Chemoattractant-induced membrane hyperpolarization in *Dictyostelium discoideum*

A possible role for cyclic GMP

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Received 17 May 1990; revised version received 23 July 1990

Membrane potentials of the cellular slime mold *Dictyostelium discoideum* were monitored after chemotactic stimulation by measuring the distribution of the lipophilic cation tetraphenylphosphonium. Stimulation with the chemoattractant cAMP induces a transient membrane hyperpolarization which reaches its most negative value between 1–3 min after stimulation. This hyperpolarization is consistent with the opening of potassium channels. Measurements in *streamer F* mutant cells reveal that cGMP likely plays a role in the regulation of the cAMP-induced hyperpolarization.

K^+ channel; H^+-ATPase; Cyclic AMP; Chemotaxis; Cyclic GMP; *Dictyostelium discoideum*

1. INTRODUCTION

In the unicellular stage the cellular slime mold *Dictyostelium discoideum* lives as single amoebae in association with their food source, bacteria. A multicellular stage is entered upon exhaustion or removal of the food source. Starvation of the cells induces periodic secretion of cyclic AMP (cAMP). cAMP mediates the aggregation of the single cells into a multicellular structure by acting as chemoattractant [1,2]. The chemotactic stimulation of *D. discoideum* cells leads to a diversity of transient intracellular responses [3,4]. Among these responses there are fluxes of different ions: H^+, K^+ and Ca^{2+} [5–10]. These fluxes are caused by stimulation of electrogenic proton pumps and the opening of ion channels [6–8], which will lead to membrane potential changes and may play a role in the regulation of the chemotactic response. In addition, the cAMP-induced ion fluxes may be regulated by the membrane potential.

Electrophysiological studies showed that the membrane potential of unstimulated *D. discoideum* cells is mainly regulated by an electrogenic proton pump, and that potassium ions play a minor role [11,12]. Membrane potential measurements in stimulated wild type and mutant cells may reveal some aspects of the cAMP-induced ion fluxes and their regulation. It has been shown that reliable steady-state membrane potential measurements with electrophysiological techniques are very difficult in *D. discoideum* [11]. Therefore, we used the uptake of the lipophilic cation TPP^+ as an alternative though indirect method to measure membrane potential [e.g. 13,14].

In the present study we report the effects of cAMP stimulation on the membrane potential of *D. discoideum*. In addition, membrane potential upon cAMP stimulation was measured in the mutants *streamer F (StmF)* [15,16] and *Synag 7* [17]. Our measurements indicate that the cAMP-induced cyclic GMP response plays a role in the regulation of the ion fluxes observed upon chemotactic stimulation.

2. MATERIALS AND METHODS

2.1. *Materials*

Tetra[^{3}H]phenylphosphonium bromide and tritiated water were obtained from Amersham Int., England. Silicon oil AR 20 and AR 200 were from Wacker Chemie, Munich, FRG. All other chemicals were from Sigma Chemical Co., St. Louis, USA, and Merck, Darmstadt, FRG.

2.2. *Cell culture conditions*

Experiments were done with *Dictyostelium discoideum* NC-4(H) grown in association with *Escherichia coli* 281 on SM medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.4 g $Na_2HPO_4 \cdot 2H_2O$, 15 g agar and 1 l H_2O at 22°C. Vegetative cells were harvested with cold 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB), and washed free from bacteria with PB by 3 washes and centrifugations at 150×*g* for 2 min. Cells used for experiments were starved by incubation on non-nutrient agar (1.5% agar in PB) for 4.5 h at 22°C, followed by 1 h shaking in suspension at 2×10^7

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Abbreviations: TPP^+, tetraphenylphosphonium cation; DES, diethylstilbestrol; E_m membrane potential; E_{hyp}, membrane potential at 2 min after cAMP stimulation

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

cells · ml^{-1} in PB. Mutant cells used were grown and treated in the same way as the wild type cells.

2.3. *TPP$^+$-measurements*

For TPP$^+$-uptake experiments, cells in suspension (8×10^7 cells/ml) in PB were incubated with 100 μM [^{3}H]TPP$^+$. TPP$^+$-incubation time was in each experiment 5 min, unless stated otherwise. Cells were stimulated with cAMP at different times after TPP$^+$ addition. At $t = 5$ min the 50 μl cell suspension samples were layered on top of 500 μl silicon oil (AR 20:AR 200 = 2:1) and 10 μl 10% sucrose. Immediately thereafter, the cells were separated from the extracellular solution by centrifugation of the cells through the oil layer at 10 000 × g for 15 s in a swingout rotor. This separation is based on the fact that the density of the silicon oil is higher than that of the extracellular fluid but lower than the density of the cells [18,19,19a]. Due to the centrifugation the cells pass the oil layer while the extracellular fluid remains on top of the oil. Thereafter, the tubes were frozen and the tips containing the sucrose and cell-pellet were cut off. The radioactivity of cell-pellet and supernatant was measured after dissolving pellet and supernatant in 2 ml scintillation fluid. Control experiments without cells showed that no radioactivity from the extracellular fluid reached the pellet, hence, all the radioactivity in the pellet was cell-associated. Experiments with HCl added to the bottom solution and pH indicators to the top solution revealed that there was no redistribution of liquid from the bottom to the top under the conditions used. From these measurements the ratio between extra- and intracellular TPP$^+$ (R_{TPP}) was determined. The ratio between [^{3}H]TPP$^+$ in the extracellular fluid and the pellet ranged between about 1 and 4. Parallel experiments at the same time with cells from the same suspensions were done with added tritiated water instead of [^{3}H]TPP$^+$ under all stimulation conditions. From these measurements the volume ratio between cells and extracellular solution (R_v) was determined. The membrane potential was calculated by substitution of R_{TPP} and R_v into the Nernst equation: $E = RT/F \ln R_{TPP}/R_v$, in which R = gas constant, T = absolute temperature and F = Faraday's constant.

Experiments were performed in duplicate at room temperature (ca 20°C). Mean values ± standard deviation (SD) are given (unless specified otherwise), with n = number of independent experiments.

3. RESULTS

3.1. *Resting membrane potential*

The resting membrane potential of starved *D. discoideum* cells in PB (pH 6.5) was measured. The mean calculated membrane potential value was -46.2 ± 7.1 mV ($n = 16$). This value is close to the estimated membrane potential based on microelectrode-induced peak potential measurements [12]. The membrane potential of *D. discoideum* is mainly determined by an electrogenic proton pump, and to a lesser extent by the potassium equilibrium potential [11,12]. Table I shows that the membrane potential as measured by the TPP$^+$ distribution is sensitive to both proton pump inhibition by DES and increase in extracellular potassium concentration. The measured values are in agreement with the results of microelectrode studies which have been published before [11,12]. In addition, the potassium channel blocker quinine depolarizes the membrane (Table I). From these measurements we conclude that the TPP$^+$ distribution can be used to measure membrane potentials in *D. discoideum*.

Table I

Resting membrane potential (E_m) and membrane potential at 2 min after stimulation (E_{hyp}) with 10^{-7} M cAMP under various conditions. Inhibitors were added together with [^{3}H]TPP$^+$ or tritiated water

Condition	E_m (mV)	E_{hyp} (mV)
Control[1]	-46.2 ± 7.1 ($n = 16$)	-58.9 ± 6.6 ($n = 12$)
20 μM DES	-28.6 ± 4.5 ($n = 3$)	-55.1 ± 12.8 ($n = 3$)
50 mM potassium[2]	-33.5 ± 5.2 ($n = 4$)	-38.4 ± 5.3 ($n = 4$)
0.2 mM Quinine	-35.0 ± 8.8 ($n = 4$)	-37.1 ± 7.0 ($n = 4$)

[1]Control solution PB (pH 6.5)
[2]Sodium/potassium phosphate buffer with 50 mM K$^+$, pH 6.5

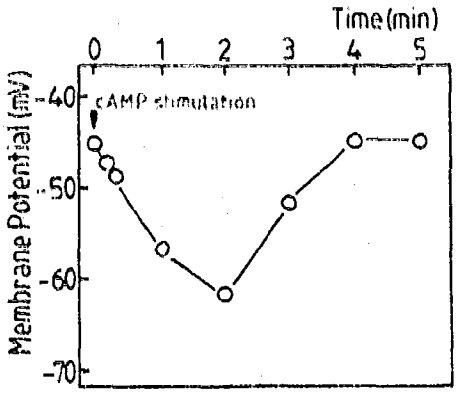

Fig. 1. Response of the membrane potential of starved *D. discoideum* cells upon stimulation with 10^{-7} M cAMP at $t = 0$. Response of one experiment (in duplicate) is shown.

3.2. *cAMP-induced hyperpolarization*

Stimulation of starved cells with 10^{-7}M cAMP induces a transient hyperpolarization of the membrane potential (Fig. 1). This hyperpolarization reaches its most negative value of about −59 mV between 1 and 3 min after stimulation. Fig. 2 shows the effect of different cAMP concentrations on the cAMP-induced membrane hyperpolarization, with a half-maximal stimulation at about 10^{-7}M cAMP. This hyperpolarization may be due to the opening of ion channels

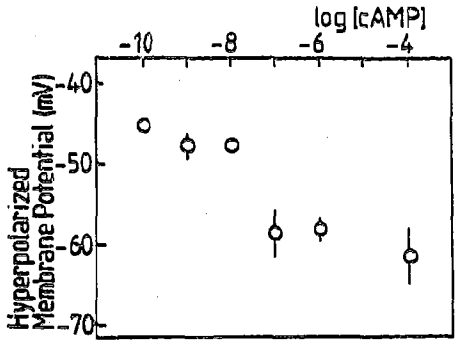

Fig. 2. Mean hyperpolarized membrane potential 2 min after stimulation of starved *D. discoideum* cells as a function of the cAMP concentration used. Results of 2 to 4 independent experiments are shown: bars indicate ± SE.

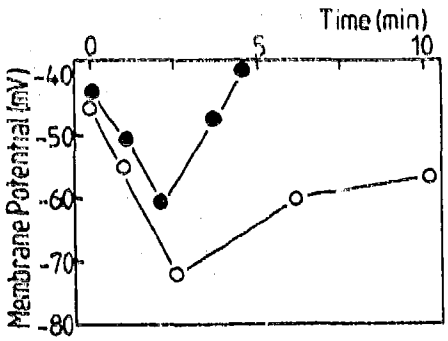

Fig. 3. Membrane potential response upon stimulation of *Synag 7* (•) and *StmF* (○) cells with 10^{-7} M cAMP at $t = 0$. In experiments where samples were obtained after more than 5 min after stimulation with cAMP, TPP^+ and cAMP were added together.

or stimulation of electrogenic ion pumps. The reported cAMP-induced efflux of potassium ions [6] as well as of protons [7] may result in membrane potential hyperpolarization.

Table I shows the effect of 20 μM DES, 50 mM K^+ and 0.2 mM quinine on the cAMP-induced membrane hyperpolarization. Although DES is able to depolarize the steady-state membrane potential, it has no effect on the cAMP-induced hyperpolarization. However, both high K^+ and quinine inhibit the hyperpolarizing response effectively.

3.3. *Regulation of the cAMP-induced hyperpolarization*

To reveal some aspects of the regulation of the cAMP-induced hyperpolarization membrane potentials upon chemotactic stimulation of mutant cells were measured. Mutant *Synag 7* cells are defective in the cAMP-induced activation of adenylate cyclase [17]. The cAMP-induced hyperpolarization, however, was not different from that in wild type cells (Fig. 3; $E_m = -42 \pm 11$ mV, $E_{hyp} = -68 \pm 9$ mV, $n = 2$).

StmF mutants do not possess cGMP-specific phosphodiesterase. This causes a persistent elevation of intracellular cGMP upon cAMP stimulation [15,16]. The cAMP-induced membrane potential hyperpolarization in *StmF* mutants differs from the normal response (Fig. 3; $E_m = -45.4 \pm 9.9$ mV, $n = 8$; $E_{hyp} = -72.3 \pm 13.2$ mV, $n = 5$). The cAMP-induced hyperpolarization in *StmF* cells is much more prolonged than in wild type cells. Six min after stimulation these cells still have a hyperpolarized membrane while the membrane potential of wild type cells already returned to the basal level (cf. Figs 1 and 3).

4. DISCUSSION

The membrane potential of *D. discoideum* cells is mainly determined by an electrogenic proton pump and to a lesser extent by the potassium equilibrium potential [11,12]. Our TPP^+-distribution measurements provided an independent method to measure the membrane potential of *Dictyostelium* and confirmed these properties of the cell membrane measured with microelectrodes (Table I). Furthermore, it is likely that the contribution of the potassium equilibrium potential to the membrane potential is due to the presence of quinine-sensitive K^+ channels, since quinine depolarizes the membrane (Table I). These can be the K^+ channels observed in patch clamp experiments, which are active at the resting membrane potential [20].

Stimulation of starved cells with cAMP leads to membrane hyperpolarization (Fig. 1). This hyperpolarization coincides with the cAMP-induced K^+, Ca^{2+} and H^+ fluxes [5–10]. However, only the K^+ and H^+ effluxes will lead to membrane hyperpolarization. Our experiments indicate that the main contribution to the hyperpolarization is due to the activation of potassium channels, since it can be effectively inhibited by high extracellular K^+ concentration and quinine. It is likely that the reported efflux of K^+ is responsible for the hyperpolarization because both responses can be inhibited by quinine (Table I, [6]), and show about the same dose-response curve (Fig. 2, [21]). The activity of the electrogenic proton pump increases with about 50% upon cAMP stimulation [7]. This increase, however, does not lead to a measurable hyperpolarization of the membrane potential since proton pump inhibition has no effect on the hyperpolarization while both quinine and high K^+ completely inhibit hyperpolarization (cf. Table I). The absence of membrane hyperpolarization while proton pump activity increases may be explained by the presence of cAMP-induced membrane conductance changes (e.g. ion channel activation), or activation of depolarizing ion pump currents. In *Neurospora* cells the presence of a membrane depolarizing K^+/H^+ co-transporter increases the efficacy of proton pumping by decreasing membrane hyperpolarization [22].

The experiments with mutant *Synag 7* cells show that the cAMP-induced elevation of the cytoplasmic cAMP level is not a mediator of the cAMP-induced hyperpolarization. The cAMP-induced cGMP response, however, may be involved in the hyperpolarizing response as indicated by the response of *StmF* mutant cells (Fig. 3). Cells with a prolonged rise of the cGMP level show a prolonged membrane hyperpolarization as well. The K^+ channels which are likely responsible for the membrane hyperpolarization are Ca^{2+}-sensitive, i.e. they are inactivated by a rise in intracellular Ca^{2+} [21]. These results may implicate that (i) increased cGMP levels prevent a rise in cytosolic Ca^{2+} and thus prevent inactivation of the K^+ channels, or (ii) increased cGMP levels keep the K^+ channels in the activated state or insensitive to Ca^{2+}. It is not likely that ion channel opening is directly regulated by cGMP since the time scale of the transient cGMP response is much shorter than that of the cAMP-induced hyperpolariza-

tion. Phosphorylation via cGMP dependent protein kinases can be a possible activation mechanism [23].

The cAMP-induced opening of K^+ channels may play a role in the regulation of the Ca^{2+} influx and efflux, and proton pump activation. The Ca^{2+} and H^+ fluxes are probably important for the regulation of chemotaxis. Membrane-potential changes which might be correlated with chemotactic movement have been reported [24]. The change of the membrane potential itself, however, likely plays no significant role in the signal transduction leading to chemotaxis, since artificially depolarized cells show normal chemotaxis [25].

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