# Possible role of the C5a receptor in cell death and neurodegeneration

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## PUBLICATIONS RELATED TO THE SUBJECT OF THIS THESIS:

### PAPERS:

I. **Farkas I.**, Baranyi L., Liposits Zs., Yamamoto T., Okada H. (1998) C5a anaphylatoxin fragment causes apoptosis in TGW neuroblastoma cells. *Neuroscience* 86(3), 903-911. Impact factor: 3.594

II. **Farkas I.**, Baranyi L., Takahashi, M., Fukuda A., Liposits Zs., Yamamoto T.,Okada H. (1998) A neuronal C5a receptor and an associated apoptotic signal transduction pathway. *Journal of Physiology (London)* 507(Pt3), 679-687. Impact factor: 3.16

III. **Farkas I.**, Baranyi L., Kaneko Y., Yamamoto T., Okada H. (1999) Effect of C5a and tumor necrosis factor-S on C5a receptor of neuroblastoma cells. *Biochemical and Biophysical Research Communication. Submitted.* Impact factor: 2.671

### ABSTRACTS:

I. **Farkas I.**, Baranyi L., Liposits Zs., Okada N., Okada H. (1996) Apoptotic effect of the PR226-MAP antisense homology box peptide. *International Symposium on Network and Evolution of Molecular Information.* Tokyo, Japan,

II. **Farkas I.**, Baranyi L., Liposits Zs., Yamamoto T., Okada H. (1996) A complement C5a fragment peptide causes apoptosis in TGW neuroblastoma cells. Fifth International Conference on Alzheimer's Disease and Related Disorders. Osaka, Japan. *Neurobiol. Aging* 1996/S17/4S/S183. Impact factor: 2.872

III. **Farkas I.**, Baranyi L., Yamamoto T., Liposits Zs., Okada H. (1997) Komplement C5a receptor-mediált apoptózis neuroblasztoma sejteken. *Magyar Idegtudományi Társaság (MITT) IV. konferenciája.* Gödöllő, Hungary.

IV. **Farkas I.**, Baranyi L., Yamamoto T., Liposits Zs., Okada H. (1997) A C5a receptor szerepe neuroblasztoma és leukémia sejtek apoptózisában. *Magyar Anatómus Társaság IX. kongresszusa.* Szeged, Hungary.

V. **Farkas, I.**, Baranyi L., Okada H., Liposits Zs. (1998) Effects of complement 5a (C5a) and tumor necrosis factor on C5a receptor of neuroblastoma cells. *Magyar*

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VI. **Farkas, I.**, Baranyi L., Okada H., Liposits Zs. (1998) Effects of complement 5a (C5a) and tumor necrosis factor on C5a receptor of neuroblastoma cells. *ENA Congress, The Forum of European Neuroscience*, Berlin, Germany

VII. **Farkas I.**, Yamamoto T., Okada H., Baranyi L. (1998) A new concept in the Alzheimer's disease: The possible role of the complement C5a receptor in the neurodegeneration. *Clinical Neuroscience* 51(1), 44-45

VIII. **Farkas I.**, Dudás B., Baranyi L., Mihály A., Yamamoto T., Okada H. (1999) Expression of the complement C5a receptor in the neurons of the control and Alzheimer brain. *Magyar Idegtudományi Társaság (MITT) V. konferenciája.* Pécs-Harkány, Hungary.

CONTENTS

## AIMS OF THIS STUDY

1. To demonstrate the expression of the complement C5a receptor in the neurons of some regions of the normal human brain;

2. To investigate the possibility of whether the C5a receptor can contribute to the progression in neurodegenerative diseases, by means of immunohistochemical detection of the C5a receptor in the brains of Alzheimer's disease patients.

3. To elaborate a model for analysis of the possible role of the C5a receptor in neurodegeneration, by means of detection of the expression of the C5a receptor in a neuroblastoma cell line.

4. To prove the role of the C5a receptor in the neurodegeneration by means of the administration of different synthetic peptides containing amino acid sequences of the C5a receptor or the C5a ligand to different cells expressing the C5a receptor. To examine whether it is possible to cause programmed cell death by these peptides and whether apoptosis is specifically associated with the C5a receptor.

5. To investigate what kinds of processes are accompanied by apoptosis. To examine the changes in the c-fos level and the roles of G-proteins and calcium in the process of programmed cell death.

## ABBREVIATIONS

MAC - membrane attack complex

CS - complement system

AHB - antisense homology box

AD - Alzheimer's disease

C5 - (5th) molecule of the complement cascade

C5a - a small fragment of the C5 molecule

C5aR - the receptor for the anaphylatoxin C5a molecule

TNFα - tumor necrosis factor-α

MAP – multiple antigenic peptide

PL37-MAP – a synthetic peptide involving amino acid sequence 37-53 of C5a

PR226-MAP – a synthetic peptide involving amino acid sequence 226-243 of C5aR

FBS – fetal bovine serum

DAB - 3,3-diaminobenzidine

TUNEL - TdT-mediated dUTP-biotin nick end labeling

HBSS - Hank's balanced salt solution

FITC - avidin-fluorescein isothiocyanate

PBS – phosphate-buffered salt solution

C5aR-IR - C5aR-immunoreactivity

IR - immunoreactive

RT-PCR – reverse transcriptase polymerase chain reaction

HIC – high irreversible inward current (electrophysiological sign of cell death)

SEM – standard error of mean

NFT - neurofibrillary tangle

FACS – fluorescence associated cell sorter

APP - amyloid precursor protein

# INTRODUCTION

## The complement system

The complement system (CS), as part of the humoral immune system consists of nearly 30 serum and membrane molecules. After the initial activation, its components (proteins of the classical pathway: C1-C4; proteins of the alternative pathway: C3, factors B and D, and properdin; proteins of the terminal pathway: membrane attack complex (MAC); and several plasma and membrane-bound regulatory molecules) interact in a highly regulated enzymatic cascade to generate an inflammatory response and to facilitate antigen clearance. One of the final products, the MAC, creates a pore in the membrane and lyses the attacked cells, the invading bacteria or viruses. Additionally, activation of the CS plays a role in several immune reactions. For example, it induces vasodilation, attracts phagocytic cells in a chemotactic way, and facilitates phagocytosis in different parts of the body, including the brain.

The brain was for a long time traditionally regarded as an immunologically isolated part of the body. The existence of the blood-brain barrier, the absence of the conventional lymphatic drainage, and the unusual tolerance of the brain to transplantation accounted for this notion. However, it has been discovered over the past decade that the brain possesses its own endogenous active immune system and that chronic inflammation of the brain can contribute to several neurodegenerative diseases. Since the brain is separated from the plasma by the blood-brain barrier, the components of this immune system, including the molecules of the CS, are obviously expressed by the different cells of the central nervous system.

## Expression of the molecules of the complement system in the brain

Since activation of the CS can be involved in certain neurodegenerative processes, the question naturally arises of what kind of cells are the main sources of the complement molecules?

### *Expression of the complement molecules by astrocytes*

In the past, the primary role of the mature astrocytes was thought to be structural, providing a supporting framework for neurons and contributing to the blood-brain barrier by wrapping processes around the microvessels in the brain. However, recent evidence suggests

that astrocytes are immunologically active cells with an important role in host defence in the brain, while functioning as immunocompetent cells as well. They not only produce, but also respond to several cytokines in an autocrine way (Fabry et al., 1994). Upon stimulation with inflammatory cytokines, astrocytes have been induced to express adhesion molecules and major histocompatibility complex antigens *in vitro*, raising the possibility that astrocytes present antigens *in vivo*, too (Fontana et al., 1984). Astrocytes have been demonstrated to be motile cells; they can respond to chemotactic stimuli and ingest foreign particles, mimicking professional phagocytes (DeGroot et al., 1994).

Astrocytes are capable of synthesizing elements of the entire complement cascade, as well. The first experimental evidence was provided by Levi-Strauss and Mallat (1987): they showed that murine astrocyte cell lines and primary murine astrocytes in culture produced some molecules of the CS. In succeeding years, these findings were extended to astrocytes and astrocyte cell lines of rat and human origin (Gasque et al., 1992a; Rus et al., 1992; Gasque et al., 1995b). It has been shown that human astrocyte cell lines express and secrete all the components of the classical, alternative and terminal pathways. The functional activities of all the component proteins were confirmed by demonstrating that it was possible to assemble a lytic complement pathway from the protein products generated in culture (Gasque et al., 1995a). Although without stimulation, most components were expressed at a low level; the synthesis of the components could be enhanced by inflammatory cytokines such as interferon-$\gamma$, interleukin-1$\beta$ and tumor necrosis factor-$\alpha$ (TNF$\alpha$ ) (Gasque et al., 1995a). These observations indicate that astrocytes and astrocyte-derived cell lines can, in the presence of the appropriate cytokines, produce a complete, functional CS. Since these cells can themselves synthesize inflammatory cytokines (Fabry et al., 1994) this raises the possibility that astrocytes switch on complement biosynthesis in an autocrine manner.

In addition to the components of the different pathways of the complement cascade, astrocytes synthesize and secrete fluid-phase regulators of the classical, alternative and terminal pathways, restricting activation of the complement cascade in the local environment (Morgan and Gasque, 1996). Membrane-bound complement regulators are also expressed on the membrane of the astrocytes (Gasque et al., 1995a).

Most of the activities of the complement are mediated by the interaction of complement fragments with specific receptors on the effector cell membranes (Liszewski and

Atkinson, 1993; Kuby, 1994). Astrocytes have been reported to express almost all of these receptors, including receptors for the anaphylatoxin C5a (Gasque et al., 1995b, 1996).

*Expression of the complement molecules by microglia*

The microglia are highly active cells capable of chemotaxis and phagocytosis (Morgan and Gasque, 1996). Although they probably make a substantial contribution to the local biosynthesis of the complement molecules in the brain (Pasinetti et al., 1992), the total expression of the microglia is presumably minor in volume relative to that of the astrocytes, since astrocytes are a more abundant cell type in the brain. Moreover, they also bear a receptor for the C5a anaphylatoxin (Lacy et al., 1995).

*Expression of complement regulatory molecules in the oligodendrocytes*

At present, it is thought that oligodendrocytes do not contribute to the expression of the elements of the complement cascade. Nevertheless, they express and secrete either the fluid phase or the membrane-bound regulatory proteins (Piddlesden and Morgan, 1993; Vedeler et al., 1994; Zajicek et al., 1995). It is worthy of mention that rat oligodendrocytes do not express all of these molecules, whereas human oligodendrocytes do (Piddlesden and Morgan 1993); rat oligodendrocytes are therefore more vulnerable than human cells to homologous complement attack.

*Expression of the complement molecules by neurons*

It was earlier believed that the brain neurons are not subjects rather passive objects of the CS. This was based on a long-lasting inability to demonstrate the expression of most of the elements of either the classical or the alternative pathway, excluding C4, which has long been predominantly associated with neurons (Johnson et al., 1992; Kalaria, 1993; Pasinetti 1996). However, the lytic product of the terminal pathway, the MAC, was found in brain neurons under pathological conditions (McGeer et al., 1989a). Not surprisingly, many complement regulatory molecules have been demonstrated in the nerve cells, showing that, although they were regarded as passive objects of the complement attack, they are ready to decrease the effects of any unwanted complement activation (McGeer and McGeer, 1992). The expression of fluid-phase and membrane-bound complement inhibitor molecules was reported in the brain neurons in certain neurodegenerative diseases (Akiyama et al., 1991;

McGeer et al., 1991) and in human neuroblastoma cells (Shen et al., 1995; Zhang et al., 1998) regulating the complement-mediated neurotoxicity. The old belief has recently started to decline, since it has been discovered that complement proteins of the classical pathway are also expressed by neurons in the Alzheimer's disease (AD) brain (Shen et al., 1997; Terai et al., 1997). Nevertheless, the function of the complement molecules expressed by the neurons remains to be elucidated.

Although oligodendrocytes and neurons express complement regulatory molecules, either secreting them into the fluid phase or expressing them on their membrane surface, these cells are much more vulnerable to killing by complement *in vitro* (Morgan and Gasque, 1996). However, the exact role of the complement *in vivo* is less well understood, especially in the neurodegenerative diseases, where activation of the complement cascade occurs.

**Activation of the complement system in the neurodegenerative diseases**

The term neurodegeneration covers a rather heterogeneous group of disorders characterized by the loss of active, functioning neurons. The blood-brain barrier remains more or less intact in these diseases. One of the most prominent representatives of these disorders is the AD. The histological hallmarks of AD are the presence of senile plaques and neurofibrillary tangles (NFTs) both induced by the accumulation of abnormal protein fibrils. The observation that these pathological protein accumulations were stained strongly for the complement molecules led several groups to suggest that the activated CS is involved in the pathogenesis of AD (reviewed by Morgan et al., 1997; McGeer and McGeer, 1998). The search for the activating factor of the complement cascade in the AD brain was obviously focussed on the pathological protein accumulations. The senile plaques in AD contain an abnormal amyloid peptide fragment termed β-amyloid. It was demonstrated that β-amyloid itself can directly trigger activation of the classical (Rogers et al., 1992; Velazquez et al., 1997) and the alternative complement pathways *in vitro*, as well (Bradt et al., 1998). The experiments lend support to the hypothesis that the β-amyloid binds complement molecules and activates both the classical and alternative pathways, which may drive inflammation-like processes in the AD brain and may contribute to the neurodegeneration. This theory is supported by the recent discovery that all the elements of the classical and the terminal complement pathway are upregulated in the pyramidal cells of the hippocampus and the

temporal cortex of the AD brain (Shen et al., 1997; Terai et al., 1997).

Although pathological activation of the CS has been reported mostly in AD, some authors have described the possible involvement of the CS in another neurodegenerative disorder too, in the Pick's disease (Morgan et al., 1997). The histological hallmark of Pick's disease is the presence of the Pick body in the brain neurons; this consists of insoluble debris, and particularly abnormal neuroskeletal proteins (Ulrich et al., 1987). Neuronal loss and astrocyte proliferation occur in certain areas, which appear to be restricted to the frontal and temporal lobes. It has recently been reported that the MAC and other elements of the complement cascade are also present in the Pick bodies (Yasuhara et al., 1994; Shingrao et al., 1996).

Although MAC can lyse the cells and the presence of the MAC has been demonstrated in the neurons of the AD brain, a territorial correlation has not yet been reported between the regions where the neurodegeneration occurs and the areas where the MAC can be found. Therefore, further examination is required to reveal relations between the neurodegeneration and the complement activation, including the role of the anaphylatoxin molecules and their receptor.

**Expression of C5aR and the presence of C5a in the brain**

During activation of the CS, numerous peptides are released and interact with cellular components to amplify the inflammatory processes. These peptides, called anaphylatoxins, include C5a, a 74 aminoacid long fragment of C5 (Rother and Till, 1988; Liszewski and Atkinson, 1993; Kuby, 1994). C5a is regarded as the most potent chemoattractant anaphylatoxin of the CS. Among others, it stimulates the release of cytokines, for example TNFα and histamine, causes contraction of the smooth muscle cells and induces phagocytosis (Goldstein, 1988; Rollins et al., 1991; Konteatis et al., 1994; Gasque et al., 1995). C5a binds specifically to its receptor (C5aR) on the cell membrane, which has seven transmembrane segments and intracellularly is coupled to pertussis toxin-sensitive and insensitive G-proteins (Amatruda et al., 1991; Gerard and Gerard, 1991). C5aR is mainly expressed on neutrophils and eosinophils. However, during recent years it has been found in other cell types, such as vascular smooth muscle cells, liver parenchymal cells, lung alveolar and bronchial epithelial cells, endothelial cells and human astrocytes (Huey and Hugli, 1985; Gerard et al., 1989;

Gasque et al., 1995; Havilland et al., 1995). Stahel and his coworkers recently demonstrated the presence of C5aR in the hippocampal and cortical pyramidal neurons of the mouse and rat brain (Stahel et al., 1997a, 1997b). It was shown that, although the expression of C5aR is low under physiological conditions, it is upregulated in injuries and inflammatory conditions. TNFα was also identified as an essential mediator of the neuronal expression of C5aR in the mouse brain. Besides its pro-inflammatory anaphylactic properties, C5a has well-characterized chemotactic and aggregating roles in neutrophils, while it stimulates macrophages too (Liszewski and Atkinson, 1993; Kuby, 1994). C5a is also chemotactic for astrocytes and brain-derived microglia *in vitro* (Yao et al., 1990). Thus, the complement-derived anaphylatoxin(s) might contribute indirectly to the pathogenesis of the neurodegenerative diseases, e.g. AD by modulating the chemoattraction of the reactive glia into AD plaques and by damaging neighboring neurons. However, this cannot explain the degeneration of neurons which are not in the neighborhood of the plaques.

Our main purpose was to determine the expression and distribution of C5aR in the neurons, both in the normal human brain and in the AD brain. Our second goal was to investigate whether there is any possible direct degenerative effect of the C5a anaphylatoxin and/or its receptor besides their possible indirect contribution to the neurodegeneration found in the AD. In order to analyse these questions, the so-called antisense homology box (AHB) peptides were used. AHBs are short (8-15 aminoacid long) motifs in the peptides, representing intra- and/or intermolecular sense-antisense regions (Baranyi et al., 1995). According to the molecular recognition theory (Blalock, 1992), the sense-antisense peptides encoded in the same reading frame on opposite strands of DNA can bind to each other because of their hydropathic (hydrophilic/hydrophobic) complementarity. Since sense-antisense sequences might represent putative interaction sites between proteins, the AHB peptides might be a useful approach to the prediction of intra- and/or intermolecular interaction sites. The AHB regions in C5aR and C5a have recently been analysed (Baranyi et al., 1996). Through use of these regions, AHB peptides were synthesized in oligomeric (MAP = multiple antigenic peptide) form, using the aminoacid sequences of C5aR (aminoacids 226-243, termed PR226-MAP) and the C5a ligand (aminoacids 37-53, termed PL37-MAP). PR226-MAP was used to raise monoclonal antibody against the 227-243 aminoacid region of C5aR. In addition, PR226-MAP was found to induce a C5aR-specific inhibitory effect when

administered in low doses to U-937 promyelocytic leukemia cells bearing C5aR, while higher doses induced cell death in U-937 cells. PL37-MAP was reported to increase and maintain a relatively high intracellular calcium level for a prolonged period in U-937 cells (Baranyi et al., 1996). We used this monoclonal antibody to examine the distribution of C5aR in human brain neurons, and PL37-MAP and PR226-MAP to investigate the function of C5aR on C5aR-bearing cells of neuronal or non-neuronal origin (Farkas et al., 1998a, 1998b, 1999c).

## MATERIALS AND METHODS

### Cell culturing

The culture medium used for TGW human neuroblastoma cells, $Ltk^-$ mouse fibroblast cells and $Ltk^-$/C5aR cells ($Ltk^-$ cells transfected with human C5aR cDNA and expressing C5aR) (Watanabe et al., 1995) was RPMI-1640 supplemented with 10 % fetal bovine serum (FBS). The cells were kept in an incubator at 37 $^oC$, in a 95 % air + 5 % $CO_2$ atmosphere.

### RT-PCR analysis

The total RNA was isolated from TGW, $Ltk^-$ and $Ltk^-$/C5aR cells by an acid guanidium thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987). The mRNA was then converted to cDNA by reverse transcriptase (Superscript II, Life Technologies, Grand Island, USA). The cDNA was subjected to polymerase chain reaction (PCR) analysis for human C5aR, using the sense primer 5'-ATGAACTCCTTCAATTATACC-3' and antisense primer 5'-TGGTGGAAAGTACTCCTCCCG-3' (Gerard and Gerard, 1991). The reaction mixture for PCR amplification consisted of 250 ng of cDNA, 100 ng of each primer, 200 μM of each of the four deoxynucleotide triphosphates and 1.0 U of Taq polymerase (Wako Pure Chemicals Co., Osaka, Japan) in 25 μl of 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 10 mM mercaptoethanol and 2 mM $MgCl_2$. The thermal cycle protocol used was denaturation at 94 $^oC$ for 3 min., followed by cycles at 94 $^oC$ for 1 min., at 55 $^oC$ for 1 min., and at 72 $^oC$ for 1.5 min., and was terminated by a final 7 min. incubation at 72 $^oC$. Controls were run

without reverse transcriptase or without template cDNA to ensure that the results were not due to amplification of any genomic or contaminating DNA. Each reaction mixture (10 μl) was electrophoresed through a 1.5 % NuSieve 3:1 (FMC BioProducts) agarose gel for 30 min. and visualized by incubation for 10 min. in a solution containing 100 ng of ethidium bromide per ml.

**Nick end labeling of the apoptotic cells**

The MEBSTAIN apoptosis kit (Medical & Biological Laboratories Co, LtD., Nagoya, Japan) was used for TdT-mediated dUTP-biotin nick end labeling (TUNEL) method as suggested by the manufacturer, except that instead of avidin-fluorescein isothyiocyanate (FITC) the samples were incubated with streptavidin-peroxidase conjugate (1:1000 dilution; Southern Biotechnology Associates, Birmingham, AL, USA) and visualized with a solution containing 3,3-diaminobenzidine (DAB) and nickel ammonium sulfate (Wako Fine Chemicals, Tokyo, Japan). The cells were treated with 10 μg/ml (600 nM) PR226-MAP (TGW cells) or with 10 μM PL37-MAP (TGW, $Ltk^-$ and $Ltk^-$/C5aR cells) extracellularly in Hank's balanced salt solution (HBSS) at 37 $^oC$. In the control culture, the medium was changed to AHB peptide-free HBSS. The TGW cells were counterstained with neutral red.

**c-fos immunostaining**

TGW cells were treated with 10 μg/ml (600 nM) PR226-MAP or with 10 μM PL37-MAP extracellularly in HBSS at 37 $^oC$ for 30 min. and 1 h, respectively. For control cells, the culture media was also changed to AHB peptide-free HBSS for 30 min. or 1 h. After treatment, the cells were fixed in 4 % paraformaldehyde at 4 $^oC$ for 20 min. and then exposed to 0.2 % Triton X-100 for 30 min. at room temperature. For immunostaining, rabbit anti c-fos serum (Santa Cruz Biotechnology, Santa Cruz CA) was used. The secondary antibody was biotinylated goat anti-rabbit IgG (Vector Laboratories Inc.). The immunoreactive sites were visualized with streptavidin-peroxidase and DAB-nickel solution, similarly to the procedure used for nick-end labeling.

**C5aR immunochemistry**

In order to prepare monoclonal C5aR antibody, a peptide was synthesized in MAP form. The amino acid sequence of the peptide corresponded to the intracellular loop (amino acids 227-243) of the human C5a receptor (Gerard and Gerard, 1991). Antiserum was raised against the peptide in mouse, hybridomas were then made and the monoclonal antibody was purified. The binding of antibody to C5aR was examined by flow cytometry (FACScan, Becton Dickinson, USA) on neutrophils expressing high-affinity C5aR; the binding of the antibody to the peptide was verified by ELISA. The isotype of the antibody was identified as IgM.

Human brains of AD and aged control patients (82-89 years) were received from the brain bank of the Fukushimura Hospital, Toyohashi, Japan. The brain samples, including the hippocampus and the cortex, were dissected (post mortem time: 1-3 h) and fixed in 4% formaldehyde and 30 μm sections were then cut from frozen samples. The sections were treated with 10% thioglycolic acid, 0.2 % Triton X-100 and 3% hydrogen peroxide solution in that sequence. After each treatment, the sections were washed with phosphate-buffered salt solution (PBS). After blocking with FBS, the sections were incubated with the C5aR antibody (1:100 dilution, 2 days at 4 °C). Biotinylated goat-anti mouse IgM antibody (1:200 dilution, Vector Laboratories Inc.) was used as secondary antibody. After washing with PBS, the sections were incubated in diluted peroxidase-conjugated streptavidin (1:2000 dilution, Jackson Immunochemicals, USA). The cells were visualized with DAB-nickel and the reaction product was intensified by the silver intensification method of Gallyas as modified by Dobó et al. (Dobó et al., 1996).

Two different antibodies of IgM isotype raised in mouse, monoclonal anti-mouse Thy 1.2 (SIGMA Chemical Company, St. Louis, USA) and monoclonal anti-beta galactosidase (SIGMA) were used for the negative control of our C5aR antibody. Neither of them has previously been reported to label any neuronal structures in the human brain. Human brain sections obtained from the control patients were pretreated as described in the C5aR immunohistochemistry procedure. They were then incubated with these antibodies (1:1000, 2 days at 4 °C). After incubation in primary antibodies, the sections were processed as described earlier.

**Toluidine blue staining**

The sections obtained from the brains of the AD patients were mounted on glass slides and air-dried. The sections were then briefly dipped into 1 % toluidine blue solution for 1-2 sec., washed in PBS, dehydrated in graded series of ethanol and covered.

**Patch clamp measurements**

TGW, $Ltk^-$ and $Ltk^-$/C5aR cells were voltage clamped at a -70 mV holding potential at room temperature using a whole-cell clamp configuration. The instruments used for electrophysiology were as follows: Axopatch 200-A patch clamp amplifier, Digidata-1200 data acquisition system and pCLAMP 6.02 software from Axon Instruments Inc., Foster City, U.S.A. The resistance of the patch electrodes was 8-10 MΩ. The solutions were as follows: standard extracellular solution (Hepes 10 mM, NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, glucose 10 mM, pH=7.34); standard 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); Ca-free extracellular solution (Hepes 10 mM, NaCl 140 mM, KCl 5 mM, $MgCl_2$ 10 mM, EGTA 2 mM, pH=7.34); BAPTA-containing intracellular solution (Hepes 10 mM, KCl 40 mM, NaCl 15 mM, $CaCl_2$ 0.1 mM, $MgCl_2$ 2 mM, BAPTA-tetrapotassium salt 20 mM, pH=7.25). For pertussis toxin treatment, the cells were cultured overnight in complete RPMI-1640+FBS medium containing 500 ng/ml pertussis toxin before patch clamp measurement. For C5a (human recombinant, SIGMA Chemical Company, St. Louis, USA) treatment, the cells were exposed to 1 μg/ml (120 nM) C5a, added to the extracellular solution for 5 min just before whole-cell recording. Extracellularly, PR226-MAP was applied by puff pipette from a distance of 300-500 μm for 1.5 min at a concentration of 3.5 μg/ml; PL37-MAP was also applied by puff pipette from a distance of 300-500 μm for 2 min if not indicated otherwise, with 100 nM in the case of TGW cells, or 500 nM for $Ltk^-$ and $Ltk^-$/C5aR cells. The recordings started simultaneously with drug application. When PR226-MAP was applied two or three times, the second or third application of the peptide after 6 or 11 min is indicated by arrows in the figures. The intracellularly applied PR226-MAP or PL37-MAP was diluted directly in the pipette solution. The reversal potential of the steady current was determined by applying positive voltage pulses.

**Flow cytometry**

TGW cells were harvested and washed 3 times in 14 ml of calcium-free PBS at room temperature. Following the last centrifugation, the pellet (2-5x$10^7$ cells/tube) was resuspended in 200 µl of calcium-free PBS and 1 µM Fluo-3 was added to the suspension for each $10^6$ cells (Fluo-3AM, 1 mM stock in DMSO, WAKO Chemicals Ltd., Tokyo, Japan). After loading the cells with fluorescent dye and washing 3 times in 14 ml of calcium-free PBS, the cells were resuspended in ice-cold HBSS supplemented with 1% bovine serum albumin. The efficiency of Fluo-3AM loading was tested by adding Ca-ionophore (A23187, Calbiochem, La Jolla, CA) to 1 ml aliquots of the cell suspension (5x$10^5$/ml). The increase in fluorescence due to changes in intracellular calcium content was measured on a FACScan flow cytometer (200 events/sec, Becton-Dickinson, Mountain View, Ca, USA), using Chronys software. In each measurement, the baseline fluorescence activity was measured for 20 sec, 200 nM C5a (Sigma, USA) was then added and the time course of the change in fluorescence intensity was measured for an additional 700 sec. To minimize the non-specific binding of C5a, all solutions were prepared in HBSS containing 1% BSA and the solutions and cells were incubated in 96-well, flat-bottomed, precoated, polystyrene tissue culture plates (Falcon, USA).

**Statistical analysis**

Analyses were performed by the Student's t-test, using Microsoft Excel 4.0 (Microsoft Inc., Richmond, USA) and GraphPad Prism 2.01 (GraphPad Software, Inc., San Diego, USA) software. In order to calculate integrated areas of the currents recorded in the patch whole-cell clamp experiments, the ClampFit unit of PClamp 6.02 software (Axon Instruments) was used. Integrated areas were corrected for baseline shifts. In TUNEL staining, statistical analysis of apoptotic cells was performed in 6 different areas of 3 culture dishes for the control and for each different peptide treatment condition.

## RESULTS

### C5aR immunoreactivity in human brain neurons

Immunohistochemical analysis of the normal human brain sections using the monoclonal anti-human C5aR antibody demonstrated the expression of C5aR in the hippocampus. C5aR-immunoreactivity (C5aR-IR) was restricted to the layers of the pyramidal cells and the granular cells (Fig. 1.A, magnification: 4x). Higher magnification (10x) showed that the immunoreactive (IR) cells could be clearly identified as neurons in the fascia dentata and the CA1-4 layers of the pyramidal cells (Fig. 1.B) and granular cells, due to their characteristic location and morphology. Sections of the temporal cortex were also stained by the C5aR antibody: layers III and V of the cortical pyramidal cells were found to be IR (Fig. 1.C, magnification: 10x). C5aR-IR was found in the soma, whereas the processes were mostly devoid of it.

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**Figure 1. C5aR immunostaining of the control human hippocampus and cortex.**

**A.** Low magnification (4x) showed C5aR-IR in the granular and pyramidal layers of the hippocampus. Bar = 200 μm. **B.** Higher magnification (10x) of the pyramidal layer of the hippocampus demonstrates that the C5aR-IR cells are pyramidal cells. **C.** The cortical pyramidal cells of the cortex also showed IR when the C5aR antibody was used. **D.** No staining was observed in the cortex when anti-beta galactosidase was used as control IgM antibody. **E.** Positive staining was not observed with the other control IgM antibody (anti-mouse Thy 1.2) in the cortex. **F.** When anti-mouse Thy 1.2 was used, positive immunostaining also cannot be detected in the hippocampus.

Bar = 40 μm in each figure, if not stated otherwise.

**Figure 2. C5aR immunostaining and toluidin-blue staining of the hippocampus and cortex of the Alzheimer brain.**

**A.** C5aR immunostaining showed a decreased expression of C5aR in the pyramidal layers of the hippocampus of the AD brain. **B.** A similar result was found in the cortex: C5aR-IR was absent in the pyramidal cells of the cortex. **C.** Toluidine blue staining revealed the presence of the pyramidal cells of the hippocampus. **D.** The pyramidal cells are also present in the temporal cortex, as revealed by toluidin blue staining.

Bar = 40 μm in each figure.

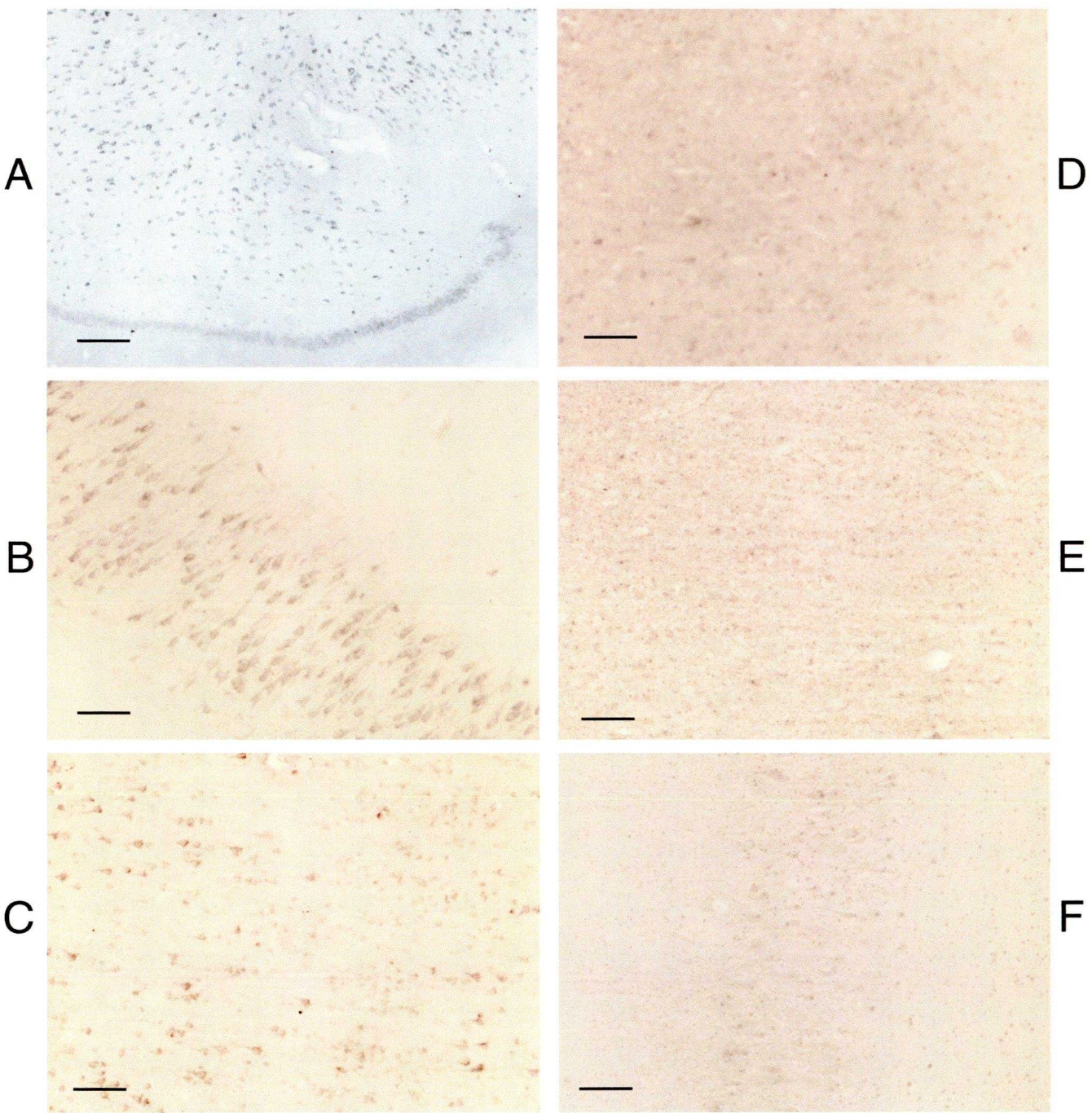


Fig. 1.

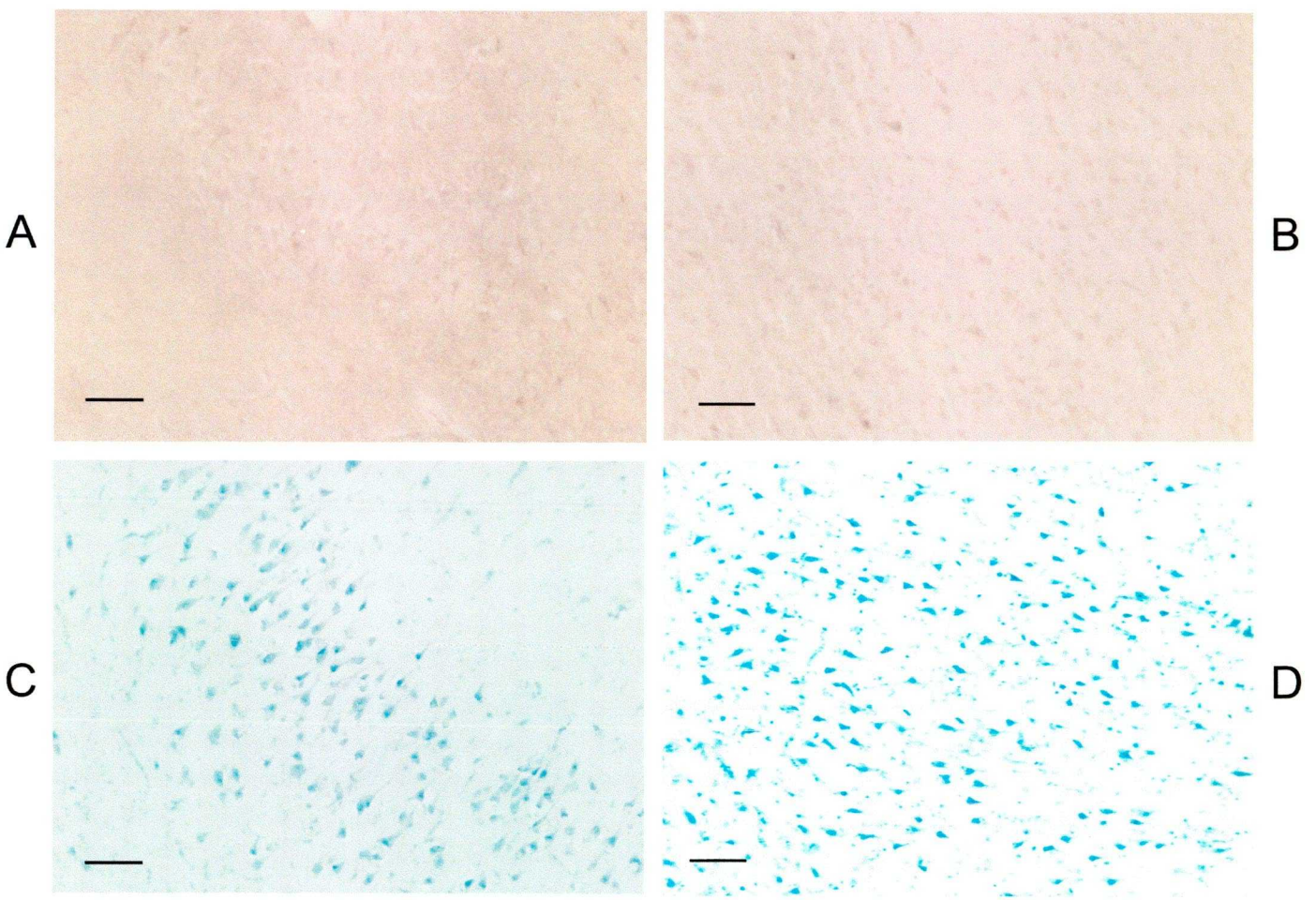


Fig. 2.

Sections of the hippocampus and the temporal cortex obtained from control patients were incubated with the control IgM antibodies. Labeling of the temporal cortex with the anti-beta-galactosidase did not reveal any staining (Fig. 1.D). The use of anti-mouse Thy 1.2 did not reveal any staining either in the temporal cortex (Fig. 1.E) or in the hippocampus (Fig. 1.F).

**C5aR immunoreactivity disappears in the neurons of the Alzheimer brain**

In order to investigate the role of C5aR in the neurodegenerative diseases, brain sections of AD patients were also incubated with the anti-C5aR antibody. The staining intensity was decreased dramatically in both the layers of the pyramidal cells of the hippocampus (Fig. 2.A) and the pyramidal cells of the temporal cortex (Fig. 2.B).

Since the disappearance of C5aR-IR in the AD can be due either to the loss of C5aR-IR neurons or to a decreased expression of the receptor in the membrane of the nerve cells, the AD brain sections were stained with toluidine blue. The Nissl staining of the sections revealed that the majority of the pyramidal neurons of both the hippocampus (Fig. 2.C) and the temporal cortex (Fig. 2.D) are present in the sections, although many of them show abnormal morphology of the shrunken, atrophied cell body.

**C5aR expression in TGW neuroblastoma cells**

In order to have a proper model for analysis of the role of C5aR in the neurons, the expression of C5aR was examined in TGW neuroblastoma cells, and Ltk$^-$ and Ltk$^-$/C5aR fibroblast cells by RT-PCR (Fig. 3). The C5aR mRNA was demonstrated in both TGW and

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**Figure 3. RT-PCR analysis of C5aR in TGW, Ltk$^-$ and Ltk$^-$/C5aR cells.**

RT-PCR showed a single band (552 bp) for C5aR in lanes 5 and 7 in TGW and Ltk$^-$/C5aR cells. Lane 1: molecular weight markers; lanes 2-4: controls with no reverse transcriptase for TGW, Ltk$^-$ and Ltk$^-$/C5aR cells, respectively; lanes 5-7: with reverse transcriptase for TGW, Ltk$^-$ and Ltk$^-$/C5aR cells, respectively; lane 8: control with no addition of template cDNA.

**Figure 4. Flow cytometry of TGW cells during C5a administration.**

Flow cytometry showed a transient increase in the intracellular free calcium content of the TGW cells in response to the administration of 200 nM C5a.

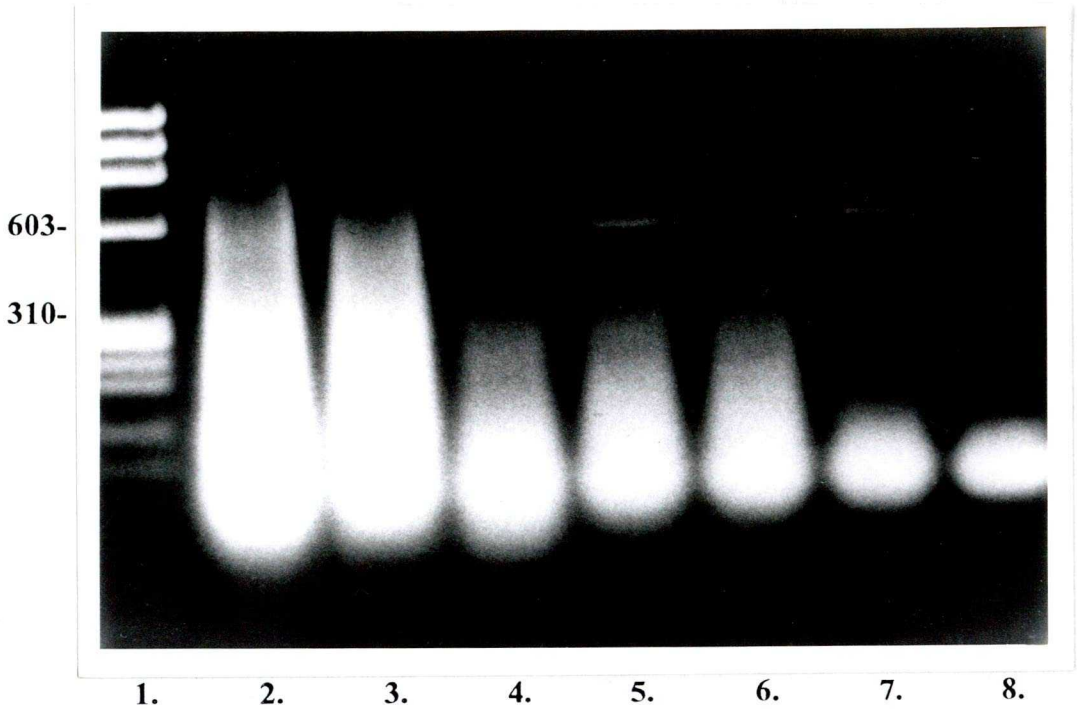
603-
310-
1.
2.
3.
4.
5.
6.
7.
8.

Fig. 3.

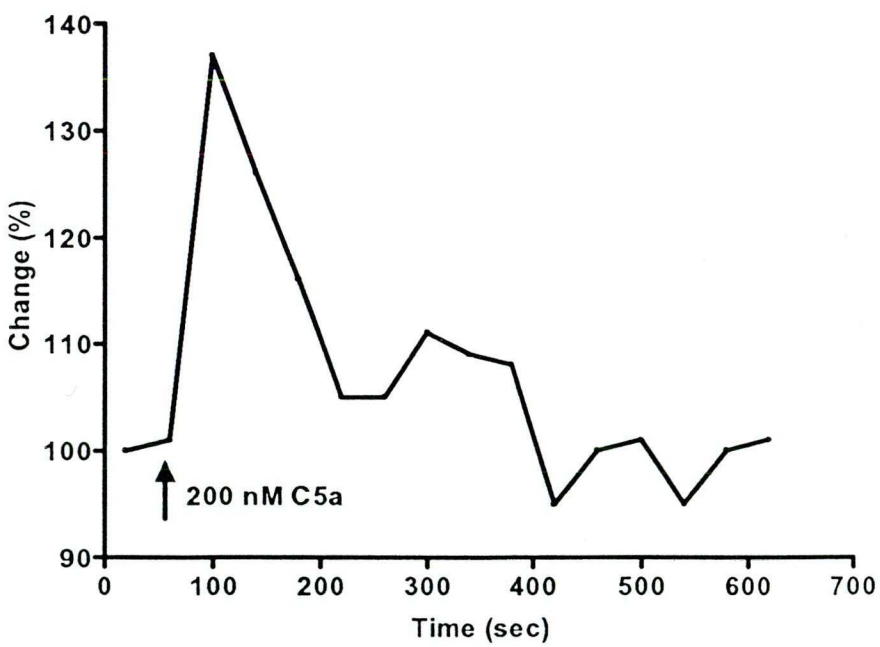
140
130
120
110
100
90
Change (%)
200 nM C5a
0
100
200
300
400
500
600
700
Time (sec)

Fig. 4.

Ltk⁻/C5aR cells (lanes 5 and 7, respectively) with a molecular size of 552 bp. However, it was absent from Ltk⁻ cells (lane 6). None of the controls without reverse transcriptase exhibited bands corresponding to C5aR mRNA (lanes 2-4), excluding the possibility of genomic DNA contamination.

Flow cytometry demonstrated a slow, transient increase in the intracellular calcium concentration when 200 nM C5a was administered to TGW cells (Fig. 4).

**Apoptosis and c-fos immunostaining of TGW cells**

*Effects of the PR226-MAP peptide*

Application of the TUNEL method demonstrated that spontaneous apoptosis (as indicated by dark nuclear staining) is a negligible event in the culture of the control TGW cells (0.61 ± 0.17 %, mean ± SEM) (Fig. 5.A). The photograph of the control culture reveals intact neurons with a well-developed network as shown by the neutral-red counterstaining. In the PR226-MAP treated culture (10 μg/ml), the light-brown color in the nucleus of 7.4 ± 0.95 % of the cells showed the onset of apoptosis as early as after 30 min of treatment, indicating significant rapid DNA fragmentation in these cells ($p<0.001$) (Fig. 5.B). Following 2 h of treatment, the shape of the cells has changed, they have retracted their processes and the proportion of dead cells has increased up to 11.9 ± 2.75 % ($p<0.05$), while the nucleus of the

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**Figure 5. TUNEL staining and c-fos immunostaining in TGW cells after PR226-MAP administration.**

**A.** The TUNEL staining of control TGW cells demonstrated a low spontaneous apoptosis rate, as shown by the dark nuclear staining. **B.** After 30 min, PR226-MAP treatment caused DNA fragmentation. The light-brown nuclear staining demonstrated the beginning of apoptosis. **C.** The DNA damage became more extensive after 2 h, as indicated by the dark staining in the nuclei of the cells. **D.** Higher magnification showed chromatin aggregation. Bar=10 μm. **E.** Increased c-fos immunoreactivity was found in the nuclei of the cells after 1 h of PR226-MAP treatment. **F.** Without peptide treatment, c-fos immunostaining of the control cells is weak and homogeneous.

Bar=25 μm in each figure, if not stated otherwise.

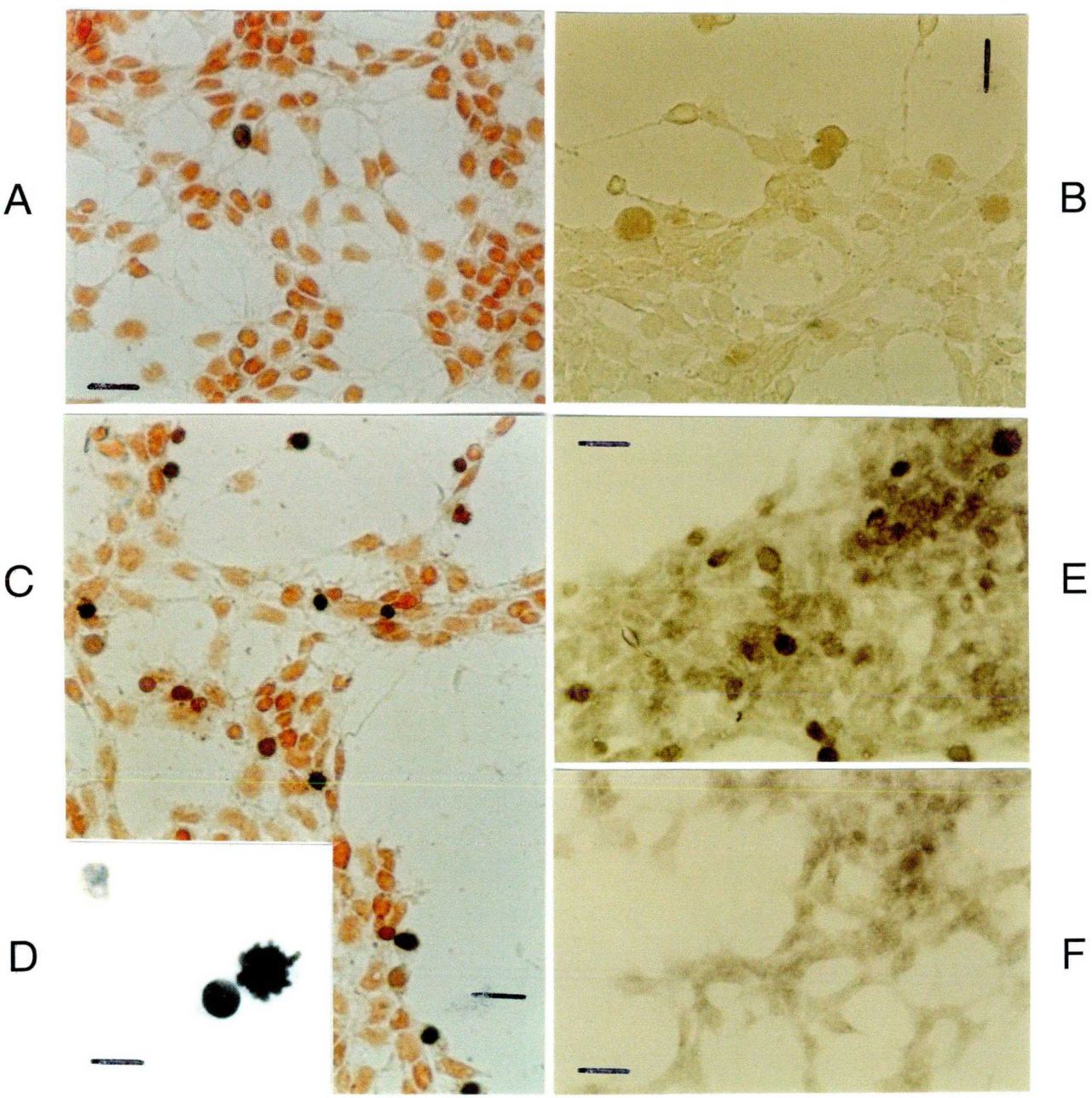


Fig. 5.

cells has become more intensely stained (Fig. 5.C). In the insert (Fig. 5.D), condensed, aggregated chromatin in the nucleus of TGW cells can be seen at high magnification (100 x).

In control cultures, c-fos immunostaining showed a uniform staining with low intensity (Fig. 5.F). In cultures treated with PR226-MAP, weak nuclear staining was encountered after 30 min of treatment. After 1 h, staining of the nucleus was greatly enhanced (Fig. 5.E), demonstrating an elevated level of nuclear c-fos immunoreactivity.

*Effects of the PL37-MAP peptide*

Similarly to PR226-MAP, the administration of PL37-MAP also caused apoptosis and elevation of the c-fos level in TGW cells. As compared to the control culture (Fig. 6.A), the morphology of the PL37-MAP treated cells changed and the length of their neurites was reduced in 1 h (Fig. 6.B), as demonstrated by means of the TUNEL method and the neutral red counterstaining. After 4 h of PL37-MAP treatment, intense dark staining was revealed in the nucleus of 19.6 ± 2.6 % ($p<0.001$) of TGW cells, indicating that extensive DNA fragmentation had occurred in these cells (Fig. 6.C). After 6 h, the number of cells dead with apoptosis had increased up to 26.7 ± 1.8 % (Fig. 6.D), while peptide treatment for 8 h caused a majority of the cells to die (65.2 ± 6.9 %) (Fig. 6.E).

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**Figure 6. Apoptosis and c-fos immunostaining in TGW cells after PL37-MAP administration.**

**A.** In a control culture, a TGW cell exhibited dark apoptotic staining in its nucleus (arrow), indicating a rare event of spontaneous apoptosis. **B.** After 1 h of PL37-MAP treatment, the cells had become rounded and retracted their neurites. **C.** The intense dark nuclear staining of many TGW cells showed that DNA fragmentation had become extensive after 4 h of treatment. **D.** The number of strongly stained apoptotic cells was increased further after 6 h. **E.** After 8 h, a majority of the cells showed intense apoptotic staining. **F.** c-fos immunostaining of the cells in the control cultures revealed a homogeneous background staining. **G.** An elevated nuclear c-fos level was detected after 30 min of PL37-MAP treatment. **H.** The proportion of the cells exhibiting enhanced nuclear c-fos staining was increased further after 1 h.

Bar=25 μm in each figures.

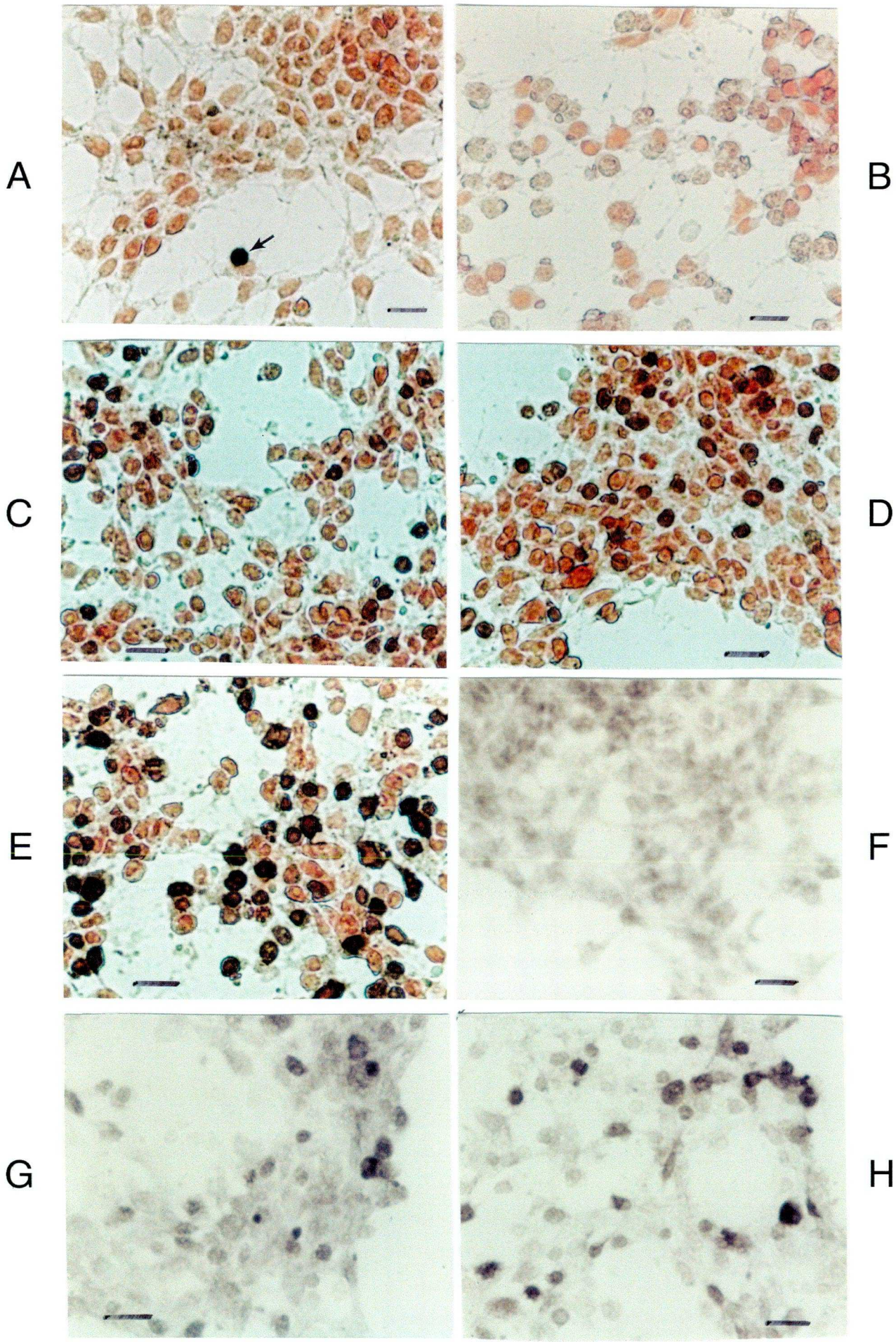


Fig. 6.

The c-fos immunostaining showed that, relative to the control culture (Fig. 6.F), an elevation of the nuclear c-fos level occurred due to the PL37-MAP treatment of TGW cells in 30 min (9.6 ± 1.8 %, $p<0.01$) (Fig. 6.G). After 1 h, the proportion of cells presenting intense nuclear c-fos staining was increased further (17.2 ± 2.1 %) (Fig. 6.H).

**The apoptotic effect of PL37-MAP is associated with C5aR**

In order to prove that the apoptosis triggered by the AHB peptide is associated with the presence of C5aR in the cell membrane, PL37-MAP was applied to Ltk$^-$/C5aR and Ltk$^-$ cells, and DNA fragmentation was then demonstrated by means of the TUNEL method. Weak DNA fragmentation was found in untreated control cultures of Ltk$^-$/C5aR cells, showing sporadic spontaneous apoptosis (Fig. 7.A) . No DNA fragmentation was seen in the control cultures of Ltk$^-$ cells (Fig. 7.B). Upon treatment with PL37-MAP (10 μM, 8 h), apoptosis failed to occur in Ltk$^-$ cells lacking C5R (Fig. 7.C). However, PL37-MAP (10 μM, 8 h) induced apoptosis in a majority of Ltk$^-$/C5aR cells expressing C5aR (86.3 ± 6.9 %) (Fig. 7.D), demonstrating that the presence of C5aR was necessary for the induced apoptosis.

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**Figure 7. TUNEL staining of Ltk$^-$/C5aR and Ltk$^-$ cells after PL37-MAP administration.**

**A.** In the control culture of Ltk$^-$/C5aR cells, TUNEL staining showed sporadic spontaneous apoptotic cell death. **B.** There was no sign of DNA fragmentation in the control culture of Ltk$^-$ cells. **C.** Administration of 10 μM PL37-MAP did not induce apoptosis in Ltk$^-$ cells during 8 hours. **D.** Application of PL37-MAP caused extensive DNA fragmentation in Ltk$^-$/C5aR cells, as demonstrated by the intense dark staining in the nucleus of a majority of the cells.

Bar=25 μm in each figure.

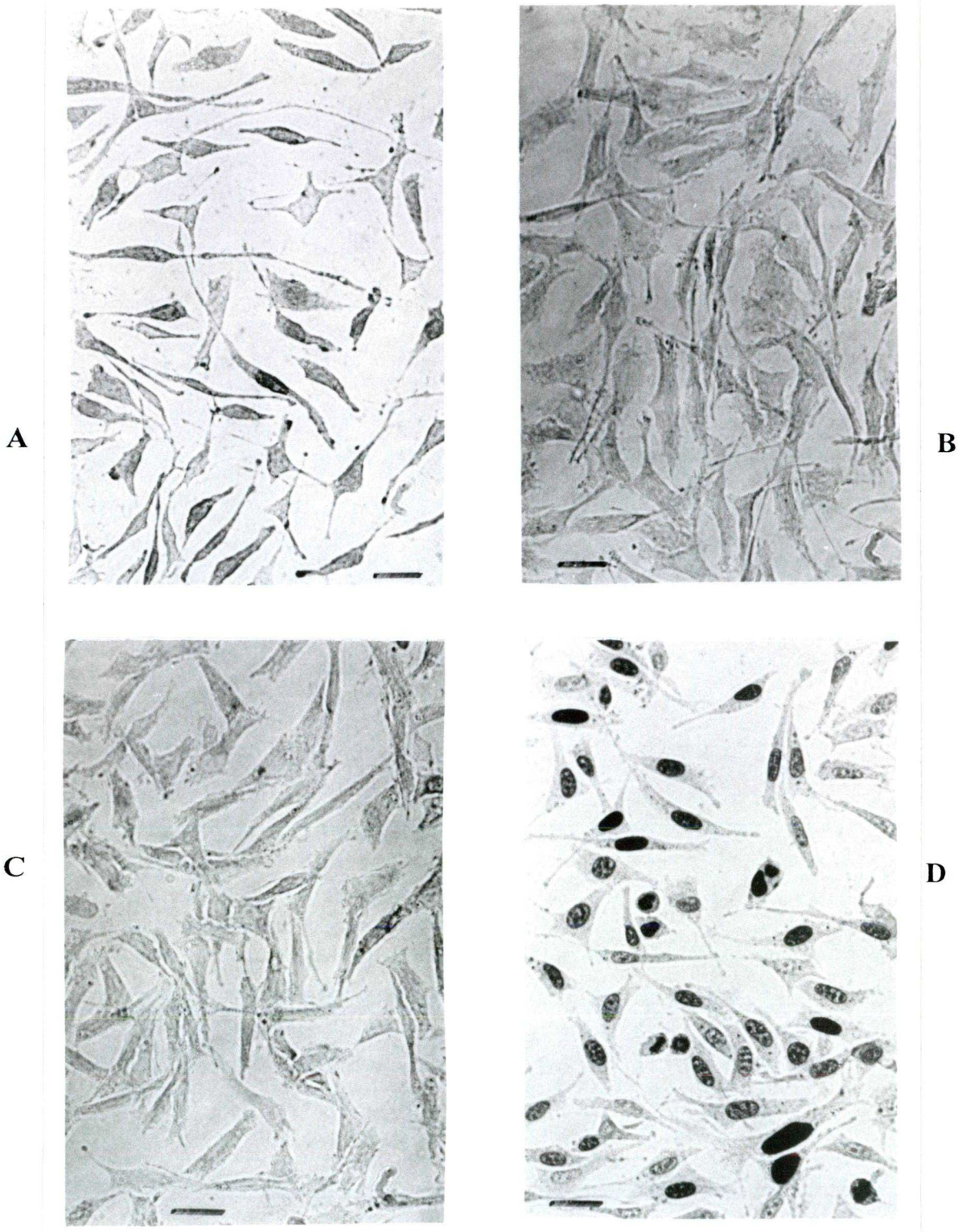


Fig. 7.

**Electrophysiological examination of TGW cells. Involvement of calcium and G-proteins in the process of C5aR-associated apoptosis**

*Effects of the PR226-MAP peptide*

Whole-cell clamp measurements on TGW cells demonstrated that treatment with PR226-MAP (3.7 μg/ml) resulted in the appearance of repetitive inward current pulses in 1 min with an amplitude of 150-200 pA (-192 ± 33 pA, mean ± SEM) as an early sign of apoptosis (Fig. 8.A). Successive administration of the peptide was indicated by a small downward-pointing arrow. The integrated area, representing the net electric charge passing through the membrane is –8982 ± 2906 pA*s (mean ± SEM) in the first 5 min. In 10 min, a high irreversible inward current (HIC) occurred, marking an irreversible change in the cell membrane, which was considered an indicator of the electrophysiological sign of cell death.

On application of 0.2 mM cobalt chloride, a calcium channel blocker, to the extracellular solution, the onset of the inward pulses was blocked (Fig. 8.B). Similarly, the repetitive pulses were abolished in calcium-free extracellular solution (Fig. 8.C). These results demonstrated that the recorded inward pulses were results of calcium ion influx.

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**Figure 8. Whole-cell clamp recordings in TGW cells during PR226-MAP administration.**

**A**. Within 1 min PR226-MAP administration caused repetitive inward current pulses recorded by means of the whole-cell clamp method. The arrow shows successive peptide administration **B**. A calcium channel blocker ($CoCl_2$) inhibited the onset of the current pulses. **C**. Administration of PR226-MAP in a Ca-free extracellular solution did not induce a repetitive current, showing that the repetitive current represents calcium influx. **D**. Intracellularly applied PR226-MAP did not evoke current or cell death, demonstrating that PR226-MAP requires an extracellular or appropriately oriented intramembrane structure to bind to. **E**. Pertussis toxin pretreatment attenuated the amplitude of the PR226-MAP-evoked repetitive current. This shows the involvement of the C5aR associated G-proteins in the apoptotic signal transduction pathway. **F**. C5a pretreatment delays the PR226-MAP-evoked calcium influx and inhibits the appearance of the HIC. This suggests that the apoptotic signal transduction pathway is associated with C5aR. **G**. In Ca-free extracellular solution and with intracellularly used BAPTA salt, the repetitive inward pulses and HIC did not occur.

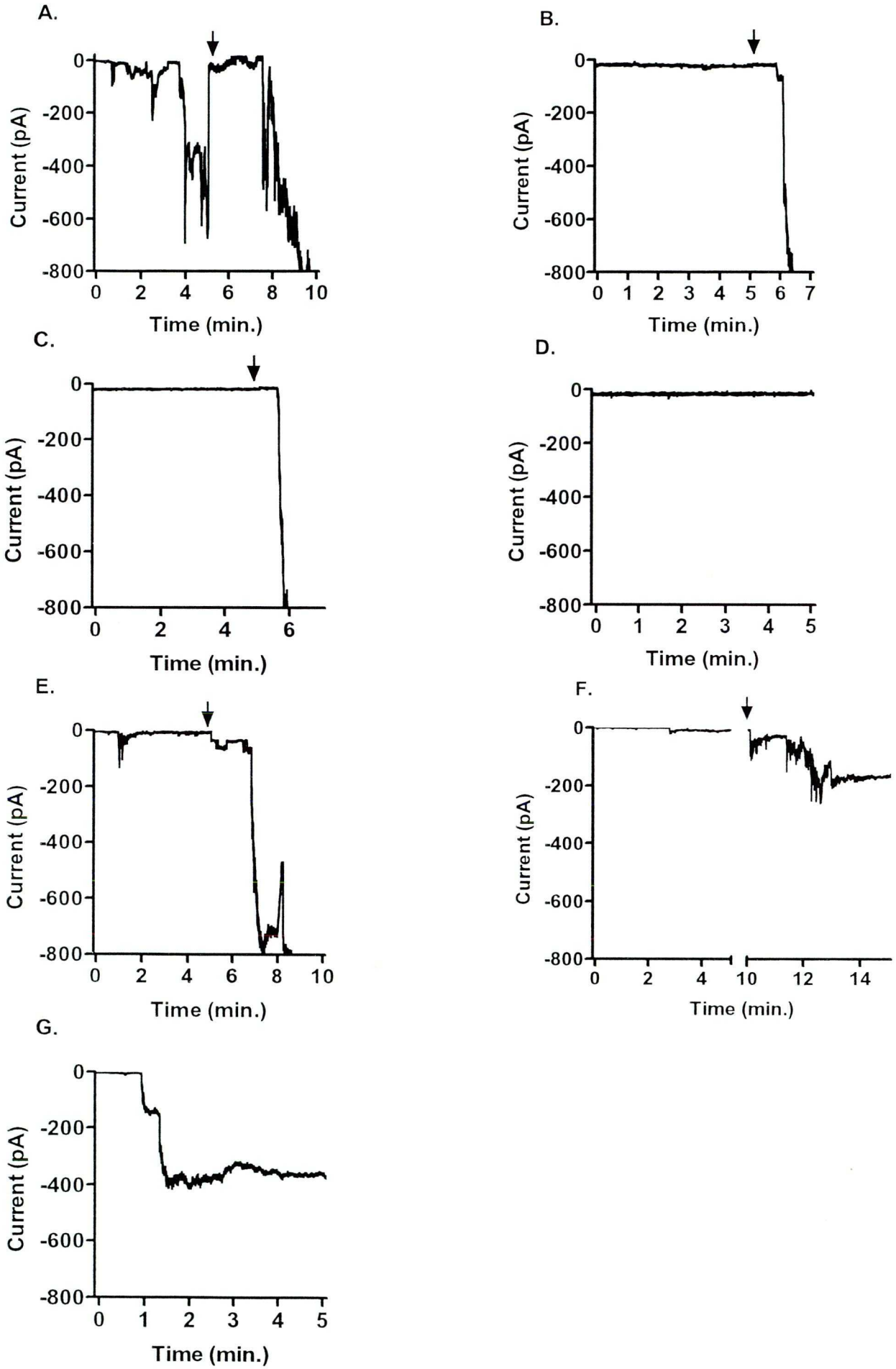


Fig. 8.

However, HIC could be recorded either in the presence of cobalt chloride or in calcium-free extracellular solution, showing that neither of them could inhibit PR226-MAP induced cell death.

In order to investigate the orientation-specificity of the effect of PR226-MAP, it was administered to TGW cells intracellularly, through the patch pipette. Although the concentration of the peptide was double that used extracellularly, the peptide did not trigger any current (Fig. 8.D). This result reveals that PR226-MAP requires an extracellular structure, which is responsible for its apoptotic effect.

TGW cells were pretreated with pertussis toxin, a G-protein inhibitor, and PR226-MAP was then administered to these TGW cells. The pretreatment decreased both the amplitude of the calcium current pulses (-105 ± 13 pA; mean ± SEM) and the integrated area (-3101 ± 269 pA) ($p<0.05$) (Fig. 8.E). This result shows that the apoptotic signal transduction pathway involves pertussis-toxin sensitive G-proteins. Nevertheless, pertussis toxin pretreatment did not eliminate the onset of the HIC.

TGW cells were also pretreated with 120 nM C5a. Administration of C5a alone did not cause any significant ion current. However the pretreatment delayed the onset of the repetitive calcium influx and blocked the onset of the HIC as well (Fig. 8.F). Even successive administration of PR226-MAP failed to evoke the HIC. Nevertheless, the calcium pulses were followed by a steady, irreversible inward current with an amplitude of 200-400 pA and a reversal potential of 1.2 ± 1.4 mV, which indicates that the steady current is due to a non-specific ion flow through the membrane of the cell.

Cobalt chloride and the application of PR226-MAP in calcium-free extracellular solution inhibited the inward calcium current, but not the onset of the HIC, which was regarded as a sign of PR226-MAP induced cell death. In order to investigate the role of the intracellular calcium sources in cell death, PR226-MAP was applied in calcium-free extracellular solution while the intracellular free calcium content was chelated by 20 mM BAPTA in the intracellular solution. Calcium depletion eliminated the onset of the HIC completely (Fig. 8.G), but a steady inward current was recorded with an amplitude of 250-500 pA and a reversal potential of 0.8 ± 1.2 mV, similarly to the current recorded in the case of C5a pretreatment.

*Effects of the PL37-MAP peptide*

Ion current responses of TGW cells to PL37-MAP treatment were also measured by whole-cell clamp recordings. Inward current pulses were evoked by extracellularly applied PL37-MAP (100 nM) with an amplitude of -256.9 ± 92.69 pA as early as after 1 min (Fig. 9.A). The integrated area of the current was -17.82 ± 8.16 nA*s. Inward current pulses with higher amplitude were evoked by a higher concentration of PL37-MAP (500 nM), which was followed by a HIC exceeding 2 nA (Fig. 9.B). This HIC was again considered a sign of electrophysiological cell death. Dose-response graphs showed a linear correlation between the concentration of PL37-MAP and the amplitude of the evoked current pulses (Fig. 9.C, left vertical axis, open squares), while the net electric charge flowing through the membrane also increased with the applied dose (Fig. 9.C, right vertical axis, closed squares).

We presumed that the current pulses evoked by PL37-MAP are due to a calcium influx. Therefore, in order to abolish the calcium component of the current, 0.2 mM cobalt

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**Figure 9. Whole-cell clamp recordings in TGW cells during PL37-MAP administration.** **A**. Within 1 min, PL37-MAP evoked inward current pulses in TGW cells, as demonstrated by whole-cell clamp recordings. **B**. At a higher dose, PL37-MAP evoked current pulses with higher amplitude, followed by a HIC. **C**. The dose-response graph showed that both the amplitude (open squares, linear scale) and the integrated area (closed squares, semilogarithmic scale) of the PL37-MAP evoked current pulses increase with the applied peptide concentration. **D.** Application of a calcium channel blocker ($CoCl_2$) in the extracellular solution completely abrogated the onset of the current pulses in TGW cells. **E.** Pertussis toxin pretreatment can attenuate the PL37-MAP-evoked current. **F.** C5a pretreatment also decreased the PL37-MAP evoked current. **G.** The intracellularly applied PL37-MAP did not evoke current or cell death in TGW cells.

**Figure 10. Whole-cell clamp recordings of Ltk$^-$/C5aR and Ltk$^-$ cells during PL37-MAP administration.**

**A.** PL37-MAP induced the onset of inward current pulses in Ltk$^-$/C5aR cells expressing C5aR. **B.** Administration of PL37-MAP did not evoke an ion current in Ltk$^-$ cells not expressing C5aR, showing the C5aR-specificity of the PL37-MAP evoked current.

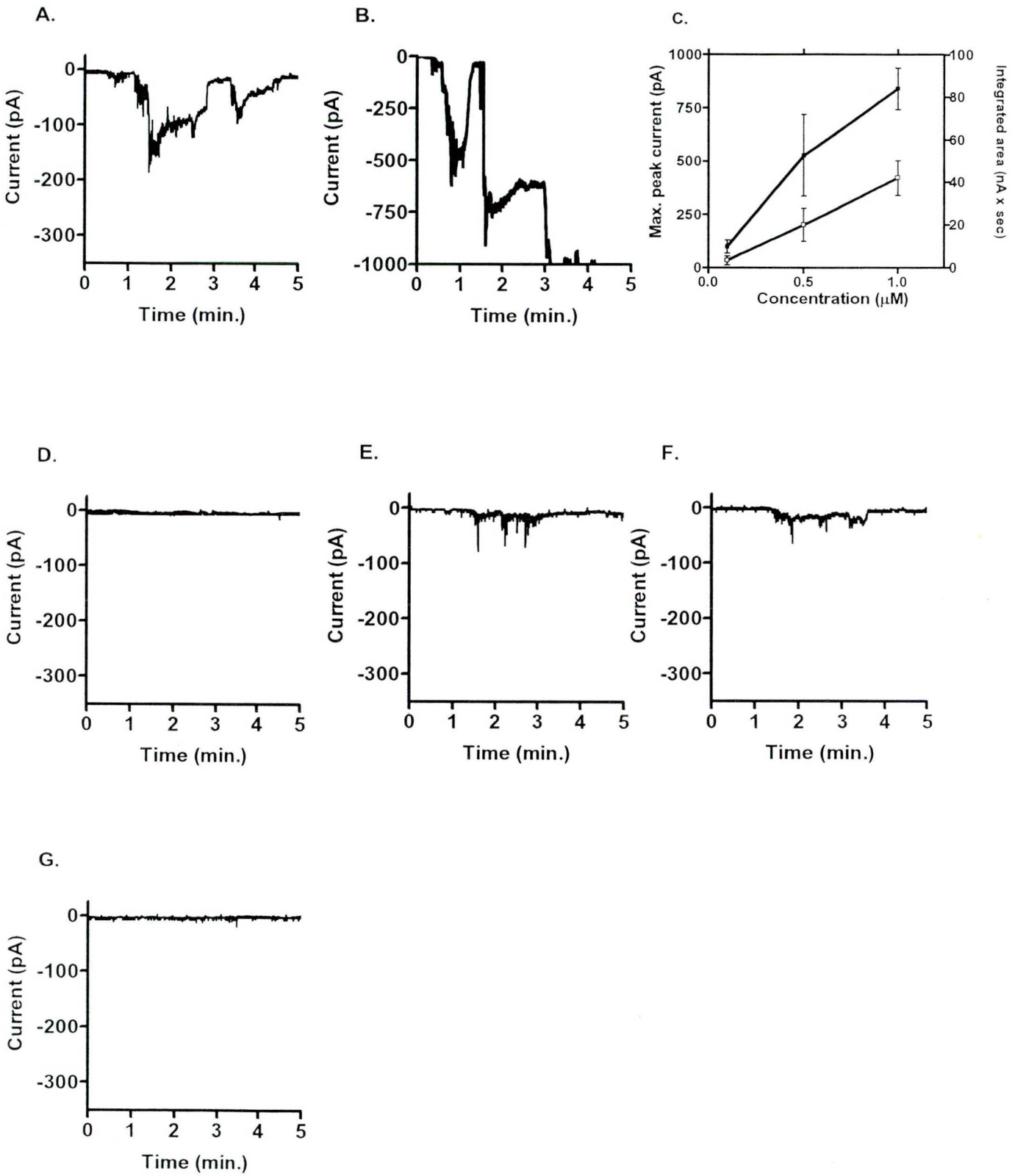
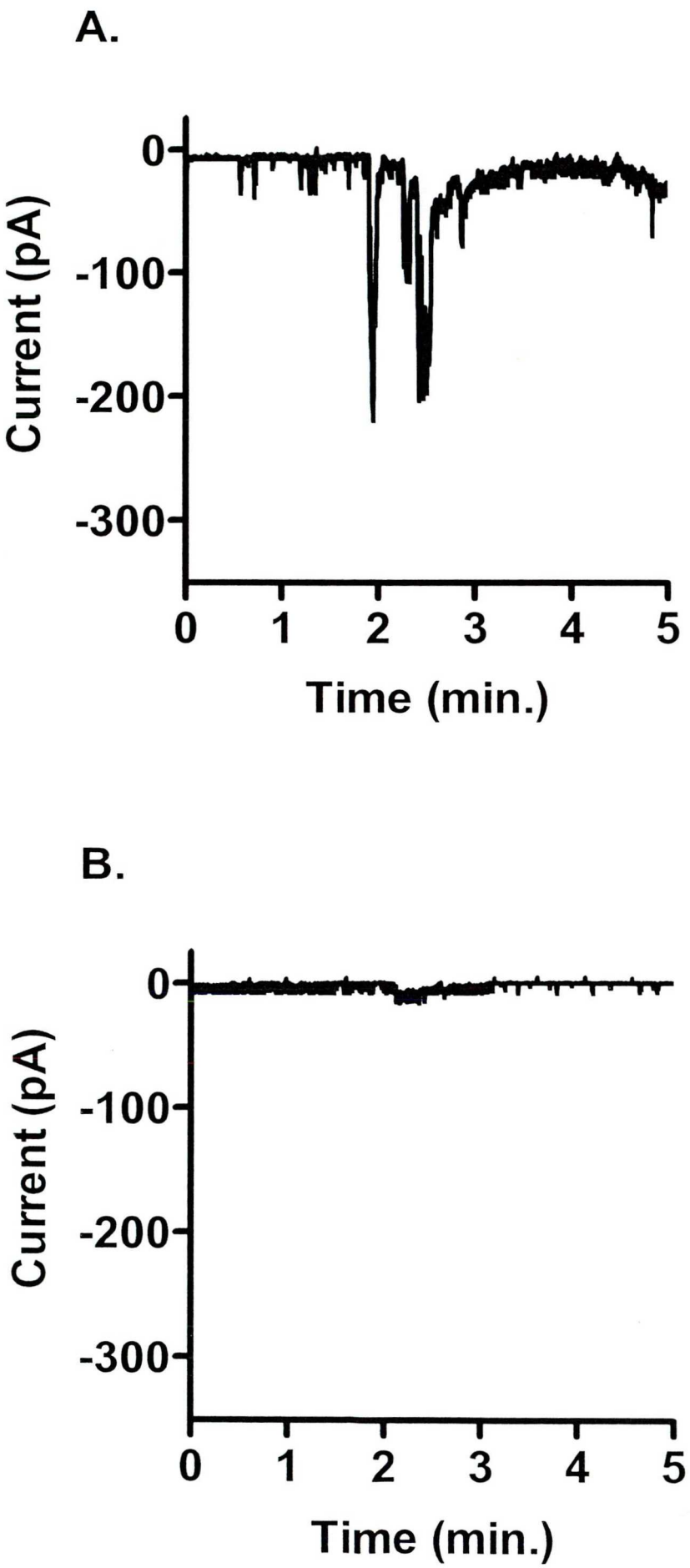

A.
Current (pA)
0
-100
-200
-300
0 1 2 3 4 5
Time (min.)
B.
Current (pA)
0
-250
-500
-750
-1000
0 1 2 3 4 5
Time (min.)
C.
Max. peak current (pA)
1000
750
500
250
0
0.0
0.5
1.0
Concentration (μM)
Integrated area (nA x sec)
100
80
60
40
20
0
D.
Current (pA)
0
-100
-200
-300
0 1 2 3 4 5
Time (min.)
E.
Current (pA)
0
-100
-200
-300
0 1 2 3 4 5
Time (min.)
F.
Current (pA)
0
-100
-200
-300
0 1 2 3 4 5
Time (min.)
G.
Current (pA)
0
-100
-200
-300
0 1 2 3 4 5
Time (min.)

Fig. 9.


A.
0
-100
-200
-300
Current (pA)
0
1
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3
4
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Time (min.)
B.
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-100
-200
-300
Current (pA)
0
1
2
3
4
5
Time (min.)

Fig. 10.

chloride was applied extracellularly to TGW cells prior to administration of the peptide (100 nM). Application of the calcium channel blocker inhibited the inward current pulses (Fig. 9.D), demonstrating that the recorded current is due to the calcium ions flowing into the cell through the cell membrane.

It was also presumed that the effect of PL37-MAP is associated with C5aR. Therefore, TGW cells were pretreated with pertussis toxin, and PL37-MAP (100 nM) was then administered. The recorded current shows that, as compared to the influx without pertussis toxin, the pretreatment significantly attenuated ($p<0.05$) both the amplitude (-117 ± 38.5 pA) and the integrated area (-2.33 ± 0.55 nA*s) of the evoked current (Fig. 9.E).

Further evidence of the relation between C5aR and the effect of PL37-MAP is the fact that C5a pretreatment (1 μg/ml; 120 nM) significantly decreased ($p<0.05$) both the amplitude (-0.6 ± 23.8 pA) and the integrated area (-1.14 ± 0.36 nA*s) of the PL37-MAP evoked ion current (Fig. 9.F).

Similarly to PR226-MAP, the orientation-specificity of the effect of PL37-MAP was also examined. The patch pipette was filled with intracellular solution in which high a concentration of PL37-MAP (1 μM) was diluted prior to filling. Although the extracellular administration of such a high concentration of PL37-MAP caused extremely rapid cell death, intracellular application of the peptide did not cause any significant effect in TGW cells. The calcium current pulses were absent even after 20 min (Fig. 9.G).

In order to demonstrate that the calcium current pulses evoked by PL37-MAP are due to the interaction between the peptide and the C5aR expressed on the membrane of the cells, PL37-MAP (500 nM) was applied to $Ltk^-$/C5aR and $Ltk^-$ fibroblast cells. Inward current pulses were evoked in $Ltk^-$/C5aR cells (expressing C5aR) with an amplitude of -222 ± 33.1 pA (Fig. 10.A), while PL37-MAP did not induce any significant current in $Ltk^-$ fibroblast cells not expressing C5aR (Fig. 10.B).

## DISCUSSION

### C5aR immunoreactivity in human brain neurons

In our studies, the pyramidal cells and granular cells of the hippocampus and the pyramidal cells of the temporal cortex of the human brain were found to be immunoreactive

(IR) for C5aR. Our observation, that C5aR may be expressed in typical neuronal cell layers is supported by the findings of other authors: the stratum pyramidale and the stratum granulosum in the hippocampus or the Purkinje cells of the cerebellum of mice and rats have already been reported to express C5aR (Pasinetti 1996; Stahel et al., 1997a, 1997b). Although a few glial cells cannot be excluded as a source of the C5aR expression in these brain regions with a more diffuse morphologic staining pattern, the morphology showed that neurons appeared to be the most prominent candidate for this C5aR immunostaining.

**C5aR immunoreactivity disappears in the neurons of the Alzheimer brain**

Our results that the hippocampal and cortical pyramidal cells and the hippocampal granular cells express C5aR acquire special importance from the finding that these cells are involved in the pathogenesis of Alzheimer's disease (AD). AD is primarily considered to be a disorder of the cytoskeleton of a few nerve cell types (for a review, see Braak et al., 1996). The cytoskeletal changes mainly consist of neurofibrillary tangles (NFTs) and neuropil threads and with many characteristic extracellular senile plaques containing mostly β-amyloid peptide. The NFTs showing degeneration of the cells in the hippocampus first appear in the CA1-4 pyramidal cells. In the later stages of the disease, NFTs develope in the granule cells of the fascia dentata too. NFTs can also be found in the pyramidal cells of the cortex, in the earlier stages of the disease in the entorhinal-transentorhinal region, but later they spread to the temporal and other cortical regions as well (Braak et al., 1996).

These findings prompted us to examine the expression of C5aR in these cells in AD. Our results demonstrated that both the hippocampal formation and the cortical region of the AD brain showed low or absent C5aR-IR relative to the control brain. In order to determine, whether the absence of staining is due to the disappearance of the cells expressing C5aR or to the low or absent C5aR expression of the individual cells, both the hippocampal and the cortical sections of the AD brain were stained with toluidine blue. The toluidine blue staining showed many pyramidal cells in the tissue, in either the hippocampal or the cortical areas. Therefore, we conclude that the missing staining is mainly due to the decreased level of C5aR expression in the cells. One possibitility for this low expression is that the C5aR production of the cell is lower because of the degenerative processes. Another possible explanation is that C5aR was activated by some ligand binding to it and, due to the well-known internalization

processes of C5aR (Rother and Till, 1988) the density of C5aR in the membrane of the cells decreased; although the cell probably continues to express C5aR, a new dynamic balance is established at a lower level due to the continuous internalization.

**C5aR expression in TGW neuroblastoma cells**

Since the pyramidal cells of the hippocampus and the cortex are involved in AD, and these cells seem to express C5aR in the control brain, but not in the AD brain, we were interested in whether this receptor can be involved in any degenerative process or cell death. For this purpose, we first had to find an appropriate neuronal model bearing C5aR. Although C5aR has already been demonstrated in neurons of the rodent brain (Pasinetti, 1996; Stahel et al., 1997a, 1997b), other cells of the brain, e.g. astrocytes and fibroblasts, also express C5aR (Gasque et al., 1995b), thereby disturbing experimental observations of the neuronal C5aR.

Therefore, we set out to find a better model with neuronal characters only. Our RT-PCR experiments showed that TGW neuroblastoma cells are suitable candidates for this purpose, since they express C5aR. In addition, our FACS measurements showed that the C5aR expressed in TGW cells is active since its ligand (C5a) could evoke an increased intracellular calcium level. Nevertheless, the required concentration of C5a was much higher than in other cell types (Gennaro et al., 1984; Konteatis et al., 1994; Gasque et al., 1995b), suggesting that the function of the C5aR of the neurons can differ from that of the other cell types, and/or a pharmacologically distinct C5aR subtype can be found in TGW cells. However, the function of the C5aR expression in the neurons remains to be elucidated. In myeloid cells, C5aR mediates the potent anaphylatoxic and chemotactic activities of C5a (Liszevski and Atkinson, 1993; Kuby, 1994). Within the central nervous system, the C5aR expression mediates the chemotaxis of astrocytes and microglia (Armstrong et al., 1990; Yao et al., 1990). An enhanced C5aR expression has also been demonstrated on the glial cells in acute and chronic active lesions in patients with multiple sclerosis (Müller et al., 1996). Additionally, C5a exerts psychopharmacologic effects on the drinking and eating behavior of rats, suggesting that C5a/C5aR mediates adrenergic activity in the hypothalamus and implying the expression of C5aR on the neurons innervating the hypothalamic areas responsible for the eating and drinking habits (Williams et al., 1985). Nevertheless, these studies also suggest that the C5aR-IR substances in the neurons may extend beyond immunologic functions.

**C5aR-associated apoptosis**

*Considerations concerning TUNEL staining*

By using TUNEL apoptosis staining and measuring the evoked ion currents with whole-cell clamp electrophysiology, we demonstrated that both PR226-MAP and PL37-MAP can induce apoptosis in TGW and $Ltk^-$/C5aR cells, respectively, which express C5aR, while apoptosis could not be triggered in $Ltk^-$ cells not bearing C5aR. This result suggests that the C5aR-associated signal transduction pathway is coupled to the apoptotic signal transduction pathway.

*Electrophysiological observations. Neuroprotective-like effect of C5a*

The effect of C5a pretreatment in attenuating the ion current evoked by PL37-MAP also supports this suggestion. Although certain fragments of C5a and C5aR can cause apoptosis, no data have yet been published about any apoptotic effect of the non-fragmented, intact C5a molecule, the physiological ligand of C5aR. Therefore, we can conclude that, although the normal activation of C5aR cannot trigger apoptosis, abnormal activation of C5aR can lead to cell death in the cells bearing C5aR. Our results demonstrated that, instead of triggering cell death, C5a delayed the onset of the calcium influx and abolished that of the HIC evoked by PR226-MAP. This suggests that C5a has a neuroprotective-like effect in this apoptotic process, rather than causing the death of the cell. A possible explanation of this neuroprotective-like effect of C5a is that the antisense homology box (AHB) peptides used in these experiments and the C5a-induced signals could interfere with each other, modifying the apoptotic signal. Another explanation could be the competitive binding of C5a and AHB peptides to C5aR (Baranyi et al., 1996).

*Roles of G-proteins and calcium in C5aR-associated apoptosis*

C5aR is coupled to pertussis toxin-sensitive G-proteins in different cells (Amatruda et al., 1991; Gerard and Gerard, 1991; Rollins et al., 1991; Vanek et al., 1994; Lee et al., 1995). Our measurements demonstrated that pertussis toxin diminished the ion influx evoked by either PR227-MAP or PL37-MAP. This result suggests that the G-proteins of the C5aR-associated signal transduction pathways are involved in the apoptotic signal transduction pathway activated by peptide treatment. Further, it can be concluded that, while triggering the

apoptosis, PR227-MAP and PL37-MAP utilize similar, but not necessarily the same signal transduction pathways. This conclusion is supported by the fact that, although extracellular calcium is required only for the effect of PL37-MAP, PR226-MAP utilizes both extracellular and intracellular calcium sources to exert its apoptotic effect, as concluded from the electrophysiological measurements in which calcium-free extracellular and BAPTA-containing intracellular solutions were used. Nevertheless, the experiments showed that calcium is necessary for the induction of apoptosis by both peptides, probably for the nucleases degrading the DNA and/or the second messenger signaling system.

*Possible correlation between an elevated c-fos level and apoptosis*

Triggering apoptosis usually needs some days or at least some hours, and it is therefore almost impossible to observe the apoptotic processes by intracellular electrophysiology. However, both PL37-MAP and PR226-MAP induce extremely rapid programmed cell death (possibly the most rapid apoptosis discovered so far) in the cells expressing C5aR. The earliest electrophysiological signs occur as soon as in 1-2 min. An elevated nuclear c-fos level and the first signs of DNA fragmentation can be observed in 30 min, although the intensive c-fos and TUNEL staining can be seen later. The distribution patterns of the c-fos and TUNEL staining are very similar, suggesting that the abnormal activation of C5aR, the elevated c-fos and the DNA fragmentation may be correlated. This idea is supported by the findings of other authors that cell injury can be accompanied by c-fos activation (Trump and Berezesky, 1992) and that elevation of the intracellular calcium level can activate c-fos transcription (Thompson et al., 1995).

**The possible involvement of neuronal C5aR in the pathomechanism of AD**

Our results that 1) the pyramidal and granular cells of the hippocampus and the pyramidal cells of the cortex express C5aR; 2) the level of C5aR expression is diminished in these cells in AD; 3) and abnormal activation of C5aR can lead to apoptosis, can be related to the data of other authors investigating AD. It has already been reported that the complement cascade is activated by β-amyloid, the main component of the senile plaques of the AD brain (Eikelenboom et al., 1989; McGeer et al., 1989b; Rogers et al., 1992). Each of the elements of the classical pathway was found in the AD brain, as demonstrated at either the mRNA or

the peptide level (Shen et al., 1997; Terai et al., 1997). Additionally, the result that the administration of anti-inflammatory drugs can serve as a putative agent to maintain the condition of AD patients lends support to the significance of the local inflammation in the brain (McGeer and Rogers, 1992). It has also been reported that inflammatory processes in the brain can increase the proportion of the isoforms of the amyloid precursor protein (APP) containing Kunitz-type protease inhibitory domains (751 and 770 residues, respectively) as compared to the shorter isoform (695 residues) not having such a domain (Brugg et al., 1995). The abundance of the Kunitz-type protease inhibitors in AD (Neve et al., 1988, 1990; Tanzi et al., 1988) suggests an impaired protease function in the AD brain. The isoforms of APP containing Kunitz-type protease inhibitory domains can inhibit the proteolytic processing of APP and degradation of the β-amyloid molecule (Naidu et al., 1995; Saporito-Irwin and Van Nostrand, 1995). It may be presumed that the cleavage and degradation of other molecules, such as C5a, are also inhibited or modified, and abnormal fragments of C5a might therefore also exist in the AD brain, causing abnormal activation of the C5aR expressed on the membrane of certain cell types. Since our results demonstrated that the abnormal activation of C5aR can trigger apoptosis, this cell death can contribute to the neurodegeneration of the neurons bearing C5aR. Therefore, it can be presumed that certain cells, including neurons expressing C5aR, are more vulnerable to the apoptosis associated with C5aR. These data present a possible explanation of why certain cell types, e.g. pyramidal cells, are so susceptible to the cell death in neurodegenerative diseases where local inflammation and impaired protease functions can be found, e.g. in AD. Our results further suggest the possibility that an abnormal fragment(s) of C5a might be involved in the neurodegeneration found in AD.

## SUMMARY

1. Our results suggest that certain types of neurons in the normal human brain, e.g. the hippocampal pyramidal and granular cells and the cortical pyramidal cells, express the complement C5aR immunoreactive substances.
2. However, we found that in these brain regions from AD patients, the expression of C5aR in the neurons decreases or even disappears completely.
3. We utilized TGW neuroblastoma cells bearing C5aR, on the supposition that TGW cells

are a suitable model for the examination of putative functions of C5aR in the neurons.

4. Through the use of TGW cells and synthetic peptides derived from the antisense homology boxes of C5a and C5aR, we found that the abnormal activation of C5aR can trigger the most rapid programmed cell death examined so far.
5. We demonstrated that apoptosis is associated with C5aR via pertussis toxin-sensitive G-proteins coupled to C5aR.
6. We also showed that calcium is an important component in the signal transduction processes of apoptosis.
7. We found that the level of nuclear c-fos increases as apoptosis proceeds.

As a final conclusion, our results suggest that C5aR and fragments of C5a might be involved in the neurodegeneration, especially in diseases where local inflammation occurs, e.g. in AD.

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