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Tumor Necrosis Factor Inhibits K^+ Current Expression in Cultured Oligodendrocytes

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Summary. The effects of tumor necrosis factor-α (TNF-α), a cytokine secreted by activated macrophages, on the electrical membrane properties of cultured adult ovine oligodendrocytes (OLGs) were investigated using the whole-cell voltage-clamp technique. Treatment with recombinant human TNF-α (rhTNF) for 24 to 72 hr produces (i) process retraction in some but not all OLGs, (ii) a reduction in the resting membrane potential with no significant change in membrane capacitance or input resistance over control cells and (iii) a decrease in the expression of both the inwardly rectifying and outward K^+ current. The magnitude of the membrane potential change as well as K^+ current inhibition was larger in cells with retracted processes. The electrophysiological effects of rhTNF were attenuated when rhTNF was neutralized with a polyclonal anti-rhTNF antibody. The binding of rhTNF to its receptor has been reported to increase GTP binding, to increase GTPase activity of a pertussis-sensitive G protein, and to produce an elevation in intracellular cAMP in other cell types. However, pretreatment of OLGs with activated pertussis toxin failed to attenuate or mimic the effects of rhTNF. Chronic exposure of OLGs to the membrane permeant analogue of cAMP, 8-bromo-cAMP, resulted primarily in an inhibition of the inwardly rectifying K^+ current, an effect which was less than that produced by rhTNF alone and without any of the associated rhTNF-induced morphological changes. This indicates that the effects of rhTNF cannot be entirely accounted for by an elevation in intracellular cAMP. Cycloheximide (CHX), an inhibitor of protein synthesis, mimicked the effects of rhTNF; however, the effects of rhTNF and CHX were not additive. The finding that both ionic current expression and membrane potential were reduced in cells treated with rhTNF that appeared morphologically normal suggests that abnormal ion channel expression in OLGs precedes and may contribute to eventual myelin swelling and damage.

Key Words cytokines · glial cells · ion channels · myelin · demyelination

Introduction

The concept of an integrated neural-immune network finds its strongest basis in recent studies of immune system regulation of neuroendocrine function. Cytokines, the interferons and the interleukins secreted by activated cells of the immune system, have been reported to modulate the excitability of central neurons as well as regulate the function of hypothalamic-pituitary-adrenal axis (Bernton et al., 1987; Sapolsky et al., 1987; Hori et al., 1988). Cytokines also appear to regulate glial cell proliferation, activation, and differentiation; the functional effect dependent upon the maturity of the target cells (Giulian, Vaca & Johnson, 1988). Of the cytokines, tumor necrosis factor-α (TNF-α), a 17-kDa polypeptide secreted by activated macrophages (Old, 1990), is of particular interest due to its possible role in immune-mediated demyelination (Robbins et al., 1987; Selmaj & Raine, 1988). TNF-α induces MHC class I antigen expression on the surface of astrocytes, but not on oligodendrocytes (OLGs) (Lavi et al., 1988). TNF-α has also been reported to be cytotoxic for rat and mouse OLGs (Robbins et al., 1987; Selmaj & Raine, 1988), yet appears to be mitogenic for cultured astrocytes isolated from mature bovine brain (Selmaj et al., 1990). Such a differential effect on these two types of glial cells may contribute to the loss of OLGs and development of reactive gliosis found in demyelinating diseases.

Although there is some evidence for the activation of protein kinases and G proteins in the cellular response to TNF-α in nonglial systems (Hensel et al., 1987; Imamura et al., 1988; Zhang et al., 1988), the molecular events in the signal transduction cascade remain largely unknown. Insight into glial cell responses to TNF-α comes from the histological studies of Selmaj and Raine (1988) on spinal cord explant cultures, in which recombinant human TNF (rhTNF) caused dilatation or ballooning of the myelin sheath prior to any visible OLG degeneration. This finding suggests that a change in the ionic microenvironment, possibly resulting from the dysfunction of ion channels, occurred in the initial stages of the pathological response. It remains unclear as to whether the TNF-induced morphological effects reported for the spinal cord explants resulted from a direct rather than an indirect effect on OLGs. We, therefore, undertook the present studies to deter-

mine whether rhTNF could alter the electrophysiology of mature OLGs maintained in pure culture.

Cultured adult ovine OLGs express predominantly K^+ channels: an inwardly rectifying K^+ current and an outward K^+ current (Soliven et al., 1988*a*). The inward and outward K^+ currents are differentially modulated by cytoplasmic protein kinases known to regulate myelin metabolism in the cultured cells (Vartanian et al., 1986; Soliven et al., 1988*b*; Hertz et al., 1990). The Ba^{2+}-sensitive inwardly rectifying K^+ channels are activated at membrane potentials near rest in OLGs, and may play an important role in K^+ homeostasis during neuronal firing (Barres, Chun & Corey 1990; Hertz et al., 1990). Therefore, a dysfunction of OLG ion channels could lead to abnormal K^+ homeostasis in the ionic microenvironment at the paranodal region. Using whole-cell voltage-clamp techniques on cultured adult ovine OLGs, we investigated the possibility that rhTNF-α may be (i) affecting the expression of or (ii) inducing dysfunction in OLG ion channel activation. We found that incubation of OLGs with rhTNF for 24 to 72 hr resulted in process retraction and an inhibition in the expression of both inwardly rectifying and outward K^+ currents. Both the electrophysiological and morphological findings are consistent with evidence suggesting that cytokines acting directly on OLGs are capable of inducing myelin disruption observed in demyelinating plaques.

Materials and Methods

Cell Culture

OLGs were isolated from the brains of 4–6 month old lambs as described previously (Szuchet, Arnason & Polak, 1980; Szuchet & Yim, 1984). Cells were plated on polylysine-coated petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% horse serum, 2 mM glutamine, and antibiotics (0.3 μg/ml of amphotericin B and 2.4 μg/ml of garamycin). Culture purity was ascertained to be 98–99% using a monoclonal antibody against galactocerebroside (a gift from Dr. B. Ranscht) as well as a polyclonal antibody against CNPase (a gift from Dr. T. Sprinkle). Electrophysiological experiments were performed 3–21 days following OLG attachment.

TNF Receptors: Immunofluorescence and Binding Assays

The presence of TNF receptors on OLGs was investigated by two different approaches: (i) indirect immunofluorescence; and (ii) direct binding of ^{125}I-TNF. For immunofluorescence, cells were treated successively with rhTNF (2.8 nM), rabbit polyclonal anti-TNF antibody and FITC-goat anti-rabbit IgG. Control cultures were treated with rhTNF followed by FITC-goat anti-rabbit IgG or with the anti-TNF antibody followed by FITC-goat anti-rabbit IgG.

Binding assays were performed on OLGs maintained for 2 weeks in adherent cultures in 12-well plates (1×10^6 cells/well). After the removal of the growth medium, OLGs were incubated with increasing concentrations of ^{125}I-TNF (50 pM–5 nM) in DMEM containing 10% horse serum for 2 hr at 4°C. After the incubation period, OLGs were washed four times with ice-cold phosphate buffered saline, solubilized in 1% SDS, and cell-bound radioactivity was determined in a Beckman Gamma 4000 counter (Beckman Instruments, Fullerton, CA). An aliquot was also taken from each sample for determination of total protein content using micro BCA protein assay reagent (Pierce, Rockford, IL). Specific binding was defined as the difference between total binding and nonspecific binding in the presence of a 100-fold excess of unlabeled rhTNF. Each determination was made in duplicate. The data were analyzed using the Equilibrium Binding Data Analysis program (McPherson, 1983).

Cellular Viability

Trypan blue exclusion was used as a probe of cellular viability following exposure of the OLGs to rhTNF during adherence. After allowing 48 hr for attachment, the number of floating versus attached OLGs that excluded trypan blue were determined using a hemocytometer. Attached OLGs were detached with 0.25% trypsin in 1 mM Na-EDTA solution for 5 min prior to counting.

For OLGs maintained in adherent cultures for 2–3 wks, the effect of rhTNF on cellular viability was determined either with trypan blue exclusion or with fluorescein diacetate/propidium iodide double staining procedure. Cells were incubated for 3 min at room temperature with fluorescein diacetate (15 μg/ml) and propidium iodide (4.5 μg/ml) (Favaron et al., 1988). The stained cells were examined immediately following staining with a standard epi-illumination microscope (Leitz, 450 nm excitation). Cells that enable propidium iodide to penetrate and intercalate with DNA yield a red fluorescence and represent nonviable cells, while cells that contain esterases to hydrolyze fluorescein diacetate to produce a green-yellow fluorescence represent viable cells. Thus, the percentage of nonviable cells could be determined easily on the basis of color.

Electrophysiology

Current recordings were obtained using the whole-cell configuration of the patch-clamp technique as previously described (Soliven et al., 1988a). The pipette resistance ranged from 2–5 MΩ. Cells were studied at room temperature. The bathing solution consisted of the following (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH = 7.3. Pipette (intracellular) solutions contained (in mM): 140 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, pH = 7.3. In some experiments, 0.5 mM ATP and 0.5 mM GTP were included in the pipette solution. Current records were filtered at 2 kHz using an eight-pole Bessel filter and sampled at 5 kHz. Data were not leak or capacity corrected unless specified. Membrane capacitance and input resistance were determined in experiments using 140 mM CsCl in the pipette filling solution, with 1 mM $BaCl_2$ added to the bath solution when needed to block both inward and outward K^+ currents. Membrane capacitance was measured by integrating the current during a voltage

step to either −40 or +30 mV and subtracting the baseline established 10 msec after the step.

The drugs used in this study were obtained from the following sources: recombinant human TNF-α, polyclonal anti-TNF antibody (Genzyme, Boston, MA); recombinant human IL-2 (Boehringer Mannheim, IN; Genzyme, Boston, MA); 8-bromo-cAMP, cycloheximide (Sigma, St. Louis, MO); pertussis toxin (List Biologicals, Campbell, CA). RhTNF had a specific activity of 2×10^7 U/mg while rhIL-2 had a specific activity of 2×10^6 U/mg. Endotoxin levels were less than 0.1 ng/10 μg. ^{125}I-TNF (Amersham, Arlington Heights, IL) had a sp act of 660 Ci/mmol.

Where three or more experiments were performed for a given experimental condition, values are reported as either the mean ± SEM or as the range with the number of experiments in parentheses.

Results

Presence of TNF Receptors on Ovine OLGs

Virtually all somatic tissues express receptors for TNF-α either constitutively or following activation (Old, 1990). We have used anti-TNF antibody in an indirect immunofluorescence technique to detect the binding of TNF to OLGs. Positive staining was obtained only when rhTNF, anti-TNF antibody and FITC-goat anti-rabbit IgG were added sequentially. No staining was observed when rhTNF or anti-TNF antibody was added alone followed by FITC-goat anti- rabbit IgG (*data not shown*).

To further characterize TNF binding to OLGs and obtain a quantitative assessment of receptors per cell, OLGs were incubated with increasing concentrations of ^{125}I-TNF. Based on kinetics of TNF binding to other cell types, incubation for 2 hr at 4°C was chosen to produce maximal binding with minimal internalization. Specific binding approached saturation at a concentration of ≥5 nM. Scatchard plot analysis of the binding to OLGs under these conditions indicated the presence of approximately 2400 receptors per cell, with a K_d of 1.24 nM.

Effect of rhTNF on OLG Adherence and Morphology

Cytokine regulation of cell adhesion receptors has been reported (Heino et al., 1989). Exposure of OLGs to rhTNF (100 ng/ml to 1 μg/ml) (2.8 to 28 nM) during the first 24–48 hr of adherence did not have a significant effect on OLG attachment to polylysine-coated dishes. In three sets of experiments, 69.1 ± 6.3% of control OLGs attached, to be compared to 62 ± 6.7% attachment in rhTNF-treated cells. Only viable cells (cells which excluded trypan blue, *see* Materials and Methods) were counted to determine the percentage of attachment. However, incubation of 2-week-old cultured OLGs with rhTNF for 48 hr resulted in unique morphological changes. While OLGs remained attached and the soma appeared phase-bright, a significant fraction of the cells exhibited retracted processes as seen in Fig. 1. The percentage of OLGs with retracted processes, which appeared to be culture condition dependent, varied from 10–50%. TNF-treated cells with or without retracted processes were determined to be viable on the basis of trypan blue exclusion and fluorescein diacetate/propidium iodide staining (*data not shown*). In both control OLG cultures and rhTNF-treated cultures, 1% or less of the OLGs were nonviable. Cells treated with recombinant human interleukin-2 (rhIL2) (20–60 ng/ml) (1.3–3.9 nM) appeared morphologically normal.

Passive Membrane Properties of rhTNF-Treated OLGs

To ensure adequate voltage control, electrophysiological experiments were performed on process-bearing OLGs which had not made contact with other cells nor formed an extensive network of processes, i.e., in areas of lower cell density. Membrane capacitance was measured in control (CTRL) cells and compared to that observed for cells treated with rhTNF which had retracted processes (TNF-ABP). The capacitance ranged from 8.8 to 32.4 pF in cells with somata 10–15 μm in diameter. It should be noted that capacitance would be much higher in OLGs that had formed extensive processes, i.e., in older cultures. Mean capacitance in rhTNF-treated cells with retracted processes was 16.3 ± 1.1 pF (n = 23) compared to 16.2 ± 1.3 pF (n = 25) observed in the control cells. The observation that total capacitance is unchanged suggests that rhTNF causes retraction and not dissolution of processes. Membrane input resistance was determined as the reciprocal of the slope conductance measured between −120 and −75 mV in experiments in which K^+ currents were blocked in the presence of internal Cs^+ and external Ba^{2+} (*see* Materials and Methods). There was no significant difference in the input resistance of control [2.8 ± 0.4 GΩ (n = 23)] versus that observed for TNF-ABP cells [3.2 ± 0.5 GΩ (n = 22)], suggesting that rhTNF-treated cells were viable (a decrease in input resistance would be expected to accompany cytotoxicity). Input resistance values also demonstrate that ionic currents were adequately blocked since input resistance measured in control OLGs in normal bath and pipette solutions

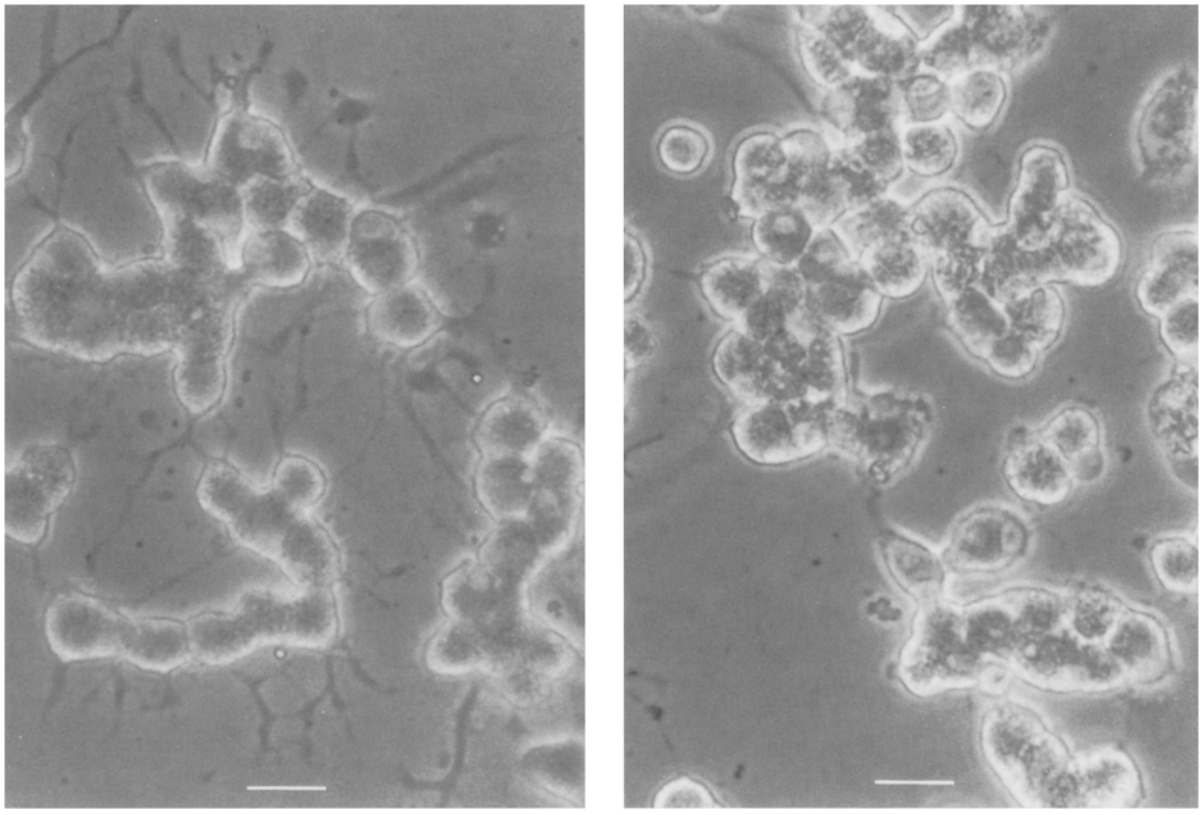

Fig. 1. Phase micrographs and fluorescence photomicrographs of 2-week-old cultured OLGs that have been incubated with rhTNF (2.8 nM) for 48 hr, showing process retraction (right panel), as compared to control cells (left panel). Calibration bar represents 22 μm.

was 0.36 ± 0.07 GΩ (n = 15), due to the activation of inwardly rectifying K^+ current.

rhTNF Produces Membrane Depolarization and Inhibits the Expression of Both Inwardly Rectifying and Outward K^+ Conductance

Resting membrane potential (RMP) was estimated as the zero current potential from current-voltage plots. Incubation of OLGs with rhTNF (2.8–28 nM) produced membrane depolarization, as summarized in Fig. 2. The RMP in control OLGs ranged from −60 to −83 mV with a mean of −68.9 ± 0.9 mV (n = 62), whereas the mean RMP in TNF-ABP cells was −21.0 ± 2.7 mV (n = 30) ($P < 0.0005$). TNF-treated cells that retained apparent normal processes (TNF-NP) likewise exhibited a depolarized membrane potential (−53.5 ± 2.7 mV, n = 29, $P <$ 0.005), suggesting that membrane potential changes were detectable prior to process retraction.

Cultured adult ovine OLGs express predominantly K^+ channels: (i) an outward K^+ current that

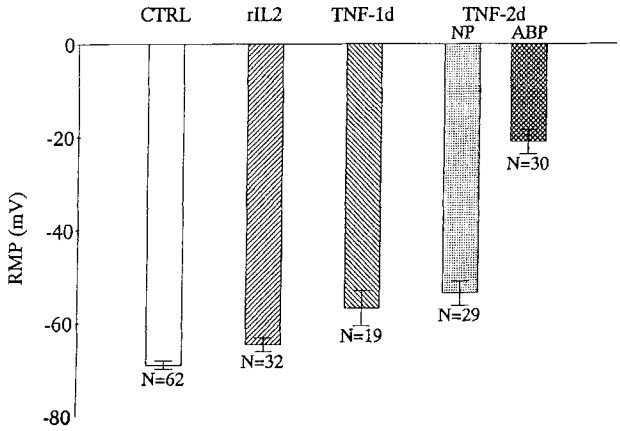

Fig. 2. The effect of rhTNF on the resting membrane potential (*RMP*) of OLGs. RMP was estimated as the zero current potential obtained from the current-voltage relationships. Cells treated with rhTNF (2.8–28 nM) showed significant membrane depolarization when compared to either control cells (*CTRL*) or cells treated with interleukin-2 (*rIL2*). *TNF-1d* refers to cells treated with rhTNF for 24 hr, while TNF-2d refers to cells treated with rhTNF for 48–72 hr. *NP* refers to TNF-treated cells with normal processes, while *ABP* refers to TNF-treated cells with retracted processes.

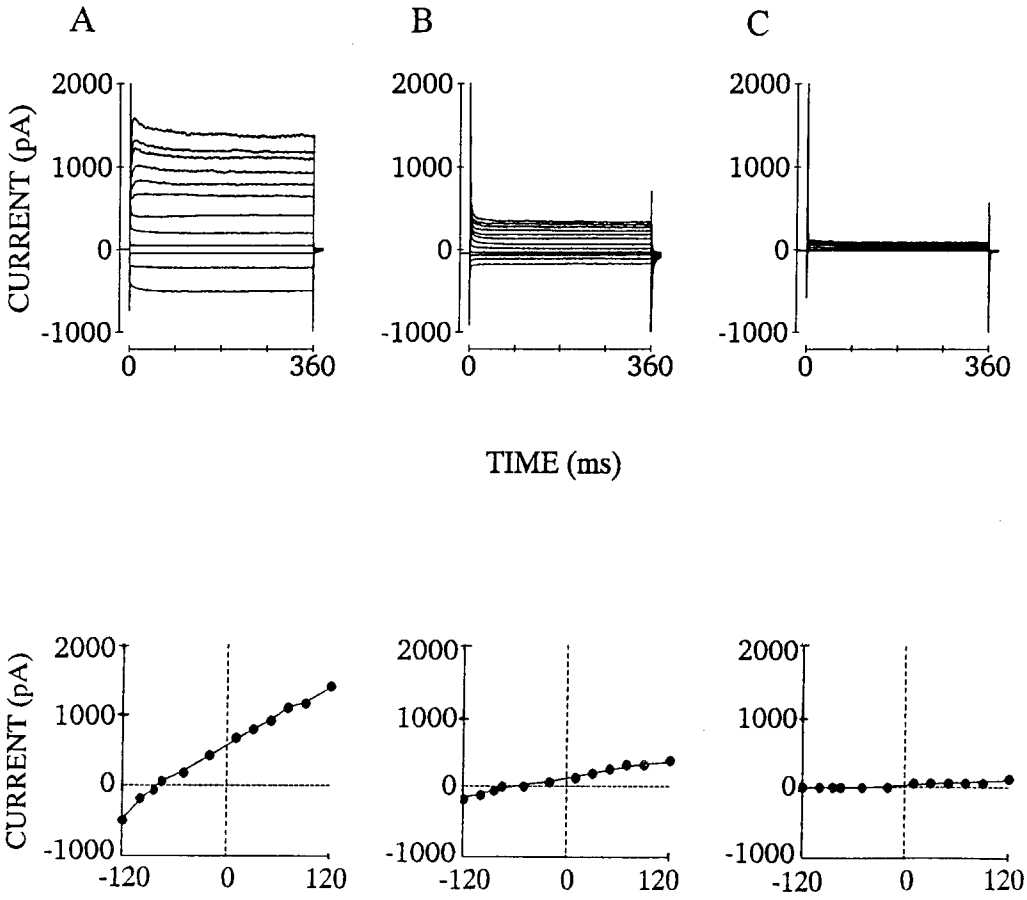

Fig. 3. Representative whole-cell current recordings from a control OLG (*A*), a TNF-treated cell that retained normal processes (*B*), and from a cell with retracted processes (*C*). (*A*) Whole-cell current record from a control oligodendrocyte illustrating the presence of outward currents that consisted of a transient component and a steady-state component. In addition, an inwardly rectifying K^+ current was observed. Pulses of 360 msec in duration were stepped to the following potentials (in mV): −120, −100, −85, −75, −50, −20, 10, 30, 50, 70, 90, 120 from a holding potential of −80 mV at 10-sec intervals. Below each current record is the corresponding current-voltage plot constructed from steady-state current values measured at 336 msec following the initiation of the voltage pulses. (*B*) TNF-treated cell with normal processes which exhibited K^+ currents similar to that observed in control cells, but with a reduction in current amplitudes. (*C*) Current record and the corresponding current voltage plot from TNF-treated cell with retracted processes illustrating absence of inward rectifier K^+ current and a significant reduction in the amplitude of outward K^+ current.

is comprised of a transient/inactivating component and a steady-state/noninactivating component; and (ii) an inwardly rectifying K^+ current that is blocked by 1 mM Ba^{2+} in the external solution. The characteristics of these currents and their modulation have been analyzed in previous studies (Soliven et al., 1988*a*,*b*). Due to overlapping K^+ current activation ranges, current measurements in the present studies were not leak subtracted. The expression of both outward and inward K^+ currents was significantly decreased in OLGs treated with rhTNF (2.8–28 nM). Examples of whole-cell current records and the corresponding current-voltage plot obtained from a control OLG are depicted in Fig. 3*A* and compared to current records and the current-voltage plots from two rhTNF-treated OLGs (TNF-NP in Fig. 3*B* and TNF-ABP in Fig. 3*C*). Voltage pulses of 360 msec in duration were applied to varying potentials from a holding potential of −80 mV at 10-sec intervals. The rhTNF-inhibition of outward and inward current amplitude was more prominent in TNF-ABP cells, but current inhibition could be detected even in TNF-NP cells. Mean current amplitude at −120 mV in controls was −460.2 ± 36.7 pA (n = 62), to be compared to −64.5 ± 9.1 pA (n = 30) in TNF-ABP cells and −233.3 ± 30.5 pA (n = 29) in TNF-NP cells. The reduction in inward rectifier current amplitude was clearly evident after 24 hr of treatment with TNF, while the inhibition of outward K^+ current amplitude was not detectable until 48–72 hr following rhTNF exposure. The effect was also observed when OLGs were treated with 1.4 nM

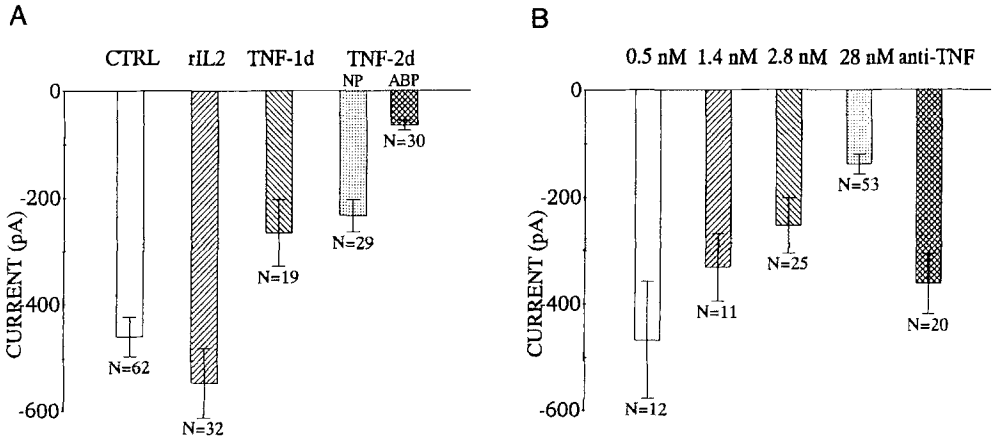

Fig. 4. Summary of the effect of rhTNF on inwardly rectifying K^+ current. Steady-state current amplitudes were measured at −120 mV at 336 msec following the initiation of the voltage pulse. (*A*) The reduction in inwardly rectifying K^+ current amplitude was evident after incubation of the cells with rhTNF for 24 hr (*TNF-1d*). Current inhibition was more prominent in cells that had retracted processes (*ABP*) than in cells that retained normal processes (*NP*). (*B*) Concentration-dependence of TNF effect. Current amplitudes were reduced only at concentrations in the nM range. Neutralization of rhTNF with rabbit anti-TNF antibody attenuated the effect of rhTNF on current amplitude.

rhTNF (mean current amplitude at −120 mV was −331.5 ± 63.4 pA), but not in OLGs treated with 0.5 nM rhTNF (n = 12). Incubation with rIL-2 (1.3–3.9 nM) had no effect on either RMP or current amplitude. When rhTNF (2.8–28 nM) was preneutralized with polyclonal anti-TNF antibody for 4 hr prior to addition to OLGs for 48 hr, the morphological changes in OLGs were prevented and the effect on K^+ current expression attenuated (Fig. 4). The effect of rhTNF was not reversible upon reincubation in normal media for 3 days.

The rhTNF-induced reduction in K^+ current could not be accounted for by a shift in the voltage dependence of either outward or inward current activation, as seen in Fig. 5. In recordings where inward rectifier current was completely absent, the steady-state parameters of activation of outward K^+ current were determined. Normalized conductance versus voltage curves were averaged from five experiments and fitted with a Boltzmann function of the form

$$g_K(V) = g_{K,max}/[1 + e^{(V-V_{1/2})/k_n}]$$

where $V_{1/2}$ is the voltage at the midpoint of the activation curve and k_n is the steepness of the voltage dependence. In the cells treated with rhTNF, the $V_{1/2}$ for outward K^+ current activation was −3.8 mV with a slope factor k_n of −17.9. In control cells, the parameters of outward K^+ activation were determined from conductance-voltage curves obtained from OLGs maintained in adherent culture for only 2–3 days since these cells often lack inward rectifier expression. The $V_{1/2}$ in control cells was −4.7 mV, with a slope factor of −17.5.

The kinetics of inward rectifier activation were determined using a double pulse protocol. Channel closure was induced by conditioning pulses of 60 msec to a series of depolarized potentials and then stepped to −120 mV where channel activation is maximal. Although outward K^+ current is activated at depolarized potentials, e.g., −20 mV, the contribution of the outward K^+ channel tail current to the inward rectifier current would be minimal due to the rapid time course of the outward K^+ channel deactivation during the hyperpolarized test pulse to −120 mV. As was observed with the outward K^+ current, there was no significant difference in the parameters of inward rectifier activation for OLGs treated with rhTNF as compared to that obtained for control cells (Fig. 5*B*).

Possible Mechanism of Action of rhTNF on the Electrophysiological Properties of OLGs

Since pertussis-sensitive G proteins and cAMP have been implicated in the signal transduction process following binding of TNF to its receptors (Hensel et al., 1987; Imamura et al., 1988; Zhang et al., 1988), we examined the possibility that similar mechanisms may play a part in the action of TNF on OLGs. OLGs were exposed to activated pertussis toxin (10 μg/ml) for 16 hr prior to treatment with rhTNF for 72 hr. Pertussis toxin alone had no effect on current amplitude. It also failed to inhibit or attenuate rhTNF-induced changes in morphology or current expression, suggesting that a pertussis toxin-sensitive G protein is not involved in the action of

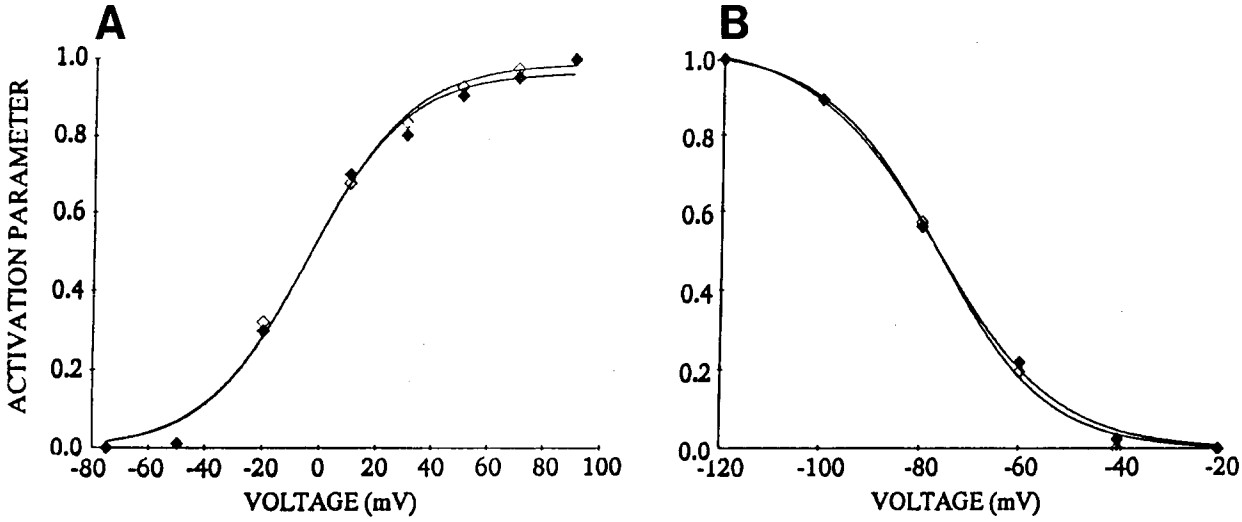

Fig. 5. Steady-state parameters of activation for outward K^+ current (*A*) and inwardly rectifying K^+ current (*B*) in control OLGs and rhTNF-treated OLGs. (*A*) The activation parameters of outward K^+ current were determined from leak-subtracted conductance-voltage curves which were normalized, averaged and fitted with a Boltzmann function of the form

$$y = g_{K,max}/[1 + e^{(V-V_{1/2})/k_n}]$$

where $V_{1/2}$ is the voltage at the midpoint of the activation curve and k_n is the steepness of the voltage-dependence. $V_{1/2} = -4.7$ mV and $k_n = -17.5$ ($n = 5$) for CTRL (filled diamond); $V_{1/2} = -3.8$ mV and $k_n = -17.9$ ($n = 5$) for TNF-treated cells. (*B*) The activation parameters of the inward rectifier K^+ current were obtained using a double pulse protocol where channel closure was induced by conditioning pulses of 60 msec to a series of depolarized potentials and then stepped to -120 mV where channel activation is maximal. The fraction of the channels available for instantaneous activation of inwardly rectifying K^+ current was normalized, averaged and fitted with the Boltzmann equation. $V_{1/2} = -77.5$ mV and $k_n = 12.4$ ($n = 4$) in CTRL; $V_{1/2} = -77.1$ mV and $k_n = 11.2$ ($n = 4$) in TNF-treated cells.

rhTNF. Mean current amplitude at -120 mV in cells pretreated with pertussis toxin and subsequently treated with rhTNF was -146.5 ± 39.6 pA ($n = 10$).

In contrast, cells that were exposed to the membrane permeant 8-bromo-cAMP (1 mM) for 48 hr exhibited depolarized membrane potentials (-42 ± 5.4 mV, $n = 26$) similar to that observed for rhTNF-treated cells. Mean K^+ current amplitudes measured at -120 and at 10 mV in 8-bromo-cAMP treated cells were also reduced, the magnitude of inwardly rectifying K^+ current reduction being greater than that of K^+ outward current (-292 ± 35 pA at -120 mV and 377 ± 58 pA at 10 mV, respectively) (Fig. 6). There were no obvious morphological changes in the cAMP-treated cells.

To determine whether the protein kinase C pathway participates in the post-receptor signal transduction events, we examined the response of OLGs to phorbol myristate acetate (PMA), an activator of protein kinase C. Continuous exposure of 2–3 wk old cells to PMA (100 nM) for 24–48 hr induced cell clumping in addition to process retraction, a phenomenon different from that seen with rhTNF. In addition, we have previously shown that galactosylsphingosine (psychosine), a protein kinase C inhibitor does not affect K^+ current expression, although it has a direct lytic effect on OLG processes (Vartanian et al., 1989).

It has been reported that the response to TNF is regulated by protein synthesis (Pohlman & Harlan, 1989). Treatment of the cells with sublethal concentrations of cycloheximide (CHX) (1–2 μg/ml), an inhibitor of protein synthesis, for 24–48 hr mimicked the effect of rhTNF on OLG current. Mean inward rectifier current amplitude in CHX-treated cells was -266.1 ± 56 pA ($n = 31$). RMP in these cells was -52.1 ± 3.1 mV. It is of interest to note that these values are close to that of rhTNF-treated cells which retained processes. No additive or synergistic effects were observed when cells were treated with both rhTNF and CHX for 24 hr. No cytotoxicity was observed with CHX alone or with addition of both TNF-α and CHX. These results are summarized in Fig. 6.

Discussion

This study demonstrates that prolonged exposure of cultured adult ovine OLGs to rhTNF produces process retraction in a subpopulation of cells, the

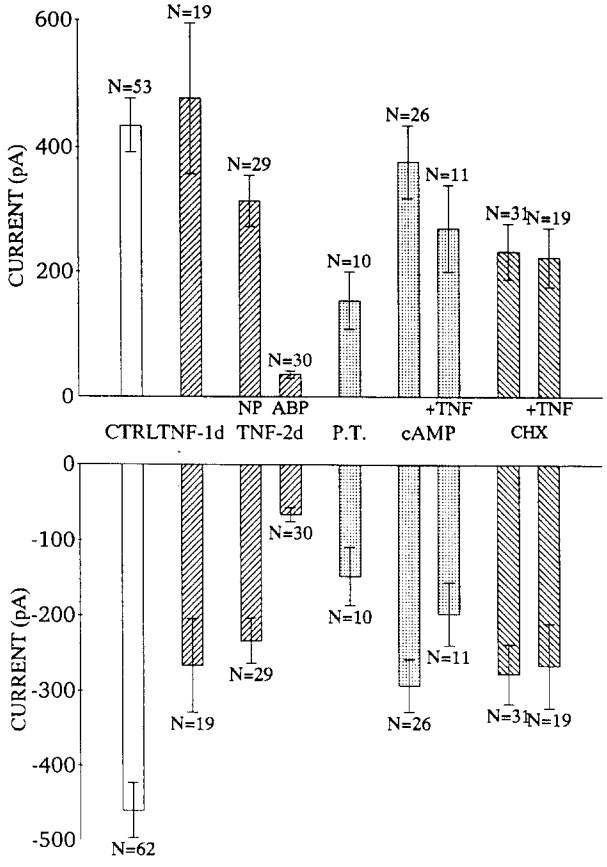

Fig. 6. Effects of pertussis toxin (*P.T.*), 8-bromo-cAMP (*cAMP*), and cycloheximide (*CHX*) on OLG currents. Steady-state current amplitudes were measured at −120 mV for inwardly rectifying K^+ current and at +10 mV for outward K^+ current. Pre-exposure of OLGs to pertussis toxin (10 μg/ml) for 16 hr prior to treatment with TNF did not block the response to rhTNF. Exposure to 8-bromo-cAMP (1 mM) resulted predominantly in a reduction in the amplitude of inwardly rectifying K^+ current, while exposure to CHX (1–2 μg/ml) mimicked rhTNF-induced effect on both inward rectifier and outward K^+ current. No additive or synergistic effects were observed when cells were treated with both rhTNF and CHX.

extent of which is dependent upon the exposure duration. A reduction both in the resting membrane potential and in the expression of the inwardly rectifying and outward K^+ current was observed in all cells exposed to rhTNF; however, the magnitude of the reduction was more pronounced in those cells with retracted processes. The reduction in current amplitude for both K^+ conductances was not due to a shift in the voltage dependence of activation. Both membrane depolarization and inhibition of inward current amplitude preceded obvious process retraction. The action of TNF is specific in that neutralization with anti-TNF antibody attenuated the effects of rhTNF and treatment with rhIL-2 failed to mimic either the electrophysiological findings or the morphological changes.

Modulation of ion channel expression as well as membrane potential by chronic exposure to soluble growth factors and cytokines has been reported for both glial and nonglial cells. Ca^{2+} currents are detected in cortical astrocytes only when the cultured cells are pre-exposed to "permissive" lots of serum for 48 hr and subsequently treated with substances that increase cAMP, e.g. isoproterenol or forskolin (Barres, Chun & Corey, 1989). Transforming growth factor β-1, on the other hand, reversibly suppresses the expression of Ca^{2+} and Na^+ channels in the developing skeletal myoblast (Caffrey, Brown & Schneider, 1989). TNF has been reported to induce a decline in membrane potential in rat skeletal muscle both in vitro as well as in vivo (Tracey et al., 1986). Prior to this investigation, cytokines have not been shown to affect ionic current expression in central glial cell types. However, interferons and interleukins have been demonstrated to induce surface major histocompatibility complex antigens on glial cells (Wong et al., 1984), to exhibit growth promoting effect on astrocytes (Giulian et al., 1988) and to affect OLG proliferation (Benveniste & Merill, 1986; Saneto et al., 1986; Suzumura & Silberberg, 1989).

TNF has been implicated in the development of inflammatory demyelinating disease processes in the central nervous system. Robbins et al. (1987) reported that stimulated rat astrocytes produced a factor cytotoxic for neonatal rat OLGs and that the cytotoxicity could be mimicked by high concentration of rhTNF (200 ng/ml, 2360 U/ml). We did not observe cytotoxicity in the cultured adult ovine OLGs exposed to similar concentration of TNF for 48–72 hr. This may reflect a differentiation-dependent susceptibility of OLGs to TNF. Cultured OLGs exposed to rhTNF remained phase bright, excluded trypan blue as well as propidium iodide, and exhibited the same passive membrane properties as observed in control cells. Process retraction observed in OLGs was not associated with a change in total capacitance. Similarly, process retraction in Schwann cells has been reported to be accompanied by an increase in somal capacitance (Howe & Ritchie, 1990) but not the total capacitance.

The extent of K^+ current reduction and the depolarization induced by chronic exposure to TNF far exceeds that previously observed in electrophysiological experiments employing bath superfusion of forskolin or PMA, activators of second messenger pathways involved in the regulation of myelin metabolism in the cultured cells (Soliven et al., 1988b). It is unlikely that the membrane depolarization seen in TNF-treated cells is due to an increased leak con-

ductance, since the mean input resistance in TNF-treated cells was not significantly different from controls. Furthermore, a depolarization associated with increased leak conductance would result in an increase in total current, not a decrease, as was observed in the case of the TNF-treated cells.

The inhibition of K^+ channel expression and membrane depolarization which accompanied process retraction in TNF-treated OLGs is to be contrasted with the electrophysiological changes accompanying process dissolution induced by galactosylsphingosine (psychosine), a protein kinase C inhibitor (Vartanian et al., 1989). Psychosine accumulates in OLGs as an alternative catabolite of galactosylceramides in patients lacking galactosylceramide β-galactosidase (Krabbe disease), a disorder characterized by the degeneration of OLGs and hypomyelination. In spite of the process dissolution, as confirmed by a decrease in the total membrane capacitance, psychosine has no significant effect on RMP or ionic current expression (Vartanian et al., 1989), suggesting that most of the K^+ channels are present in the soma. Thus, the lack of processes *per se* cannot account for the effect of TNF on K^+ currents. Furthermore, both inward and, to a lesser extent, outward current amplitude was reduced and significant membrane depolarization was observed even in TNF-treated cells that retained apparent normal processes. It should be noted that reference to TNF-treated cells that retained normal processes may represent an underestimate of TNF-induced morphological changes, since small morphological changes would not have been quantitatively resolvable in our studies.

The mechanism linking TNF-cell binding to functional effects remains obscure. In agreement with previous studies (Robbins et al., 1987; Selmaj & Raine, 1988), the effects of TNF were observed at relatively high concentrations. However, electrophysiological and morphological changes induced by TNF cannot be explained on the basis of nonspecific binding or generalized cytotoxicity for the following reasons: (i) OLGs express TNF receptors that are saturable and have a K_d of 1.24 nM, comparable to previously reported K_d values (0.1–1 nM) on a variety of cell types (Tsujimoto, Yip & Vilcek, 1985; Scheurich et al., 1986; Stauber, Aiyer & Aggarwal, 1988). (ii) Although TNF-α induced an apparent deleterious effect on OLGs, it did not affect cell viability nor did it perturb the initial OLG-substratum interactions thought to be important in the initiation of a myelinogenic metabolism (Szuchet, Yim & Monsma, 1983; Szuchet, Polak & Yim, 1986; Vartanian et al., 1986; Yim, Szuchet & Polak, 1986).

Some of the important changes observed in other cell types following exposure to TNF include (i) the accumulation of intracellular cAMP with a concomitant increase in protein kinase activity (PKA) (Zhang et al., 1988), (ii) the phosphorylation of a 26 to 28 kD protein (Schutze et al., 1989), and (iii) activation of phospholipase A_2 with mobilization of arachidonic acid metabolites (Granger et al., 1990) (for review, *see* Old, 1990). In our studies, chronic exposure of OLGs to 8-bromo-cAMP for 48–72 hr resulted in greater inhibition of the expression of inward rectifier over that observed for the outward K^+ current. The interpretation of the cAMP-induced inhibition of the inwardly rectifying K^+ current cannot be accounted for solely on the basis of an activation of PKA since we have previously shown (Hertz et al., 1990) that acute exposure of voltage-clamped cells to membrane permeant analogues of cAMP has no effect on inward K^+ current amplitude. Whether or not this represents the difference in acute versus chronic effects remains to be studied. The effect of 8-bromo-cAMP on K^+ currents was not as dramatic as that seen with TNF, and no obvious morphological changes were observed, suggesting that the cAMP alone may not account for all the actions of TNF.

There is evidence that TNF-α is toxic to certain cell types only when protein synthesis is inhibited by cycloheximide (Pohlman & Harlan, 1989). Incubation of OLGs with low concentrations of cycloheximide (1–2 μg/ml) for 24–48 hr mimicked the effect of TNF on OLG currents. No additive or synergistic effects were seen when cells were treated with both the TNF-α and CHX, suggesting both may be acting through the same mechanism. Cycloheximide at higher concentrations (10–20 μg/ml) inhibits synthesis of myelin proteins and produces focal accumulation of membrane vesicles in OLGs, accompanied by distortion and vesiculation of paranodal myelin sheaths (Cullen & Webster, 1989).

Our finding that abnormal ion channel expression and membrane depolarization in OLGs treated with rhTNF precede process retraction supports the morphological observations of Selmaj and Raine (1988) showing that widening of periaxonal space occurs initially without overt myelin destruction in spinal cord explant cultures exposed to TNF. It is tempting to speculate that the inhibition of OLG K^+ channels leads to a disturbance in ionic homeostasis in the periaxonal space and precipitates ensuing myelin damage. Alternatively, process retraction could be due to inhibition of the synthesis of cytoskeletal proteins which occurs independent of the inhibition of ion channel expression. There is evidence that a close relationship exists between the myelin components, MBP and CNPase, with cytoskeletal components, e.g. tubulin and microfilaments (Wilson & Brophy, 1989). Although the exact mechanism of

action of TNF remains to be elucidated, our observations bear relevance to the pathogenesis of inflammatory demyelinating diseases.

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