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ELECTROPHYSIOLOGY OF PHAGOCYTIC MEMBRANES

III. EVIDENCE FOR A CALCIUM-DEPENDENT POTASSIUM PERMEABILITY CHANGE DURING SLOW HYPERPOLARIZATIONS OF ACTIVATED MACROPHAGES

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Summary

The roles of potassium and calcium in the slow hyperpolarizations of membranes of activated macrophages are investigated using standard intracellular electrical recording techniques.

The amplitude of spontaneous slow hyperpolarizations decreases as a logarithmic function of the external potassium concentration in the culture medium. Similar dependence on the potassium gradient is observed when different levels of membrane potentials are imposed by constant current injection. The reversal potential for electrically evoked slow hyperpolarizations is —90 mV. A 10-fold increase in external potassium concentration causes a 60 mV shift of the reversal potential towards zero.

Divalent cation ionophores (A23187 and X537A) can induce slow hyperpolarization responses in quiescent cells or permanent hyperpolarization in spontaneously active cells. The amplitude of the ionophore-induced hyperpolarizations is reduced by an increase in external potassium concentration in a manner consistent with data on slow hyperpolarization responses in the absence of ionophore.

The calcium antagonist, verapamil, depresses the slow hyperpolarization responses at the concentration of 10^{-5} M.

It is suggested that the development of the hyperpolarizing response is due to a calcium-dependent potassium channel. The data support the assumption that spontaneous and artificially elicited slow hyperpolarization responses share a common calcium-dependent mechanism.

Introduction

Macrophages are essential to immune functions such as antigen presentation, destruction of transformed cells, phagocytosis, chemotaxis and enzyme secretion. The plasma membrane plays a fundamental role in these phenomena and is the structure through which direct cell-to-cell communication is established [1,2].

The application of electrophysiological techniques to immunocompetent cells is a new addition to the approaches used to probe their membranes. Lymphocytes [3—5] and macrophages [6—9] have recently been subjected to microelectrode studies. The acceptance of an electrophysiological record still depends considerably on the subjective judgement of the researcher at the time of the measurement. In the preceding paper of this series [9] we have attempted to define objective criteria to evaluate the electrical recordings from the very motile phagocyte membrane. It was also possible to demonstrate the cytoplasmic location of the electrode's tip by means of a fluorescent tracer.

Macrophage membranes exhibit slow hyperpolarizations which have been ascribed to an increase in potassium permeability [6,7]. The functional role of the permeability changes is still unknown. Previous studies suggest among other possibilities that a similar mechanism may occur in association with chemotaxis [10] or with enzyme secretion [11].

The experiments described here were designed to probe further into the mechanism of the slow hyperpolarization responses. The results provide suggestive evidence for a calcium-dependent potassium channel that is responsible both for spontaneous and evoked slow hyperpolarization responses.

Materials and Methods

Cells. Macrophages from oil-induced guinea-pig or mouse peritoneal exudates were obtained and cultivated as previously described [9]. Culture medium was RPMI 1640 (Grand Island Biological Company, NY) with 10% fetal bovine serum and antibiotics (streptomycin, 10 μg/ml; penicillin, 100 μg/ml; fungizone 0.25 μg/ml). Experiments were performed after incubation from 72 to 96 h in 5% CO_2 at 37°C.

Results from mouse cells are shown only for the verapamil experiments (Figs. 4 and 5).

Intracellular recordings. About 15 min before the experiments, Hepes-buffered medium was added to the culture and changed at intervals of 10 min. A single microelectrode allowed simultaneous current injection and voltage recordings [4]. Glass microelectrodes were filled with 3 M KCl and their resistances ranged from 35 to 70 MΩ. The tip diameter of the electrodes under our conditions is less than 200 nm [12].

A conventional electrophysiological set-up as previously described [7] was used. Transmembrane voltage and injected current were displayed on a dual-beam oscilloscope and registered on a strip-chart recorder.

Solutions. The complete culture medium averaged 4.5 mM potassium as measured by flame photometry. KCl was added to the medium to obtain the desired concentration. No attempt to compensate for osmotic changes was

made. The addition of sucrose (up to 90 mM) for 30 min did not produce detectable alterations in cell morphology or in the electrical parameters monitored (membrane potential, input resistance and slow hyperpolarization firing). The ionophores A23187 (a gift of Eli Lilly & Co.) and X537A (a gift of Hoffmann-La Roche Co.) were dissolved in dimethyl sulfoxide and the solution added to the medium (final concentration 0.1%). Controls with dimethyl sulfoxide, added to a final concentration of 0.2%, had no conspicuous effect on morphology or electrical parameters. The experiments with salt solutions were performed by replacing the culture medium with a solution containing 125 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 20 mM glucose and 15 mM Hepes. Verapamil was a gift of the Laboratório Knoll SA (Rio de Janeiro).

Results

Spontaneous hyperpolarizations at different potassium gradients

The great variability of slow hyperpolarizations prompted us to establish the following criteria for the selection of amplitude measurements: (a) spontaneous repetitive responses stable for periods of at least 1 min; (b) maximal response as compared to other records obtained from the same cell; (c) stability of both membrane potential and input resistance during quiescent periods. Using these standards 25 cells were selected in different external potassium concentrations.

The scatter diagram of this cell population is shown in Fig. 1. The fitted regression line has a regression coefficient of 0.86 and a slope of 47 mV/decade. The analysis of the slow hyperpolarization records reveals a large variability in amplitudes from cell to cell and also in the same cell. Even using our criteria, the maximal recorded slow hyperpolarizations may not indicate the equilibrium potential as shown by the persistence of a measurable input resistance at the peak of the slow hyperpolarizations (Fig. 1B). Electrically induced

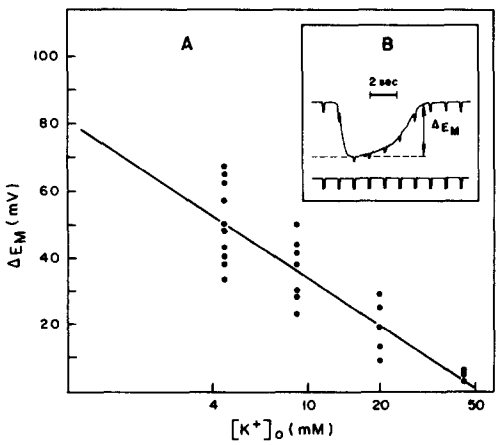

Fig. 1. Effect of external potassium concentration on spontaneous slow hyperpolarization amplitude. (A) Fitted regression line for different cells. The slope is 47 mV and the calculated regression coefficient is 0.86. (B) Sample of strip-chart recording illustrating the measurement of ΔE_M and of input resistance. Upper trace, transmembrane voltage record; lower trace, applied current monitor. ΔE_M in this case is 43 mV.

responses have a smaller diversity and a more precise quantitative approach is presented in the next section.

Reversal potential of electrically induced slow hyperpolarizations

To determine the reversal potential of the slow hyperpolarization responses, the resting potential of cells kept in salt solution was changed to the desired level (E_M^*) by application of long constant current pulses. During this procedure, the slow hyperpolarization responses could be evoked electrically by superimposing a single pulse of $1-3 \cdot 10^{-9}$ A (150 ms duration) on the injected constant current.

Fig. 2 illustrates the correlation of the applied constant current with the changes in membrane potential at rest and during slow hyperpolarizations for two different external K^+ concentrations in the same cell. The interrupted lines represent the imposed resting membrane potentials (E_M^*) and the continuous lines represent the corresponding peak value of the slow hyperpolarizations (E_{SH}). The reversal potential, which corresponds to the interaction of the two lines, is —90 mV at normal potassium concentrations (arrow 1). At an external potassium concentration of 45 mM the reversal potential shifts to —30 mV (arrow 2). This cell shows an example in which the shift of the reversal potential for a 10-fold increase in external [K^+] was 60 mV. In six other experiments similar shifts were obtained using different increases of external [K^+]. The average of this shifts was 59.5 mV/decade.

In six out of eight experiments the resting input resistance decreased in high [K^+] (Fig. 2). In the other cases, no significant alteration of the resistance was seen.

In another series of experiments, NaCl of the salt solution was substituted by choline chloride. The measured reversal potentials (five cells in 4.5 mM KCl) were not significantly alterated (mean —85 mV) and the slow hyperpolarization time course was not modified by the absence of external sodium. It is con-

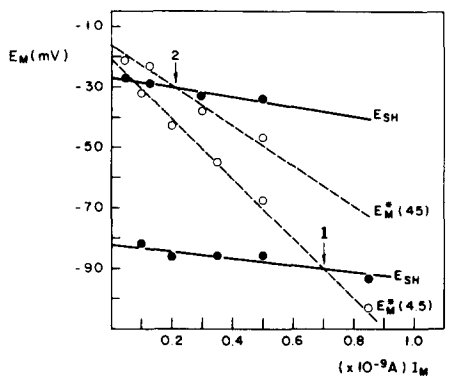

Fig. 2. Reversal potential of electrically elicited slow hyperpolarization. The total current injected (I_M) is plotted against the transmembrane potential (E_M). Open circles represent the imposed 'resting' membrane potential (E_M^*) and solid circles the corresponding peak amplitude of slow hyperpolarization, at two KCl concentrations (4.5 and 45 mM). The continuous lines represent the equilibrium potentials (E_{SH}) for the slow hyperpolarization responses. The reversal potentials obtained by the intersection of the two lines (1 and 2) are respectively, —90 and —30 mV.

cluded that there is no significant contribution of a change in sodium permeability to the reversal potential of slow hyperpolarizations.

It is noteworthy that the 60 mV shift for the reversal potential is very close to the slope predicted from the Nernst equation at 37°C (61 mV). The discrepancy with the slope found in Fig. 1A (47 mV) may be due to the inclusion of submaximal responses in the cell population selected or to the participation of other ions in the slow hyperpolarizations.

The reversal potential of slow hyperpolarizations is interpreted as the voltage at which no ion movement occurs in the channel, of which the conductance (or permeability) changes during the response. The constant current necessary to bring the resting membrane to the reversal potential is therefore exactly that necessary to balance out any background shunting of the slow hyperpolarization mechanism by other fixed ion channels; as the slow hyperpolarization mechanism itself cannot produce ion flow at its own equilibrium potential, the membrane voltage remains unaltered even though a slow hyperpolarization response is in fact taking place (as evidenced by membrane resistance measurement). This reasoning, taken together with the observed slope of 60 mV/decade for the K^+ sensitivity of the reversal potential, allows one to assert that the slow hyperpolarization response is due to an increase in K^+ conductance partly shunted by other ions.

Effect of divalent cation ionophores

The carboxylic acid antibiotic A23187 is widely used as a divalent cation ionophore [13]. When this substance is added to quiescent cells in a final concentration of $5 \cdot 10^{-7}$ M small slow hyperpolarizations are observed with amplitudes ranging from 10 to 35 mV and a duration of 8—16 s (Fig. 3, upper trace). At higher concentration ($3 \cdot 10^{-6}$ M) the slow hyperpolarization responses induced by the ionophore are of higher amplitude (20—55 mV) and longer durations (12—40 s). In a few cases (three out of ten experiments) a permanent hyperpolarization was observed.

The other carboxylic ionophore tried was X537A [14]. At the concentration of $2 \cdot 10^{-6}$ M six out of eight quiescent cells exhibited slow hyperpolarization responses of 15—50 mV amplitude and durations from 20 to 50 s. In three cases out of the six a permanently hyperpolarized state was induced. Fig. 3 (lower trace) illustrates one of these cases in which a spontaneously active cell remained hyperpolarized. The similarity in the effects of the two ionophores used and the fact that the same behavior was seen in quiescent and spontaneously active cells, indicate that an increase in cytoplasmic calcium is a step involved in the increase in potassium permeability.

Effect of divalent cation ionophore in different potassium gradients

The next step was to test if the slow hyperpolarizations induced by the ionophore were also dependent on the potassium gradient. Cells were impaled in salt solution containing A23187 at a concentration of $3 \cdot 10^{-6}$ M and different concentrations of potassium. The results are shown in Table I. It is observed that the amplitude of the slow hyperpolarizations obtained under these conditions is also affected by the potassium gradient. The K^+ effects on the amplitudes of the different hyperpolarizing responses described (spontaneous, iono-

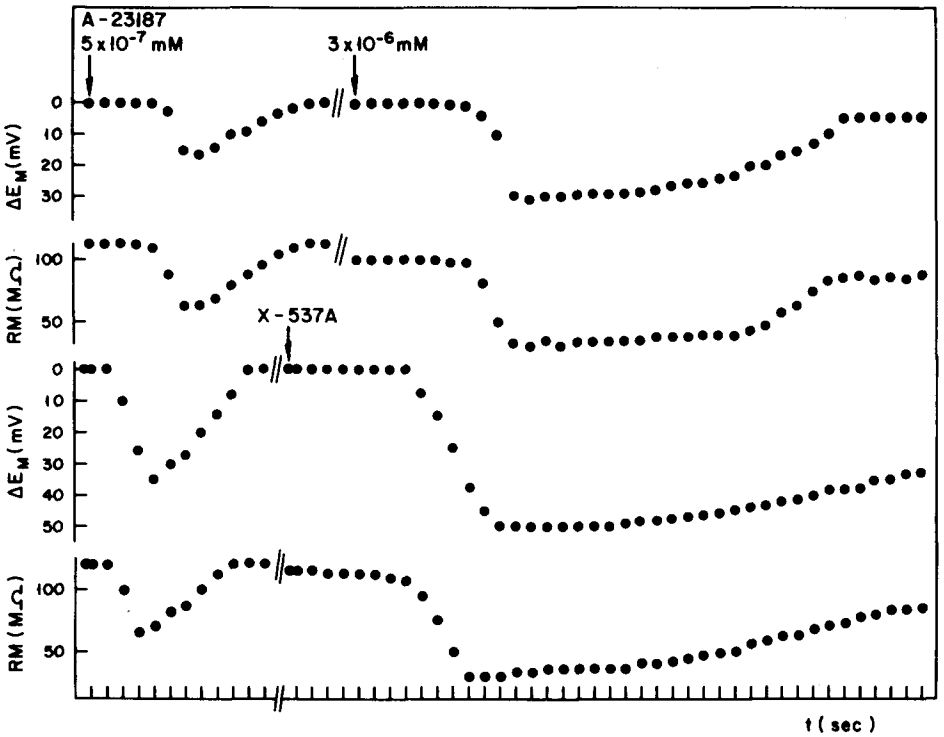

Fig. 3. Effect of divalent cation ionophores. The changes in membrane potential (ΔE_M) and the input resistances (R_M) of two cells are plotted as a function of time. The upper traces refer to A23187 at the given concentration applied to a quiescent cell as indicated by arrows. The lower traces are from an experiment with a spontaneously active cell which was permanently hyperpolarized after addition of X537A (arrow), at a final concentration of $2 \cdot 10^{-6}$ M.

phore-induced and electrically evoked) are therefore in good agreement. The evidence suggests that the three kinds of slow hyperpolarization are caused by a selective increase in potassium permeability. Furthermore, the ionophore effect also suggests that this is triggered or mediated by an increase in calcium permeability or cytoplasmic calcium concentration.

Mechanically induced slow hyperpolarizations in quiescent cells

The variety of shape and amplitude of slow hyperpolarizations makes a more

TABLE I

EFFECT OF $[K^+]_o$ ON THE AMPLITUDE OF IONOPHORE-INDUCED SLOW HYPERPOLARIZATION

$[K^+]_o$ (mM)	ΔE_M * (mV)	Number of cells
4.5	48 ± 9	6
9.0	34 ± 10	4
20.0	19 ± 8.5	3

* Mean ± S.D.

direct quantitation impossible. It is then necessary to use qualitative observations to describe further these capricious events of the macrophage membrane.

The penetration of the microelectrode frequently causes triggering of slow hyperpolarizations. This occurs in quiescent as well as in spontaneously active cells. In the former category, one may obtain responses to electrical or mechanical stimulation, but in some cells the slow hyperpolarizations only occurred upon electrode penetration. In these cases it was observed that the amplitude and duration of the single response increased approximately in accordance with the use of larger electrode tips (assessed from the higher electrode resistances).

It is noteworthy that in 34 quiescent cells that exhibited only one slow hyperpolarization response upon microelectrode penetration, the hyperpolarization always started when the cell was impaled. The slow hyperpolarization was never recorded in the middle of its time course, as would be expected if this production were not related to the impalement.

Depression of spontaneous slow hyperpolarizations by verapamil

Verapamil is a selective Ca^{2+} antagonist that has been shown to block transmembrane Ca^{2+} influx in the heart fiber [15]. Fig. 4 illustrates an experiment with mouse macrophage in which a clear depressing effect was observed in the amplitude of spontaneous slow hyperpolarizations. This record was taken from a cell that was spontaneously firing slow hyperpolarizations of approximately constant shape and amplitude. The final concentration of the drug was 10^{-5} M. Not shown in the figure is a parallel depression of the membrane resistance decrease during the slow hyperpolarizations. Some time after the second depressed response shown in Fig. 4 (dotted line), another depressed slow hyperpolarization was produced by the cell with identical shape and time course. There was no sign of cellular damage as morphology and resting transmembrane potential remained unchanged during the impalement. In addition, no change in transmembrane resistance indicating membrane damage was observed (records not shown). Depression of spontaneous responses by verapamil was a consistent finding in three additional experiments. The variability of slow hyperpolarizations described in the preceding sections makes difficult further

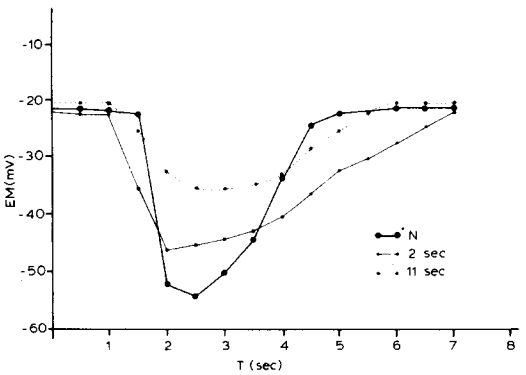

Fig. 4. Depression of slow hyperpolarization by verapamil. Membrane potential (E_M) is plotted as a function of time. Large circles indicate the control (N) and small circles are from records after the indicated time intervals. Final verapamil concentration, 10^{-5} M.

TABLE II

COMPARISON OF DATA ON Ca-DEPENDENT K CHANNELS

	Present work	Gallin et al. [6]	Nelson and coworkers [16—18]	Okada and coworkers [19—22]
Cell system	macrophage	macrophage	L-cell	L-cell
Reversal potential [a] (mV)	—90	—53	—80	—90
Shift of reversal potential with 10-fold increase of $[K^+]_o$ (mV)	60	50	57 [b]	c
Slope of E_M for each log unit of $[K^+]_o$ (mV)	47	d	e	64[c]
Proposed source(s) of calcium	external and internal [f]	d	primary source: internal	main source: external
Effect of Ca antagonists on slow hyperpolarization	verapamil blocks	not performed [g]	Co^{2+} blocks; D-600 has no effect	verapamil, Mg^{2+}, Ba^{2+} and Ruthenium red block
Contribution of Cl^- or Na^+ to slow hyperpolarization	not detectable	not performed	not performed	not detectable

[a] Obtained by superimposing different E_m with current injection.

[b] Estimated from data.

[c] A thorough analysis with a model is presented by Okada et al. [22].

[d] Effect of $[K^+]_o$ on slow hyperpolarization without varying E_M was not studied. The authors refer to variation of cytoplasmic calcium without speculating as to the source of the ion (see Ref. 5).

[e] Results are in accordance with a potassium electrode behavior for the membrane during slow hyperpolarization.

[f] Our results are compatible with an influx of Ca^{2+} through the membrane which could trigger the release of additional Ca^{2+} from intracellular stores.

[g] EGTA inhibited spontaneous and evoked slow hyperpolarization. Mg^{2+} inhibited evoked slow hyperpolarization.

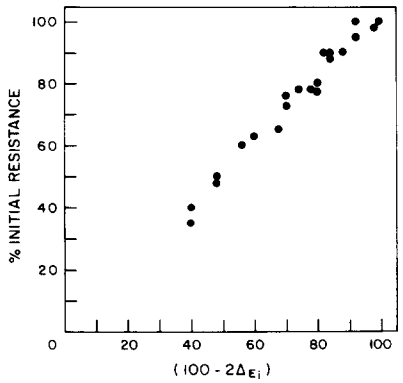

Fig. 5. Linear correlation of instant voltage change and instant decrease of the original resistance. The measurements included here were taken from successive slow hyperpolarizations (third to sixth) in the course of verapamil application. Abcissa: linear transformation equivalent to the instant voltage variation during slow hyperpolarization, given by: $100 - 2\Delta E_i$. Ordinate: instant percentage of initial resistance.

attempts to extend this finding to a more quantitative dose-effect relationship. Fig. 5 illustrates the parallel changes in voltage and resistance at different intervals during depression by verapamil. Ordinates are the percentage of the membrane resistance value before the slow hyperpolarizations. An empirical parameter indicating the corresponding percent change in voltage at the peak of the slow hyperpolarizations is plotted on the abcissae ($100 - 2\Delta E_i$). The fact that a linear correlation exists supports our interpretation of the verapamil effect. The resting membrane potential and input resistance remained unchanged, suggesting that the substance is acting on the slow hyperpolarization mechanism. Taken together with the evidence presented by the ionophore experiments, the present findings suggest that a transmembrane influx of Ca^{2+} can be implicated in the firing of the slow hyperpolarization responses.

Discussion

The effects of increasing the external K^+ concentration on the slow hyperpolarization amplitude, the reversal potentials for the slow hyperpolarizations and the shifts observed for a 10-fold increase in $[K^+]$ indicate that slow membrane hyperpolarizations in macrophage are produced by the activation of a voltage-independent potassium channel. No effect of the increase in osmolarity was observed in cell morphology or in the electrophysiological parameters. This observation may be related to the ability of inflammatory macrophages to resist adverse environments.

It is also suggested that the activation of this voltage-independent K^+ channel is either triggered or wholly supported by admission of external Ca^{2+} into the cytoplasm. The following results point in this direction. (a) Calcium ionophores induce slow hyperpolarization responses of similar characteristics (Fig. 3); (b) verapamil, a calcium-entry antagonist, depresses spontaneous slow hyperpolarizations; (d) indirect evidence also suggests that the slow hyperpolarizations induced by electrode penetration in quiescent cells could result from a leakage of calcium into the cell. Our findings with verapamil are in accordance with

those of Okada, who observed complete blocking of hyperpolarizing responses in L-strain fibroblasts with $2 \cdot 10^{-4}$ M (Okada, Y., personal communication).

The data shown here support our previous findings [7,9] in which we reported that: (1) slow hyperpolarization is blocked by tetraethylammonium, a specific blocker of potassium conductance increase in excitable tissues; (2) a permanent hyperpolarized state is induced by the potassium ionophore valinomycin; (3) mechanical stimulation with a second microelectrode or by a gentle tap on the table can induce slow hyperpolarization.

Table II is a comparison with previous work on macrophages and L-cells, included as possibly the best example of slow hyperpolarization study found in the literature [16—22]. Our results support the assumption that at the peak of slow hyperpolarization the membrane behavior closely approximates that of a potassium electrode. Gallin et al. [6] have obtained evidence consistent with this interpretation. The use of different methods may explain quantitative differences which are not very large, with the exception of the reversal potential. Although the large L-cells included here are distinct from macrophages, the two models seem to function similarly.

The main difference in the results of the two groups that worked with L-cells, is that Okada et al. [22] suggest that external Ca^{2+} is the main source of the divalent cation involved in the triggering of slow hyperpolarization whereas Henkart and Nelson [18] believe that internal stores could supply Ca^{2+} for the slow hyperpolarization. A possible explanation for the observed differences could be that the cell normally uses external Ca^{2+} as the source of divalent cation. However, after prolonged periods in Ca^{2+}-depleted medium (as occurred in the experiments of Henkart and Nelson), Ca^{2+} could be released from internal compartments. We cannot of course exclude the possibility that both mechanisms can act in the normal reponse at physiological Ca^{2+} concentrations in the external medium. For the case of L-cells the proposed model [22] is more precise because: (a) permeabilities to Na^+, Cl^- and K^+ have been measured with success [23], and data are in accordance with electrophysiological measurements; (b) the estimated intracellular [K^+] correlates well with the reversal potential for slow hyperpolarization. Intense cytoplasmic compartmentalization renders difficult the estimation of the free K^+ concentration in the macrophage cytoplasm. The great motility, irregular cell shape and extensive folding have so far thwarted the efforts to estimate with accuracy the resistance of a unit of macrophage membrane area.

Our data suggest that in macrophages external Ca^{2+} may trigger the slow hyperpolarization induced upon impalement. Mechanical stimulation described previously [9] could be subsequent to electrode vibration. This would affect membrane sealing around the microelectrode and allow influx of calcium leading to slow hyperpolarization triggering.

It was not possible to obtain reliable records from macrophages in calcium-free medium, but using macrophage polykaryons it was observed that slow hyperpolarizations are present in the absence of external calcium (Persechini, P.M., Araujo, E.G. and Oliveira-Castro, G.M., unpublished data). The available evidence is insufficient to assess the contribution of internal stores in the production of slow hyperpolarizations by macrophages.

The fact that calcium controls a number of functions is well established in

several cells: erythrocytes [24], neurons [25], pancreatic beta cells [26], salivary gland cells [27] and lacrymal gland cells [28], to name only a few. A critical and comprehensive review of the problem is found in the work of Lew and Ferreira [29].

In the case of macrophages the more direct evidence for the Ca-dependent potassium channel comes from the finding that slow hyperpolarizations can be triggered regularly when Ca^{2+} is injected into the cytoplasm (Persechini, P.M., Araujo, E.G. and Oliveira-Castro, G.M., unpublished data). Preliminary results show that in all cases where calcium iontophoretic injections are performed, the slow hyperpolarization response follows.

The physiological role of slow hyperpolarizations in macrophage is still an open question. From several speculative possibilities we can select: (1) the well established requirement for Ca^{2+} in cell motility (see the discussion of a parallel between excitation-contraction coupling in muscle and the possible role in locomotion of L-cells by Henkart and Nelson [18]); (2) the fact that chemotactic factors induce slow hyperpolarizations has prompted Gallin et al. [10] to propose an interesting model for neutrophil orientation which could also be applicable to the case of macrophages; (3) that degranulation of macrophages is induced by Ca ionophores [11] making enzyme secretion another possible function for the calcium-controlled slow hyperpolarizations. Thus, although at the moment the role of a calcium increase in the macrophage cytoplasm is unclear, there is sufficient evidence to examine further its participation in functions such as random motility, phagocytosis, chemotaxis and secretion.

Acknowledgments

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