Tumor necrosis factor-α downregulates sodium current in skeletal muscle by protein kinase C activation: involvement in critical illness polyneuromyopathy

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Guillouet M, Gueret G, Rannou F, Giroux-Metges MA, Gioux M, Arvieux CC, Pennec JP. Tumor necrosis factor-α downregulates sodium current in skeletal muscle by protein kinase C activation: involvement in critical illness polyneuromyopathy. Am J Physiol Cell Physiol 301: C1057–C1063, 2011. First published July 27, 2011; doi:10.1152/ajpcell.00097.2011.—Sepsis is involved in the decrease of membrane excitability of skeletal muscle, leading to polyneuromyopathy. This effect is mediated by alterations of the properties of voltage-gated sodium channels (NaV), but the exact mechanism is still unknown. The aim of the present study was to check whether tumor necrosis factor (TNF-α), a cytokine released during sepsis, exerts a rapid effect on NaV. Sodium current (I Na) was recorded by macro-patch clamp in skeletal muscle fibers isolated from rat peroneus longus muscle, in control conditions and after TNF-α addition. Analyses of dose-effect and time-effect relationships were carried out. Effect of chelerythrine, a PKC inhibitor, was also studied to determine the way of action of TNF-α. TNF-α induced a reversible dose- and time-dependent inhibition of I Na. A maximum inhibition of 75% of the control current was observed. A shift toward more negative potentials of activation and inactivation curves of I Na was also noticed. These effects were prevented by chelerythrine pretreatment. TNF-α is a cytokine released in the early stages of sepsis. Besides a possible transcriptional role, i.e., modification of the channel type and/or number, we demonstrated the existence of a rapid, posttranscriptional inhibition of NaV by TNF-α. The downregulation of the sodium current could be mediated by a PKC-induced phosphorylation of the sodium channel, thus leading to a significant decrease in muscle excitability.

voltage-gated sodium channel; muscle excitability; sepsis

Critical Illness Polyneuromyopathy (CIP) is the most frequently observed neuromuscular disorder in intensive care units (ICU) (25, 46, 47). Consequences include difficulties in weaning from mechanical ventilation, prolonged time in an ICU, and increased hospital costs. It can also lead to decreased exercise capacity months or even years after the event (13, 54). Both critical illness neuromyopathy (CIN) and myopathy (CIM) are described. The first is characterized by an axonopathy, while the second is defined by the early occurrence of muscle fiber atrophy (34) and membrane inexcitability (52). In fact, it appears that myopathy is often present in conjunction with neuromyopathy (20). This leads to another descriptive term: critical illness polyneuromyopathy. The development of this illness is linked to the intensity of the underlying disease as showed by a high Simplified Acute Physiology (SAPS) II score (52). Many risk factors have been reported, such as immobilization and treatments involving neuromuscular blocking agents and/or corticoids. Nevertheless, sepsis and associated systemic inflammatory response syndrome seem to have a major role in the triggering of this pathology (6, 7, 52). Clinical signs include flaccid and symmetrical weakness and possible reduction of deep tendon reflexes. Facial weakness, if present, is mild, and cranial nerves are preserved (24). The clinical diagnosis can be done with the help of the Medical Research Council score, but only when the patients are conscious. In sedated patients, diagnosis can be performed with an electrophysiological approach (52). Indeed, membrane muscle inexcitability allows an early diagnosis in unconscious patients by electromyography and recording of the muscle conductive speed (3, 17, 22, 23, 48, 52). A study of the nerve current-threshold relationship in critically ill patients has shown a reduction in neuromuscular excitability (53). In an animal model of sepsis, an increase in the reexcitation delay at the motor endplate level was observed (33, 39).

In skeletal muscle, voltage-dependent sodium channels (NaV) are responsible for the initiation and propagation of the action potential (AP), which is a determinant in muscle contractile properties. Membrane excitability of the skeletal muscle is rapidly reduced in the first stage of CIP: an early alteration of NaV is highly possible. It could correspond to a lower number of potentially excitable sodium channels to trigger membrane AP, thus leading to a decrease in muscle excitability (40). The exact mechanism is still mostly unknown. In experimental models of CIP, it was previously shown that chronic sepsis led to NaV functional alterations (40). The main effect of chronic sepsis observed on NaV was a decrease in maximal sodium current associated with a shift in fast inactivation curve toward negative potentials (12, 36, 37). Two nonexclusive mechanisms could be involved: a transcriptional one and a posttranscriptional one. On the one hand, an increase in muscle NaV1.5 was observed in a rat model of chronic sepsis (37), suggesting that a change in the ratio NaV1.4/NaV1.5 is quite possible. On the other hand, Haeseler et al. (20), using human embryonic kidney cells expressing NaV1.4, showed that acute administration of lipopolysaccharides (LPS), which is another model of sepsis, reduces availability of NaV1.4 during membrane depolarization, confirming a posttranscriptional effect of sepsis. They suggest an involve-
ment of channel phosphorylation by PKC activation. It has been shown that the α-subunit of the sodium channel had several phosphorylation sites on the intracellular loops and that the phosphorylation of these sites can lead to a decrease of the sodium current, as well as modifications of the inactivation process (20, 44).

TNF-α is an important mediator of the inflammatory response released in the early stages of sepsis. It is involved in the decrease of muscle force production (35). To our knowledge, an early effect of TNF-α on Nav properties in skeletal muscle has not yet been described. However, another cytokine also released during sepsis, ciliary nervous trophic factor (CNTF), decreased the sodium current within 5 min (44). This effect was attributed to Nav phosphorylation induced by PKC activation.

The aim of our study was to evidence a rapid alteration of the Nav properties induced by TNF-α, in isolated rat skeletal muscle fibers and to determine a potentially involved mechanism.

MATERIALS AND METHODS

Muscle Fiber Isolation

All procedures were performed according to our ethical regional committee recommendations. The experiments were authorized by departmental agreement no. A29-019-03 and were performed according to the recommendations of European Community directive no. 86/609. Female Wistar rats (n = 35; averaging 300 g) were obtained from Centre d’Élevage Janvier (Le Genest Saint Isle, France) and were used in the present study. The whole surgical procedure was carried out under deep anesthesia (pentobarbital sodium, 40 mg/kg, injected intraperitoneally).

A mixed type muscle, the peroneus longus (PL), was dissected free from the hindlimb of the rat and placed in standard medium (HEPES-buffered saline: 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 30 mM HEPES, and pH 7.3) supplemented with collagenase (3 mg/ml, type II, Invitrogen) for 2 h at 37°C to dissociate the fibers. Then, isolated fibers were sampled and rinsed before being placed in standard medium before currents were again recorded. The patches with no reliable peak current amplitude were discarded.

Patch Clamp

Fast sodium currents were recorded in cell-attached configuration (21) with macropatch clamp technique at room temperature (22 ± 2°C). This value was selected because a temperature <20°C would induce modifications of voltage dependence of gating kinetics in Nav (43). A GeneClamp 500B amplifier equipped with a CV-5-1 GU headstage was used (Axon Instruments, Foster City, CA). Micropipettes were pulled and polished from GC150TF-10 borosilicate glass (Harvard) using a horizontal pipette puller (Zeitz Instruments). They had resistances averaging 1.7 MΩ when filled with standard medium. Voltage-clamp protocols and data acquisition were performed with WinWCP software (version V4.1.2; Whole Cell Program, University of Strathclyde, Glasgow, UK) through a 16-bit analog-to-digital interface card (6024E, National Instruments). Currents were low pass filtered at 5 kHz and digitized at 48 kHz. To eliminate residual capacitance transient and leak current, a P/4 subtraction procedure was used. Visualization of the seal was possible with Hoffman contrast on an inverted microscope (Olympus IX70) and with a progressive-scan digital camera (XC5800CE, Sony). Because sodium channel density is five- to tenfold higher on the endplate border than away from the endplate (42), sodium currents were recorded away (>200 μm) from the extrajunctional membrane: this location ensured more reproducible levels in sodium currents. Before recordings, fibers were placed in CsCl-containing medium (145 mM CsCl, 5 mM EGTA, 1 mM MgCl₂, and 10 mM HEPES, pH 7.3) which brings membrane to a resting potential value close to zero by blocking the potassium channels. This was preliminary checked by measuring the resting potential of the muscle fibers with intracellular glass microelectrodes in standard and CsCl-containing media. The resting potential value was −59.1 ± 0.2 mV in control medium and −57 ± 0.6 mV in CsCl medium. Simultaneous measurements of resting potential and Nav currents were not made used in our study because it was previously shown that this technique can induce modifications of Nav properties (11). Moreover, with CsCl- and EGTA-containing medium, the recorded current was the sodium one. Then, the holding potential was fixed at −100 mV. This value was higher than the physiological value in intact skeletal muscle fibers, but at this potential, almost all the channels are in a closed-activable state, leading few sodium channels to be nonconductive during the depolarizing pulses (4).

A pulse protocol was then applied to the patch membrane, and sodium currents were recorded. The first stimulation program was a cycle of 20-ms test pulses from −60 to 0 mV in 2-mV increments, immediately followed by 20-ms test pulses from 0 mV to +100 mV in 10-mV increments. These protocols were used to improve the voltage control during the activation of the channels. Each test pulse was preceded by four hyperpolarizing pulses allowing leak current calculation (P/4 protocol). The intervals between each test pulse were long enough (3 s) to allow complete recovery of the sodium channels from inactivation. This protocol was repeated at least three times for each patch to ensure sodium current stability. A second program was used to determine the inactivation curve of the sodium channels by applying 50-ms depolarizing pulses to the membrane before application of a 100-mV test pulse from the holding potential, thus giving a membrane potential of 0 mV. This value of the membrane potential is near to the value corresponding to the maximum sodium current. The patches with no reliable peak current amplitude were discarded.

After the current was recorded in control conditions, rat recombinant TNF-α (ref. TS9-44, Sigma) was added to the medium at various final concentrations: 2.5, 5, 10, 25, 50, and 100 ng/ml. The recordings were performed after 5, 10, 15, 20, and 25 min following TNF-α addition. Recordings were also done after addition in the bath of a specific PKC inhibitor, chelerythrine (25 μM, Sigma), before and after TNF-α addition. Finally, reversibility of the TNF-α effect was also checked: after Na current was recorded, fibers were then carefully removed from TNF-α-containing medium and returned to the control medium before currents were again recorded.

Analysis of the Results

Current-voltage curve and maximal sodium conductance. $I_{Na\ max}$ corresponds to the maximal sodium current related to membrane area under the patch pipette and is expressed in absolute value (NA). The current-voltage relationship or I-V curve represents the relationship between imposed membrane potential and maximal sodium current registered. The maximal sodium conductance ($g_{Na\ max}$) is given by the slope of the quasi-linear part of the current-voltage relationship.

Activation determination. The normalized activation curve was plotted according to the relationship between $g_{Na}$ and $g_{Na\ max}$ in the −60 to +30 mV range according to the relationship:
where $E_m$ is imposed membrane potential and $E_{Na}$ is Nernst potential for sodium, determined from the $I$-$V$ curve. 

Calculated values of $g_{Na}/g_{Na\ max}$ were fitted onto the Boltzmann equation:

$$g_{Na}/g_{Na\ max} = I_{Na}/[g_{Na\ max}(E_m - E_{Na})]$$

where $V_{m1/2}$ is potential at which half of the channels are activated and $K_m$ is slope factor.

**Fast inactivation curve.** The normalized fast inactivation curve was obtained by plotting the ratio $I_{Na}/I_{Na\ max}$ as a function of the membrane potential. $I_{Na}$ was obtained according to the inactivation program previously described (40). The obtained inactivation curve was fitted onto a Boltzmann equation.

**Time constant determination.** Time constants corresponding to activation ($\tau_m$) and inactivation ($\tau_h$) of the sodium current were calculated by fitting $I_{Na\ max}$ onto the following Hodgkin-Huxley relation:

$$I_{Na\ max}(t) = A[1 - \exp(-(t/\tau_m))]^p \cdot [h_{inf} - (h_{inf} - 1) \cdot \exp(-(t/\tau_h))]$$

where $A$ is the voltage-dependent part of the current, $P$ is exponent of $m$, $h_{inf}$ is equilibrium value reached by $h$ according to the potential, and $t$ is time. This was carried out by using the curve fitting functionality of the WCP program.

**Data analysis and statistics.** Excel (Microsoft, Redmond, WA) was used to analyze the experimental data and to perform Boltzmann curve fittings. All values are given as means ± SE. Statistical differences were determined by Student’s $t$-test or the Aspin-Welsh test after the normality of distribution (Lilliefors test) and the equality of the variances (ANOVA test) were checked. Differences were considered significant at $P < 0.05$.

**RESULTS**

**Maximum Sodium Current**

TNF-α induced a decrease in maximum sodium current ($I_{Na\ max}$) compared with control fibers (Fig. 1, A and B). The current-voltage relationship (Fig. 1C) also showed a decrease in maximum current and conductance. The percentage of sodium current inhibition was plotted according to TNF-α concentration showing that the decrease of $I_{Na\ max}$ was concentration dependent in a range from 5 to 50 ng/ml (Fig. 2). For a concentration of 2.5 ng/ml, no significant effect was observed. A plateau effect, corresponding to a maximal inhibition of 75% of the control current, was observed for concentrations equal to or above 50 ng/ml (Fig. 2).

After addition of TNF-α, a time-dependent effect was also observed showing a relatively rapid decrease of sodium current leading to an inhibition of 50% of $I_{Na\ max}$ after the first 5 min (Fig. 3). Then an inhibition averaging 75% of the initial current was observed after 7–8 min when the maximum effect was obtained after 15 min.

**Activation Curves**

Activation curves were shifted by TNF-α with a concentration-dependent effect toward negative potentials (Fig. 4A), leading to an opening of sodium channels for more negative potentials. In fact, the activation potential was not strongly modified, but the slopes of the curves were increased according to TNF-α concentration, leading to a premature activation of sodium channels. The effect of the 100 ng/ml concentration was more marked than the 50 ng/ml on the activation curves, whereas the effects of these concentrations on $I_{Na\ max}$ were not...
significantly different. Activation plateau was then reached for more negative potentials, in a concentration-dependent manner.

**Inactivation Curves**

In the same way, TNF-α induced a shift toward more negative potentials of the inactivation curves from concentrations of 10 ng/ml and above (Fig. 4B). This resulted in an earlier closure of Nav induced by TNF-α. It can be noticed that, as for the activation curve, the shift of the inactivation curve corresponding to 100 ng/ml is stronger than the others. In the opposite, the effects observed for 25 and 50 ng/ml were not different. The slope is only slightly increased when concentration of TNF-α increased. In summary, we noted a dose-dependent effect of TNF-α on the inactivation curves, but not as strong as the effect observed on activation curves.

**Time Constants**

For all of the TNF-α concentrations tested, calculation of time constants $\tau_m$ and $\tau_h$ showed no modification when compared with control values (Fig. 5).

**Effects of Chelerythrine**

Chelerythrine alone, at a concentration of 25 μM, showed no significant effect on Nav properties. When chelerythrine was added in the bath solution 10 min before TNF-α, the effects of TNF-α on the sodium maximal current and on the other Nav properties were completely suppressed (Fig. 2).

**Reversibility**

Removing the fibers from TNF-α-containing medium and putting them in control (TNF-α free) medium allowed recovery of the sodium current within 30 min. The obtained values were not different from the control ones, as shown in Fig. 6.

**DISCUSSION**

It has been shown that TNF-α can compromise muscle contractility by altering the excitation/contraction coupling mechanism (35). However, up until now, there is no evidence of a direct effect of TNF-α on muscle excitability and especially of a rapid effect on Nav.
Effect on $I_{Na} \text{max}$

Our study clearly shows that TNF-α had a significant effect on Nav corresponding to a decrease in $I_{Na} \text{max}$ and then in muscle excitability: a computer simulation of the muscle AP based on Hodgkin and Huxley type equations (1) confirmed that when sodium current was reduced by 50%, a stimulation corresponding to the excitability threshold in control conditions could not trigger an AP in TNF-α conditions (25 ng/ml). The current required to trigger an AP was increased by >15% in these conditions. The decrease observed in $I_{Na} \text{max}$ was roughly dose dependent. The concentration of 2.5 ng/ml induced no effect but from 5 ng/ml the effect on $I_{Na} \text{max}$ was significant. This corresponds to a threshold effect already observed with CNTF (44). These concentrations are in the physiological range, as concentrations up to 3 ng/ml of TNF-α are reported during sepsis in the plasma (31). There was a plateau effect from 50 ng/ml and above corresponding to a maximal inhibition of 75% of the control current. It should be noticed that even with the maximal used concentration of TNF-α, a total inhibition of the sodium current was never observed. In fact, the maximum TNF-α concentration used in the present study (100 ng/ml) could appear rather extraphysiological. However, the TNF-α concentrations reported in the literature during sepsis (31) are measured in plasma and not in the muscle interstitium. As some muscle fibers can produce TNF-α in response to LPS infusion (26), the concentration of TNF-α can also be somewhat different between plasma and muscle. Moreover, as TNF-α concentration presents a peak and then decreases, the measured concentration may be lower than the true peak concentration. We first studied the effects of physiological concentrations (2.5, 5, and 10 ng/ml) and observed an increasing effect, then we used higher doses to draw the reported dose-response curve and evidence a plateau effect of TNF-α. It could also be noticed that the dose of 100 ng/ml induced a stronger effect on the activation an inactivation curves than the 50 ng/ml concentration (Fig. 4, A and B) but without inducing a stronger decrease in $I_{Na} \text{max}$ (Fig. 2). Moreover, in a study about the effects of TNF-α on neuronal NaV, some authors used concentrations up to 100 ng/ml (10).

The decrease of $I_{Na} \text{max}$ induced by TNF-α was very rapid: the half-time of the effect was near 5 min, and the maximum effect was reached in 15 min. After that, the effect remained stable with TNF-α present in the bath. This points out that TNF-α could have a role in the inhibition of NaV and in an early decrease of excitability as it could exert its effect in the very first minutes of its release. This rapid effect also precluded a possible transcriptional effect like modifications of the channel number or of the channel type. In fact, our study shows that this effect should be related to a posttranscriptional mechanism. Our results are in agreement with Haeseler et al. (20), who showed, in another model, that acute application of LPS rapidly and partially reduced sodium current in a reversible manner by altering NaV1.4 properties.

Effect on Activation and Inactivation Curves

Our results showed that activation and inactivation were both affected by TNF-α. When TNF-α concentration was increased, the activation curve was shifted to more negative potentials. This means that NaV were opened from more negative potentials than in control conditions, then earlier. This effect is not in favor of a decrease of the sodium current induced by TNF-α. However, the inactivation curve was also shifted to more negative potentials, leading to a stronger inactivation of the NaV at the same potential. According to that, channels were closed for a more negative potential, then earlier. This shift could partly explain the decrease of the maximal sodium current obtained with TNF-α. Such an effect on the inactivation curve was also observed by Haeseler et al. (20) in their LPS sepsis model (20). Our results are also in agreement with Teener et al. (45), who reported a shift toward more negative potentials in both activation and inactivation curves in CIP. By this effect, roughly dose dependent, TNF-α
could reduce the opening time of NaV. This shift in inactivation curves should lead to an increased number of sodium channels in a nonactivable state and then contribute to the muscular inexcitability observed in CIP (24). However, the decrease of \( I_{Na_{max}} \) induced by TNF-\( \alpha \) could not be totally explained by the observed shift in activation and inactivation curves because these shifts were not significant for TNF-\( \alpha \) at 5 ng/ml and also because TNF-\( \alpha \) at 100 ng/ml still increased the shift when \( I_{Na_{max}} \) showed no supplemental decrease. Then the observed decrease in sodium current could involve different mechanisms. Among the possibilities is an internalization of NaV leading to a decrease in their total number at the membrane level during CIP (12, 36, 37). According to our results, inactivation seemed to be the prominent reason for the decrease of the sodium current in sepsis because of the speed of action observed. Then the observed effect on NaV is mostly related to disregulation of the channel rather than transcriptional modifications as suggested by Teener et al. (45).

**Effect On Time Constants**

The lack of modifications in time constants showed that TNF-\( \alpha \) did not modify the activation and inactivation speeds. Then the time-dependent part of the gating mechanisms is not affected; only the voltage dependence is involved. This could be due to an effect of TNF-\( \alpha \) on a specific site of the channel related to voltage-dependent mechanisms.

**Potential implication of a Phosphorylation Mechanism**

Previous papers showed that PKC activation by 1-oleoyl-2-acyethyl-sn-glycerol (OAG) could lead to NaV inhibition (30, 32). PKC can be inhibited by several compounds like staurosporine or chelerythrine (50). Our present study showed that a pretreatment of the fibers with chelerythrine prevented the effects induced by TNF-\( \alpha \). Indeed, in fibers pretreated with chelerythrine, no significant effect in sodium maximal current or in the activation-inactivation curves was noted. We can conclude that TNF-\( \alpha \) induces a decrease of the sodium current by a phosphorylation process involving PKC activation. These results are in agreement with the hypothesis of a rapid effect of TNF-\( \alpha \) mediated by phosphorylation of a domain of the NaV involved in gating mechanism. Modulation of NaV by protein kinases is known to be critically important in the regulation of channel function. Gating of NaV isoforms is related to molecular mechanisms like phosphorylation (9, 10, 36). The exact mechanisms will have to be checked. PKC interacts with a site in the channel inactivation gate: internal loop between domains III and IV. This site is highly conserved and is present in all of the isoforms (5). In addition, NaV have various phosphorylation sites, and three signaling pathways are described leading to PKC activation by TNF-\( \alpha \). The first one is an activation of the A2 phospholipase, then arachidonic acid and PKC. The second one involves a phospholipase C then the diacid glycerol and the PKC. The last one is mediated through the activation of a protein kinase A which can stimulate the PKC.

Gating of NaV isoforms is related to molecular mechanisms like phosphorylation (8, 9, 38). Previous studies showed that another cytokine (CNTF) also decreased the sodium currents by a PKC-mediated mechanism (44). It is known that inhibition of NaV by PKC activation is dependent on channel type (28), so effects could also be different according to the target. The observed shift could be related to the existence of the two types of sodium channels in the peroneus longus associated with a preferential inhibition of one type by TNF-\( \alpha \) (12). This point is interesting because the effects of sepsis are known to be different according to muscle type (49). However, the myocardium, which expresses NaV1.5, is also affected during CIP (24).

**Physiopathology and Clinical Implications**

Pathophysiology of CIP and especially the role of sepsis are not already fully understood. Apart of the rapid effects described here, we cannot exclude that a prolonged contact with inflammation factors released by sepsis and especially TNF-\( \alpha \) leads to transcriptional effects including a progressive change in sodium channel isoform. This could explain why some patients have long-lasting aftereffects (15, 51). Nevertheless, the reversibility of membrane inexcitability observed in humans (2) as in rat (29) supports the hypothesis that the effects of sepsis on NaV in CIP are mainly posttranscriptional. Our results showing a reversible effect of TNF-\( \alpha \) are in keeping with this hypothesis.

Similarly in humans, this acute and reversible effect could be responsible for the quick recovery observed in some patients. This mechanism was already evoked in Novak's study (29), and our results show that this effect could be at least partially explained by TNF-\( \alpha \).

To conclude, our findings are in support of an acquired channelopathy mediated by TNF-\( \alpha \), which induces a rapid decrease in sodium current and muscle excitability, both of which are involved in the first stage of critical illness polynuromyopathy.

**Conclusion**

This study shows that, in skeletal muscle fibers, TNF-\( \alpha \) reversibly decreases the maximal sodium current in a dose- and time-dependent manner. The use of chelerythrine indicates that a pathway involving PKC-mediated phosphorylation of the channel is involved. Our results support the hypothesis that TNF-\( \alpha \), a factor related to inflammation and released during early stages of sepsis, plays a role in CIP triggering. Indeed, CIP could then be assimilated to an acquired sodium channelopathy. Further studies are needed to elucidate the mechanisms involved and to increase our knowledge of the physiopathology of CIP.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**