Voltage Clamp Studies in Macrophages from Mouse Spleen Cultures

Abstract. *Voltage clamp studies of macrophages from cultures of mouse spleen macrophages produced N-shaped steady-state current-voltage curves containing a region of negative slope resistance. Some macrophages exhibit two stable states of membrane potential, having current-voltage relationships that cross the voltage axis at three points. Outward currents that turn on at voltages of +15 millivolts or greater were noted in several cells. The addition of barium chloride to the bathing medium abolished the negative slope resistance and reduced the inward currents in response to hyperpolarizing voltage steps. These data provide direct evidence that macrophages exhibit at least two different voltage-dependent conductances and demonstrate that voltage clamp techniques can be useful in studying the membrane properties of leukocytes.*

Although a great deal is known about the importance of the macrophage in host defenses and its role in the immune system, relatively little is known about the ionic permeabilities and the electrical properties of the macrophage membrane. Macrophages or macrophage-like cells exhibit a number of interesting electrophysiological properties (*1–3*). In particular, recent current clamp studies have shown that cultured mouse peritoneal macrophages can exhibit nonlinear current-voltage (*I-V*) relationships containing a region of transitional or unstable voltage (*3*). The currents in this unstable region, estimated from the rate of rise and fall of the voltage responses to injected current pulses, suggested the existence of a region of negative slope (increasing resistance) (*4*). The region of voltage instability was unaffected by tetrodotoxin and cobalt, but was abolished by both barium and rubidium ions. In addition, increasing the external potassium concentration shifted the region of unstable voltage to more depolarized levels (*4*). These results indicated that the unstable region is probably produced by a voltage-dependent potassium conductance similar to that reported in egg cell membranes (*5*).

For direct measurement of the currents in transitional or unstable regions of the *I-V* relationship of macrophages, voltage clamp studies are necessary. Voltage clamp techniques requiring impalement with two microelectrodes have at present been precluded because macrophages are extremely sensitive to microelectrode damage, which causes rapid loss of the transitional region of the *I-V* curves. I now describe voltage clamp data from cultured spleen macrophages. The data were obtained with the use of a single-electrode voltage clamp (*6*), which has proven to be effective in both vertebrate and invertebrate cells (*7*). The data presented here demonstrate that a single-electrode voltage clamp can be used to study slow membrane currents in macrophages and that, under voltage clamp, these cells exhibit N-shaped steady-state *I-V* relationships, a property usually associated with excitable cells. Cells that exhibited two stable membrane potential levels had N-shaped *I-V* curves that crossed the voltage axis at three points. Addition of barium ions to the bathing medium abolished the negative slope resistance of the *I-V* curves, thereby eliminating the two stable states of membrane potential.

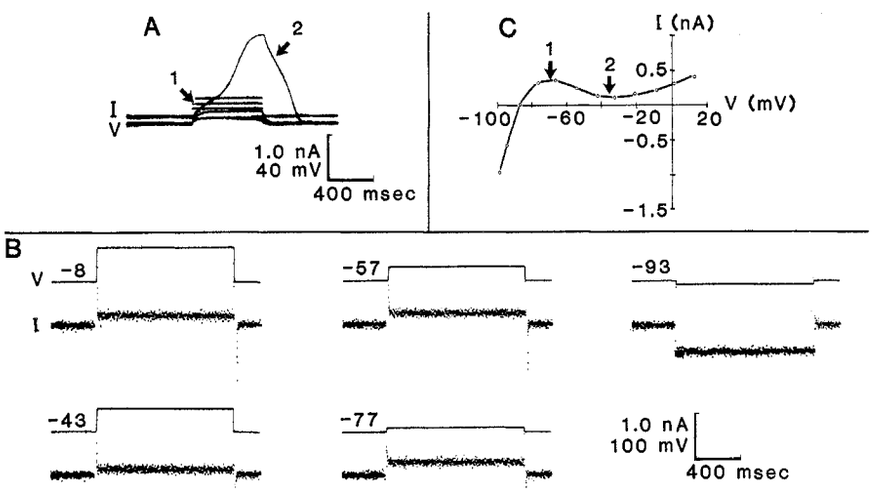


Fig. 1. Voltage clamp and current clamp data from a cultured spleen macrophage. (A) Current clamp records showing three voltage responses of cell to injected currents. The cell had a resting potential of −87 mV. The top tracing monitors current and the bottom tracing, voltage. (B) Voltage clamp data showing currents resulting from 1.2-second step commands from zero current holding potential. (C) Current-voltage plot of voltage clamp data shown in (B). Steady-state currents are measured at the end of the step command.

The macrophages (*8*) used in this study were derived from B6D2F1 mouse spleen cell suspensions that were cultured at 37°C in RPMI-1640 (Flow Laboratories) supplemented with 5 percent fetal calf serum, 1 percent glutamine, and penicillin streptomycin (100 U/ml) for 10 to 20 days before recordings. Experiments were performed at 35° to 37°C in Hanks solution containing 133 m*M* $NaCl_2$, 4.6 m*M* KCl, 1.6 m*M* $CaCl_2 \cdot H_2O$, 0.3 m*M* $MgCl_2 \cdot 6H_2O$, 10 m*M* glucose, 10 m*M* Hepes, and 0.2 percent albumin fraction V (Sigma). In four experiments, the calcium concentration of the bathing medium was raised to 4.0 m*M*. Raising the extracellular calcium seemed to increase the viability of the cells during recordings. Cultures were overlaid with light mineral oil to prevent evaporation of the bathing medium during the experiment. Intracellular recordings were obtained with fiber-filled glass microelectrodes containing 3*M* KCl with resistance of 25 to 50 megohms. The current-passing ability of the electrodes was evaluated before and after each experiment; data were used only when the electrodes behaved linearly with the injection of current. The bath was connected to the recording setup by means of a 10-kilohm 3*M* KCl agar bridge. Voltage and current clamping was achieved with a single-electrode voltage clamp (*6*), a 1-kHz switching frequency being used between voltage-recording and current-passing modes. This switching frequency is faster than the time constants of the cells, which averaged 17 msec. Measurements of the steady-state currents were made at the end of voltage steps that ranged in duration from 0.4 to 1.5 seconds. These steps settled to a stable potential within 5 to 10 msec.

Previous current clamp studies have shown that macrophages from cultures of mouse peritoneal cells exhibit nonlinear responses to injected current pulses, and, in some instances, actually show two stable states of membrane potential (*3*). In these studies, cultured macrophages obtained from spleen cell suspensions exhibited electrical properties under current clamp that were similar to those previously described in peritoneal

0036-8075/81/1023-0458$01.00/0

macrophages. Some macrophages exhibited relatively linear *I-V* relationships under current clamp, but 10 to 20 percent of the cells on which recordings were made had markedly nonlinear *I-V* relationships. Seven cells that showed nonlinear *I-V* relationships under current clamp were successfully voltage clamped (Fig. 1). The voltage responses to injected current pulses are shown in Fig. 1A. The largest voltage response is clearly nonlinear. The points in this response at which the rate of rise and fall of the voltage response first changes are marked by arrows 1 and 2, respectively. Plotting the amplitude of the voltage response against the injected current, results in an S-shaped *I-V* curve containing a transitional region under current clamp similar to those previously published (*3*). When the voltage of this cell was clamped at the resting membrane potential and then decreased stepwise to potentials in the range of −65 to −30 mV, the amplitude of the outward currents actually decreased; this is evident from the tracings of currents produced by voltage steps of different amplitude (Fig. 1B) and from the steady-state *I-V* curve for this cell determined from these and other voltage steps (Fig. 1C). The arrows in Fig. 1C point to the voltages corresponding to the inflection points that define the transitional region in the voltage response shown in Fig. 1A. It is apparent that these points determined under current clamp agree with the beginning and end of the negative slope region measured under voltage clamp. This cell had a conductance of 71 nS for inward currents in the voltage range −83 to −100 mV and 8.6 nS for outward currents in the voltage range −10 to +14 mV. The *I-V* curve is confined to a limited voltage range in this and subsequent figures because clamping to larger depolarizing or hyperpolarizing voltage steps was difficult and usually resulted in noisy, unstable records and inadequate clamping. In three cells, voltage clamp data were obtained for voltage steps to potential levels of +15 mV or greater. In these cells, slowly developing outward currents were noted for voltage steps greater than +15 mV. One of these cells is shown in Fig. 3B.

Previous experiments have demonstrated that the unstable or transitional region of the *I-V* relationship measured under current clamp is unaffected by the addition of cobalt or tetrodotoxin; this indicates that a noninactivating voltage-dependent inward sodium or calcium current is probably not responsible for producing the region of negative slope resistance in macrophages (*4*). In egg

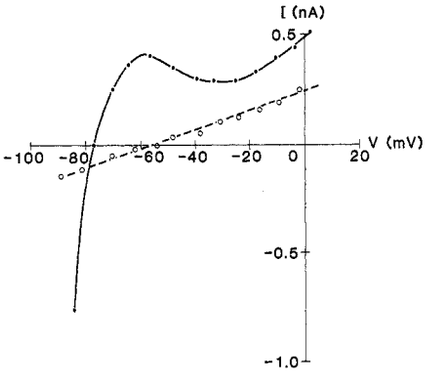


Fig. 2. Current-voltage plot of cell in normal Hanks solution (solid line) and 5 minutes after addition of 3 m*M* barium chloride (dashed line) to the bathing solution. Currents were measured at the end of 700-msec voltage steps from a holding potential of −77 mV. The cell is in Hanks solution containing 2.5 m*M* excess calcium chloride.

cells, a voltage-dependent potassium conductance that inactivates with depolarization produces a region of negative slope resistance (*9*). Barium, an agent that blocks inward potassium rectification in a number of different cell types (*10*), did eliminate the transitional region in macrophages under current clamp (*2*). The *I-V* relationships of a cell measured in normal Hanks solution and after addition of 3 m*M* barium chloride are shown in Fig. 2. The cell was voltage clamped at a holding potential of −77 mV, and the currents were measured at the end of 700-msec voltage steps. Addition of barium rapidly abolished the region of negative slope resistance, shifted the resting membrane potential from −77 to −51 mV, and decreased the inward currents for hyperpolarizing voltage steps. Those results are in agreement with previous current clamp data (*4*).

Five of the seven macrophages that were voltage clamped had N-shaped *I-V* plots similar to those shown in Figs. 1 and 2. In two of the cells, however, the *I-V* curves actually crossed the voltage axis in three places. Both of these cells exhibited two stable states of membrane potential. The voltage responses of one of these cells to injected currents under current clamp conditions are shown in Fig. 3A. Injecting a depolarizing current pulse at the membrane potential level of −83 mV (left portion of Fig. 3A) resulted in a depolarization outlasting the current pulse and stabilizing at a new membrane potential level (−21 mV). Applying a hyperpolarizing current step at the more depolarized membrane potential caused the cell to revert to the original −83 Mv membrane potential (right portion of Fig. 3A). Because this cell exhibited two stable states of membrane potential, it could be voltage clamped at either potential level (−21 mV and −83 mV) with no

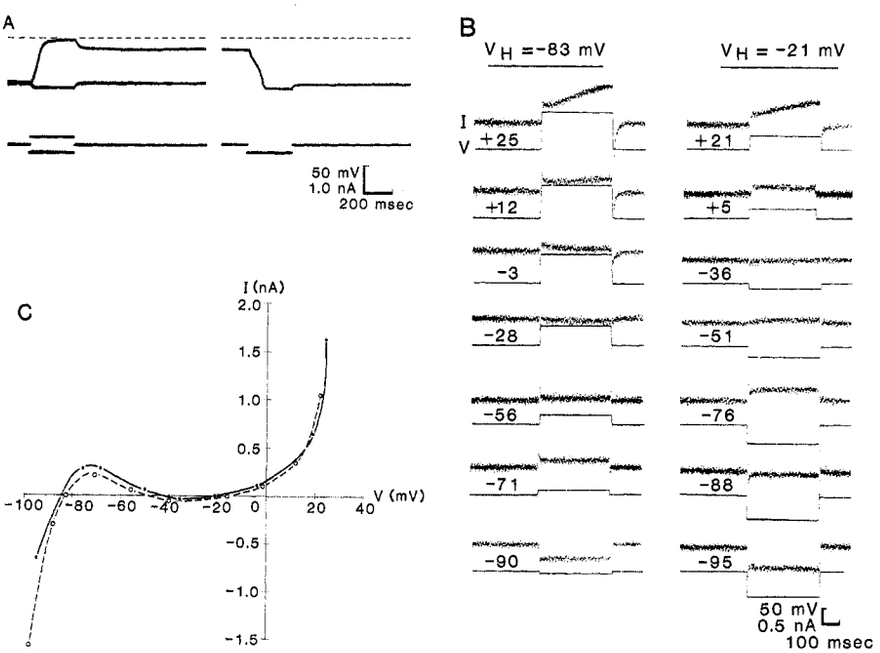


Fig. 3. Voltage clamp and current clamp data from a cultured spleen macrophage exhibiting two stable states of membrane potential. The cell was in Hanks solution containing 2.5 m*M* excess calcium chloride. (A) Current clamp records showing voltage responses of cell to injected current. Dotted line indicates zero potential. The bottom tracing monitors current and the top tracing, voltage. (B) Voltage clamp data showing currents resulting from 400-msec step commands from the two zero-current holding potentials. (C) Current-voltage plot of voltage clamp data shown in (B). Steady-state currents were measured at the end of the step command. Voltage clamp data were obtained at a holding potential of −83 mV (solid line) and −21 mV (dashed line).

net current flowing. Voltage clamp data obtained at these two potential levels, by applying step depolarizing and hyperpolarizing commands, are shown in Fig. 3B. At the holding potential (V_H) of −83 mV, increasing the level of the depolarizing voltage steps from −71 to −28 mV resulted in decrease in outward current. At the V_H of −21 mV, hyperpolarizing steps to −51 and −76 mV actually produced a small outward current. The current-voltage plots derived from the data obtained at the two different holding potentials are very similar, exhibiting a region of negative slope resistance in the voltage range −75 to −35 mV (Fig. 3C). It is evident from the plot that there is a small net inward current at voltages of −20 to −40 mV, which results in the *I-V* curve crossing the voltage axis in three places. In addition to the negative slope resistance (at −70 to −35 mV) and small net inward current, this cell exhibited an increase in outward currents for voltage steps more positive than +12 mV (evident in the voltage steps to +25 and +21 mV in Fig. 3B). This current is time-dependent, becoming activated approximately 30 msec after the depolarizing step to +25 mV from the holding potential of −83 mV (Fig. 3B) and slowly increasing, just saturating at the end of the 500-msec voltage step. The voltage clamp became unstable with larger depolarizing voltage steps, thereby preventing the outward current from being studied in greater detail.

This study demonstrates that voltage clamp techniques useful in the study of membrane properties of many types of cells can be used to study currents across the membranes of leukocytes. The region of negative slope resistance in the steady-state *I-V* curves, which is abolished by addition of barium to the bathing medium, and the outward currents that become activated at positive voltages, indicate that leukocytes exhibit at least two voltage-dependent conductances. Examining these conductances should provide information about the physiology of macrophages. Various nerve and muscle cells (*11*) as well as egg cells (*9*) exhibit N-shaped steady-state *I-V* curves. In excitable cells, N-shaped steady-state *I-V* curves have been implicated in pacemaker activity (*12*). The significance of this type of *I-V* curve in nonexcitable cells such as egg cells is not clear (*5*). Only 10 to 20 percent of the macrophages recorded from spleen cultures exhibit N-shaped *I-V* curves. Whether these cells are functionally different from other macrophages or simply reflect the absence of microelectrode-induced damage remains to be determined. Macrophages display a number of complex membrane-related functions such as motility, secretion, and endocytosis that may influence or be influenced by these conductances. The ability to directly study currents across the membranes of leukocytes and control membrane potential makes possible new experiments for the study of the role of ionic conductances in leukocyte function.

ELAINE K. GALLIN
Physiology Department, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814

References and Notes

1. E. K. Gallin, M. L. Wiederhold, P. E. Lipsky, A. S. Rosenthal, *J. Cell. Physiol.* **86**, 653 (1975).
2. E. K. Gallin, *ibid.* **107**, 21 (1981).
3. _____ and D. R. Livengood, *J. Cell Biol.* **85**, 160 (1980).
4. _____, *Am. J. Physiol.: Cell Physiol.* **214**, C9 (1981).
5. S. Hagiwara and L. Jaffe, *Annu. Rev. Biophys. Bioeng.* **8**, 385 (1980).
6. W. A. Wilson and M. M. Goldner, *J. Neurobiol.* **6**, 411 (1975).
7. D. Johnston, J. Hablitz, W. A. Wilson, *Nature (London)* **286**, 391 (1980); T. Pellmar and W. A. Wilson, *ibid.* **269**, 76 (1977).
8. The macrophages from which recordings were made were well-spread large cells (> 30 μm), often containing more than one nucleus and were identified as macrophages by morphological criteria during the recording period. Identification was confirmed at the end of each experiment by staining the cell with a nonspecific esterase stain [I. R. Koski, D. G. Poplack, R. M. Blaese, in *In Vivo Methods in Cell-Mediated and Tumor Immunity*, B. R. Bloom and J. R. David, Eds. (Academic Press, New York, 1976), p. 359]. In two cases cells were exposed to opsonized red blood cells after recordings were made to determine if they were still capable of endocytosis. Both cells ingested four or more red cells within 30 minutes, indicating that they were viable after the voltage clamp studies. The electrophysiological data from one of these cells is shown in Fig. 2.
9. S. Miyazaki, K. Takahashi, K. Tsuda, M. Yoshi, *J. Physiol. (London)* **238**, 55 (1974).
10. N. B. Standen and P. R. Stanfield, *ibid.* **280**, 169 (1978); S. Hagiwara, S. Miyasaki, W. Moody, J. Patlak, *ibid.* **279**, 167 (1978).
11. T. G. Smith, W. K. Stell, J. E. Brown, *Science* **162**, 454 (1968); D. C. Gadsby and P. F. Cranfield, *J. Physiol. (London)* **254**, 129 (1977).
12. W. A. Wilson and H. Wachtel, *Science* **186**, 932 (1974).
13. I thank T. Pellmar, D. Livengood, and J. Freschi for critical review of the manuscript and T. Pellmar for helpful advice on the use of the single-electrode voltage clamp. Supported by Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00020. The views presented in this report are those of the author. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

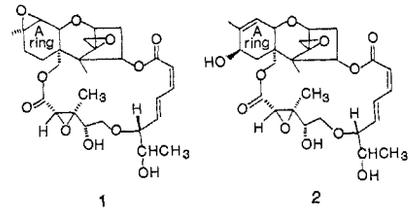
20 April 1981; revised 12 June 1981

Interaction Between the Antibiotic Trichothecenes and the Higher Plant *Baccharis megapotamica*

Abstract. *The Brazilian shrub* Baccharis megapotamica *contains significant amounts of antibiotic trichothecenes. When these plants are grown in the United States, they are devoid of the mycotoxins. Feeding experiments with fungus-produced trichothecenes show that* Baccharis megapotamica *absorbs, translocates, and chemically alters these compounds to ones with structures analogous to those found in the plant in its native habitat. The mycotoxins, which have no apparent ill effect in* Baccharis megapotamica, *kill tomatoes, peppers, and artichokes.*

We report that mycotoxins, presumably produced by a soil fungus, are absorbed, translocated, and stored in the higher plant *Baccharis megapotamica* Spreng (Asteraceae), a shrub found in Brazil. This situation, in which a significant quantity of a potent antibiotic is taken up, chemically modified, and stored by a higher plant with no apparent ill effect to that plant, appears to be unique (*1–4*).

Kupchan *et al.* (*5*) reported that an extract of *B. megapotamica* exhibited high activity in vivo against P388 mouse leukemia. From this extract was isolated a series of potent antileukemic sesquiterpenes belonging to the well-known class of trichothecene antibiotics, which previously had been isolated only from cultures of various saprophytic soil fungi (*6–8*). These particular compounds, the baccharinoids (**1** and **2**), are closely related to the macrocyclic trichothecenes known as roridins [for example, roridin A (**5a**)] and verrucarins [for example, verrucarin A (**6a**)]. The baccharinoids exhibit higher activity in vivo against P388 than do either the roridins or verrucarins. In addition, the presence of the trichothecene baccharinoids in *B. megapotamica* is curious because such compounds are normally highly phytotoxic (*6–8*), yet the baccharinoids are present



1 2

in *B. megapotamica* in such a high concentration (0.02 to 0.03 percent by dry weight) (*9*) that their presence seems unlikely to be the result of simple contamination by surface fungi.

0036-8075/81/1023-0460$01.00/0