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Exogenous heat shock protein hsp70 activates potassium channels in U937 cells

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Abstract

With the use of patch clamp technique, the effect of exogenous heat shock protein hsp70 on ion channel properties in the plasma membrane of human promonocyte U937 cells has been examined. Cell-attached experiments showed that the addition of 30–100 μg/ml hsp70 to the pipette solution resulted in an activation of outward currents through potassium-selective channels of 9 pS unitary conductance. The activity of K^+-selective channels did not depend on membrane voltage and could be controlled by the intracellular free calcium concentration as revealed in inside-out recordings. K^+ channels with similar conductance and kinetic behaviour were found in normal cell-attached patches very rarely. Outside-out experiments showed that the addition of hsp70 to the external solution induced a channel-like stepwise increase of inward current which may provide cation entry from the extracellular medium. The interaction of extracellular hsp70 with the membrane surface of the native cell and of the excised fragment was found to be different. The results suggest that hsp70-induced activation of Ca-dependent K channels in monocyte-macrophage cells may be due to a local increase of free Ca^{2+} concentration just near the inner membrane side.

Keywords: Heat shock protein hsp70; Ionic channel; Potassium conductance; Patch clamp; U937 cell

1. Introduction

Molecular chaperones are known to participate in a large variety of cellular processes [1]. For the last few years, data have appeared indicating that such well-studied chaperones as members of hsp70 family may play the role of extracellular effectors. It has been recognized that hsp-related proteins belong to the main immunogens in a number of infectious diseases (for review, see Ref. [2]). Pure hsp70 was found to increase production of interleukin-1 (IL-1), IL-6 and other monokines [3,4]. Exogenous hsp70 purified from bovine brain could associate with cell surface as shown by Johnson and Tytell [5] on primary culture of rabbit arterial smooth muscle cells. Alder et al. [6] found previously that heat shock proteins induced ion conducting pores in planar lipid bilayers. Our preliminary results have indicated that the addition of pure hsp70 from bovine muscle to the culture of U937 human promonocytes causes changes in the surface antigen pattern, affects the rate of DNA synthesis, viability and proliferation of the cells (unpublished data). The mechanisms of the interaction of exogenous heat shock proteins with native cells remain unknown. One can assume the extracellular hsp70 possibly appearing in blood or inflammatory sites due to the disruption of infectious or host cells may affect membrane properties in immune cells.

The human promonocyte U937 cell line established from a histiocytic lymphoma by Sundstrom and Nilsson [7] has been used as a suitable model to study the role of heat shock proteins in a cellular response to stress conditions as well as in normal cell activities such as growth and differentiation. On the other hand, electrophysiological measurements on U937 cells were performed to investigate membrane properties of monocytes-macrophages [8,9].

In the present work, we have examined whether ion channels are involved in the interaction of extracellular hsp70 with membrane surface in U937 cells. Patch clamp technique allowed to study very early events including some changes of ionic permeability and channel activity in the plasma membrane in response to heat shock proteins. Moreover, single current measurements on excised fragments may help to estimate a possible role of hsp70-induced pore formation observed in bilayers [6] in the real effects on cell membranes. Using cell-attached and inside-

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out configurations, we have found outward currents through potassium-selective channels activated by the application of hsp70 to the extracellular surface of the patch. The results of outside-out experiments revealed direct hsp70 effect on membrane fragments and suggested possible mechanisms of the hsp-induced channel activity in native cells.

2. Materials and methods

2.1. Cells

Human histiocytic lymphoma U937 cells obtained from Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia) were maintained in RPMI-1640 containing 10% fetal bovine serum and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin) at 37°C. Cells were plated on coverslips (0.4 × 0.4 cm) 1–3 days before experiment.

2.2. Electrophysiology

We used the patch-clamp method in the cell-attached, inside-out and outside-out modes [10]. The recordings were performed at room temperature (22–23°C) on the stage of inverted microscope with Nomarsky optics (magnification of 256 ×). At the beginning of the experiment, the recording chamber (volume of 0.1 ml) was filled with normal external solution in which giga-seal was formed. When working in excised configurations, the pipette tip with the membrane fragment was transferred to a compartment of a smaller volume (about 20 μl).

Ionic current measurements were performed essentially as described earlier [11]. Membrane voltage was the potential of the intracellular membrane side minus the potential of the extracellular one. Patch pipettes were fabricated with Pirex capillary tubing, coated with Sylgard 184, polished immediately before use and had resistance of 10–15 mΩ. Current signals recorded on magnetic tape were low-pass filtered with Bessel 4-pole filter using a cut-off frequency ranging from 200 to 1.000 Hz. The signals transferred to the computer were digitized at 1 ms/pt with 12-bit accuracy and analysed off-line. Averaged data are given as the mean ± S.D. (number of experiments).

2.3. Solutions

The bath solution containing (in mM) 145 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/TrisOH (pH 7.3) was used

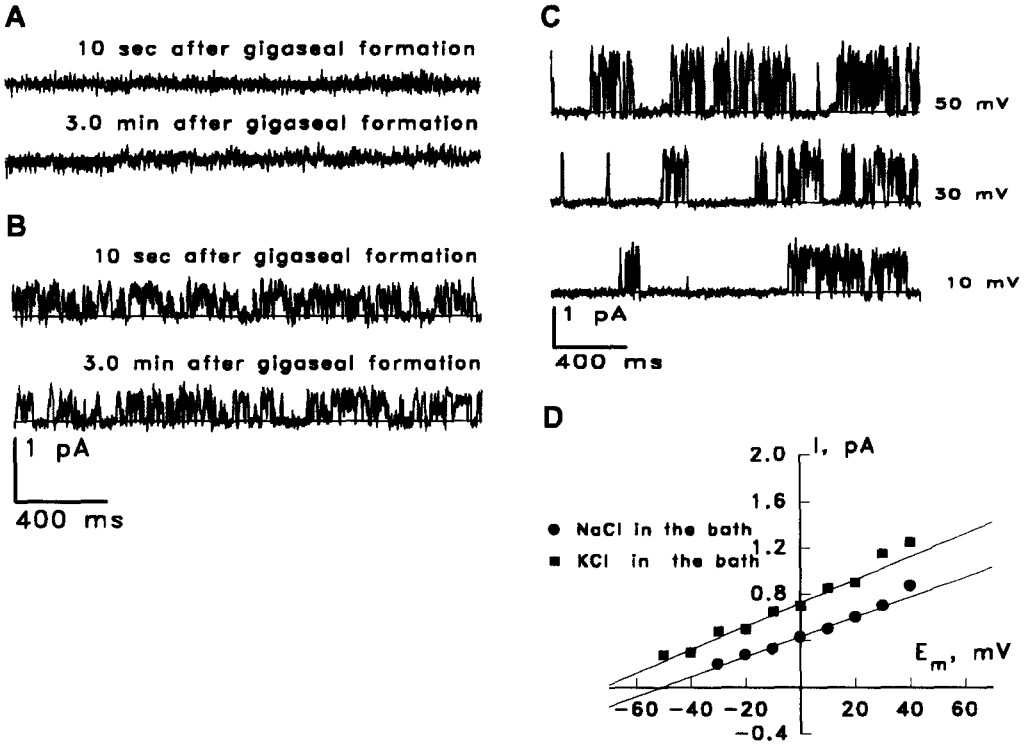

Fig. 1. Effect of hsp70 on single channel activity in a cell-attached membrane patch of U937 cells. (A) Typical cell-attached recording obtained with normal Na-containing pipette and bath solutions at resting potential showing no channel activity. (B) Current records obtained with 100 μg/ml hsp70 in the pipette showing outward currents through potassium channels. Ionic conditions were the same as in A. Filter was 200 Hz. (C) Cell-attached recordings of single channel activity in the same patch (100 μg/ml hsp70 in pipette) obtained in potassium bath solution. Membrane potentials are indicated near traces. (D) Current–voltage relationships of hsp70- activated channels measured in Na- and then in K-containing bath solution. Unitary conductance is 9.0 pS.

in cell-attached measurements to nullify the resting membrane potential. In case of cell-attached and inside-out recordings, patch pipettes were filled with the external solution containing (in mM) 145 (or 125) NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/TrisOH (pH 7.3). Bath cytozol-like solution contained (in mM) 140 KCl (or 10 KCl + 63 K_2SO_4), 20 Hepes-KOH (pH 7.3), 1 $MgCl_2$, 2 EGTA or HEDTA and an appropriate quantity of $CaCl_2$ calculated accordingly to [12] to establish the final free calcium concentration at the desired level in the range from 0.01 μM to 10 μM. The same solution (with 0.1 or 0.01 μM free Ca) was in pipette in the case of outside-out recordings. Hepes, EGTA, HEDTA, bovine serum albumin (fraction V) were from Sigma.

Heat shock protein 70 kDa (hsp70) was isolated from bovine muscle by the method described in Ref. [13]. The preparation referred to as hsp70 was of approx. 98% purity and contained about 60% of hsp72 and 40% of hsp73 as revealed by electrophoresis under denaturating conditions. Aliquots of hsp70 stock solution were added to the normal Na-containing bath or pipette solution in patch experiments.

3. Results

3.1. Hsp70 evoked activity of K^+-selective channels in cell-attached patches of U937 cells

We studied single channel activity in U937 cell membrane using patch-clamp measurements on intact cells. Most of the cell-attached patches (80%, $n = 40$) were silent when normal solution was in the pipette. Fig. 1 shows the results of representative experiments demonstrating the effect of hsp70 on channel activity in the plasma membrane fragment of native cell. When hsp70 (100 μg/ml) was added to the pipette solution, unitary currents of outward direction were observed just after giga-seal formation representing open-closed state transitions of ionic channels (Fig. 1B). Initially, to study the effect of hsp70, transmembrane currents were recorded in the Na-containing bath solution that is similar to native conditions (Fig. 1A,B). Then, to evaluate correctly conducting properties of channels activated by hsp70 application in cell-attached experiments, ionic currents were measured in the potassium-containing bath solution. Fig. 1C demonstrates single channel recordings with hsp70 in pipette on the same patch at different levels of membrane potential. The corresponding current–voltage relationship shifted to negative potentials along the voltage axis as compared to the relationship obtained in the Na-containing bath (Fig. 1D). This shift reflected the value of resting potential of the native cell in the normal sodium extracellular solution. The slope of the linear part of current–voltage curve corresponded to the unitary conductance of 9.0 pS. Ionic currents were outward at the whole voltage range (Fig. 1C,D); the reversal potential value obtained by extrapolation was −84 mV, indicating potassium selectivity of channels. K^+ channels found in U937 cell membrane in presence of hsp70 in pipette were not inactivated and could be continuously active at all voltages. The kinetic behaviour of this channel type was characterized by a rapid flickering during open-channel bursts alternated with silent periods.

In this series of experiments with hsp70 (30–100 μg/ml) in the pipette, 42 cell-attached patches were tested and 40 of these displayed typical K^+ channel activity similar to that shown in Fig. 1B,C. The mean conductance value was 8.6 ± 2.7 pS ($n = 5$). In a number of patches, the development of leakage conductance reversed at zero potential prevented precise measurements of the amplitude of K^+ channel openings in the wide voltage range. In these cases, outward K^+ currents were recorded reliably only near zero holding potential.

In some experiments with hsp70 in the pipette, several K^+ channels were activated in patch just after giga-seal formation so it was difficult to identify individual openings. Cell-attached recordings at different holding potentials most probably indicated the potassium nature of induced conductance. In a few minutes, channel activity usually decreased in such a way that single events could be recorded. Following inside-out recording also made it possible to estimate reliably parameters of individual openings.

After excising the patch with hsp70 in the pipette, the activity of K^+ channels could also be observed in the inside-out recording (Fig. 2). Fig. 2A shows current records at different holding potentials obtained in the bath 'intracellular' solution containing 145 mM KCl and 1 μM free

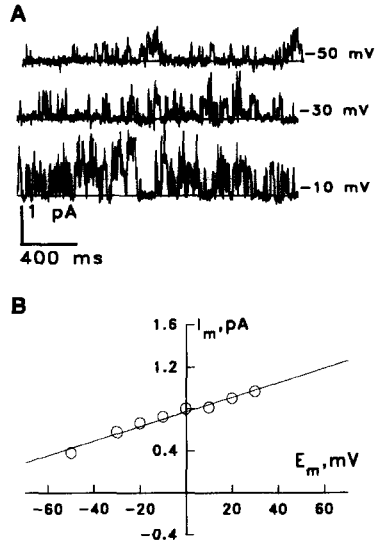

Fig. 2. The activity of K^+ channels in the inside-out patch with hsp70 (50 μg/ml) in the pipette. (A) Current records at different holding potentials, indicated near traces; filter was 200 Hz. Bath intracellular solutions contained 140 mM KCl and 1 μM free $[Ca_i^{2+}]$ ($pCa_i = 6$). (B) Current–voltage relation: unitary conductance is 8.3 pS.

$[Ca_i^{2+}]$; corresponding unitary current–voltage relation is presented in Fig. 2B. Inside-out recordings revealed outward currents through K^+ channels characterized by voltage insensibility. The kinetic behaviour of channels was also similar in cell-attached and inside-out measurements. It should be noted that transitions between open and closed states within the bursts accelerated markedly with depolarization, making it difficult to get a real value of the open state level (see Fig. 1C and Fig. 2A). Thus, both in inside-out and in cell-attached recordings, some deviations of the current–voltage relations from the linear character were often observed with increase of positive potentials. The unitary conductance determined from the linear part of current–voltage curve in the range of negative potentials (Fig. 2B) was 8.3 pS. The mean value was 8.7 ± 3.6 pS ($n = 8$).

Inside-out experiments with known composition of the solutions from the outer and inner side of the membrane fragment confirmed directly the cationic specificity of channels functioning in the presence of hsp70 and their high potassium selectivity. As shown in Fig. 2A,B, unitary currents were outward in the whole voltage range and the reversed potential obtained by extrapolation was about -97 mV; the mean value was -93 ± 15 mV ($n = 8$). The substitution of chloride anions with sulphate in the bath cytozol-like solution did not change amplitude of currents. On the other hand, the equimolar substitution of K^+ cations with Na^+ in the bath solution resulted in a fully reversible abolishment of outward currents induced with hsp70 in the whole voltage range (not shown).

Inside-out recordings revealed that functioning of hsp-activated channels was dependent on free ionized calcium concentration $[Ca_i^{2+}]$ in the bath solution (Fig. 3). After excising the patch with 100 μg/ml hsp70 in the pipette, typical activity of K^+ channels was observed at 1 μM $[Ca_i^{2+}]$ in cytozol-like solution. K^+ currents disappeared when the level $[Ca_i^{2+}]$ was reduced to 0.1 μM and reappeared when calcium concentration was again elevated up

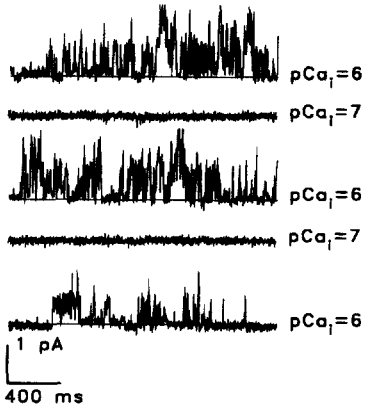

Fig. 3. Inside-out recordings of K^+ channel activity with hsp70 in the pipette at different free ionized calcium concentrations $[Ca_i^{2+}]$ in the bath solution; pCa_i values are indicated near traces. Membrane potential was 0 mV. Filter was 200 Hz.

to 1 μM. Thus, the activity of voltage-independent K channels induced by hsp70 in cell-attached patch may be regulated by the intracellular calcium concentration. However, in three inside-out patches, K^+ currents were recorded at the decrease of $[Ca_i^{2+}]$ to 0.01 μM. This can be accounted for by variability of inside-out patch properties when taking into consideration possible preservation of some intracellular structures after the excising of membrane fragment from the cell [14]. The existence of two populations of K channels characterized by similar conductance and different calcium sensitivity in U937 cells also could not be excluded. This question was not specially examined here.

It was found that the addition of hsp70 (100 μg/ml) to the bath extracellular solution did not evoke channel activation in cell-attached patch. In some experiments an increase of membrane noise was recorded.

As pointed out above, the major part of cell-attached patches with normal pipette and bath solutions displayed no channel openings for at least 5–10 min after the giga-seal formation. In the other 8 out of 40 normal patches we could rarely observe outward currents in cell-attached recordings, evidently representing the background activity of voltage-independent K^+ channels in U937 cells. Among them, there were four patches in which the activity was rather high and sufficient to measure current–voltage relation. The unitary conductance determined from the slope of current–voltage curve was 7.6 ± 2.3 pS ($n = 4$) and the reversal potential estimated by extrapolation was -85 ± 9 mV. These cell-attached recordings obtained with normal solution in the pipette and bath (not shown) revealed typical outward currents through potassium-selective channels. They appeared to be very similar to K^+ currents described actually in every cell-attached patch with hsp70 in the pipette (Fig. 1B–D)). This similarity was confirmed in the inside-out recordings of K^+ channel activity in normal patches; the unitary conductance was equal to 9.2 ± 2.3 pS ($n = 7$) and the extrapolated reversal potential was -81 ± 8 mV.

In two of these experiments with normal patches displaying K^+ currents in cell-attached configuration, the subsequent inside-out recordings also showed that intracellular $[Ca_i^{2+}]$ could be a regulator of K^+ channel activity. Moreover, in 10 experiments with normal pipette solution we have observed characteristic outward K^+ currents activated by increase of $[Ca_i^{2+}]$ (up to 1 μM) in the inside-out configuration after excising the cell-attached patches displaying no activity (Fig. 4). K^+ currents in normal patch and their Ca dependence (Fig. 4) were similar to those presented in Fig. 3 (for inside-out patch with hsp70 in the pipette).

On the whole, these data indicate that exogenous hsp70 increases drastically the activity of K^+-selective channels in the membrane fragment of the native U937 cell. One of possible mechanisms responsible for the channel activation might be a rise in the free Ca level near the intracellular

membrane side caused by hsp application to the external surface.

We carried out a series of control experiments to study, under similar conditions, the effect of another protein, – bovine serum albumin, – on channel activity in plasma membrane of U937 cells in cell-attached and inside-out configurations. 18 of 21 cell-attached patches with 50 μg/ml albumin in the pipette displayed no activity. In 3 patches, outward K^+ currents with conductance of 7–8 pS were observed. It can be concluded that the addition of albumin resulted in no change of the background channel activity and, particularly, did not evoke activation of the outward currents through potassium-selective channels in membrane fragment of native cell. This confirms that the effect of hsp70 on channel properties is related to a specific interaction of extracellular heat shock protein with the surface of U937 cells.

3.2. Outside-out experiments showed direct effect of exogenous hsp70 on plasma membrane properties in isolated fragment

In our cell-attached experiments, we did not observe any channel events induced by hsp70 which could provide a cation influx from the extracellular medium and, particularly, Ca entry to the cytoplasm. To elucidate the nature of the hsp70 interaction with the extracellular membrane side, its direct effect in outside-out patch configuration was studied.

Being a suspension cell line, U937 cells are weakly adhered to the surface of coverslips. This makes it very difficult to excise outside-out patch from the cell membrane. We had ten successful attempts to obtain reliable outside-out preparation by extremely careful excising of membrane fragment from the cell. Under normal conditions, the results of these experiments were in agreement with our previous data obtained in cell-attached measurements. In one outside-out patch, we observed an outward current activity of the K^+-selective channel of 7.2 pS (not shown) similar to those described above, as measured under identical ionic conditions (K^+ inside, Na^+ outside). Then, in symmetrical K^+ solutions, outward and inward single currents were measured displaying reversion near 0 mV and unitary conductance about 30 pS.

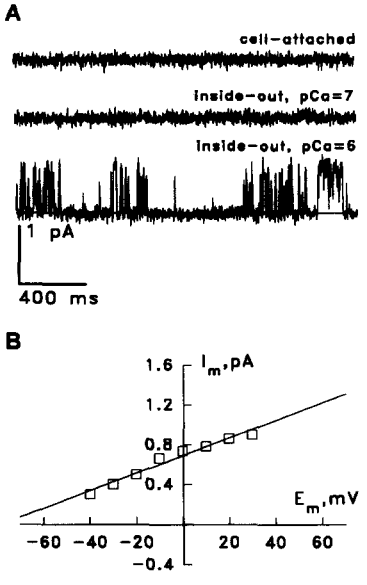

Fig. 4. Ca-activated K^+ channels in U937 cell membrane (without hsp70 application). (A) Cell-attached and inside-out recordings obtained on the same patch with normal pipette solution. Holding membrane potential was 0 mV. Activity of K^+ channels was observed only after excising the patch and increasing the level of $[Ca_i^{2+}]$ that is pointed near traces. (B) Correspondent current–voltage relation shows unitary conductance of 8.9 pS.

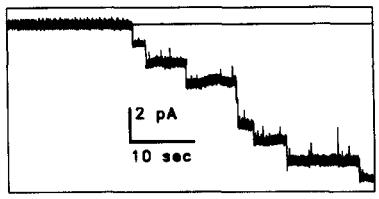

Fig. 5. Addition of 50 μg/ml hsp70 to the normal Na-containing external solution induced channel-like increase of the ionic conductance in outside-out patch of U937 cell. Membrane potential was −10 mV. Zero current level is indicated by solid line. Single step of inward current was about 1.1 pA.

In other 9 experiments, outside-out recordings revealed no channel events in the normal bath solution. These patches were tested with the external hsp70 addition. Fig. 5 shows the effect of hsp70 in representative experiment. In 1–2 min after the application of 50 μg/ml hsp70 to the extracellular surface of membrane fragment, outside-out recording displayed a stepwise increase of transmembrane current of inward direction. The amplitude of single transitions between current sublevels proved to be identical; it was about 1.1 pA at holding potential −10 mV. This hsp70-induced stepwise increase of the inward current resembled successive openings of the same channels.

Current steps of back direction corresponding to the channel transition from the open to closed state were rarely recorded. The reversion of currents induced by hsp70 in outside-out patch appeared to be close to zero potential. As a rule, it was difficult to measure single transitions at higher potentials, far from 0 mV because of great shifts of the transmembrane current.

It was found that hsp-induced stepwise increase of inward current stopped when hsp70-containing external solution was washed out with 100 mM $CaCl_2$. Fig. 6A shows outside-out recording of single channel activity at different holding potentials measured in 100 mM $CaCl_2$-containing external bath after the hsp70 treatment of excised membrane fragment. Corresponding current–voltage relation is presented in Fig. 6B. These inward currents recorded in outside-out patch after hsp70 application represented the activity of Ca-selective channels characterized

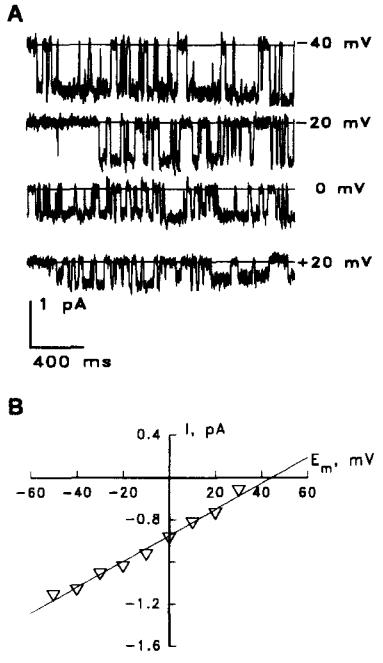

Fig. 6. Activity of Ca-permeable channels in outside-out patch treated previously with hsp70 (50 μg/ml). (A) Outside-out recordings of inward Ca currents in the external solution containing 100 mM $CaCl_2$. Holding potentials are indicated near traces. (B) Corresponding current voltage relation confirmed Ca nature of single currents observed. Unitary conductance: 12.3 pS.

by voltage insensibility and unitary conductance of 12 pS; reversal potential value obtained by extrapolation was about +47 mV. Calcium currents were found to be inhibited by the external addition of cadmium ions (10 μM $CdCl_2$). These channels proved to be permeable to barium cations (not shown) that is also typical for known Ca-specific channel types. In 100 mM $BaCl_2$-containing external solution, the unitary conductance was about 22 pS that was approximately twice higher than the value in 100 mM $CaCl_2$.

We have never observed the activity of Ca-permeable channels in the normal outside-out patches without previous hsp70 application. In our experiments on U937 cells, calcium currents were not recorded in other patch configurations either.

4. Discussion

In the present work, direct effects of exogenous heat shock protein hsp70 on plasma membrane properties in promonocyte U937 cells have been examined using different modes of patch clamp method. The main finding is that under physiological conditions hsp70 application to membrane fragment of the native cell caused activation of K^+-selective channels in it. K^+ currents with similar characteristics were observed very rarely in normal cell-attached patches. This confirms our conclusion that hsp70 increased drastically the activity of channels preformed in normal U937 cells but did not induce a novel type of K^+-selective pores. Inside-out experiments showed that K currents in excised fragments could be activated by a rise in free ionized Ca level up to 1 μM in the bath 'intracellular' solution. K^+-selective channels described here were voltage-independent and had a rather small conductance (about 9 pS). This value was determined in cell-attached and inside-out configuration with sodium solution in the pipette under the ionic conditions similar to physiological ones. As shown in outside-out recordings, K channels of this type display a higher conductance value (about 30 pS) with reversion of currents near zero in equilibrium potassium solutions.

Patch-clamp studies on monocyte-macrophages and other leukocyte cells have revealed a great diversity of K^+-selective channels identified primarily by their conductance value, voltage and Ca dependence [16]. The characteristics of channels found in our experiments appeared to be rather similar to properties of Ca-activated K^+ channels reported by Kanno and Takishima [9] in single channel study on U937 cells. Similar channels have been also described in human T- and B-lymphocytes [17,18]. Non-inactivating channel of 8.2 pS observed infrequently in cell-attached recording on T-lymphocytes [19] belonged most probably to the same type of K^+ channels. These data confirm our observations showing that the activity of K^+ channels was very low in plasma membrane of native promonocyte-like U937 cells. It should be noted that during cell-attached measurements the disturbances of cells were minimum, if any; the intracellular structures were not directly affected. So we can use the term native or intact cell in that case in contrast with experiments on excised fragments (outside- or inside-out configurations) or whole-cell recordings.

Electrophysiological measurements described here revealed very early events in the interaction of exogenous heat shock protein with plasma membrane of promonocyte U937 cells. Mechanisms of interaction of extracellular hsp70 with immune cells have been paid great attention due to their antigenic and chaperonic properties [2,15]. The effect of purified hsp-related proteins on immune characteristics of cells was reported [3,4]. Our patch-clamp data are in agreement with these immunological results and recent findings indicating association of extracellular hsp70 with the cell surface [5].

Outside-out experiments showed a channel-like stepwise increase of the inward transmembrane current in the excised patch induced by hsp addition to Na-containing external medium. The formation of ion-conducting pores by hsp70 and other proteins of hsp group in planar lipid bilayers was reported [6]. Obviously, some common features occur in the effects of hsp70 on artificial lipid membranes and excised fragment of cell membranes. One of the possible explanations of hsp effect is that the appearance of the non-selective channel-like conductance

may be due to an insertion of protein molecules into the lipid bilayer.

Our study has indicated an evident difference between hsp70 effect on membrane properties in native U937 cells and in excised fragments. In our cell-attached experiments with hsp, we have never observed development of inward currents through non-selective channels. It seems reasonable to assume that living cells possess of special mechanisms to prevent such disturbing effects of some physiologically important agents present in the extracellular fluid. At the same time, small or short-lasting cation currents due to the hsp70 application to cell-attached patch might have occurred but could not be recorded.

Mechanisms of K^+-selective channel activation in cell-attached patches with hsp70 addition remain to be elucidated. We suggest that hsp effect on K^+ channel activity may be due to a local increase of cytozol Ca near intracellular membrane side. Data of outside-out experiments provide some evidence concerning a possibility of hsp-induced Ca entry from the external solution. The openings of non-selective cation or Ca-selective channels would ensure an elevation of intracellular Ca level. It remains unknown whether extracellular hsp70 actually causes the formation of Ca-permeable pores in membranes of living cells at least for a short period of time.

We have obtained interesting data on stable normal patches showing the role of local cation influx through single pores in the regulation of Ca-dependent channel activity (Fig. 7). The entry of a minimum quantity of extracellular ions including calcium was likely to activate K^+ channel(s) in the fragment. At the same time, no changes of the intracellular Ca concentration in the whole cell volume would be recorded. This phenomenon may occur in case of hsp70 effect on K^+ channel activity in cell-attached patches described in our work. We emphasize a probable role of the local increase of intracellular Ca just near the inner membrane side evoked by hsp70. This assumption is also in agreement with the results of cell-attached experiments when hsp was added to the bath solution. In general, our data also confirm that specific localization and space correspondence of membrane structures is of great importance both in channel regulation and in other intracellular processes.

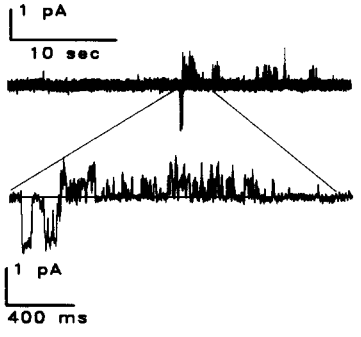

Fig. 7. An example of cell-attached recording in normal stable patches displaying minimal channel activity during 5–10 min. These records revealed occasionally some inward openings followed by a short period of outward current activity possibly representing Ca-activated K^+ channels. Spontaneous inward current due to membrane defects or other unknown mechanisms may provide cation entry from the extracellular side and result in a local elevation of free Ca^{2+} just near the inner membrane side.

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