Estimation of the Membrane Potential of Cultured Macrophages from the Fast Potential Transient upon Microelectrode Entry

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ABSTRACT Analysis of membrane potential recordings upon microelectrode impalement of four types of macrophages (cell lines P388D1 and PU5-1.8, cultured mouse peritoneal macrophages, and cultured human monocytes) reveals that these cells have membrane potentials at least two times more negative than sustained potential values (E_s) frequently reported. Upon microelectrode entry into the cell (P388D1), the recorded potential drops to a peak value (E_p) (mean −37 mV for 50 cells, range −15 to −70 mV) within 2 ms, after which it decays to a depolarized potential (E_n) (mean −12 mV) in about 20 ms. Thereafter, the membrane develops one or a series of slow hyperpolarizations before a final sustained membrane potential (E_s) (mean −14 mV, range −5 to −40) is established. The mean value of the peak of the first hyperpolarization (E_h) is −30 mV (range −10 to −55 mV). The initial fast peak transient, measured upon microelectrode entry, was first described and analyzed by Lassen et al. (Lassen, U. V., A. M. T. Nielson, L. Pape, and L. O. Simonsen, 1971, *J. Membr. Biol.* 6:269–288) for other cells. It indicates that the microelectrode introduces a leakage into the membrane, causing a change in the membrane potential from its real value before impalement to a sustained depolarized value. This was shown to be true for macrophages by two-electrode impalements of single cells. Values of E_p, E_n, E_h, E_s, and membrane resistance (R_m) measured for the other macrophages were similar to those of P388D1. From these results we conclude that E_p is a better estimate of the true membrane potential of macrophages than E_s, and that the slow hyperpolarizations upon impalement should be regarded as transient repolarizations back to the original membrane potentials. Thus, analysis of the initial fast impalement transient can be a valuable aid in the estimation of the membrane potential of various sorts of small isolated cells by microelectrodes.

Over the past 10 years, interest has been growing in membrane electrophysiological processes in macrophages during various functions such as chemotaxis (1, 2) and phagocytosis (3). Many studies were made on single cells with glass microelectrodes. In addition, membrane potential measurements on populations of monocytes (4) and neutrophilic granulocytes (5) using voltage-sensitive dyes have been reported. The present study is a critical evaluation of membrane potential measurements on single macrophages by use of glass microelectrodes. The relatively small size of macrophages has made membrane potential measurements with microelectrodes difficult and cell damage is often visible (6, 7). To obtain large cells, investigators have used thioglycolate- or oil-induced peritoneal exudates (6, 8–10), or they have increased cell size by fusion techniques (11).

Upon microelectrode entry into a cell, a single or a series of spontaneous hyperpolarizations lasting a second or more may occur in macrophages and L-cells (a fibroblast cell line) (7, 8, 12–14). Since these hyperpolarizations are slow compared with membrane potential changes in excitable cells (i.e. action potentials), investigators have studied membrane potential changes at slow chart record speeds. Hyperpolarizations eventually damp out and a constant membrane potential is sustained for the duration of the measurement. Reported values of sustained membrane potentials of macrophages and L-cells are generally within the range of −5 to −25 mV (1, 11–13, 15–17). These relatively low values compared to membrane potentials of nerve and muscle cells, and the occasional finding of higher values for macrophages (9, 10, 18), led to the speculation

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MARCH 1983 796–801

that a membrane leakage induced by the microelectrode penetration could be a source of error in membrane potential measurements (18, 20).

To avoid errors in membrane potential measurements due to membrane leakage induced by microelectrode impalement, Lassen et al. and Lassen annd Rasmussen (21, 22) introduced a method to estimate the membrane potential existing before electrode penetration. By analysis of the potential transient occurring within the first milliseconds after microelectrode entry, they showed that microelectrode penetration of Ehrlich ascites tumor cells induced membrane leakage, and they concluded that the membrane potential before microelectrode entry is at least twice as negative as the presumed membrane potential based on the sustained potential values. Surprisingly, the significance of Lassen's findings has not been recognized in the field of macrophage electrophysiology.

The objective of our study was to provide an estimate of the macrophage membrane potential, which takes into account microelectrode-induced membrane leakage, if present. We used the analysis of the fast potential transient upon impalement by Lassen and colleagues (21, 22) to obtain evidence for such a leakage. First, we studied cells of macrophage cell line P388D1 (12, 15) and found fast initial peak transients in potential upon microelectrode entry. To verify that this transient can be used to estimate the membrane potential prior to microelectrode entry, we performed double electrode measurements on single cells of this cell line. Finally, the more general significance of the fast initial impalement transient was shown in microelectrode measurements on another macrophage cell line (PU5-1.8), on cultured mouse peritoneal macrophages, and on cultured human monocytes.

MATERIALS AND METHODS

Cell Cultures: All cells were cultured on glass coverslips (24 mm diameter) in 35-mm diameter Falcon tissue culture dishes (Falcon Labware, Oxnard, CA). The cells (with the exception of human monocytes) were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, CA.) supplemented with 10% fetal bovine serum (Flow Laboratories), 200 mM glutamine (Microbiological Associates, Bethesda, MD), 1,000 U/ml penicillin G (Mycofarm, Delft, The Netherlands) and 50 μg/ml streptomycin (Mycofarm). Human monocytes were cultured in medium 199 (Microbiological Associates), supplemented with 20% inactivated newborn calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 2,000 U penicillin G/ml (Mycofarm), and 50 μg streptomyocin/ml (Mycofarm).

Macrophage cell lines P388D1 (23) and PU5-1.8 (24) were maintained by incubating ~ 5×10^5 cells and replating the cells every 5–7 d. P388D1 cells were used after 5–7 d of culture and PU5-1.8 cells after 3–5 d of culture. Mouse peritoneal macrophages were harvested and cultured as described elsewhere (25). Adherent peritoneal macrophages were cultured for 3 wk before use. Human monocytes were obtained from the blood of healthy donors using standard differential centrifugation on Ficoll-Hypaque (26). 5×10^5 cells/ml was suspended in 2 ml of medium and adherent cells were cultured for 12 d. All cells were cultured in 5% CO_2 humidified atmosphere at 37°C, and the culture medium was replaced every 2–3 d. This way, cells reached diameters between 10 and 40 μm.

Electrophysiology: For electrophysiological experiments, glass coverslips with adherent cells were secured to an open bottom Teflon dish (outer diameter 35 mm) and transferred to a micro-CO_2 incubator on the stage of an inverted Zeiss microscope (31). Then, 3.5 ml of medium was added and covered by a 2-ml layer of light gas-permeable mineral oil (Klearol, Sonneborn Div., Witco Chemical, NY) to prevent evaporation of the medium. The micro-CO_2 incubator keeps the culture at 37°C and heats and directs a gas mixture of 95% air plus 5% CO_2 over the surface of the oil. This way, the temperature gradient between the center and the side of the dish was less than 0.5°C and the pH of the medium was maintained at 7.3.

Glass microelectrodes containing glass fibers were backfilled with 4 M K-acetate (27). The resistances of the microelectrodes in the medium ranged from 100 MΩ to 400 MΩ. Membrane potentials were measured with a dual input microelectrode amplifier with capacitance compensation (WPI Series 700 Micro Probe Model 750, WP Instruments, New Haven, CT), so that two simultaneous microelectrode measurements could be made from the same cell. A slight modification of the amplifier enabled us to pass constant current pulses through the microelectrode in order to measure membrane resistances.

The membrane potential recordings were stored on FM magnetic tape and analyzed thereafter. A chart recorder was used (high frequency cut-off at about 1 Hz) to plot the slow time course of the membrane potential after impalement. The fast transients, occurring within milliseconds after impalement, were analyzed from the screen of a storage oscilloscope. Membrane potential records were accepted for further analysis if a final potential was sustained for at least 1 min; the microelectrode resistance and the base line after the measurement were equal to the values before impalement; and no visible cell damage occurred. These criteria are generally used for acceptance of a membrane potential recording (7, 11, 15). The criterion of the "1-min sustained potential" was chosen, since we found in preliminary experiments that cells which sustained a constant membrane potential for 1 min could frequently sustain it for longer than 5 min, sometimes up till 45 min (even with two microelectrodes in the cell). An additional criterion used by us was that the rise-time of the sudden potential deflection, as a result of microelectrode-entry, was not larger than the rise-time of the microelectrode.

RESULTS

Slow Membrane Potential Recordings

A record of the slow membrane potential changes occurring after microelectrode entry into a P388D1 cell is shown in Fig. 1 *a*. Upon touching the cell with the microelectrode, one sometimes sees a positive prepotential. The amplitude of this prepotential is graded depending on the pressure exerted on the cell by the microelectrode. All potential values have been measured with respect to the base line before the prepotential. After touching the cell membrane with the microelectrode, a slight tap to the set-up drives the microelectrode into the cell, as is evident from the sudden potential drop of the baseline. The notch (E_n) to which the voltage drops is sustained for a fraction of a second, after which the membrane hyperpolarizes to a peak value E_h of −44 mV. After a while, the potential returns to a depolarized stable level (E_s) of −10 mV and remains there until the microelectrode is withdrawn from the cell. Sometimes the cell goes into a series of hyperpolarizations (temporary oscillation) before it establishes the final sustained level of E_s. The amplitude of these hyperpolarizations, however, never exceeds E_h.

Membrane resistance (R_m) was monitored by passing current pulses of 0.2 nA through the microelectrode. Current pulse injection before impalement served to measure the microelectrode resistance R_e (Fig. 1). After microelectrode entry, R_e plus R_m was measured so that R_m could be calculated. This procedure proved to be acceptable, as was shown by two-electrode experiments in which R_m was also measured by the second electrode, independently of the measurement by the current injecting electrode (Fig. 2 *a*). The mean value of R_m for the P388D1 cells measured during E_s was 51.2 MΩ (Table I).

The slow membrane potential changes after impalement are accompanied by slow changes in membrane resistance. As the cell hyperpolarizes, R_m decreases with the same time course as the membrane potential; R_m is therefore at its smallest value at E_h and, when the membrane depolarizes, R_m increases. Once the membrane potential has come to rest at E_s, R_m reaches its maximum level, which is either sustained throughout the rest of the measurement or slowly increases before reaching a sustained level (Fig. 1 *a*).

Fast Impalement Transients

To investigate whether microelectrode penetration induces a membrane leak, we analyzed the potential transient within the first milliseconds after microelectrode entry on a fast time scale of a storage oscilloscope. If no leakage were introduced, a step-wise negative deflection to the real resting membrane

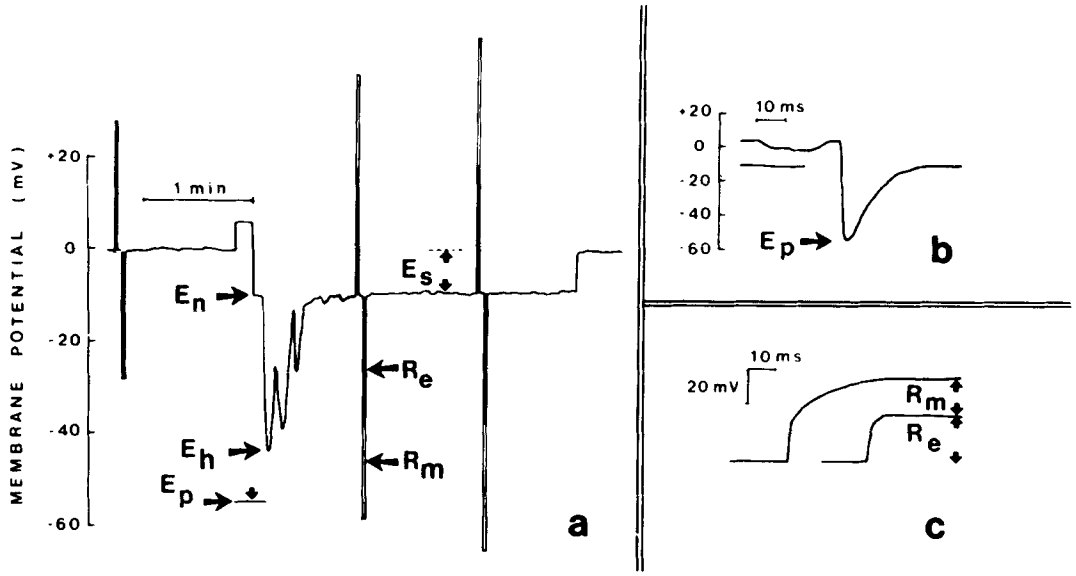

FIGURE 1 (*a*) Membrane potential recording of a P388D1 cell with a pen recorder (slow time scale). The pulses seen before and after impalement result from current pulses (0.2 nA) injected to measure microelectrode plus membrane resistance (R_m). The dark (R_e) and light (R_m) parts of the pulses during the sustained membrane potential (E_s) indicate the size of R_e and R_m. (*b*) The impalement transient of the cell in *a* on a fast time scale reveals that even though a steady state E_s of −10 mV is achieved during the recording of the cell of *a*, the membrane potential has rapidly decayed after microelectrode entry from an E_p of −54 mV to an E_n of −10 mV within 20 ms. The level of E_p is also indicated in *a*. (*c*) The lower trace is the voltage response of the microelectrode to the current pulse, indicating a microelectrode time constant (τ_e) of about 2 ms. The upper trace is the first voltage response of electrode plus membrane to the current pulse during E_s (see *a*). The time constants of the microelectrode (τ_e) and that of the membrane ($\tau_m \simeq 20$ ms) can be measured from the current response.

potential with approximately the time constant of the microelectrode ($\tau_e = R_eC_e$) is expected. If the microelectrode instantaneously introduces a leakage conductance into the membrane, the time course of the potential record is expected to consist of a steep negative deflection with time constant $\tau \simeq \tau_e$ to a peak potential value (E_p), followed by a slower decay to a depolarized membrane potential. If the current voltage relationship of the membrane were linear, the tail of this decay would be exponential with a time constant $\tau \simeq \tau_m = R_mC_m$, where R_m is the resistance of the leaky membrane and C_m the capacitance of the membrane (22). Since the time constant of the capacitively compensated microelectrode is generally at least 10 times smaller than that of the membrane of an impaled cell, it is possible to detect such an impalement transient. The fast impalement transient, taken from the record already shown on a slow time base in Fig. 1 *a*, is shown in Fig. 1 *b*. Upon impaling the cell, a sharp drop in potential is seen to a peak (E_p) of −54 mV. The potential then decays with a slower time course to a depolarized level (E_n), which appears on the chart recorder as a notch (Fig. 1 *a*). Such a fast impalement transient cannot be detected by the chart recorder. In Fig. 1 *c*, the current pulse response of the microelectrode (bottom trace) and the microelectrode plus membrane response during E_s (upper trace) is shown. Comparison of Fig. 1 *b* and *c* shows that the time constant of the downgoing deflection of Fig. 1 *b* is about equal to τ_e (lower trace in Fig. 1 *c*), while that of the upgoing deflection is approximately equal to the time constant of the impaled cell (upper trace of Fig. 1 *c*). Only 5% of the impalement transients showed initial baseline shifts much slower than τ_e. These transients were probably movement artifacts caused by the tap on the set-up. Such penetrations were rejected from the analysis.

Two-electrode Impalements

To prove the validity of the interpretation of the peak transients, we made simultaneous measurements on the same cell using two microelectrodes. After impalement of the cell with the first microelectrode, the value of the membrane potential is known prior to the second microelectrode penetration. The peak value of the second impalement transient (E_{p2}) should give an estimate of E_s, measured by the first microelectrode prior to the entry of the second microelectrode. The simultaneous records on a slow time scale of microelectrode 1 (ME1) and microelectrode 2 (ME2) are shown in Fig. 2 *a*. Both records show that the introduction of ME2 evokes spontaneous membrane potential oscillations which hyperpolarize with respect to the preceding E_s. The initial impalement transient has a peak value (E_{p1}) of −62 mV (Fig. 2 *b*); this level is also indicated in Fig. 2 *a*. Simultaneous potential transients, as measured by ME1 and ME2, as ME2 impales the cell, are shown in Fig. 2 *c*. ME2 shows the characteristic transient consisting of a peak followed by a leakage decay. ME1 only measures a decay as is expected from the interpretation of the single impalement peak transient. These records also show that the E_{p2} measured by ME2 is an underestimation of the membrane potential before ME2 enters the cell. 10 double impalement experiments gave similar results. The peak of the impalement transient of ME2 was always smaller in amplitude than the preceding E_s measured by the first microelectrode. These double electrode experiments clearly reveal the natureof the peak transients to be the result of the introduction of a leakage conductance to the membrane by the electrode. One can also see the leakage that ME2 causes, by noticing that R_m, measured by ME1, is larger both before and after the presence of ME2 in the cell (Fig. 2 *a*).

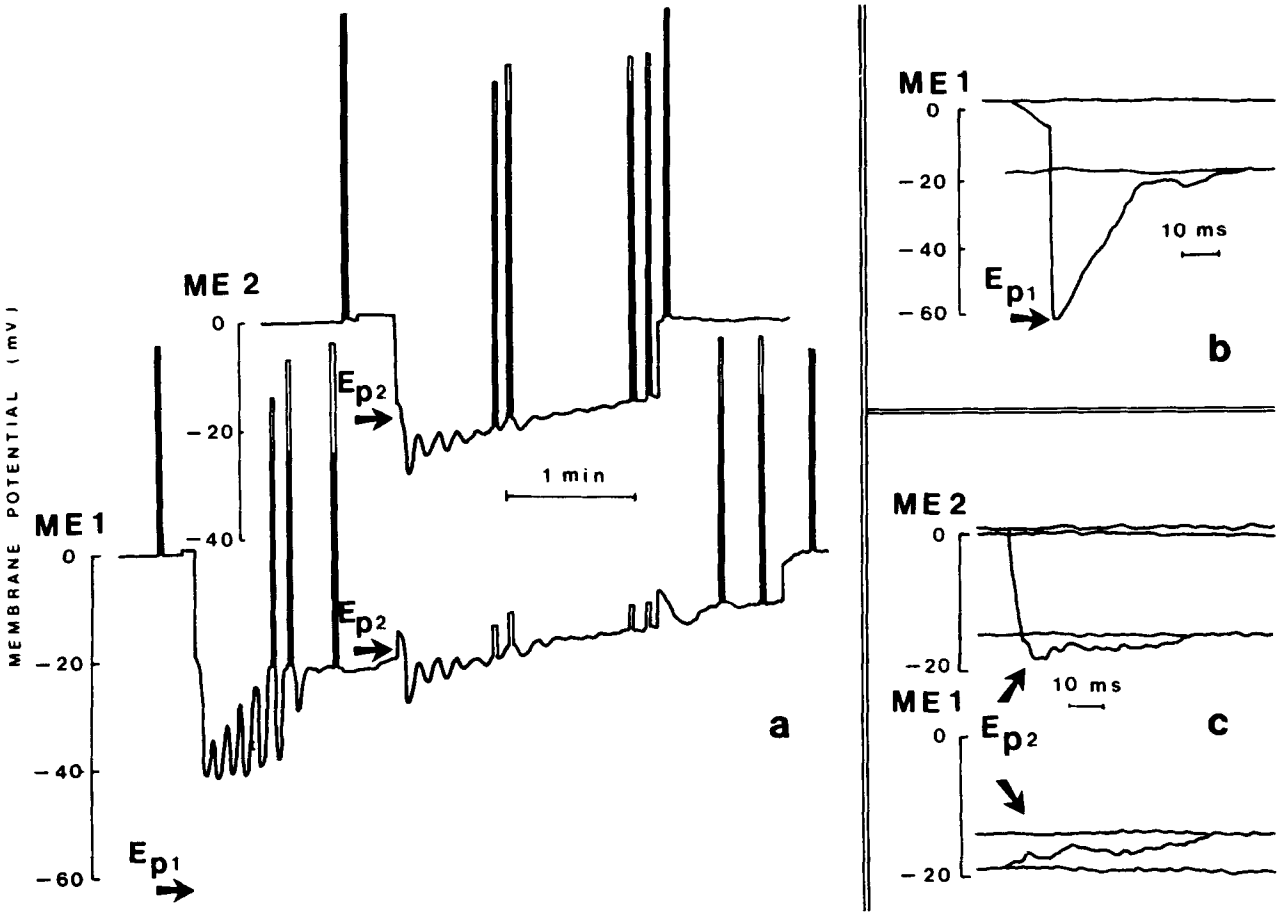

FIGURE 2 (a) The slow time course of the membrane potential record from a P388D1 cell as two electrodes successively impale the same cell. The membrane resistances (R_m) measured by ME1 as a response to current injection through ME2 have the same sizes as the resistances measured by ME2. In this particular experiment the hyperpolarizations upon entry of ME2 are smaller than those upon ME1. (*b*) The fast initial peak transient seen when the first microelectrode (ME1) enters the cell indicates that the membrane potential before electrode entry must have been at least −64 mV (E_{p1}). (*c*) Simultaneous recordings of both electrodes, on a fast time scale, as the second microelectrode (ME2) enters the cell, show that the E_{p2} value as measured by ME2 is a good estimate of the membrane potential (as measured by ME1) before ME2 entry.

Electrophysiological Characteristics of P388D1

The distribution of E_s and E_p values of the P388D1 cells is shown in Fig. 3*a*. The mean value of E_s for 50 cells was −14.5 mV (SD 7.7), which is not much different from the value of −11 mV found by Gormley et al. (15) for the P388D1. The mean value of E_p was −36.6 mV (SD 11.8). For each cell, the ratio E_p/E_s was determined so that the mean underestimation of the membrane potential could be calculated. The distribution of E_p/E_s ratios revealed that in all but two cells E_p is greater than E_s (Fig. 3*b*). From this distribution, it can be concluded that the membrane potential before microelectrode entry is on the average a factor 3.4 (SD 2.7) more negative than E_s (Table I). The distribution of E_p values from cells in which no satisfactory E_s was established (data not shown; 31 impalements resulted in a mean E_p value of −31 mV, SD 11.2), showed no significant difference from that in Fig. 3*a*. This means that even peak transients of "bad impalements" can be used to estimate the membrane potential. On the other hand, cells with E_s values sustained for periods longer than 10 min exhibited similar fast potential transients upon impalement. Thus, practically all cells suffer from an initial impalement leakage. However, many cells still reach and sustain a steady-state depolarized potential E_s. In practically all records, the membrane hyperpolarized from E_n to a peak level of E_h near E_p. To determine whether E_p exceeds E_h, we calculated the ratio E_p/E_h for each cell. The distribution of E_p/E_h is plotted in Fig. 3*c* with a mean value of 1.39 (SD 0.7) for 50 cells.

Electrophysiological Characteristics of Other Macrophages

To determine whether microelectrode induced leakage is a general problem in macrophage membrane potential measurements, we investigated macrophage cell line PU5-1.8, cultured mouse peritoneal macrophages, and cultured human monocytes. All three cell types exhibited fast and slow membrane potential transients as described for P388D1. The mean values of the different parameters are listed in Table I. In all cell types, E_p is larger than E_s. The value of E_n is about equal to E_s and the mean R_m measured was about the same for all types of cell.

DISCUSSION

The present study shows that the membrane potential of the macrophage cells investigated is more negative than the sustained potential (E_s). This difference is shown to be due to a

membrane leakage introduced by the microelectrode. A better estimate of the resting membrane potential is the peak potential (E_p), which is reached within the first milliseconds after microelectrode entry. Measurements of E_p showed that the true membrane potential of macrophages can be at least a factor two to three times more negative than has been presumed on the basis of sustained potential recordings, reported by others (1, 11, 12, 15, 16). The result of general interest for small-cell electrophysiology is the demonstration (by two-electrode impalements) that Lassen's interpretation (21, 22) of the initial fast peak transient upon impalement is essentially correct and that, therefore, his analysis can be used to evaluate the role of leakage during microelectrode measurements.

Since the membrane potential, estimated by E_p, is more negative than the peak (E_h) of the hyperpolarization following impalement, these hyperpolarizations should rather be regarded as (transient) repolarizations back to the original membrane potential. Hyperpolarizations are probably due to an increase in the potassium conductance of the membrane as a result of a rise in intracellular calcium (7, 11). It could well be that the calcium needed to trigger this response enters the cell via the leakage pathway introduced by the microelectrode (20). The intracellular calcium level could then be modulated by intracellular calcium uptake and/or release mechanisms, as suggested previously (7, 11, 28, 29), which results in slow membrane potential oscillations.

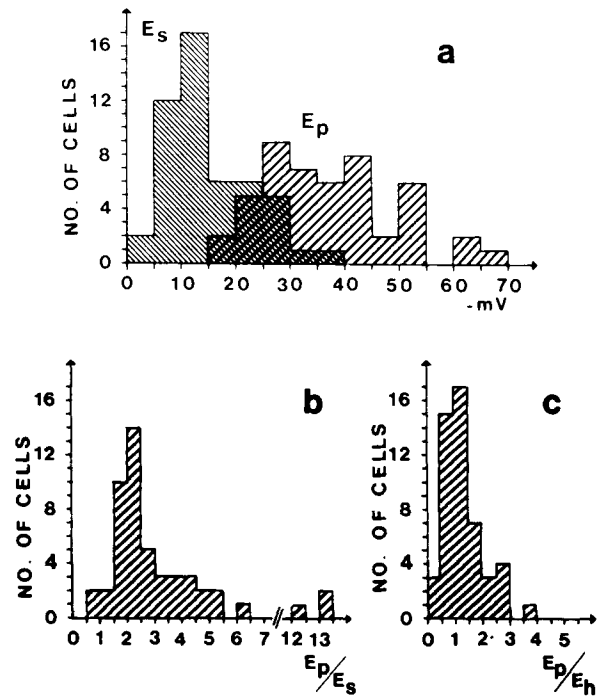

FIGURE 3 (*a*) The distribution of the sustained membrane potentials (E_s) and their initial peak values (E_p) of the P388D1 (50 cells) shows that E_s is generally less negative than E_p. (*b*) The distribution of E_p/E_s (same data as in *a*) indicates that in all but two cases E_p is more negative than E_s. (*c*) The distribution of E_p/E_h shows that E_h approaches E_p, implying that hyperpolarizing responses after impalement are repolarizations back to the original membrane potential.

A sudden entry of calcium ions through the leak pathway around the electrode tip (giving rise to an increased potassium conductance) cannot be the cause of the fast initial potential transient upon impalement. If that was the case, then the first electrode should record such a peak transient, when the second electrode enters the cell. This was never observed (cf. Fig. 2*c*).

The value of E_p remains an underestimation of the membrane potential before microelectrode entry. One of the reasons for this is that during the downward deflection to E_p the cell has time to leak (21, 30). We did not try to exactly determine the real (i.e. preimpalement) membrane potential by curve fitting of the peak transient, since the tail of this transient often did not seem perfectly exponential (see Fig. 2*b*). This may have been due to small movement artifacts (we did not use a piezo-stepper to drive the microelectrode into the cell), to nonlinear membrane properties (10, 18), or to a time dependent introduction of the membrane leakage (22). Another problem in such a curve-fitting procedure is that it is unknown whether the leakage conductance also introduces a leakage reversal potential (21). If the observed E_s were close to this leakage reversal potential, it would mean that the leakage conductance would be much larger than the preimpalement membrane conductance. This would also explain that the peak of the second electrode impalement transient does not overshoot the final E_s as much as does the peak of the first impaling electrode.

The method we have applied to determine the membrane potential before microelectrode entry has also been used to prove that sea urchin eggs have a much more negative membrane potential (−60 to −70 mV) than had been generally presumed (−8 to −11 mV) (30). Gallin (9, 10, 18) has identified a subpopulation of macrophages that also has more negative sustained membrane potentials (−60 to −70 mV) than the rest and whose nonlinear current-voltage characteristics are almost identical to those of sea urchin eggs (10). Gallin is uncertain, however, whether this subpopulation is functionally different from the rest or whether these cells simply lack microelectrode leakage (18). Lassen et al. (22) estimated that the leakage of a 3 Å hydration mantle around the glass could have a resistance in the order of 100 MΩ. If one adds this parallel conductance to the current-voltage curve that Gallin (18) found for cells with high membrane potentials, the resultant curve becomes close to linear and the resulting resting membrane potential is then between −10 and −20 mV. This calculation substantiates Gallin's view that the high resting membrane potentials could reflect the absence of microelectrode leakage. Analysis of the fast potential transients upon cell impalement would be the obvious method to determine the extent of microelectrode leakage.

Since microelectrode penetration not only lowers the steady-state membrane potential but also activates repolarizing con-

TABLE I

Electrical Characteristics of Different Types of Macrophages

Cell types	*n*	$E_p \pm$ SD (−mV)	$E_n \pm$ SD (−mV)	$E_h \pm$ SD (−mV)	$E_s \pm$ SD (−mV)	$R_m \pm$ SD (MΩ)	$E_p/E_s \pm$ SD
P388D1	50	36.6 ± 11.8	12.3 ± 4.4	30.3 ± 11.2	14.5 ± 7.7	51.2 ± 32.9	3.4 ± 2.7
PU5-1.8	35	24.6 ± 6.7	9.7 ± 4.6	25.9 ± 11.1	12.9 ± 5.9	40.3 ± 21.3	2.2 ± 0.9
Mouse peritoneal macrophages	30	33.7 ± 9.1	10.6 ± 3.6	28.0 ± 10.8	15.8 ± 5.4	42.9 ± 17.0	2.5 ± 1.4
Cultured human monocytes	16	30.4 ± 9.1	14.7 ± 3.1	35.1 ± 13.9	21.0 ± 12.7	45.5 ± 21.8	1.8 ± 0.7

ductance changes (known as hyperpolarizations), both processes must be taken into consideration for the interpretation of electrophysiological data of macrophages obtained with glass microelectrodes. The analysis of impalement transients introduced by Lassen et al. (21) is of great importance in this respect. This analysis can also be used to estimate the conductance of the unperturbed cell and to determine the ionic mechanism of its membrane potential (21).

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