**CA$^{2+}$- OR PHORBOL ESTER - DEPENDENT EFFECT OF ATP ON A SUBPOPULATION OF cAMP CELL-SURFACE RECEPTORS IN MEMBRANES FROM D. DISCOIDEUM.**  
A ROLE FOR PROTEIN KINASE C

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**D. discoideum** cells contain surface receptors for the chemoattractant cAMP which are composed of fast and slowly dissociating binding sites with half-lives of respectively about 1 s and 15 s (Van Haastert and De Wit, J. Biol. Chem. 259, 13321-13328). In membranes prepared by shearing the cells through a Nucleopore filter, ATP has no effect on cAMP-binding at equilibrium, but the number of slowly dissociating sites is increased about 2-fold by ATP while their apparent affinity and off-rate are not altered by ATP. The effect of ATP is stimulated about 3-fold by Ca$^{2+}$ with a half maximal effect at 100 µM Ca$^{2+}$. The tumor promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), increases this Ca$^{2+}$-sensitivity of the ATP effect to about 0.2 µM Ca$^{2+}$. These data suggest that a specific subpopulation of cAMP receptors in membranes from *D. discoideum* is altered by the action of protein kinase C.

**D. discoideum** cells detect the chemoattractant cAMP by means of cell surface receptors (1), which lead to several reactions, such as the rapid activation of guanylate cyclase (2), the rapid uptake of extracellular Ca$^{2+}$ (3,4), and the slower activation of adenylate cyclase (5,6). Intracellular cAMP and Ca$^{2+}$ are supposed to be involved in the chemotactic reaction (7,8), while the cAMP is secreted in the medium (6).

Binding of cAMP to these cells is heterogeneous (9). A small portion of the binding sites has high affinity for cAMP ($K_d = 15$ nM) and release bound cAMP slowly with a half-life of about 15 s. The majority of the binding sites release cAMP with a half-life of about 1 s. These fast dissociating binding sites are composed of high affinity ($K_d = 60$ nM) and low affinity ($K_d = 450$ nM) sites which interconvert during the binding reaction (9). The transition of high to low affinity sites in cells is pro-
moted in isolated membranes by guanyl nucleotides with a specificity sug-
gestig the action of a GTP-binding protein (10). Guanyl nucleotides also
reduce the number and affinity of the slowly dissociating sites. During
these experiments it was observed that ATP does not alter the total
population of binding sites (10), but that the binding to the slowly dis-
sociating sites was increased about 2-fold. The observation that ADP was
without effect and the non-hydrolyzable analog App(NH)p even slightly in-
hibited CAMP binding to the slowly dissociating sites may suggest a phos-
phorylation reaction.

Recent data suggest that phosphorylation of insulin (11), EGF (12,13)
and β-adrenergic (14,15) receptors is mediated by the phospholipid-depend-
ent, Ca²⁺-stimulated protein kinase (protein kinase C, for review see (16)).
It has been shown that the putative phosphorylation does not alter the num-
ber of receptors, but that the affinity and/or responsiveness of the recep-
tors are diminished (17-19). These conclusions are based on the observations
that tumor promoting phorbol esters, which are regarded as specific activa-
tors of protein kinase C, induce the effects described above, and that phos-
phorylation of receptors is correlated with desensitization. The cell sur-
face CAMP receptor from D. discoideum has not been well characterized bio-
chemically, which complicates a study on its possible phosphorylation. There-
fore we have elaborated the indirect effect of ATP on CAMP binding, and show
that this effect is Ca²⁺- or phorbol ester dependent.

Materials and Methods

[2,8-³H]cAMP (1.5 TBq/mmol) was from the Radiochemical Centre, the
silicon oils AR20 and AR200 were obtained from Wacker Chemie, phorbol 12-
myristate 13-acetate (PMA), GTP, GDP, and CAMP were obtained from Sigma,
and GTPyS was a generous gift of Dr. F. Eckstein. ATP was from Boehringer.
D. discoideum cells were grown and starved as described (9). Membranes were
prepared by shearing the cells through a Nucleopore filter (diameter 25 mm,
pore size 3 μm), see (10). CAMP-binding was measured at 20°C by incubating
membranes derived from 10⁷ cells with usually 2 nM [³H]cAMP and 5 mM dithio-
threitol in a total volume of 100 μl. Bound and free [³H]cAMP were separated
by centrifugation through silicon oil (9).

Results

A Scatchard plot of the binding of different CAMP concentrations in the
absence or presence of 1 mM ATP is shown in figure 1. The main figure re-
Figure 1. Scatchard plot of cAMP binding to membranes in the absence (o) or presence (•) of 1 mM ATP. Membranes were prepared and incubated with different concentrations [3H]cAMP as described in Materials and Methods. Main figure: [3H]cAMP-binding after an incubation period of 75 s. Inset: [3H]cAMP-binding after an incubation period of 75 s and a chase with 0.1 mM cAMP during 10 s.

Figure 2. Dissociation of the cAMP-receptor complex in the absence (o) or presence (•) of 1 mM ATP. Membranes were incubated with 2 nM [3H]cAMP with or without 1 mM ATP. Then (at t = 0 in the figure) 0.1 mM cAMP was added, and membranes were centrifuged through silicon oil at the times indicated.

presents the binding at equilibrium, and ATP has no effect as was shown previously for 2 nM [3H]cAMP (10). The inset of figure 1 shows the residual [3H]cAMP binding after a chase with excess cAMP for 10 s, and represents the binding to the slowly dissociating sites (9). The affinity of these sites is about 16 nM; ATP induces an about 2-fold increase in the number of S sites, and has no detectable effect on the affinity.

The effect of ATP on the rate of dissociation of bound [3H]cAMP is shown in figure 2. In control membranes the dissociation is biphasic; at 2 nM [3H]cAMP about 30% of the bound radioactivity dissociates with a half life of about 15 s (k1 = 0.04 s⁻¹). In the presence of ATP this rate constant is virtually identical, but the fraction of slowly dissociating [3H] cAMP is increased to about 60%. These results show that ATP increases the number of a subpopulation of slowly dissociating sites without affecting their apparent kinetic constants Kd and k⁻¹.

The stimulation of [3H]cAMP binding by ATP is inhibited by 2 mM EGTA from 97% to 61% (Table 1), which suggests that the effect of ATP is Ca²⁺-stimulated. The tumor promoting phorbol ester PMA, a specific activator
Table I. Ca\(^{2+}\)- or phorbol ester-dependent stimulation of cAMP binding to the slowly dissociating sites

<table>
<thead>
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<th>incubation</th>
<th>% stimulation by ATP</th>
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<tr>
<td>control</td>
<td>97 ± 7</td>
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<tr>
<td>2 mM EGTA</td>
<td>61 ± 12**</td>
</tr>
<tr>
<td>1 μM PMA</td>
<td>107 ± 14</td>
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<tr>
<td>2 mM EGTA + 1 μM PMA</td>
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</tr>
<tr>
<td>1 μM trifluoroperazin</td>
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<td>10 μM chlorpromazine</td>
<td>102 ± 10</td>
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<tr>
<td>100 μM chlorpromazine</td>
<td>109 ± 11</td>
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Membranes were prepared as described in Materials and methods. cAMP binding was measured with 2 nM \(^{3}H\)cAMP with and without 1 mM ATP in the presence of the drugs as indicated. The incubation period was 75 s, which is followed by a chase with 0.1 mM cAMP during 10 s. The data shown are the means and standard deviations of four independent experiments each with triplicate incubations.

** significant below control (P < 0.02).

of the Ca\(^{2+}\)-dependent, protein kinase C, restores the EGTA-induced inhibition of the ATP effect. Trifluoroperazine and chlorpromazine, inhibitors of Ca\(^{2+}\)-calmodulin-dependent protein kinase, do not alter the ATP-induced stimulation of cAMP-binding to the slowly dissociating cAMP binding sites.

The Ca\(^{2+}\)-dependency of the ATP effect was measured in membranes prepared in the presence of EGTA (figure 3A). In the absence of Ca\(^{2+}\), ATP stimulates cAMP-binding by 25±5%. This stimulation is increased to 70±6% at 1 mM Ca\(^{2+}\); a half-maximal effect is observed at 100 μM Ca\(^{2+}\). The phorbol ester PMA shifts the Ca\(^{2+}\)-dependency to about 500-fold lower Ca\(^{2+}\) concentrations; a half-maximal effect is observed at 0.2 μM Ca\(^{2+}\). The effect of PMA occurs at low concentrations (figure 3B); it saturates at about 1 μM, and a half-maximal effect occurs at approximately 5 nM PMA.

Previously we have shown that guanyl nucleotides decrease the binding of \(^{3}H\)cAMP to the slowly dissociating sites (10). Does ATP prevent the
Figure 3. Effect of PMA on the Ca\textsuperscript{2+}-dependency of the ATP-mediated increase of cAMP-binding. Membranes were prepared in the presence of 10 mM EGTA, centrifuged and resuspended in phosphate buffer containing 0.5 mM EGTA (final concentration). cAMP-binding was measured with 2 nM [\textsuperscript{3}H]cAMP in the absence and presence of 1 mM ATP at different concentrations of free Ca\textsuperscript{2+} and different concentrations of PMA. [\textsuperscript{3}H]cAMP binding was detected at 10 s after a chase with 0.1 mM cAMP. A: (●), without PMA; (○) with 1 nM PMA. B: with 10 nM free Ca\textsuperscript{2+}. The data shown are the means and standard deviations of three independent experiments with triplicate incubations.

action of guanyl nucleotides? The results of table 2 reveal that the guanyl nucleotides GDP, GTP and GTP\textsubscript{y}S inhibit cAMP binding to the slowly dissociating sites by about 80%; the 2-fold stimulation by ATP is completely counteracted by 100 \mu M of the guanyl nucleotides.

Discussion

The main findings of the present report are that i.) ATP, but not ADP or the nonhydrolyzable analog App(NH)p, stimulates cAMP binding to a sub-

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<th>incubation</th>
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<tr>
<td></td>
<td>- ATP</td>
</tr>
<tr>
<td>control</td>
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</tr>
<tr>
<td>100 \mu M GDP</td>
<td>22 ± 4</td>
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<tr>
<td>100 \mu M GTP</td>
<td>18 ± 3</td>
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<tr>
<td>100 \mu M GTP\textsubscript{y}S</td>
<td>17 ± 2</td>
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\textsuperscript{3}H]cAMP-binding was measured in an incubation medium containing 2 nM [\textsuperscript{3}H]cAMP with or without 1 mM ATP and the guanyl nucleotides at the indicated concentrations. [\textsuperscript{3}H]cAMP-binding was detected at 10 s after a chase with excess cAMP. The data are presented as relative to the control which did not contain guanyl nucleotides or ATP. Means and standard deviations of three experiments are shown.
population of slowly dissociating binding sites. ii) ATP increases the number of the sites, while the affinity and off-rate are not altered. iii) The effect of ATP is potentiated by Ca^{2+} with EC_{50} = 100 \ \mu M \ Ca^{2+}. iv) The phorbol ester PMA enhances the Ca^{2+}-sensitivity to an EC_{50} = 0.2 \ \mu M \ Ca^{2+}.

Tumor promoting phorbol esters are regarded as specific activators of the phospholipid-dependent, Ca^{2+}-stimulated protein kinase C (see 16). Phorbol esters, and the natural activator diacylglycerol, stimulate protein kinase C by increasing the sensitivity to Ca^{2+} from the submillimolar to the submicromolar range. The observation that PMA reduces the Ca^{2+}-dependency of the ATP stimulation of cAMP-binding from 100 \ \mu M to 0.2 \ \mu M \ Ca^{2+} strongly suggests that the ATP effect is caused by a phosphorylation reaction mediated by protein kinase C. The substrate of protein kinase C could be the receptor as in (14,15) or a protein which interacts with the receptor, such as a GTP-binding protein. The latter possibility seems unlikely since the effect of ATP is completely counteracted by guanyl nucleotides (table 2).

Experiments which would suggest that protein kinase C can be activated in vivo (i.e. that diacylglycerol is formed by cAMP-stimulated hydrolysis of inositol phospholipids) have been negative thus far. cAMP does not induce an increased turnover or hydrolysis of inositol phospholipids in cells labeled with [^{32}P]-inorganic phosphate. cAMP does also not induce an increase of diacylglycerol in cells labeled via bacteria with [^{3}H] glycerol. Unfortunately, we are unable to label the cells with [^{3}H] inositol (unpublished observations).

What could be the function of the putative phosphorylation of a subpopulation of cAMP receptors? In other organisms it has been suggested that phosphorylation of \beta-\adrenergic receptors by protein kinase C correlated with desensitization (14,15). In D. discoideum the cAMP-induced cAMP and cGMP responses rapidly desensitize (20,21). It is interesting to notice that cAMP induces the rapid phosphorylation of a protein with a molecular weight of about 47 kD in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22). Furthermore, photoaffinity labelling of surface receptors
with [\(^{32}\)P]-8 azido-cAMP yields two labeled bands with apparent molecular weight of 40 and 43 kD. The proportioning of these two bands changes during the binding reaction; the band with the higher molecular weight prevails when cells are desensitized (23). It may well be that these two bands represent the same protein in a different state of phosphorylation. The present data indicate that the action of protein kinase C alters a specific population of cAMP binding sites, which may suggest that covalent modification of the cAMP receptor by phosphorylation could be the basis of desensitization in D. discoidum.

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References