REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

FORM P.8
(to be lodged in duplicate)

A&A Ref: **143058**

PUBLICATION PARTICULARS AND ABSTRACT
(Section 32(3)(a) - Regulations 22(1)(g) and 31)

21	01	PATENT APPLICATION NO	22	LODGING DATE	43	ACCEPTANCE DATE
		2001/0656		**23 January 2001**		~~22.8.2001~~ 25.4.05

51	INTERNATIONAL CLASSIFICATION	NOT FOR PUBLICATION
	A61K A61P C07K	CLASSIFIED BY: **ISA**

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EARLIEST PRIORITY CLAIMED	COUNTRY		NUMBER		DATE	
	33	**EPO (BE)**	31	**98870180.1**	32	**14 August 1998**

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

54	TITLE OF INVENTION

TNF-derived peptides for use in treating oedema

57	ABSTRACT (NOT MORE THAN 150 WORDS)

NUMBER OF SHEETS 32

The sheet(s) containing the abstract is/are attached.

If no classification is furnished, Form P.9 should accompany this form.
~~The figure of the drawing to which the abstract refers is attached.~~

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The present invention relates to the finding that peptides derived from a specific domain of tumor necrosis factor-alpha (TNF-α) can efficiently be used to treat oedema. More specifically, the present invention relates to the usage of peptides derived from the region of human TNF-α from Ser^{100} to Glu^{116} to treat pulmonary oedema. Moreover, the present invention concerns a circularized peptide having amino acid sequence CGQRETPEGAEAKPWYC which is very efficient in inducing oedema resorption.

TNF-DERIVED PEPTIDES FOR USE IN TREATING OEDEMA

FIELD OF THE INVENTION

The present invention is based on the finding that peptides derived from a specific domain of tumor necrosis factor-alpha (TNF-α) can efficiently be used to treat oedema. More specifically, the present invention relates to the usage of peptides derived from the region of human TNF-α from Ser^{100} to Glu^{116} to treat pulmonary oedema. For example, the circularized peptide having amino acid sequence CGQRETPEGAEAKPWYC is shown to be very efficient in inducing oedema resorption.

BACKGROUND OF THE INVENTION

Pulmonary transplantation is shown to be successful in the treatment of patients with end-stage pulmonary disease. However, pulmonary oedema or edema (both terms can be used interchangeably) following reperfusion of the transplant is a major clinical problem for which no efficient drug exists at this moment. In addition, recent evidence indicates that the endothelium plays an essential role in regulating the dynamic interaction between pulmonary vasodilatation and vasoconstriction and is a major target during ischemia/reperfusion and acute respiratory distress syndrome (ARDS)-related lung injury. Thus, given that pulmonary edema often results in lung transplant rejection and that there is a persistent shortage of lungs available for transplantation, there is an urgent need to efficiently prevent or treat pulmonary edema.

During ischemia and reperfusion (I/R), a typical induction of inflammatory cytokines like tumor necrosis factor-alpha (TNF) occurs. TNF is a pleiotropic cytokine, mainly produced by activated macrophages, that is synthesized as a transmembrane molecule that can be released by metalloproteinases from the cell surface into the circulation (Gearing *et al.*, 1994). TNF has been shown to bind to at least two types of membrane-bound receptors, TNF receptor 1 (55 kD) and TNF receptor 2 (75 kD), that are expressed on most somatic cells, with the exception of erythrocytes and unstimulated T lymphocytes. TNF can be considered as a two-edged sword: indeed, when overproduced, TNF has been shown to be implicated in the pathology of various infectious diseases, such as LPS-induced sepsis (Beutler *et al.*, 1985), cerebral malaria (Grau *et al.*, 1987), as well as treatment-associated mortality in African trypanosomiasis (Lucas *et al.*, 1993). In contrast, TNF was shown to be one of the most efficient protective agents against cecal ligation and puncture-induced septic peritonitis in mice and rats (Echtenacher *et al.*, 1990, Alexander *et al.*, 1991; Lucas *et al.*,

1997) and to be implicated in host defense during pneumococcal pneumonia in mice (van der Poll *et al.*, 1997). Moreover, mice deficient in TNF receptor 1 were shown to be significantly more sensitive to *Listeria monocytogenes* (Rothe *et al.*, 1993; Pfeffer *et al.*, 1993) and *Mycobacterium tuberculosis* infection (Flynn *et al.*, 1995) as well as against fungal (Steinshamn *et al.*, 1996) and *Toxoplasma* infections (Deckert-Schluter *et al.*, 1998). Therefore, it becomes clear that apart from its detrimental effects during overproduction or during prolonged chronic secretion, TNF is also one of the most potent protective agents against infections by various pathogens. In this regard, peptides derived from TNF have been suggested to be used as treatment against disease (DE 3841759 to Böhm et al.)

Apart from exerting a plethora of effects mediated by the activation of its two types of receptors (TNF receptor 1, 55kD, and TNF receptor 2, 75 kD), TNF can also mediate receptor-independent activities. The tip domain of TNF is located on the top of its bell-shaped structure and is spatially distinct from its receptor binding sites, that are localized at the base of the trimeric molecule (Lucas *et al.*, 1994). This domain has lectin-like affinity for specific oligosaccharides, such as trimannose and diacetylchitobiose. Both TNF and the tip peptide of TNF are capable of mediating a trypanolytic activity by interfering with the lysosomal integrity of the trypanosome, a pH-dependent effect probably involving the insertion of TNF into the lysosomal membrane (Magez *et al.*, 1997). Moreover, mutants of the tip peptide in which three critical amino acids (T(105); E(107); E(110)) were replaced by A, were completely unable to mediate this activity (Lucas *et al.*, 1994). A mouse TNF (mTNF) triple mutant, T105A-E107A-E110A (referred to hereafter as triple mTNF), lacks the trypanolytic and lectin-like affinity to oligosaccharides as compared to wild type TNF. The triple mTNF has significantly reduced systemic toxicity as compared to wild-type mTNF *in vivo*, but retains its peritonitis-protective effect in a murine model (Lucas *et al.*, 1997).

Another receptor-independent activity of TNF is its membrane-inserting and sodium channel forming capacity (Baldwin *et al.* 1996). Indeed, others have shown that TNF forms a Na^+-channel in an artificial lipid bilayer model, an activity that is pH-dependent, probably because it requires the «cracking» of the trimer, thus exposing hydrophobic residues to the membrane (Kagan *et al.*, 1992).

Recent observations have indicated that instillation of anti-TNF-neutralizing antibody into the lungs of rats 5 min before bacterial infection inhibits the increase in alveolar liquid clearance, which is known to be driven by a change in intracellular sodium content in the alveolar epithelial cells. Moreover, instillation of TNF in normal rats increases alveolar liquid clearance by 43% over 1 hour (Rezaiguia *et al.*, 1997). Although the latter findings indicate that TNF might be used to induce alveolar liquid clearance, wild type TNF cannot be used therapeutically due to its high systemic

toxicity. The present invention relates to the usage of a selected group of TNF-derived peptides which can, to our surprise, efficiently be used to induce edema resorption and which have, compared to wild type TNF, lost systemic toxicity.

AIMS OF THE INVENTION

It is clear that there is an urgent need to efficiently prevent or treat pulmonary edema. Although some data demonstrate that TNF might be involved in oedema resorption, it is clear that this pleiotropic and potentially toxic molecule can not be used to treat oedema.

In this repect, the present invention aims at providing a non-toxic molecule with the same oedema resorption-inducing capacity as TNF. More specifically, the present invention aims at providing non-toxic peptides derived from TNF which can be used to prevent or treat oedema. Moreover, the present invention aims at providing a pharmaceutical composition comprising TNF-derived peptides which induce oedema resorption. In essence, the present invention aims at providing a new medical use of the TNF-derived, trypanocidal peptides as described by Lucas *et al.* (1994) and fragments and variants thereof.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: **(A)** Current-voltage relationship in murine lung microvascular endothelial cells, preincubation for 30 min with wt mTNF (100 ng/ml) or NES buffer at pH 6 and at pH 7.3. The values represent the means of ≥ 5 cells $\pm$ SEM (*: $P \leq 0.05$). **(B)** Characteristic current traces of a lung MVEC pretreated with medium (top) or with 100 ng/ml of TNF (bottom) at pH 6.0.

Figure 2: Current-voltage relationship in resident peritoneal macrophages isolated from **(A)** control and **(B)** TNFR $\frac{1}{2}^{0/0}$ C57BL/6 mice. cells were pretreated for 30 min with medium, wt mTNF (100 ng/ml) or Ltip peptide (100µg/ml). The values indicate the means of ≥ 5 cells $\pm$ SEM (*: $P \leq 0.05$).

Figure 3: Effect of amiloride (100 µM), added for 30 min during the preincubation step, on wt mTNF-induced increase in membrane conductance in MVEC. Comparison of the effect of triple mTNF (100 ng/ml) and wt mTNF (100ng/ml), upon 30 min preincubation with lung MVEC. Values

indicate the means of ≥ 5 cells ± SEM (*: P ≤ 0.05).

Figure 4: (A) Effect of Ltip (100 µg/ml) *versus* controls in CBA lung MVEC at pH 6 and pH 7.3. (B) Comparison of the effect of 30 min preincubation of MVEC with Ltip peptide, mutTip peptide, and scramblTip peptide at pH 6. Effect of amiloride (100 µM) added during the preincubation, on Ltip peptide-induced increase in membrane conductance in MVEC. Values indicate the means of ≥ 5 cells ± SEM (*: P ≤ 0.05).

Figure 5: Effect of mTNF tip peptide (1mg/lung) on lung weight change (in g) during an isolated lung perfusion experiment lasting 150 min.

Figure 6: Effect of wild type mTNF (●,1µg/lung) or mTNF tip peptide (▲,1mg/lung) *versus* controls [○, NaCl] on lung weight change (in % versus baseline lung weight at 30 min) during isolated lung perfusion experiments after 150 min. Each symbol [○, ● or ▲] represents one lung.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention relates to the use of a peptide comprising a chain of 7 to 17, preferably a chain of 11 to 16, more preferably a chain of 13 to 15 and most preferably a chain of 14 contiguous amino acids derived from the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115} for the manufacture of a medicament for treating oedema. More specifically the present invention relates to the use of a peptide as described above wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY and PKDTPEGAELKPWY as described by Lucas *et al.* (1994). The latter sequences are given in the well-known one-letter code for amino acids (the three-letter code is sometimes used further).

The term "peptide" refers to a polymer of amino acids (aa) derived from the trypanolytic TNF domain having lectin-like affinity as described by Lucas *et al.* (1994). Moreover, the latter term relates to a polymer of 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 contiguous amino acids derived from

the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115}. The latter TNF regions also refer to the regions shown in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987). However, it should be clear that the region of human TNF-α from Ser^{100} to Glu^{116} is identical to human TNF-α from Ser^{99} to Glu^{116} in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987) and that the region of mouse TNF-α from Ser^{99} to Glu^{115} is identical to mouse TNF-α from Ser^{98} to Glu^{115} in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987). The term "peptide" more specifically relates to a peptide comprising the hexamer TPEGAE of the latter TNF regions or any peptide comprising the corresponding amino acids T, E and E of the latter hexamer which were shown to be three critical amino acids by Lucas *et al.* (1994). It should be clear that the present invention relates to any peptide derived from the latter TNF regions and does not exclude post-translational modifications of the peptides such as glycosylation, acetylation, phosphorylation, modifications with fatty acids and the like. Included within the present invention are, for example, peptides containing one or more analogues of an aa (including unnatural aa's), peptides with substituted linkages, mutated versions or natural sequence variations of the peptides, peptides containing disulfide bounds between cysteine residues, as well as other modifications known in the art. The peptides of the present invention are also defined functionally, that is, the present invention relates to any peptide which can be used to treat oedema or which can be used for the manufacture of a medicament for treating oedema. In essence, the present invention relates to any molecule, obtained by any method known in the art, with the same or very similar characteristics as the trypanolytic peptides defined by Lucas *et al.* (1994).

The peptides of the present invention can be prepared by any method known in the art such as classical chemical synthesis, as described by Houbenweyl (1974) and Atherton & Shepard (1989), or by means of recombinant DNA techniques as described by Maniatis et al. (1982) and, more specifically, by Lucas et al. (1994).

The term oedema (or edema) relates to any abnormal excess accumulation of (serous) fluid in connective tissue or in a serous cavity. In particular, the latter term relates to pulmonary oedema (see also *Examples* section).

Furthermore, the present invention concerns the use of a peptide as described above wherein said peptide is circularized. More specifically, the present invention relates to the use of a peptide as described above, wherein said peptide is circularized by replacing the NH_2- and COOH-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines. In this regard, the present invention concerns the use of a peptide as described above wherein said

circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAELKPWYC as described by Lucas *et al.* (1994).

The present invention finally relates to a pharmaceutical composition for treating oedema comprising a peptide as described above. The terms "a pharmaceutical composition for treating oedema" relates to any composition comprising a peptide as defined above which prevents, ameliorates or cures oedema, in particular pulmonary oedema. More specifically, the terms "a pharmaceutical composition for treating oedema" or "a drug or medicament for treating oedema" (both terms can be used interchangeably) relate to a composition comprising a peptide as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat oedema. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. The "medicament" may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parenteral administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the peptide of the present invention is given at a dose between 1 μg/kg and 10 mg/kg, more preferably between 10 μg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used. If so, the medicament may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and can not be construed as to restrict the invention in any way.

EXAMPLES

Example 1:

MATERIAL AND METHODS

Animals, cells and reagents. Male CBA/J or C57BL/6 mice, as well as male TNFR ½ $^{0/0}$ C57BL/6 mice deficient in TNF receptors (Bruce et al., 1996) provided by H. Bluethmann, F. Hoffmann-La Roche, Basel, Switzerland, were used at the age of 8-10 weeks. Their care was in accordance with institutional guidelines. Lung microvascular endothelial cells were isolated from CBA/J mice and characterized as described (Jackson et al., 1990) using magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway), covalently bound to a purified rat-anti-mouse PECAM-1 monoclonal antibody (donated by B. Imhof, University of Geneva). Microvascular lung endothelial cells were resuspended in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, 20% FCS, 40 U/ml heparin and 100 mg/ml endothelial cell growth supplement (Brunschwig Chemie, Basel, Switzerland). For patch clamp experiments, cells were plated onto 35x10 mm easy grip Petri dishes (Beckton Dickinson, Plymouth, UK), pre-coated with 0.2% gelatin (Sigma, Buchs, Switzerland). Resident peritoneal macrophages, isolated in ice cold RPMI containing antibiotics and 10 U/ml Heparin, were left to adhere onto 35x10 mm easy grip Petri dishes for 4 h, after which the non-adherent cells were removed. Cells were grown in RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and 10 % fetal bovine serum (all from Gibco). For patch clamp, the macrophages were used 24 h after isolation.

TNF and peptides. *E.coli*-derived recombinant murine TNF (further referred as TNF in the text) and an *E.coli*-derived recombinant (T104A-E106A-E109A) triple TNF mutant (mutTNF) were synthesized as described elsewhere (Lucas et al., 1997). TNF-derived peptides were synthesized with the use of Fmoc-a-amino group protection (Fields et al. 1990), and purified with a C18 reversed-phase high-performance liquid chromatography column.

The following TNF-derived peptides were synthesized:

Long tip peptide 99-115 (LTip) GG-CGPKDTPEGAELKPWYC

Mutated tip peptide 99-115 (mutTip)	GG-CGPKDAPAGAALKPWYC
Scrambled tip peptide (scamblTip)	GG-CGTKPWELGPDEKPAYC
Short tip peptide (STip)	CTPEGAEC

To theoretically retain the original TNF conformation as much as possible, Ltip, MutTip and ScamblTip peptides were circularized. Ser^{99} of the TNF sequence was replaced by Cys, and Cys^{100} by Gly so that the disulfide bridge could be formed between Cys^{99} and Cys^{115} in the peptides. The STip peptide could not be circularized. The peptides were NH_2-biotinylated.

Electrophysiology. Cells were pretreated for 30 min with TNF, mutTNF and tip peptides at 37° C in a buffer consisting of 145 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM D-glucose, and 10 mM Hepes, and pH-adjusted with NaOH to required value. Cells were then washed with the same buffer pH-adjusted at 7.3, and experiments were performed using the tight-seal, whole-cell recording technique. Currents were recorded with an Axopatch-200A amplifier (Axon Instrument Inc, Foster City, CA, USA), low pass-filtered at 1 kHz. Digitalization and off-line analysis was performed using the WCP program (J. Dempster, Strathclyde Electrophysiology Software, Glasgow, UK). Patch pipettes were pulled from borosilicate glass and fire polished to have an open resistance of 3-5 MW with an internal solution containing 130 mM CsCl, 2 mM MgCl2, 10 mM EGTA, 20 mM TEA-Cl, 10 mM D-glucose, 10 mM Hepes, pH-adjusted to 7.3 with CsOH. Series resistances were kept under 10 MW. Capacitance and series resistance compensation were applied and set to 70%. All experiments were done at room temperature. Results are given as mean ± SEM, unless otherwise indicated. Analysis of variance was performed on currents and membrane conductance values, with post-hoc Dunn-Bonferroni test for significance of differences observed between two groups. A P value of 0.05 was considered significant.

Tryptophan fluorescence. Fluorescence measurements were made with a PTI spectrofluorimeter. The excitation wavelength was 295 nm and slit widths were 5 nm and 2.5 nm for excitation and emission respectively. For each recorded spectrum, the Raman scatter contribution was removed by subtraction of a buffer blank. All buffers contained 150 mM NaCl, and 20 mM of N-[2-morpholino] ethane-sulfonic acid (MES) buffer at the desired pH. The

samples were allowed to incubate for 1h 30 at the desired pH before measuring the emission spectrum. The wild type and mutant TNF concentrations were 6 μg/ml.

Preparation of liposomes. Large unilamellar liposomes were prepared by reverse phase evaporation as previsouly described (Vecsey-Semjen et al., 1996). Liposomes were prepared of either 100% egg phosphatidylglycerol (EPG) or a mixture of EPC and EPG (1:1 W/W) in a buffer containing 100 mM KCl, 20 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid] (HEPES), pH 7.4 and 1.5 mg/ml of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ).

Choride efflux measurements. All fluorescence experiments were carried out using a PTI spectrofluorometer equipped with a thermostated cell holder (37° C). The dye was excited at 350 nm and emission was recorded at 422 nm, both excitation and emission band widths were set to 5 nm. Liposomes were diluted to a final concentration of 50 μg/ml in a solution containing 100 mM KNO3 and 20 mM MES pH 6.1 or 20 mM HEPES, pH 7.4. Wild type and mutant TNF were added to a final concentration of 3 μg/ml.

Proinflammatory activity of TNFs and TNF tip peptides. Proinflammatory activity of TNF and derived peptides was tested using a bioassay measuring their capacity to induce the surface upregulation of intercellular adhesion molecule (ICAM)-1 in alveolar type II-like epithelial A549 (Pugin et al., 1996). Briefly, A549 cells were plated at confluence in a microtiter plate, and incubated with the various concentrations of TNF, mutTNF, and peptides for 18 hrs at 37° C. Surface upregulation of ICAM-1 was detected by direct ELISA on cells using a first anti-ICAM-1 antibody (R&D systems, Abdington, UK), a second donkey-anti mouse IgG-peroxidase conjugated antibody (Jackson), revealed by o-phenylenediamine (Sigma), and stopped by H2SO4. Optical densities (O.D.) were read at 490 nm, with subtraction of 620 nm O.D. readings.

RESULTS

Example 1.1: **Effect of TNF on membrane conductance in murine cells**

We first investigated whether TNF modified the whole cell current in primary murine cells. A 30 min preincubation of resident peritoneal macrophages and lung microvascular endothelial cells with 100 ng/ml of TNF resulted in a significant increase in outward and, to a lesser extent, inward current in the case of microvascular endothelial cells, as measured by means of whole-cell patch clamp, as compared to cells unexposed to TNF (endothelial cells, Fig. 1A; and macrophages, Fig. 2). A reduction in preincubation time (down to 5 min) or in dose of TNF (down to 10 ng/ml) gave similar results (data not shown). This effect required acidic preincubation conditions, since it did not occur when the preincubation was performed at pH 7.3 (Fig. 1). The conductance induced by TNF was voltage-independent and showed a reversal potential of about 0 mV in the case of endothelial cells. In order to investigate whether the ion current increase induced by TNF was TNF receptor-dependent, resident peritoneal macrophages were isolated from mice deficient in both TNF receptor-1 and -2 ($TNFR1/2^{0/0}$), and tested in the whole cell patch clamp assay. TNF induced a voltage-dependent current in cells lacking TNF receptors (Fig. 2B). This critical experiment showed that the TNF-induced conductance in mammalian cells occurred in a TNF-receptor independent manner. These results also indicated that the TNF-induced current is not cell type specific.

Since the lectin-like domain of TNF is spatially and functionally distinct from its receptor binding sites, we next investigated whether it was implicated in the observed ion channel activating effect of TNF in mammalian cells. Therefore, the effect of a TNF mutant (mutTNF), in which the three critical residues for the lectin-like activity of TNF were replaced by an alanin, was compared with TNF in endothelial cells. As shown in Fig. 3, mutTNF completely lacked the conductance activating effect of TNF, even at a 100-fold higher dose (1 µg/ml mutTNF *versus* 10 ng/ml of TNF, data not shown). In contrast, the native and the mutated TNF molecules showed similar potencies in the induction of ICAM-1 in A549 epithelial cells (Fig. 4). This indicated that despite a conserved TNF receptor-mediated activity, mutTNF was unable to increase ion permeability. In order to test the hypothesis that TNF gated a sodium channel, we performed additional experiments in the presence of amiloride, an epithelial sodium channel blocker. One hundred µM amiloride added during the pretreatment at pH 6.0 abrogated the TNF-induced increase in conductance (Fig. 3).

Example 1.2: **The tip domain of TNF mediates its membrane conductance increasing effect**

Since the tip domain of TNF seemed to be critical for its activation of ion permeability, we next tested whether a peptide mimicking this region was sufficient for increasing membrane conductance, as observed with native TNF. Treatment of endothelial cells and macrophages with the 17 amino acid (aa) circularized long tip peptide (Ltip peptide), that mimics the lectin-like domain of TNF, resulted at acidic pH in increased outward, and inward currents in the case of microvascular endothelial cells. In contrast to TNF, the effect persisted at neutral pH, although less pronounced (Fig. 2A and 4A). Similarly to TNF, the effect was blocked by 100 μM amiloride (Fig. 4B). A mutant (T104A-E106A-E109A) 17 aa circularized peptide (mutTip peptide) and a 17 aa circularized peptide containing the same aa as Ltip peptide in a random sequence (scramblTip peptide) were inactive with regard to the ion channel activity (Fig. 4B). These results indicated that the tip domain of TNF was mediating its membrane conductance increasing activity, and confirmed that residues T104, E106 and E109 were essential for this effect. Ltip peptide was also active in cells deficient in both TNFR-1 and -2 receptors (Fig. 2B). However, a short tip hexapeptide containing the 3 critical aa failed to induce a voltage-dependent current in microvascular endothelial cells (data not shown), suggesting that this peptide was below the minimal structure carrying the ion channel effect. Importantly, none of the peptides induced ICAM-1 in A549 cells, indicating that they lacked a TNF receptor-mediated activity.

Example 1.3: **Native and mutated TNF undergo partial unfolding at acidic pH**

It was previously shown that TNF interacted with lipids in a pH dependent manner and that this membrane interaction correlated with partial unfolding of the protein (Hlodan et al., 1994) (Baldwin et al., (1996). We therefore investigated whether the lack of activity of mutTNF on lung MVEC at acidic pH was due to its inability to undergo partial unfolding and to interact with membranes. The conformation of mutTNF at various pH values was followed by measuring the intrinsic tryptophan fluorescence of the molecule. The fluorescence intensity dropped upon acidification of the medium, and the maximum emission underwent a red shift from 318 nm at pH 6 to 339 nm at pH 4.6. These observations indicated that the initially buried tryptophan residues became exposed to the solvent. The protein was however not fully unfolded since the

spectrum at pH 4.6 was not as red shifted as that of mutTNF in 6 M GuHCl. These results show that mutTNF was able to undergo acidic unfolding. Acidic unfolding of mutTNF was in fact more rapid and slightly more extended than that of wild type TNF.

Example 1.4: **Both native and mutated TNF interact with membranes at acidic pH**

We next investigated whether mutTNF was able to interact with membranes at acidic pH by following its ability to induce chloride leakage from liposomes containing the chloride sensitive dye SPQ. These experiments were performed using liposomes containing 100 % egg phosphatidyl glycerol (EPG). Native TNF induced chloride efflux at pH 6.1. MutTNF was still folded at pH 6; we have however previously shown that the pH at the surface of 100% EPG vesicles was far lower than that of the bulk pH, and more specifically that at a bulk pH of 6, the surface pH was 4.35. Therefore, mutTNF is likely to have undergone partial unfolding at the surface of the EPG vesicles. The effect of mutTNF on SPQ fluorescence was even more pronounced than that of wild type TNF, in agreement with the fact that its acidic unfolding was more rapid than that of wild type TNF. As previously observed for native TNF (Baldwin et al., 1996), mutTNF did not interact with membranes at neutral pH.

In order to investigate whether chloride efflux was due to membrane binding or membrane insertion of TNF, we have analyzed whether brominated lipids were able to quench the intrinsic fluorescence of TNF and mutTNF upon membrane interaction. Brominated lipids have been useful in determining the topology of membrane proteins (Bolen et al., 1990) (Markello et al., 1985) as well as studying the membrane interaction of pore-forming toxins (Gonzalez-Manas et al., 1992) (Van der Goot et al., 1991) (Vecsey-Semjen et al., 1997). TNF contains two tryptophan residues, one at the top of the receptor binding domain and one at the top of the so called tip domain. If the tip of the TNF trimer were to insert into the lipid bilayer, the fluorescence of Trp-113 should be quenched upon insertion into liposomes composed of dioleoylphosphatidylglycerol that had bromines attached at positions 9 and 10 of the acyl chains. We have indeed previously observed that tryptophans located at the boundary between the lipid head groups and the acyl chains were succeptible to bromide quenching. We were however unable to see any fluorescence quenching when adding either TNF or mutTNF at acidic pH to vesicles formed of brominated lipids.

The observations described above show that mutTNF undergoes partial unfolding at acidic pH and is then able to interact with membranes. The lack of quenching by brominated lipids however

suggests that chloride release was due to binding of the partially unfolded TNF molecules to the lipid bilayer rather then to membrane insertion of the molecule.

We next tested whether the TNF tip peptides were able to induce chloride efflux from SPQ containing vesicles and whether tryptophan quenching could be observed upon interaction with brominated lipids. Liposomes containing either 100 % neutral lipids, 100 % acidic lipids or a 1:1 mixture of both were used. For none of the lipid compositions and for peptide concentrations up to 300 µg/ml could we observe any change in SPQ fluorescence nor any quenching by brominated lipids, and this with all 4 peptides. These experiments suggested that the LTip as well as the modified tip peptides were unable to interact with membranes.

Example 2:

Isolated Lung Perfusion Experiments

Lungs of female Whistar rats weighing about 300 g were isolated as described in DeCampos *et al.* (1993). The lungs were injected intratracheally with either 500 µl of sterile 9% NaCl, wild type murine TNF (1 µg/lung) or mTNF tip peptide (Ltip, see above; 1mg/lung). Subsequently, the lungs were perfused with blood isolated from the same rat. Thirty minutes later, the lungs were injected intratracheally with 2 ml of sterile 9% NaCl solution which leads to a weight increase of about 2 g (FIG. 5). The weight evolution was then followed continuously for 150 min (FIG 5).

The weight of control lungs (pretreated with NaCl) did not decrease with time whereas, in contrast, the lungs that had been pretreated with either wt TNF or tip peptide showed a significant decrease of weight of 25 to 50 % after 150 min (FIG. 5 & 6) which corresponds with a diminished presence of hydrostatic oedema. In the case of the TNF tip peptide, the weight loss started immediately upon injection of the 2 ml of Na Cl solution (FIG. 5).

These experiments demonstrate that the tip peptide of mTNF, like the wild type molecule, can lead to oedema resorption. However, the tip peptide, in contrast to wt mTNF, does not interact with the TNF receptors and does not lead to an increased expression of adhesion molecules in lung endothelial- and epithelial cells. Consequently, the tip peptide induces less lung toxicity if compared to wt mTNF.

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CLAIMS

1. Use of a peptide comprising a chain of 7 to 17 contiguous amino acids derived from the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115} for the manufacture of a medicament for treating oedema.

2. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 11 to 16 contiguous amino acids.

3. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 13 to 15 contiguous amino acids.

4. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 14 contiguous amino acids.

5. Use of a peptide according to claim 4, wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY and PKDTPEGAELKPWY.

6. Use of a peptide according to any of claims 1 to 5, wherein said peptide is circularized.

7. Use of a peptide according to claim 6, wherein said peptide is circularized by replacing the NH_2- and COOH-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines.

8. Use of a peptide according to claim 7, wherein said circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAELKPWYC.

9. Use of a peptide according to any of claims 1 to 8, wherein said oedema is pulmonary oedema.

10. A pharmaceutical composition for treating oedema comprising a peptide according to any of claims 1 to 9.

11. A substance or composition for use in a method for treating oedema, said substance or composition comprising a peptide comprising a chain of 7 to 17 contiguous amino acids derived from the region of human TNF-α from Ser^{87} to Glu^{96} or from the region of mouse TNF-α from Ser^{87} to Glu^{96}, and said method comprising administering an effective amount of said substance or composition.

12. A substance or composition for use in a method of treatment according to claim 11, wherein said peptide comprises a chain of 11 to 16 contiguous amino acids.

13. A substance or composition for use in a method of treatment according to claim 11, wherein the peptide comprises a chain of 13 to 15 contiguous amino acids.

14. A substance or composition for use in a method of treatment according to claim 11, wherein said peptide comprises a chain of 14 contiguous amino acids.

15. A substance or composition for use in a method of treatment according to claim 14, wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY and PKDTPEGAELKPWY.

16. A substance or composition for use in a method of treatment according to any of claims 11 to 15, wherein said peptide is circularized.

17. A substance or composition for use in a method of treatment according to claim 16, wherein said peptide is circularized by replacing the NH_2- and C00H-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines.

18. A substance or composition for use in a method of treatment according to claim 17,

wherein said circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAELKPWYC.

19. A substance or composition for use in a method of treatment according to any of claims 11 to 18, wherein said oedema is pulmonary oedema.

20. Use as claimed in claim 1, substantially as herein described and illustrated.

21. A composition as claimed in claim 10, substantially as herein described and illustrated.

22. A substance or composition for use in a method of treatment as claimed in claim 11, substantially as herein described and illustrated.

23. A new use of a peptide as defined in claim 1, a new composition, or a substance or composition for a new use in a method of treatment, substantially as herein described.

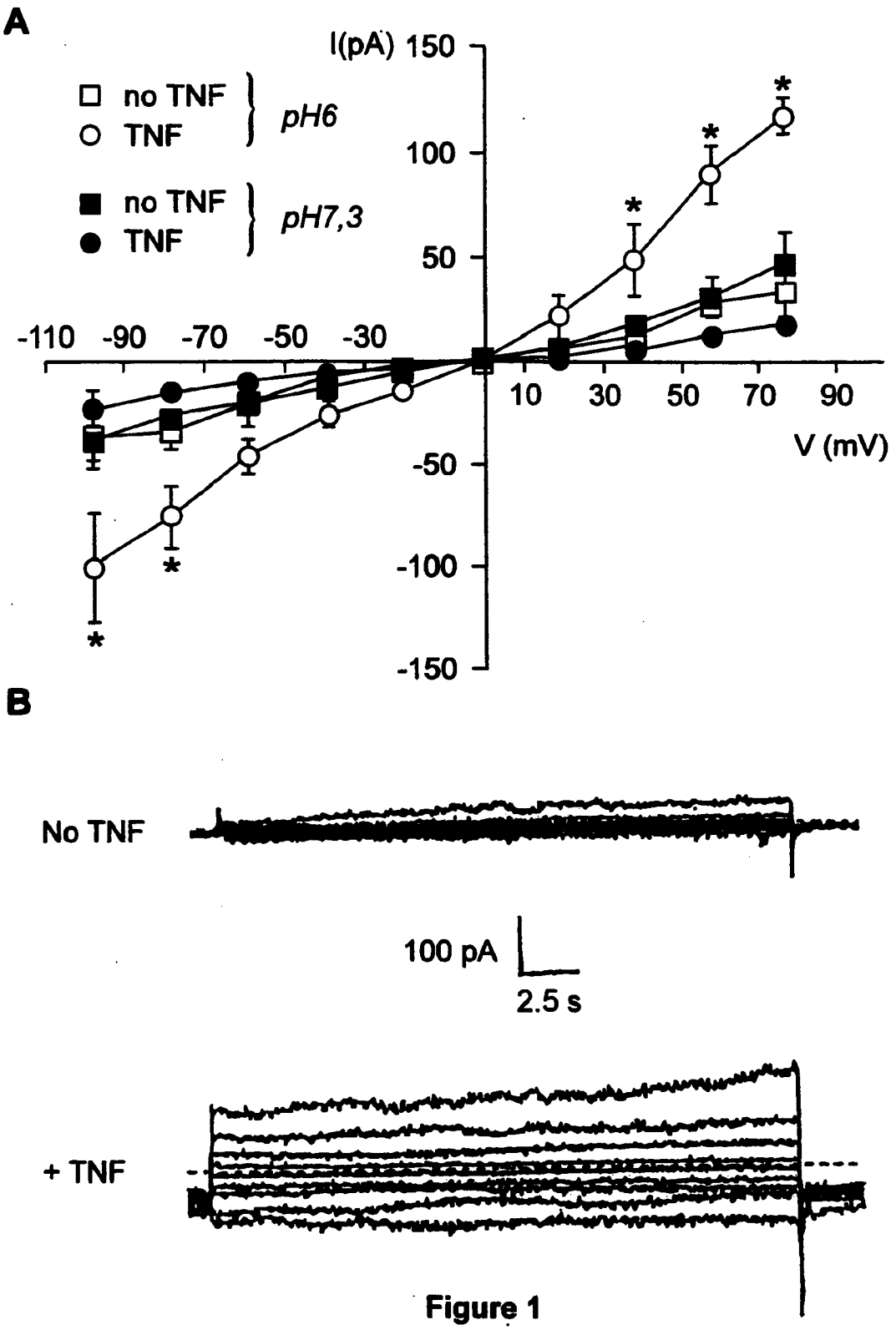
A
I(pA)
150
100
50
-50
-100
-150
-110
-90
-70
-50
-30
10
30
50
70
90
V (mV)
no TNF
TNF
pH6
no TNF
TNF
pH7,3
*
B
No TNF
100 pA
2.5 s
+ TNF

Figure 1

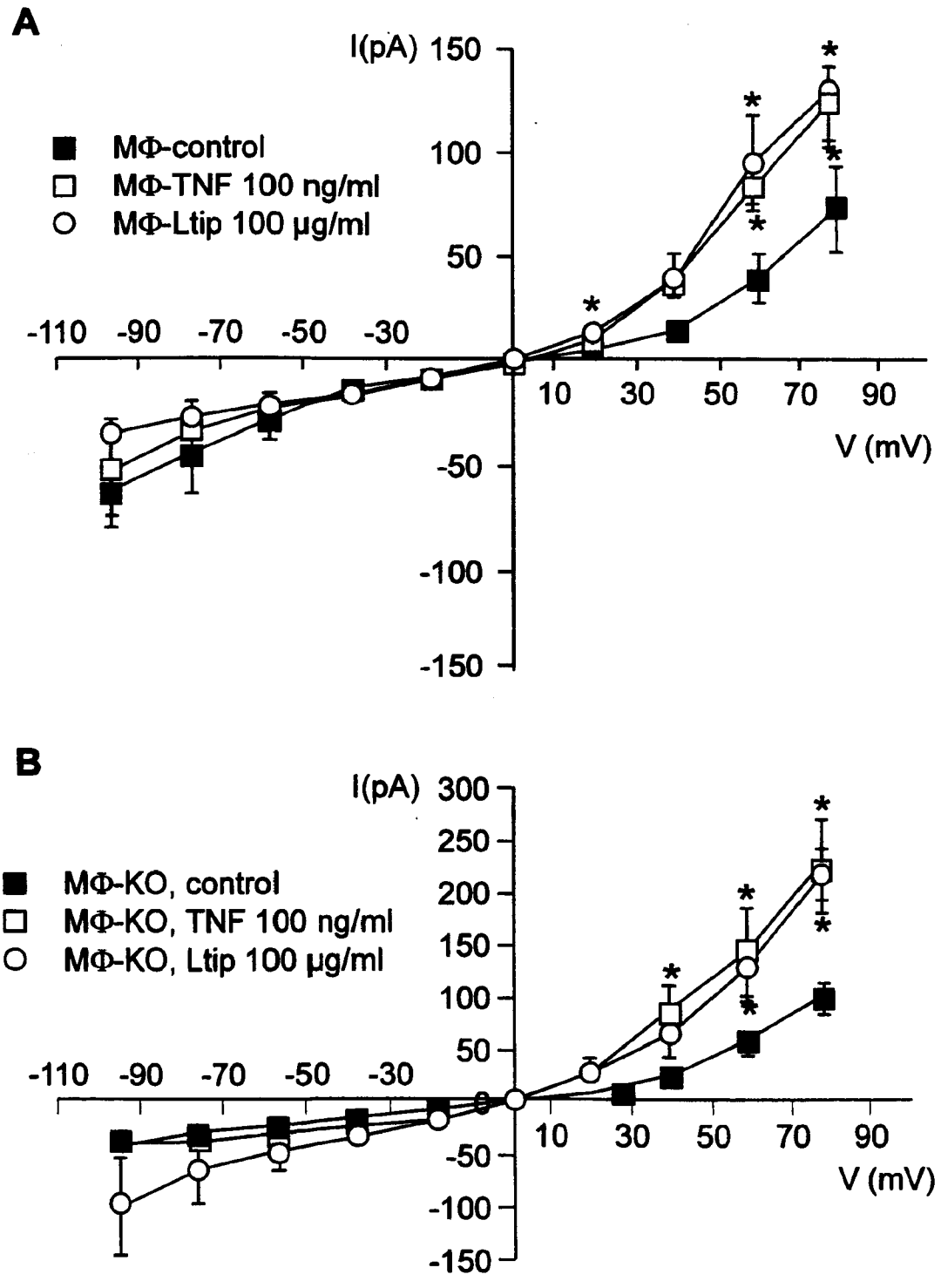
A
I(pA)
150
100
50
-50
-100
-150
-110 -90 -70 -50 -30
10 30 50 70 90
V (mV)
MΦ-control
MΦ-TNF 100 ng/ml
MΦ-Ltip 100 µg/ml
B
I(pA)
300
250
200
150
100
50
-50
-100
-150
-110 -90 -70 -50 -30
10 30 50 70 90
V (mV)
MΦ-KO, control
MΦ-KO, TNF 100 ng/ml
MΦ-KO, Ltip 100 µg/ml

Figure 2

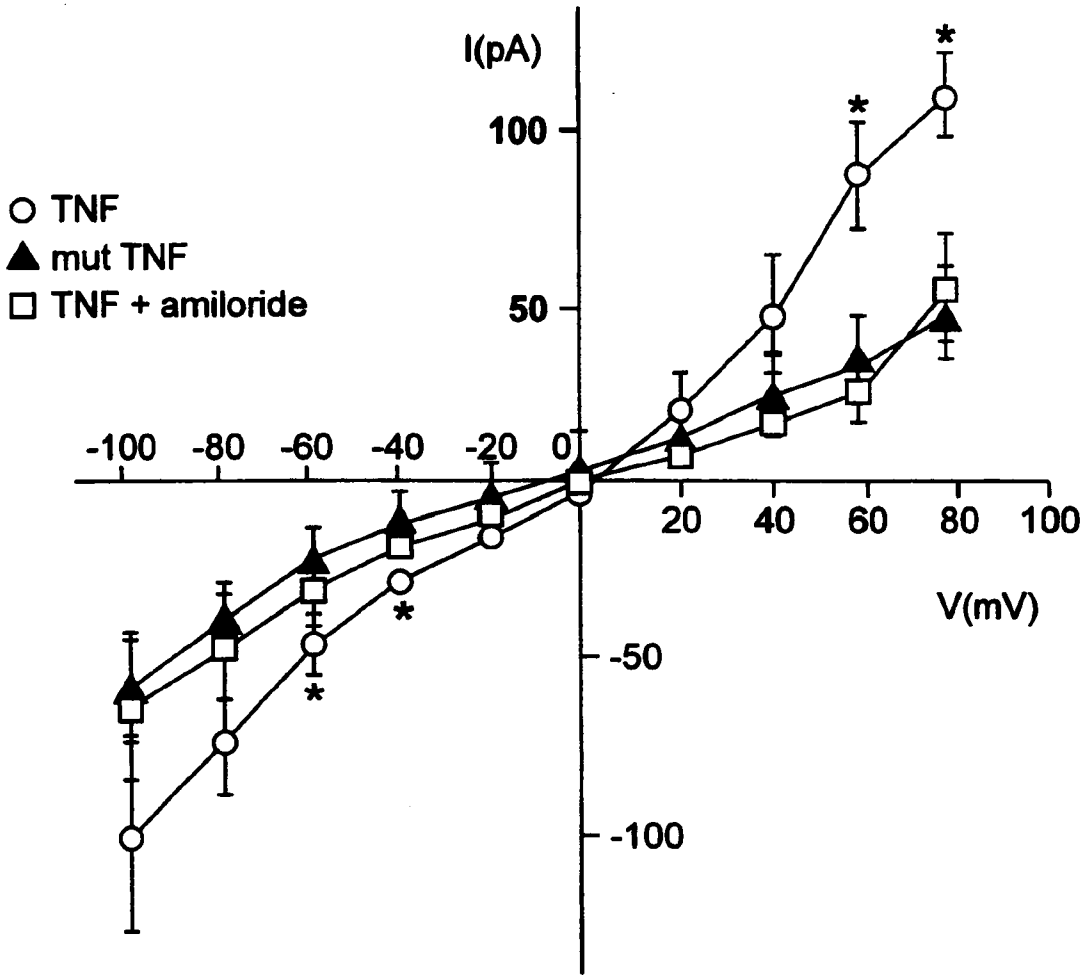
I(pA)
100
50
-50
-100
-100 -80 -60 -40 -20 0
20 40 60 80 100
V(mV)
TNF
mut TNF
TNF + amiloride
*
*
*
*

Figure 3

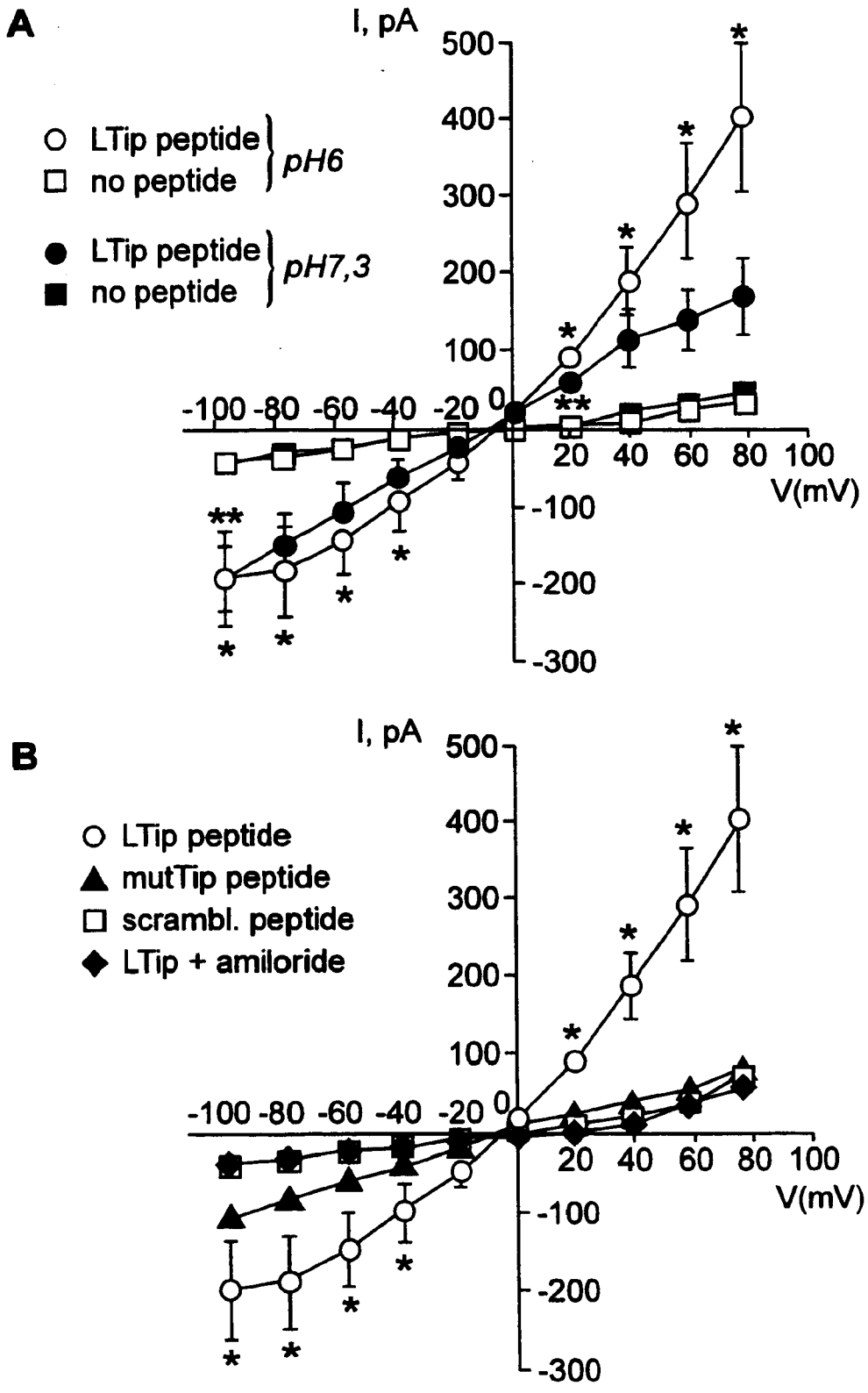
A
I, pA
LTip peptide
no peptide
pH6
LTip peptide
no peptide
pH7,3
500
400
300
200
100
-100 -80 -60 -40 -20 0
20 40 60 80 100
V(mV)
-100
-200
-300
B
I, pA
LTip peptide
mutTip peptide
scrambl. peptide
LTip + amiloride
500
400
300
200
100
-100 -80 -60 -40 -20 0
20 40 60 80 100
V(mV)
-100
-200
-300

Figure 4

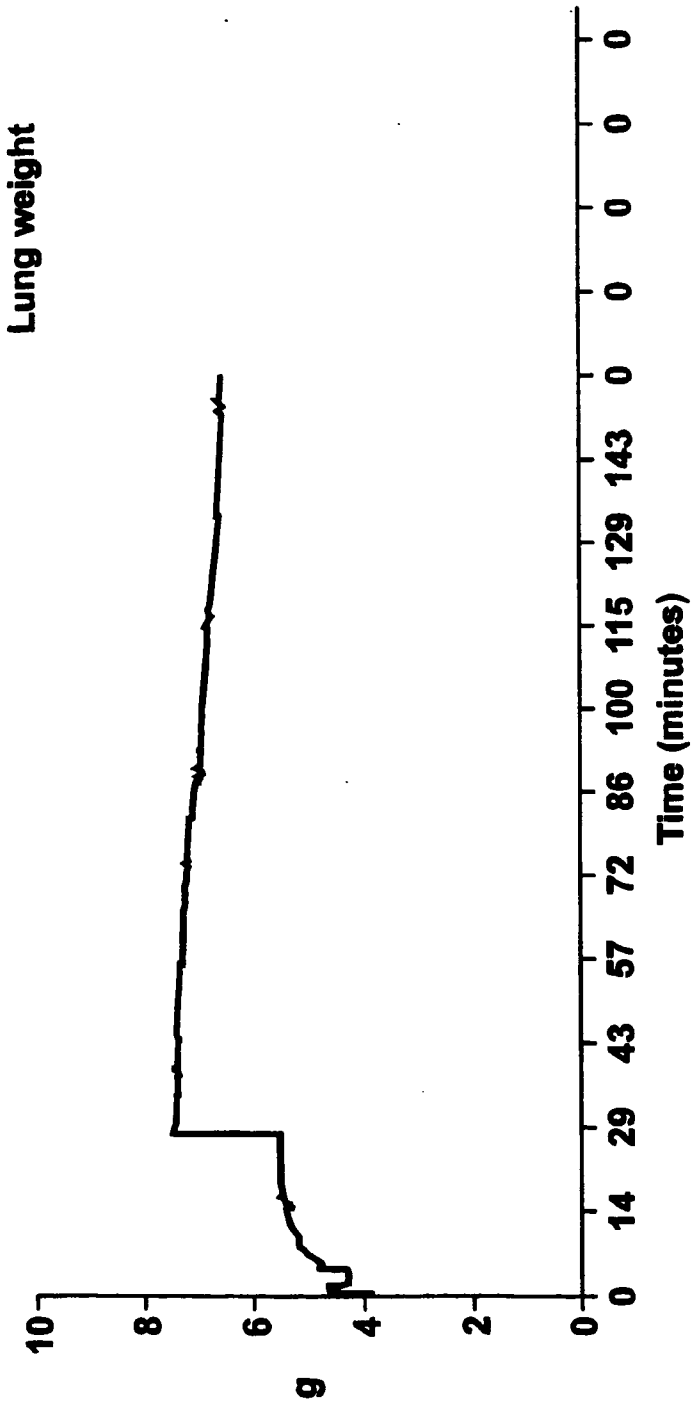
Lung weight
g
10
8
6
4
2
0
0 14 29 43 57 72 86 100 115 129 143 0 0 0 0 0 0 0
Time (minutes)

Figure 5

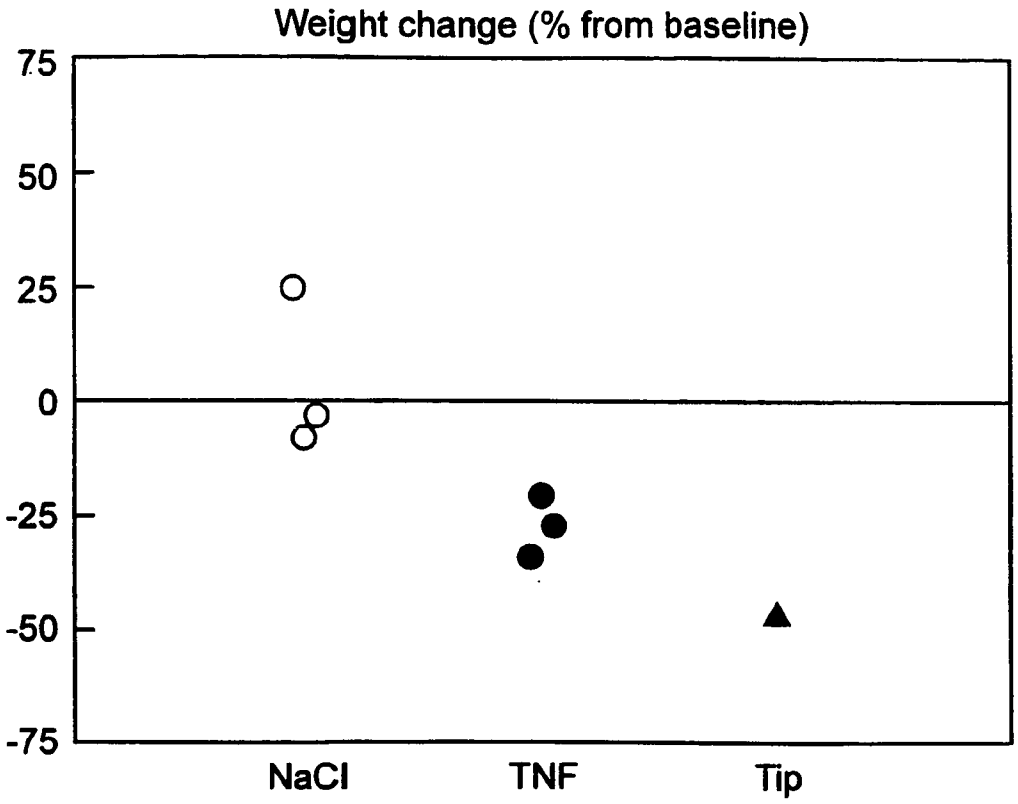

Figure 6