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（分担）研究報告書

AcPepAの安定性及び代謝物に関する研究

研究分担者　前田康博　名古屋市立大学大学院薬学研究科

研究要旨　C5a アナフィラトキシン阻害相補性ペプチドである AcPepA の EDTA 血漿中での安定性を検討した。また生体中における AcPepA の代謝物を同定した。

A 研究目的

AcPepA は生体中での分解速度が非常に速い（半減期約２分)。血漿中 AcPepA の安定性を調べ保存条件の検討を行う。また，代謝物を同定し，AcPepA の体内動態を調べることを目的とする。

B 研究方法

ヒト EDTA 血漿に AcPepA を加え，0, 25, 37 ℃で incubate した。これまでに確立した HPLC-MS/MS 法により経時的に定量し，AcPepA の安定性を検討した。

ラットに AcPepA を 5mg/Kg で急速静注後，経時的に採血し、1mM EDTA 水溶液に加え氷冷した。HPLC-MS/MS により代謝物を同定し，AcPepA およびその代謝物の動態を検討した。

C 実験結果

0, 25, 37 ℃での EDTA 血漿中 AcPepA の半減期は 3.2 日，2.8 時間，1.1 時間であった。

ラットにおける AcPepA の代謝物は MS/MS において *m/z* 679.78 > 1030.0 及び 1104 > 608.1 が検出された。これらは AcPepA の C 末端からアミノ酸が２つ脱離したペプチド(15AA)または４つ脱離した(13AA)であると同定された。これらのペプチドは AcPepA 投与後すぐに生成し始め 50-70 秒で最大濃度となった後，減少した。15AA の半減期は約１分，13AA の半減期は約６分であった。これらのペプチドは更に切断され代謝されると考えられる。

D 考察

AcPepA は体内において非常に速く分解がされるが，EDTA 血漿中 0 ℃では半減期 3 日程度と比較的安定である。また AcPepA は体内で C 末端のアミノ酸がペプチダーゼの作用によって切断されていくと考えられる。以上から，EDTA が金属イオンをキレートすることでペプチダーゼが阻害され安定に存在したと考えられる。

E 結論

血中 AcPepA 分析を行うには，EDTA 血漿とし-20 ℃以下で保存する。
AcPepA はペプチダーゼにより速やかに代謝される。

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研究成果の刊行に関する一覧表レイアウト（参考）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
岡田秀親	補体系の全体像	大井洋之 木下タロウ 松下操	補体への招待	メジカルビュー社	東京	2011	9-14
今井優樹 岡田則子	補体活性化と制御 後期経路	大井洋之 木下タロウ 松下操	補体への招待	メジカルビュー社	東京	2011	26-38

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HMGB1 release by C5a anaphylatoxin is an effective target for sepsis treatment

Noriko Okada*, Masaki Imai*, Alan Okada[&], Fumiko Ono[#] and Hidechika Okada*[,&]

*Department of Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, [&]Choju Medical Institute, Fukushimura Hospital, Toyohashi 411-8124, and [#]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba 305-0843, Japan

Address correspondence and reprint request to Dr. Hidechika Okada, Choju Medical Institute, Fukushimura Hospital, Noyori-cho, Yamanaka 19-14, Toyohashi 441-8124, Japan E-mail: hiokada@med.nagoya-cu.ac.jp

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Key words: complement; C5a; anaphylatoxin; inflammation; sepsis; peptide; endotoxin-shock; C5a receptor; C5L2; HMGB1; cytokine storm.

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Antibodies to C5a have proven to be effective in treating experimental septic primate models[1,2]. A 17 amino acid peptide (ASGAPAPGPAGPLRPMF) named PepA binds to C5a and prevents complement-mediated lethal shock in rats[3]. AcPepA harboring an acetyl group at the N-terminal alanine showed increased inhibitory activity against C5a[4]. Cynomolgus monkeys destined to expire from a lethal dose of bacterial endotoxin (4mg/kg) were rescued by intravenous administration of AcPepA. AcPepA could have interfered with the ability of C5a to stimulate C5L2[5,6] which is responsible for HMGB1 release and stimulation of TLR4[7-9] as an endogeneous ligand with LPS behavior. The suppression of HMGB1 release by AcPepA administration to LPS-shock monkeys is likely responsible for rescuing the animals.

Sepsis is a systemic inflammatory response syndrome (SIRS) that causes disseminated intravascular coagulation (DIC) and multiple organ failure (MOF). Antibodies to C5a have proven to be effective in treating experimental septic primate models[1,2]. We generated an inhibitory peptide of C5a composed of an amino acid sequence ASGAPAPGPAGPLRPMF named PepA[3] . Acetylation at the N-terminal alanine of PepA improved the C5a inhibitory capacity and was named AcPepA[4].

Under anesthesia with sodium pentobarbital, 10 cynomolgus monkeys (weighing about 5 kg) were intravenously administered 4 mg/kg LPS within 30 min. Three monkeys for the control group were infused with 15 ml saline during 3 hrs after the LPS injection. Seven experimental group monkeys were infused intravenously with 15 ml of 2 mg/ml AcPepA starting at 30 min after LPS injection for 3 hrs (2 mg/kg/hr for 3 hrs). Six hrs after LPS administration, anesthesia was terminated when the blood samples showed leukocytosis and increased CPK in all monkeys. Monkeys were observed for their status. All of the 7 AcPepA treated monkeys returned to a healthy condition by the following day, while the 3 control monkeys died within two days.

Despite the increased TNFα and other cytokine levels, high mobility group box 1 (HMGB1)[5,6] which is an endogenous stimulator of TLR4[7-9] did not increase in the AcPepA infused animals (Fig. 1).

Furthermore, AcPepA could suppress pathophysiological events and prolonged survival time of sepsis piglets induced by cecal ligation and perforation (CLP)[10]. Survival times were longer in the AcPepA treated group than in the CLP alone group (19.3hrs ± 2.7hrs vs. 9.9 hrs ± 0.7 hrs, $P<0.005$). In this case, AcPepA also delayed the HMGB-1 surge.

These above results indicate that suppression of C5 anaphylatoxin interferes with

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the induction of a cytokine storm. Since C5a has the capacity to cause release of HMGB1 following stimulation of the second C5a receptor termed C5L2 generated on activated monocytes[11-13], inhibition of C5a successfully interferes with the above release which has the capacity to generate inflammatory cytokines stimulating TLR4 as an endogenous ligand (Fig. 2).

Recently, thrombomodulin (TM) administration has been shown to rescue septic shock animals[14]. The enhanced activity of thrombin when complexed with TM should have caused activation of thrombin activatable fibrinolysis inhibitor (TAFI) which then inactivates C5a anaphylatoxin by removing the C-terminal arginine[15,16] resulting in suppression of HMGB1 release. Therefore, the therapeutic effect of TM on sepsis should also be due to inactivation of C5a anaphylatoxin which initiates a cytokine storm through HMGB1 release.

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(Fig. 1)

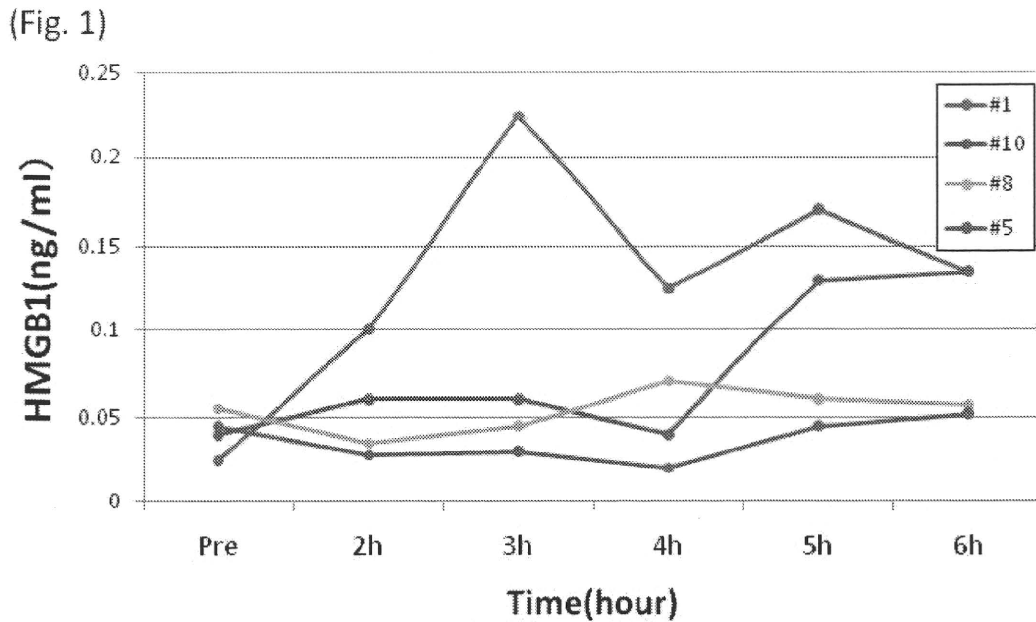

Figure 1. Increase in HMGB1 in plasma of LPS- injected monkeys.

Six cynomolgus monkeys intravenously infused with a lethal dose of bacterial LPS (4mg/kg) destined to death were treated with intravenous administration of 2 mg/kg/h of AcPepA for 3h starting 30 min after the lethal LPS injection (#5 and #8).. Control monkeys (#1 and #10) were infused only saline in stead of AcPepA following LPS injection. Despite the increased TNFα and other cytokine levels, high mobility group box 1 (HMGB1) which is an endogenous stimulator of TLR4[7] did not increase in the AcPepA infused animals (#5 and #8).

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(Fig. 2)

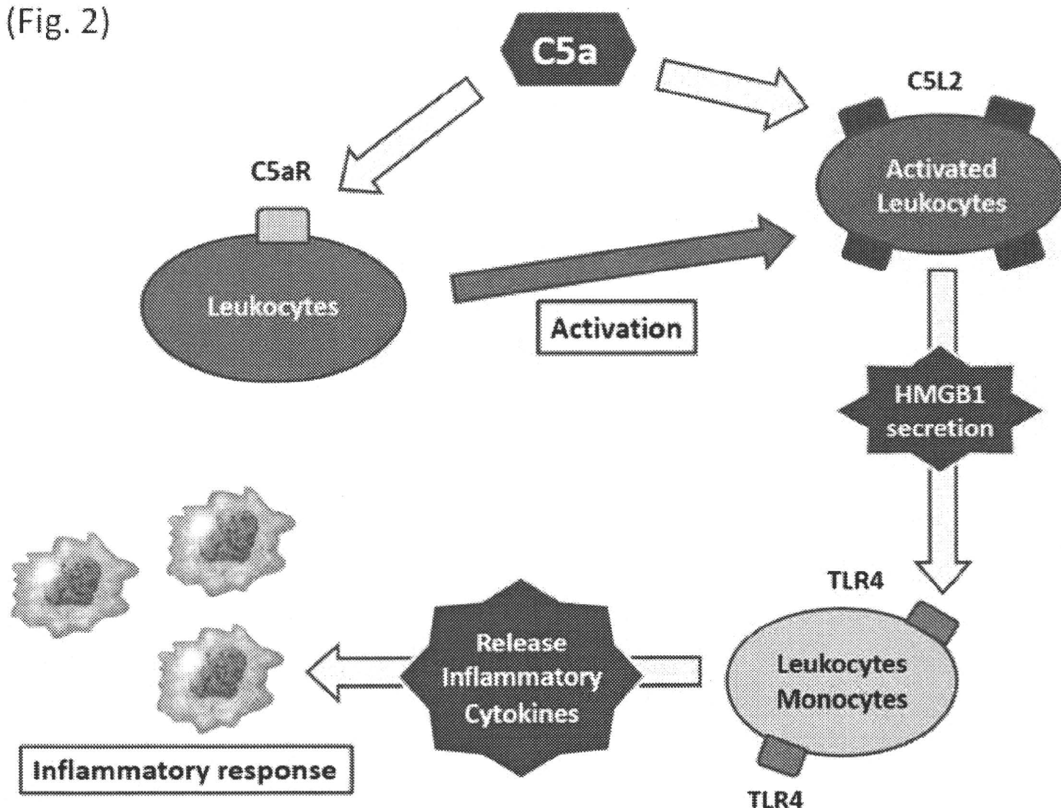

Figure 2. Possible role for C5a in a positive feedback inflammatory circuit.

Following bacterial infection, LPS stimulates TLR4, and C5a generated during complement activation stimulates C5aR resulting in expression of C5L2 on leukocyte membranes. Stimulation of C5L2 by C5a on activated leukocytes induces release of HMGB1 which then reacts with TLR-4 on other leukocytes, as did LPS, resulting in further recruitment of activated leukocytes that express C5L2. These reactions create an inflammatory amplification circuit.

相補性ペプチドによる炎症の制御

今井優樹[1)]/岡田則子[2)]

〔KEYWORDS〕 相補性ペプチド，アナフィラトキシンC5a，炎症

1．はじめに

生物は，細菌やウイルスなどの微生物の体内への感染・侵入に対して，補体やToll-like receptor(TLR)に代表される自然免疫系の生体内防御機構を活性化させ，それらを体内からすばやく排除する仕組みを備えている．自然免疫がひとたび病原体の感染を感知すると，免疫応答に必須な炎症性サイトカインが産生され，生体内で炎症反応を引き起こし，免疫系にかかわる細胞を，感染局所に動員して病原体を排除する．しかし一方で，これらの防御反応の異常亢進により，アレルギーや自己免疫疾患が誘発されたり，重症化した場合には敗血症，播種性血管内凝固症候群(disseminated intravascular coagulation；DIC)，多臓器不全(multiple organ failure；MOF)などの全身性炎症反応症候群(systemic inflammatory response syndrome；SIRS)病態へと進展する．現在まで，様々な敗血症の動物実験モデルが作られ，またいくつもの薬剤の臨床試験が行われてきたが，いまだに臨床の場における敗血症などの過剰な炎症反応に起因する致死的病態を改善する薬剤は登場していない．

本稿では過剰な炎症反応を制御することを目的に創出された相補性ペプチド(complementary peptide)について概説するとともに，相補性ペプチドの今後の可能性について述べる．

2．相補性ペプチド

ポリペプチド鎖から構成される蛋白質では，分子内でアミノ酸配列自身が情報化され，分子内にセンスペプチド・アンチセンスペプチドの関係によって，相互に対応する配列情報をもつ部分が散在することが，1995年Baranyiらにより見いだされ[1)]，この部分をアンチセンスホモロジーボックス(antisense homology box；AHB)と命名した．アンチセンスペプチドはその特徴としてセン

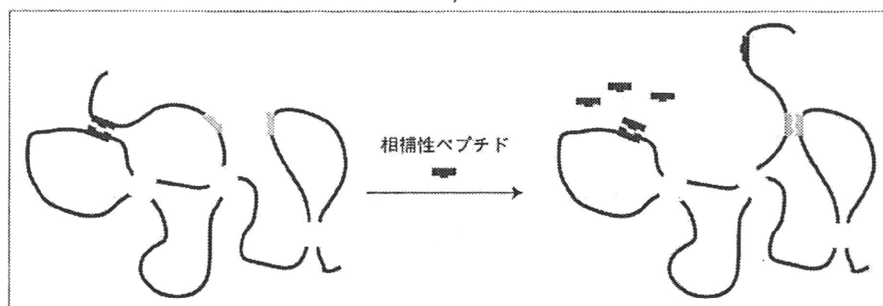

図1　AHBペプチドによる標的蛋白質分子の機能制御

1）IMAI Masaki　名古屋市立大学大学院医学系研究科病態医科学講座免疫学分野・講師
2）OKADA Noriko　同分野・教授

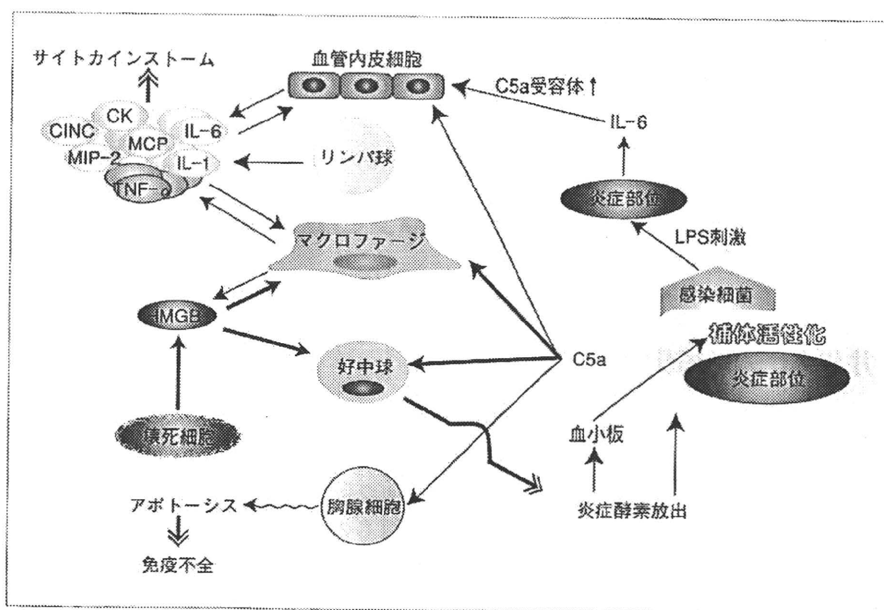

図2　C5aアナフィラトキシンの敗血症ショックにおける役割

スペプチド間でのハイドロパシーが逆相関値となることが知られている．AHBは対応するセンスペプチド間で相互作用することにより，高次構造の形成と維持に重要な働きをしているであろうことが推察された(図1)．そこでわれわれは，AHBに対応する相補性ペプチドを設計，合成して作用させることにより，蛋白質の機能制御が可能となることを立証してきた．相補性ペプチドとは，標的ペプチドに対して，疎水性パターンが逆値になって相補性になっていること，対応する位置のアミノ酸側鎖の容積(bulkiness)が対応性でアミノ酸同士のα炭素が0.5 nm以内に接近できること，ペプチド骨格のバックボーン並列性(backbone alignment)の一致性などを指標にして作成するペプチドであり，われわれは相補性ペプチドを自動設計するコンピュータプログラムMIMETICを独自に開発することに成功した[2]．現在までに，MIMETICで設計合成した相補性ペプチドの約30%が標的蛋白質の機能を制御する活性をもち，相補性ペプチドの蛋白質機能制御の有効性が検証できており[3~5]，これらの中から最適な炎症過反応制御性ペプチドを選出して解析を進めている．

3. アナフィラトキシンC5aの機能

C5a(fifth component of complement)は補体活性化反応の中間産物であり，アナフィラトキシン(anaphylatoxin)と呼称されるごとく，血管透過性を高めてショック症状を引き起こす．また，極微量(pgオーダー)で単球やマクロファージを活性化して炎症局所に遊走集積させ，interleukin (IL)-1β，IL-6，tumor necrosis factor (TNF)-αなどの炎症性サイトカインを分泌する．一方で，肥満細胞や好塩基球を活性化し，アレルギー性の炎症を引き起こすヒスタミンやセロトニンなど種々のメディエーターを放出させる．さらに，C5aは血管内皮細胞に直接作用し，P-セレクチンなどの接着分子を発現させる．C5a刺激によりマクロファージから放出されたIL-1β，TNF-αは血管内皮細胞表面にintercellular adhesion molecule(ICAM)-1やE-セレクチンを，IL-6はC5aレセプターの発現を増強させる．通常ではこのような免疫応答で炎症を引き起こされた後，炎症性サイトカインやそれらを放出するマクロファージ，好中球は時間の経過とともに沈静化されるが，過剰な炎症反応ではマクロファージや好中球，血管内皮細胞から大量の炎症性サイトカインが放出され，サイトカイン・ストームを誘発する(図2)．敗血症などにおいては，細菌により宿主の正常な免疫応答力が阻害された免疫不全の状態にも陥り，DICやMOFなどの重篤な病態に進行

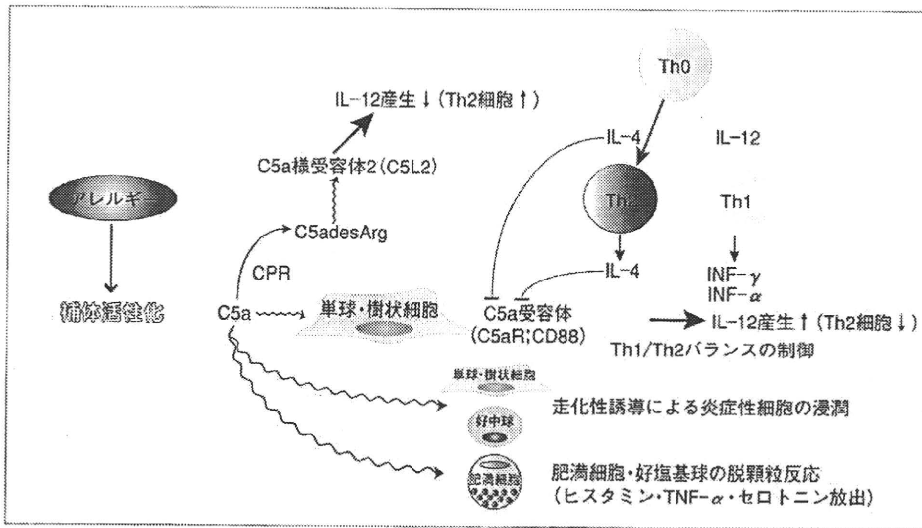

図3　アレルギー症状発症におけるC5aアナフィラトキシンの役割

すると考えられる[6]．

4. C5aに対する相補性ペプチドのエンドトキシンショックに対する治療効果

Baranyiらは C5a と C5a 受容体に対する分子間 AHB を検索し，AHB ペプチドが C5a 刺激による細胞内へのカルシウムイオンの流入を抑制することを見いだし，C5a-C5a 受容体の相互作用に影響を及ぼすことを証明した[7]．その中で最も強い阻害活性を示した C5a の AHB ペプチドである PL37 に対する相補性ペプチドを MIMETIC で検索し，C5a に反応してその相互作用を阻害する相補性ペプチド PepA を創出した．LPS(lipopolysaccharide：リポ多糖)投与によるラットエンドトキシンショック実験モデルにおいて，コントロールでは生存率 0% であったのに対し，4 mg/kg の PepA を静脈注射すると全例を救命できた[4]．ペプチド剤は蛋白分解酵素の作用を受け，速やかに分解されるため半減期が短いという欠点がある．そこで，PepA の N 末端アミノ酸をアセチル化した AcPepA を作成して検討した結果，AcPepA はさらに強力な効果を発揮することが確かめられた[8]．さらに AcPepA は，致死量の LPS を投与したエンドトキシンショック病態のカニクイザルを救命できることも証明されており，臨床応用への道が開かれつつある．

5. C5aに対する相補性ペプチドの抗アレルギー効果

炎症性疾患の一つである喘息などのアレルギー病態にも，C5a アナフィラトキシンが深く関与している．Th2 細胞より放出されたサイトカインが B 細胞を刺激し，IgE 抗体を産生させる．IgE 抗体は好酸球や肥満細胞の受容体に結合し，そこに抗原が結合すると，ヒスタミンやセロトニンなどのメディエーターを放出させ炎症を引き起こす．アレルギー反応が補体系を活性化するメカニズムとして，アレルゲンが糖鎖構造をもっている場合は直接レクチン経路または第二経路で，アレルゲン—抗体複合体形成の場合は古典経路で活性化されることが知られている．補体の活性化により生じた C5a は好中球，単球，好酸球，肥満細胞，好塩基球や T 細胞などの炎症性の細胞に認識され，それらの細胞を炎症局所に集積させ，単球を樹状細胞へ分化誘導や，サイトカインおよびヒスタミンなどを放出させる．さらに，近年 C5a が抗原提示細胞を介して，T 細胞の Th1/Th2 反応を制御していることが明らかになってきた[9]．C5a は単球や樹状細胞上に発現している C5a 受容体に結合し，IL-12 を産生することにより Th1 反応を誘導するのだが，アレルギー症状のような Th2 優位の際は，Th2 細胞から産生される IL-4 により，単球や樹状細胞の C5a 受容体の発現を

低下させる．また，C5aからアルギニンがとれた一次代謝物であるC5adesArgがその受容体であるC5L2（C5a-like receptor 2：C5a様受容体）に結合すると，IL-12の産生を抑制するように働く（図3）．これらの報告から，アレルギー反応におけるC5aの制御はその症状を緩和するのに有効な方法の一つであると考えられる[10]．濱らはヒト肺組織を用いた喘息モデルにおいて，AcPepAが肥満細胞から放出されるメディエーターの一つCysLTsの産生を強力に抑制することを明らかにしており[11]，相補性ペプチドの抗喘息薬としての可能性も検討されている．

6．おわりに

現在，C5a阻害相補性ペプチドAcPepAはブタ新生児を用いたCLP（cecal ligation and purcture：腸管穿孔モデル）実験においても著明な延命効果やサイトカインの過剰放出抑制効果が認められている．その結果を基に，敗血症やSIRSなどの患者の救命効果を臨床治療実験で明らかにするトランスレーショナルリサーチへと進めることが可能であると考えている．また，相補性ペプチド創出技術を用いて，サイトカインストームの進行で形成されるTNF-αやHMGB1などほかの起炎性因子に対する相補性ペプチドも創生し，病態が進行した患者も救命できる治療薬としての開発を目指している．ペプチド剤は蛋白質分解酵素の作用を受けて速やかに分解されるため，蓄積毒性などの副作用リスクが少ないと考えられる．これらの特性を生かし，将来的には，相補性ペプチドが臨床病態における重要な因子を制御する戦略に幅広く応用されることになり，さらに多くの疾患における有効な治療薬の開発手段として発展していくことを期待している．

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1954年以来、臨床検査技師の技術・学識研鑚の象徴である資格認定試験問題集待望の刊行。至近の5年間（第90～94回、2002-2006年）の、微生物（寄生虫含む）、病理（細胞診検査含む）、臨床化学、血液、免疫血清、循環生理、神経生理、呼吸生理の各科目の問題・解答を全文掲載。受験者にとって利便性が高く、国家試験受験者にとっても、あるいは生涯学習用テキストとしても十分に役立つ問題集。

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Estrogen enhances expression of the complement C5a receptor and the C5a-agonist evoked calcium influx in hormone secreting neurons of the hypothalamus

Imre Farkas [a,*], Patricia Varju [a], Emese Szabo [a], Erik Hrabovszky [a], Noriko Okada [b], Hidechika Okada [c], Zsolt Liposits [a,d]

[a] *Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony u. 43, 1083 Budapest, Hungary*
[b] *Department of Biodefense Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi 467-8601, Japan*
[c] *Choju Medical Institute, Fukushimura Hospital, Toyohashi, Aichi 441-8124, Japan*
[d] *Department of Neuroscience, Information Technology Faculty, Péter Pázmány Catholic University, Budapest, Hungary*

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Abstract

In the present study we examined presence of the complement C5a receptor (C5aR) in hypothalamic neurosecretory neurons of the rodent brain and effect of estrogen on C5aR expression. Whole cell patch clamp measurements revealed that magnocellular neurons in the supraoptic and paraventricular nuclei of hypothalamic slices of the rats responded to the C5aR-agonist PL37-MAP peptide with calcium ion current pulses. Gonadotropin-releasing hormone (GnRH) producing neurons in slices of the preoptic area of the mice also reacted to the peptide treatment with inward calcium current. PL37-MAP was able to evoke the inward ion current of GnRH neurons in slices from ovariectomized animals. The amplitude of the inward pulses became higher in slices obtained from 17β-estradiol (E2) substituted mice. Calcium imaging experiments demonstrated that PL37-MAP increased the intracellular calcium content in the culture of the GnRH-producing GT1-7 cell line in a concentration-dependent manner. Calcium imaging also showed that E2 pretreatment elevated the PL37-MAP evoked increase of the intracellular calcium content in the GT1-7 cells. The estrogen receptor blocker Faslodex in the medium prevented the E2-evoked increase of the PL37-MAP-triggered elevation of the intracellular calcium content in the GT1-7 cells demonstrating that the effect of E2 might be related to the presence of estrogen receptor. Real-time PCR experiments revealed that E2 increased the expression of C5aR mRNA in GT1-7 neurons, suggesting that an increased C5aR synthesis could be involved in the estrogenic modulation of calcium response.

These data indicate that hypothalamic neuroendocrine neurons can integrate immune and neuroendocrine functions. Our results may serve a better understanding of the inflammatory and neurodegeneratory diseases of the hypothalamus and the related neuroendocrine and autonomic compensatory responses.
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Keywords: Hypothalamus; Complement C5a receptor; Neurosecretory neurons; Estrogen

1. Introduction

Multidirectional communication exists between the neuroendocrine and immune systems. Inflammation, for example, suppresses pulsatile gonadotropin-releasing hormone (GnRH) secretion resulting in disruption of the ovarian cycle and fertility (Karsch et al., 2002). Reversely, endocrine hormones also affect functions of the classical immune organs. In addition to its known effects modulating, for example, the electric and endocrine functions of neuroendocrine cells (Farkas et al., 2007; Thakur and Sharma, 2007), estrogen increases the levels of inducible nitric oxide synthase, nitric oxide and interferon-γ in splenocytes (Karpuzoglu and Ahmed, 2006). However, very little is known about the mechanism whereby hormones of endocrine glands modulate immune responses in the brain.

The central nervous system (CNS) utilizes its own protection systems including the complement system (C) to eliminate invading microorganisms. Numerous cell types, such as

* Corresponding author. Tel.: +36 1 210 9400x368; fax: +36 1 210 9944.
E-mail address: farkas@koki.hu (I. Farkas).

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neurons, endothelial cells, astrocytes, microglia and oligodendrocytes (Thomas et al., 2000; Gasque et al., 2000) have been identified as sources of complete and functional C in the brain. In addition, expression of various components of C has also been detected in affected neurons of Alzheimer's disease (AD) patients (Shen et al., 1997; Terai et al., 1997; Strohmeyer et al., 2000). Anaphylatoxins including C5a, that can be generated during activation of C, have also been identified in the brain. C5a is a 74 amino acid-long peptide, which is cleaved from the C5 component of C during inflammation (Rother et al., 1998). Binding of C5a to its receptor (C5aR) evokes several responses such as increased intracellular calcium content, phagocytosis, chemotaxis, degranulation and the synthesis and release of various inflammatory mediators (Konteatis et al., 1994; Rother et al., 1998). Expression of C5aR has been revealed in astrocytes and microglia of the CNS (Gasque et al., 1995, 1997). Neurons of the hippocampal formation, pyramidal cells of the cerebral cortex, Purkinje cells in the cerebellum and a subset of thalamic neurons equally synthesize this receptor in rodents (Stahel et al., 1997a,b; VanBeek et al., 2000). Likewise, C5aR has been revealed in human hippocampal and cortical pyramidal neurons and neuroblastoma cells (Farkas et al., 1998b, 1999, 2003; O'Barr et al., 2001). In contrast, only limited information is available about the expression and role of C5aR in the hypothalamus, the main central regulator of neuroendocrine axes and autonomic functions. The axonal processes of the hypophysiotrophic parvicellular neurons and also those of the magnocellular neurosecretory cells terminate outside the blood–brain barrier, in the median eminence and the posterior pituitary, respectively. Therefore, these neuroendocrine cells occupy an ideal anatomical position to sense and mediate the peripheral immune signals to various endocrine axes.

While the anti-inflammatory and neuroprotective effects of estrogen are well established (Kovacs, 2005; Suzuki et al., 2006; Turgeon et al., 2004) recent studies have demonstrated that this hormone plays a rather complex role in the immune response of the brain (Sohrabji, 2005; Morale et al., 2006). Compelling evidence has been provided to prove that estrogen is required for a proper immune response in the CNS (Soucy et al., 2005) including the hypothalamus that is known to control the neuroendocrine axes and the autonomic nervous system. In the present study we addressed the issue of whether neuroendocrine cells synthesize functional C5aR. Whole cell clamp electrophysiology, real-time PCR and calcium imaging were used to examine the effects of C5aR activation upon various hypothalamic neurosecretory cells, such as magnocellular neurons and GnRH-producing neurons. In addition, the modulatory effect of estrogen was studied on the response triggered by activation of C5aR in hypothalamic neurons.

2. Methods

2.1. Experimental animals and cell line

Rats and mice were housed in light- and temperature controlled environment with free access to food and water and treated in accordance with the legal requirements of the Animal Care and Use Committee of the Institute of Experimental Medicine and the European Community (Decree 86/609/EEC). All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine. All efforts were made to minimize animal suffering and the number of animals used.

Magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei and GnRH-producing cells of the preoptic area were chosen as model systems to examine the effects of C5aR activation on neuroendocrine cells. Magnocellular neurons of the PVN and SON could be easily visualized in the rat brain slice by their characteristic location, shape and size. The immortalized GT1-7 neuronal cell line which produces GnRH was generated and kindly provided for these studies by Dr. Pamela L. Mellon (Mellon et al., 1990). In order to visualize GnRH neurons in the brain slices, GnRH-enhanced green fluorescent protein (GnRH-GFP) transgenic mice (kind gift by Dr. Suzanne Moenter) were chosen in which the GnRH promoter drives selective GFP expression in the majority of GnRH neurons (Suter et al., 2000).

2.2. Estrogen treatment paradigms

Six adult female GnRH-GFP mice at the age of 100 days were ovariectomized (OVX) bilaterally under pentobarbital anesthesia (35 mg/kg bw, i.p.) and allowed to survive for 1 week to decrease endogenous sex steroid levels. Then three of them (OVX group) received a single subcutaneous (s.c.; 100 μl) injection of sunflower oil vehicle. The other three mice (OVX + E2 group) were injected s.c. with 17β-estradiol (E2; 2 ng/g body weight) in vehicle. This dose of estradiol was chosen because a saturating dose of estradiol is approx. 3.6 ng/g bodyweight (BW) in rats (Brown et al., 1992). Applying the 2 ng/g subsaturating dose we could avoid pharmacological responses due to supraphysiological estradiol levels.

The animals were sacrificed 24 h after receiving injection. Brain slices were then prepared for the electrophysiological recording.

2.3. C5aR-agonist peptide (PL37-MAP)

The amino acid sequence (RAARISLGPRCIKAFTE) of the C5aR-agonist peptide (PL37-MAP) is a fragment of C5a and represents antisense-homology-box (AHB) region of C5a (Baranyi et al., 1995, 1996). The peptide was synthesized in multiple antigenic peptide (MAP) form.

The main reason to choose PL37-MAP instead of C5a was the carboxypeptidase activity existing in the brain slice (Che et al., 2005). Carboxypeptidase removes Arg from the C-terminal of C5a, therefore, the effective concentration of the intact C5a changes quickly. In addition, due to the carboxypeptidase activity, two active forms of C5a (the intact C5a and the desArg-C5a, both of them having different effect when activating the C5aR) would exist simultaneously in the brain slice during the experiment. The unpredictably changing ratio of the two forms would make the sound measurement difficult. We could avoid this difficulty by using the C5aR-agonist PL37-MAP.

During the experiments the PL37-MAP peptide was pipetted directly into the bath fluid of the cells to be recorded.

2.4. Cell culture

GT1-7 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing high-glucose and supplemented with 10% fetal calf serum (FCS) and 5% horse serum (HS). Before estrogen treatment the culturing medium was replaced with a steroid/thyroid- and phenol red-free one and cells were cultured in this medium for 48 h. Subsequently, the cells were treated with water-soluble E2 (SIGMA) at 20 nM and then used at various time points in PCR ("t" = 0.5, 2, 8, 24 and 48 h) and calcium imaging experiments (24 h). The control cells were used after the steroid-withdrawal period of 48 + "t" hours. In order to determine if estrogen receptor was involved in the observed effects, other control cells were co-treated with E2 and the estrogen receptor blocker Faslodex (ICI 182,780; 1 μM; Tocris Inc.).

2.5. Reverse transcription

Total RNA samples from control and E2 treated GT1-7 cells were isolated with TRIzol LS reagent (Invitrogen) according to the manufacturers instructions.

RNA from three equivalent cultures were mixed and the RNA solutions were diluted to reach a final concentration of 1 μg/μl. From each treatment group 2 μg total RNA was used for cDNA synthesis. cDNA reaction mixtures (40 μl) contained oligodT, random hexamers and 1.5 mM $MgCl_2$. Reverse transcription was performed in a Perkin-Elmer thermal cycler with the ImProm II Reverse Transcription System (Promega) according to instructions by the manufacturer.

2.6. Real-time quantitative PCR

Real-time polymerase chain reactions (PCR) were carried out in a Light Cycler PCR machine (Roche) with the DNA Master SYBR Green I mix (Roche) following the manufacturer's protocols. Each RT-PCR experiment was performed in triplicate. The 10 μl reaction volumes were placed in Light Cycler glass capillaries (Roche) and composed of 1 μl DNA Master SYBR Green I (Roche), 1 μl cDNA mix, 4 mM $MgCl_2$ and 0.3 μM specific primers. The transcript of the house keeping gene hypoxanthine-guanine phosphoribosil transferase (HPRT), which is not regulated by estrogen, was used as an internal control to compensate for variations in amplification efficiency when the amounts of the C5aR amplicon were calculated.

The standard curves were created by amplifications of oligonucleotides containing partial sequences of the HPRT and C5aR genes with the same primer sequences as used for the amplification of experimental samples. The oligonucleotide standards were prepared using serial 1:10 dilutions with TE buffer, over a concentration range spanning the sample concentrations. For quantification, the test oligonucleotide was used at a dilution close to the sample concentrations and the reverse transcribed RNA isolated at 0, 0.5, 2, 8, 24 and 48 h after estrogen treatment. H_2O was included as no template control. Real-time PCR conditions were as follows: HPRT: 95 °C, 5 min for denaturation; 65 cycles: 94 °C, 5 s; 57 °C, 7 s; 72 °C, 10 s; C5aR: 95 °C, 5 min; 65 cycles for 94 °C, 5 s; 57 °C, 7 s and 72 °C, 10 s and cooling to 40 °C, 30 s. Primer sequences used in the PCR reactions were as follows: HPRT forward 5′-tgt aat gat cag tca acg ggg-3′, reverse 5′-tgg cct gta tcc aac act tcg-3′; the C5aR primer was 5′-tgc cct ggt ggt gtg ggt ga-3′ (forward) and 5′-agg acg gaa tgg tga gga gc-3′ (reverse).

The relative amount of the products was determined from the log phase of the reaction.

2.7. Calcium imaging

Cultured GT1-7 cells were loaded with the calcium-sensitive fluorescent dye Fura-2 AM (1 μM; Molecular Probes, Eugene, OR, USA) in loading buffer containing 0.1% DMSO (Molecular Probes) in 1.5 h at room temperature (RT). After washing with Hanks' Balanced Salt Solution (HBSS), the experiments were carried out at RT. The PL37-MAP peptide (62.5–250 nM) was pipetted directly onto the cells in HBSS after a 4 min baseline recording and then the diluted peptide remained in the HBSS during recording. In the case of E2 pretreatment, the cells were pretreated with E2 as described in Section 2.4 and all of the rinsing and extracellular solutions contained the same concentration of E2. After the 4 min baseline recording the PL37-MAP peptide (62.5–125 nM) was introduced into the bath fluid containing E2 and then the diluted peptide remained in the HBSS–E2 mixture during recording.

The experiments were carried out with a Deltascan Model 4000 calcium imaging system (Photon Technology International, Princeton, NJ, USA), using the ratio of the fluorescent signals obtained at excitation wavelengths of 340 and 380 nm to determine changes in the intracellular calcium concentration.

2.8. Brain slice preparation

Animals (22 ± 5-day-old male Wistar rats for recording in the SON, PVN and anterior hypothalamic area [AHA] and 110 ± 12-day-old female mice for recording GnRH-GFP neurons) were anaesthetized with pentobarbital (35 mg/kg bw, i.p.) and decapitated. The brains were removed, and then immersed in ice cold artificial cerebrospinal fluid (ACSF; NaCl 140 mM, KCl 3 mM, $MgSO_4$ 1.3 mM, NaH_2PO_4 1.4 mM, $CaCl_2$ 2.4 mM, glucose 11 mM, HEPES 5 mM, pH 7.25) bubbled with 95% O_2–5% CO_2. Hypothalamic blocks were dissected from the rat and mouse brains and 300 μm thick slices containing the PVN, SON and AHA of rats or GnRH neurons in the preoptic area of mice, were sectioned with a VT-1000S vibratome (Leica GmBH, Germany) using a sapphire knife (Delaware Diamond Knives Inc., Wilmington, DE, USA) in ice cold and oxygenated ACSF. The slices were bisected along the third ventricle and equilibrated in ACSF saturated with O_2/CO_2 mixture at RT for 1.5 h. In order to record the neurons, the equilibrated hemi-slices were placed in an immersion-type recording chamber.

2.9. Whole cell clamp experiments

The cells were voltage clamped at RT using a whole cell clamp configuration. The instruments used for electrophysiology were as follows: Axopatch 200B patch clamp amplifier, Digidata-1322A data acquisition system and pCLAMP 9.2 software (Axon Instruments-Molecular Devices Co., Sunnyvale, CA, USA), the headstage of the amplifier was fitted to a MHW-3 hydraulic micromanipulator (Narishige Co., Japan). The cells were visualized by a BX51WI upright microscope (Olympus Co., Japan) equipped with an epifluorescent filter set (excitation filter: U-HQ450–490; dichroic mirror U-Q495LP; emission filter: U-HQ490–540) capable of visualizing the GnRH-GFP neurons in the brain slice and a Cohu 4912 CCD camera (Cohu Inc., San Diego, CA, USA) driven by a Scion Image for Windows Beta 4.0.2 software (Scion Co., Frederick, MD, USA). The microscope and the micromanipulator were fitted to a S'Table antivibration table equipped with a Petra platform (Supertech Co., Hungary-Switzerland). Softwares were run on an IBM compatible personal computer. The patch electrodes (o.d. = 1.5 mm, thin wall, Garner Co., USA) were pulled with a Flaming-Brown P-97 horizontal puller (Sutter Instrument Co., Novato, CA, USA) and polished with an MF-830 microforge (Narishige). Resistance of patch electrodes was 8–10 MΩ for GT1-7 cells and 2–3 MΩ for the neurons in the brain slices.

The solutions for electrophysiological recording were as follows: extracellular solution for GT1-7 cells (HEPES 10 mM, NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, glucose 10 mM, pH 7.34) and ACSF for brain slices (see Section 2.8 for composition of ACSF); intracellular pipette solution (HEPES 10 mM, KCl 110 mM, NaCl 15 mM, $CaCl_2$ 0.1 mM, $MgCl_2$ 2 mM, EGTA 1 mM, pH 7.25). The brain slices were oxygenated by bubbling the extracellular solution with O_2/CO_2 gas mixture during recording at RT.

Using the epifluorescent filter set, GnRH-GFP neurons were identified in the acute brain slices by their green fluorescence, typical fusiform shape and apparent topographic location in the preoptic area.

Holding potential was −50 mV at the GT1-7 cells and −70 mV at the neurons in the brain slices. Pipette offset potential, series resistance and capacitance were compensated before recording. Only cells with low leakage were used for electrophysiological measurements. The cells requiring any leak subtraction were omitted.

Electrophysiological recordings started simultaneously with the peptide treatment.

Complement C5a (5 μg/ml, human recombinant; Sigma) treatment was carried out by adding C5a to the extracellular solution. The cells were exposed to C5a for 10 min before the patch clamp recording.

$CoCl_2$ was added to the bath solution at 0.5 mM.

2.10. Statistical analyses

Data are presented as mean ± S.E.M.

Statistical analyses (one-way parametric ANOVA and Newman–Keuls multiple comparison test) of the calcium imaging measurements were carried out on $n \geq 14$ cells at each recording using a Prism software package (GraphPad Software, San Diego, CA, USA). Integration and determining maximum of the recorded curves were carried out after subtractive baseline correction.

Electrophysiological recordings were carried out on at least eight cells for each experiment. The average of maximum values of the recorded ion currents was calculated using the PClamp 9.2 software then Student's t-test of the Prism software was applied to perform the statistical analysis.

3. Results

3.1. Calcium imaging

Experiments showed that extracellularly applied C5aR-agonist PL37-MAP peptide evoked elevation in the intracellular

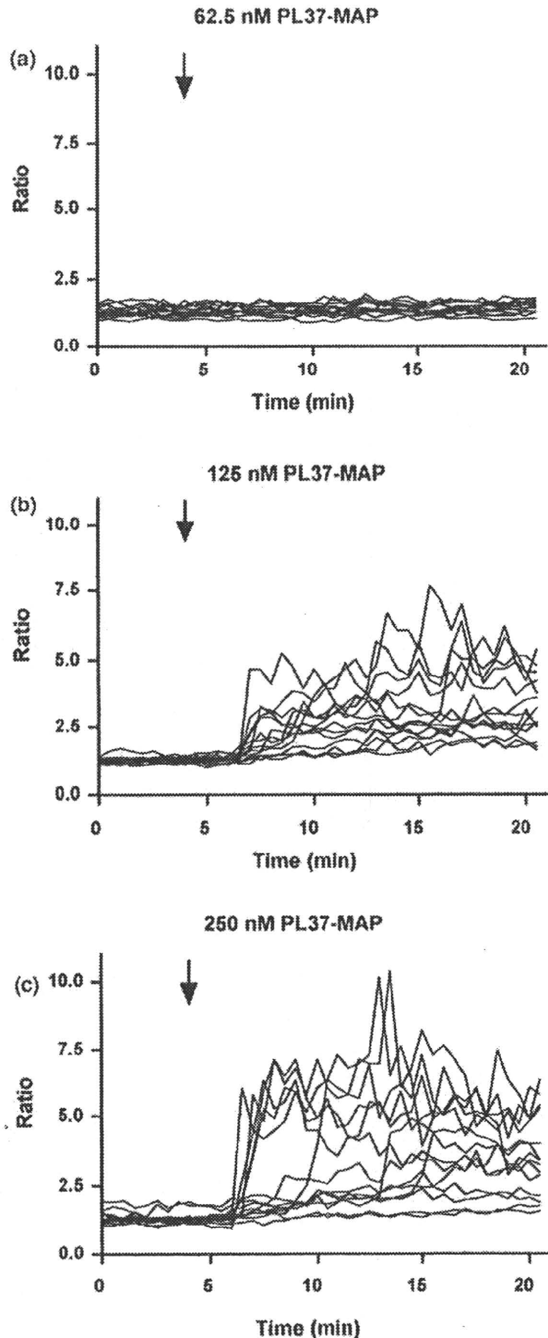

Fig. 1. Calcium imaging measurements of the GT1-7 cells upon treatment with various concentration of the C5a-agonist PL37-MAP peptide. Application of the peptide is shown with arrow. Low concentration of the peptide (62.5 nM) (a) failed to evoke calcium influx. Higher concentrations of PL37-MAP (125 nM) (b) and 250 nM (c) triggered significant elevation of the intracellular calcium content. "Ratio" (vertical axis) refers to the ratio of the fluorescent signals obtained at excitation wavelengths of 340 and 380 nm to determine changes in the intracellular calcium concentration.

calcium content in the fura 2-AM loaded neurons of the gonadotropin-releasing hormone (GnRH) producing GT1-7 cell line (Fig. 1a–c). The peptide did not elevate the calcium content at 62.5 nM (Fig. 1a) whereas change could be recorded at higher concentration of PL37-MAP (125 and 250 nM) (Fig. 1b and c). Elevation started within 2–5 min after introducing the peptide into the bath fluid. Peak amplitude of the ratio value increased with increasing concentration of the PL37-MAP added. After baseline correction, the average maximum peak amplitude of the ratio value was 0.2 ± 0.03 ($n = 14$) at 62.5 nM peptide, 2.35 ± 0.24 ($n = 17$) at 125 nM peptide and 4.55 ± 0.8 ($n = 17$) at 250 nM peptide (Fig. 2a), showing significant increase in the calcium signal (ANOVA: $p < 0.0001$ and $F = 19.26$; Newman–Keuls: $p < 0.01$ for each comparison). The integrated area of the recorded curves representing the net changes in the intracellular free calcium content also revealed significant increase in the calcium signal (Fig. 2b) (ANOVA: $p < 0.0001$ and $F = 179.3$; Newman–Keuls: $p < 0.001$ for each comparison).

In order to examine whether estrogen interacted with the calcium signal mediated via the C5aR, the GT1-7 cells were pretreated with E2 (20 nM) then effect of the PL37-MAP was recorded (Fig. 3a–d) at two concentrations of the peptide (62.5 and 125 nM). In contrast to the result shown in Fig. 1a, at 62.5 nM the peptide elevated the intracellular calcium concentration if the cells were pretreated with E2 (Fig. 3a) showing that E2 potentiated the C5aR-related response of the cells. Similarly, when 125 nM PL37-MAP was applied, E2 increased the calcium influx (Fig. 3c). In order to demonstrate that the effect of E2 could be mediated via estrogen receptor, E2 was co-administered with the estrogen receptor blocker Faslodex (1 μM). Under this condition the response of the cells to 62.5 and 125 nM PL37-MAP did not differ from the calcium signal recorded without E2 showing that Faslodex inhibited the potentiating effect of E2 (Fig. 3b and d). Faslodex alone did not affect the response and the recorded curve was similar to the one measured without E2 (not shown). After baseline subtraction, the average maximum of the recorded curves was 3.8 ± 0.9 (125 nM PL37-MAP + E2; $n = 18$; Newman–Keuls test comparing with the control: $p < 0.05$), 3.5 ± 1.2 (62.5 nM PL37-MAP + E2; $n = 15$; Newman–Keuls test comparing with the control: $p < 0.01$), 2.0 ± 0.4 (125 nM PL37-MAP + E2 + Faslodex; $n = 15$; Newman–Keuls test comparing with the control: $p > 0.05$) and 0.3 ± 0.06 (62.5 nM PL37-MAP + E2 + Faslodex; $n = 14$; Newman–Keuls test comparing with the control: $p > 0.05$), showing that E2 significantly increased the average maximum amplitude (ANOVA for 125 nM PL37-MAP: $p = 0.0098$ and $F = 4.196$; for 62.5 nM: $p = 0.0065$ and $F = 4.571$) and that effect of E2 was abolished by Faslodex (Fig. 4a and c). The normalized areas-under-curve representing the net changes in the intracellular free calcium content also showed significant increase when PL37-MAP was administered to GT1-7 cells pretreated with E2 in the absence of Faslodex (Fig. 4b and d). Increase in the calcium signal of the E2-pretreated cells started in 2–5 min after applying the peptide. The time to start did not differ significantly from the curves recorded without E2.

3.2. *Whole cell clamp measurements*

Electrophysiology provided further evidence that hypothalamic neurons express functional C5aR and estrogen interacts with the signal activated by the C5a. Whole cell clamp