CHOLERAGEN-INDUCED CYCLIC NUCLEOTIDE RESPONSE IN A LOWER EUKARYOTE

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1. Introduction

Choleragen, the exoenterotoxin produced by *Vibrio cholerae* [1–3] reacts with almost all vertebrate cells tested to produce a sustained increase in the cAMP level of tissues, cells [4,5] and isolated membranes [6,7]. The cellular cAMP level elevated by choleragen is often associated with the acquisition by many cultured cells of differentiation characteristics and/or inhibition of proliferation [8,9], though in some instances this elevation has been associated with initiation of proliferation [10].

Steps in the mechanism of action of the toxin, as now understood, include the characteristic binding of the choleragen B protomer to the G_{M1} ganglioside [11,12] located in the plasma membrane, the disulfide reduction to release the A_1 subunit from the A protomer [13,14] and the A_1 subunit-facilitated ADP-ribosylation of a GTP-binding regulatory unit of the membrane-bound adenyl cyclase. ADP ribosylation, with NAD as donor [15], is thought to interdict the normal control function of the regulatory unit of adenyl cyclase, leaving the enzyme in the activated state.

We have found that choleragen induces a change in the calcium-controlled H^+-permeability [16,17], mimicking that produced by light [18] as well as by dibutyryl cAMP or caffeine [19–21]. This reaction appears to be closely related to photoreception in the light-induced sporulation of *Physarum polycephalum* [17]. Light also rapidly induces a transient elevation of plasmodial cAMP [22]. In the plasmodial phase, choleragen [23] increases the mitotic delay induced by γ-irradiation [24–26]; thus, the toxin acts in a manner opposite to caffeine, which increases the levels of both cyclic nucleotides (J. W. D., N. L. O., in preparation) and reduces or eliminates mitotic delay [23].

We report here that choleragen reacts with the lower eukaryote syncytial slime mold, *Physarum polycephalum*, to elevate the plasmodial cAMP level, but not that of cGMP, in a uniquely characteristic manner.

Abbreviations: cAMP, adenosine-3′,5′-monophosphate; cGMP, guanosine-3′-5′-monophosphate; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide

2. Methods and materials

Physarum polycephalum strain M_3C VI was employed. Cultures were prepared and maintained at 26°C as in [27] using a medium with modified salts [28]. Microplasmodia were grown in shaken flasks, and large surface plasmodia were prepared by fusing microplasmodia on filter paper in Petri dishes. The Petri dish cultures were used ~24 h later, usually in the latter part of the G_2 period preceding mitosis III (the third mitosis after fusion).

Culture sampling and the extraction and assays for cyclic nucleotides were performed as in [29]. Briefly, 1/8th of a surface culture (~5 mg protein) was removed, blotted and the plasmodium quickly removed with a spatula to liquid nitrogen and lyophilized. Care was taken to minimize the length of time cultures were out of the incubator and their exposure to light, since light elevates the cAMP level in this organism [17,22,30]. In an ice bath, the lyophilized samples were extracted by homogenization in M $HClO_4$, the extract neutralized with 0.5 M K_2CO_3 and assayed for cAMP and cGMP by radioimmunoassay employing the New England Nuclear kits and the high sensitivity acetylation procedures. For each of the cyclic nucleotide assays, 125% of the specified

antibody reagent volumes was used. Substantial improvement in reproducibility of replicate assays was achieved. Extracted pellets were dissolved in 0.4 N NaOH and analyzed for protein by a modification of the Lowry procedure [31].

The monosialoganglioside G_{M1} (Supelco) (440 μg) in $CHCl_3$–methanol was taken to dryness under high purity nitrogen at room temperature then dispersed in culture medium (220 μl, pH 7.0), and 2.20 μg choleragen in 220 μl were added and incubated 60 min before use [32]. Choleragen (Schwarz-Mann) was freshly reconstituted from a lyophilized preparation to contain 1.0 mg toxin/ml and stored at 4°C.

3. Results

3.1. *Response of cAMP to choleragen*

Fig.1B shows that choleragen, added to large surface cultures of *Physarum polycephalum*, rapidly induces a marked increase in the plasmodial cAMP content. Table 1 summarizes the data plotted in fig.1B. The onset of the initial phase occurs rapidly (within 6 min for 1 μg choleragen) and is transient. The initial high level rapidly returns to a new basal level about twice that of the initial untreated plasmodium. This level was maintained approximately through the duration of the experiment. The same pattern was found in replicate experiments. In another experiment when the plasmodium was exposed to choleragen for 10 min, well rinsed, and transferred to conditioned medium (from another control culture), and then analyzed for cyclic nucleotide content, a new sustained level, 2.4-fold higher than the original basal level, was observed. The kinetics of the transient increase in cAMP with 0.5 μg/ml of choleragen follows a much less rapid course (fig.1B; table 1), reaching a maximum after ~15 min and declining to the new elevated value ~45 min after

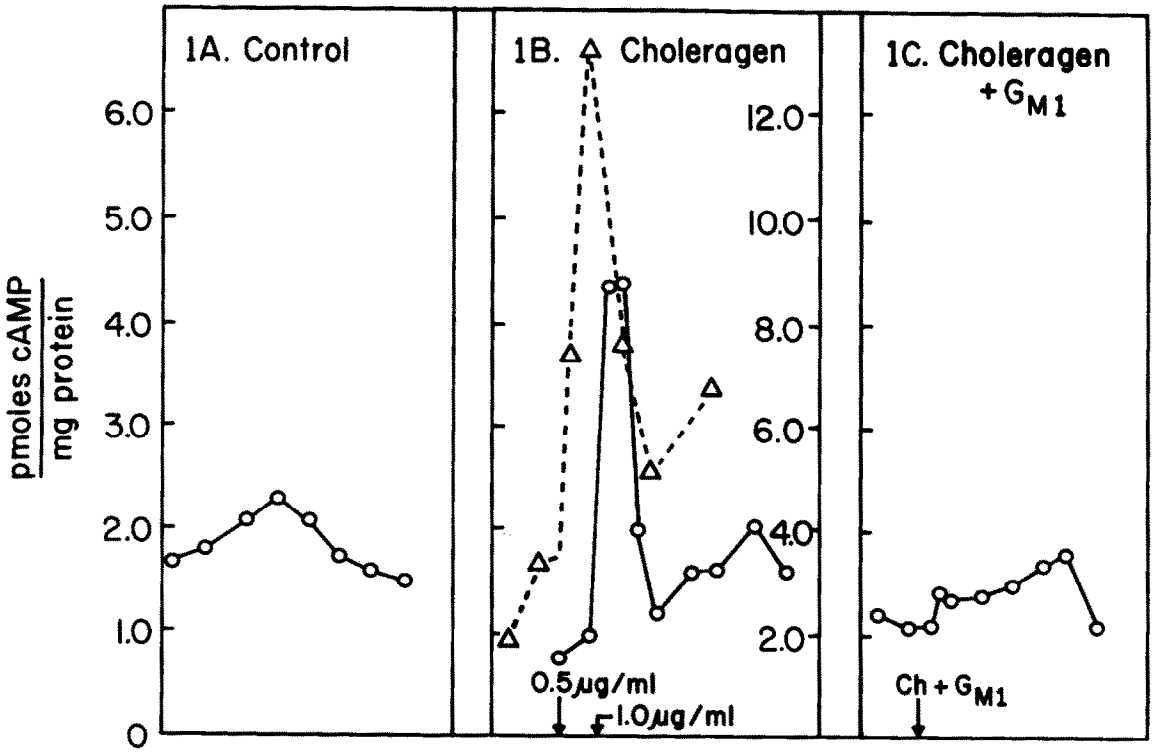

Fig.1. Response of plasmodial cAMP to choleragen. Plasmodia for each experimental condition were prepared, sampled and assayed for cyclic nucleotides as in section 2. Each sample when analyzed for both cAMP and cGMP yielded values plotted in the paired graphs (e.g., 1A,2A). Each series of points (fig.1A,B or C) is derived from the same plasmodium. Total protein/plasmodium varied from 37–45 mg. Additions were made at the indicated times (↓) to give the following final concentrations: choleragen, 0.5 μg/ml (–△– and values on right ordinate, fig.1B) or 1.0 μg/ml (–○– and values on left ordinate, fig.1B); 1.0 μg choleragen/ml + 200 μg G_{M1}/ml (fig.1C), and no addition (fig.1A). The choleragen–G_{M1} mixture was prepared as in section 2. See table 1 for characteristics of choleragen action.

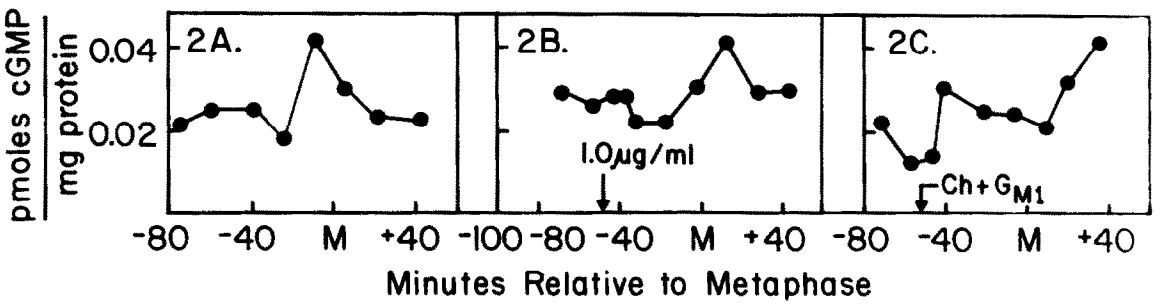

Fig.2. Response of plasmodial cGMP to choleragen. Conditions in fig.1 apply exactly except that in fig.1B only, data for 1.0 μg/ml addition of choleragen is shown. Figure designations (A–C) correspond to their uses in fig.1.

choleragen addition. Theophylline (2 mM), which when added alone leaves the plasmodial cAMP level unaltered (J. W. D., N. L. O., in preparation), suppresses the choleragen-induced cAMP elevation by 48% (also see [8]) when added just prior (1 min) to choleragen addition. The abscissa of fig.1B also indicates the time relative to mitosis (each syncytial plasmodium is mitotically synchronous) as well as the time elapsed (see also table 1). The induction pattern is not unique to the late G_2 period of the mitotic cycle in this organism, but is also elicited at other cycle times.

Fig.1C indicates that prior reaction of choleragen with the G_{M1} ganglioside nullifies the elevation of the cAMP level produced when the untreated toxin is employed. Fig.1A displays a typical control cAMP pattern found during this period. Individual cultures may exhibit differing basal cAMP levels which with time show fluctuations about an average value. However, no progressive transient pattern related to cell cycle position has been detected [29,30].

3.2. *Response of cGMP to choleragen*

The plasmodial cGMP response to choleragen was observed to be in agreement with the specificity of choleragen action on other cells (fig.2B). Fig.2A and 2C indicate the control responses when no choleragen is added and when choleragen is reacted with G_{M1} ganglioside before addition to the cultures, respectively.

4. Discussion

The data presented indicate that choleragen reacts with *Physarum polycephalum* to give qualitatively typical responses for both the cAMP and cGMP levels. Except for insect larval cells [33], we are not aware of reported choleragen reactivity with any other lower eukaryotes. These studies further indicate the presence of a hormone-like receptor system found earlier in our photoreception studies [17,22]. Choleragen mimics the light-induced H^+ uptake [16,17] in

Table 1
Characteristics of choleragen action on plasmodial cAMP content[a]

Exp. no.	Time (fig.1B abscissa)	Choleragen	Time after choleragen addition (min)	[cAMP] (pmol/mg protein)	Increase (-fold)
	−92 to −67	−	−	2.7 (av.)	(0)
I. 0.5 μg choleragen/ml	−67 to −52[b]	+	15[b]	13. (peak)	4.3
	−37 to +8	+	30–75	6.6 (av.)	2.4
	−68 to −48	−	−	0.89 (av.)	(0)
II. 1.0 μg choleragen/ml	−48 to −39.5[b]	+	8.5[b]	3.8 (peak)	4.3
	−17 to +43	+	31–91	1.7 (av.)	1.9

[a] Data from fig.1B; [b] Time for generation of peak [cAMP]

Physarum during induction of sporulation, suggesting, with other evidence, that an adenyl cyclase is involved in both responses.

If the elevation of the cAMP level in *Physarum* occurs by the same general mechanism found in higher eukaryotes, choleragen binds to the G_{M1}, or a similar, ganglioside in the plasma membrane and subsequently activates an adenyl cyclase. The striking characteristic of rapid elevation of the plasmodial cAMP level, though not normally observed with mammalian tissue cells where the latent period is usually 20–30 min, has been reported with such cells in culture [7,8]. While the mechanism of the rapid activation in *Physarum* is presently unknown, the simplest explanation may reside in an accelerated activation sequence following the membrane binding of choleragen. Activation of adenyl cyclase in membrane preparations from vertebrate cells by the A or A_1 protomer occurs essentially without a lag [8,13,34]. In *Physarum*, reactivity with the whole plasmodium may be accelerated by, e.g., ready accessibility of the A protomer to the regulatory unit of the cyclase. The molecular basis for the latent period between choleragen binding and the earliest detectable rise in cAMP level is still not understood in the vertebrate cells.

At the pH (4.7) of the growth medium, choleragen reversibly dissociates into subunits and thus becomes partially inactivated [2]; however, the activation of adenyl cyclase is much faster (~4.3-fold increase of cAMP in 8.5 min; table 1, fig.2B) than is dissociation [2], and therefore dissociation should not play an important role if choleragen binding is essentially irreversible as it is with vertebrate cells. However, if choleragen does dissociate appreciably under these conditions, for *Physarum*, the A subunit may be sufficient for adenyl cyclase activation, as reported for two vertebrate cells [6,34]. Proteolytic activities in the growth medium may also be important.

The novel characteristic not observed with vertebrate cells is the rapid decline of the transiently elevated cAMP level to a new level higher than the initial basal level. Whether this drop in cAMP level is caused by a deactivation of adenyl cyclase or, e.g., by an elevation of a phosphodiesterase activity is presently unknown. However, caffeine and several phosphodiesterase inhibitors now under study, or light [19,22], also elicit similar patterns (J. W. D., N. L. O., in preparation).

As with other cells, choleragen affects only the cAMP level without altering the cGMP level. The 'normal' levels of cAMP (1–2 pmol/mg protein) or of cGMP (0.03–0.1 pmol/mg protein) observed are typical of this organism during rapid growth [29,30]. In rabbit small intestine epithelial cells, choleragen activity is reported to suppress guanyl cyclase and phosphodiesterase [35] to produce a new steady state cGMP level.

As with other choleragen-responding tissues, complexation with G_{M1} ganglioside neutralizes the ability of the toxin to activate adenyl cyclase since membrane-bound G_{M1} is the normal toxin receptor [11,12], while the preformed choleragen–G_{M1} complex is inactive. Although choleragen binding to G_{M1} is very rapid and essentially irreversible, until the choleragen receptor in *Physarum* is positively identified, the possibility of other receptors such as homologous oligosaccharides [36] or glycoproteins [37] must be considered.

When choleragen is added to γ-irradiated plasmodia, it slightly increases the radiation-induced mitotic delay [23]. Thus, as proposed in [23,28], elevation of intracellular cAMP alone cannot be responsible for alleviating this radiation syndrome. It appears that the elevation of cGMP induced by caffeine, but not by choleragen, may better correlate with the ability of caffeine, but not of choleragen, to promote progression of irradiated plasmodia to metaphase.

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