A neuronal C5a receptor and an associated apoptotic signal transduction pathway

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1. We report the first experimental evidence of a neuronal C5a receptor (nC5aR) in human cells of neuronal origin. Expression of nC5aR mRNA was demonstrated by the reverse transcriptase-polymerase chain reaction (RT-PCR) in TGW human neuroblastoma cells.

2. Expression of a functional C5aR was supported by the finding that C5a evoked a transient increase in the intracellular calcium level as measured by flow cytometry (FACS).

3. To analyse the function of the nC5aR, an antisense peptide fragment of the C5aR was used. Previous data showed that a C5aR fragment (a peptide termed PR226) has C5aR agonist and antagonist effects in U-937 cells depending on the concentration of the peptide. We found that a multiple antigenic peptide (MAP) form of the same peptide (termed PR226-MAP) induced rapid elevation of nuclear c-fos immunoreactivity and resulted in DNA fragmentation, a characteristic sign of apoptosis, in TGW cells.

4. Early electrophysiological events characteristic of apoptosis were also detected: intermittent calcium current pulses were recorded within 1–2 min of peptide administration. C5a pretreatment delayed the onset of this calcium influx.

5. We also demonstrated that the apoptotic pathway is linked to nC5aR via pertussis toxin-sensitive G-proteins.

6. Although the function of C5a and its receptor on neurons is unknown, these results suggest that an abnormal activation of this signal transduction pathway can result in apoptosis and, subsequently, in neurodegeneration.

Activation of the complement system is accompanied by the release of a seventy-four amino acid long amino terminal fragment of the complement factor C5. This fragment, C5a, is one of the most potent mediators of inflammation, acts as a chemoattractant for leukocytes and activates phagocytosis (reviewed by Rother & Till, 1988). The C5a receptor (C5aR) is a member of the rhodopsin superfamily of proteins bearing seven transmembrane regions and it is associated with both pertussis toxin-sensitive and -resistant G-proteins (Amatruda, Steele, Slepak & Simon, 1991; Gerard & Gerard, 1991). Earlier, expression of C5aR was reported in leukocytes; however, it has also been demonstrated in other cell types, such as liver parenchymal cells, lung bronchial and alveolar epithelial cells, vascular smooth muscle and endothelial cells as well as in human astrocytes (Huey & Hugli, 1985; Gerard, Hodges, Drazen, Weller & Gerard, 1989; Gasque *et al.* 1995; Havilland *et al.* 1995). In this report, we demonstrate for the first time that cells of a TGW human neuroblastoma cell line express a C5aR-like molecule termed neuronal C5aR (nC5aR).

In order to analyse the function of C5aR, antisense homology box peptides were generated to modify the activity of the receptor. Antisense homology boxes (AHBs) in C5aR and its ligand C5a have been analysed recently (Baranyi, Campbell & Okada, 1996). AHBs are relatively short eight to fifteen amino acid long regions in proteins related in a sense–antisense fashion as if encoded by complementary DNA strands and have been found to be hydropathically complementary. The peptides corresponding to complementary AHBs are predicted to interact (Baranyi, Campbell, Ohshima, Fujimoto, Boros & Okada, 1995). One of the peptides representing an AHB in C5aR (amino acids 226–243, termed PR226) was synthesized and found to induce a C5aR-specific agonist effect when administered at

low doses or alternatively behaves as an antagonist at high doses and influences C5a binding in U-937 cells (Baranyi *et al.* 1996). We used the oligomeric (multiple antigenic peptide, MAP) form of the PR226 peptide ($Peptide_8$-K_4K_2K, termed PR226-MAP) to modulate C5aR-specific responses in cells since the MAP form of peptides has been shown to be more potent than the monomers of the respective peptides (Fassina, Consonni, Zetta & Cassani, 1992). Furthermore, the PR226MAP peptide was shown to be a potent inhibitor of C5aR, while at high doses it induced apoptotic cell death in U-937 cells (L. Baranyi, W. Campbell, T. Soji, I. Farkas, K. Baranji, N. Okada & H. Okada, unpublished data).

METHODS

Cell culturing

The culture media used for the TGW human neuroblastoma cells, the Ltk^- mouse fibroblast cells and Ltk^-/C5aR (Ltk^- cells transfected with human C5aR cDNA and expressing C5aR) cells (Watanabe, Kuraya, Kasukawa, Yanagisawa, Yanagisawa & Fujita, 1995) was RPMI-1640 (Nipro Company, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA). The cells were kept in an incubator at 37 °C in a 5% CO_2-containing atmosphere.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from TGW, Ltk^- and Ltk^-/C5aR cells by an acid guanidium thiocyanate–phenol–chloroform procedure (Chomczynski & Sacchi, 1987). The mRNA was then converted to cDNA by reverse transcriptase (Superscript II, Life Technologies, Grand Island, NY, USA). The cDNA was subjected to the polymerase chain reaction (PCR) analysis for human C5aR, using the sense primer 5′-ATGAACTCCTTCAATTATACC-3′ and the antisense primer 5′-TGGTGGAAAGTACTCCTCCCG-3′ (Gerard & 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 sulphate, 10 mM mercaptoethanol and 2 mM $MgCl_2$. The thermal cycle protocol used was denaturation at 94 °C for 3 min followed by cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1·5 min, and was terminated by a final 7 min incubation at 72 °C. Controls were run without reverse transcriptase or without template cDNA to ensure that results were not due to amplification of any genomic or contaminating DNA. Each reaction mixture (10 μl) was subject to electrophoresis through a 1·5% NuSieve 3:1 (FMC BioProducts, Rockland, ME, USA) agarose gel for 30 min and visualized by incubation for 10 min in a solution containing 100 ng ethidium bromide per millilitre.

Nick-end labelling of the apoptotic cells

The MEBSTAIN apoptosis kit (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) was used as suggested by the manufacturer except that instead of avidin–fluorescein isothiocyanate (FITC) the samples were incubated in streptavidin–peroxidase conjugate (1 : 1000 dilution; Southern Biotechnology Associates, Birmingham, AL, USA) and visualized with a solution containing 20 mg 3,3-diaminobenzidine (DAB), 6 ml 1% nickel–ammonium sulphate (Wako Fine Chemicals, Tokyo, Japan) and 120 μl 2% H_2O_2 diluted to 40 ml in Tris buffer (pH 7·6). The cells were treated with 10 μg ml^{-1} (600 nM) PR226-MAP extracellularly in Hank's balanced salt solution (HBSS), at 37 °C. For control cells the culture medium was changed to PR226-MAP-free HBSS. The control and 2 h treated cells were counterstained with Neutral Red.

c-fos immunostaining

Cells were treated with 10 μg ml^{-1} (600 nM) PR226-MAP extracellularly in HBSS at 37 °C for 30 min or 1 h. For control cells, the culture medium was also changed to PR226-MAP-free HBSS for 30 min or 1 h. After treatment the cells were fixed in 4% paraformaldehyde at 4 °C for 20 min and then exposed to 0·2% Triton X-100 for 30 min at room temperature (20–25 °C). For immunostaining, rabbit anti c-fos serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The secondary antibody was biotinylated goat, anti rabbit IgG (Vector Laboratories Inc.). Visualization of the immunoreactive sites was performed with streptavidin–peroxidase and DAB–nickel solution similar to the procedure used for nick-end labelling.

Patch clamp measurements

The TGW cells were voltage clamped at a holding potential of −70 mV 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; the headstage of the amplifier was fitted to an MHW-3 hydraulic manipulator produced by Narishige Inc., Tokyo, Japan; the cells were visualized on an Olympus IMT-2 inverted microscope. Software was run on an IBM compatible personal computer with a 90 MHz Pentium processor. The patch electrodes (o.d. = 1·5 mm, thin wall, Garner Co. USA) were pulled with a PP-83 puller and fire polished with a MF-83 microforge (Narishige Inc.). Resistance of the patch electrodes was 8–10 MΩ. The solutions were as follows: standard extracellular solution ((mM): Hepes, 10; NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; pH 7·34); standard intracellular pipette solution ((mM): Hepes, 10; KCl, 110; NaCl, 15; $CaCl_2$, 0·1; $MgCl_2$, 2; EGTA, 1; pH 7·25); Ca^{2+}-free extracellular solution ((mM): Hepes, 10; NaCl, 140; KCl, 5; $MgCl_2$, 10; EGTA, 2; pH 7·34); BAPTA-containing intracellular solution ((mM): Hepes, 10; KCl, 40; NaCl, 15; $CaCl_2$, 0·1; $MgCl_2$, 2; BAPTA-tetrapotassium salt, 20; pH 7·25). For pertussis toxin treatment, the cells were cultured overnight in complete RPMI-1640 (Nissui, Tokyo, Japan) + FBS medium containing 500 ng ml^{-1} pertussis toxin (Sigma) before patch clamp measurements. For C5a (human recombinant, Sigma) treatment the cells were exposed to 1 μg ml^{-1} C5a in 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^{-1}. The recordings started simultaneously with drug application. The second or third application of the peptide at the 6th or the 11th minute, respectively, is indicated by arrows in Fig. 4. The intracellularly applied PR226-MAP was diluted directly in 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 calcium-free phosphate-buffered saline (PBS) at room temperature. Following the last centrifugation, the pellet ($2–5 \times 10^7$ cells per tube) was resuspended in 200 μl calcium-free PBS and 1 μM Fluo-3 AM (1 mM stock in DMSO, Wako Fine Chemicals) was added to the suspension for each 10^6 cells. After loading the cells with fluorescent stain and washing 3 times in 14 ml calcium-free PBS, the cells were resuspended in ice-cold Phenol Red-free Hank's solution supplemented with 1% bovine serum albumin. The efficiency of Fluo-3 AM loading was tested by adding the Ca^{2+} ionophore A23187 (Calbiochem, La Jolla, CA, USA) to 1 ml aliquots

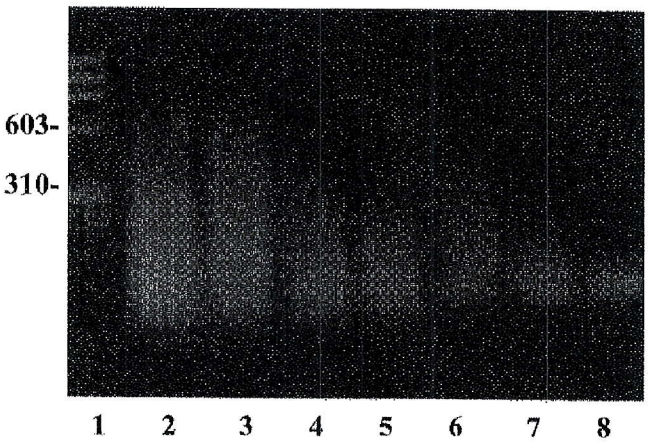

Figure 1. Expression of C5aR

Human C5a-receptor from TGW and in Ltk$^-$/C5aR cells is detected in a single band (552 bp) in lanes 5 and 7 by RT-PCR. Lane 1 contains molecular weight markers; lanes 2–4 are controls with no reverse transcriptase for the TGW, Ltk$^-$ and Ltk$^-$/C5aR cells, respectively; lanes 5–7 are with reverse transcriptase for the TGW, Ltk$^-$ and Ltk$^-$/C5aR cells, respectively; lane 8 is a control with no addition of template cDNA.

of the cell suspension (5×10^5 ml^{-1}). The increase in fluorescence due to changes in the intracellular calcium content was measured on a FACScan flow cytometer using Chronys software (200 events s^{-1}; Becton-Dickinson, Mountain View, CA, USA). For each measurement, baseline fluorescence activity was measured for 20 s, then 200 nM C5a was added and the time course of the change in fluorescence intensity was measured for an additional 700 s. To minimize non-specific binding of C5a, all solutions were prepared in Phenol Red-free Hank's solution containing 1% bovine serum albumin (BSA) and the solutions and cells were incubated in 96-well, flat-bottomed, precoated, polystyrene tissue culture plates (Falcon).

Statistical analysis

Analyses were performed with Student's *t* test using Microsoft Excel 4.0 (Microsoft Inc.) and GraphPad Prism 2.01 (GraphPad Software, Inc., San Diego, CA, USA) software. In order to calculate integrated areas of the currents recorded in the whole-cell clamp experiments the ClampFit unit of the pCLAMP 6.02 software (Axon Instruments) was used. Integrated areas were corrected for baseline shifts. In TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick-end labelling) staining, statistical analysis of apoptotic cells was performed in six different areas of three culture dishes for the control and every different peptide treatment condition.

RESULTS

Expression of nC5aR in TGW cells was demonstrated by the reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 1). The single band in lane 5 shows nC5aR mRNA expression in TGW cells. Using the same primers, C5aR mRNA was demonstrated in human C5aR transfected Ltk$^-$/C5aR cells (lane 7) while it was absent in Ltk$^-$ (lane 6) (Watanabe *et al.* 1995). The molecular size of transcripts is identical (552 bp). Without reverse transcriptase none of the lanes showed bands corresponding to C5aR mRNA (lanes 2–4), excluding the possibility of genomic DNA contamination.

Administration of an aphysiologically high concentration (200 nM) of C5a to TGW cells induced a weak and relatively slow, transient increase in the intracellular calcium level

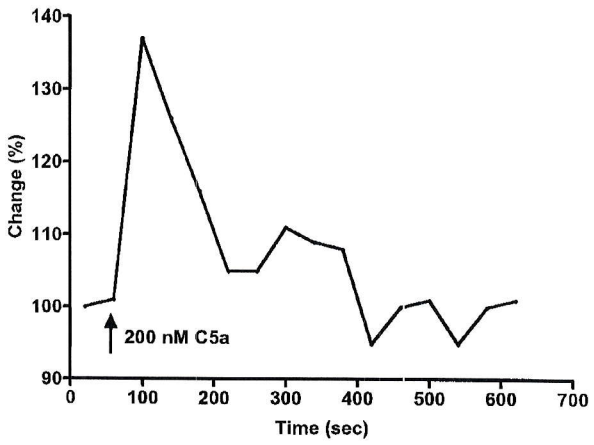

Figure 2. Ca5-induced oscillation of Ca^{2+} level in TGW cells

Flow cytometric measurement shows a transient increase (40 s) in the intracellular free calcium content in response to administration of 200 nM C5a.

(135–140% of the control level) that was measured by flow cytometry (FACS) (Fig. 2).

Spontaneous apoptosis of TGW cells, as demonstrated by the TUNEL method, was negligible in the control culture (0·61 ± 0·17% (mean ± S.E.M.) in 6 different areas of 3 culture dishes; Fig. 3*A*). Neutral Red counterstaining showed a well-preserved neuronal network. The cells possessed long processes and well-established cell-to-cell type connections. In the PR226-MAP peptide-treated culture, light brown staining was found in the nuclei of 7·4 ± 0·95% of the cells within 30 min, indicating rapid DNA fragmentation in a subpopulation of TGW cells ($P < 0·001$) (Fig. 3*B*). After 2 h of PR226-MAP treatment, the shape of the cells became rounded and the cells retracted most of their processes (Fig. 3*C*). The proportion of cells with apoptotic staining increased to 11·9 ± 2·75% ($P < 0·05$) and the nuclei of these cells were more intensely stained. At higher power, chromatin aggregation could be seen (Fig. 3*D*).

In control cultures, c-fos immunostaining showed a low intensity uniform staining (Fig. 3*F*). In cultures treated with peptide, weak nuclear staining was exhibited within 30 min. After 1 h, a more enhanced nuclear staining was evident (Fig. 3*E*) demonstrating an elevated level of nuclear c-fos immunoreactivity.

The ion current response of TGW cells to PR226-MAP treatment was measured by whole-cell clamp recording (Fig. 4*A–G*). In 1 min, upon treatment with PR226-MAP, repetitive, inward current pulses of 150–200 pA (mean ± S.E.M.; 192 ± 33 pA) appeared, as an early sign of apoptosis (Fig. 4*A*). Subsequent pulses have an even higher amplitude

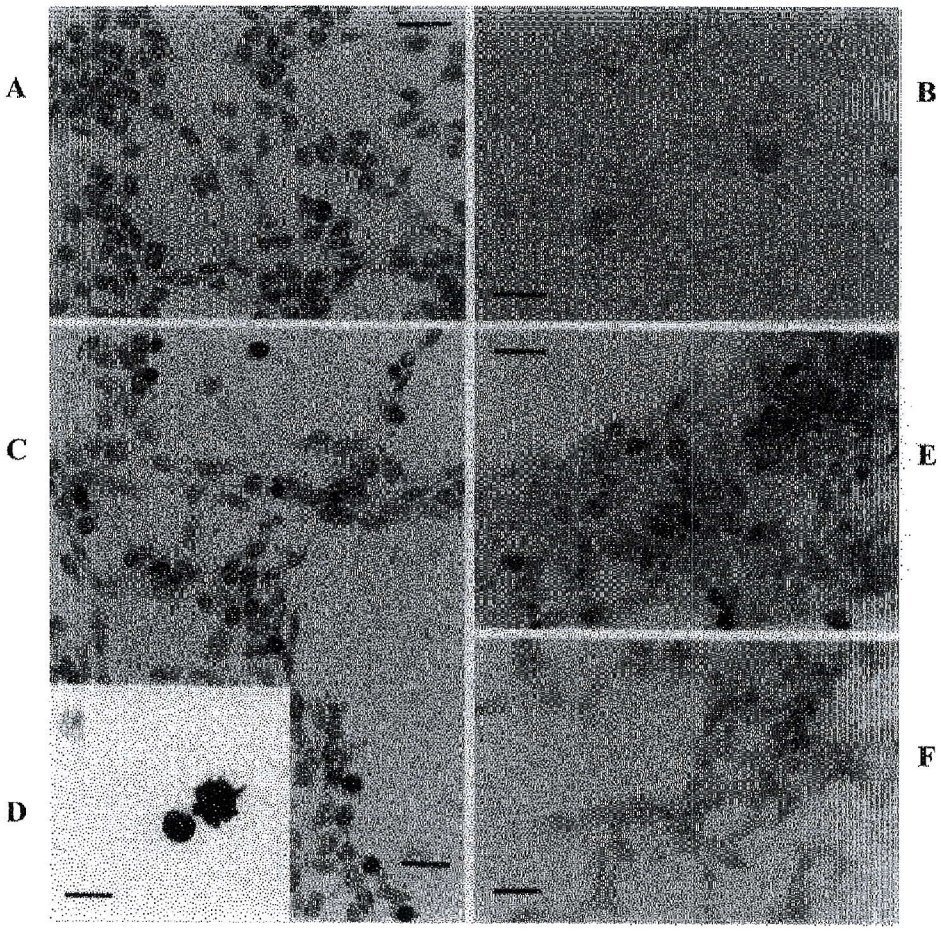

Figure 3. PR226-MAP-induced apoptosis

A, MEBSTAIN staining of control TGW cells demonstrates that about 0·6% of the cells show dark staining in their nuclei reflecting low spontaneous apoptosis rate. *B*, after 30 min, PR226-MAP treatment caused DNA fragmentation. Light brown staining of the nucleus in 7·4% of the cells demonstrates apoptosis. *C*, the DNA damage becomes more extensive after 2 h, marked by dark staining in the nuclei of 11·9% of the TGW cells. *D*, higher magnification shows chromatin aggregation (treatment for 2 h) in the nuclei of the cells. Scale bar, 10 μm. *E*, increased c-fos immunoreactivity can be found in the nuclei of the cells after 1 h PR226-MAP treatment. *F*, without peptide treatment, c-fos immunostaining of the control cells is weak and homogeneous. Scale bar, 25 μm in each panel if not stated otherwise.

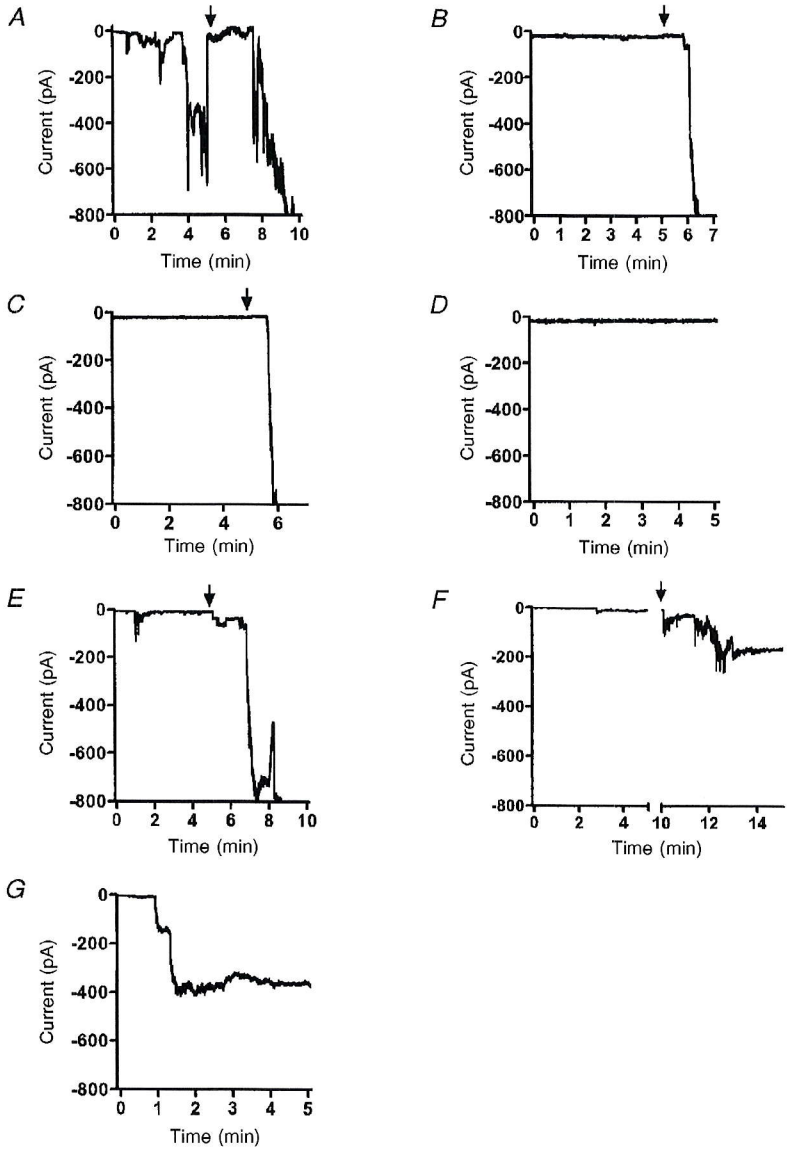

Figure 4. Electrophysiological changes during apoptosis

A, within 1 min PR226-MAP administration caused repetitive inward current pulses recorded by means of the whole-cell clamp method. The peak amplitude reached 150–200 pA. The usual width of the pulses was several seconds. The amplitude of the subsequent current pulses increased as PR226-MAP was administered. *B*, 0·2 mM $CoCl_2$ inhibited onset of the current pulses. *C*, administering PR226-MAP in a Ca^{2+}-free extracellular solution did not induce a repetitive current. Experiments with cobalt and in Ca^{2+}-free extracellular media show that the repetitive current evoked by PR226-MAP represents calcium influx. *D*, the intracellularly applied PR226-MAP did not evoke current or cell death even at a higher concentration (7·5 $\mu g\ ml^{-1}$), demonstrating that PR226-MAP requires an extracellular or properly oriented intramembrane structure to bind to. *E*, pertussis toxin pretreatment could attenuate the amplitude of the PR226-MAP-evoked repetitive current. This shows involvement of the nC5aR-associated pertussis toxin-sensitive signal transduction component (probably 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 the nC5aR or a nC5aR-linked structure. Nevertheless, in 15 min an irreversible steady current appeared (amplitude, 200–400 pA). *G*, in Ca^{2+}-free extracellular solution and using BAPTA-salt intracellularly to chelate the intracellular calcium, the repetitive inward pulses and high irreversible current did not occur. Rather, an irreversible steady current was evoked with an amplitude of 300–500 pA that flowed through non-selective ion channels. PR226-MAP was applied extracellularly (3·7 $\mu g\ ml^{-1}$) except in *D*, where it was applied intracellularly. The figure shows representative results obtained in 6–10 experiments. Untreated (PR226-MAP-negative) controls were also recorded by applying the extracellular solution by puff pipette (not shown).

(250–400 pA). The integrated area on the graph representing the net electric charge transferred through the membrane is -8982 ± 2906 pA s (mean ± S.E.M.) during the first 5 min. In 10 min, a high, irreversible inward current (HIC) exceeding 2 nA marked an irreversible change in the cells, which we consider to be an electrophysiological sign of cell death.

By adding a calcium channel blocker (0·2 mM cobalt chloride) to the extracellular solutions (Fig. 4*B*) or applying PR226-MAP in calcium-free extracellular solution (Fig. 4*C*) the onset of the repetitive current pulses can be blocked demonstrating that this current was a result of calcium ion influx. However, neither cobalt nor a calcium-free extracellular environment could inhibit PR226-MAP peptide-induced cell death, as indicated by the onset of HIC under these experimental conditions (Fig. 4*B* and *C*).

PR226-MAP was also applied intracellularly through the patch pipette. Although we applied PR226-MAP at a higher concentration (7·5 μg ml^{-1}) than extracellularly, the calcium current pulses were absent even after 20 min (Fig. 4*D*). This result indicates that PR226-MAP requires an extracellular (or properly oriented intramembrane) structure to exert its apoptotic effect.

In order to demonstrate that the PR226-MAP-induced response uses a signal transduction pathway involving G-proteins, we pretreated the TGW cells with pertussis toxin and then recorded its effect on the PR226-MAP-evoked current. Pertussis toxin pretreatment attenuated the calcium current (maximum current amplitude during the first 5 min: 105 ± 13 pA (mean ± S.E.M.); integrated area: -3101 ± 269 pA s) (Fig. 4*E*). Both the maximum current amplitude and integrated area were significantly lower than those measured without pertussis toxin pretreatment ($P < 0{\cdot}05$). Nevertheless, in 10 min the HIC appeared indicating that PR226-MAP is fatal, even under these conditions.

C5a (1 μg ml^{-1}, 120 nM) did not evoke an ion current in TGW cells. The only change observed by patch clamp measurements was attenuation of baseline 'noise' (not shown). Nevertheless, pretreatment of the cells with C5a abrogated the onset of calcium influx and only after a third application of the PR226-MAP peptide could this inhibition be overcome (Fig. 4*F*). In addition, C5a not only delayed the onset of the repetitive calcium current but inhibited onset of the HIC. Even successive administration of the PR226-MAP failed to evoke the HIC. Instead, the calcium current pulses were followed by a steady, irreversible, inward current with an amplitude of 200–400 pA. The reversal potential of this steady current was $1{\cdot}2 \pm 1{\cdot}4$ mV, indicating a non-specific ion flow through the cell membrane.

Our earlier experiments showed that the cobalt chloride and Ca^{2+}-free extracellular solution did not inhibit PR226-MAP-induced cell death, i.e. intracellular calcium stores might also be activated to supply calcium necessary for nucleases. Therefore PR226-MAP was administered in Ca^{2+}-free extracellular medium and the intracellular calcium was chelated by 20 mM BAPTA in the intracellular solution. Calcium depletion completely abolished the onset of the HIC. Nevertheless, instead of HIC, a steady inward current was recorded (Fig. 4*G*). Its amplitude (250–500 pA) and reversal potential ($0{\cdot}8 \pm 1{\cdot}2$ mV) were similar to the one observed in the case of PR226-MAP treatment in the presence of C5a (Fig. 4*F*). Substituting NaCl with an equimolar amount of *N*-methyl-D-glucamine in the Ca^{2+}-free extracellular solution did not affect it.

In order to analyse the role of energy-dependent transport in the apoptosis induced by PR226-MAP, 2 mM ATP was applied in the BAPTA-containing intracellular solution. However, ATP did not affect the ion current evoked by the peptide (not shown). To block ATP-dependent transport we pretreated the cells with 0·04% sodium azide for 30 min and then recorded the PR226-MAP-evoked ion currents in standard and Ca^{2+}-free extracellular solution. In the case of the Ca^{2+}-free extracellular environment, we used a BAPTA-containing intracellular solution. However, sodium azide also did not influence the onset of either calcium influx and HIC or that of the steady inward current (not shown).

DISCUSSION

Our RT-PCR experiment showed expression of a nC5aR in TGW neuroblastoma cells. Recently, polyclonal and monoclonal antibodies developed to C5aR also demonstrated the presence of the receptor in other neuroblastoma cell lines (although the staining was weaker than in the case of TGW cells). C5a receptor expression has been demonstrated in human neurons of CNS origin (H. Okada, M. Takahashi, N. Okada, N. Matsukawa, L. Baranyi & T. Yamamoto, unpublished data), and rat and murine neuronal cells. FACS measurements demonstrated that the expressed nC5a receptor is functionally active, since its respective ligand can evoke an increase in intracellular calcium levels in TGW cells. Nevertheless, the C5a concentration required to evoke this response was much higher (10- to 100-fold) than for other cell types (Gennaro, Pozzan & Romeo, 1984; Konteatis *et al.* 1994; Gasque *et al.* 1995) indicating that either a pharmacologically distinct C5a receptor subtype has been found or the TGW cells are lacking some accessory molecules required for efficient functioning of the receptor, i.e. the presence of an impaired nC5aR signal transduction system in TGW cells.

C5a can evoke different ion fluxes in different cell types. Ichinose *et al.* (1992) reported a potassium current response in mouse peritoneal macrophages, and a chloride ion efflux was found in C5aR-transfected oocytes (Murphy, Gallin & Tiffany, 1990). An increase in the intracellular free Ca^{2+} concentration, as a response to C5a in bovine neutrophil granulocytes, was also reported (Gennaro *et al.* 1984). Since

patch clamp measurements failed to show ion currents, intracellular calcium pools should be responsible for the increase in the cytosolic calcium level detected by FACS.

PR226-MAP administration induced rapid DNA fragmentation and elevation in nuclear c-fos immunostaining. Similar distribution of nuclear c-fos activation and that of the DNA fragmentation was found upon treatment of TGW cells with PR226-MAP. This suggests a possible correlation between activation of *c-fos* and the process of DNA fragmentation. This notion is further supported by the observation that *c-fos* activation can occur following cell injury (Trump & Berezesky, 1992).

Earlier data showed that the monomeric PR226 peptide can function as a C5aR agonist and influence C5a binding by the receptor in U-937 cells as well (Baranyi *et al.* 1996). The authors concluded that these effects may be mediated either by interaction with C5aR, or by modulation of the C5aR-associated signal transducing apparatus. C5aR is associated with several types of G-protein that are pertussis toxin sensitive or pertussis toxin insensitive (Amatruda *et al.* 1991; Gerard & Gerard, 1991; Rollins *et al.* 1991). We found that pertussis toxin pretreatment attenuated the calcium current evoked by PR226-MAP showing that at least one of the signals triggered by the peptide utilizes a pertussis toxin-sensitive component, probably G-proteins. However, onset of HIC suggests involvement of a pertussis toxin-insensitive signal transduction element also.

The nC5aR specificity of PR226-MAP-induced apoptotic cell death was demonstrated by the effect of C5a anaphylatoxin. C5a pretreatment delayed the onset of the calcium influx and abolished that of the HIC, suggesting a possible protective effect of C5a on these cells under these artificial conditions. Furthermore, this suggests that the observed apoptotic signal transduction pathway is coupled directly to nC5aR or to a nC5aR-associated signal transduction pathway. One possible explanation for the effect of C5a pretreatment is that it desensitizes nC5aR, and internalization of nC5aR may also decrease the number of nC5aRs accessible for the PR226-MAP (Rother & Till, 1988). Another possibility is provided by the observation that PR226-MAP can integrate into membranes spontaneously (L. Baranyi, W. Campbell, T. Soji, I. Farkas, K. Baranji, N. Okada & H. Okada, unpublished data). Upon insertion into the cell membrane, it may interact with a molecule that is involved in the nC5aR-associated signal transduction pathway, while C5a binds directly to the receptor. In this case PR226-MAP and the C5a-induced signals could interfere with each other, modifying the apoptotic signal, resulting in a state represented by the onset of the steady current.

For apoptosis to occur, free intracellular calcium is required to activate endonucleases that are responsible for DNA fragmentation (Orrenius, McConkey, Bellomo & Nicotera, 1989; Fawthrop, Boobis & Davies, 1991; Mah, Zhong, Liu, Roghani, Edwards & Bredesen, 1993; Kroemer & Martinez-A, 1995; Kroemer, Petit, Zamzani, Vayssiere & Mignotte, 1995), although an elevation in calcium content is not always a strict prerequisite for apoptosis (Lennon, Kilfeather, Hallett, Campbell & Cotter, 1992). Increase in the intracellular free calcium level also correlates with *c-fos* activation following cell injury (reviewed by Trump & Berezesky, 1992). Both the extracellular medium and intracellular calcium stores can serve as a calcium source to activate endonucleases and mediate DNA fragmentation (Nagata & Golstein, 1995; Oshimi & Miyazaki, 1995). Calcium depletion abrogated onset of HIC showing that in our experimental system the supply of free calcium is essential for PR226-MAP-induced rapid cell death.

The steady current evoked by PR226-MAP in the calcium-depleted environment was not influenced when NaCl was removed from the extracellular solution. This observation and a reversal potential close to 0 mV excludes the transport of ions such as calcium, sodium or chloride through the plasma membrane. Based on this observation, it is thought that this current represents a steady transport of negatively charged molecules from the cell interior into the intercellular space. A similar current has been reported by Furukawa *et al.* (1994) in the case of β-amyloid-treated rat cortical neurons and was considered as a sign of developing neurodegeneration. Nevertheless, onset of the steady current is earlier in the calcium-depleted environment than in the case of C5a pretreatment.

Mitochondrial changes occurring in apoptosis suggest that ATP depletion might be involved in the process of cell death (reviewed by Kroemer *et al.* 1995). This depletion abrogates ATP-dependent ion pumps in the cell membrane and/or in the endoplasmic reticulum. Nevertheless, PR226-MAP-induced cell death is mediated neither by an excess nor depletion of ATP since intracellular application of ATP or pretreatment with sodium azide did not affect the ion currents evoked by PR226-MAP. From these results we concluded that the early phase of the PR226-MAP-induced apoptosis does not require ATP and that the steady inward current represents passive transport of ions, probably by opening of non-selective ion channels.

The importance of our results, that cells of neuronal origin express a C5aR-like receptor, and that the associated signal transduction system is linked to an apoptotic pathway may be enhanced by the data of Rogers *et al.* (1992), that the classical pathway of the complement cascade is activated in cerebral amyloid deposits found in Alzheimer's disease (reviewed in Kalaria, 1993) and by the results of Pasinetti (1996) that neurons of the hippocampal formation and parietal cortex express C5aR. Our data may provide a link between neurodegeneration in Alzheimer's disease and complement activation. Moreover, the involvement of apoptotic cell death in several neurodegenerative disorders including Alzheimer's disease and Parkinson's disease has

already been suggested (Thompson, 1995). The apoptotic changes that occur after β-amyloid treatment of cultured neurons (Loo, Copani, Pike, Whittemore, Walencewicz & Cotman, 1993; Takashima, Noguchi, Sato, Hoshino & Imahori, 1993) or induced by a mutant amyloid precursor protein, utilizing a G-protein-associated pathway (Yamatsuji *et al.* 1996), also support this suggestion. Moreover, our unpublished data indicate that not only the C5aR fragment but another peptide, representing an antisense homology box in C5a, can abnormally activate nC5aR and induce apoptotic cell death (L. Baranyi, W. Campbell, T. Soji, I. Farkas, K. Baranji, N. Okada & H. Okada, manuscript in preparation). Since C5aR is expressed in neurons of the CNS (Pasinetti, 1996), we can predict a higher vulnerability of C5aR-expressing neurons in pathological situations associated with complement activation associated with improper protein metabolism in the central nervous system due to a possible abnormal activation of cells via nC5aR or its associated signal transduction pathway.

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