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Activation of anion channels by zymosan particles in membranes of peritoneal macrophages

H.-A. Kolb and J. Ubl

University of Constance, Faculty of Biology, Constance (F.R.G.)

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Patch-clamp recordings were used to study the effect of zymosan adsorption on membranes of freshly isolated peritoneal macrophages of mouse. Superfusion of adherent macrophages by zymosan in the on-cell pipette configuration stimulated the appearance of anion channels after a varying time delay in the minute range. The channel is activated by passing through a stage of fluctuations of increasing amplitude. Once the full channel current has been reached, the fluctuations become transformed into the typical current pattern of three well-defined conducting channel states. The adoption of the two substates appeared to be dependent on zymosan. Up to nine simultaneously open anion channels could be observed with a single channel conductance of 220–400 pS. Absence of external Ca^{2+} had no inhibiting influence on the effect of zymosan. Anion channels could in some cases be observed under control conditions, after attachment of the pipette to the membrane. The channel activation could be mimicked by addition of A23187 to calcium-containing bath solutions. There is evidence that a zymosan-mediated rise of intracellular Ca^{2+} might be involved in the stimulus response coupling. The activation of calcium-dependent potassium channels was not observed.

Introduction

The role of extra- and intracellular Ca^{2+} as well as changes of the membrane potential by particle-induced phagocytosis have been the subject of some controversy [1]. Kouri et al. [2] have observed that rat peritoneal macrophages show sustained hyperpolarization correlated with phagocytosis of latex beads. On rat alveolar macrophages, ingestion of zymosan, an immunological reagent extracted from the cell walls of *Saccharomyces cerevisiae* yeast, causes increased depolarization [3]. Using IgG-coated red blood cells, Gallin [4] found no visible changes of membrane potential or conductance during the phagocytic process of peritoneal macrophages. In a variety of phagocytic cells, phagocytosis was followed by a Ca_i^{2+}-dependent slow hyperpolarization due to potassium efflux [1]. An activation of potassium channels or calcium-dependent potassium channels by zymosan could not be observed in the present study. But, as outlined in a recent review [5], the appearance of the channels is complex and virtually depends on the species of macrophage, the corresponding state of maturation, and the type of phagocytosis.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Correspondence: H.-A. Kolb, University of Constance, Faculty of Biology, D-7750 Constance, F.R.G.

For the first time we report the monitoring of the patch-clamp current during adsorption of zymosan particles to the membrane of freshly isolated murine peritoneal macrophages. It will be shown that anion channels of large unit conductance become activated as the only channel type. A short communication has been published previously [6].

Anion-selective channels of large unit conductance (200–400 pS) have been identified in membranes of cultured rat muscle [7], chicken muscle, FO cells, thymocytes, freshly isolated peritoneal macrophages [8], cultured Schwann cells [9], in apical membranes of various epithelial cell lines [10,11] and in cultured pulmonary alveolar cells [12]. Characteristic properties of this anion channel are two gating mechanisms which control the channel-open probability. Besides the analyzed steep voltage-dependent gating, there is evidence for unidentified but calcium-dependent events within the cell which ultimately lead to modulation of channel activity [8].

Materials and Methods

Cell material

Peritoneal macrophages were isolated as follows: An NMRI mouse was killed. Through a small hole in the abdominal wall 3 ml of RPMI 1640 (Gibco) medium were injected. The abdomen was agitated on a vibrator, and about 3 ml of the solution were withdrawn. Cells were washed twice in Hepes-buffered RPMI 1640 medium (Gibco). The resuspended cells were incubated on petri dishes, which contained glass coverslips, for 1 h at 37 °C.

Patch-clamp experiments and current-potential notation

Patch-clamp experiments were carried out according to the method of Hamill et al. [13]. The patch-current was stored on an FM tape recorder (RACAL Mod. Store 4 DS) with a frequency response DC to 10 kHz. To avoid uncertainty in the actual resting membrane potential, the denoted potentials are the actually applied pipette potentials. This is based on the fact that the reported resting membrane potential of macrophages shows large variations within the range of −20 mV to −80 mV, and changes during cell stimulation by phagocytic material [1,14]. The drawn current directions are referred to the pipette filling solution. Cationic current fluxes from the pipette into the cytoplasm are drawn as upward deflections. As reference electrode, a micropipette filled with 150 mM KCl was used throughout the experiments, and placed at the solution exit of the perfusion chamber.

Superfusion and staining

The glass coverslips were broken into pieces and placed in a miniature perfusion chamber which contained an exchangeable volume of about 200 µl. The volume could be completely replaced within 15 s. At the start of all experiments, the bath contained RPMI 1640 buffered with 20 mM Hepes to pH 7.3 (control solution). The experiments were performed at 20–22 °C. For establishing the giga-seal, adherent peritoneal macrophages were selected by use of Nomarski optics. The micropipette was typically placed on the center of the spread cell. During superfusion of the cells with zymosan (Sigma) -containing solutions, the pipette was held in the cell-attached configuration (on-cell mode) and a constant pipette potential in the range of 0 mV to −15 mV was applied. To exclude a bias of the results by channel stimulation as a response of pipette attachment to the membrane, only those membrane patches were selected which showed no detectable channel activity up to at least 15 min after pipette sealing. After this period the chamber volume was replaced by the desired electrolyte which contained 50 µg zymosan per ml.

In parallel with the superfusion experiments, adherent macrophages were exposed for 20 min to the identical zymosan containing solution. The zymosan ingestion was studied by the standard fixation and Giemsa-staining assay. Fixation was performed with 2% glutaraldehyde for 30 min and staining with Giemsa (Merck). The occurrence of endocytotic vesicles within the cells was considered as evidence for phagocytosis of zymosan.

Electrolyte solutions

At the start of the experiment, the control solution (see above) was used in the bath. In some experiments, it was replaced by the following elec-

trolytes, denoted as high-K and high-Na solutions, which were also used as a pipette filling. High-K electrolyte of 145 mM KCl/1.13 mM $MgCl_2$/20 mM Hepes-KOH (pH 7.3), for he high-Na electrolyte KCl was substituted by an equal amount of NaCl. To obtain a level of $[Ca^{2+}]$ in the bath of 10^{-7} M, 1.1 mM EGTA and 0.55 mM Ca^{2+} was added to these media. For experiments with the divalent cation ionophore A23187, a stock solution of 10^{-3} M in ethanol was prepared. An aliquot of this solution was added to the control solution and diluted to 1 μM A23187. In the case of zymosan-containing solutions, a stock solution of 50 mg/ml was prepared and diluted as indicated. Zymosan was finally dispersed by sonification of the medium for 1 min.

Amplitude histograms and channel-open probabilities

Mean current amplitudes of conducting and non-conducting channel states were determined from current amplitude histograms. The histograms were derived on line with a digital signal analyzer (Hewlett Packard (HP) 5420A Signal Analyzer) at a real sampling time of 9.8 μs. The area of the Gaussian-like distributed current amplitudes of the channel in the closed state and the conducting channel levels were calculated on line by an HP9825A. The ratio of corresponding area divided by the total area was taken as estimate for the open probability of the channel to be in one of the channel states. Values are given as mean ± S.E. (n = number of independent experiments).

Results

Zymosan-mediated channel activation

For the investigation of the effect of zymosan, freshly isolated peritoneal macrophages were used. Adherent and spread macrophages were selected and the patch-current in the on-cell mode of the pipette was analyzed. Fig. 1a shows a typical record after replacement of the control solution in the bath by the identical solution with 50 μg/ml zymosan added. The record presented starts 1 min after the replacement. For 15 min prior to the addition of zymosan, no channel-induced current fluctuations could be observed. The figure indicates a transient and fluctuating increase of the

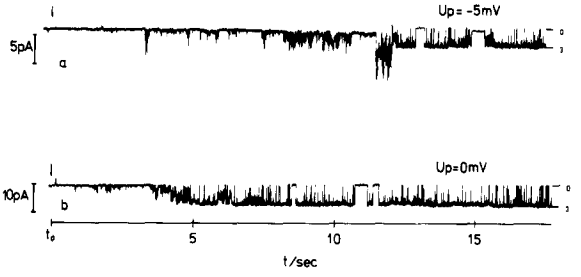

Fig. 1. On-cell patch-clamp records of the transient appearance of current fluctuations. a. Previous to the exchange of the bath solution by an identical one containing 50 μg zymosan/ml, only the leak current was recorded for 15 min. The replacement was completed about 1 min prior to the presented trace. b. The spontaneous occurrence of channel-induced current fluctuations is shown. The presented trace is started at t_0, about 5 min after establishing the giga-seal. Previously, only the leak current was observed. The peritoneal macrophages were incubated in RPMI 1640 and 20 mM Hepes at pH 7.3. The pipette was filled with high-K electrolyte. In both cases, a pipette potential was applied as indicated. The downward deflections denote an anion influx into the cytoplasm (Materials and Methods). On the right-hand side, the fully open channel state (3) and the channel closed state (0) are marked.

patch-current. About 10 s after the first occurrence of increased current fluctuations, the current starts to switch between well-definable current levels. The later current pattern can be observed thereafter. The transient and fluctuating increase of the patch-current is characteristic for the zymosan-mediated channel activation. After further incubation of the cell in the presence of zymosan, it is possible to observe the appearance of additional channels with a similar kinetical pattern. This is shown in Fig. 2. Up to six channels of equal unit amplitude are simultaneously activated. The corresponding channel showed anion selectivity (see below). No other type of current pattern was induced by the presence of zymosan. Variation of the zymosan concentration in the range of 5–500 μg/ml had no significant effect on the mean number of activated channels. The mean time delay, after which an increase of current fluctuations could be observed, decreased slightly from 5.0 ± 2.5 min (n = 8) at 5 μg zymosan/ml to 2.9 ± 2.3 min (n = 8) at 500 μg zymosan/ml. Variation of the constantly applied pipette potential for different experiments in the range of

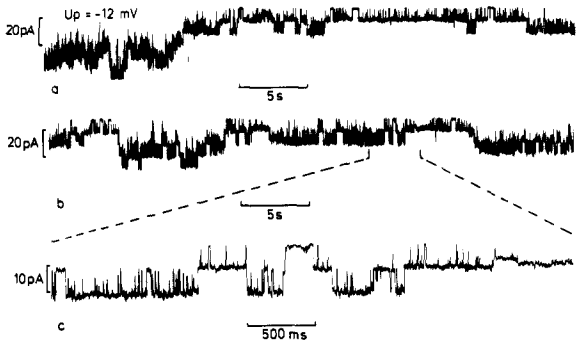

Fig. 2. Current fluctuations generated by the presence of zymosan. The record was obtained in the on-cell mode. After a silent phase of about 15 min, the bath was replaced by a solution containing 50 μg zymosan/ml in addition. About 2 min after the replacement several anion channels became transiently activated. The record presented was taken about 6 min after the first occurrence of channel activity. The records of a and b are one continuous trace. In c, a section of trace b, which is marked, is shown on an expanded time scale. A pipette potential of −12 mV was applied. The bath and pipette filling was the same as used for Fig. 1.

−20 mV to +20 mV had no significant influence on the time course of channel activation.

After 1 day of cell culture, addition of zymosan induced the described effect in one experiment out of 13, whereas after 2 days no channel activation by zymosan could be observed in 15 experiments. Analogous to this observation, the result of the staining procedure on zymosan-exposed macrophages (Materials and Methods) indicated a failure of phagocytic activity. But after 1 day of cell culture, the increased appearance of a voltage-gated inward-rectifying potassium channel with a slope conductance of $\gamma = 39 \pm 3$ pS ($n = 16$) could be observed in the cell-attached configuration with high-K electrolyte in the pipette and control solution for the bath. Using high-Na electrolyte in the pipette, an outward-rectifying potassium channel with a slope conductance of $\gamma = 16 \pm 1$ pS ($n = 3$) was observed (Somogyi and Kolb, unpublished observation; see also Ref. 14).

Since zymosan was added while the pipette was held in the on-cell mode, there is evidence that a second messenger like cytoplasmic free calcium (Ca_i^{2+}) might be involved in channel activation. Therefore, in analogy to the addition of zymosan, 1 μM A23187 was added to the bath. Within a time delay of 5 ± 3 min ($n = 9$) a similar transient current increase could be observed (see also Ref. 8).

It has to be mentioned that under control conditions in the absence of zymosan, the activation of the described current pattern can be observed occassionally (see (Fig. 1b). The attachment of the pipette tip on the membrane surface probably activates the macrophage and induces an increase of the patch-current. The observed time delay is in the range of at least 1 and up to about 10 min after pipette contact with the membrane. The measured time delays of channel activation are summarized in Table I. The table indicates that zymosan particles increase the frequency of channel activation by a factor of four. But it has to be considered that zymosan was added only in those experiments where no channel activity could be observed up to 15 min after sealing. Longer preincubation times could not be chosen due to the

TABLE I

Channel activation in membrane patches of macrophages in the absence (A) and presence (B, 50 μg zymosan/ml bath) of zymosan. The experiments were performed in the on-cell mode of the patch pipette. The cells were either incubated in control (RPMI 1640) medium, high-K electrolyte or high-Na electrolyte with 1.1 mM EGTA added. As pipette-filling solution, either high-K or high-Na was used. To measure the effect of zymosan on channel stimulation, those experiments were selected where no increase of the patch current could be observed up to 15 min. The corresponding delay times, after which the first channel-induced current fluctuations are measured, are given without the time-offset for the silent phase. Membrane patches which showed only the type of current fluctuation of Fig. 1 were considered as stimulated.

Electrolyte composition of bath/pipette	Number of experiments	Number of stimulated patches	Mean delay in min for stimulation
A.			
Control/high-Na	10	2	7.1 ± 2.1
High-K/high-K	27	5	5.2 ± 2.2
High-Na/high-K (10^{-7} Ca^{2+})	18	3	6.5 ± 3.5
B.			
Control/high-Na	10	8	4.5 ± 1.8
High-K/high-K	19	16	3.5 ± 2.0
High-Na/high-K (10^{-7} Ca^{2+})	8	6	7.5 ± 3.0

limited stability of the membrane patches. Furthermore, we investigated whether low calcium in the bath medium had an influence on the channel activation. As may be seen from Table I, the use of a high-Na medium with 10^{-7} M Ca^{2+} had no significant effect on either the spontaneous channel activation or the zymosan-mediated one. In analogy, the mean number of six vesicles/macrophage, which became visible after fixation and staining, was independent of external calcium as well.

It was previously shown that depolarization of the patched membrane increases the open probability of the anion channel of large unit conductance [8] in the inside-out configuration, but not in the cell-attached configuration of the micropipette. In the cell-attached configuration of the micropipette the effect of zymosan was analyzed for varying bath and pipette filling solutions. The results are given in Table I. Depolarization of the cell by replacement of the physiological bath solution by a high-K electrolyte showed no significant influence on the frequency of channel appearance, both in the absence and presence of zymosan.

Single-channel properties

The corresponding current-voltage relation of the channels fully open state shows linear behavior (see Fig. 3). For the unit single channel conductance $\gamma_3 = 332 \pm 70$ pS ($n = 30$) is derived. Besides the conducting fully open channel state which is denoted by γ_3 two further conducting states γ_1 and γ_2 ($\gamma_1 < \gamma_2$) could be identified. Fig. 3 indicates that using symmetrical electrolytes across the patched membrane the channel adopts a permanently closed state for positive as well as negative potentials beyond certain threshold values (see legend of Fig. 3). Fig. 4 shows current records after zymosan induced channel activation at different pipette potentials. It shows the occurrence of the distinguishable channel conductance states γ_1, γ_2 and γ_3. It was found that in contrast to the observed large variation of γ_3 in the range of about 220 pS to 400 pS, the range of the ratios γ_2/γ_3 and γ_1/γ_3 was significantly smaller.

For the mean ratio γ_2/γ_3 (γ_1/γ_3) a value of 0.66 ± 0.05 ($n = 28$) (0.26 ± 0.05 ($n = 22$)) is obtained. Using the inside-out configuration the ion selectivity could be determined. Within the experimental error the reversal potential of the three conducting channel states was zero millivolt for high-K as well as high-Na electrolyte. This indicates a comparable selectivity of chloride over cations for the different substates. Dilution of 145 mM KCl in the bath to 75 mM shifts the reversal potential to 15.8 ± 2.5 mV ($n = 4$) (see also Fig. 3). Replacement of 145 mM KCl in the bath by 300 mM (500 mM) KCl shifted the reversal potential to -12.5 mM (-19.2 mV), respectively. If one applies the Goldman-Hodgkin-Katz equation for potassium and chloride as transported ions, a selectivity ratio of about five to six for chloride

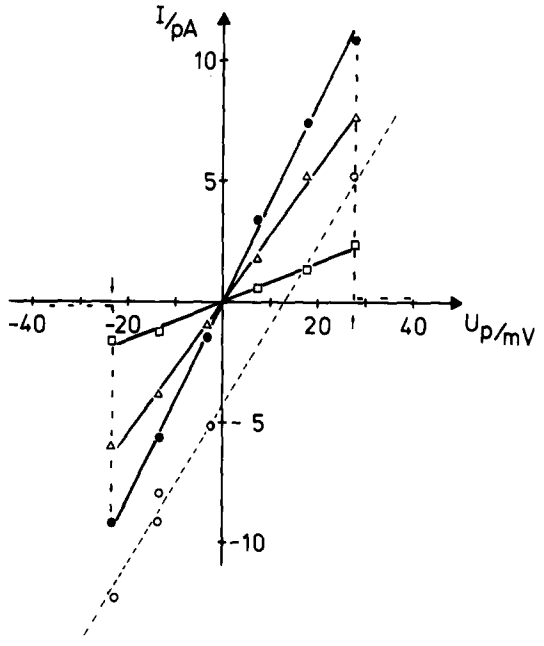

Fig. 3. Single-channel current amplitudes (i) as function of applied pipette potential (u). The data were measured in the excised-mode of the membrane after channel activation by zymosan in the on-cell mode. Both sides of the membrane were exposed to high-K electrolyte. Besides the current amplitudes of the fully open channel state (●, I_3), also those of the corresponding conducting substates (△, I_2) and (□, I_1) are shown. The corresponding conductances are: $\gamma_1 = 80$ pS, $\gamma_2 = 280$ pS and $\gamma_3 = 410$ pS. Outside certain threshold potentials (about +28 mV and −22 mV which are marked by arrows) the channel closes permanently. After substitution of 145 mM KCl in the high-K electrolyte by 75 mM KCl the dashed line (○, I_3) was obtained for the fully open channel state. For the corresponding slope conductance $\gamma_3 = 330$ pS is derived and a reversal potential of $U_p = +13$ mV. For further explanations see text.

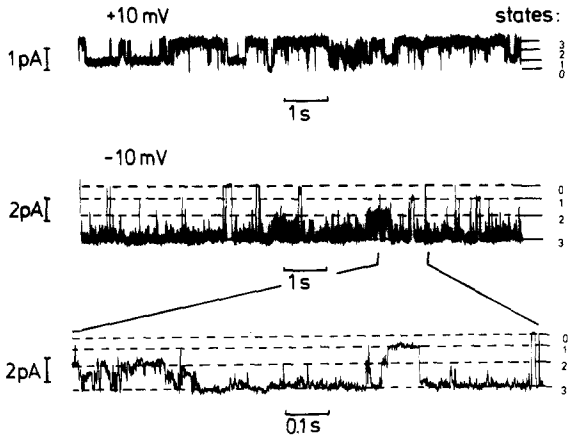

Fig. 4. Current record in the on-cell mode after zymosan mediated channel activation at a pipette potential of +10 mV and −10 mV. In the lowest trace the current fluctuations is displayed on an expanded time scale. The record shows the appearance of the fully open channel state (γ_3) and two intermediate conducting substates (γ_2, γ_1). The treatment of the cells by zymosan and the electrolyte composition were the same as described for Fig. 1a.

over potassium isderived for the fully open channel state. This result is in agreement with previously reported results [8,9]. The replacement of 145 mM KCl in the pipette by 75 mM KCl and 75 mM potassium aspartate shifted the reversal potential to about +14 mV ($n = 2$), whereas for a replacement of KCl by KNO_3 no significant shift of the reversal potential was observed.

The conducting states γ_3 and γ_2 were described previously [8]. In addition we observed state γ_1. But the frequency of adoption of state γ_1 appears to be dependent on the zymosan-mediated channel activation. The probability to adopt state γ_1 after zymosan-mediated channel activation is 0.051 compared to 0.012 in the case of spontaneously active channels. As described previously in detail, the once activated anion channel shows a steep voltage-dependent gating behavior [8] (see also Fig. 3). The voltage-gated transition to the permanently closed state can occur from any of the three conducting states. A voltage-induced stepwise adoption of the sequence $\gamma_3 \rightarrow \gamma_2 \rightarrow \gamma_1 \rightarrow \gamma_0$ was not observed.

Discussion

The zymosan-mediated activation of ion channels, and the corresponding single channel properties have been studied in membrane patches of freshly isolated peritoneal mouse macrophages. Exposure of the cell membrane of adherent macrophages to zymosan particles initiated the opening of anion channels of large single channel conductance in the patched-membrane under cell-attached configuration of the micropipette. The activation occurred after a varied time delay in the minute range. The delay decreased slightly with increased zymosan concentration of 5 to 500 μg/ml. But the mean number of simultaneously activated channels showed no variation. During the phase of activation, the fully open channel state was not reached in an all-or-nothing transition, but by a transient current increase which showed interruptions in the millisecond range. This transient behavior is characteristic only for the on-set of channel activity. While zymosan was present the simultaneous opening of up to nine channels of equal unit amplitude could be observed. For all membrane species wherein this channel type could be identified, kinetic transitions between well-defined conductance states [8] and varying intermediate current levels were reported [10,11]. Our observations indicate that the intermediate current values are preferentially adopted during the early phase of channel activation.

After a culture period of about 24 h a zymosan-induced activation of ion channels could only be observed in less than 10% of the experiments. By application of a staining procedure, a steep decrease of phagocytic activity after this time in culture could also be found.

The anion channel has a conductance of about 332 pS for the fully open state (γ_3), and varies between the extremes of 220 pS and about 400 pS in different experiments. A similar large variation of the single channel conductance has been reported for the calcium-dependent maxi-potassium channel [15]. Both channel types, the anion- and maxi-potassium channels, show the appearance of substates. In case of the anion channel, at least two conducting substates (γ_1, γ_2) could be identified (Fig. 3). Despite the large variation in γ_3 the

corresponding ratio γ_1/γ_3 and γ_2/γ_3 are about constant. For the three conducting states γ_1, γ_2 and γ_3 a comparable ion selectivity of about 5 : 1 for chloride over sodium or potassium (see also [8,9]) was found. Besides the channel activation, the presence of zymosan induced a significant frequency increase for the adoption of substate γ_1.

We have speculated about the mechanisms which regulate the activation of the large anion channel. Since zymosan was added to the bath after establishing the on-cell mode of the micropipette, there is evidence that a second messenger might be involved in the stimulus-response coupling. This is supported by the finding that the effect of zymosan could be mimicked by the addition of the divalent cation ionophore A23187 to calcium-containing bath solution. Birmelin and Decker [16] reported that isolated Kupffer cells of rat liver showed an increased Ca^{2+} uptake as an initial event after stimulation with zymosan particles. One may suggest that cytoplasmic free calcium acts as a second messenger and may be involved in phosphorylations or in the binding of specific ligands to the transport protein leading to a conducting channel [17,18]. The open probability of the once activated anion channel appears to be largely independent of a variation of Ca_i^{2+} in the range of 10^{-8}–10^{-3} M (see also Ref. 9), which was tested under inside-out configuration of the membrane patch. The question which role external calcium plays in phagocytosis remains open.

We found that a low calcium concentration in the bath ($Ca^{2+} \approx 10^{-7}$ M) had no significant influence on the activation of the anion channel by zymosan. For IgGγ2b/γ1Fc receptor-dependent phagocytosis, it was shown that free cytoplasmic calcium increases even in the absence of external supply [19]. Surprisingly we did not find an activation of Ca_i^{2+}-dependent voltage-gated maxi-potassium channel (cf. Ref. 20). This channel type was identified in human monocytes which were cultured for longer than 24 h [21], but we could not observe it in more than about 200 patch-clamp experiments in the on-cell or the excised mode, using freshly isolated peritoneal mouse macrophages. As described in a recent review [5], different types of ion channels, mainly potassium-selective channels, were identified in macrophages. But the appearance of the channels is complex and virtually dependent on the species of macrophages, their state of maturation, and the type of phagocytic material. The activation of selective outward directed potassium channels by zymosan could not be observed in more than 80 experiments. Independently of zymosan, inward and outward rectifying potassium channels with a single channel conductance of about 35 pS were found, and could be identified with increased frequency after prolonged culturing (see also Ref. 14). Therefore the activation of the anion channel of large unit conductance might be specific for zymosan-induced phagocytosis by freshly isolated and adherent peritoneal macrophages. Zymosan had a similar effect on isolated Kupffer cells of rat liver, but on human granulocytes, no channel activation could be observed (unpublished observation). In analogy to the activation of the anion channel by zymosan, it was possible to observe occasionally this channel type after a similar time delay after attachment of the pipette to the cell membrane (Table I). It cannot be excluded that in these cases the pipette-membrane attachment acts as a phagocytic signal. Further experiments are necessary to elucidate this activation mechanism.

A second mechanism which obviously controls the open probability is the potential gradient across the channel. The channel conducts in the inside-out configuration of the membrane patch at transmembrane voltages of about zero (see also Fig. 3); outside this voltage range, it reverts to a permanently closed state with characteristic voltage-dependent relaxation times (see Refs. 8 and 11, and Fig. 3). This voltage dependency appears to be an intrinsic property of this channel type. A similar result has recently been reported for this channel type in membranes of histamine-secreting cells [22].

Finally, we want to comment on the physiological function of the large anion channel. Based on the single channel parameters and gating properties, there is evidence that the anion channel might also be involved as a hemichannel in the formation of cell-to-cell channels [23]. Junctional channels have not been identified for macrophages, but, in vitro, macrophages have been found to be electrically coupled to each other [24]. A cytoplasmic bridging of macrophages by 'close' junc-

tions has been identified from electronmicrographs [25]. Therefore, the anion channel could also play a role in enabling cell-to-cell interaction of macrophages for phagocytic events to occur especially in cases where the geometric size of the particles and cells is of the same order as that of the macrophage.

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