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INCREASES IN CYTOSOLIC FREE Ca^{2+} INDUCED BY ATP, COMPLEMENT AND β-LIPOPROTEIN IN MOUSE L FIBROBLASTS

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SUMMARY: By means of Ca^{2+}- and K^+-selective microelectrodes, the changes in intracellular free Ca^{2+} and K^+ were measured during the hyperpolarizing responses induced by ATP, complement and β-lipoprotein in mouse fibroblastic L cells. The cytoplasmic Ca^{2+} concentration ($[Ca]_i$) was about 0.4 μM in the resting state. The hyperpolarizing responses always coincided with a phasic increase in $[Ca]_i$. ATP or β-lipoprotein induced about a 2-fold rise in $[Ca]_i$, and complement did up to 3-fold. Both the hyperpolarizing responses and $[Ca]_i$ increases were prevented by removal of external Ca^{2+} or by application of a Ca-channel blocker, nifedipine. Quinine, a Ca-activated K-channel inhibitor, suppressed the hyperpolarizing responses but not the $[Ca]_i$ increases. During the hyperpolarizing response, the intracellular free K^+ concentration gradually decreased from about 120 to 110 mM. Thus, it is concluded that ATP, complement and β-lipoprotein caused a transient elevation of cytoplasmic free Ca^{2+} due to Ca^{2+} influxes, thereby inducing electrical membrane responses through activation of Ca-dependent K-channels in the fibroblasts.

Mouse L-strain fibroblasts exhibit spontaneous, repeated hyperpolarizations displaying membrane potential oscillations (1,2), and respond with slow hyperpolarizations to mechanical or electrical stimuli (3). In addition, the cells show hyperpolarizing responses to β-lipoprotein (4), ATP (5) and complement (6), probably through activation of each receptor. Similar electrical responses to chemical stimuli have also been observed in human normal diploid fibroblasts (5,6).

It has been known that both the spontaneous and stimulus-induced hyperpolarizing responses result from the K^+ conductance increase in L cells (2-7). Several lines of evidence suggest that an intracellular Ca^{2+} increase, which may trigger the operation of Ca-activated K-channels, is essential

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for the genesis of spontaneous oscillatory hyperpolarizations and the hyperpolarizing responses induced by electrical or mechanical stimuli (8-11). Therefore, it is possible that the hyperpolarizing responses induced by these chemical stimuli are also brought about by a significant increase in cytoplasmic free Ca^{2+}. To test this possibility, the intracellular free Ca^{2+} and K^{+} concentrations were directly measured in the present study, using Ca^{2+}- and K^{+}-selective microelectrodes.

MATERIALS AND METHODS: L cells were cultured in the Fischer medium supplemented with 10% bovine serum. The cells were plated on the bottom of plastic dishes (5 cm in diameter). A monolayer of giant L cells was obtained by cell fusion with polyethylene glycol and subjected to electrophysiological studies.

For the recording of membrane potential standard techniques (1) were employed. The resistance of 3 M KCl-filled microelectrodes was 20 to 60 MΩ, and the tip potential less than 5 mV. Single-barrelled Ca^{2+}- and K^{+}-selective microelectrodes with tip diameter of about 1 μm (Ca- and K-electrodes) were made with a neutral ligand sensor cocktail (12) and with Corning 477317, respectively, according to the methods reported by Rink and Tsien (13) with a slight modification (11). The resistance of the Ca- or K-electrode was the order of 10 or 1 GΩ, respectively. The outer surface of Ca-electrode was coated with an electroconductive resinous paint (Dotite S-1, Fujikura Chemicals) to reduce the response time. Calibration of the Ca-electrode was carried out before and after each impalement in the presence of 130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.20). The calibration media containing 10^{-8} to 10^{-6} M Ca^{2+} were made from a Ca^{2+}-buffer containing 1 mM EGTA (Nakarai Chem. Co.). The Ca^{2+} concentrations were calculated according to Fabiato and Fabiato (14). The Ca-electrodes employed herein exhibited Nernstian responses between pCa 6.5 and 2 and sub-Nernstian responses between pCa 6.5 and 8, as shown in Fig. 1A. The

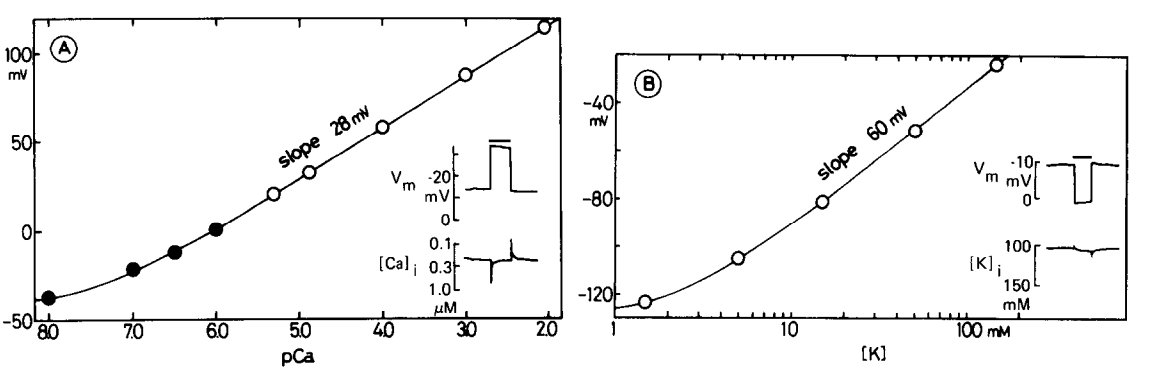

Fig. 1 Calibration of Ca^{2+}-selective and K^{+}-selective microelectrodes. A) Ca-electrode potentials in $CaCl_2$ solutions approximating the cytosol (open circles) and in Ca-EGTA buffer solutions approximating the cytosol (closed circles). B) K-electrode potentials in KCl-Na gluconate solutions. The selectivity coefficient of this K-electrode was 0.015 against Na^{+}. *Insets*: Voltage sensitivities of Ca- (A) and K-electrodes (B). Constant currents (± 5 nA) were applied (at bars) to the cell membrane for 10 sec (A) and 6 sec (B) through intracellular microelectrodes after compensating for the electrode resistances by a bridge circuit.

Ca-electrodes exhibiting super- or sub-Nernstian responses and/or hysteresis between pCa 6.5 and 2 were discarded. Calibration of the K-electrodes was made in a series of solutions containing KCl and Na-gluconate of 150 mM in total as well as 10 mM Tris-HCl (pH 7.20). The K-electrodes showed ideal responses between 10 and 150 mM K^+ (Fig. 1B). The K-electrodes never showed hysteresis and super-Nernstian responses. The response time for changes in ion concentrations or that for electrical potential changes was satisfactorily fast ($\leq$1.5 sec for Ca-electrode, $\leq$0.5 sec for K-electrode). The latter was tested within the cell by a current clamp method as shown in Fig. 1A,B (Insets). This test also confirmed that the Ca- and K-electrodes sense voltage as well, though not as fast, as a conventional 3 M KCl-filled microelectrode. Intracellular free Ca^{2+} and K^+ concentrations ($[Ca]_i$ and $[K]_i$) were measured by substracting the potential of a conventional microelectrode recorded through a WPI KS 700 amplifier from that of a Ca- or K-electrode recorded through a WPI FD 223 amplifier. All experiments were performed at room temperature (24 $\pm$ 2 °C).

The control medium was a Tris-buffered saline (TBS) containing 143 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM mannitol and 10 mM Tris-HCl (pH 7.2). In most of the experiments with Ca-electrodes, TBS devoid of $CaCl_2$ was employed to minimize a leakage entry of extracellular Ca^{2+} upon the electrode penetration. This nominally Ca^{2+}-free TBS was found to contain about 5 μM Ca^{2+} due to the contamination in the chemicals and distilled water employed.

ATP (0.1 M, disodium salt, Sigma) was dissolved with 0.54 mM $CaCl_2$ and 0.1 M Tris in distilled water (final pCa about 6, pH 7.2). Human β-lipoprotein (5 mg/ml, ICN Pharmaceutical Inc.) was dissolved in Ca^{2+}-free TBS (final pCa 6.3, pH 7.2), and guinea pig whole complement (230 CH50 units/ml, Cordis Labo Inc.) was with 4 mM EGTA in Ca^{2+}-free TBS (final pCa about 6, pH 7.2). Nifedipine (a gift from Bayer Yakuhin Ltd.) and quinine (Nakarai) were applied by adding an aliquot of these solutions (1/100 volume) into the bathing solution.

RESULTS AND DISCUSSION: Oscillations of the membrane potential (1) were observed in L cells incubated not only in a control TBS containing 0.9 mM Ca^{2+} but also in a nominally Ca^{2+}-free TBS containing about 5 μM Ca^{2+}. During the potential oscillation, synchronous oscillation of $[Ca]_i$ was observed (Fig. 2A), as found previously (11). When ATP, β-lipoprotein or complement was applied to the cell during or after subsiding the oscillation, a slow hyperpolarization was instantaneously observed (Fig. 2), as reported previously (4-6). Biphasic elevation of intracellular free Ca^{2+} was associated with the hyperpolarizing responses induced by these chemical stimulations (Fig. 2); that is, a small increase in the cytoplasmic Ca^{2+} occurred immediately after application of these chemicals (Fig. 2, asterisks), and this was followed by a remarkable $[Ca]_i$ increase. Both the hyperpolarizing response and the $[Ca]_i$ increase returned to the original resting level after 30 sec to 5 min. The peak responses of membrane potential and $[Ca]_i$ observed at about 5 μM of extracellular Ca^{2+} are summarized in Table 1. During the

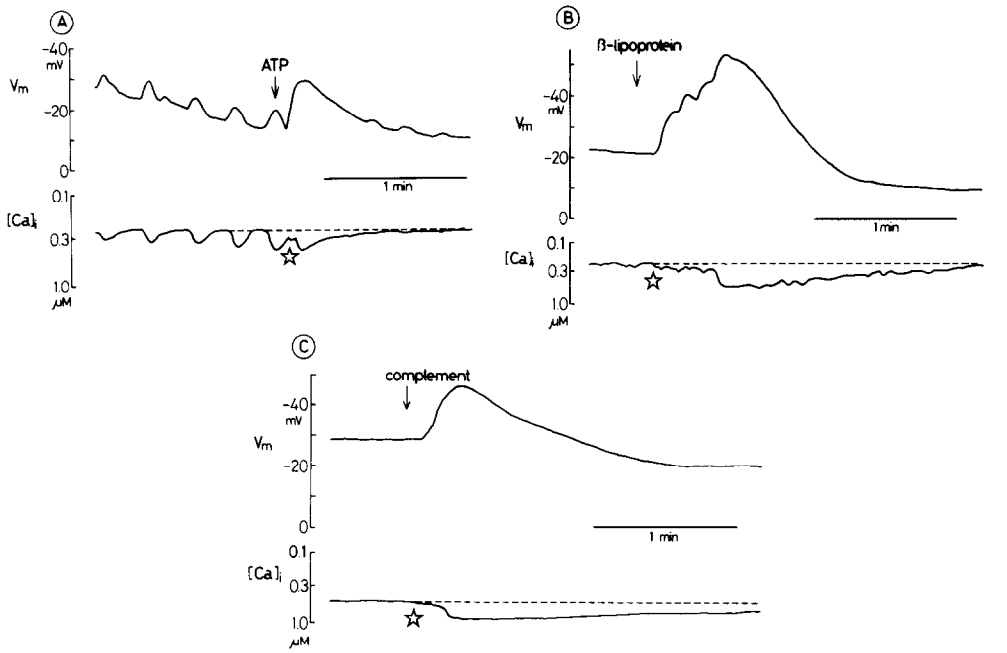

Fig. 2 Increases in intracellular free Ca^{2+} concentration in giant L cells during hyperpolarizing responses induced by ATP (final 1 mM), β-lipoprotein (final 50 μg/ml) and complement (final 2.3 CH50 units/ml). Note that small increases in $[Ca]_i$ (asterisks) from the basal levels (broken lines) occurred immediately after application of these chemicals and marked $[Ca]_i$ increases followed after them. In C, the $[Ca]_i$ level returned to the original resting level about 5 min after stimulation by complement.

Table 1. Changes in membrane potential and in intracellular Ca^{2+} concentration in response to ATP, β-lipoprotein and complement*

	number of observations	membrane potential (mV)	$[Ca]_i$ (μM)
control	7	-15.7 ± 2.4	0.45 ± 0.08
ATP	7	-30.5 ± 3.9	0.76 ± 0.13
control	7	-15.8 ± 1.8	0.36 ± 0.06
β-lipoprotein	7	-32.0 ± 4.8	0.75 ± 0.17
control	5	-19.1 ± 4.5	0.44 ± 0.04
complement	5	-34.0 ± 5.4	1.23 ± 0.17

* Steady resting values (control) and peak responses to chemicals (ATP, β-lipoprotein, complement) are tabulated.

hyperpolarizing responses, about 2-fold increases in the $[Ca]_i$ level were brought about by ATP and β-lipoprotein, whereas complement gave rise to about 3-fold increases. In the presence of 0.9 mM Ca^{2+} in the bathing solution, much higher $[Ca]_i$ levels were frequently observed both in the resting and activated states, probably due to a sizable Ca^{2+} entry through the leakage pathway around the impaled electrodes. In several experiments with extracellular 0.9 mM Ca^{2+}, however, the $[Ca]_i$ values were similar to those in Table 1 before and after application of ATP or complement (not shown).

Upon application of serum or several mitogenic serum factors, similar $[Ca]_i$ increases have been observed with optical Ca^{2+} indicator quin 2 (15-18) or aequorin (19) in fibroblasts. Complement was found to induce the hyperpolarizing response even during exposure to 10% serum. Thus, the serum factors possibly contaminated in the complement preparation are not responsible for the responses induced by complement. Formation of the membrane attack of complement on the cell surfaces may not underlie the complement response because complement was effective even after heat-inactivation. In our preliminary study, a purified complement component (C1q) was also fond to be effective (6).

A reduction of extracellular Ca^{2+} concentration down to 0.1 μM (by adding 1 mM EGTA) abolished the responses induced by ATP (Fig. 3A). In the presence of nifedipine, a Ca-channel inhibitor, both the hyperpolarizing response and $[Ca]_i$ increase induced by complement (Fig. 3B) or β-lipoprotein (not shown) were suppressed. Therefore, it is likely that the increase in cytoplasmic Ca^{2+} upon the chemical stimulation is mainly derived from the outside of the cell through Ca-channels.

In the presence of quinine, an inhibitor of Ca-activated K-channel, the hyperpolarizing responses induced by the chemicals were markedly inhibited, while the increase in $[Ca]_i$ was always preserved (Fig. 3C). These results strongly suggest that the hyperpolarizing responses to chemical stimuli are due to activation of quinine-sensitive, Ca-dependent K-channels.

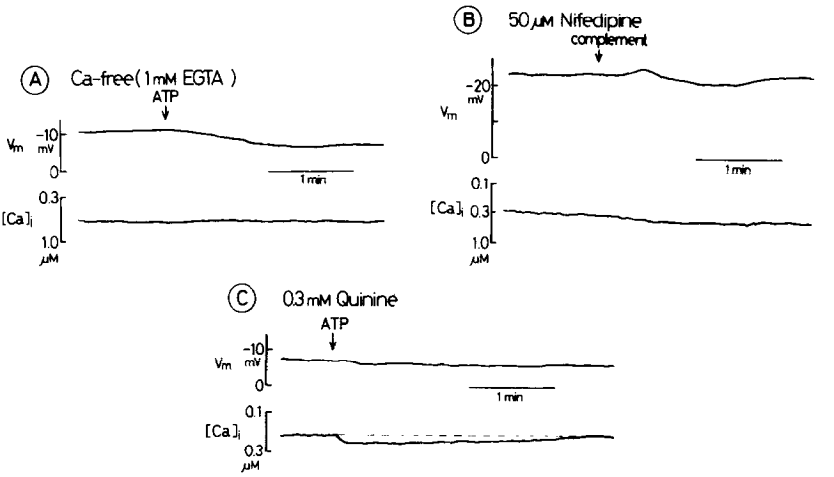

Fig. 3 Effects of extracellular applications of EGTA, nifedipine and quinine on hyperpolarizing responses and $[Ca]_i$ increases induced by ATP and complement in giant L cells. EGTA, nifedipine and quinine were added 3 to 5 min before the application of ATP or complement. The final concentrations of these chemicals and a broken line are the same as in Fig. 2. Note that the pattern of the $[Ca]_i$ increase was monophasic in the presence of quinine. Therefore, it is possible that the biphasic increases in $[Ca]_i$ observed in the absence of quinine (Fig. 2) are related to the membrane hyperpolarizations.

If this were the case, a significant decrease in cytosolic K^+ would be observed after the chemical stimulation, since the membrane of L cells is known to have a high conductance to Cl^- (7, 20), the fluxes of which can compensatively take place with the K^+ fluxes. This was examined by the $[K]_i$ measurements with K^+-selective microelectrodes. Upon stimulation with ATP, β-lipoprotein or complement, a significant decrease in $[K]_i$ from the original resting level (about 120 mM) occurred with a slight time lag behind the hyperpolarizing response (Fig. 4, Table 2). When the electrical response terminated, the $[K]_i$ value reached a new steady level (about 110 mM).

The present experiments using Ca^{2+}- and K^+-selective microelectrodes provide direct evidence for the notion that the hyperpolarizing responses to ATP, complement and β-lipoprotein are caused by a transient elevation of the cytoplasmic Ca^{2+} concentration which, in turn, stimulates Ca-activated K-channels in the fibroblasts. It was shown in L cells that the hyperpolarizing response is closely associated with the pinocytosis of β-lipoprotein (4) and the chemotactic activity to ATP or complement (6,21). Therefore,

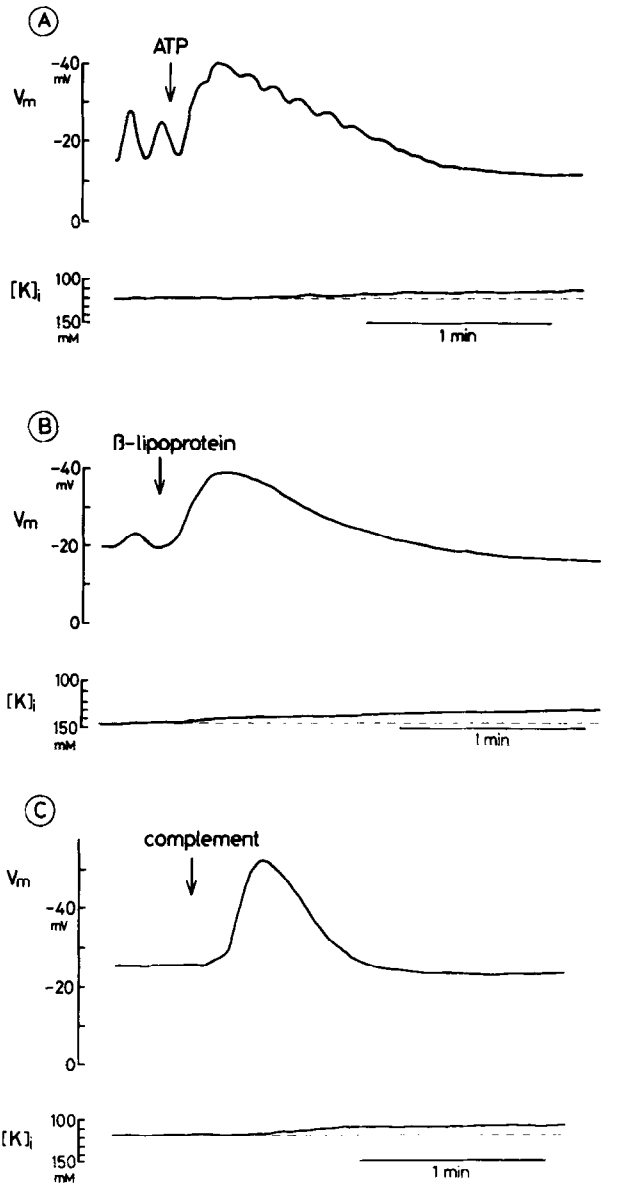

Fig. 4 Decreases in intracellular free K^+ concentration in giant L cells during hyperpolarizing responses induced by ATP, β-lipoprotein and complement. The final concentrations of these chemicals are the same as in Fig. 2. Broken lines indicate the basal $[K]_i$ level before the application of chemicals. The $[K]_i$ values stayed at new steady levels even after subsiding the hyperpolarizing responses for up to 10 min.

it is possible that the rise of $[Ca]_i$ induced by the chemical stimuli is involved in the physiological function of fibroblasts for cholesterol metabolism and wound healing.

Table 2. Changes in membrane potential and in intracellular K^+ concentration in response to ATP, β-lipoprotein and complement*

	number of observations	membrane potential (mV)	$[K]_i$ (mM)
control	6	-19.2 ± 2.0	119.9 ± 6.1
ATP	6	-39.4 ± 3.5	108.9 ± 6.3
control	3	-20.8 ± 1.0	126.3 ± 10.1
β-lipoprotein	3	-45.2 ± 6.9	114.0 ± 7.9
control	5	-21.7 ± 3.1	113.2 ± 5.4
complement	5	-46.7 ± 4.8	106.6 ± 5.7

* Steady resting values (control) and peak responses to chemicals (ATP, β-lipoprotein, complement) are tabulated.

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