Distinct Characteristics of the Basal Activities of Adenylyl Cyclases 2 and 6*

(Received for publication, May 17, 1995)

Joseph P. Pieroni, Anya Harryt, Jianqiang Chen, Ofer Jacobowitz, Ronald P. Magnusson, and Ravi Iyengar‡

From the Department of Pharmacology, Mount Sinai School of Medicine, City University of New York, New York, New York 10029-6574

Regulation of basal activities of adenylyl cyclase (AC) 2 and 6, expressed in Sf9 cells by infection with recombinant baculovirus, was studied. An antipeptide antibody that recognizes AC2 and AC6 with equal sensitivity was used to establish that equivalent levels were expressed. Basal activities of AC2 and AC6 were compared at varying concentrations of Mg$^{2+}$ or Mn$^{2+}$ ions; AC2 had 15- and 10-fold greater activity than AC6, respectively. At 20 mM Mg$^{2+}$, the $K_v$ values for ATP were 88 and 39 nM for AC2 and AC6, respectively, whereas their $V_{\text{max}}$ values were 281 and 11 pmol/mg protein-min. With 100 µM forskolin and either Mg$^{2+}$ or Mn$^{2+}$, the difference in activities between AC2 and AC6 was reduced to approximately 2-fold. Forskolin stimulated AC6 greater than 40-fold at 0.5-2 mM Mg$^{2+}$, whereas AC2 was stimulated 4-6-fold. At 20 mM Mg$^{2+}$, AC2 was stimulated 2-fold by forskolin, whereas AC6 was stimulated 18-fold. With Mg$^{2+}$ alone, activities of AC2 and AC6 were not saturable up to 20 mM and yielded curvilinear Hofstee transformations. With forskolin, activities of both AC2 and AC6 were saturable by 10 mM Mg$^{2+}$ and yielded linear Hofstee transformations. These data indicate that there are substantial differences in the basal enzymatic activities of adenylyl cyclase isoforms, due to differential regulation by Mg$^{2+}$ ions rather than intrinsic catalytic capabilities. Thus the presence and relative abundance of adenylyl cyclase subtype could greatly affect the resting cellular cAMP levels with consequent effects on important biological functions, such as differentiation and proliferation.

The ambient level of intracellular cAMP is an important regulator of biological processes, such as proliferation and differentiation. Studies from our laboratory and others recently have shown that a 2-fold increase, or less, in intracellular cAMP can be regulated, including constant stimulation of adenylyl cyclase activity (1–5). There is little evidence, however, that either of these phenomena occur. It is well documented that receptor or G protein stimulation of adenylyl cyclases leads to desensitization of the stimulatory signal at the level of the receptor (6) or at loci downstream (7, 8). This results in transitory alterations in cAMP levels. If, however, the different adenylyl cyclase isoforms have different basal activities, the ambient intracellular cAMP concentrations could be set at a certain level simply by having the appropriate isoform(s). The molecular diversity of different AC2 forms in heterotrimeric G protein-coupled systems is now established. Nine different isoforms of mammalian G$_\text{s}$-stimulated adenylyl cyclases are known (9, 10). We chose to study the basal properties of two of these adenylyl cyclases (AC2 and AC6) in an attempt to develop the hypothesis that basal properties of different isoforms could contribute to very different intracellular cAMP levels. cAMP production by AC2 is stimulated by several signals including G$_\text{s}$-α and βγ-subunits (11), and by protein kinase C (12, 13). In contrast, cAMP production by AC6 is only stimulated by G$_\text{s}$-α, and inhibited extensively by G$_\text{i}$-α (14, 15), protein kinase A, and low concentrations of Ca$^{2+}$ (Ref. 9 and references therein). Thus, as we had previously surmised that the presence of AC2 could be reflective of a cell’s need to have elevated cAMP levels in response to multiple signals (14), the presence of AC6 could maintain low levels of intracellular cAMP. An extension of this line of reasoning is that AC6 may also have very low basal activities in comparison to AC2 and hence allow the cell to keep basal cAMP levels low. To determine whether different adenylyl cyclases have distinct basal activities, we expressed both adenylyl cyclases in Sf9 cells using the baculovirus expression system and studied regulation of their basal activities by Mg$^{2+}$ and Mn$^{2+}$ in the absence and presence of forskolin.

EXPERIMENTAL PROCEDURES

AC2 cDNA was the kind gift of Dr. Randall Reed (Johns Hopkins University). Sf9 cells and pVL-1392 were obtained by the Mount Sinai Protein Expression Core facility from Dr. Max Summers. BaculoGold virus was from Pharmigen. Serum-free Sf9 medium was from Life Technologies, Inc., or Sigma. [α-32P]ATP was purchased from ICN. Forskolin was from Sigma. Sources of all other materials have been previously described (8, 14).

Insertion of Adenylyl Cyclases into Baculovirus and Expression in Sf9 Cells—AC2 was excised from pBSII-AC2 as an EcoRI fragment and inserted into pVL-1392. AC6 was excised from pBSII-AC6 as a BamHI/EcoRI fragment and inserted into pVL-1393. Orientation of the inserts in the recombinant plasmids was verified by restriction digests. Recombinant plasmids were individually transfected into Sf9 cells along with BaculoGold virus DNA. Recombinant baculovirus were purified by two rounds of limiting dilutions. Their identities were verified by dot blots.

Preparation of Membranes from Infected Sf9 Cells—Sf9 cells were grown in SF-900 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum or in serum-free insect culture medium (Sigma).

*Supported by National Institutes of Health Grants CA-44998 and DK-38761 and American Cancer Society Grant CB-132. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Supported by predoctoral National Research Service Award F31-15599.

§Supported by the Endocrinology Training Program Grant DK-07645.

1 The abbreviation used is: AC, adenylyl cyclase.

2 Y.-b. Chen, R. Iyengar, unpublished observations.
Cells were infected with a multiplicity of infection of ~1. Cells were harvested 2–4 days after infection. Cells were pelleted and washed with lysis buffer that contained 20 mM NaHepes (pH 8.0), 4 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl, and a protease mixture of 2 mg/ml aprotonin, 4 mg/ml leupeptin, 1 mM 1,10 phenanthroline, and 1 mM phenylmethylsulfonyl fluoride. For AC6, 50 mM sucrose was included in the homogenization buffer. Cells were lysed by nitrogen cavitation (600 p.s.i. for 30 min). The homogenate was centrifuged at 1,000 × g for 10 min. The low speed supernatant was then centrifuged at 100,000 × g for 60 min. The high speed pellet was resuspended in 10 mM NaHepes (pH 8.0), 1 mM EDTA, 2 mM dithiothreitol, 200 or 790 mM sucrose for AC2 or AC6, respectively, and the protease inhibitor mixture. Membranes were stored at a concentration of 3–6 mg/ml at −70 °C. The higher sucrose concentration proved to be necessary for the preservation of the activity of AC6, but did not affect the AC2 activity. For the sake of convenience, AC2 was stored at 200 mM sucrose.

Adenylyl Cyclase Assays—Adenylyl cyclase activity was measured with 5 μg of protein in a final assay volume of 50 μl at 32 °C. The assay mixture contained 20 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM [γ-32P]ATP (2,000–5,000 cpm/pmol). The concentration of Mg2+ ion used in individual experiments are specified. Mg2+ was added as MgCl2, and free Mg2+ was estimated by subtraction of the EDTA and ATP concentrations from the total MgCl2 added. All assays were performed in triplicate, and values are means of triplicate determinations. Coefficient of variance was always less than 10%. All experiments were repeated two or more times with different batches of membranes. Qualitatively similar results were obtained, and typical experiments are shown.

Immunoblotting—Membranes from control (thyroid peroxidase-infected) or AC2- and AC6-expressing Sf9 cells were resolved on 7% SDS-polyacrylamide gels and transferred to nitrocellulose paper. Unless otherwise indicated, the proteins were blotted with a 1:500 dilution of ACcomm antibody. The bands were visualized by horseradish peroxidase-coupled second antibody reaction using the ECL system from Amersham Corp. The autoradiograms were scanned by a Hoeffer densitometer as required.

RESULTS

Membranes containing expressed AC2 and AC6, and control membranes (from thyroid peroxidase-infected cells), were probed with the ACcomm antiserum to determine the levels of adenylyl cyclase expressed. This antibody is an antipeptide antibody against a 14-amino acid stretch (IGARKPQYDI-...). The antibodies have been used in the recognition of AC2 expressed in Sf9 cells (13). In control Sf9 cell expressing thyroid peroxidase, no major bands were seen. In contrast, membranes from Sf9 cells expressing AC2 or AC6 showed prominently stained bands (Fig. 1A). The size of the bands are in accordance with the estimated masses from the cDNA clones. AC2 is expressed as 106 KDa protein and AC6 is expressed as 132–133 KDa protein. It is noteworthy that both start sites of AC6 (16) appear to be used, as indicated by the presence of the doublet. When the ACcomm antibody was preincubated with the immunogen peptide, no bands were visible in the AC2- or AC6-expressing membranes (data not shown). In order to establish that both adenylyl cyclases were being recognized by the antibody with similar sensitivity, we tested different concentrations of antiserum on a low (2.5 μg) level of membrane proteins from Sf9 cells. An immunoblot with varying dilutions of antiserum is shown in Fig. 1B. The intensity of the bands was determined by densitometry (Fig. 1C). It can be seen readily from Fig. 1B and C, that the ACcomm antibody recognized AC2 and AC6 with similar sensitivity. When different amounts of Sf9 membranes were used at a fixed (1:500) antibody concentration, the signal intensity for both AC2 and AC6 varied with the amount of membrane protein used (Fig. 1D). The data in Fig. 1 indicate that the ACcomm antibody can be used to estimate the amounts of AC2 and AC6.

We made several batches of AC2- and AC6-expressing mem-

![Figure 1](image-url)
10, similar to the value seen in cyc-S49 cell membranes (17). We (8) and others (18) have shown that AC6 is present in S49 cells. In contrast, the ratio of Mn$^{2+}$/Mg$^{2+}$ activities of AC6 is in the range of 2–3.

As our standard assay mixture contains 0.1 mM ATP, it is possible that some of the observed differences in activity between AC2 and AC6 are due to differences in $K_m$ of the enzyme isoforms for the substrate. Hence we measured the $K_m$ of AC2 and AC6 for ATP at a high (20 mM) Mg$^{2+}$ ion concentration (Fig. 4). There was a 2-fold difference in the $K_m$ for ATP between AC2 and AC6; the difference in $V_{max}$, however, was greater than 25-fold.

To establish whether the differences in activities between AC2 and AC6 are due to differences in intrinsic catalytic rates or due to differential regulation of basal activities by divalent cations, we determined activities of the two enzymes in the presence of 100 μM forskolin, a direct stimulator of adenylyl cyclase (19), and varying concentrations of Mn$^{2+}$. Generally, Mn$^{2+}$ plus forskolin can be used to elicit the maximum available activity of adenylyl cyclases. A comparison of the activities of AC2 and AC6 in the presence of 100 μM forskolin and varying amounts of Mn$^{2+}$ is shown in Fig. 5. Although AC6 had less activity than AC2 at all concentrations of Mn$^{2+}$ tested, the difference in activity between AC2 and AC6 was only 2-fold. In contrast, in the absence of forskolin at 20 mM Mg$^{2+}$, AC2 had 15-fold greater activity than AC6 (Fig. 2, B and C), and in the absence of forskolin at 10 mM Mn$^{2+}$, AC2 had 10-fold greater activity than AC6 (Fig. 3, A and B). Thus the observed differences in basal activities between AC2 and AC6, in large part, are not due to intrinsic differences in catalytic capabilities of...
these two isoforms but rather due to different responses to stimulation by divalent cations. A noteworthy feature of the experiment in Fig. 5 was that, in the presence of forskolin, the AC2 activity was saturable with respect to Mn2+ ions within 10 mM, in contrast to the continuous increase observed in the absence of forskolin (Fig. 3A). Hence we determined whether AC2 and AC6 activities in the presence of forskolin were saturable with respect to Mg2+ as well.

The effect of varying concentrations of Mg2+ in the presence of 100 μM forskolin on AC2 and AC6 activities is shown in Fig. 6A. It can be readily seen that stimulation by increasing concentrations of Mg2+ is saturable by 5–10 mM Mg2+. This profile contrasts with that seen in the absence of forskolin in which the activity increases in a continuous fashion up to the highest concentration tested (20 mM). The fold stimulation by forskolin for AC2 and AC6 is shown in Fig. 6B. An interesting difference between AC2 and AC6 was observed. At all concentrations of Mg2+ ions tested, forskolin was able to stimulate AC6 activity extensively, with a 45-fold stimulation at 1–2 mM Mg2+ (Fig. 6B). In contrast, fold stimulation by forskolin for AC2 was much lower. Even at low Mg2+ ion concentrations, only a 6-fold stimulation was seen; increasing concentrations of Mg2+ ions elicited substantial amounts of activity, such that forskolin stimulation was very modest (~2-fold).

The Mg2+ concentration effect curves for AC2 and AC6 in the absence and presence of forskolin were subjected to linear transformations. In the absence of forskolin, curvilinear Eadie-Hofstee plots were obtained for both AC2 and AC6 (Fig. 7A, a, and 7B, a), indicating the possibility of multiple sites of interactions for Mg2+. In the presence of forskolin, linear transformations were obtained, indicating that there may be a single allosteric site for divalent cation regulation of the catalytic activities of AC2 and AC6 (Fig. 7A, b, and 7B, b).

**DISCUSSION**

It is now well established that the different mammalian adenylyl cyclases have unique signal recognition capabilities (9, 20). This allows the cAMP pathway to respond to a variety of G protein-coupled receptors (21) as well as receptor tyrosine kinases that stimulate protein kinase C activity. The varied capabilities for signal input, coupled with the differential expression of the adenylyl cyclase isoforms in different cell types and tissues, allow the identity of the individual adenylyl cyclases present to impart to the hormone-regulated adenylyl cyclase system distinct features that may be in consonance with the regulatory requirements of the cell type and tissue. In addition to the varied signal recognition capabilities, the data presented here indicate that the basal activities of different adenylyl cyclases can also be sufficiently different such that the presence and relative abundance of a certain adenylyl cyclase isoform can affect the basal cAMP levels in cells and tissues.

We studied AC2 and AC6. Previous studies had noted differences in basal activities between AC1 and AC3 (22). However, the reasons underlying the differences in basal activities remained unexplored until now.

The activity of AC2 is 25-fold higher than that of AC6 at optimal Mg2+ concentrations (Fig. 4). It is noteworthy that the differences between AC2 and AC6 are most pronounced at low, physiologically relevant, free Mg2+ ion concentrations. This difference in basal activities between AC2 and AC6 is not due, in large part, to intrinsic differences in the catalytic capabilities between AC2 and AC6, since in the presence of Mn2+ and forskolin there is only a 2-fold difference in activity. Rather the low basal activity of AC6 arises from the inability of Mg2+ to elicit high levels of catalytic activity from AC6. Thus under normal cellular conditions, when AC6 constitutes a significant part of the complement of adenylyl cyclases expressed, the basal cAMP production is likely to be low. This agrees well with the previously noted propensity of AC6 to lower cAMP levels in response to a variety of signals. As AC6 is a widely expressed adenylyl cyclase (16, 23), our findings may provide an explanation for the relatively low level of basal intracellular cAMP in many tissues. In contrast AC2, which has a high basal activity, is most abundant in the brain, an organ known to have relatively high basal cAMP levels (24).

Previous studies from our laboratory had noted the presence of an allosteric site for divalent cations on adenylyl cyclase that was required for the expression of Gα-stimulated activity (25). The data presented here extend those observations and indicate that occupancy at the allosteric site on adenylyl cyclase may be responsible for the expression of basal activities.
Basal Activities of Adenylyl Cyclases 2 and 6

as well. Further, from our data it appears that the regulation of basal activity of the different adenylyl cyclase isoforms by Mg\(^2+\) ion is likely to be a unique feature of each adenylyl cyclase.

What is the biological significance of these vastly different basal activities for the different adenylyl cyclases? The answer may be in the important role that intracellular levels of cAMP play in regulating many biological processes. Three examples are noteworthy. 1) Embryonic development: recently it has been shown that basal protein kinase A activity, and hence the ambient levels of cAMP, plays a crucial role in the formation of the compound eye, wing, and leg of Drosophila by regulating the activity of the morphogen Hedgehog (see Ref. 26 and references therein). Current evidence from analysis of the development of retina, leg, and wing (27, 28) supports the notion that the basal activity of protein kinase A is counteracted by the Hedgehog signaling pathway (26). Similarly in vertebrate systems, long-range induction of sclerotome by Sonic hedgehog is blocked by elevation of cAMP levels (29). Thus it appears likely that ambient levels of cellular cAMP are a crucial determinant of embryonic development in several species. It is reasonable to assume that the activity of protein kinase A in a cell is reflective of the activity of the adenylyl cyclase resident in the cell. Our data indicate that different adenylyl cyclases can have very different levels of basal activity. Thus the identity of the adenylyl cyclase present could play a determining role in regulating development by morphogens.

2) Transformation and proliferation of mammalian cells: we have shown that modest increases in cAMP levels are sufficient to block transformation of NIH-3T3 by powerful oncogenes such as H-ras (1). Thus the presence of isoforms of adenylyl cyclase that have high basal activity could work to inhibit processes that lead to neoplastic transformation. Lowering of cAMP levels has been known to trigger proliferation of certain cells such as RAT-1 fibroblasts (30), and in these cells it would be crucial to have adenylyl cyclases with low basal activities for proliferation to occur.

3) The addicted state: it has long been noted that the onset of the addicted state is accompanied by lowered intracellular cAMP levels in neurons (31, 32). Such decreased cAMP levels are thought to be responsible, in part, for the onset of the addicted state (32). It will be interesting to determine whether the lowered levels of cAMP result from decreased activity of the preexisting adenylyl cyclases or from expression of a different complement of adenylyl cyclases with lower basal activities.

The data presented in this paper, and the biological phenomena described above, indicate that the different adenylyl cyclase isoforms and their relative abundance will be able to define not only the responses of the cellular cAMP pathway to external signals but may also regulate the integral physiology of the cell by virtue of their basal activities.

 references therein). Current evidence from analysis of the development of retina, leg, and wing (27, 28) supports the notion that the basal activity of protein kinase A is counteracted by the Hedgehog signaling pathway (26). Similarly in vertebrate systems, long-range induction of sclerotome by Sonic hedgehog is blocked by elevation of cAMP levels (29). Thus it appears likely that ambient levels of cellular cAMP are a crucial determinant of embryonic development in several species. It is reasonable to assume that the activity of protein kinase A in a cell is reflective of the activity of the adenylyl cyclase resident in the cell. Our data indicate that different adenylyl cyclases can have very different levels of basal activity. Thus the identity of the adenylyl cyclase present could play a determining role in regulating development by morphogens.

2) Transformation and proliferation of mammalian cells: we have shown that modest increases in cAMP levels are sufficient to block transformation of NIH-3T3 by powerful oncogenes such as H-ras (1). Thus the presence of isoforms of adenylyl cyclase that have high basal activity could work to inhibit processes that lead to neoplastic transformation. Lowering of cAMP levels has been known to trigger proliferation of certain cells such as RAT-1 fibroblasts (30), and in these cells it would be crucial to have adenylyl cyclases with low basal activities for proliferation to occur.

3) The addicted state: it has long been noted that the onset of the addicted state is accompanied by lowered intracellular cAMP levels in neurons (31, 32). Such decreased cAMP levels are thought to be responsible, in part, for the onset of the addicted state (32). It will be interesting to determine whether the lowered levels of cAMP result from decreased activity of the preexisting adenylyl cyclases or from expression of a different complement of adenylyl cyclases with lower basal activities.

The data presented in this paper, and the biological phenomena described above, indicate that the different adenylyl cyclase isoforms and their relative abundance will be able to define not only the responses of the cellular cAMP pathway to external signals but may also regulate the integral physiology of the cell by virtue of their basal activities.

**REFERENCES**

1. Chen, J.-H., and Iyengar, R. (1994) *Science* **263**, 1278–1281
2. Wu, J., Drent, P., Jellinek, T., Woffman, A., Wider, M. J., and Sturgill, T. W. (1993) *Science* **262**, 1065–1069
3. Cook, S. J., and McCormick, F. (1993) *Science* **262**, 1069–1072
4. Graves, L. M., Bornfeldt, K. A., Raines, E., Potts, B. C., MacDonald, S. G., Ross, R., and Krebs, E. W. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10300–10304
5. Severson, B., King, X., and Lawrence, J. C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10305–10309
6. Inglese, J., Freedman, N. J., Koch, W. J., and Leftkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 23735–23738
7. Rich, K. A., Codina, J., Floyd, G., Sekura, R., Hildebrandt, J. D., and Iyengar, R. (1994) *J. Biol. Chem.* **269**, 7893–7901
8. Premont, R. T., Jacobowitz, O., and Iyengar, R. (1992) *Endocrinology* **131**, 2774–2784
9. Iyengar, R. (1993) *FASEB J.* **7**, 768–775
10. Watson, P. A., Krupinski, J., Keminski, A. M., and Frankenfield, C. D. (1993) *J. Biol. Chem.* **268**, 28893–28898
11. Tang, W.-J., and Gilman, A. G. (1991) *Science* **254**, 1500–1503
12. Premont, R. T., Chen, J., and Iyengar, R. (1993) *J. Biol. Chem.* **268**, 3829–3832
13. Jacobowitz, O., and Iyengar, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1060–10640
14. Chen, J., and Iyengar, R. (1993) *J. Biol. Chem.* **268**, 12253–12256
15. Taussig, R., Quarabyl, L. M., and Gilman, A. G. (1993) *J. Biol. Chem.* **268**, 39–12
16. Premont, R. T., Chen, J., Ma, H.-W., Pronapalli, M., and Iyengar, R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9809–9813
17. Ross, E. M., Howlett, A. C., Ferguson, K. M., and Gilman, A. G. (1978) *J. Biol. Chem.* **253**, 6401–6412
18. Krupinski, J., Lehman, P. C., Frankenfield, C. D., Zwaagstra, J. C., and Watson, P. A. (1992) *J. Biol. Chem.* **267**, 24858–24862
19. Seamon, K., and Daly, J. W. (1981) *J. Biol. Chem.* **256**, 9799–9801
20. Pieroni, J. P., Jacobowitz, O., Chen, J., and Iyengar, R. (1993) *Curr. Opin. Neurobiol.* **3**, 345–351
Basal Activities of Adenylyl Cyclases 2 and 6

21. Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R., and Bourne, H. R. (1993) J. Biol. Chem. 268, 13900–13905
22. Bakalyar, H. A., and Reed, R. R. (1990) Science 250, 1403–1406
23. Katsushika, S., Chen, L., Kawabe, J.-I., Nilakantan, R., Halnon, N. J., Homoy, C. J., and Ishikawa, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8774–8778
24. Rall, T. W. (1982) Handbook of Experimental Pharmacology 58/I, pp. 3-14, Springer Verlag, Berlin
25. Somkuti, S. G., Hildebrandt, J. D., Herberg, J. T., and Iyengar, R. (1982) J. Biol. Chem. 257, 6387–6393
26. Blair, S. S. (1995) Nature 373, 656–657
27. Pan, D., and Rubin, G. M. (1995) Cell 80, 563–572
28. Jiang, J., and Strul, G. (1995) Cell 80, 563–572
29. Fan, C.-H., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M. (1995) Cell 81, 457–465
30. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenar, W. H. (1989) Cell 59, 45–54
31. Collier, H. O. J. (1980) Nature 283, 625–629
32. Nestler, E. J. (1992) J. Neurosci. 12, 2439–2450