Biochimica et Biophysica Acta, 972 (1988) 283–292
Elsevier

BBA 12368

Depolarization of macrophage polykaryons in the absence of external sodium induces a cyclic stimulation of a calcium-activated potassium conductance

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(Received 9 March 1988)
(Revised manuscript received 18 August 1988)

Key words: Macrophage polykaryon; Calcium activated potassium channel; Electrophysiology; Sodium/calcium ion exchange; (Mouse)

Macrophage polykaryons associated with the foreign body granuloma display several electrophysiological properties when studied with intracellular microelectrodes. One of the most evident properties is the slow hyperpolarization (2–5 s long, 10–60 mV amplitude), due to transient openings of Ca^{2+}-dependent K^+ channels, that is similar to those observed in macrophages. How this oscillation of membrane potential is triggered is not well known and the only way to repeatedly activate it under experimental control is through the intracellular injection of Ca^{2+}. Although this technique is important for understanding the properties of the K^+ channels, no information has been obtained about the way Ca^{2+} levels are raised and controlled in the cytosol. Slow hyperpolarizations can also be triggered by electrical stimulation, but reproducibility is low with cells bathed in physiological solutions. We then decided to investigate the effect of depolarization on the electrophysiological properties of macrophage polykaryons exposed to bathing solutions of several ionic compositions. We show in this paper that cell membrane depolarization induced by a long current pulse can trigger several patterns of membrane potential changes and that, in the absence of extracellular Na^+, repetitive oscillations of decaying amplitudes are observed in almost all the cells. They are very similar to the slow hyperpolarizations, are dependent on the presence of extracellular Ca^{2+}, and are blocked by quinine and D-600. Whole-cell patch clamp recording under voltage clamp conditions showed an outward current that oscillates and that also exhibits decaying amplitudes. The data presented here indicate that these oscillations are a consequence of the cyclic opening of the Ca^{2+}-activated K^+ channels and support the hypothesis that favors the participation of Ca^{2+} channels and of the Ca^{2+}/Na^+ exchange system in their triggering. These two mechanisms are not enough to explain either why the K^+ channels close or why the membrane potential returns to the original level at the end of each cycle. The possibility of using these oscillations as a model to study the slow hyperpolarization is discussed.

Introduction

Membranes of macrophages and macrophage polykaryons associated with the foreign body granuloma display Ca^{2+}-dependent K^+ channels that can induce a significant decrease in the membrane conductivity and a hyperpolarization of the membrane potential (2–5 s long, 10–60 mV amplitude), as demonstrated by conventional intracellular microelectrode and patch clamp recordings [1–6]. The intracellular iontophoretic injection of either Ca^{2+} or Sr^{2+} reproducibly induces these channels to open under experimental control [2,7], but the same can be achieved in some cases by

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electrical, chemical, or mechanical stimulation [2,4,8,9]. The electrical stimuli may consist of either positive or negative current pulses without any well defined threshold for amplitude, width, and membrane potential. Transient membrane hyperpolarizations and simultaneous decrease of membrane input resistance occasionally occur during intracellular microelectrode recording of both kinds of cells without the requirement for any apparent stimulus. Each of these so-called spontaneous slow hyperpolarizations are similar to the ones induced by intracellular Ca^{2+} injection but they can occur repetitively, sequentially, or even superimposed [2,4], while the Ca^{2+}-induced slow hyperpolarization occurs only once after each stimulus [2].

The mechanisms by which the intracellular Ca^{2+} is raised and controlled during spontaneous slow hyperpolarization are not known. A participation of external Ca^{2+} and Ca^{2+} channels is suggested based on the ability of verapamil and EGTA to block these oscillations [4,10], but studies of this phenomenon are made difficult because the occurrence of the spontaneous slow hyperpolarization and the electrical and mechanical stimulation have poor reproducibility. So, any experimental manipulation that could generate oscillations of membrane potential by activating the Ca^{2+}-dependent K^+ channels, without the need for the iontophoretic Ca^{2+} injections, would be an important tool to investigate the mechanism underlying the spontaneous slow hyperpolarization and the control of intracellular Ca^{2+} on macrophages and macrophage polykaryons.

In this paper we investigated the effects of depolarizing current pulses and the effect of different ionic compositions of extracellular solution on the membrane potential of macrophage polykaryons. We present a method of inducing repetitive slow hyperpolarizations by depolarizing the cell in the absence of extracellular Na^+ and discuss its possible implications in the elucidation of the mechanism of Ca^{2+} control during the slow hyperpolarization.

Materials and Methods

Cells. Macrophage polykaryons were formed on the surface of round glass coverslips (6 mm diameter) kept in the peritoneal cavity of outbred albino or AKR and C_3H mice strains for 4–60 days [2,11]. The coverslips were removed and washed in culture medium (RPMI-1640, Gibco) containing 5% fetal calf serum and buffered with 6 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Cells were maintained in this medium at 37°C for at least 20 min before electrophysiological measurements.

Solutions and reagents. Most of the experiments were done in saline solutions. The normal solution had the following composition (in mM): NaCl = 140, KCl = 5, $CaCl_2$ = 2, $MgCl_2$ = 1, Hepes = 6, pH 7.3. In the Tris solution, NaCl was replaced by 140 mM of Tris (Sigma Chemical Co.) and the pH was adjusted to 7.3 with HCl. All other saline solutions were obtained by isosmotic replacement of either NaCl or Tris by another salt, as specified in the text and in the figure legends.

Quinine (Sigma), EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, Sigma), ouabain (Sigma), digoxin (Wellcome), tetraethylammonium (Eastman) and D-600 (Knoll) were dissolved into saline solutions 1 day before the experiments and kept at 4°C until use. Amiloride (Merck Sharp & Dohme) was prepared just before use.

Electrophysiological measurements. Simultaneous intracellular recordings of membrane potential and input resistance were done using a standard electrophysiological recording system [2]. Glass microelectrode (30–45 MΩ) were back-filled with a 2.5 M KCl solution and connected to a high input-impedance preamplifier with an active bridge circuit (M4A Electrometer, WP Instruments). For Ca^{2+} injections, a second microelectrode pulled in the same conditions and filled with a 0.5 M solution of $CaCl_2$ was connected through a 200 MΩ resistor to a voltage source, as previously described [2]. During a single-microelectrode recording, the injected current was recorded through the current monitor of the electrometer. When a Ca^{2+}-injecting microelectrode was used, a virtual ground circuit was interpolated between the bath and ground, allowing measurements of current without interfering with the electrical potential of the bathing solution.

An electrophysiological recording was accepted as valid only after the following criteria had been

fulfilled: (a) the positive pre-potential (observed when the microelectrode touches the cell surface) was less than 4 mV; (b) the change of the tip potential during each recording was less than 4 mV; (c) the resistance change of the microelectrode was less than 10% of the membrane resistance measured during each impalement and was obtained by the V/I ratio of the largest current pulse of the recording; (d) the microelectrodes should have a linear response to long pulses of current (up to 30 s); (e) the change in the input resistance of the membrane during each recording was less than 10% of the initial resting value (measured by using 0.5 nA, 0.2 s standard current pulses); (f) the membrane potential during the current pulses was always less than 100 mV.

The whole-cell patch clamp recordings were performed basically as described by Hamill et al. [12]. In brief, a boroscilicate micropipet was back-filled with an intracellular solution (concentrations in mM: KCl = 140, NaCl = 5, $CaCl_2$ = 0.2, EGTA = 0.6, $MgCl_2$ = 2, Hepes = 10, pH 7.2) and connected through a 10 GΩ probe to a WPI 7050A pre-amplifier. The pipet resistances under these conditions were in the 10–20 MΩ range and the sealing resistances 5–60 GΩ. There was no resistance compensation during the recordings which were all done within 15 min after rupture of membrane, giving no time for equilibration between the cytoplasm and the pipet solution. Except for Fig. 7, where the patch clamp technique was used, all other recordings were obtained by intracellular microelectrodes.

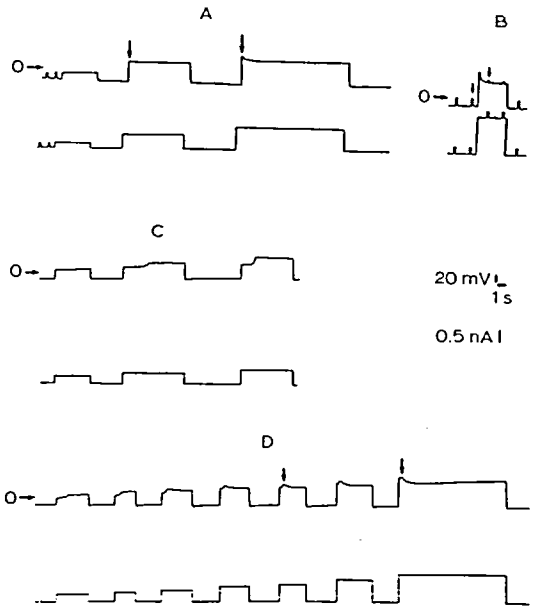

Fig. 1. Membrane potential during depolarizing pulse. (A) A peak can be seen at the beginning of the pulse when the membrane potential becomes higher than 20 mV. (B) Same as in (A). Small current pulses (0.2 nA) are included to indicate the decrease in the membrane conductivity at the plateau (arrows). (C) Step-like activity arrives above 0 mV. Note that the amplitude of the step becomes larger and the initial lag time decreases as the membrane potential increases. (D) A spike-like activity is observed at potentials higher than 20 mV. Notice that at lower potentials only the step-like activity is observed. All the cells are in culture medium. Upper trace: voltage. Lower trace: current.

Results

Membrane potential changes in response to depolarizing pulses

The effects of depolarizing current pulses on the membrane potential of macrophage polykaryons are shown in Fig. 1. When cells are impaled in normal saline or in culture medium, an ohmic response to current pulses can be obtained only if the induced membrane potentials are in the range of −80 mV to about 20/40 mV [7]. As the pulses increase, the potential responds with a peak at the beginning of the pulse and then stabilizes at a less depolarized level within 1–3 s (Fig. 1A and Ref. 7). The membrane conductance is greatly increased during application of the current, as can be seen from the small potential changes induced by the small pulses superimposed on the stimuli (Fig. 1B). In about 10% of the cells, either a spike or a step-like increase in the membrane potential can be observed during the depolarizing pulse, as shown in Fig. 1C and D. In the cells that display the step-like activity, a spike can be obtained by increasing the amplitude of the stimuli (Fig. 1D), suggesting that they may be related to each other. The patterns of these phenomena are extremely variable, can change with increasing membrane potential, and usually occur superimposed on the peak shown in Fig. 1A and B.

The patterns shown in Fig. 1 are also obtained in a variety of isotonic salt solutions in which the ionic compositions have been changed and/or drugs have been added, as summarized in Table I.

TABLE I

DIFFERENT CONDITIONS IN WHICH THE PATTERNS OF FIG. 1 WERE OBSERVED

Data represent responses to pulses that depolarize the cells to values smaller than 100 mV: '+' means observed; '−', not observed; '±', observed in less than 50% of the cells. All the ionic concentrations are in mM.

Solution	Pattern	
	initial peak (Fig. 1A)	step or spike-like [a] (Fig. 1C and D)
Culture medium	+	+
Normal solution		
without modification	+	+
$K^+ = 30$	+	+
$Mg^{2+} = 0$	+	−
$Mg^{2+} = 20$	+	−
$Cl^- = 0$ [b]	±	+
$Ca^{2+} = 0$ (EGTA = 2)	+	−
$Ca^{2+} = 20$ [c]	+	+
Tris solutions ($Na^+ = 0$)		
without modification	+	+
$Ca^{2+} = 0$ (EGTA = 2)	+	−
$Ca^{2+} = 12$	+	−
$Mn^{2+} = 5$	+	+
quinine = 0.1	+	+
Other $Na^+ = 0$ solutions		
$NH_3Cl = 140$	+	+
LiCl = 140	+	+
choline chloride = 140	+	+
saccharose = 280	+	−

[a] Although it is not clear whether the patterns of Fig. 1C and D have the same ionic origin, we cannot separate them based only on the time course of the membrane potential.

[b] Sodium isothianate substituted for NaCl.

[c] High Ca^{2+} concentration induces several modifications of the electrophysiological properties of these cells [7]. In the case considered here, the time course of the initial peak and of the step-like activity is significantly slowed, the membrane potential returns to a depolarized level at the end of the pulse and reaches the resting value only after 5–20 s. The input conductance also recovers slowly, following a time course of the potential that is in marked contrast to the normal-Ca^{2+} situation shown in Fig. 1B.

Response of cells to the absence of external Na^+

The most striking and reproducible cell response to depolarizing pulses occurs when Nacl is completely replaced by Tris-HCl in the salt solution (Tris solution). In this case the membrane potential does not stabilize after the initial peak and a series of oscillations of decreasing amplitudes is observed in more than 90% of the cells (Fig. 2). In over 300 cells studied, the first cycle begins within about 2–3 s after the onset of the stimulus, the mean number of cycles is 5, and the mean duration of each cycle lasts 3.8 s. The threshold of potential required to trigger the oscillations is around 0 mV but it is extremely variable from cell to cell and generally is in the positive range. Most of the records in this study have been obtained at an initial potential of 20–100 mV. The potential always changes in the negative direction and after the last cycle it stabilizes at the more depolarized level observed in the interval between the cycles. The membrane input resistance decreases during the oscillations and has its lowest value at the minimum of each cycle. This is more accentuated in the cycles with the greatest amplitudes.

Cyclic oscillations are also induced by depolarizing pulses when NaCl is replaced by choline chloride, ammonium chloride, or sucrose (data not shown). Remarkably, in all instance, sodium ions at concentrations greater than 5–8 mM completely abolish the oscillations (data not shown). When lithium ions are substituted for sodium ions, no oscillation is observed, indicating that this cation is probably replacing sodium as a 'repressor' of the oscillation (data not shown).

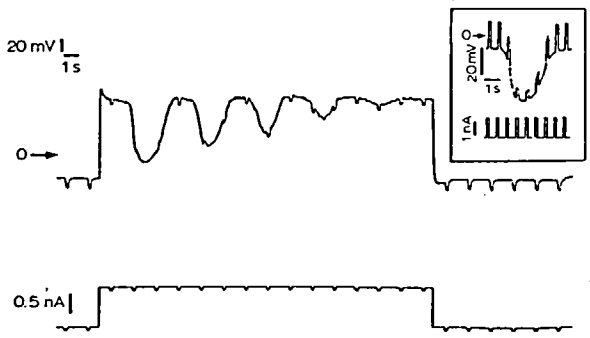

Fig. 2. Induction of cyclic oscillations. Cells were exposed to Tris solution (zero Na^+) and depolarized to about 100 mV. Notice the decrease of the input resistance during each cycle and the presence of the initial peak at the onset of the pulse. Inset: slow hyperpolarization observed in one cell bathed in culture medium without any apparent stimuli.

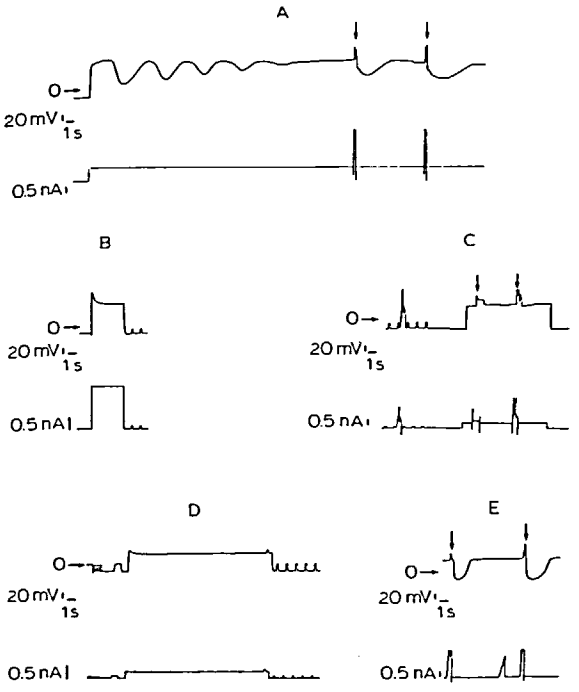

Fig. 3. A role for the Ca^{2+}-activated K^+ channels. (A) Each intracellular Ca^{2+} injection (arrows) triggers one oscillation of the membrane potential in a cell exposed to the Tris solution. Notice that these oscillations are induced after the end of the last cycle. (B) A depolarizing pulse does not trigger cyclic oscillations when quinine (0.1 mM) is present in the Tris solution. (C) Cell in Tris solution without K^+. No oscillations are induced either by the depolarizing current or by intracellular Ca^{2+} injection (arrows). (D) No oscillation can be triggered during depolarization in Ca^{2+}-depleted Tris solution (2 mM EGTA and no Ca^{2+} added). (E) Each iontophoretic Ca^{2+} injection triggers an oscillation of the membrane potential in a Ca^{2+}-depleted Tris solution (5 mM EGTA).

Role for a Ca^{2+}-activated K^+ conductance

The time course of membrane potential change and the decrease in input resistance during each cycle given a pattern that closely resembles the slow membrane hyperpolarizations already described for macrophages and macrophage polykaryons in normal culture medium in the absence of depolarization pulses (inset in Fig. 2B) [2]. In order to further substantiate this point we decided to investigate in more detail whether the depolarization-induced cyclic oscillations observed in low sodium concentrations are also a consequence of cyclic openings of the Ca^{2+}-activated K^+ channels (Fig. 3). First of all we relied on the observations that the opening of these channels in macrophages and macrophage polykaryons can be easily induced by intracellular iontophoretic injection of Ca^{2+} [2,7]. In Fig. 3A we show that during a depolarizing pulse in the presence of Tris solution each Ca^{2+} injection is followed by one cycle of hyperpolarization and by a corresponding decrease of resistance, indicating that these channels are present and can be activated by calcium under these conditions. One marked characteristic of the oscillations triggered by depolarization in the absence of extracellular Na^+ is that their amplitude decreases at each cycle and finally vanishes. Regarding the participation of the Ca^{2+}-activated K^+ channels, two questions can be asked at this point; is this progressive decay of potential amplitudes a consequence of K^+ channel inactivation or a phenomenon that results from a progressive decrease of the final activity of intracellular calcium at each cycle? The results of Fig. 3A show that a Ca^{2+} injection remains effective in triggering slow hyperpolarization even after the oscillations have vanished, indicating that the K^+ channels still respond to Ca^{2+} activation and that an inability to generate increased levels of intracellular Ca^{2+} may be involved in the potential amplitude decrease.

We also investigated the effects of quinine, a potent inhibitor of the Ca^{2+}-dependent K^+ channel of several cell types [13], including macrophage polykaryons [7]. In doses ranging from 0.1 to 1.5 mM, quinine completely abolished the pulse-induced oscillation (Fig. 3B). The action of quinine is reversible (data not shown) and occurs within 10 min of drug application. The absence of extracellular K^+ (a requisite for the activation of Ca^{2+}-activated K^+ conductance in red blood cells [14,15]) also abolishes the oscillations (Fig. 3C). The effects of quinine and the K^+ depletion favor the notion that the inhibition occurs at the level of the K^+ channel and not as a consequence of the interference with the mechanisms of intracellular Ca^{2+} increase. Accordingly, iontophoretic injection of Ca^{2+} does not induce an oscillation of the membrane potential either in the presence of quinine [7] or in the absence of extracellular K^+ (Fig. 3C).

Depletion of extracellular Ca^{2+} in the extracellular Tris solution (zero-$CaCl_2$ + 1–5 mM EGTA) also inhibits the pulse-induced oscillation (Fig.

3D). This effect is reversible (data not shown) and appears to occur at a stage that antecedes activation of K^+ channels, since iontophoretic Ca^{2+} injections given during the application of depolarization pulses induce membrane potential oscillations and corresponding decreases in input resistance that occur exactly the same way as they would in the presence of Ca^{2+} (Fig. 3E). Moreover, the Ca^{2+} channel blocker D-600 at a concentration of 100 μM (but not 10 μM) has the same effect as extracellular Ca^{2+} depletion, including the observation that its effect can be overcome by Ca^{2+} injections (data not shown).

In conclusion, the data presented here support the hypothesis that potential oscillations induced by depolarization in the absence of extracellular Na^+ are triggered by cyclic rises in the intracellular Ca^{2+} concentrations that trigger the opening of Ca^{2+}-activated K^+ channels in a way similar to that observed during the spontaneous slow hyperpolarizations of macrophages and macrophage polykaryons.

Effect of intracellular pH and amiloride

The requirement for low Na^+ in the stimulation of cyclic oscillations led us to consider the possibility that a Na^+/H^+ antiporter and intracellular pH might be involved in the control and triggering of the oscillations. Our working hypothesis argued that in zero-Na^+ condition the Na^+/H^+ antiporter would be either inhibited or reversed. In any case we should expect a tendency towards an acidulation of the cytoplasm and/or an increased depletion of the intracellular Na^+.

The effect of lowering intracellular pH was tested directly in the experiment of Fig. 4 in which we added CO_2 into the Tris solution immediately before cell impalement, a treatment that is effective in acidifying the cytoplasm of most cells. The ability of the cells to oscillate during the depolarizing pulses is highly depressed under these conditions (Fig. 4A) and the effect can be reverted by returning to the normal Tris solution without CO_2 (Fig. 4B). It should be noted that the extracellular pH was not affected significantly due to the presence of Hepes and high concentrations of Tris.

In order to investigate further the involvement of Na^+/H^+ antiporters, we studied the effect of amiloride, a known inhibitor of this ion exchange

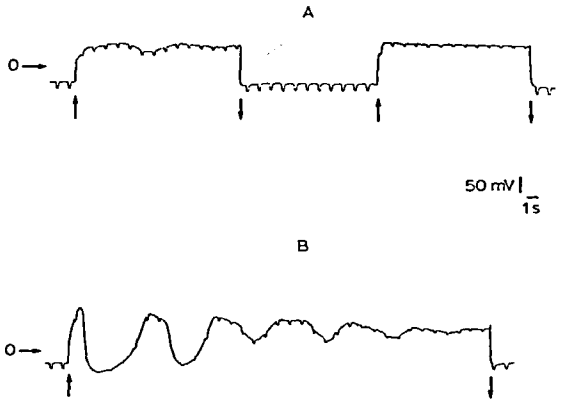

Fig. 4. Inhibition of the oscillations by lowering the intracellular pH. (A) The cell was impaled and stimulated with depolarizing pulses, 5 min after the Tris solution had been briefly exposed to CO_2. Notice the almost complete absence of oscillations. (B) The same cell was impaled again, 4 min after returning to a new Tris solution without CO_2, and cyclic oscillations were triggered upon stimulation. The amplitudes of the current (not shown) are 3 nA and 4 nA in (A), 4 nA in (B), and 0.5 nA in the small pulses. Arrows indicate the beginning and the end of the pulses.

mechanism [16,17]. As shown in Fig. 5, amiloride induces a significant increase in the number of cycles observed during depolarization in the absence of external Na^+ (10–25 cycles, 10 cells as compared to a mean of 5 cycles in the absence of the drug), but it does not stimulate the oscillations when Na^+ is present in normal concentrations (not shown).

Effect of different stimuli

We next designed a series of experiments in which different kinds of depolarizing pulses were

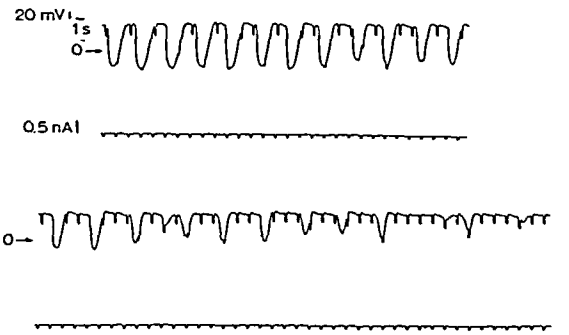

Fig. 5. Effect of amiloride. Continuous recording of a cell stimulated in Tris solution, 10 min after the addition of 1 mM of amiloride. 25 cycles can be observed.

used (Fig. 6). We show first that the occurrence of multiple cycles is uniquely associated with the application of the depolarizing pulse (Fig. 6A). If the pulse is interrupted during the course of one oscillation, the membrane potential value changes immediately to a hyperpolarized state (indicating a deviation towards the K^+ equilibrium potential) and then repolarizes to the resting membrane potential following a time course typical of the oscillation, but no subsequent cycle occurs. When the oscillation is interrupted in the interval between two cycles, the membrane potential returns directly to the resting value and no new cycle begins (not shown). We then investigated the effect of restimulating cells after the oscillations have vanished. As shown in Fig. 6B cells promptly initiate a new series of cycles if the new pulse is applied after a suitable interval, generally greater than 5 s. A cyclic response to restimulation can also occur at the end of the cycles if the current

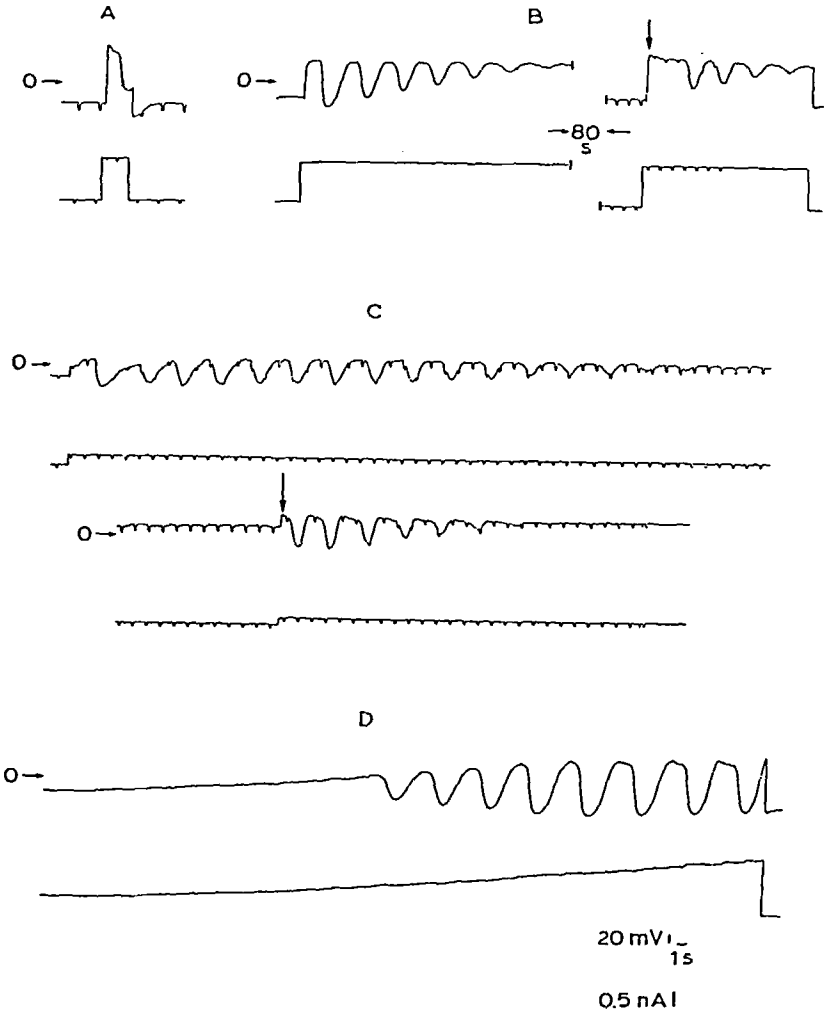

Fig. 6. Effect of different stimuli. (A) The first cycle is completed but no other cycle is observed when the stimulus is interrupted during the oscillation. (B) A second stimulus (arrow) applied 23 s after the end of the first pulse can trigger a new sequence of oscillations after the first one has been completed. During the first stimulus, the potential does not start to oscillate again, even 1 min after the end of the first series of cycles. (C) New oscillations can also be triggered by a step increase of the amplitude of the stimulus (arrow). (D) The number of cycles can increase and their amplitude kept without decaying for a longer time when the stimulus increases continuously (controlled by hand). All the cells were impaled in Tris solution.

pulse is steeply increased to a new level without returning first to the resting membrane potential (Fig. 6C). Fig. 6D shows that the amplitude of the cycle can be sustained if the depolarizing current increases continuously. In this case, there exists a limiting rate (in the range 1–5 mV/s) for the membrane potential increase below which oscillations do not occur (not shown).

Whole-cell voltage clamp

The electric current applied to stimulate the cells depolarizes the membrane but the potential is not clamped. So it is not clear whether the change in the potential observed during each cycle is necessary to complete that cycle and initiate the next one or whether there are oscillations of ionic currents through the membrane even at constant potential. To investigate this problem we performed whole-cell recordings of the macrophage polykaryons in Tris solution using the patch-clamp technique. We observed that a depolarizing shift of the clamping potential induced the occurrence of an oscillating outward current that had decreasing amplitudes and a time course similar to the cyclic oscillations of membrane potential previously described (Fig. 7). Whole-cell current clamp recordings using the same technique also displayed cyclic potential oscillation similar to those of Fig. 2 (not shown), indicating that during whole cell recordings, cells display the same phenomena observed with intracellular microelectrodes and that the oscillating current indeed corresponds to the current involved in the oscillation of the potential. This result indicates that the oscillations triggered in the absence of Na^+, and the probable oscillations of intracellular Ca^{2+} that activate the K^+ channel, are stimulated by depolarization and

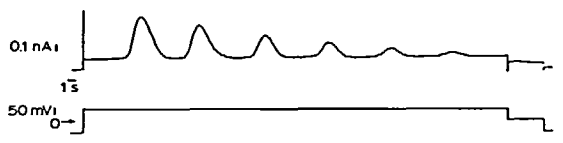

Fig. 7. Outward current during the oscillations. Whole-cell patch clamp recording was done in a cell bathed in Tris solution, 3 min after the rupture of the cell membrane. The potential inside the pipet (lower trace) was clamped at −80 mV and then shifted to +80 mV. An outward current oscillating with decaying amplitudes (upper trace) was observed during the depolarizing stage.

do not require a repolarization to start a new cycle.

Discussion

In this paper we have identified different patterns of membrane potential response during stimulating of macrophage polykaryons with depolarizing current pulses. We showed that cyclic potential oscillations of decreasing amplitudes can be induced when the extracellular sodium ion concentration is lower than 5 mM. We also observed that the sharp peak of potential recorded just after the beginning of the pulse and the less common spikes and steep depolarization can occur in several different conditions as summarized in Table I.

The peak pattern was almost always present in all the solutions tested except in the absence of Cl^-, when it was not observed in about 50% of the cells. The low incidence of the step-like and the spike patterns makes it difficult to draw any meaningful conclusion about the ionic nature of these phenomena, but the data presented here give some important information, such as their occurrence in the absence of external Na^+ and Ca^{2+}, and may be used in future experiments. Considering the evidence favoring the existence of several kinds of ion channels in the membrane of macrophages, such as an inward rectifier K^+ channel, a delayed outward rectifier K^+ channel, a Cl^- channel and a channel related to the Fc receptor [18–21], their possible involvement in the phenomena described here should be considered.

We focused attention on the oscillations that can be triggered by depolarizing current pulses when all the external Na^+ is substituted either by Tris, choline, NH_3^+, or sucrose, but not by Li^+. They are very reproducible (more than 90% of the tested cells) and are amenable to experimental control, which make them a very attractive model to investigate the triggering mechanisms of the spontaneous slow hyperpolarizations already described in macrophages and macrophage polykaryons. We presented several pieces of evidence indicating that these oscillations are in fact a consequence of the cyclic activation of Ca^{2+}-dependent K^+ channels: (a) the pattern of the time course of the changes in potential and resistance

of each cycle are similar to those already described for the slow hyperpolarizations of macrophages and macrophage polykaryons, a case where the participation of this channel has already been characterized [1–6]; (b) the Ca^{2+}-activated K^+ channel can be activated by intracellular Ca^{2+} injection even in the absence of external Na^+ (Fig. 3); (c) inhibition by quinine or absence of extracellular K^+; (d) the phenomenon is blocked by $[Ca^{2+}] = 0$ plus EGTA and also by D-600, without interfering with the response of the intracellular Ca^{2+} injection; (e) the maximal amplitude of each cycle is dislocated towards a more positive potential when the concentration of K^+ in the extracellular solution is raised to a higher level (not shown).

The question then arises concerning the mechanisms of increase and control of cytoplasmic Ca^{2+}. Two major mechanisms seem to be important: (a) the Ca^{2+} channels and (b) the Na^+/Ca^{2+} antiport. The dependence on external Ca^{2+}, the blockade by D-600 and the fact that the oscillations are triggered by depolarization suggests a role for Ca^{2+} channels [22,23]. The Na^+/Ca^{2+} exchange mechanism, first proposed by Reuter and Seitz for cardiac cells [24], also has several features that fit the phenomenon we have been describing. It can utilize the electrochemical gradient of Na^+ either to increase or to decrease the intracellular Ca^{2+} concentration [25–28]. This mechanism is not electroneutral (it exchanges three or four sodium ions for one Ca^{2+} [28]), which implies that an influx of Ca^{2+} can be induced by depolarization in the absence of external Na^+. Another important characteristic of the Na^+/Ca^{2+} antiport is the fact that its electrochemical potential can reach a new equilibrium just after depolarization. This feature could explain the decay of the amplitudes of each cycle during the oscillations, the stimulation of a new series of cycles by a step pulse (Fig. 6C), and also the non-vanishing oscillations induced by a continuously increasing pulse (Fig. 6D).

The blockage of the oscillations by increasing the CO_2 concentration of the extracellular medium establishes for the first time a relationship between the decrease of the intracellular pH and the inhibition of the slow hyperpolarizations. This result suggests that an increase in the intracellular pH could have the opposite effect, acting either as an activator or a co-activator of the oscillations. The increase in the number of oscillations observed in the presence of amiloride favors this hypothesis since in the absence of extracellular Na^+, the Na^+/H^+ antiporter [16,17,29] is expected to work in the reverse direction, loading H^+ into the cells. The presence of amiloride would then avoid this inhibitory factor and favor and increase of pH. We tried to increase the intracellular pH directly by adding ammonium chloride to the Tris solution but we were not able to observe any significant increase in the number of cycles during the depolarizing stimuli (not shown). At this moment we do not know which sites are sensitive to pH. Inhibition of the Na^+/Ca^{2+} and the Na^+/H^+ exchange mechanisms or even an direct effect on the Ca^{2+} and K^+ channels by low pH cannot be ruled out. A coupling between the Na^+/H^+ and a Ca^{2+}/H^+ exchange mechanisms [30] should also be considered.

Another important question that has to be considered concerns the mechanism by which the membrane potential returns to the highest positive value after the minimum reached at each cycle. The data presented here allow us to suggest that it is due neither to a voltage-dependent mechanism nor to an inactivation of the Ca^{2+}-activated K^+ channels since the current can oscillate under constant voltage conditions (Fig. 7) and the K^+ channels can be opened by an injection of Ca^{2+} even after the oscillations have vanished (Fig. 3A).

The similarities between the oscillations described here and the spontaneous slow hyperpolarization are evident and it is possible to assume that most of our conclusions should apply to both. The Na^+/Ca^{2+} antiporter may or may not be important for the spontaneous slow hyperpolarization that occurs in normal Na^+ culture medium and any other source of continuous influx of Ca^{2+} should fit the model.

Acknowledgements

We are indebted to Dr. G.M. Oliveira-Castro for discussions, suggestions and helping to support the experiments, and to Dr. M.O. Massuda and Dr. A.C. Carvalho for discussions, suggestions and helping in the patch clamp technique. We want to thank Dr. A. Pimentel for helpful discus-

sions and suggestions, J. Barbosa for his excellent technical assistance, and Merck Sharp & Dohme for kindly providing amiloride. We also would like to thank Dr. J.D.-E. Young for his critical comments on the manuscript. This work was supported by grants from the National Research Council (CNPq), National Fund for Development of Science and Technology (FINEP-FNDCT) and Council for Graduate Education of the Federal University of Rio de Janeiro (CEPG-UFRJ). A preliminary report of this work has been published [31].

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