Effects of calcium antagonists on cyclic AMP phosphodiesterase induction in Dictyostelium discoideum

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Summary

Previous studies have suggested that cyclic GMP and/or Ca\(^{2+}\) might function as second messengers in the induction by exogenous cyclic AMP of the cyclic AMP phosphodiesterase (PD) in Dictyostelium discoideum. To assess further the role of Ca\(^{2+}\) in PD induction we examined the effect on this process of a number of putative Ca\(^{2+}\)-channel blockers. At relatively low micromolar concentrations, TMB-8, nicardipine, nifedipine, diltiazem and verapamil all altered cell shape and inhibited PD induction in a similar dose-dependent fashion. Concentrations of these drugs that abolished PD induction had no effect on cell viability; however, higher concentrations reduced viability and caused cell lysis. All effects of these compounds on the cells were antagonized at least partially by 5–10 mM Ca\(^{2+}\). Other cations tested were considerably less effective. Like the organic inhibitors, La\(^{3+}\) also altered cell shape, inhibited PD induction and reduced cell viability at elevated concentrations, but its effect on the cells appeared to be more complex. Inhibition of PD induction by the organic antagonists could not be attributed solely to an impaired uptake of extracellular Ca\(^{2+}\), a reduction of ATP pools in the cells or a direct effect on calmodulin. Concentrations of TMB-8 that inhibited PD induction had little effect on the cyclic GMP response. Therefore, this compound did not inhibit PD induction indirectly by blocking cyclic GMP synthesis. While the sites of action of these drugs could not be determined precisely, the results are consistent with the hypothesis that intracellular Ca\(^{2+}\) functions as a regulator of PD induction in this organism.

Key words: Ca\(^{2+}\) antagonists, cyclic AMP phosphodiesterase, Dictyostelium.

Introduction

During early development, amoebae of the cellular slime mould Dictyostelium discoideum undergo aggregation and differentiation in response to endogenously generated waves of extracellular cyclic AMP (for a review, see Devreotes, 1982). The level of cyclic AMP outside (and perhaps inside) the cells is regulated, in part, by a variety of phosphodiesterase (PD) activities: a soluble extracellular enzyme (ePD) (Riedel & Gerisch, 1971; Orlow et al., 1981), a soluble intracellular enzyme (iPD) and a cell surface membrane-bound enzyme (mPD) (Malchow et al., 1972). All three phosphodiesterases appear to be functionally and structurally related (Malchow et al., 1973; Kessin et al., 1979; Brown & Rutherford, 1980; Shapiro et al., 1983) and the iPD is probably a precursor of the ePD (Klein & Darmon, 1975). When early developing amoebae are exposed to high concentrations of cyclic AMP, there is a dramatic induction of the iPD and ePD (but not mPD) enzymes (Tsang & Coukell, 1977; Lappano & Coukell, 1982). Enhanced secretion of ePD reduces the level of cyclic AMP in the extracellular environment and ensures that the cells detect the natural signals as pulses (Yeh et al., 1978).

Although PD induction by exogenous cyclic AMP appears to be mediated via the plasma membrane cyclic AMP receptor (Van Haastert et al., 1982; Coukell et al., 1983), the nature of the second messenger(s) regulating enzyme synthesis has not been established. Owing to a close correlation between the ability of various chemoattractants to increase cyclic GMP pools and to induce iPD and ePD activity, it has been suggested that these enzymes might be regulated by changes in the level of intracellular cyclic GMP (Lappano & Coukell, 1982; Van Haastert et al., 1982). Other...
studies, however, have implicated Ca²⁺ in the regulation of PD induction (Yamasaki & Hayashi, 1982; Hashimoto & Hayashi, 1985). Recently, Bumann et al. (1984) reported that treatment of early developing cells with exogenous cyclic AMP, stimulated the cells to take up Ca²⁺ from the extracellular medium. Moreover, the cells retained this Ca²⁺ as long as the external cyclic AMP stimulus remained constant. Thus, incubation conditions that induce PD and elevate intracellular cyclic GMP (Lappano & Coukell, 1982) also appear to increase levels of cellular Ca²⁺. This observation suggested further that the messenger role in PD induction initially attributed to intracellular cyclic GMP might in fact be played by intracellular Ca²⁺ or by both substances acting in concert. In support of this latter idea, Europe-Finner & Newell (1984) found that the putative intracellular Ca²⁺ antagonist, TMB-8, blocked both cyclic AMP-stimulated cyclic GMP production and chemotaxis in D. discoideum. Therefore, to learn more about the role(s) of intracellular Ca²⁺ and cyclic GMP during PD induction in this organism, we have examined the effects of TMB-8 and other putative Ca²⁺-channel blockers on this process and on the physiology of the cells during early development.

Materials and methods

Materials

Chemicals used in this study were purchased from the following companies: [8-³H]cyclic AMP (27 Ci mmol⁻¹; 1 Ci = 37 GBq) and ⁴⁵CaCl₂ (9-7 mCi mg⁻¹ Ca²⁺) (ICN); cyclic GMP RIA kits (Amersham); cyclic AMP, ATP, Tris, EGTA, bovine serum albumin (BSA) (fraction V), CaCl₂, LaCl₃, nicardipine, nifedipine, diltiazem, verapamil, rotenone, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and luciferin/luciferase extract (FLE-250) (Sigma); TMB-8 (Calbiochem or Sigma); dimethylsulphoxide (DMSO), HCIO₄ and KHCO₃ (Fisher). Trifluoperazine was a generous gift from Smith Kline & French (Toronto). Materials used in this study were purchased from BDH Chemicals, except for the bacteriological peptone, which was purchased from Oxoid, and the agar, which was from Scott.

Strains and growth conditions

Most experiments were performed with D. discoideum strain HC91 (Coukell, 1975). In a few experiments, a cyclic GMP phosphodiesterase-deficient mutant, NF368 (Ross & Newell, 1979; Coukell & Cameron, 1986) was used. Stocks were maintained on SM agar plates (Sussman, 1966) in association with Klebsiella aerogenes, which was purchased from Amersham. All media components were obtained from BDH Chemicals, except for the bacteriological peptone, which was purchased from Oxoid, and the agar, which was from Scott.

Materials and methods

Phosphodiesterase induction

To induce the iPd and ePD enzymes, 2-ml samples of cell suspension were shaken in 10-ml flasks at 22°C with 1 mM cyclic AMP, and other chemicals where indicated. After 2 h, 1-5 ml of each suspension was removed and centrifuged at 1000 g for 5 min at room temperature. The supernatants were removed, dialysed overnight at 4°C against a 500-fold excess of 50 mM-Tris•HCl, pH 7.5 (buffer B) and assayed for ePD activity. The cell pellets were washed once in 10 ml of cold buffer B, resuspended in 0.5-1.0 ml of the same buffer and stored at -70°C. The supernatants were removed and assayed for iPd activity. PD activities were determined in duplicate as described (Tsang & Coukell, 1977). One unit of PD activity was defined as the amount of enzyme required to degrade 1 nmol of cyclic AMP min⁻¹ at 35°C. Protein concentration was determined according to the method of Lowry et al. (1951) using BSA as a standard. The kinetics of iPd and ePD induction under these conditions are shown in Fig. 1.

Stock solutions (40 mM) of the putative Ca²⁺-channel blockers were prepared in buffer A (TMB-8, diltiazem, verapamil and LaCl₃) or DMSO and stored at -20°C. When present during an induction experiment, they were usually added 5 min prior to the cyclic AMP. Control experiments revealed that addition of TMB-8 up to 30 min before the cyclic AMP did not enhance its effect on PD induction. The

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Fig. 1. Kinetics of phosphodiesterase induction in strain HC91. Amoeae were grown and starved for 2 h as described in Materials and methods. At zero time, cyclic AMP was added to a 10 ml cell suspension to give an initial concentration of 1 mM (●, ■) while a second 10 ml suspension received an equal volume of buffer (O, □). Every 30 min, 1-5 ml samples of suspension were removed from each flask, processed and assayed for iPd (●, O) and ePD (■, □) activity.
concentration of DMSO in the suspensions never exceeded 2%; this concentration had no effect on PD induction.

**ATP measurements**

A 500 μl sample of each cell suspension (2×10^7 cells ml^-1) was transferred to a microcentrifuge tube and spun at 12 000 g for 10 s. The cell pellets were resuspended in 100 μl of ice-cold buffer A and immediately lysed by the addition of 100 μl of cold 3·5% HClO₄. The samples were neutralized with 50 μl of 50% saturated KHCO₃, left on ice for 10–15 min, centrifuged, and the supernatants were stored at −70°C. Duplicate samples of each supernatant were analysed for ATP using the luciferase assay of Cole et al. (1967). Light emission was measured in a Beckman model LS-233 spectrophotometer set at maximum sensitivity. The significance of differences in data was assessed by Student's two-tailed t-test.

**Ca²⁺-uptake assays**

Ca²⁺ uptake by intact cells was measured in the presence of low and high concentrations of extracellular Ca²⁺. (1) *Low Ca²⁺*: duplicate 2-ml samples of cell suspension, supplemented and unsupplemented with Ca²⁺ antagonists, were shaken for 10 min under induction conditions. 45Ca²⁺ (≈10 μCi) was then added to each suspension and a 100 μl sample was immediately withdrawn and added to 1 ml of ice-cold 20 mM-Tris·HCl, 1 mM-EGTA, pH 7·2, in a microcentrifuge tube. The suspensions were spun for 5 s, decanted and the cell pellets were resuspended in another 1 ml of Tris/EGTA and centrifuged. At various times up to 2 h, additional 100-μl samples were removed from the suspensions and processed in the same manner. The washed cell pellets were then solubilized in 100 μl of 2% SDS and counted in 1 ml of PCS scintillant. Addition of 45Ca²⁺ to the suspensions increased the extracellular Ca²⁺ concentration by about 6 μM.

(2) *High Ca²⁺*: uptake of extracellular Ca²⁺ in the high micromolar to millimolar range was measured by the lanthanum stopping technique of Europe-Finner & Newell (1985), except that after terminating Ca²⁺ transport, the cells were immediately washed twice with 1 ml of ice-cold 100 μM-LaCl₃. The cell pellets were then solubilized in 0·3 ml of NCS and counted in 1 ml of PCS.

**Table 1. Effect of the Ca²⁺-channel blockers and trifluoperazine on PD induction and cell viability**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>Antagonism of iPD inhibition by Ca²⁺ (-fold increase)</th>
<th>Concentrations causing cell lysis (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB-8</td>
<td>24 ± 4</td>
<td>10 ± 3</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>10</td>
<td>20 ± 0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>50</td>
<td>17 ± 2</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Verapamil</td>
<td>75</td>
<td>7 ± 5</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>75</td>
<td>8 ± 1</td>
<td>&gt;250</td>
</tr>
<tr>
<td>LaCl₃</td>
<td>N.D. †</td>
<td>N.D.</td>
<td>&gt;150</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>15</td>
<td>0±6</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

*IC₅₀ values were estimated from inhibition curves (see Fig. 2A). Results are the average of values obtained in two experiments with each compound except for TMB-8, which is the average ± S.E. of five experiments.

† Cells were incubated under induction conditions in the presence or absence of 5 mM-Ca²⁺ and a concentration of inhibitor sufficient to reduce PD induction by >90%. Results are expressed as the -fold increase in iPD specific activity in the presence of Ca²⁺ and are average values for two or more experiments with each compound.

 Effects of Ca²⁺ antagonists on Dictyostelium

**Cyclic GMP changes in cyclic AMP-stimulated cells were measured by a radioimmunoassay as described (Coukell & Cameron, 1986). Cell viability was estimated by diluting a sample of cell suspension in SS and plating 0·1 ml with an equal volume of suspension of *K. aerogenes* on each of three SM agar plates. The average number of clones/plate was determined after 4–6 days incubation at 22°C.**

**Results**

**Effects of the Ca²⁺ antagonists on PD induction**

To assess the role of Ca²⁺ during PD induction in *D. discoideum*, we examined the effects on this process of various compounds reported to be Ca²⁺-channel blockers in vertebrate cells. All five organic Ca²⁺ antagonists employed inhibited both iPD and ePD induction in a dose-dependent fashion with IC₅₀ values (i.e. the concentration required to inhibit induction by 50%) in the low micromolar range (Table 1). In each case, the inhibitory effect was greater on ePD than on iPD production; this probably reflects an effect of the drugs on secretion. Inhibition curves obtained with TMB-8 are shown in Fig. 2A. Similar results were observed with the other organic inhibitors (data not shown). The five organic Ca²⁺ antagonists completely abolished PD induction at concentrations that had no influence on cell viability (determined by plate counts). However, with each drug, as the concentration was increased, the cells became spherical, dissociated from clumps to single cells and eventually lysed (Table 1; Fig. 2). Compared to the organic Ca²⁺ antagonists, LaCl₃ had a more complex effect on the cells. At concentrations >50 μM, this compound also inhibited PD production, altered cell shape and eventually induced cell lysis; however, at lower concentrations its
Fig. 2. Effect of increasing concentrations of TMB-8 (A) or LaCl$_3$ (B) on phosphodiesterase induction and cell viability. Amoebae of strains HC91 (○, •) and NP368 (△, ▲) were grown and starved as described in Materials and methods. Cell suspensions in the presence of different concentrations of TMB-8 or LaCl$_3$ were treated with 1 mM-cyclic AMP and shaken. After 2 h, the suspensions were assayed for iPD (○, △) and ePD (●, ▲) activity, and cell viability (---). Results are the average of two experiments with each strain.

major effect seemed to be to impair secretion (Fig. 2B; Table 1).

To determine if the organic inhibitors were interfering with Ca$^{2+}$ metabolism, an attempt was made to overcome the inhibitory effects of these drugs by adding millimolar concentrations of extracellular Ca$^{2+}$. As shown in Table 1, inhibition of iPD induction by all five compounds was effectively antagonized by 5 mM-Ca$^{2+}$, e.g. iPD activities were elevated 7.5- to 20-fold. Under the same conditions, ePD activities were usually increased two- to threefold (data not shown). In addition to antagonizing the inhibition of PD induction, millimolar extracellular Ca$^{2+}$ also prevented the deleterious effects of these drugs on cell viability observed at higher concentrations (data not shown). As reported by Hashimoto & Hayashi (1985), we found that the calmodulin antagonist, trifluoperazine, also inhibited PD induction and caused cell lysis at elevated concentrations. However, unlike the putative Ca$^{2+}$ antagonists, the effects of trifluoperazine on the cells were not antagonized by millimolar Ca$^{2+}$; in fact, they were often enhanced slightly (Table 1).

To examine the specificity of Ca$^{2+}$ in this process, we compared the effectiveness of various cations (at 5 mM) in overcoming the inhibition of PD induction by 80 μM-TMB-8 (Fig. 3). Addition of Ca$^{2+}$ increased iPD activity 10-fold. Zn$^{2+}$ and Mg$^{2+}$ were considerably less effective at preventing iPD inhibition, while Mn$^{2+}$ and Cu$^{2+}$ had no significant effect. None of the
divalent cations was particularly effective at overcoming inhibition of ePD induction by TMB-8, but Ca\textsuperscript{2+} increased the activity slightly. Na\textsuperscript{+} or K\textsuperscript{+} at 5-10 mM had no influence on either iPD or ePD induction (data not shown). The concentration dependence of Ca\textsuperscript{2+} and Zn\textsuperscript{2+} in antagonizing inhibition of PD induction by TMB-8 is shown in Fig. 4. Ca\textsuperscript{2+} prevented inhibition of both iPD and ePD most effectively at concentrations of 5-10 mM. Zn\textsuperscript{2+}, at the same concentrations, partially overcame iPD inhibition, but concentrations up to 20 mM failed to prevent inhibition of ePD induction.

**Effects of the Ca\textsuperscript{2+} antagonists on Ca\textsuperscript{2+} transport**

To determine if the Ca\textsuperscript{2+} antagonists inhibited PD induction by blocking transport of Ca\textsuperscript{2+} across the plasma membrane, we examined the effects of these compounds on the uptake of extracellular Ca\textsuperscript{2+}. Fig. 5A shows the uptake of \textsuperscript{45}Ca\textsuperscript{2+} by intact cells under PD induction conditions, where the concentration of extracellular Ca\textsuperscript{2+} is probably in the low micromolar range (Bumann et al. 1984). Under these conditions, Ca\textsuperscript{2+} uptake was half-maximal at 4-5 min and it reached a plateau after 20-30 min. The level of \textsuperscript{45}Ca\textsuperscript{2+} associated with the cells then remained relatively constant for at least 2 h (data not shown). Omission of cyclic AMP had no effect on the rate or magnitude of Ca\textsuperscript{2+} uptake. While the kinetics of \textsuperscript{45}Ca\textsuperscript{2+} transport were similar in every experiment, the level of radioactivity associated with the cells varied by as much as fourfold; this was probably due to differences in the specific activity of the extracellular Ca\textsuperscript{2+} in the cell suspensions. Therefore, in each experiment, values were normalized to the radioactivity in the cells after 30 min. Over this time period, the presence of 100 \muM-TMB-8 partially inhibited both the rate and magnitude of Ca\textsuperscript{2+} transport (Fig. 5A). After 30 min, the level of radioactivity associated with the TMB-8-treated cells generally increased slowly towards control values (not shown). Verapamil at 200 \muM had a similar but slightly less inhibitory effect on Ca\textsuperscript{2+} uptake (data not shown). In contrast, diltiazem (300 \muM) (Fig. 5A), nifedipine (200 \muM) and nicardipine (80 \muM) (not shown) had little or no effect on Ca\textsuperscript{2+} transport. The effect of La\textsuperscript{3+} (100 \muM) could not be assessed because it caused severe cell clumping and erratic results. Unlike the Ca\textsuperscript{2+} antagonists, trifluoperazine (40 \muM) stimulated transport, and Ca\textsuperscript{2+} uptake continued for at least 30 min (Fig. 5A).

The organic inhibitors were also examined for an effect on Ca\textsuperscript{2+} uptake by the low-affinity Ca\textsuperscript{2+} transport system recently described by Europe-Finner & Newell (1985). As illustrated in Fig. 5B, 100 \muM-TMB-8 had no effect on the rapid uptake of 1-5 mM or 150 \muM-Ca\textsuperscript{2+} by this system. These experiments were performed in the absence of cyclic AMP, but identical results were obtained under induction conditions. Nicardipine (80 \muM), nifedipine (200 \muM), diltiazem (300 \muM) and verapamil (200 \muM) also failed to inhibit Ca\textsuperscript{2+} uptake (data not shown).

**Effects of the Ca\textsuperscript{2+} antagonists on ATP pools**

TMB-8, at relatively high concentrations, has been reported to inhibit respiration in *D. discoideum* (Europe-Finner et al. 1985). Therefore, TMB-8 and the other putative Ca\textsuperscript{2+} antagonists might inhibit PD induction by reducing energy levels in the cells. To investigate this possibility, we examined the effect of these compounds on the level of the ATP pools in cells incubated for 2 h under PD induction conditions. As shown in Table 2, neither the Ca\textsuperscript{2+} antagonists nor trifluoperazine had any effect on the ATP pools in these cells.
cells. In contrast, the electron transport inhibitor, rotenone (at 200 μM), significantly reduced the pools, and the uncoupler of oxidative phosphorylation, CCCP (at 10 μM), almost completely abolished them.

**Does TMB-8 block PD induction by inhibiting cyclic GMP production?**

Europe-Finner & Newell (1984) suggested that TMB-8 might inhibit chemotaxis by reducing cyclic GMP synthesis. Therefore, experiments were performed to determine if the inhibition of PD induction by TMB-8 could be explained by a decrease in cyclic GMP production. Since cyclic AMP-stimulated cells of strain NP368 possess higher levels of intracellular cyclic GMP than HC91 cells (Coukell & Cameron, 1986; Lappano & Coukell, 1982), one might expect PD induction in strain NP368 to be more resistant to TMB-8, if TMB-8 acts by inhibiting cyclic GMP

![Figure 5](image_url)

**Fig. 5.** Effect of Ca^2+ antagonists on the uptake of extracellular Ca^{2+} by amoebae of strain HC91. A. Cells were incubated under PD induction conditions (low extracellular Ca^{2+}) and assayed for ^{45}Ca^{2+} uptake as described in Materials and methods. Ca^{2+} uptake values in the absence of drugs (○) are the average ± s.e. of results obtained in 19 experiments and are expressed relative to the 30 min time-point values. Ca^{2+} uptake values in the presence of drugs are expressed relative to the 30 min values for the corresponding control cells. (Δ) 100 μM-TMB-8 (n = 3); (□) 300 μM-diltiazem (n = 3); (□) 40 μM-trifluoperazine (n = 4). In all experiments, counts in zero time samples (5–15 % of maximum) were subtracted from other values. B. Cells were harvested, washed in calcium-free SS, starved for 2 h in buffer A and assayed for Ca^{2+} uptake as described by Europe-Finner & Newell (1983). ^{45}Ca^{2+} transport was measured in the absence (filled symbols) and in the presence (open symbols) of 100 μM-TMB-8. The drug was added to the suspensions 5 or 10 min before the labelled Ca^{2+}. Extracellular Ca^{2+} concentrations: (○, □) 1.5 mM; (Δ) 150 μM. Results are the average ± S.E. of values obtained in three independent experiments.

<table>
<thead>
<tr>
<th>Table 2. Effect of various compounds on ATP pools in HC91 cells incubated under PD induction conditions</th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>TMB-8</td>
</tr>
<tr>
<td>Diltiazem</td>
</tr>
<tr>
<td>Nifedipine</td>
</tr>
<tr>
<td>Verapamil</td>
</tr>
<tr>
<td>Trifluoperazine</td>
</tr>
<tr>
<td>CCCP</td>
</tr>
<tr>
<td>Rotenone</td>
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</tbody>
</table>

*P < 0.001 (versus control).
**P < 0.05 (versus control).
† The Ca^{2+} antagonists and trifluoperazine were added to give a concentration that inhibited iPD and ePD induction by >95 %.
‡ Values are expressed as nmol ATP/10^7 cells ± s.e. and are the average of (n) experiments.

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production. However, as shown in Fig. 2A, strains NP368 and HC91 appeared to be equally sensitive to this drug.

Fig. 6 presents results of an experiment designed to determine directly if TMB-8 inhibits the cyclic GMP response under conditions where iPD and ePD induction is strongly inhibited. To ensure that there were sufficient cells to measure cyclic GMP levels accurately, the cell concentration in these experiments was increased to $6 \times 10^7$ cells ml$^{-1}$ and TMB-8 was added to give a concentration of 100 $\mu$M. Also, PD was induced by the addition of 1 $\mu$M-cyclic AMP at zero time and after 1 h of incubation. Under these conditions, TMB-8 inhibited both iPD and ePD production by >90% (Fig. 6A,B). When aggregation-competent cells were stimulated with 100 $n$M-cyclic AMP under similar conditions (i.e. same cell population, cell density and TMB-8 concentration), cyclic GMP production was reduced by no more than 15% (Fig. 6C).

Since Europe-Finner & Newell (1984) found that 2 mM-TMB-8 abolished the cyclic GMP response, we examined the effect of this concentration of the drug on HC91 cells incubated under their conditions. As reported in the earlier paper, 2 mM-TMB-8 completely inhibited cyclic GMP production (data not shown). However, during these experiments it was observed that the high concentration of TMB-8 dramatically reduced the viability of the cells and slightly lowered the levels of their ATP pools (Table 3). Although not statistically significant, due to the variability of the results with the drug-treated cells, in all three experiments ATP pools were lower in cells incubated with 2 mM-TMB-8 than in control cells. In addition to these effects, it was observed that an appreciable fraction of the treated cells had lost their phase-halo when viewed microscopically (data not shown). All of these effects of TMB-8 on the cells were largely overcome by the presence of 7 mM-Ca$^{2+}$.

Fig. 6. Effect of TMB-8 on phosphodiesterase induction and the cyclic GMP response in strain HC91. Amoebae were washed and resuspended in buffer A to $2 \times 10^7$ cells ml$^{-1}$. PD induction (A,B): A portion of the cells were starved in shaken suspension for 2 h, washed, resuspended in buffer A to $6 \times 10^7$ cells ml$^{-1}$ and placed in two flasks. One suspension (O) was treated with 100 $\mu$M-TMB-8 (final concentration) while the other suspension (●) served as the control. Both suspensions received 1 $\mu$M-cyclic AMP at zero time and after 1 h. At the times indicated, samples of suspension were removed, processed and assayed for iPD (A) and ePD (B) activity. Cyclic GMP response (C): the remainder of the cells were treated in shaken suspension for 4–5 h with 100 $n$M pulses of cyclic AMP to induce aggregation competence, washed, resuspended in buffer A to $6 \times 10^7$ cells ml$^{-1}$ and transferred to two flasks. TMB-8 (100 $\mu$M) was added to one suspension (□) while the control (●) was untreated. Both suspensions were shaken vigorously at room temperature and treated with 100 $n$M-cyclic AMP pulses at 5 min intervals to synchronize the cell population. After 30 min, 100-$\mu$l samples of suspension were removed, stimulated with 100 $n$M-cyclic AMP for the periods indicated, acidified/neutralized and assayed for cyclic GMP. Results are an average ± S.E. of values obtained in three separate experiments.
Table 3. Effect of 2 mM-TMB-8 on the ATP pools and viability of HC91 cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ATP pools (nmol ATP/10^7 cells)</th>
<th>Cell viability (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.40 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>+2 mM TMB-8</td>
<td>1.96 ± 0.26*</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>+2 mM TMB-8</td>
<td>1.44 ± 0.01*</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>+7 mM Ca^2+</td>
<td></td>
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</table>

Cells were grown, starved and treated with 2 mM-TMB-8 for 40 min as described by Europe-Finner & Newell (1984). Results are the average ± S.E. of three experiments.

Discussion

A variety of chemicals reported to act as Ca^{2+}-channel blockers in vertebrate cells (see Janis & Trigg, 1983) were found to be potent inhibitors of PD induction in Dictyostelium (Table 1). At relatively low micromolar concentrations, which had no effect on cell viability, all five organic Ca^{2+} antagonists were observed to alter cell shape and inhibit PD induction in a similar dose-dependent fashion (Fig. 2A). At higher concentrations, cell viability decreased and the cells lysed. In all cases, the effects of these drugs on the cells were at least partially alleviated by the presence of 5–10 mM extracellular Ca^{2+}. Like the organic inhibitors, La^{3+} also altered cell shape and reduced cell viability at higher concentrations, but had a more complex effect on PD induction (Fig. 2B). These observations suggest that the different organic Ca^{2+} antagonists might act at the same site(s) in the cells while La^{3+} probably acts at different (or additional) cellular sites.

While millimolar Ca^{2+} was most effective at antagonizing the actions of these drugs on the cells, other divalent cations also had some effect (Fig. 3). For example, Zn^{2+}, and to a lesser extent Mg^{2+}, was capable of partially overcoming the inhibition of iPD induction by TMB-8. However, unlike Ca^{2+}, none of the other cations was able to antagonize the effect of TMB-8 on ePD production (Figs 3, 4). This probably reflects a requirement for Ca^{2+} in secretion (Foreman et al. 1976). Interestingly, Yamasaki & Hayashi (1982) also found that, apart from Ca^{2+}, only Zn^{2+} could effectively counteract the inhibition of iPD (but not ePD) by millimolar EGTA. While the role of Zn^{2+} in PD induction is unknown, these observations suggest that TMB-8 and EGTA might impair the same process(es) in the cells.

Where might TMB-8 and the other organic Ca^{2+} antagonists act in Dictyostelium cells? Originally, TMB-8 was reported to stabilize intracellular membrane-bound Ca^{2+} in muscle cells (Malagodi & Chiov, 1974). As a result of this observation, TMB-8 has been used as a tool in a variety of experimental systems including Dictyostelium to assess the involvement of intracellular Ca^{2+} in the regulation of agonist-induced cellular responses (e.g. see Smith & Iden, 1979; Owen & Villereal, 1982; Europe-Finner & Newell, 1984). Unfortunately, very few of these studies actually examined the effects of TMB-8 on intracellular Ca^{2+}. Recently, however, these experiments have been performed and they failed to support the notion that TMB-8 inhibits the mobilization of Ca^{2+} from intracellular stores (Simpson et al. 1984; Kojima et al. 1985; Willems et al. 1986). Thus, TMB-8 probably does not inhibit PD induction by blocking the release of intracellular Ca^{2+}. Since all of the organic antagonists have been reported to be plasma membrane Ca^{2+}-channel blockers in other systems (Janis & Trigg, 1983; Kojima et al. 1985), they might inhibit PD induction in Dictyostelium by preventing the uptake of extracellular Ca^{2+}. Such an effect would be expected to impair PD induction since this process is strongly inhibited by the presence of millimolar EGTA (Yamasaki & Hayashi, 1982; Coukell & Cameron, unpublished). Cells incubated under PD induction conditions were observed to take up 45Ca^{2+} by a relatively slow, cyclic AMP-independent process (Fig. 5A); this is probably the same process reported by others (Parish & Weibel, 1980; Yamasaki & Hayashi, 1982). However, addition of TMB-8 or verapamil to the cells, at concentrations that completely inhibited PD induction, only partially blocked Ca^{2+} transport by this system (<35%), and the other Ca^{2+} antagonists had no significant effect. The organic inhibitors also failed to block the more rapid uptake of extracellular Ca^{2+} that occurs at higher Ca^{2+} concentrations (Fig. 5B). On the basis of these results, it seems unlikely that any of the organic antagonists inhibit PD induction solely by blocking Ca^{2+} transport into the cells. However, it is possible that TMB-8 and verapamil exert part of their effect in this way. A third possibility is that the organic antagonists might inhibit PD induction indirectly by decreasing energy levels in the cells. In fact, TMB-8 has been reported to inhibit respiration partially in Dictyostelium (Europe-Finner & Newell, 1985). To determine directly the effect of these compounds on the energy level in the cells, we measured the ATP pools in cells incubated under induction conditions. Results of these experiments (Table 2) revealed that neither the Ca^{2+} antagonists nor trifluoperazine, at concentrations that strongly inhibited PD induction, significantly affected ATP levels in the cells. However, consistent with the earlier report, TMB-8 did appear to reduce the ATP pools when present at much higher concentrations (Table 3). These findings indicate that the effects of the Ca^{2+} antagonists on the cells cannot be attributed entirely to an impairment of energy metabolism. Finally, it is possible that these compounds interact with a Ca^{2+}-binding protein(s) that...
function(s) in a process other than ion transport. One such protein, which can probably be excluded, however, is calmodulin. While the calmodulin inhibitor, trifluoperazine, had many effects on the cells similar to those of the organic Ca\(^{2+}\) antagonists, these effects were enhanced rather than alleviated by the addition of extracellular Ca\(^{2+}\) (Table 1). This is not surprising, since the affinity of calmodulin for trifluoperazine is increased by Ca\(^{2+}\) (Levin & Weiss, 1977). If the Ca\(^{2+}\) antagonists act by binding to calmodulin, they must interact with the protein at a different site from trifluoperazine and in a Ca\(^{2+}\)-reversible manner. Recently, it has been reported that TMB-8 is an inhibitor in vitro of protein kinase C, another Ca\(^{2+}\)-binding protein (Simpson et al., 1984; Kojima et al., 1985). While it is tempting to speculate that the organic Ca\(^{2+}\) antagonists might be acting at this site in Dictyostelium, there is no direct evidence at present for protein kinase C in this organism (Hashimoto & Hayashi, 1985). Although the results presented here are consistent with the idea that the organic Ca\(^{2+}\) antagonists inhibit PD induction by interacting with a Ca\(^{2+}\)-dependent process, the complexity of Ca\(^{2+}\) metabolism in general and the lack of basic information on the function of this ion in Dictyostelium make it difficult to identify the major site(s) of action of these drugs.

Earlier work (Europe-Finner & Newell, 1984; Europe-Finner et al., 1985) suggested that high concentrations (2\,mm) of TMB-8 inhibited chemotaxis in Dictyostelium by blocking the release of Ca\(^{2+}\) from intracellular stores and impairing cyclic GMP synthesis. In the present study, lower concentrations of TMB-8, which almost completely abolished PD induction, had little effect on the cyclic GMP response (Fig. 6). Therefore, TMB-8 would appear not to inhibit PD induction indirectly by reducing cyclic GMP production. While the hypothesis that high concentrations of TMB-8 prevent cyclic GMP synthesis by immobilizing intracellular Ca\(^{2+}\) cannot be discarded, the failure of recent studies to support this mechanism of action of TMB-8 (see above) as well as the observations that high concentrations of this drug alter the cell surface, impair mitochondrial function and reduce cell viability (see Results; Table 3) suggest that inhibition of the cyclic GMP response by TMB-8 is more likely to be the result of generalized damage to the cells.

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References


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