In Situ Measurements of External pH and Optical Density Oscillations in Dictyostelium discoideum Aggregates

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Abstract. In situ measurements of extracellular pH by means of microelectrodes and in situ measurements of optical density were performed on aggregating cells of Dictyostelium discoideum. Early aggregation stage AX2 cells showed sinusoidal pH oscillations, which could be inhibited by the specific relay inhibitor caffeine, indicating that they were coupled to cAMP oscillations. Sometimes biphasic pH oscillations were found, which can be explained by the superposition of two harmonic pH oscillations. These harmonic oscillations might arise by gating of the cAMP signal; a part of the cells respond to every cAMP signal and another subpopulation to every second cAMP pulse. Late aggregation-stage cells showed complex changes of the extracellular pH, which could be inhibited by caffeine.

Optical density measurements of wave propagation in aggregation streams of HG220 also revealed gating behavior. In addition to sinusoidal optical density oscillations, biphasic and still more complex oscillations were observed.

Upon starvation, individual amebas of the cellular slime mold Dictyostelium discoideum become organized into multicellular structures. The cells aggregate to form a hemispherical cell mass. Aggregation is mediated by the outward propagation of cyclic AMP (cAMP) waves (Gerisch et al., 1977; Tomchik and Devreotes, 1981; Loomis, 1982) that originate from an aggregation center consisting of cells that periodically release pulses of cAMP. These autonomously produced cAMP pulses are relayed throughout the population by the other amebas (Shaffer, 1975; Roos et al., 1975).

In addition, the amebas respond chemotactically to cAMP. This chemotactic response to the propagating cAMP waves leads to a periodic movement of the cells, which is oriented in the direction of the aggregation center. The outward moving cAMP waves organize an inward movement of the cells, which is also wavelike (Devreotes, 1982). The period length of chemotactic aggregation waves seen in time-lapse observations is not constant, but decreases from ~10 min initially to ~2 min during aggregation (Durston, 1974; Gross et al., 1978).

It is known from experiments performed in cell suspension that periodically released cAMP pulses are accompanied by sinusoidal oscillations of the extracellular pH with an amplitude of 0.01 pH units (Malchow et al., 1978). These cAMP pulses in cell suspension are also coupled to oscillations in optical density (Gerisch and Hess, 1974). Thus in situ measurements of both extracellular pH and optical density can be used as indicators for cAMP waves.

In this paper, we present in situ measurements of the extracellular pH and optical density in aggregates. The results provide evidence for gated responses to cAMP waves.

Materials and Methods

Cells and Strains

AX2 cells were cultivated as described elsewhere (Watts and Ashworth, 1970). Cells in 1 ml of cell suspension (cell titer, 4 × 10⁶/ml to 8 × 10⁶/ml) were harvested, vitally stained with 10 μl 0.1% neutral red, and immediately thereupon washed in KK2 buffer (potassium phosphate buffer, pH 6.8). The cells were resuspended in KK2 buffer to a final volume of 30 μl and this drop of cells was put on a 1% water agar plate. Under these conditions, development to the tipped aggregate stage took 12−15 h.

The mutant HG220 (an axenic streamer mutant strain derived from HL220 and kindly supplied by Dr. G. Gerisch) was cultivated by streaking spores on one side of a bacterial (Klebsiella aerogenes) growth plate (5 g bacto peptone [Difco Laboratories Inc., Detroit, MI], 0.5 g yeast extract [Difco Laboratories Inc.], 5 g glucose, 2.31 g KH₂PO₄, 1.7 g K₂HPO₄·3H₂O, and 20 g agar per liter). Under these conditions, the mutant cells clear the lawn of bacteria over a period of several days giving rise to large aggregation streams just behind the growth zone.

Measurement of the Extracellular pH with Microelectrodes

pH was measured with the aid of pH-sensitive microelectrodes filled with a liquid ion exchanger (Ammann et al., 1981). The measuring set up consisted of a pH-sensitive electrode and a separate reference electrode (filled with 3 M KCl in 1% agar). pH-sensitive microelectrodes were constructed from glass micropipettes with a tip diameter of 5 to 10 μm. The tips were siliconized by immersion in a 10% siliclad (Serva 35130) solution, baked for 1 h at 100°C, and filled with a proton selective ionophore (World Precision Instruments, Inc., New Haven, CT; IE-010). The electrode contained 3 M KCl, 0.2 M K₂HPO₄ (pH 6.6) as reference solution. These electrodes showed a response of 50 mV/pH unit in the range of pH 6−8. The electrode potential was recorded by a guarded operational amplifier (Burr-Brown 3528) connected as a unity gain follower. pH was measured as the potential difference between the ion selective electrode and filled with a proton selective ionophore (World Precision Instruments, Inc., New Haven, CT; IE-010).
and the grounded reference electrode. Total noise measured by inserting the pH-sensitive electrode into the agar was ≤ 0.2 mV. All measurements were performed in a Faraday cage under dark and moist conditions. In situ measurements of the extracellular pH were performed by inserting the pH-sensitive electrode in the extracellular space of aggregation competent AX2 cells densely packed in a 30-µl droplet.

**Optical Density Measurements**

Optical density was measured as the transmission of white light through an HG220 aggregation stream. The measurements were made with the aid of a microscope equipped with a photomultiplier and a variable measuring slit (Leitz MPV compact). The intensity of transmitted light was measured in a 30 × 10-µm area, oriented perpendicular to the aggregation stream. Measurements were made at a 25× magnification with semi-darkfield illumination.

**Power Spectrum Analysis**

The recorded signals were digitized by a Kontron microcomputer. Sample time was 0.2 s; data were averaged over 2.8 s and stored as one data point on floppy disk. Data analysis was performed on sets of 512 data points.

The data were first normalized by subtracting the mean of the data set and then filtered through a second order digital butterworth band pass filter (Schwiger, 1983) with cut off frequencies of 0.1 Hz and 1.666 × 10⁻³ Hz, respectively, to eliminate long-term drift of the recorded signals.

Power spectra of the filtered data were calculated by the use of a Fast Fourier Transform algorithm (Bloomfield, 1976). The power at each frequency was expressed as percentage of total power.

**Results**

**Sinusoidal Oscillations of the Extracellular pH in Early Aggregation–Stage Cells**

To test the sensitivity of the microelectrode system, we attempted to measure pH oscillations in suspensions of aggregation-competent cells (Malchow et al., 1978). 3 h after the onset of starvation, we started recordings of extracellular pH in 30-µl drops of AX2 cells. The extracellular pH of 3–5-h-starved cells showed sustained sinusoidal oscillations in 17 of 21 measurements (Fig. 1a). Oscillations were highly periodic, with a mean period length of 5.1 ± 1.0 min. Noise was normally very low and even the rare measurements, which showed a greater noise amplitude, clearly revealed periodic pH oscillations (Fig. 1b). As expected, even these oscillations showed a clear single peak in their power spectrum (Fig. 1c). The amplitude of pH oscillations was quite constant with a mean of 1.2 ± 0.7 mV, which corresponds to 0.024 pH units. Transient variations of the amplitude were observed in very few cases (Fig. 2a).

In addition to the normal sinusoidal oscillations, we also detected spike-shaped responses in a few cases (Fig. 2b).

**Coupling of pH Oscillations to cAMP Waves**

To investigate whether these pH oscillations were coupled to the relay of cAMP waves, we tested the effect of caffeine upon oscillations, since caffeine has recently been shown to be a relatively specific inhibitor of cAMP relay (Brenner and Thoms, 1984; Theibert and Devreotes, 1983). Fig. 3 shows that caffeine added to a final concentration of 10 mM inhibited the oscillations completely. This finding shows that the pH oscillations are coupled to cAMP relaying.

**Biphasic Oscillations of the Extracellular pH in Early Aggregation–Stage Cells**

In addition to the sinusoidal pH oscillations described above we observed biphasic pH oscillations in 4 of 21 measurements performed on 3–5-h-starved cells (Fig. 4a). Power spectrum analysis yielded two dominant frequencies (Fig. 4b). Mean period lengths were 6.8 ± 1.1 and 3.6 ± 1.0 min, respectively. The two frequencies observed are most likely harmonics. Note that the short period of the biphasic oscillations seemed to be shorter than the period of the sinusoidal oscillations. The amplitude of biphasic pH oscillations was in the same range as the amplitude of the sinusoidal oscillations.

**Complex Changes of the Extracellular pH in Late Aggregation–Stage Cells**

The sinusoidal oscillations measured in 3–5-h-starved cells changed after 5–6 h of development into a complex mode (Fig. 5). After 7 h of starvation, only complex changes of the extracellular pH were seen (Fig. 6a). At this time, the cells had aggregated into clumps, which were still contained within the liquid droplet. Power spectrum analysis of these signals yielded several oscillation frequencies, with period lengths equal to and shorter (up to ~1 min) than the period length of sinusoidal oscillations (Fig. 6b). Thus the change in signal mode was characterized by the appearance of higher frequency signals of reduced amplitude. The amplitude of the

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**Figure 1.** Extracellular pH of early aggregation–stage cells (3–5 h starved). (a) Sinusoidal oscillations of the extracellular pH (mean period length, 5.1 min; mean amplitude, 0.024 pH units). (b) Noisy pH oscillations. (c) Power spectrum of noisy pH oscillations revealing a clear single periodicity.
Figure 2. (a) Transient variations of the amplitude of sinusoidal pH oscillations. These were observed only in very few cases. (b) In a few cases, spike-shaped responses were detected instead of sinusoidal pH oscillations.

Figure 3. Inhibition of sinusoidal oscillations of the extracellular pH after addition of caffeine (10 mM). The time of addition of caffeine is marked by an arrowhead.

low frequency components was in the same range as the amplitude of the sinusoidal oscillations.

To test whether the complex changes in pH were also coupled to cAMP relaying, we tried to inhibit them by adding caffeine. In 12 of 14 experiments, the complex changes could be inhibited by the addition of caffeine to a final concentration of 10 mM (Fig. 6c).

We have observed similar complex pH changes in migrating AX2-slugs (Weijer, C. J., and K. Gottmann, manuscript in preparation).

Observations of Chemotactic Waves in HG220 Aggregation Streams

In the mutant HG220, propagating optical density waves can be seen in aggregation streams. These waves are presumably caused by the chemotactic response of aggregating cells to propagating cAMP waves (Fig. 7a). There are instances where the pattern of wave propagation is highly periodic with a frequency of ~1–0.5 min⁻¹ and a propagation velocity of ~100 µm/min (Gerisch et al., 1985; Gottmann, K., and C. J. Weijer, unpublished observations). Sometimes, however, the waves did not occur in this very regular pattern, but in groups of two or three (see Figs. 9a and 10, respectively) regularly spaced (~100 µm) waves separated from each other by greater distances. We have also observed patterns with about twice the normal period (Fig. 8a). All patterns occurred simultaneously on the same agar plate and often the wave pattern in the same aggregation stream changed. Using time-lapse video recording, the disappearance and reappearance of individual waves during propagation was observed.

To determine the frequency of the optical density oscillations quantitatively, we made measurements of the optical
density changes in aggregation streams and analyzed the power spectrum. The regular pattern shown in Fig. 7a had a frequency of 1–0.5 min⁻¹ (Fig. 7b). The frequency and amplitude of these oscillations showed slight variations resulting in a rather broad power spectrum (Fig. 7c). In aggregation streams showing visible waves with about twice the period as above, we measured biphasic optical density oscillations (Fig. 8b). The power spectrum of these oscillations revealed two dominant frequencies, which appeared to be harmonics (Fig. 8c). Patterns which consisted of groups of two waves gave rather complex optical density oscillations. Two big peaks were periodically separated by a much smaller one (Fig. 9b). The power spectrum of these oscillations showed several harmonics (Fig. 9c).

**Mean Extracellular pH**

The extracellular pH of aggregating cells showed a mean value of 6.9 ± 0.24 pH units.

**Discussion**

**Sinusoidal pH Oscillations**

Using pH-sensitive microelectrodes, we have measured sinusoidal pH oscillations in early aggregation-stage cells (Fig. 1a). These pH oscillations resemble the pH oscillations found in cell suspensions (Malchow et al., 1978). However the period length was shorter than that found in cell suspensions (5 min in situ as compared to 8 min in suspension). Therefore the period was closer to the period length of aggregation waves in time-lapse movies of early Dictyostelium development (Durston, 1974). It is very likely that the observed early pH oscillations are coupled to the cAMP waves, since addition of the relay inhibitor, caffeine, abolished the oscillations (Fig. 3).
Figure 8. (a) Regular wave pattern with about twice the period length as seen in Fig. 7a. (b) Optical density measurements showed biphasic oscillations resembling those found in pH measurements (see Fig. 4a). (c) Power spectrum analysis of these oscillations showed two harmonic frequencies.

Furthermore, it has been shown that pH oscillations found in cell suspensions can be phase shifted by the addition of cAMP strongly suggesting the coupling of pH oscillations to cAMP waves (Malchow et al., 1978).

Gating of cAMP Waves as Revealed by Extracellular pH Oscillations

Sometimes biphasic pH oscillations were found in early aggregation-stage cells (Fig. 4a). This oscillation mode can be explained by a simple gating mechanism.

The general explanation of cAMP wave propagation is that there are few cells which oscillate autonomously with a given frequency; i.e., periodically produce pulses of cAMP. These cells form the aggregation centers, from which propagating waves are initiated. The other cells are responders; i.e., they produce a pulse of cAMP only when they are stimulated with cAMP. This behavior can be described in terms of the Devreotes excitation–adaptation model (Dinauer et al., 1980; Devreotes, 1982).

Upon stimulation by cAMP, two processes, excitation and adaptation, are activated. The magnitude of the cAMP stimulus determines the final level of excitation and adaptation. Excitation rises faster than adaptation and this leads to a transient difference in excitation and adaptation levels. The activity of the cAMP-synthesizing enzyme, adenylate cyclase, is proportional to the difference between excitation and adaptation. Thus cAMP is produced and secreted. The secreted cAMP binds to the cAMP receptors of the same cell, thereby causing an autocatalytic self-stimulation. Upon adaptation,
cAMP synthesis stops, and excitation and adaptation levels decrease to pre-stimulation levels. The decrease in adaptation is slower than the decrease in excitation. Therefore, a cell which has just relayed a cAMP pulse and is not yet completely de-adapted, cannot respond to a small stimulus provided by a propagating cAMP wave, since a small stimulus will lead to a small rise in excitation, which does not exceed the prevailing adaptation level. This cell can only respond to a small CAMP stimulus when the adaptation level has decreased to a lower level; i.e., after a refractory period. The length of the refractory period is determined by the time it takes the adaptation level to fall below the excitation level determined by a new stimulus.

Using these concepts of adaptation, deadaptation, and autocatalytic self-stimulation, it is possible to explain how a population of cells showing a gaussian distribution of deadaptation time constants (i.e., refractory periods) can give a biphasic relay response to a single driving frequency.

If the period of CAMP oscillations created by the aggregation center is shorter than the refractory period of a fraction of the cells, then two distinct populations will develop. One population can relay every CAMP wave initiated by the aggregation center, while the cells with long deadaptation times will be unable to respond to every CAMP wave but will respond to every second CAMP wave. Thus, one population oscillates with twice the frequency of the other. This leads to biphasic pH oscillations as a result of the superposition of protons secreted into the extracellular space.

As expected, the biphasic oscillations showed two dominant oscillation frequencies with a ratio of 1:2 in their power spectrum (Fig. 4b). This oscillation pattern can be generated by the addition of two sine waves with a frequency ratio of 1:2. Furthermore, the fast component of the biphasic oscillations, which probably reflects the signalling frequency of the aggregation center, was higher than the frequency of the sinusoidal oscillations (period length, 3.6 compared to 5.1 min). This suggests that at this stage all cells can still respond to a 5-min signal with a 5-min period, while only a part of the population is able to respond to a driving signal with a 3.6-min period.

**Gating of cAMP Signals as Revealed by Chemotactic Response**

Observations on wave propagation in HG220 aggregation streams revealed gating behavior similar to that found in early aggregation-stage amebas. Sometimes waves were visible with about twice the period length found normally (Fig. 8a). Gating of cAMP signals by the relaying cells can explain this behavior. The refractory phase of relaying cells might be too long to respond to every cAMP pulse produced by the aggregation center and therefore only every second cAMP pulse is relayed. However, the more sensitive optical density measurements on such patterns revealed a small peak between two big ones (Fig. 8b). These biphasic oscillations appear to result from a chemotactic response to biphasic CAMP oscillations, which are created by gating. One population relays every CAMP wave initiated by the aggregation center while the other has a longer refractory period and therefore responds to every second CAMP wave. Power spectrum analysis of these biphasic optical density oscillations confirms the presence of two dominant frequencies with a ratio of 1:2 (Fig. 8c).

A further example of gating was also observed in HG220 aggregation streams. Sometimes we observed waves traveling in groups of two or three (Figs. 9a and 10, respectively). This type of phenomenon is expected to occur if a small fraction of cells propagate every CAMP wave coming from the aggregation center while most cells have a refractory period which is slightly too long to permit them to respond to every wave.

Because of the longer refractory period, cells drift out of phase with the driving stimulus; i.e., they respond to a second CAMP wave in a later phase of the wave than they did to the previous wave and may not respond at all to a third or a fourth CAMP wave. After skipping one wave, such cells will again respond at the beginning of the next wave.

The quantitative optical density measurements in Fig. 9b support this interpretation. They indicate that the two visible waves are quantitatively different in size as expected if the second response is somewhat out of phase with the driving CAMP wave. Furthermore, they indicate that the 'skipped wave' in the photograph actually corresponds to a small response as expected if it is even further out of phase with the driving CAMP wave.

Gating was also observed directly, i.e., we noted the disappearance of individual waves during propagation. This disappearance of individual waves can be attributed to a local heterogeneity in the distribution of the refractory periods along aggregation streams. The observation of reappearing waves can be explained in a similar manner. If a minor population of cells responds to every wave, i.e., does not gate the CAMP waves initiated by the aggregation center, then this population will propagate all the waves through zones where most of the cells are unable to relay every wave. During propagation through such zones, the CAMP wave will have a low amplitude and the wave may be invisible, because the cells only show a weak chemotactic response. However, the optical density wave will reappear in zones of cells having shorter refractory periods.

Additional evidence for the ability of amebas to respond in a gated mode comes from the observation that *Dicyostelium discoideum* cells, which are artificially stimulated with high-frequency CAMP pulses, respond to these CAMP pulses with lower frequency (Robertson and Drage, 1975).

**Complex pH Changes in Late Aggregation-stage Cells**

We have measured complex changes of the external pH in late aggregation-stage cells (Fig. 6a), which in contrast to early pH oscillations did not show a clear single periodicity.
(Fig. 6 b); these complex signals were inhibited by the addition of the specific relay inhibitor caffeine (Fig. 6c), suggesting that these complex pH changes were also coupled to cAMP relay.

The following are several possible factors that might contribute to signal complexity.

(a) The contribution of noise. Contribution of noise to the complexity of the measured signals seems to be low, because early pH oscillations were also clearly periodic in the rare measurements showing enhanced noise amplitude (Fig. 1 b). Furthermore the inhibitory effect of the specific relay inhibitor caffeine makes it unlikely that more than a minor portion of the signal results from noise.

(b) Complexity might result from complex pH responses to simple sinusoidal cAMP waves. In early aggregation-stage cells, spike-shaped pH responses occurred in a few cases. These spike-shaped responses may represent an alternative response mode. The pH spikes varied in their amplitude and shape and are therefore much more complex than the sinusoidal pH oscillations (Fig. 2b). Therefore, spike-shaped pH responses could contribute much to the complexity of the pH changes in late aggregation stage cells, if spike-shaped pH responses are a common mode of response during later aggregation.

Although transient variations in amplitude of early sinusoidal pH oscillations were seldom observed (Fig. 2a), it is possible that such transient variations in amplitude are more common in late aggregation and thereby contribute to signal complexity.

(c) Complexity might result from complex modes of relaying of cAMP waves. As shown by time-lapse observation of aggregation waves, the frequency of cAMP waves increases during aggregation (Durston, 1974). This frequency increase might result in the formation of distinct subpopulations of cells, which differ in their ability to relay cAMP waves. Some cells might relay every cAMP wave, while others relay every second wave. Thus, distinct populations of cells might be created relaying cAMP waves with different, but harmonic frequencies. In consequence, they would produce pH oscillations with different, but harmonic frequencies, which are superimposed in the extracellular space and might contribute to signal complexity.

(d) Variations in timing of the cAMP pulses produced by the aggregation center (chaotic attractors; see Martiel and Goldbeter, 1985), might also contribute to complex pH changes. Variations in frequency of cAMP waves were observed in HG220 aggregation streams (Fig. 7), and therefore may play a role in the generation of complex pH signals.

**Conclusion**

In summary, we conclude that the cells can alter the frequency of the relay signals by a gating process. Thus it is possible that an apparently homogeneous population of cells is split up in two subpopulations that respond at different frequencies. One of these subpopulations gates cAMP waves, and therefore oscillates with a different period length than the other population.

In the above discussion, all the results are explained by gating of the relay signal. Since it is not known whether the pH response is part of the relay response or the chemotactic response and the light scattering change is almost certainly due to the chemotactic response, it is equally possible to explain our results by gating of the chemotactic response. The cAMP signal might be relayed by all cells, but only a subpopulation of cells can show a chemotactic response to every wave. Gating at both levels might also occur simultaneously. If gating occurs on the level of the chemotactic response, this could lead to cell sorting. Cells that can respond to every wave might move faster than cells, which show gated responses. This possibility is now under investigation.

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