Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 86-93

MOLECULAR CLONING AND EXPRESSION OF AN ADENOSINE A2b RECEPTOR FROM HUMAN BRAIN †

Kerrie D. Pierce, Timothy J. Furlong, Lisa A. Selbie and John Shine*

Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney 2010, N.S.W., Australia

Received July 13, 1992

A novel receptor cDNA was isolated from a human hippocampal cDNA library. The encoded polypeptide contains structural features consistent with its classification as a G protein-coupled receptor and shares 45% homology with the human A1 and A2a adenosine receptors. Chinese hamster ovary K1 cells expressing this receptor showed marked stimulation of adenylate cyclase when treated with 1mM adenosine. There was no response to ligands selective for A1 and A2a receptors but the general adenosine agonist N-ethylcarboxyamidoadenosine (NECA) caused a 10 fold increase in cyclic AMP accumulation with an EC_{50} of approximately 0.9μM. This effect was inhibited by the adenosine receptor antagonist theophylline. Specific binding of A1 and A2a selective agonists and NECA was not detected. It is proposed that the novel receptor is a human brain adenosine A2b receptor subtype.

Adenosine has two important roles in mammalian physiology. As well as being an integral component of intracellular energy metabolism adenosine activates specific plasma membrane receptors in an autocrine or paracrine fashion (1). In the central and peripheral nervous systems extracellular adenosine is an important modulator of cell function. For example adenosine alters the activity of ion channels and inhibits the release of neurotransmitters such as serotonin, dopamine and acetylcholine (2). Adenosine also modulates the activity of other ligand-receptor-messenger systems such as histamine receptor mediated inositol phospholipid hydrolysis (3,4). In this way adenosine is an important regulator of neuronal function, particularly in conditions of ATP depletion (5). In addition, adenosine receptors are the primary site of action of alkylxanthine antagonists such as caffeine and theophylline (6).

Adenosine receptors belong to the G protein-coupled class of plasma membrane-associated receptors (7) and have been broadly divided into two main classes, A1 and A2, based on the rank order of affinity of synthetic adenosine analogues and coupling to second messenger systems (8). Pharmacological evidence also points to the existence of additional subclasses. For example, some groups have proposed that A1a and A1b subtypes exist in the central and peripheral nervous systems respectively (9) and a further A3 assignment has been suggested

† Sequence data from this article have been deposited with the GenBank Data library under accession No. M97759.

*To whom correspondence should be addressed.

0006-291X/92 $4.00

(10). The existence of high and low affinity A2 receptors, A2a and A2b respectively, however, is widely accepted (11,12). Both receptors are associated with an elevation of intracellular cAMP concentrations but the A2b subtype is characterized by its low affinity for adenosine analogues and its widespread distribution in the brain (11,12). A similar low affinity receptor, also designated A2b, has been described in fibroblasts but appears to differ in coupling mechanisms from the brain A2b receptor (12,13,14).

The molecular basis of these pharmacological distinctions has recently become clearer with the molecular cloning and characterization of the dog thyroid and rat and bovine brain A1 subtype (15,16,17,18,19) and the A2a subtype from dog thyroid and rat and human brain (15,20,21,22). In addition, other members of the adenosine receptor family have recently been cloned including a low affinity A2 receptor from rat with unique distribution in brain (23) and a novel receptor from rat testis (24). To date there has been no description of the widely distributed brain low affinity A2b receptor. We present here the molecular cloning of a human brain low affinity adenosine receptor which exhibits characteristics consistent with its classification as a brain A2b subtype.

MATERIALS AND METHODS

PCR amplification

DNA was prepared from a human hippocampal cDNA library (Stratagene, La Jolla, CA) by standard methods. Oligonucleotide primers were designed and synthesized after analyzing the second and fourth transmembrane regions (TM2 and TM4) of a number of G protein-coupled receptors. These regions have conserved amino acid sequences and encompass a portion of the polypeptide relatively conserved in length. The oligonucleotide primers

TM2 5'-CAGAACGAATTCAATGTT(C/T)TT(A/G)TG(G/T)GGTCTTTG (G/T)CI(G/T)CI(G/A)C(A/C/T)GA-3'

TM4 5'-GAGTCCGAAGCTTAGTGGGCA(G/A)GAGATGG(T/C)(G/A) AAIG(A/C)IA(G/A)IA(G/A/C)CCA-3'

were 192 and 96 fold degenerate and contained 2 and 3 inosine residues respectively. Amplification of human hippocampal cDNA was performed by PCR on a Hybaid thermal cycler (Teddington, UK) with Tth DNA polymerase (Toyobo Co., Osaka, Japan) and commercially available reagents. An initial denaturation period (95°C for 5 minutes) was followed by 5 cycles of denaturation (95°C for 2 min), annealing (55°C for 1 min) and extension (70°C for 2 min); followed by 5 cycles of denaturation (92°C for 2 min), annealing (50° C for 1 min) and extension (70°C for 2 min); followed by 30 cycles of denaturation (92°C for 2 min) , annealing (42°C for 1 min) and extension (70°C for 2 min). Reaction products were separated using electrophoresis in 3% NuSieve GTG agarose (FMC BioProducts, Rockland, ME). DNA fragments, 300-330bp in size, were isolated, subcloned into M13mp19 and single stranded sequencing performed using standard dideoxy nucleotide chain termination methods.

cDNA library screening, DNA sequencing and subcloning

Approximately 10^6 clones from a human hippocampal Lambda ZAP II cDNA library (Stratagene, La Jolla, CA) were screened with one randomly primed ^{32}P-labelled cloned PCR fragment (Random Priming Kit, BRL, Gaithersburg, MD).

Positively hybridizing plaques were plaque purified and the nested Bluescript plasmids were isolated with helper phage according to the manufacturer's protocol. Inserts were subcloned into M13mp19 and sequenced by standard methods. Sequence analysis and hydropathy plots based on the method of Kyte and Doolittle (25) were performed using Macvector 3.5 Sequence Analysis Programs (International Biotechnologies, Inc., Newhaven, CT). The 1.7kb full length cDNA insert was then subcloned from the excised pBluescript plasmid into the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA) using standard methods.

Transfection and expression

Chinese hamster ovary K1 cells (ATCC: CCL 61) were cultured under 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) supplemented with Hams F12 nutrient mixture (1:1), 10% fetal calf serum and 2mM glutamine and containing 100 IU penicillin and streptomycin. Transfections were performed according to the method of Chen and Okayama (26). Selection with the neomycin analog G-418 (8μg/ml for 1 week then 1.6μg/ml) was commenced 48 hours later and continued for 1 month.

Radioligand binding assays

Cells were washed once with PBS, allowed to detach in PBS containing 0.5mM EDTA, collected by centrifugation at 1000xg for 5 min and washed with PBS. Following a further centrifugation at 1000xg for 5 min cells were collected and gently resuspended in buffer containing 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 10mM $MgCl_2$, 50mM Tris HCl pH 7.4 and 2 IU/ml adenosine deaminase (EC 3.5.4.4; Sigma, St. Louis, MO). Cells were allowed to equilibrate in this solution for 60 min.

Binding assays were performed on intact cells (10^6 per aliquot) with the adenosine A1 receptor specific radioligand ^{3}H-CCPA (2-chloro-N6-cyclopentyladenosine; 42.8Ci/mmol, NEN/Dupont, Sydney, Australia), the adenosine A2a receptor specific radioligand ^{3}H-CGS21680 (2-[4-(2-carboxyethyl)pheneythlamino]-5'-N-ethylcarboxamido adenosine hydrochloride; 48.6Ci/mmol, NEN/Dupont, Sydney, Australia) and the non-specific adenosine receptor radioligand ^{3}H-NECA (5'-N-ethylcarboxamidoadenosine; 15.1Ci/mmol, NEN/Dupont, Sydney, Australia). Unlabelled NECA was obtained from Research Biochemicals Inc. (Natick, MA). The unlabelled compound was dissolved in dimethylsulphoxide on the day of the experiment and diluted in the above Tris buffer so that the final concentration was 100μM and solvent was 1%. Binding was determined in the above buffer at room temperature over a period of 90 minutes. Cells were harvested on Whatman GF-B filters and washed with 10ml ice-cold PBS. Radioactivity on the filters was counted in 5ml BCS liquid scintillant (Amersham, Sydney, Australia).

Determination of cAMP production

Cells were harvested in the usual manner and incubated in modified Krebs Buffer (135mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 5mM $NaHCO_3$, 1mM $CaCl_2$, 2.8mM glucose, 10mM Hepes pH7.4, adenosine deaminase 2IU/ml) at room temperature for 45 minutes. One million cells were then equilibrated with the non-xanthine phosphodiesterase inhibitor RO 20-1724 for 15 min at 37°C before incubation in the above buffer with or without agonist and/or antagonist for 20 min at 37°C. When adenosine (Calbiochem corporation, La Jolla, CA) was used adenosine deaminase was omitted. The agonists CGS21680 and CCPA, the antagonist theophylline (1,3-Dimethylxanthine) and RO 20-1724 were obtained from Research Biochemicals Inc. (Natick, MA). The reaction was terminated by the addition of HCl (final concentration 0.1M). The solution was then incubated at room temperature for 30 minutes and then neutralized and diluted in 50mM sodium acetate pH 6.2. The solution was then centrifuged and the supernatant stored at -20°C. cAMP levels were quantitated using a commercial radioimmunoassay (NEN, Sydney, Australia).

Data for cAMP accumulation were fitted to a sigmoidal curve with a Hill co-efficient of 1 using non-linear regression analysis (GraphPAD InPlot, GraphPAD Software, San Diego, CA).

RESULTS

We exploited the high degree of sequence homology within the putative transmembrane regions of the G protein-coupled receptor family to design degenerate oligonucleotide primers for use in PCR amplification of novel receptor cDNA fragments (15,27,28). One such cDNA fragment showed greatest similarity to the cloned dog adenosine A1 and A2a receptor sequences (15,16,20). This fragment was used to isolate a single full length cDNA clone designated H3.2 from a human hippocampal cDNA library. Sequence analysis of the subcloned 1.7kb insert showed an open reading frame encoding a polypeptide of 328 amino acids in length (Figure 1).

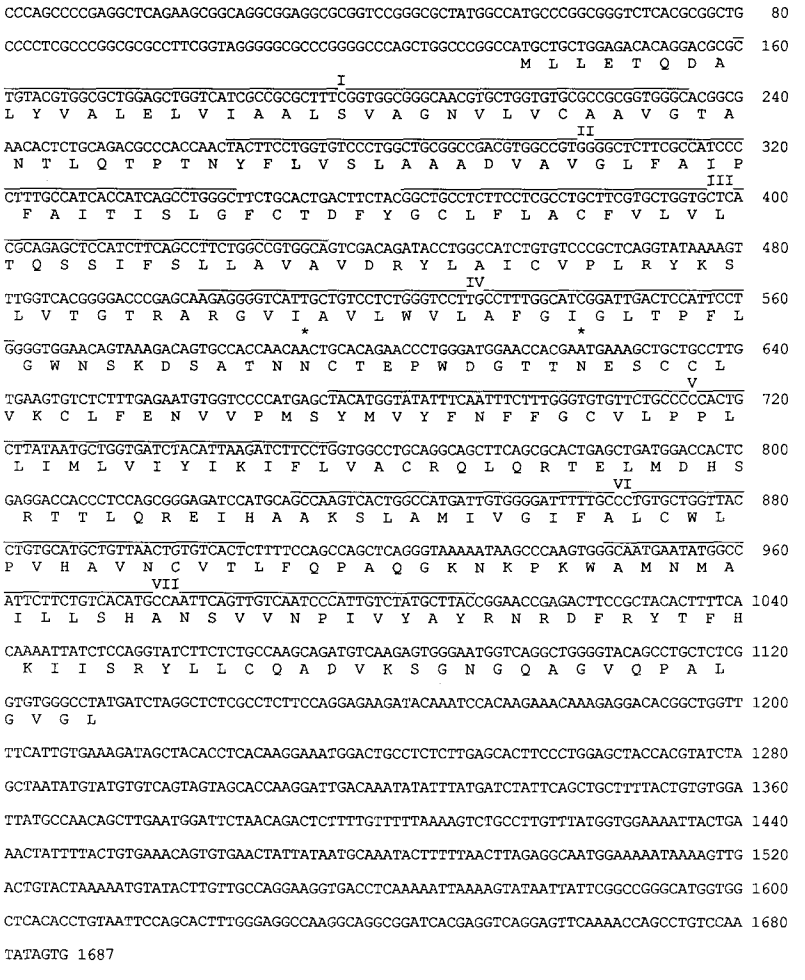

FIG. 1. Nucleotide and predicted amino acid sequence of H3.2. The putative transmembrane domains are overlined. Consensus sites for N-linked glycosylation are indicated with an overlying asterisk.

Hydropathy analysis confirmed that the encoded polypeptide has the seven hydrophobic regions believed to represent membrane spanning domains (indicated as overlined in figure 1) characteristic of the G protein-coupled receptor family (7). The predicted polypeptide shows greatest homology (45%) to the adenosine A1 and A2a receptors. In common with both previously cloned adenosine receptor subtypes, H3.2 contains potential N-glycosylation sites in the second extracellular loop (indicated * in figure 1). H3.2 shares a relatively short third intracellular loop with both previously cloned adenosine receptor subtypes, however it is more like the A1 subtype in possessing a short carboxy-terminal tail. These regions are believed to be involved in intracellular coupling to G proteins (7). In contrast, sequence alignment of H3.2 with the adenosine A2a and A1 receptors (figure 2) shows greater similarity with the A2a subtype (73% vs 59%) within the transmembrane domains where ligand binding is believed to occur (7).

```
                    ____________I____________          ____________II____________        _________III_
H3.2   MLLETQDALYVALELVIAALSVAGNVLVCAAVGTANTLQTPTNYFLVSLAAADVAVGLFAIPFAITISLGFCTDFYGCLFLACFVLVLT
A2a      MPIMGSSV-ITV--A--V-AIL------W--WLNSN--NV----V-------I---VL---------T---AACH----I--------
A1     MPPAISAFQ-A-IGI-VL--LV--P-----IW--KVNQA-RDA-FC-I----V------ALV--L--L-NI-PR-Y-HT--MV--P--I--

       ___________                                _________IV_________
H3.2   QSSIFSLLAVAVDRYLAICVPLRYKSLVTGTRARGVIAVLWVLAFGIGLTPFLGWNSKDSATNNCTEPWDGTTNESCCLVKCLFENVVPM
A2a    ---------I-I---I--RI----NG-------K-I--IC---S-A-----M----NCGQPKEGKNHSQGCGEGQVA--....--D----
A1     ----LA---I------RVKI-----TV--PR--AVA--GC-I-S-VV----LF---RLGE-QRAWAANGS-GEPVIK-E....--K-IS-

        ____________V_______________                                   ____________VI_________
H3.2   SYMVYFNFFGCVLPPLLIMLVIYIKIFLVACRQLQRTELMDHS....RTTLQREIHAAKSLAMIVGIFALCWLPVHAVNCVTLFQPAQGK
A2a    N--------A---V---L--GV-LR---A-R---KQM-SQPLPGERA-S---K-V-------I---L-------L-II--F-F-C-DCSH
A1     E--------VW------L-VL--LEV-YLIR---GKKVSASSG..DPQKYYGK-LKI-----L-LFL---S---L-IL--I---C-SCR-

          __________VII__________
H3.2   NKPKWAMNMAILLSHANSVVNPIVYAYRNRDFRYTFHKIISRYLLCQADVKSGNGQAGVQPALGVGL
A2a    APL.-L-YL--V---T------FI----I-E--Q--R---RSHV-R-QEPFKAA-TSARVL-AHGSDGEQVSLRLNGHPPGVWANGSAPH
A1     PSI..L-YI--F-T-G--AM------F-IQK--V--L--WNDHFR--PTPPVDEDPPEEA-HD

A2a    PERRPNGYALGLVSGGSAQESQGNTGLPDVELLSHELKRVCPEPPGLDDPLAQDGAGVS
```

FIG. 2. Amino acid sequence alignment of H3.2 with human A2a and dog A1* adenosine receptors. Gaps have been introduced to improve alignment and are indicated by dots. Homology with H3.2 is indicated by a dash (-). Putative transmembrane regions are overlined. *Dog and human A1 sequences are 94% homologous (Dr A.Townsend-Nicholson, personal communication).

In order to determine the identity of the putative adenosine receptor subtype, the full length cDNA was stably expressed in Chinese hamster ovary K1 (CHOK1) cells. We observed no significant binding of 4nM [^{3}H]CCPA, an A1 agonist, or 4nM [^{3}H]CGS21680, an A2a selective agonist, to cells expressing H3.2 compared to untransfected or vector transfected cells (data not shown). Significant binding was also not detected for up to 20nM [^{3}H]NECA, a non-specific adenosine receptor agonist. From these results we concluded that H3.2 does not represent a high affinity form of either subtype.

To examine low affinity interactions we assessed H3.2 transfected cells for functional responses to adenosine and adenosine analogues. It has been shown that the hippocampus exhibits negligible response to the A2a selective agonist CGS21680 but shows a strong stimulation of cAMP accumulation when treated with high (10μM) concentrations of NECA, a response attributed to the low affinity subtype A2b (29). When H3.2R transfected cells were treated with 1mM adenosine a 10 fold increase in cAMP levels (4±0.3 to 39±1.2 pmol/10^6 cells) was observed. No significant positive or negative effect on cAMP accumulation was observed in response to the A1 and A2a agonists CCPA and CGS21680 (data not shown) but a >10 fold increase in cAMP accumulation was observed in response to high concentrations of NECA (figure 3). This stimulation was inhibited 82% by the addition of the adenosine receptor antagonist theophylline. The small increase in cAMP accumulation of control cells in the presence of theophylline was presumably related to the antiphosphodiesterase activity of high concentrations of theophylline (6). The dose response curve for NECA suggested an EC_{50} of approximately 0.9μM (figure 4).

DISCUSSION

Adenosine has many actions in the nervous system. The molecular basis for this diversity of action of adenosine remains obscure. It is likely to be due to molecular distinctions

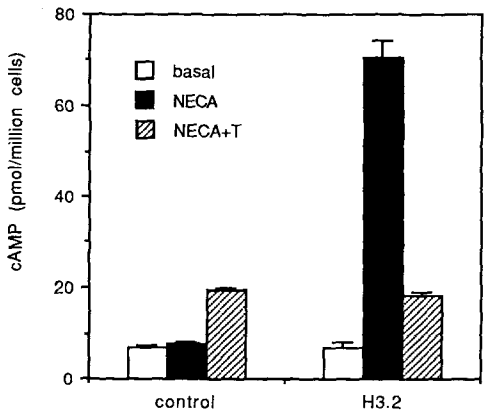

FIG. 3. Effect of NECA on cAMP accumulation in control and H3.2 transfected CHOK1 cells. Cells were treated with 10μM NECA with or without 1mM theophylline (T) and cAMP was measured by radioimmunoassay. Values are the mean of triplicate measurements ± SE.

at the level of the adenosine receptor but may also involve G protein or effector systems within the cell. Two major classes of adenosine receptor have been defined on the basis of pharmacological and biochemical evidence (A1 and A2). The A1 subtype is associated with inhibition of adenylate cyclase activity while the A2 subtype is associated with stimulation of adenylate cyclase activity. The high (A2a) and low (A2b) affinity A2 subtypes differ

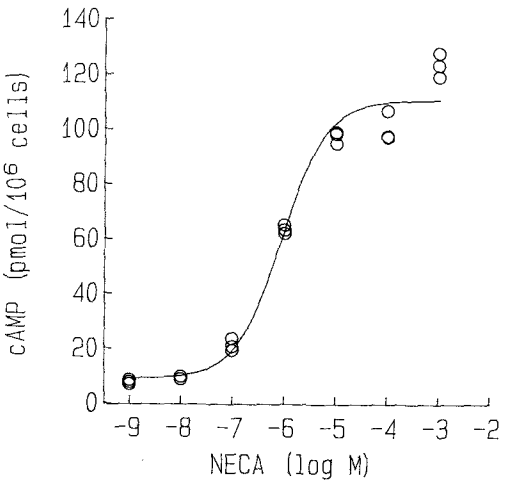

FIG. 4. Dose-response curve for NECA stimulation of cAMP accumulation in H3.2-transfected CHOK1 cells. Cells were treated with 10^{-9} to 10^{-3} M NECA for 20 minutes and cAMP was measured by radioimmunoassay. Data from a single representative experiment is presented. Triplicate measurements are depicted as open circles. Data was analysed as described in Materials and Methods.

approximately 100 fold in their affinity for adenosine (11). These subtypes also differ in their distribution with the A2b subtype found throughout the brain and the A2a subtype limited to the striatum, olfactory tubercle and nucleus accumbens (30). The availability of selective ligands has facilitated the pharmacological characterization of the A1 and A2a receptors and their cloning has permitted a molecular description. However the lack of a selective ligand for the A2b adenosine receptor has impeded its study and the structure and role of the A2b receptor in neuronal function remains obscure.

In this report we describe a new adenosine receptor subtype. It does not bind A1 or A2a receptor specific ligands or the general ligand NECA (at 20nM) but receptor-transfected cells show a marked increase in adenylate cyclase activity in reponse to adenosine and NECA (EC_{50}=0.9μM). The response is antagonized by theophylline indicating that it is a receptor mediated effect. These results suggest that H3.2 encodes a low affinity adenosine A2 receptor, possibly the previously described A2b subtype, although studies of its distribution in brain are required to substantiate this classification.

A closely related adenosine A2 receptor (RFL9) has recently been cloned from rat hypothalamus (23). It is of identical length, shares 86% amino acid sequence homology with H3.2 and has low affinity for NECA. It is not clear whether RFL9 represents the rat homologue of H3.2 or is a distinct receptor subtype. RFL9 shows a distribution inconsistent with any previously described adenosine receptor subtype and in contrast to our results, binds 20nM NECA.

It is also unclear whether the brain and fibroblast A2b receptors are distinct receptors. There appear to be differences in the effector mechanisms of both systems (12,13,14) and preliminary results from our laboratory (data not shown) indicate that H3.2 transcripts are not seen in human embryonic kidney fibroblasts in which a low affinity adenosine receptor is expressed.

The cloning and expression of a human brain A2b adenosine receptor provides the opportunity to resolve these questions and to further examine the role of adenosine receptor heterogeneity in the human nervous system.

ACKNOWLEDGMENTS

We are indebted to Yvonne Hort, Liis Tranberg and Vikki Falls for assistance with tissue culture and Marjorie Liu for sequencing. We would also like to thank Dr Robert Vandenberg and Dr Andrea Townsend-Nicholson for valuable discussion during the course of this project. This work was supported by the National Health and Medical Research Council of Australia and by a Generic Technology Grant from the Department of Industry, Technology and Commerce.

REFERENCES

1. Olsson, R.A., and Pearson, J.D. (1990) Physiol. Rev. 70, 761-845.
2. Fredholm, B.B., and Dunwiddie, T.V. (1988) Trends Pharm. Sci. 9, 130-134.
3. Alexander, S.P.H., Kendall, D.A., and Hill, S.J. (1989) Br. J. Pharmacol. 98, 1241-1248.
4. Kendall, D.A., and Hill, S.J. (1988) J. Neurochem. 50, 497-502.
5. Newby, A.C. (1984) Trends Biochem. 9, 42-44.

6. Sattin, A., and Rall, T.W. (1970) Mol. Pharmacol., 6, 13-23.
7. Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J., and Sealfon, S.C. (1992) DNA and Cell Biology 11, 1-20.
8. Stiles, G.L. (1992) J. Biol. Chem. 267, 6451-6454.
9. Gustafsson, L.E., Wiklund, C.U., Wiklund, N.P., and Stelius, L. (1990) In Purines in Cellular Signaling-Targets for New Drugs (Jacobson, K.A., Daly, J.W., and Manganiello, V., Eds.), pp.200-205. Springer-Verlag, New York.
10. Ribeiro, J.A., and Sebastiao, A. (1986) Progr. Neurobiol. 26, 179-210.
11. Daly, J.W., Butts-Lamb, P., and Padgett, W. (1983) Cell. Mol. Neurobiol., 1, 69-80.
12. Bruns, R.F., Lu, G.H., and Pugsley, T.A. (1986) Mol. Pharmacol. 29, 331-346.
13. Clark, R.B., and Seney, M.N. (1976) J. Biol. Chem. 251, 4239-4246.
14. Yeung, S.M., and Green, R.D. (1983) J. Biol. Chem., 258, 2334-2339.
15. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M-J., Dumont, J.E., and Vassart, G. (1989) Science 244, 569-572.
16. Libert, F., Schiffman, S.N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J., Vanderhaeghen, J-J., and Vassart, G. (1991) EMBO J. 10, 1677-1682.
17. Mahan, L., McVittie, L.D., Smyk-Randall, E.M., Nakata, H., Monsma Jr, F.J., Gerfen, C.R., and Sibley, D.R. (1991) Mol. Pharmacol. 40, 1-7.
18. Tucker, A.L., Linden, J., Robeva, A.S., D'Angelo, D.D., and Lynch, K.R. (1992) FEBS Lett. 297, 107-111.
19. Olah, M.E., Ren, H., Ostrowski, J., Jacobson, K.A., Stiles, G.L. (1992) J. Biol. Chem. 267, 10764-10770.
20. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaeghen, J-J., Dumont, J.E., Vassart, G., and Schiffmann, S. (1990) Biochem. Biophys. Res. Commun. 173, 1169-1178.
21. Fink, J.S., Weaver, D.R., Rivkees, S.A., Peterfreund, P., Pollack, A., Adler, E., and Reppert, S.M. (1992) Mol. Brain Res. (in press).
22. Furlong, T.J., Pierce, K.D., Selbie, L.A., and Shine, J. (1992) Mol. Brain Res. (in press).
23. Stehle, J.H., Rivkees, S.A., Lee, J.J., Weaver, D. R., Deeds, J. D., and Reppert, S.M. (1992) Mol. Endocrinol. 6, 384-393.
24. Meyerhof, W., Muller-Brechlin, R., and Richter, D. (1991) FEBS Lett. 284, 155-160.
25. Kyte, J, and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105-132.
26. Chen, C., and Okayama, H. (1987) Mol. Cell Biol. 7, 2745-2752.
27. Selbie, L.A., Hayes, G., and Shine, J. (1990) In The Biology and Medicine of Signal Transduction (Y. Nishizuka et al., Eds.), pp. 9-14. Raven Press, New York, NY.
28. Selbie, L.A., Townsend-Nicholson, A., Iismaa, T.I., and Shine, J. (1992) Mol. Brain Res. 13, 159-163.
29. Lupica, C.R., Cass, W.A., Zahniser, N.R., and Dunwiddie, T,V. (1990) J. Pharm. Exp. Ther. 252, 1134-1141.
30. Jarvis, M.F., and Williams, M. (1989) Eur. J. Pharmacol. 168, 243-246.