Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 185-192

# $CA^{2+}$- 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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Received March 1, 1985

*D. discoideum* cells contain surface receptors for the chemoattractant cAMP which are composed of fast and slowly dissociating binding sites with half-lifes 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 cGMP 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-

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moted in isolated membranes by guanyl nucleotides with a specificity suggesting 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 dissociating sites was increased about 2-fold. The observation that ADP was without effect and the non-hydrolyzable analog App(NH)p even slightly inhibited cAMP binding to the slowly dissociating sites may suggest a phosphorylation reaction.

Recent data suggest that phosphorylation of insulin (11), EGF (12,13) and β-adrenergic (14,15) receptors is mediated by the phospholipid-dependent, $Ca^{2+}$-stimulated protein kinase (protein kinase C, for review see (16)). It has been shown that the putative phosphorylation does not alter the number of receptors, but that the affinity and/or responsiveness of the receptors are diminished (17-19). These conclusions are based on the observations that tumor promoting phorbol esters, which are regarded as specific activators of protein kinase C, induce the effects described above, and that phosphorylation of receptors is correlated with desensitization. The cell surface cAMP receptor from *D.discoideum* has not been well characterized biochemically, which complicates a study on its possible phosphorylation. Therefore we have elaborated the indirect effect of ATP on cAMP binding, and show that this effect is $Ca^{2+}$- or phorbol ester dependent.

## Materials and Methods

[2,8-$^3$H]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 GTPγS 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 $10^7$ cells with usually 2 nM [$^3$H]cAMP and 5 mM dithiothreitol in a total volume of 100 μl. Bound and free [$^3$H]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-

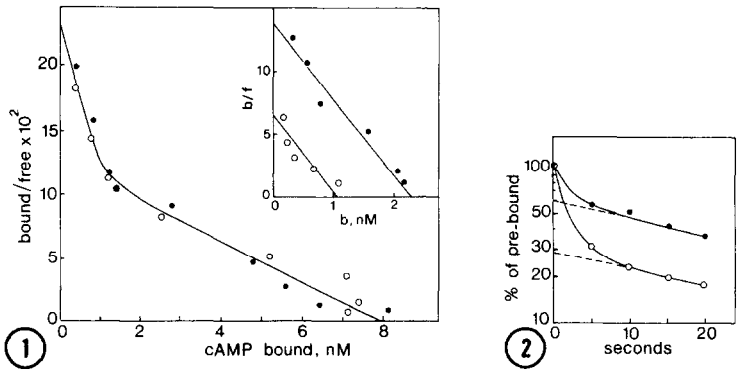


Figure 1. Scatchard plot of cAMP binding to membranes in the absence (o) or presence (•) of 1 mM ATP. Membranes were prepared and incubated with different concentrations [$^3$H]cAMP as described in Materials and Methods. Main figure: [$^3$H]cAMP-binding after an incubation period of 75 s. Inset: [$^3$H]cAMP-binding 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 [$^3$H]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 [$^3$H]cAMP (10). The inset of figure 1 shows the residual [$^3$H]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 [$^3$H]cAMP is shown in figure 2. In control membranes the dissociation is biphasic; at 2 nM [$^3$H]cAMP about 30% of the bound radioactivity dissociates with a half life of about 15 s ($k_1$ = 0.04 $s^{-1}$). In the presence of ATP this rate constant is virtually identical, but the fraction of slowly dissociating [$^3$H]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 $K_d$ and $k_{-1}$.

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

Table 1. $Ca^{2+}$- or phorbol ester-dependent stimulation of cAMP binding to the slowly dissociating sites

| incubation | % stimulation by ATP |
|---|---|
| control | 97 ± 7 |
| 2 mM EGTA | 61 ± 12** |
| 1 µM PMA | 107 ± 14 |
| 2 mM EGTA + 1 µM PMA | 99 ± 12 |
| 1 µM trifluoroperazin | 101 ± 12 |
| 10 µM trifluoroperazin | 99 ± 8 |
| 10 µM chlorpromazin | 102 ± 10 |
| 100 µM chlorpromazin | 109 ± 11 |

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

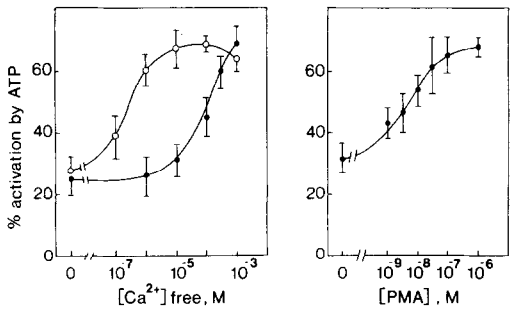


Figure 3. Effect of PMA on the $Ca^{2+}$-dependency of the ATP-mediated increase of cAMP-binding. Membranes were prepared in the presence of 10 mM EGTA, centrifuged and resuspended in phosphate buffer containing 0.5 mM EGTA (final concentration). cAMP-binding was measured with 2 nM [$^{3}$H]cAMP in the absence and presence of 1 mM ATP at different concentrations of free $Ca^{2+}$ and different concentrations of PMA. [$^{3}$H]cAMP binding was detected at 10 s after a chase with 0.1 mM cAMP. A: (●), without PMA; (○) with 1 µM PMA. B: with 10 µM free $Ca^{2+}$. 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 GTPγS inhibit cAMP binding to the slowly dissociating sites by about 80%; the 2-fold stimulation by ATP is completely counteracted by 100 µM 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-

Table 2. Cross effect of guanyl nucleotides and ATP

| incubation | % of control | |
|---|---|---|
| | - ATP | + ATP |
| control | 100 | 208 ± 10 |
| 100 µM GDP | 22 ± 4 | 24 ± 5 |
| 100 µM GTP | 18 ± 3 | 17 ± 4 |
| 100 µM GTPγS | 17 ± 2 | 19 ± 4 |

cAMP-binding was measured in an incubation medium containing 2 nM [$^{3}$H]cAMP with or without 1 mM ATP and the guanyl nucleotides at the indicated concentrations. [$^{3}$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 µM $Ca^{2+}$. iv) The phorbol ester PMA enhances the $Ca^{2+}$-sensitivity to an $EC_{50}$ = 0.2 µM $Ca^{2+}$.

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 µM to 0.2 µM $Ca^{2+}$ 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 β-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. discoideum*.

Acknowledgements

We gratefully acknowledge Theo Konijn for stimulating discussions. This work was supported by the Foundation for Fundamental Biological Research (BION) and the C. and C. Huygens Fund which are subsidized by the Netherlands Organization for the Advancement of Pure Scientific Research (ZWO).

References

1. Malchow, D., and Gerisch, G. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2423-2427.
2. Mato, J.M., and Malchow, D. (1978) FEBS Lett. 90, 119-122.
3. Wick, U., Malchow, D., and Gerisch, G. (1978) Cell Biol. Int. Rep. 2, 71-79.
4. Bumann, J., Wurster, B., and Malchow, D. (1984) J. Cell biol. 98, 173-178.
5. Roos, W., and Gerisch, G. (1976) FEBS Lett. 68, 170-172.
6. Dinauer, M.C., McKay, S.A., and Devreotes, P.N. (1980) J. Cell Biol. 86, 537-544.
7. Van Haastert, P.J.M., Van Lookeren Campagne, M.M., and Ross, F.M. (1982) FEBS Lett. 147, 149-152.
8. Europe-Finner, G.N., and Newell, P.C. (1984) FEBS Lett. 171, 315-319.
9. Van Haastert, P.J.M., and De Wit, R.J.W. (1984) J. Biol. Chem. 259, 13321-13328.
10. Van Haastert, P.J.M. (1984) Biochem. Biophys. Res. Commun. 124, 597-604.
11. Jacobs, S., Sahyoun, N.E., Saltiel, A.R., and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6211-6213.
12. Cohet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A., and Hunter, T. (1984) J. Biol. Chem. 259, 2553-2558.
13. Iwashita, S., and Fox, C.F. (1984) J. Biol. Chem. 259, 2559-2567.
14. Sibley, D.R., Nambi, P., Peters, J.R., and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 121, 973-979.
15. Kelleher, D.J., Pessin, J.E., Ruoko, A.E., and Johnson, G.L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4316-4320.
16. Nishizuka, Y. (1984) Nature 308, 693-698.
17. Garte, S.J., and Belman, S. (1980) Nature 284, 171-173.

18. Grunberger, G., and Gordon, P. (1982) Am. J. Physiol. 243, E 319-E 324.
19. Mallorga, P., Tallman, J.F., Hennebury, R.C., Hirata, F., Strittmatter, W.T., and Axelrod, J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1341-1345.
20. Devreotes, P.N., Derstine, P.L., and Steck, T.L. (1979) J. Cell Biol. 80, 291-299.
21. Van Haastert, P.J.M., and Van der Heijden, P.R. (1983) J. Cell Biol. 96, 347-353.
22. Lubs-Haukeness, J., and Klein, C. (1982) J. Biol. Chem. 257, 12204-12208.
23. Theibert, A., Klein, P., and Devreotes, P.N. (1984) J. Biol. Chem. 259, 12318-12321.