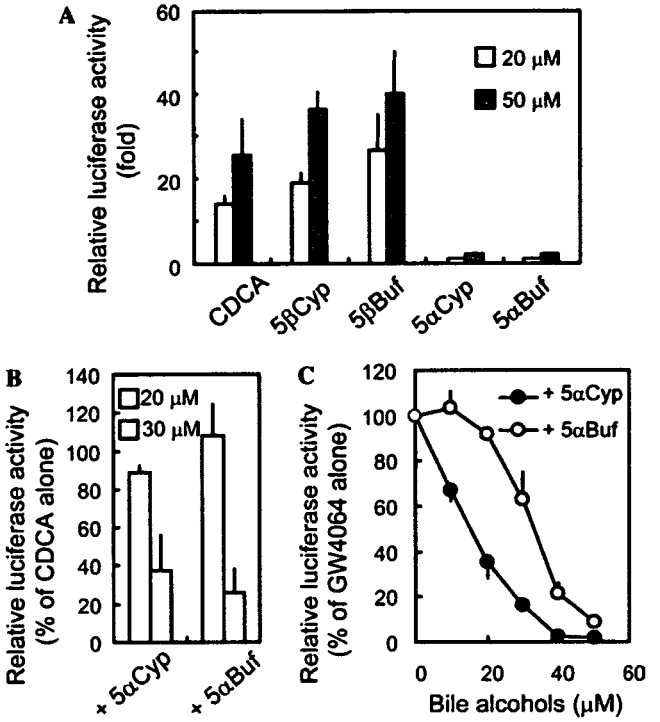

Fig. 2. 5β-Bile alcohols activate FXR, but 5α-bile alcohols function as antagonists in the cellular transactivation assay. (A) HepG2 cells were transfected with expression plasmids for human FXR and RXRα, and the $FXRE_{PLTP}$x4-tk-luc reporter plasmid together with a *Renilla* luciferase plasmid as a control. Cells were exposed to vehicle alone or to 20–50 μM of the bile alcohols indicated. Luciferase activity in the cell extract was normalized to *Renilla* luciferase activity and expressed as fold induction relative to vehicle-exposed cells. The values are means ± SD of three experiments. (B,C) Cells were transfected as in (A), except that β-Gal was used as an internal control, and exposed to 50 μM CDCA (B) or 1 μM GW4064 (C) in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. Exposure of cells to CDCA or GW4064 alone caused a 70- or 115-fold induction, respectively, relative to vehicle-exposed cells. The values are the means ± SD of three experiments.

Discussion

Bile acids and bile alcohols are produced as the terminal catabolites of cholesterol, and as amphipathic steroids they also play important roles in intestinal lipid absorption. Their unusual 5β-A/B *cis* ring juncture provides a structure that allows them to function as excellent detergents [5]. In addition, by activating FXR as physiological ligands, bile acids and alcohols directly modulate expression of genes involved in the biosynthesis/catabolism, excretion, and absorption of bile acids and cholesterol [6]. In the present study, we investigated the role of the A/B ring juncture configuration of bile salts in ligand-activation of FXR.

The results of both cell-based and in vitro assays showed that 5β-cyprinol and 5β-bufol, but not their 5α-counterparts, 5α-cyprinol and 5α-bufol, function as potent FXR agonists (Figs. 2A and 3A), indicating that the *cis*-orientation of the A ring is essential for FXR activation. It was noteworthy that, when assayed with CDCA, the 5α-alcohols potently inhibited agonist-elicited FXR transactivation

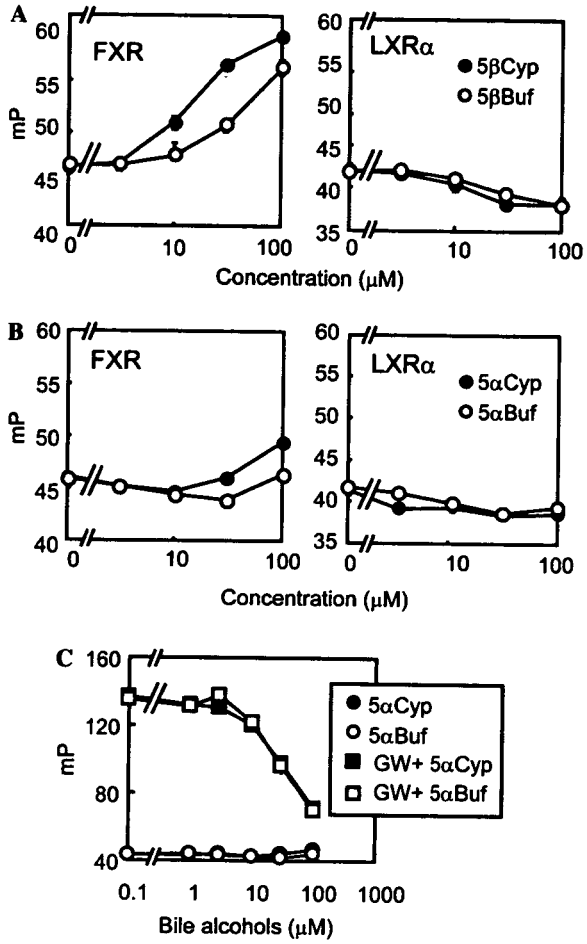

Fig. 3. 5β-Bile alcohols promote association between FXR and SRC-1 peptide in vitro, whereas 5α-bile alcohols function as antagonists, as determined by fluorescence polarization assay. (A,B) A fluorescence-tagged SRC-1 peptide (0.1 μM) was incubated with 1.5 μM GST-FXR or GST-LXRα in the presence of various concentrations of 5β-cyprinol (5β Cyp), 5β-bufol (5β Buf), 5α-cyprinol (5α Cyp), or 5α-bufol (5α Buf) indicated. Ligand-induced SRC-1 peptide association with the receptor was monitored by increases in millipolarization fluorescence units (mP). (C) Changes in fluorescence polarization caused by 3 μM GW4064 were measured in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. The values are means ± SD of three experiments. Some error bars are not visible within symbols.

(Fig. 2B). These two 5α-bile alcohols might have inhibited CDCA transport into the cells, and 5α-cyprinol has actually been shown to inhibit taurocholate uptake by *asbt*, the ileal conjugated bile acid transporter [25]. However, their inhibition of GW4064-elicited FXR transactivation and coactivator-association (Figs. 2C and 3C) indicated that they both competitively inhibit agonist-induced FXR activation. The results of the in vitro experiment (Fig. 3) also indicated that these bile alcohols directly activate FXR as ligands without being metabolized. These findings clearly show that 5α-cyprinol and 5α-bufol function as FXR antagonists.

The ability of 5β-cyprinol and 5β-bufol, and inability of their 5α-counterparts to promote coactivator-association to the receptor (Fig. 3) indicate that the A/B *cis* ring junc-

ture (5β) is required for this process. However, the inhibition of GW4064-induced coactivator-association by the 5α-bile alcohols indicates that they are capable of binding to the receptor. Crystal structure studies have demonstrated interaction between the *cis*-oriented A ring of 5β-bile acids and residues on helix 12, corroborating the association between coactivator peptide and the receptor [10,11]. It is conceivable that the *trans*-oriented A ring in the 5α-bile alcohols destabilizes this agonist conformation of helix 12, thereby preventing coactivator-association.

Although 5α-cyprinol and 5α-bufol efficiently inhibited agonist-induced FXR transactivation and FXR-target gene expression, they had no effect in the absence of agonists. In a FXRE-dependent transactivation assay using HepG2 cells, luciferase activity of no-ligand control was very low, suggesting that the level of endogenous FXR ligands is negligible. Indeed, the bile acids produced in hepatic cells in vitro do not accumulate within the cells but are rapidly released to the medium [26], whereas in vivo the liver is constantly supplied with bile acids via the enterohepatic circulation. It is possible that the 5α-bile alcohols may inhibit FXR activation in the liver.

5α-Cyprinol and its sulfate are toxic and sometimes cause renal and hepatic failure after ingestion of goldfish or carp gallbladders [27,28]. A study has shown that 5α-cyprinol inhibits taurocholate uptake [25], but the mechanism of its toxicity is not well understood. By down-regulating BSEP and up-regulating CYP7A1 antagonizing FXR may lead to an increase in the intracellular level of toxic bile acids. In this study, we found that 5α-cyprinol and 5α-bufol antagonize CDCA-induced BSEP mRNA expression in HepG2 cells at concentrations that do not affect cell viability. Because BSEP plays the major role in bile acid excretion by the liver into the bile [29], and genetic defects in BSEP have been shown to cause progressive familial intrahepatic cholestasis [30], reduced BSEP expression may be involved in the mechanism of the toxicity.

The enhancement of CDCA-induced CYP7A1 mRNA repression by 5α-cyprinol and 5α-bufol was an unexpected finding (Fig. 4B). FXR antagonists should inhibit CDCA-elicited SHP induction and thereby diminish CYP7A1 repression. Exposure of cells to these 5α-bile alcohols alone also led to repression of CYP7A1 mRNA (Fig. 4A), although the SHP mRNA elevation was small or insignificant, suggesting a FXR/SHP-independent mechanism. It is noteworthy that ursodeoxycholic acid has been shown to repress CYP7A1 expression despite its negligible ability to activate FXR [31]. Studies have shown that CYP7A1 can be repressed by bile acids via redundant pathways, including repression through activation of the xenobiotic receptor pregnane X receptor (PXR) or activation of c-Jun N-terminal kinase mediated by TNFα or FXR-inducing FGF19 production [5,32–34]. A recent study has shown that 5α-cyprinol activates mouse PXR, but not human PXR [35], suggesting that the activation of a PXR-mediated pathway is unlikely in our experiments on HepG2 cells.

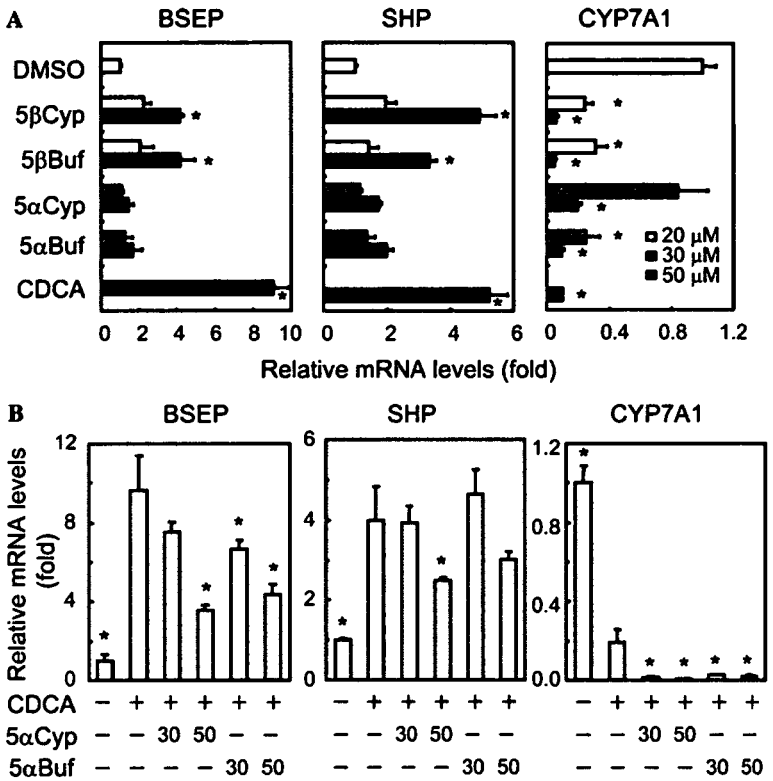

Fig. 4. Regulation of gene expression by various bile alcohols. HepG2 cells were treated for 20 h with vehicle (DMSO) alone, 50 μM CDCA, or the concentrations (20–50 μM) of bile alcohols indicated (A), or with 50 μM CDCA in the presence of the concentrations (μM) of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated (B). Total RNA was isolated from the cells, and the levels of BSEP, SHP, and CYP7A1 mRNA were measured by real-time quantitative RT-PCR. Data were normalized to 18S rRNA levels. The values represent the means ± SD relative to vehicle-exposed cells (taken as 1) from three experiments. Statistically significant differences from respective controls (A) or the cells exposed to CDCA alone (B) are indicated by an asterisk (*$P < 0.01$).

A naturally produced FXR antagonist that lowers cholesterol has been identified [36], and an FXR agonist has been shown to prevent cholesterol gallstone formation [37]. Our findings may provide insights that will be useful for drug development.

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Possible Involvement of Increase in Spinal Fibronectin Following Peripheral Nerve Injury in Upregulation of Microglial $P2X_4$, a Key Molecule for Mechanical Allodynia

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KEY WORDS
ATP; purinergic; glia; extracellular matrix; pain

ABSTRACT
We have recently demonstrated that the $P2X_4$ receptor, an ATP-gated cation channel, in spinal microglia is a key molecule that mediates the mechanical allodynia induced by peripheral nerve injury. Although microglial $P2X_4$ receptor expression is increased after peripheral nerve injury, the molecular mechanism(s) underlying its upregulation remains largely unknown. Fibronectin is a member of the extracellular matrix molecules and is actively produced in response to injury and diseases in the CNS. Here, we describe the influence of fibronectin on $P2X_4$ receptor expression in microglia and the upregulation of fibronectin after peripheral nerve injury. Microglia that were cultured on fibronectin-coated dishes showed a marked increase in $P2X_4$ receptor expression, both at the mRNA and protein levels, as compared to those cultured on control dishes. Fibronectin also enhanced the microglial Ca^{2+} responses mediated by $P2X_4$ receptors. Moreover, Western blot examination of the spinal cord from a rat with spinal nerve injury indicated that fibronectin was upregulated on the ipsilateral side. Interestingly, intrathecal injection of ATP-stimulated microglia to the rat lumber spinal cord revealed that microglia cultured on fibronectin-coated dishes was more effective in the induction of allodynia than microglia cultured on control dishes. Taken together, our results suggest that spinal fibronectin is elevated after the peripheral nerve injury and it may be involved in the upregulation of the $P2X_4$ receptor in microglia, which leads to the induction of neuropathic pain.

INTRODUCTION

Extracellular nucleotides act as signaling molecules in numerous tissues. Two groups of purinoceptors with distinct signal transduction mechanisms are known to exist. P2X purinoceptors are ligand-gated ion (cation) channels, whereas P2Y purinoceptors are members of the superfamily of G protein-coupled receptors. The P2X family consists of seven different subunits that can form homo- or hetro-oligomeric assemblies, and each subunit has two transmembrane regions with intracellular N- and C-termini. The $P2X_4$ receptor has a broad expression pattern in the periphery, and it predominates in the CNS (Le et al., 1998; Soto et al., 1996). With regard to the physiological and pathological importance of $P2X_4$ in the CNS, we have recently showed that $P2X_4$ receptors in the spinal cord are upregulated after peripheral nerve injury, which is responsible for the induction of mechanical allodynia in rats (Tsuda et al., 2003). Interestingly, the $P2X_4$ receptor is upregulated in microglia but not in neurons in the spinal cord. Allodynia is a form of neuropathic pain that is caused by normally innocuous stimuli, such as touch, and although the symptom has been recognized for over a century, its cellular mechanisms are largely unknown. Microglial $P2X_4$ receptors in the spinal cord could be a key molecule that induces the mysterious neuropathic pain, allodynia.

Microglia are brain-specific macrophages, and their activation is a general response to pathological processes in the CNS. They are in a quiescent state in the normal brain, but become rapidly activated upon brain injury, inflammation, or diseases, transforming from ramified microglia into an amoeboid macrophage-like phenotype. 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 (Milner and Campbell, 2002, 2003). Fibronectin is one of the extracellular matrix (ECM) molecules, and it is a large, multi-domain glycoprotein existing both as a cell surface protein and in plasma. Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion (Adams and Watt, 1993; Hynes, 1992; Raghow, 1994). The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the normal CNS. Some ECM molecules including fibronectin, however, are upregulated following adult CNS injury (Jones, 1996). These

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data suggest that fibronectin is a key molecule involved in the overexpression of $P2X_4$ in microglia after nerve injury.

In the present study, we demonstrate that: (1) culturing primary microglia on fibronectin induces the upregulation of functional $P2X_4$ receptors on the cell surface in vitro, (2) increased expression of fibronectin was observed in the ipsilateral side of the spinal cord taken from allodynia rats, and (3) intrathecal administration of ATP-stimulated microglia that had been treated with fibronectin enhanced the allodynic response in rats in vivo. All these findings suggest that the increase in spinal fibronectin after a spinal nerve injury is a critical event in the upregulation of microglial $P2X_4$ receptors, which would be important for the onset of mechanical allodynia.

MATERIALS AND METHODS

Isolation of Microglia

The primary cultures of rat microglia were derived from the forebrains of neonatal Wistar rats (Nakajima et al., 1992). In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and with 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 the flasks for 2 min.

Quantitative RT-PCR

Microglia were plated on tissue culture dishes that had been coated with fibronectin (Sigma, Missouri, USA) at 10 μg/ml or non-treated, and kept at 37°C for 3 h. Then, the cells were washed with warm DMEM twice and the total RNA was extracted using the RNeasy mini kit (QIAGEN Japan, Tokyo, Japan). Real time RT-PCR was performed using the TaqMan One-Step RT-PCR Master Mix Kit (Applied Biosystems, CA), $P2X_4$ primers, and TaqMan GAPDH Control Reagents (Applied Biosystems). The forward and reverse primer pairs for $P2X_4$ were:

F: 5′-TGGCGGACTATGTGATTCCA-3′

R: 5′-GGTTCACGGTGACGATCATG-3′

The PCR reaction was carried out by One Step RT-PCR in a total volume of 25 μl using the ABI PRISM 7700 Sequence Detection system (Applied Biosystems). All values were normalized with the GAPDH expression.

Western Blotting

Microglia were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% SDS, 5 mM EDTA, protease inhibitors cocktail) and mixed with Laemmli sample buffer. For the rat spinal cord homogenates, the L5 corresponding spinal cord was collected from control or allodynia rats of 1-, 3-, 7-day post operation and the area of dorsal horn was excised. Then the tissue was homogenized in homogenization buffer (PBS, 1% NP-40, 1% Triton X-100, 5mM EDTA, protease inhibitors cocktail) for 20 s on ice, centrifuged thoroughly to remove cell debris, and mixed with Laemmli sample buffer. All samples were subject to BCA assay to adjust the loading protein amount. Cell lysates or tissue homogenates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, CA). The membrane was blocked with TBS-Tween 0.05%, 1% BSA, 0.02% NaN_3, and probed with primary antibodies: anti-$P2X_4$ (Alomone, Jerusalem, Israel, 1:200 dilution), anti-β-actin (Sigma, 1:1000 dilution), anti-ERK2 (Santa Cruz, CA, 1:200 dilution), or anti-fibronectin (Dako, Glostrup, Denmark, 1:100 dilution). The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (Amersham Biosciences, NJ, 1:1000 dilution) and visualized with the ECL system (Amersham Biosciences). Bands were quantified using NIH Image J 1.33u software.

Intracellular Calcium Concentration ($[Ca^{2+}]i$) Measurement

Microglia were cultured for 24 h at 37°C on an appropriately coated Flexiperm cover glass. Then the culture medium was replaced with balanced salt solution (BSS at pH 7.4: 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, 10 mM D-glucose). Cells were loaded with fura-2 by incubating them with 5 μM fura-2-acetoxymethylester in BSS for 1 h at room temperature. Changes in $[Ca^{2+}]i$ were assessed by ratiometric images (F340/F380) of fura-2 fluorescence, which were detected with Aquacosmos/HiSca (Hamamatsu Photonics, Hamamatsu, Japan). For TNP-ATP(100 μM), PPADS (10 μM), or 0 Ca^{2+} (removal of extracellular Ca^{2+}) experiments, cells were treated with these antagonists or the 0 Ca^{2+} solution 2 min before and during ATP-applications.

Chung Model

All experiments were performed using 8-week-old male Wistar rats. All surgeries were performed under inhalation anesthesia using Forene in 100% O_2, induced at 5% and maintained at 2%. The spinal nerve on the left side was exposed at a proximal location under an aseptic condition. Then the 5^{th} lumber spinal nerve was tightly ligated with a silk suture (5-0) and its peripheral side was completely transected. The muscle and the skin were sutured closed, and the animal was allowed to recover before the behavioral testing. To evaluate allodynia, von Frey filaments were applied to the plantar surface of the hindpaw, and the withdrawal from mechanical stimulus was monitored as previously reported (Tsuda et al., 2003).

Intrathecal Catheterization and Injections of Microglia

For intrathecal microglia administration, intrathecal catheterization was performed on Wistar rats (12 weeks, male) (Tsuda et al., 2003). Briefly, with the rat under inha-

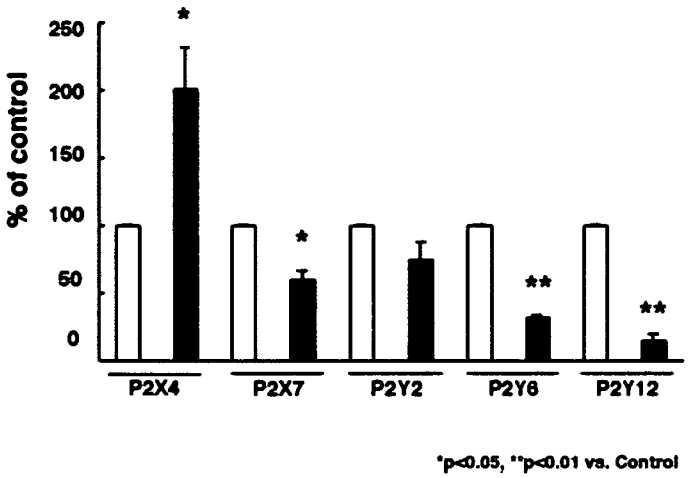

Fig. 1. The effect of fibronectin on the mRNA expression of microglial $P2X_4$. Fibronectin increased the expression of $P2X_4$ in microglia at the mRNA level. Microglia were cultured on fibronectin for 3 h at 37°C, and the expression of $P2X_4$ was assessed by quantitative RT-PCR. $P2X_4$ was markedly upregulated by fibronectin, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ purinoreceptors were significantly decreased. Data are mean ± SE of 3 separate experiments. Asterisks show significant difference from control (*$P < 0.05$, ** $P < 0.01$ vs. control, Student's t-test).

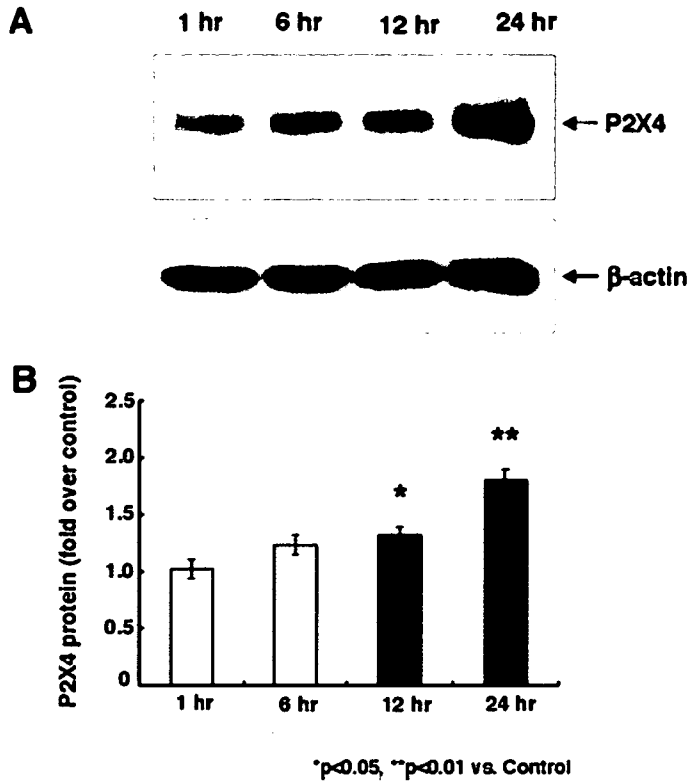

Fig. 2. Time-course study of the microglial $P2X_4$ upregulation. Fibronectin increased the expression of $P2X_4$ in microglia at the protein level. Microglia were cultured on fibronectin for 1, 6, 12, and 24 h at 37°C, and the protein expression of $P2X_4$ receptors was analyzed by Western blotting. The protein expression of $P2X_4$ receptors began to increase after 12 h of incubation and increased strongly after 24 h of incubation. The intensity of the bands was quantified with a computing densitometer using NIH Image J 1.33u image analysis software. Asterisks show significant difference from control (*$P < 0.05$, ** $P < 0.01$ vs. control, Student's t-test).

lation anesthesia, an incision was made in the atlanto-occipital membrane and the catheter was inserted caudally to the lumber enlargement (close to L4-L5 segments) of the spinal cord. Verification of the catheter placement was made by the observation of hind limb paralysis after intrathecal injection of lidocaine (2%, 5 μl) 3 days after catheterization. Animals that failed the verification for the catheter placement were not included in the data analyses. Microglia were cultured on uncoated- or fibronectin-coated dishes for 24 h at 37°C, washed twice with PBS, and harvested. After adjusting their concentrations, cells were stimulated with ATP at 0, 0.5, and 5 μM, incubated for 1 h at 37°C, and subsequently microinjected. Animals were subject to the behavioral testing 5 h after the injection.

Statistical Analysis

The von Frey test results were analyzed by the Mann-Whitney U-test and values with $P < 0.05$ were considered statistically significant. For the other data, the Student's t test was performed, and values with $P < 0.05$ (or $P < 0.01$ where appropriate) were considered statistically significant as compared to controls.

RESULTS

Fibronectin Increased the Expression of $P2X_4$ Receptors in Microglia at both the mRNA and Protein Levels

Microglia were plated onto fibronectin or control plastic, and their P2 receptor expression was studied by quantitative RT-PCR (Fig. 1). To normalize the results, we used the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and, therefore, the P2 receptor gene expression was given as the ratio P2X(Y)/GAPDH. As shown in the figure, incubation of microglia on fibronectin at 10 μg/ml for 3 h resulted in the marked upregulation of $P2X_4$ gene expression, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ were all rather diminished (Fig. 1), suggesting that the $P2X_4$ receptor is unique among the purinoceptors on microglia.

To confirm this effect of fibronectin on the $P2X_4$ receptor at the protein level, we examined its expression by Western blotting using anti-$P2X_4$ antibody (Fig. 2). As seen in Fig. 2, microglial $P2X_4$ appeared as a single band at ~75 kDa, and since its predicted molecular weight from its protein sequence is 43 kDa, the molecule seems to be heavily glycosilated (Soto et al., 1996). We previously reported that fibronectin induces profound microglial proliferation through β1 integrin (Nasu-Tada et al., 2005), and thus the protein amount loaded on the gel was carefully adjusted. In addition, β-actin was used as the endogenous control to normalize the Western blot data. Each band was quantified using computing software, and the basal value of β-actin was subtracted from the $P2X_4$ results. The increase in $P2X_4$ expression became evident after 12 h of fibronectin stimulation (Fig. 2) (1.3-fold as compared to 1 h incubation, $P < 0.05$), and the increase continued until it reached an

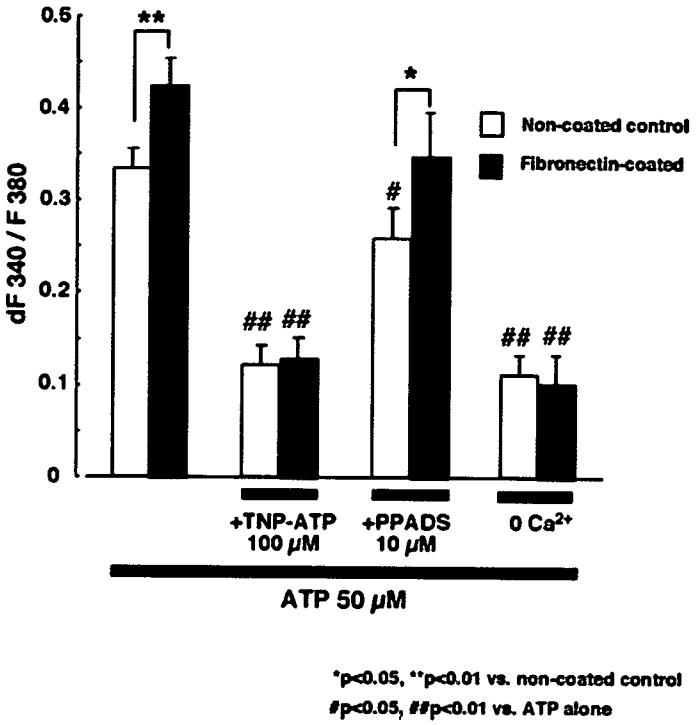

Fig. 3. Enhancement by fibronectin of the function of $P2X_4$ receptors in microglia. The function of microglial $P2X_4$ was assessed by fura-2 based $[Ca^{2+}]i$ imaging (ratio of F340/F380). Microglia cultured on fibronectin showed an increase in the Ca^{2+} response to stimulation with ATP 50 μM. Microglia were cultured for 24 h on fibronectin or on a control, and pretreated with TNP-ATP (100 μM) or PPADS (10 μM) for 2 min where required. Flexiperm cover glass (i.e., non-coated) and the ATP (50 μM)-evoked increase in $[Ca^{2+}]i$ was monitored. 0 Ca^{2+} indicates removal of Ca^{2+} from the extracellular medium. Asterisks and #s show significant difference from non-coated control and ATP alone, respectively (*$P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. ATP alone, Student's t-test).

approximately 2-fold increase after 24 h incubation ($P < 0.01$).

Microglia Cultured on Fibronectin Showed an Increase in $[Ca^{2+}]i$ in Response to ATP Stimulation

To confirm that fibronectin upregulates functional $P2X_4$ receptors on microglia, the ATP-evoked increases in $[Ca^{2+}]i$ were subsequently studied. Microglia were cultured for 24 h on fibronectin or on uncoated Flexiperm cover glass (control), and the changes in $[Ca^{2+}]i$ in response to ATP (50 μM) were detected by the conventional fura-2 method., i.e., the ratiometric images of fura-2 fluorescence. The nucleotide receptors that are known to be expressed in microglia include $P2X_4$, $P2X_7$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$ (Inoue, 2002; Sasaki et al., 2003; Tsuda et al., 2003), and possibly $P2Y_{13}$ due to its abundant mRNA in the brain and the immune system (Zhang et al., 2002). $P2Y_{12}$ and $P2Y_{13}$ receptors are Gi-coupled P2 receptors, and the activation of these receptors, in general, does not cause an elevation in $[Ca^{2+}]i$ but decreases the intracellular cAMP.

In the absence of extracellular Ca^{2+} (Fig. 3, 0 Ca^{2+}), neither the control nor microglia cultured with fibronectin showed much response to ATP 50 μM stimulation, suggesting that Gq/11-phospholipase C coupled P2Y receptors, which are dependent on intracellular Ca^{2+} storage, were not relevant to this case. In contrast, in the presence of extracellular Ca^{2+}, microglia on fibronectin showed a significant increase ($P < 0.01$) in the Ca^{2+} response to ATP 50 μM (Fig. 3, ATP alone), indicating that the expression of the ion-channel type purinoceptors, i.e., P2X receptors, is augmented by fibronectin and that these are likely to be $P2X_4$ receptors, since the $P2X_7$ receptor is activated at a relatively high concentration of ATP (i.e., concentrations greater than 100 μM) (Ralevic and Burnstock, 1998). Pretreatment of cells with TNP-ATP (an antagonist of $P2X_{1-4}$ receptors) dramatically reduced the $[Ca^{2+}]i$ response in microglia on both control and fibronectin-coated dishes, indicating that basal response to ATP at 50 μM as well as its augmented response on fibronectin substrate mostly result from microglial $P2X_4$ receptor. On the other hand, pretreatment with PPDAS (an antagonist of $P2X_{1,2,3,5,7}$) did not fundamentally affect but only slightly reduced the $[Ca^{2+}]i$ response in both populations. PPADS is known to inhibit $P2Y_{1-2}$ receptors as well and, therefore, the result indicates that microglial $P2Y_2$ receptor also constitutes the $[Ca^{2+}]i$ response to ATP stimulation. In conclusion, fibronectin upregulated the functional $P2X_4$ receptors on the microglial surface, and this lead to an enhancement of the increase in $[Ca^{2+}]i$ evoked by ATP 50 μM via $P2X_4$ receptors.

Fibronectin Was Upregulated in the Allodynia Rat Spinal Cord

As described earlier, the importance of the microglial $P2X_4$ receptor in the induction of mechanical allodynia after nerve injury has recently become evident (Tsuda et al., 2003). We sought to determine the profile of fibronectin expression in the spinal cord of nerve-injured rats, where the microglial $P2X_4$ receptor expression is increased. L5 spinal cord segments were harvested from rats of control, 1-, 3- and 7-day post nerve injury, and the expression of fibronectin was assessed by Western blotting. There has been little evidence for fibronectin in the normal CNS other than in the basement membrane of endothelial, pial, and ependymal cells, and our result showed that Naive and Day 1 rats exhibited slight signs of fibronectin (Fig. 4). However, spinal fibronectin became evident on the ipsilateral side at 3 and 7 days following the nerve injury (Fig. 4, Day 3 ipsi, Day 7 ipsi). The contralateral side remained unchanged throughout the experiment.

Upregulation of Microglial $P2X_4$ Receptors Lowered the Threshold of Pain Responses Caused by Intrathecal Transfer of the Cells

In the study by Tsuda et al. (2003), the intrathecal transfer of ATP-treated microglia induced mechanical allodynia

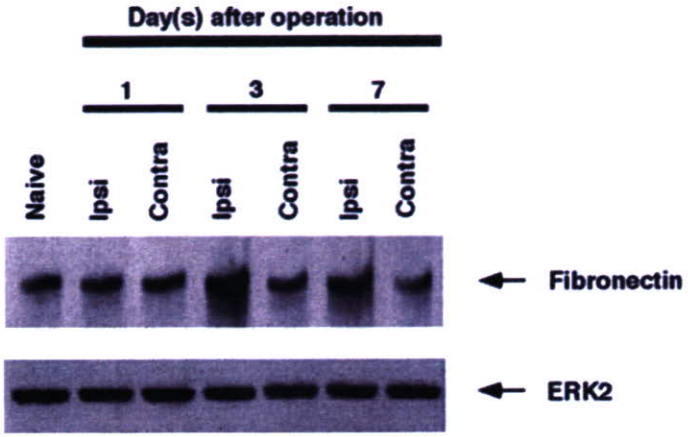

Fig. 4. Time-course study of fibronectin expression in the allodynia rat spinal cord. The rat spinal nerve on the left side was exposed, tightly ligated with a silk suture, and its peripheral side was completely transected. On Days 1, 3, and 7 post-operation, L5 spinal cords from control and allodynia rats were collected and the tissues were subjected to homogenization and Western blotting. Anti-fibronectin (Dako, 1:100 dilution) antibody and HRP-conjugated anti-rabbit IgG (Amersham) antibody were used for the detection. The data represent 3 independent experiments.

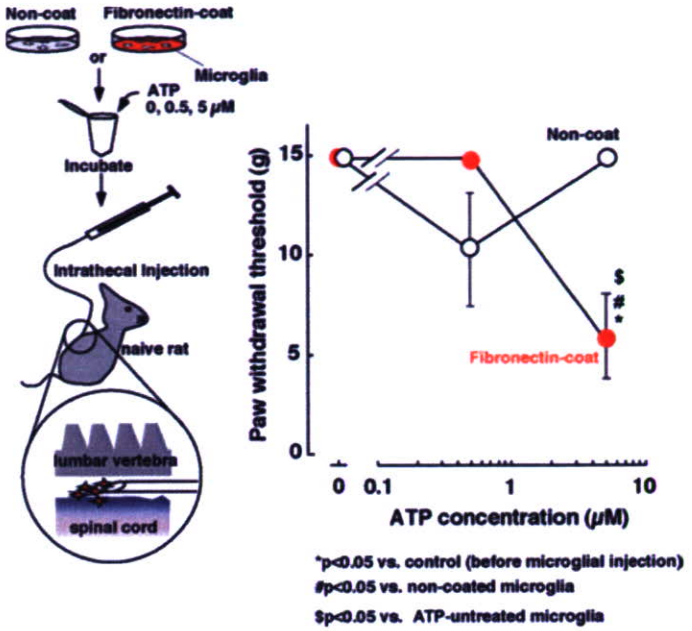

Fig. 5. Changes in nociceptive response after intrathecal transfer of microglia with elevated expression of $P2X_4$ receptors. Microglia were cultured either on fibronectin (red circle) or on control plastic (white circle) for 24 h at 37°C and both groups were subsequently stimulated with ATP at 0 (control), 0.5, and 5 μM for 1 h at 37°C. Without intrathecal microinjection of microglia, no rats showed pain behavior. Then the cells were intrathecally transferred to the lumbosacral spinal cord of a normal rat. Five h after the microinjection, nociceptive responses were evaluated by measuring the 50% paw withdrawal threshold to mechanical stimuli. Six rats were used in each group for this study. The Mann-Whitney U-test was performed and statistical significance was set at $P < 0.05$ [$*P < 0.05$ vs. control (before microglial injection); $\#P < 0.05$ vs. non-coated microglia; $\$P < 0.05$ vs. ATP-untreated microglial injection]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in normal rats, and microglial $P2X_4$ receptors were mainly responsible for this effect. Therefore, we hypothesized that microglia with more $P2X_4$ receptors expressed on the surface are capable of causing severer mechanical allodynia. To examine this hypothesis, microglia were cultured either on fibronectin or on control plastic for 24 h, stimulated with 0.5 or 5 μM of ATP for 1 h or left untreated as the control, then intrathecally transferred to normal rats, and their pain behavior was monitored 5 h after the microinjection using von Frey hairs to calculate the 50% paw withdrawal threshold (Fig. 5). Without intrathecal injection of microglia, no rat showed any pain behavior (data not shown). As seen in Fig. 5, no pain response was observed at ATP 0 (control) or 0.5 μM. An interesting difference, however, was seen at ATP 5 μM, where a significant decrease in the 50% withdrawal threshold was observed with fibronectin-treated microglia as compared with the non-treated microglia. Additionally, it was clearly demonstrated that intrathecal transfer of microglia that were treated with ATP at 50 μM, in the absence of fibronectin, was capable of inducing allodynia in the recipient rat (Tsuda et al., 2003). Collectively, these results suggest that upregulation of $P2X_4$ receptors by fibronectin lowered the threshold for the response to mechanical allodynia.

DISCUSSION

In the present study, we demonstrated that: (1) the treatment of microglia with fibronectin enhanced the expression of functional $P2X_4$ receptors, (2) the spinal fibronectin was upregulated after the peripheral nerve injury, and (3) the fibronectin treatment of microglia lowered the concentration of ATP that was necessary to cause mechanical allodynia by intrathecal transfer. Because the upregulation of $P2X_4$ receptors in spinal microglia is a critical event in the induction of mechanical allodynia after peripheral nerve injury (Tsuda et al., 2003, 2005), our present results may partially clarify the mechanism of mechanical allodynia.

The Correlation Between Fibronectin and Microglial $P2X_4$ Receptor

The $P2X_4$ receptor displays a broad tissue distribution, especially in the CNS (Soto et al., 1996). Although the precise physiological roles of $P2X_4$ receptors remain unknown, it has been reported that the upregulation of $P2X_4$ receptors is linked to several pathological conditions, such as nerve injury (Tsuda et al., 2003), ischemia (Cavaliere et al., 2003), and muscular dystrophy (Yeung et al., 2004). In the present study, we showed that fibronectin increased the microglial $P2X_4$ expression at the mRNA level by more than 2-fold. The elevation was also confirmed at the protein level, and these upregulated $P2X_4$ receptors were shown to be functional by the increase in $[Ca^{2+}]i$ mediated by $P2X_4$ receptors.

These findings are of interest because some ECM molecules, including fibronectin, are known to be upregulated following adult CNS injury (Jones, 1996). Fibro-