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Outward potassium current oscillations in macrophage polykaryons: extracellular calcium entry and calcium-induced calcium release

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Abstract

Outward current oscillations associated with transient membrane hyperpolarizations were induced in murine macrophage polykaryons by membrane depolarization in the absence of external Na^+. Oscillations corresponded to a cyclic activation of Ca^{2+}-dependent K^+ currents (IK_{Ca}) probably correlated with variations in intracellular Ca^{2+} concentration. Addition of external Na^+ (8 mM) immediately abolished the outward current oscillations, suggesting that the absence of the cation is necessary not only for their induction but also for their maintenance. Oscillations were completely blocked by nisoldipine. Ruthenium red and ryanodine reduced the number of outward current cycles in each episode, whereas quercetin prolonged the hyperpolarization 2- to 15-fold. Neither low molecular weight heparin nor the absence of a Na^+ gradient across the membrane had any influence on oscillations. The evidence suggests that Ca^{2+} entry through a pathway sensitive to Ca^{2+} channel blockers is elicited by membrane depolarization in Na^+-free medium and is essential to initiate oscillations, which are also dependent on the cyclic release of Ca^{2+} from intracellular Ca^{2+}-sensitive stores; Ca^{2+} ATPase acts by reducing intracellular Ca^{2+}, thus allowing slow deactivation of IK_{Ca}. Evidence is presented that neither a Na^+/Ca^{2+} antiporter nor Ca^{2+} release from IP_3-sensitive Ca^{2+} stores participate directly in the mechanism of oscillation.

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- Membrane potential oscillations
- Calcium-induced calcium release
- Ca^{2+} ATPase

Introduction

Transmembrane potential oscillations have been encountered in many cell types, including both excitable and non-excitable cells. They are often associated with oscillations in intracellular Ca^{2+} concentrations that activate Ca^{2+}-dependent K^+ currents (IK_{Ca}) (1-3) and can be useful as an alternative to direct measurement of intracellular Ca^{2+} concentration (4,5). This correlation has been established by simultaneous recordings of membrane potential and intracellular Ca^{2+} in fibroblasts (2) and endothelial cells (3). In non-excitable cells, Ca^{2+} oscillations are generated by different mechanisms: cyclic Ca^{2+} release from intracellular sources, either by inositol triphosphate (IP_3) as in *Xenopus* oo-

cytes and megakaryocytes (6-8), or by cytoplasmic Ca^{2+}-activated mechanisms as in hamster eggs (9), or Ca^{2+} entry-dependent oscillations as in endothelial cells (3). These different mechanisms may also interact to modulate Ca^{2+} oscillations, as is the case for IP_3- and Ca^{2+}-sensitive mechanisms in pancreatic acinar cells (10), hepatocytes and endothelial cells (1). The presence of extracellular Ca^{2+} is also important, since oscillations cannot be sustained when Ca^{2+} is removed from the extracellular medium. Extracellular Ca^{2+} allows replenishment of intracellular stores via Ca^{2+} influx pathways that are not yet fully understood (1). In non-excitable cells, the oscillation patterns vary widely (1). In a single cell type, the pattern of oscillations can vary depending on the stimulus and on the individual cell. However, the response of the same cell to repeated exposure to the same agonist can be remarkably similar (1).

In macrophages, spontaneous membrane potential oscillations due to a Ca^{2+}-dependent increase in K^+ conductance have been described (11). Because of their episodic nature, these spontaneous oscillations are virtually inaccessible experimentally. However, qualitatively similar oscillations can be induced reproducibly in macrophage polykaryons when the cells are depolarized in Na^+-free medium. These oscillations consist of cyclic hyperpolarizations of membrane potential of progressively decreasing amplitude. Each hyperpolarization lasts 3 to 5 s. Each oscillation event, on average, is composed of 5 cycles. These oscillations have been shown to depend on extracellular Ca^{2+}, and the underlying mechanism is a cyclic opening of Ca^{2+}-dependent K^+ channels (12). Thus, these oscillations may serve as a model for studying the mechanisms underlying spontaneous oscillations.

However, there is no agreement with respect to the role of Ca^{2+} oscillations or the physiological relevance of electrical activity in macrophages. While Ca^{2+} is a known second messenger, it is not clear how oscillatory changes in internal Ca^{2+} would have an advantage over step changes (4). Thus, there are few well-established links between Ca^{2+} oscillations and cellular responses. A role for Ca^{2+} oscillations has been proposed for fluid secretion in pancreatic acinar cells (13) and in neutrophil migration (14) and in terms of allowing replenishment of intracellular calcium stores (4). Calcium oscillations are also detected during frustrated phagocytosis and adherence in macrophages (15). Nonetheless, the fact that an oscillatory signal can vary in amplitude and frequency, as well as in position (calcium waves (16)), suggests that Ca^{2+} oscillations have an unusual potential for encoding cellular functions (1,4,16).

There are also indications of a link between electrical activity and macrophage functions: ionic currents are activated by the Fc fraction of immunoglobulin (17) and by extracellular ATP (18-20); oscillations of potassium currents are induced in mouse peritoneal macrophages by C5a (21) and activation of a Ca^{2+}-dependent, non-selective current is associated with superoxide release subsequent to phagocytosis (22).

In the present study, we examined the genesis and modulation of the depolarization-induced outward K^+ current oscillations in macrophage polykaryons and evaluated the role of the various mechanisms involved in intracellular Ca^{2+} homeostasis, including calcium channels, Na^+/Ca^{2+} antiporter, Ca^{2+} ATPase and intracellular Ca^{2+} stores. Knowledge of these mechanisms can help elucidate the physiological role of electrical activity and Ca^{2+} oscillations in macrophages.

Material and Methods

Cells

Murine peritoneal macrophage polykaryons were obtained as previously described (23). Briefly, a glass coverslip was implanted into the mouse peritoneal cavity. After 7 to

15 days, coverslips were removed from the animal and washed in RPMI-1640 (Gibco, Grand Island, NY) buffered with 6 mM HEPES, pH 7.3, and containing 5% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C. Coverslips were transferred to a 5-ml culture chamber on the stage of a phase-contrast Leitz microscope equipped with a Heineke condenser and a UMK 50/0.60 objective. The large macrophage polykaryons were easily identified on the surface of the coverslips.

Solutions

Unless otherwise stated, experiments were performed in standard extracellular saline containing 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM HEPES and 140 mM Tris-HCl, pH 7.3. In whole-cell patch-clamp recordings the internal solution contained 140 mM KCl, 5 mM NaCl, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES-KOH, 0.6 mM K_2EGTA, pH 7.2, and pCa 7.04 (calculated according to Fabiato and Fabiato (24)). When sodium concentration was increased (both in the external and internal saline) osmolarity was corrected by decreasing the major cation Tris in the external saline and potassium in the internal saline. No osmolarity correction was made for the sodium-free internal saline.

Reagents

Nisoldipine (Bayer, Munich, Germany) and ryanodine (K & K Laboratories, California) were dissolved in dimethyl sulfoxide (DMSO), quercetin (Sigma, St. Louis, MO) was dissolved in absolute ethanol, low molecular weight heparin (Sigma) was dissolved in internal saline, and ruthenium red (British Drug Houses, Poole, England) in water.

Electrophysiological technique

Experiments were carried out at room temperature (23-28°C). Whole-cell patch-clamping was performed using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard procedures (25). A giga-ohm seal was formed using a heat-polished micropipette back-filled with the internal saline solution. Standard electrophysiological recordings were performed using glass microelectrodes (30 to 60 MΩ tip resistance). The electrodes were back-filled with a 2.5 M KCl solution and connected to a high-input-impedance preamplifier with an active bridge circuit (M4A Electromer, WP Instruments, New Haven, CT) allowing simultaneous recording of the transmembrane potential and of the injected currents.

Iontophoretic intracellular calcium injections were performed through a second glass microelectrode back-filled with 0.5 M $CaCl_2$, as previously described (23).

The statistical tests used are specified together with the corresponding data in the Results section. In all cases differences were considered to be significant when $P<0.05$.

Results

Membrane potential oscillations and sodium dependence

Oscillations of membrane potential and current were obtained reproducibly when macrophages were depolarized in the absence of external Na^+ (Figure 1). Both responses were similar in periodicity, number and duration of cycles and progressive amplitude decrease. The most common pattern has been described (12) and is shown in Figure 1. However, there were variations in frequency and in shape (progressive amplitude increase or no change in amplitude of the successive cycles during an oscillatory episode). Oscillation was observed in 58% of the cells (N = 325). Both current and voltage oscillations were immediately interrupted when the depolarization pulse was turned off and a similar pattern was elicited by a new depolarization (data not shown).

The presence of 8 mM NaCl in the external saline was sufficient to prevent membrane potential oscillations (N = 8). Addition of Na^+ to the external medium during an oscillation abolished both hyperpolarizing voltage (Figure 2A) and outward current oscillations (Figure 2B) in 88% of the cells tested (N = 8).

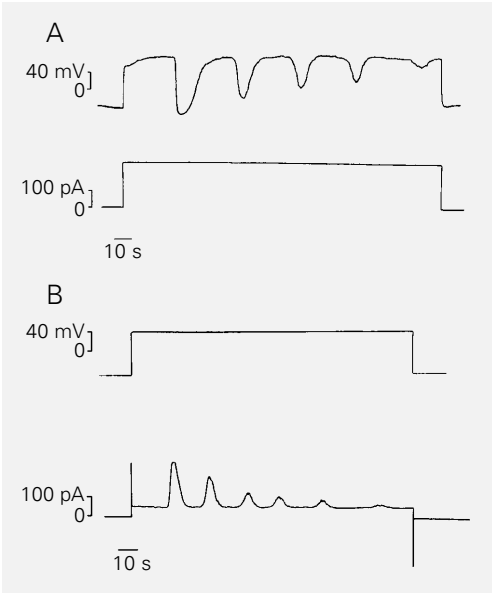

Figure 1 - Voltage and current oscillations induced by depolarization in Na^+-free medium. *A*, Depolarization by constant outward current injection induced membrane potential oscillations in macrophage polykaryons bathed in Na^+-free saline solution. Resting membrane potential = -48 mV. *B*, Outward current oscillation elicited when the transmembrane potential is clamped at +40 mV. Holding potential = -50mV. Note that current and voltage oscillations have similar patterns. Records were obtained by means of whole-cell patch-clamp under current clamp (A) and voltage clamp (B) conditions in two different cells. Calibration bars in all figures were positioned in such a way that the current and voltage values presented correspond to actual values in the tracings.

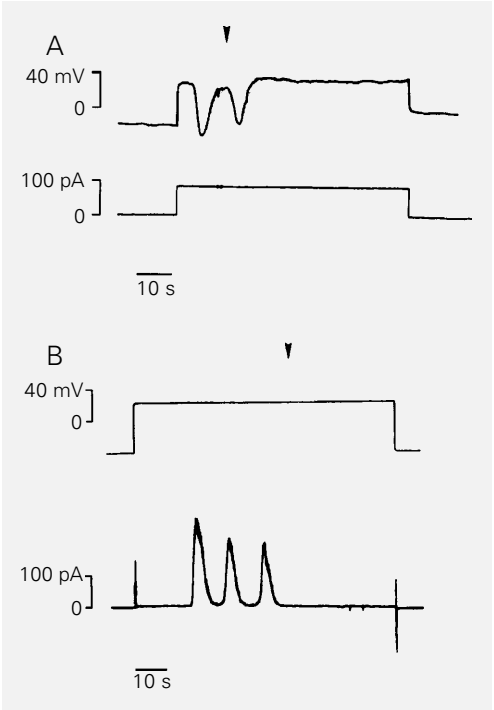

Figure 2 - Effect of external Na^+. Both voltage (A) and current (B) oscillations were interrupted by the addition of NaCl to the external medium to a final concentration of 8 mM (arrowheads). Intracellular saline was Na^+ free in both cases. Note that oscillation retains its characteristic pattern until the addition of NaCl. Whole-cell patch-clamp under current clamp conditions (A), with a resting membrane potential = -20 mV, and under voltage clamp conditions (B), with a holding potential = -40 mV.

Participation of the Na^+/Ca^{2+} antiporter in the Ca^{2+} influx may explain the dependence on the absence of external Na^+ (26). The inverted Na^+ gradient could result in reversal of this antiporter, leading to Ca^{2+} influx. This hypothesis was tested using Na^+-free internal saline to eliminate any Na^+ gradient across the membrane. An increase in the amplitude of the resting membrane potential was observed under these experimental conditions (Table 1). However, the same percentage of cells exhibited depolarization-induced oscillations with characteristics similar to those observed for cells maintained under control conditions (Figure 2 and Table 1). An increase in the internal Na^+ concentration to 15 mM did not significantly change resting potential or the pattern of the oscillatory response (Table 1).

Extracellular calcium

The possible participation of an L-type Ca^{2+} channel in the genesis of the oscillations was investigated. Nisoldipine, a blocker specific for L-type Ca^{2+} channels (27), completely blocked the oscillations when added to the external medium (N = 6) (Figure 3C). This effect persisted even when the depolarizing pulse amplitude was increased and was reversed by washing out the nisoldipine (data not shown). Addition of the solvent (DMSO) did not affect membrane potential oscillation (data not shown). The calcium-activated potassium efflux in human erythrocytes (28) and N-type potassium channels (K_n channels) in human and murine T lymphocytes (29,30) is blocked by dihydropyridine and other Ca^{2+} channel blockers. In order to test whether nisoldipine acts by blocking IK_{Ca}, calcium was injected iontophoretically before (Figure 3B) and after (Figure 3D) the addition of the blocking agent. Calcium injection induced hyperpolarization in both situations.

Calcium ATPase

Quercetin is a flavone of plant origin that blocks Ca^{2+} ATPase in plasma membranes as well as in sarcoplasmic reticulum (31,32). Figure 4 illustrates one experiment where quercetin, added to the external medium after membrane potential oscillation had been induced, generated a long-lasting hyperpolarization (~30 s). An increase in duration of hyperpolarization ranging from 2- to 15-fold was seen in 48% of the cells tested (N = 21). Hyperpolarizing cycles lasted 9.5 ± 5.0 s (mean ± SD; N = 21) before quercetin addition and increased to 25.2 ± 28.9 s in the presence of quercetin (paired *t*-test after log-transformation, P<0.05). A single, long-lasting hyperpolarization similar to that shown in Figure 4 was observed in 60% of the cells that responded to quercetin addition. Quercetin seems to prolong the time during which intracellular Ca^{2+} concentration is high enough to activate IK_{Ca}, thus maintaining a hyperpolarized membrane potential.

Intracellular calcium stores

There are two mechanisms of Ca^{2+} release from the endoplasmic reticulum, one sensitive to IP_3 and the other to Ca^{2+} itself (33). Both mechanisms were tested using specific inhibitors.

Low molecular weight heparin (200 µg/ml), which blocks Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores (33), did not significantly alter the membrane potential, the percentage of cells responding to depolarization or the characteristics of the membrane potential oscillations (Table 2).

The possible involvement of Ca^{2+}-induced Ca^{2+} release was tested by using ruthenium red in the patch-clamp pipette, or ryanodine in the external medium (6,33). A comparison of cells from the same population indicated a striking decrease in the number of hyperpolarizing cycles in the presence of ruthenium red (50 µM, Table 2). Ryanodine

Table 1 - Effect of internal sodium on membrane potential and oscillations.

Data are reported as mean ± SD or median [min, max] for the number of cases given in parentheses. *P<0.05 between the two groups (ANOVA). No statistically significant differences in the number of cycles were observed among groups (Kruskal-Wallis test).

Internal sodium (mM)	Membrane potential (mV)	Number of cycles
0	-62.1 ± 17.6 (13)*	4 [1, 7] (5)
5	-38.6 ± 20.6 (19)*	3.5 [2, 10] (24)
15	-30.5 ± 22.4 (8)	2 [2, 3] (3)

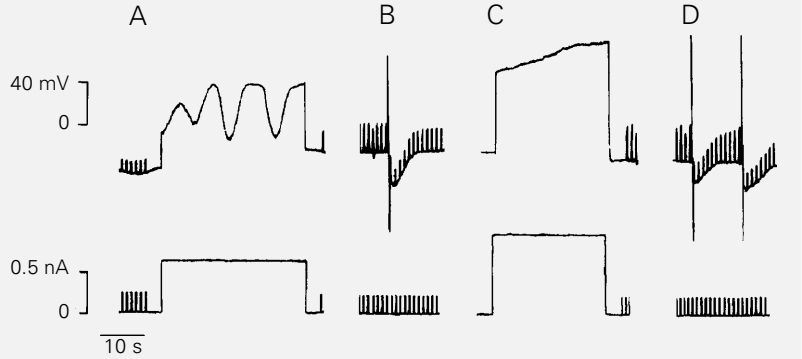

Figure 3 - Inhibition of membrane potential oscillation by nisoldipine. Control situation: depolarization-induced membrane potential oscillation (A) and hyperpolarization induced by iontophoretic intracellular calcium injection (B). Transmembrane potential oscillation was blocked by nisoldipine (20 µM) in extracellular saline (C). Hyperpolarizations induced by iontophoretic intracellular calcium injection were not blocked by nisoldipine (D). Records (A-D) were obtained from a single cell using standard intracellular microelectrodes. In *B* and *D*, iontophoretic injections of calcium were performed through a second microelectrode containing $CaCl_2$. Current pulses of 0.20 nA were injected in order to monitor membrane resistance. Resting membrane potential = -20 mV.

Table 2 - Effect of intracellular calcium release blockers on oscillations.

Data are reported as median [min, max] for the number of cells given in parentheses. *P<0.05 (Mann-Whitney test); +P<0.05 (Wilcoxon test; data from the same cells before (control) and during ryanodine treatment (experimental)).

	Number of cycles	
	Control	Experimental
Ruthenium red (50 µM)	3 [0, 7] (13)	1 [0, 3] (17)*
Heparin (200 µg/ml)	3 [0, 7] (13)	3 [1, 13] (8)
Ryanodine (1 µM)	3 [2, 4] (9)	0 [0, 1] (9)+

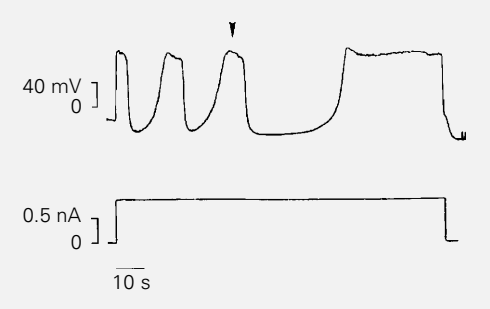

Figure 4 - Effect of quercetin on membrane potential oscillation. Quercetin (0.1 mM) applied during the oscillation (arrowhead) prolonged the subsequent hyperpolarizing cycle to almost three times the control value. The record was obtained using a standard electrophysiologic recording technique. Resting membrane potential = -20 mV.

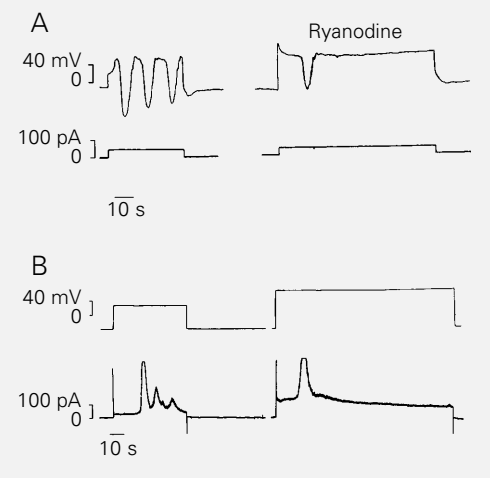

Figure 5 - Effect of ryanodine on membrane potential and outward current oscillations. The number of hyperpolarizing cycles of transmembrane potential (A) and outward current (B) oscillations was reduced by the addition of ryanodine (1 µM) to the extracellular saline. Records were obtained using a whole-cell patch-clamp under current clamp (A) and voltage clamp conditions (B). Resting membrane potential = -12 mV. Holding potential = -40 mV(B).

(1 µM) also induced a significant decrease in the number of oscillations in the same cell (Figure 5, Table 2). Ryanodine opens the Ca^{2+}-sensitive Ca^{2+} channel of the internal stores at low concentrations (nanomolar) but blocks them at the concentration used here (micromolar) (6). The results obtained with heparin, ruthenium red and ryanodine indicate that oscillations depend on the release of intracellular Ca^{2+} from Ca^{2+}-sensitive stores but not from IP_3-sensitive stores.

Discussion

In the present study, we investigated the participation of various processes related to cytoplasmic Ca^{2+} homeostasis in the regulation of the outward potassium current oscillations activated by depolarization in Na^+-free saline. Our working hypothesis proposes that the cyclic activation of the IK_{Ca} would depend on cyclic increases in cytoplasmic free Ca^{2+} activity produced by an influx through the plasma membrane, by release from intracellular stores, or by both events.

Here, we showed that nisoldipine, an L-type Ca^{2+} channel blocker, abolished membrane potential oscillations and that this effect was not due to the direct inhibition of IK_{Ca} by nisoldipine (28,30), since intracellular Ca^{2+} injection restored membrane hyperpolarization in the presence of the drug (Figure 3D). Other evidence for the participation of Ca^{2+} influx in the genesis of these membrane potential oscillations has been presented previously, and includes their dependence on extracellular Ca^{2+} and blockade by D-600 (an L-type Ca^{2+} channel blocker) (12).

These findings suggest that Ca^{2+} influx through a dihydropyridine-sensitive, depolarization-activated pathway is essential for the oscillations. However, a voltage-dependent Ca^{2+} channel, such as the L-type Ca^{2+} channel, has not yet been found in the macrophage membrane (29,34) or in the majority of non-excitable cells (1). Calcium influx pathways in non-excitable cells include channels activated by second messengers such as Ca^{2+}, inositol 1,3,4,5-tetrakisphosphate (IP_4) (16) and IP_3 (29), channels activated by depletion of intracellular Ca^{2+} stores (1), and non-selective cation channels (35). In macrophages, a Ca^{2+}-selective current activated by depletion of intracellular Ca^{2+} stores (36) and an ATP-activated Ca^{2+} channel (18) have been described. Voltage-sensitive, non-selective cation channels (37) and another ATP-activated cation channel (19) are also present. Thus, one possible pathway for Ca^{2+} influx in macrophage polykaryons would be a voltage-dependent cation channel which, in a Na^+-free environment, allows Ca^{2+} permeation. An analogous situation has been reported for the potassium channel in guard cell membranes of *Vicia faba*, where Ca^{2+} enters the cell in the absence of potassium (38). However, the fact that we did not detect

an inward current associated with depolarization is puzzling. Either this current is too small to be detected under our experimental conditions or some outward current is activated simultaneously.

The reversal of the Na^+/Ca^{2+} antiporter was another possible Ca^{2+} influx pathway studied. Reversal of this antiporter in the absence of extracellular Na^+ has been demonstrated in aortic smooth muscle cells (26). However, the use of Na^+-free internal solution in the patch-clamp experiments in order to minimize the Na^+ gradient had no influence on the induction of oscillations by depolarization (Figure 2). Nevertheless, indirect participation of this exchanger cannot be ruled out, since reversal or blockade would raise the background cytoplasmic Ca^{2+} concentration.

When macrophage polykaryons were exposed to the sodium-free saline pipette in patch-clamp experiments, a significant increase in the resting membrane potential was observed. This increase cannot be attributed to the expected decrease in the electrochemical sodium gradient or to the inhibition of the sodium potassium pump activity since both conditions would depolarize the cell. One possibility is that the decrease in internal sodium would somehow increase resting potassium conductance and thus the resting potential. Actually, a blocking effect of intracellular sodium on a calcium-dependent potassium current has been reported in bovine adrenal chromaffin cells (39).

The intracellular Ca^{2+} stores may participate in the genesis of oscillations through Ca^{2+}- and/or IP_3-sensitive mechanisms, both known to be functional in macrophages (40). Ruthenium red and ryanodine, which block Ca^{2+}-induced Ca^{2+} release at the concentrations used (6,33), significantly decreased the number of oscillation cycles in macrophage polykaryons, thus indicating the participation of this mechanism in the genesis of the oscillations. This observation reinforces the importance of Ca^{2+} influx for increasing cytoplasmic Ca^{2+} and triggering Ca^{2+} release from this store. On the other hand, low molecular weight heparin, a blocker of IP_3-induced Ca^{2+} release (16), did not have a significant effect on the oscillations, excluding the participation of this Ca^{2+} pool in its genesis.

The prolongation of the duration of the hyperpolarization of the first oscillation cycle following quercetin addition (Figure 4) suggests that each hyperpolarizing cycle is terminated normally by a decrease in cytoplasmic free Ca^{2+}. Such a decrease, mediated by Ca^{2+} ATPase activity, would deactivate IK_{Ca} and allow membrane potential repolarization.

The fact that the amplitudes of the hyperpolarizing cycles usually decrease progressively and finally subside after a mean of 5 cycles may be the result of several factors, including voltage-dependent inactivation of the Ca^{2+} influx pathway, depletion of intracellular Ca^{2+} stores, inactivation of IK_{Ca}, or desensitization of the intracellular Ca^{2+}-release mechanism. At present, we do not know which of these processes contribute to the termination of the oscillations. The fact that new oscillations can be induced by a further increase in depolarization (data not shown) rules out a voltage-dependent inactivation of the Ca^{2+} influx pathway and of IK_{Ca}. Inactivation of IK_{Ca} is also unlikely because intracellular calcium injection restored one hyperpolarization cycle (data not shown). Considering that intracellular Ca^{2+} stores are only partially repleted after each hyperpolarizing cycle, it is conceivable that a sustained depolarization leads to a progressive decrease in the Ca^{2+} content of those stores, with the end of oscillations coinciding with their depletion.

Thus, we propose the following hypothesis for the genesis of the depolarization-induced membrane potential oscillations in macrophage polykaryons in Na^+-free medium. Upon depolarization, Ca^{2+} enters the cell through a dihydropyridine-sensitive path-

way. The resulting increase in cytoplasmic Ca^{2+} activity induces the release of Ca^{2+} from Ca^{2+}-sensitive intracellular stores. This increase in Ca^{2+} activates IK_{Ca} which hyperpolarizes the cell membrane. The Ca^{2+} ATPase pumps Ca^{2+} out of the cell and/or into intracellular stores, decreasing cytoplasmic Ca^{2+} and deactivating IK_{Ca}. Additionally, the Ca^{2+}-sensitive intracellular stores are modulated by cytoplasmic calcium itself (41): above a certain intracellular calcium concentration, the open state probability of the calcium-sensitive calcium channels is reduced (42), further decreasing calcium release and consequently IK_{Ca}, allowing the membrane potential to return to its previous level. If the depolarizing pulse is sustained, intracellular Ca^{2+} may rise again, so that new cycles occur until one or more of the mechanisms discussed above terminates the oscillation.

The present study demonstrates that the depolarization-induced oscillation of membrane potential in macrophage polykaryons depends on events occurring at both the plasma membrane and the endoplasmic reticulum levels. In addition, we propose that a similar mechanism may account for the spontaneous oscillations that occur in macrophages and macrophage polykaryons. The reproducible oscillatory phenomena presented here can be very useful as a model for the study of the mechanisms for generation of oscillations and their role in cell responses, including those of macrophages.

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