Inhibition of cyclic GMP formation and aggregation in *Dictyostelium* by the intracellular Ca^{2+} antagonist TMB-8

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Aggregation in *Dictyostelium discoideum* was shown in previous studies employing EGTA to require Ca^{2+}, but the intra- or extracellular site of action of this ion and its role in chemotaxis were not determined [1]. In this investigation we show that the intracellular Ca^{2+} immobilising agent TMB-8 does not affect binding of the signalling nucleotide, cAMP, to the cell surface receptors but abolishes the rapid accumulation of intracellular cGMP and subsequent chemotactic aggregation. We infer that movement of Ca^{2+} from membrane-bound stores is triggered by binding of cAMP to the cell-surface receptor and that this plays a primary role in stimulating cGMP formation and chemotaxis.

Dictyostelium *cGMP* *Aggregation* *Intracellular calcium*

1. INTRODUCTION

Aggregation of amoebae of the cellular slime mould *Dictyostelium discoideum* is brought about by a rhythmically pulsating signal of cAMP emitted from collecting centres and relayed from cell to cell as a series of expanding concentric or spiral waves. One of the first events identified in amoebae after they have received a pulse of cAMP is the production of a brief pulse of intracellular cGMP that peaks 10 s after cAMP binding to the cell surface receptors [2,3]. This pulse, which is broken down rapidly in normal wild-type cells by a specific cGMP phosphodiesterase [4] has been correlated with the amoebal chemotactic movement response [5–7]. A possible intermediary messenger between the cAMP receptor and guanylate cyclase is Ca^{2+}. Uptake of this ion by amoebae in response to stimulation by cAMP has been observed, although the significance of this observation was uncertain as short-term incubation with EDTA did not block the chemotactic response measured as amoebal shape changes [8]. Despite previously conflicting reports concerning the sensitivity of aggregation to the presence of Ca^{2+} the need for this ion has recently been confirmed [1]. Here we used the Ca^{2+} antagonist TMB-8 that has been found in other systems to specifically block release of Ca^{2+} from cellular membrane-bound locations [9–12]. We find that, although the binding of cAMP to the cell surface receptor is not adversely affected by incubation of amoebae with TMB-8, the formation of cGMP in response to an exogenous cAMP pulse and subsequent aggregation are rapidly inhibited.

Abbreviations: TMB-8, 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate HCl; cAMP, 3′,5′-cyclic adenosine monophosphate; cGMP, 3′,5′-cyclic guanosine monophosphate

2. MATERIALS AND METHODS

2.1. *Media and culture conditions*

Nutrient 'SM' agar was prepared as in [13,14] and KMMP agar as in [15]. *D. discoideum* strain NP187 was grown in association with *Klebsiella aerogenes* strain OXF1 on SM agar in the dark at 22°C. Salt solution 'SS' contained per litre: NaCl, 600 mg; KCl, 750 mg; $CaCl \cdot 2H_2O$, 300 mg. Salt solution '4CS' contained per litre: NaCl, 600 mg; KCl, 750 mg; $CaCl_2 \cdot 2H_2O$, 600 mg (4 mM).

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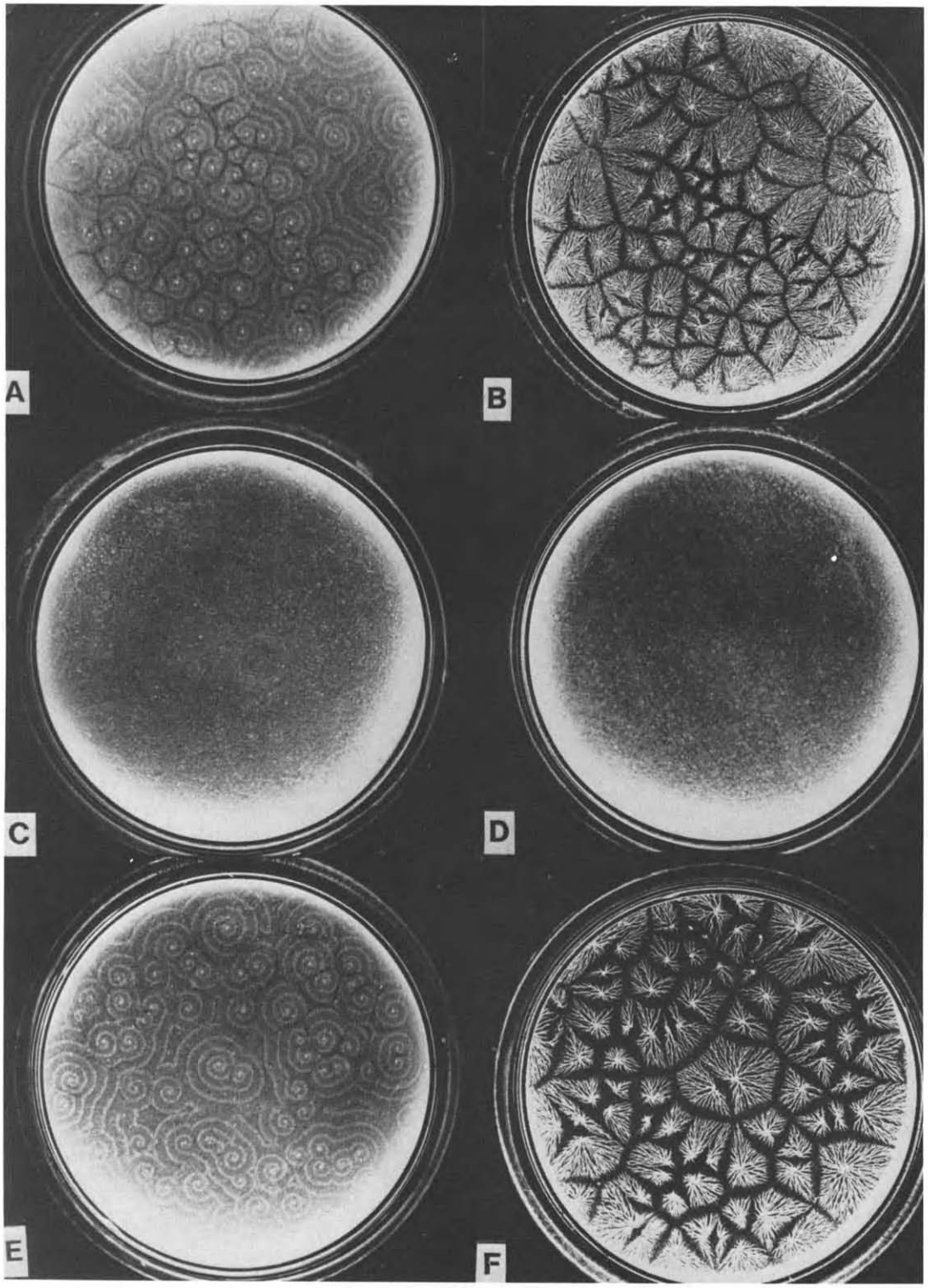
A
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Calcium-free salts 'CFS' contained per litre: NaCl, 600 mg; KCl, 750 mg. TMB-8 was obtained from Calbiochem.

2.2. *Treatment of aggregation-competent amoebae with TMB-8*

Amoebae were harvested from SM agar plates after 24 h growth as the amoebae cleared the bacterial lawn, washed free from bacteria by centrifugation (3 spins at 190 × *g* for 2 min) and resuspended in SS. Aliquots of the amoebae were then diluted to 10^7/ml with: (a) SS; (b) TMB-8 solution (1 mM) in CFS; (c) in TMB-8 solution (1 mM) in 4CS. These cell suspensions were shaken for 15 min at 22°C and plated onto KMMP agar in 50-mm petri dishes and allowed to settle for 20 min. After decanting excess liquid, the plates were air-dried in a sterile hood until they appeared matt. Amoebae were incubated overnight (17 h) at 7°C then transferred to 22°C to initiate aggregation. Aggregation was observed and recorded using darkfield optics with 35 mm time-lapse photography.

2.3. *Preparation of amoebae for cGMP assay*

Starving amoebae from 24 h SM agar plates were harvested as described above and resuspended in 17 mM K_2HPO_4/KH_2PO_4 (pH 6.1, KP buffer) at 2×10^7/ml. After 5 h shaking at 22°C in a rotary shaker (170 rpm) the amoebae were centrifuged and resuspended at 10^8/ml in KP buffer. Aliquots were then incubated with: (a) KP buffer; (b) 2 mM TMB-8 solution in KP buffer; (c) 2 mM TMB-8 and 7 mM Ca^{2+} in KP buffer, and shaken at 22°C for 20 min. The aliquots were then pulsed with 100 μl pulses of cAMP (final concentration 50 nM) 4 times at 5-min intervals to synchronize the cells. The cGMP assay was carried out as in [16] using the radio-immunoassay kit from Amersham and toluene/Triton X-100/PPO scintillant.

2.4. *cAMP binding assay*

Amoebae were prepared as described in section 2.3. Binding of [^{3}H]cAMP to amoebal cell surface receptors was assayed using a silicone oil separation method [17].

3. RESULTS

3.1. *Effect of TMB-8 on chemotactic aggregation*

When starving lawns of amoebae were incubated on KMMP agar they formed bands of light (moving) and dark (stationary) cells that eventually gave rise to centrally moving streams as observed (fig.1A,B) [18]. After incubation in the presence of 1 mM TMB-8, however, aggregation was seriously delayed and no banding or aggregation streams were formed within 5 h of those seen with the controls (fig.1C,D). Incubation in the presence of 4 mM Ca^{2+} as well as 1 mM TMB-8 nullified the inhibitory action of the drug (fig.1E,F), indicating that TMB-8 had its action on Ca^{2+} as in other systems.

3.2. *Inhibition of cGMP formation by TMB-8*

The effect of TMB-8 on the production of cGMP in response to cAMP pulses was observed using starving amoebal suspensions incubated for 40 min in the presence or absence of the drug (see section 2). Under these conditions TMB-8 at 2 mM was found to completely inhibit cGMP formation in response to 50 nM pulses of cAMP, and 7 mM Ca^{2+} almost completely annulled this inhibition (fig.2). Such effective concentrations of TMB-8 and Ca^{2+} are higher than those found to be necessary to affect amoebal aggregation on agar plates (1 mM TMB-8 and 4 mM Ca^{2+}) but this is likely to be due to the longer (17 h) incubation period in the presence of TMB-8 used in the aggregation tests. When EGTA was substituted for TMB-8 in this experiment little or no inhibition of cGMP formation was observed showing that a powerful external Ca^{2+} chelating agent cannot

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Fig.1. Inhibition by TMB-8 of aggregation of starving lawns of *D. discoideum* strain NP187 seen with darkfield optics. Amoebae were preincubated in salt solution (SS) (A,B), Ca^{2+}-free salt solution (CFS) with 1 mM TMB-8 (C,D), and in salt solution with 4 mM Ca^{2+} (4CS) plus 1 mM TMB-8 (E,F), and were then plated on KMMP agar and photographed at 1.5 h (left-hand column) and 2 h (right-hand column) after initiation of aggregation by transfer from 7 to 22°C. The petri dishes were 50 mm in diameter.

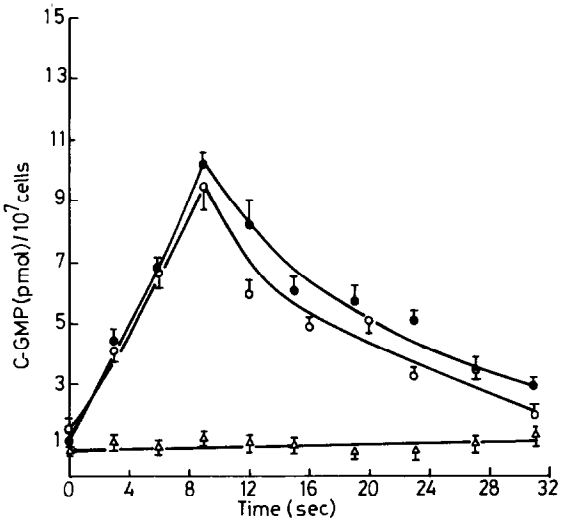

Fig.2. Effect of TMB-8 and addition of extracellular Ca^{2+} on cAMP-stimulated cGMP formation. Amoebae were preincubated in KP buffer (●), KP buffer containing 2 mM TMB-8 (△) and KP buffer containing 2 mM TMB-8 and 7 mM Ca^{2+} (○). Results are means of 10, 7, and 6 experiments, respectively. Bars indicate SE.

Table 1

Binding of cAMP to cell surface receptors in the presence of TMB-8, Ca^{2+} or EGTA

Additions	Molecules cAMP bound/cell	Probability of difference from control (significance: $p < 0.05$)
None (control)	27520 + 2110 (5)	–
2 mM TMB-8	35780 + 3060 (5)	0.25
2 mM TMB-8 plus 7 mM Ca^{2+}	60110 + 4770 (5)	0.008
7 mM Ca^{2+}	44780 + 3270 (5)	0.037
7 mM EGTA	31050 + 4490 (3)	0.40

Amoebae were incubated with the additions shown for 20 min then pulsed 4 times at 5-min intervals with cAMP to give a final concentration of 50 nM after each pulse. After a total incubation time of 40 min cAMP binding was assayed at 0°C in quadruplet aliquots. Results show SE with the number of observations in parentheses. Significance of the difference between sample and control was assessed by Student's *t*-test

mimic the action of TMB-8 (not shown). Controls also showed that the effect of TMB-8 was not on the binding of cGMP to the specific antiserum used in the cGMP assay.

3.3. *Effect of TMB-8 on cAMP binding*

To test the possibility that TMB-8 was acting through inhibition of the cell surface cAMP receptors, the binding of 50 and 95 nM [^{3}H]cAMP to cell surface receptors was ascertained in the presence and absence of TMB-8. It was found that TMB-8 did not inhibit cAMP binding (table 1). In some experiments there was a slight stimulation of binding but over 5 experiments this effect was not statistically significant. An unexpected and so far unexplained finding was that non-specific cAMP binding (binding that is not released by excess unlabelled cAMP) was increased in the presence of TMB-8 and that this effect was abolished by 7 mM Ca^{2+}. Additionally it was noticed that the effect of 7 mM Ca^{2+} concentrations was highly stimulatory on specific cAMP binding in the presence or absence of TMB-8, and further studies on this effect seem warranted.

4. DISCUSSION

Based on work done with a number of cellular systems, it has been deduced that TMB-8 acts by stabilizing intracellular membrane-bound Ca^{2+}. Early studies demonstrated that it blocked contraction in response to stimuli that released Ca^{2+} from intracellular stores in smooth muscle [10,11]. It was also shown to be a potent inhibitor of skeletal muscle contractibility by acetylcholine or by caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum [9,11]. It has been shown to inhibit release of granule enzymes by phorbol myristate acetate (PMA) from human neutrophils in a Ca^{2+}-reversible manner [19] and to inhibit growth factor stimulated Na^+ influx in human fibroblasts, an effect that was overcome by addition of Ca^{2+} plus the Ca^{2+} ionophore A23187 [20]. Work with platelets has shown that 750 μM TMB-8 almost completely inhibits aggregation induced by prostaglandin H_2 and that this is substantially reversed by 5 mM Ca^{2+} [12].

Our study has indicated that TMB-8 inhibits aggregation of *Dictyostelium* and this effect is reversed by high Ca^{2+} concentrations. It shows no effect

Our study has indicated that TMB-8 inhibits aggregation of *Dictyostelium* and this effect is reversed by high Ca^{2+} concentrations. It shows no effect on binding of the chemotactic signalling molecule cAMP to the external cell surface cAMP receptor but completely blocks the subsequent rapid and transient formation of cGMP in a Ca^{2+} reversible manner. The site of action of TMB-8 would therefore seem to lie between the cAMP receptor and guanylate cyclase. From its action in other systems and its reversibility by high added Ca^{2+} concentrations it seems likely to be acting by inhibition of release of Ca^{2+} from membrane-bound locations. The idea of internal rather than external Ca^{2+} being involved in the chemotactic response is suggested by the failure of Ca^{2+} chelating agents such as EGTA to mimic the rapid action of TMB-8 on cGMP formation.

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