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Somatostatin alters β-adrenergic receptor-effector coupling in cultured rat astrocytes

D.L.Niehoff[1] and A.W.Mudge

MRC Neuroimmunology Programme, Department of Zoology, University College London, Gower Street, London WC1E 6BT, UK

[1]Present address: Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

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The neuropeptide somatostatin potentiates β-adrenergic receptor-mediated cAMP formation in astrocytes derived from neonatal rat cortex but does not affect cAMP levels by itself. β-Adrenergic receptors in these cells can be specifically labeled with the high affinity antagonist [^{125}I]cyanopindolol ([^{125}I]CYP). In addition, astrocytes display both high and low affinity binding sites for the agonist isoproterenol, which are thought to represent receptors which are coupled or uncoupled, respectively, to the guanine nucleotide regulatory protein. We find that somatostatin does not modify β-receptor density, nor receptor affinity for either the antagonist ([^{125}I]CYP) or for the agonist isoproterenol. In the presence of the guanine nucleotide analogue, Gpp(NH)p, only low affinity (uncoupled) displacement of [^{125}I]CYP binding by isoproterenol is observed. However, somatostatin (1 μM), when added to the cells together with Gpp(NH)p, prevents the nucleotide-induced loss of the high affinity (coupled) component of agonist displacement. This result suggests that somatostatin increases noradrenaline-induced cAMP production by enhancing coupling between the β-receptor and the stimulatory guanine nucleotide regulatory protein.

Key words: somatostatin/β-adrenergic receptors/astrocytes/receptor-receptor interactions/receptor-effector coupling

Introduction

Receptors for neurotransmitters and neuropeptides have been demonstrated recently on glial cells or glial cell lines in culture, using functional assays, or direct radioligand binding. For example, cultured astrocytes derived from neonatal rat cortex bind radiolabeled β-adrenergic antagonists with high affinity (McCarthy, 1983; Maderspach and Fajszi, 1983) and respond to noradrenergic agonists by producing cAMP (Narumi *et al.*, 1978; Cummins *et al.*, 1983; Rougon *et al.*, 1983). Rougon *et al.* (1983) have shown that the response to noradrenaline is stimulated by the neuropeptides somatostatin and substance P, and inhibited by met-enkaphalin, all of which have little or no effect on basal cAMP levels on their own. Modulatory effects of neuropeptides on the actions of 'classical' neurotransmitters, in the absence of direct effects of the peptides on their own, have been demonstrated for other types of cells. Thus, substance P and somatostatin inhibit nicotinic reponses in adrenal medullary cells (Mizobe *et al.*, 1979; Role *et al.*, 1981), and VIP potentiates responses at muscarinic cholinergic receptors in the salivary gland (Lundberg, 1981). These observations raise the question of how the binding of neuropeptides modulates the response to a primary ligand. One possibility is that the binding of the peptide to its receptor is able to modify directly the number or affinity of the first receptor. Such direct alteration of receptor number or affinity by the binding of a ligand to a second receptor has been demonstrated previously. For example, GABA enhances benzodiazepine receptor affinity (Karobath and Sperk, 1979; Martin and Candy, 1978; Wastek *et al.*, 1978); several groups have shown that the peptide cholecystokinin (CCK) alters the affinity and/or density of dopamine receptors (Fuke *et al.*, 1981; Bhoola *et al.*, 1982; Murphy and Schuster, 1985), and VIP has been shown to increase the binding of muscarinic agonists (Lundberg *et al.*, 1982).

Another possibility is that the peptide could alter receptor coupling with an effector component, such as the guanine nucleotide regulatory protein, N. Modulation of catecholamine receptor-N coupling by muscarinic agonists has previously been observed in dog (Watanabe *et al.*, 1978) and rat (Yamada *et al.*, 1980) myocardium. In these preparations, muscarinic agonists reverse a guanine nucleotide-induced shift in catecholamine agonist affinity. However, such an effect on receptor-N coupling has not been reported to date in the brain or for any peptide.

We now report that somatostatin does not alter the affinity or density of β-receptors on astrocytes labeled with the antagonist [^{125}I]cyanopindolol ([^{125}I]CYP). In intact cells we have demonstrated biphasic displacement curves for isoproterenol against the lipophilic [^{125}I]CYP, indicating the existence of receptors which are coupled (high affinity) or uncoupled (low affinity) to the stimulatory guanine nucleotide regulatory protein, N_s. Furthermore, the observation that the astrocyte membrane is intrinsically permeant to nucleotides (Trams, 1974) has allowed us to demonstrate inhibition of high affinity isoproterenol binding by the guanine nucleotide analogue guanyl-5′-yl-imidophosphate [Gpp(NH)p], which promotes uncoupling of the receptor from N_s. We find that somatostatin alone does not alter isoproterenol displacement of [^{125}I]CYP; however it does reverse the Gpp(NH)p-induced reduction of high affinity (coupled) component of isoproterenol displacement. This result suggests that somatostatin can stimulate cAMP production by enhancing the coupling between the β-adrenergic receptor and N_s, and represents the first demonstration of a neuropeptide modification of the interaction of a neurotransmitter receptor with its second messenger system.

Results

Binding of [^{125}I]CYP to intact astrocytes – effect of somatostatin

Figure 1 illustrates the saturable binding of [^{125}I]CYP to living astrocytes. Computer-assisted analysis of this data indicated the presence of a single homogeneous class of binding sites with an average K_D of 29.0 ± 4.0 pM and B_{max} of 1.2 ± 0.1 fmol/well (Table I, line 1). Since the astrocyte density increased to ~8 x 10^4 cells/well during the 3–6 days in

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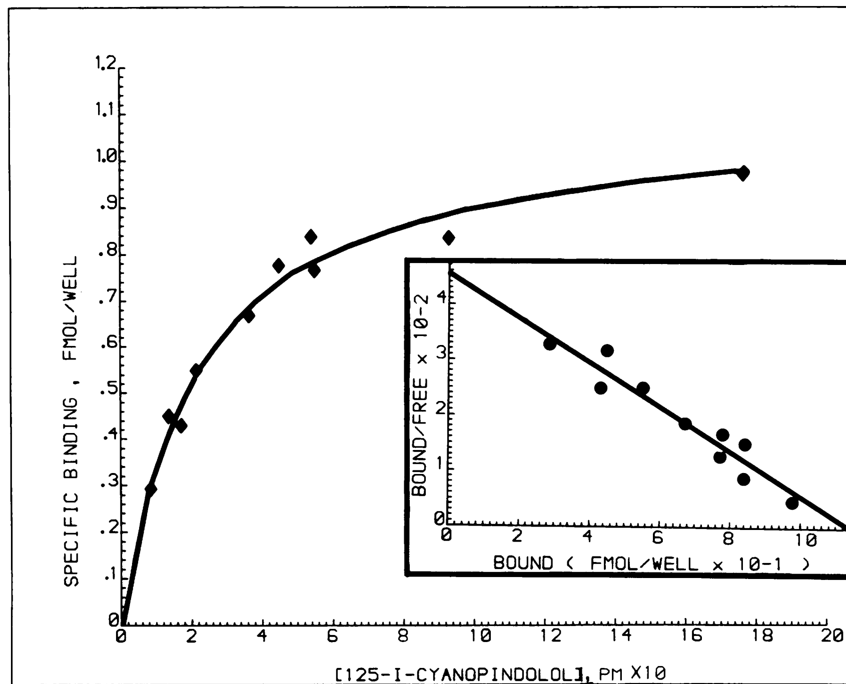

Fig. 1. Saturation of [^{125}I]CYP binding to intact rat cortical astrocytes. Shown is a representative experiment (the inset is a Scatchard analysis of the saturation data) which was repeated six additional times with similar results.

culture prior to assay (data not shown), this B_{max} value represents ~8000 receptors/cell, assuming a homogeneous distribution of receptors. Our affinity constant is similar to the value reported by Petrovic *et al.* (1983) for [^{125}I]CYP binding to homogenates of rat cortex, while we observe approximately four times the number of sites/cell as that reported for [^{3}H]dihydroalprenolol-labeled β-receptors in chick glial cultures (Maderspach and Fajszi, 1983).

As can be seen in Table I (line 2), somatostatin, at a concentration (1 μM) which was maximally effective at enhancing cAMP production (Rougon *et al.*, 1983), did not alter either the affinity or the density of β-receptors. Nor did somatostatin induce heterogeneity in the receptor population, as the data were still better fit to a one-site model.

Somatostatin modification of Gpp(NH)p effects on agonist, but not antagonist, binding

In most cyclase-coupled systems, the addition of guanine nucleotide has no effect on antagonist binding. However, in S49 cells (Contreras *et al.*, 1982), L6 cells (Wolfe and Harden, 1982) and rat lung membranes (Contreras *et al.*, 1982), GTP has been reported to increase antagonist affinity, while in mouse cortex (Hullard and Bloom, 1983) GTP produces a decrease in affinity. We assessed the effects of the non-hydrolysable GTP analogue, Gpp(NH)p, in intact astrocytes, and observed that at a concentration of 500 μM, Gpp(NH)p caused a 3-fold increase in the K_D for [^{125}I]CYP (Table I, line 3). Concomitant addition of 1 μM somatostatin failed to reverse the Gpp(NH)p-induced reduction in antagonist binding (Table I, line 4).

Indirect receptor regulation, affecting coupling between the β-adrenergic receptor and N_s, was investigated by examining the effect of somatostatin on the displacement of [^{125}I]CYP binding by the agonist isoproterenol, in the presence and absence of Gpp(NH)p. In radioligand binding studies, the existence of the receptor-N_s complex is revealed in biphasic

Table I. Binding parameters for [^{125}I]CYP in various drug treatment conditions

Drug added	K_D, (pM)	B_{max} (fmol/well)
None (n = 7)	29.0 ± 4.0	1.2 ± 0.1
1 μM somatostatin (n = 3)	28.3 ± 6.5	1.2 ± 0.4
500 μM Gpp(NH)p (n = 3)	105.2 ± 21.6[b]	1.5 ± 0.4
500 μM Gpp(NH)p + 1 μM somatostatin (n = 3)	132.4 ± 63.0[a]	1.5 ± 0.2

Numbers in parentheses are the number of replicate determinations; the effect of the various additives was assessed in the same experiment utilizing astrocytes from the same preparation. Values for K_D and B_{max} were obtained by computer analysis, using a 1-site model, and are displayed as means ± S.E.M.
[a]$p < 0.05$ *versus* control, Student's t-test.
[b]$p < 0.001$ *versus* control.

agonist displacement curves, where the high affinity population represents coupled receptors. The addition of a high concentration of GTP or Gpp(NH)p promotes the dissociation of the receptor-N_s complex. Consequently, most of the receptors exist in the uncoupled low affinity state, and monophasic agonist displacement curves are observed.

Since the effect of guanine nucleotides has been reported to take place at the inner face of the plasma membrane (Rodbell, 1980; Gilman, 1984), and we are working with intact cells, it might be expected that translocation of Gpp(NH)p would present a problem, as most cell membranes are minimally permeable to guanine nucleotides. However, it has been reported that in astrocytes, guanine nucleotides themselves

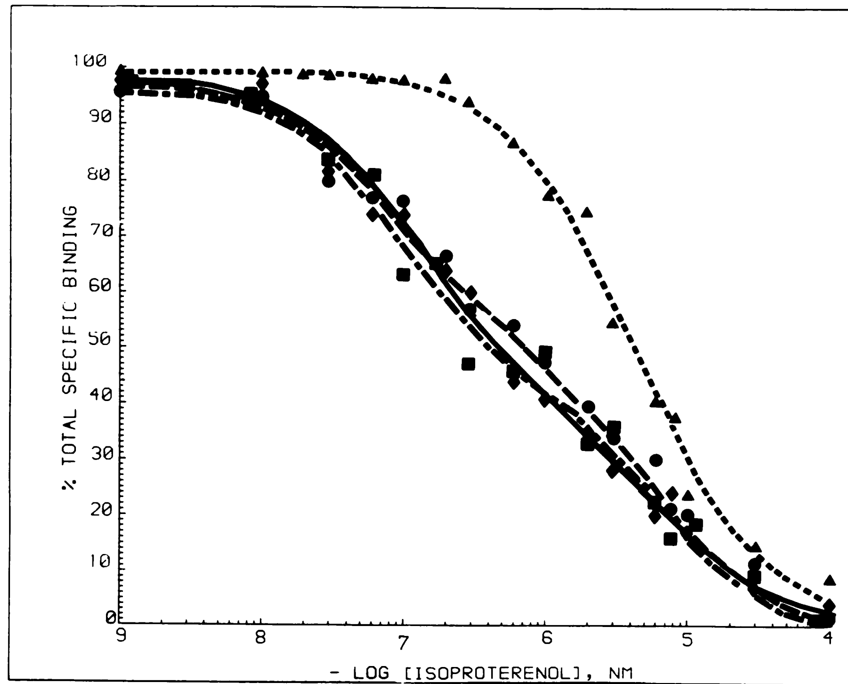

Fig. 2. The reversal of Gpp(NH)p-induced alterations in agonist displacement of [^{125}I]CYP binding by somatostatin. Astrocyte cultures were incubated with 45 pM [^{125}I]CYP in the presence of increasing concentrations of (−) isoproterenol, and the presence or absence of 10^{-6} M somatostatin, 5 x 10^{-4} M Gpp(NH)p, or a combination of peptide and guanine nucleotide. Agonist-displaceable binding is illustrated as the percentage relative to specific binding determined in the absence of agonist by the addition of 5 x 10^{-7} M (±) propranolol, and is the computer-generated best fit of pooled data from four individual experiments with essentially similar results. ◆ — ◆: control; ● — — ●. 1 μM somatostatin; ▲· · · ·▲: 500 μM Gpp(NH)p; ■·—·—■: 1 μM somatostatin + 500 μM Gpp(NH)p.

Table II. Computer-fitted values for isoproterenol displacement

Drug addition	K_{high} nM	K_{low} nM	% $bound_{high}$ fmol/well	% $bound_{low}$ fmol/well
None	53.9 ± 20.3	2785.9 ± 1246.6	59.6 ± 0.08	40.4 ± 0.08
1 μM somatostatin	36.2 ± 13.4	2569.0 ± 667.2	48.0 ± 0.06	52.0 ± 0.07
500 μM Gpp(NH)p	–	2750.5 ± 205.7	–	100.0 ± 0.01
500 μM Gpp(NH)p + 1 μM somatostatin	72.1 ± 35.0	4359.5 ± 2031.3	56.1 ± 0.11	43.9 ± 0.12

Initial estimates for [^{125}I]CYP affinity and maximum binding capacity in the various conditions were obtained from the data in Table I. Values in the table represent the computer-fitted parameter estimates for the pooled data, ± the individual standard error for each parameter. In the control, somatostatin alone, and somatostatin + Gpp(NH)p conditions, the data were statistically better fit to a 2-site model, while in the Gpp(NH)p alone condition, a 1-site model was sufficient.

can induce membrane permeability (Trams, 1974). In addition, Gomperts (1983) has shown that in the absence of external Mg^{2+}, nucleotides induce lesions in mast cell membranes. Others have made cells hyperpermeable to ions (Winegrad, 1971; McClellan and Winegrad, 1978) or bioluminescent proteins (Morgan and Morgan, 1982; Snider *et al.*, 1984) by incubating in the presence of chelators of divalent cations. Thus, we omitted Mg^{2+} from our incubation medium (see Materials and methods) to enhance the ability of Gpp(NH)p to cross the astrocyte membrane. As Mg^{2+} would still be present inside the cells, this omission would not be expected to interfere with processes such as receptor-effector coupling, which are regulated by Mg^{2+} (Bird and Maguire, 1979; Williams and Lefkowitz, 1977).

As shown in Figure 2, the displacement of [^{125}I]CYP binding to intact astrocytes by the agonist isoproterenol was biphasic in the absence of guanine nucleotides. The high affinity component accounted for ~60% of the total receptor population (Table II, line 1). The addition of 500 μM Gpp(NH)p resulted in a monophasic displacement curve which was best fit by a one-site model (Figure 2), and which had an affinity similar to that of the low affinity site in the control condition (Table II, line 3). Somatostatin (1 μM) did not alter the biphasic isoproterenol displacement curve when added alone. The affinities and relative proportions of the two sites did not differ from those observed in the absence of peptide (Figure 2; Table II, line 2). However, when somatostatin was added together with 500 μM Gpp(NH)p, the peptide inhibited the effect of the guanine nucleotide on agonist affinity, resulting in the reappearance of a biphasic displacement function (Figure 2). The proportion of high to low affinity sites was essentially the same as control values, as was the affinity of the high affinity site, while that of the low affinity site was somewhat lower (Table II, line 4).

Discussion

Our data confirm the work of McCarthy (1983) demonstrating the existence of β-adrenergic receptors on highly purified cultures of rat cortical astrocytes. Although the peptide, somatostatin, potentiates the noradrenaline-induced production of cAMP in these cells (Rougon *et al.*, 1983), it failed to increase either the affinity or maximum capacity of β-adrenergic receptor antagonist, [^{125}I]CYP. Thus, the stimulatory effect of somatostatin cannot be due to a direct modification of the catecholamine-binding domain of the receptor or to a change in the number of receptors on the cell surface.

In a variety of membrane preparations, biphasic displacement curves have been described when unlabeled β-adrenergic agonists are utilized to compete against radiolabeled antagonist binding (for review, see Hoffman and Lefkowitz, 1980). Such curves are thought to reflect the multi-step process of catecholamine activation of adenylate cyclse, in which the receptor assumes a high or low affinity state depending on whether or not it is associated with N_s. Prior to catecholamine stimulation, the inhibitory nucleotide GDP is bound to N_s, and the uncoupled receptor exists in a low affinity state (Cassel and Selinger, 1978; Hoffman and Lefkowitz, 1980). The binding of catecholamine (Step 1) promotes the formation of a ternary ligand-receptor-N_s complex, in which the receptor is converted to a state having a high affinity for agonist (Hoffman and Lefkowitz, 1980), and N_s can be activated by receptor-catalysed subunit dissociation (Gilman, 1984) and the exchange of GDP for the activating nucleotide GTP (Step 2) (Cassel and Selinger, 1978). The binding of GTP destabilizes the receptor-N_s complex, and dissociation of the ternary complex causes receptor reversion to a state with low affinity for agonist (Step 2), while N_{GTP} can go on to activate adenyl cyclase (Step 3). Subsequent to cyclase activation, GTP is hydrolysed to GDP by N_s and N_{GDP} dissociates from the cyclase to complete the cycle (Step 3).

(Step 1) $R^{LOW} + C \rightleftharpoons R^{LOW} - C$

(Step 2) $R^{LOW} - C + N_{GDP} \rightleftharpoons R^{HIGH} - C - N_{GDP} \xleftrightarrow{GTP} R^{LOW} + N^*_{GTP} + GDP$

(Step 3) $N^*_{GTP} + AC \rightleftharpoons N^*_{GTP} - AC^* \rightarrow N_{GDP} + AC$

(where R represents the β-adrenergic receptor in either the LOW or HIGH affinity state, C the catecholamine agonist, N the stimulatory guanine nucleotide regulatory protein in either the inactive or * activated state, and AC adenylate cyclase, in either the native or * activated state).

To our knowledge, the present study represents the first demonstration of high-affinity β-adrenergic agonist binding in living, intact cells under equilibrium binding conditions and physiological temperature utilizing a lipophilic radioligand. This result is probably due to our manipulations to increase cellular permeability, which would be expected to increase accessibility of the hydrophilic agonist, as well as to enhance the removal of non-specific binding to intracellular components (Staehlin *et al.*, 1983). This explanation is supported by the observation of McCarthy (1983) of high affinity agonist binding in fixed, permeabilized astrocytes, using the lipophilic antagonist [^{125}I]pindolol.

In other studies using living, intact cells, including a variety of cell lines and cardiac cells, high-affinity agonist binding under physiological conditions using lipophilic radioligands such as [^{125}I]CYP (Terasaki and Brooker, 1978; Insel and Sanda, 1979; Pittman and Molinoff, 1980; Porzig *et al.*, 1982; Toews *et al.*, 1983; Staehelin *et al.*, 1983; Hoyer *et al.*, 1984) has not been demonstrated. Such ligands may label receptors which have been internalized as a result of exposure to agonist (Chuang and Costa, 1979; Harden *et al.*, 1980; Staehelin and Simons, 1982; Staehelin *et al.*, 1983) and non-specific binding sites within cells (Staehelin *et al.*, 1983) as well as cell-surface receptors. Since only cell-surface receptors are accessible to the hydrophilic agonists, such as isoproterenol, a component of lipophilic antagonist binding is not displaceable by agonists which results in an apparent reduction in their affinity. An additional complication is the possibility that these antagonists can induce receptor alteration such that it no longer binds agonists with high affinity (Porzig *et al.*, 1982). Recently, Staehelin *et al.* (1983) have utilized the hydrophilic ligand [^{3}H]CGP12177 to label β-receptors in intact C6 glioma cells, and were able to observe high affinity isoproterenol displacement of this ligand. Others have utilized reduced temperatures (Insel and Sanda, 1979; Toews *et al.*, 1983), pre-equilibrium incubation periods (Toews *et al.*, 1983; Hoyer *et al.*, 1984), or fixation and detergent permeabilization (McCarthy,1983) to demonstrate high-affinity agonist binding. Taken together, these studies indicate that such high-affinity sites, presumably coupled to N_s, do indeed exist in intact cells, and can be observed if appropriate measures are taken to inhibit receptor internalization, increase the access of the hydrophilic agonists and promote the dissociation of non-specifically bound antagonist or if a hydrophilic antagonist such as [^{3}H]CGP-12177 is utilized. Furthermore, our results may indicate important differences between astrocytes and the other cell types studied to date, many of which are cell lines, either in the time course or in the extent of receptor internalization or inactivation. Finally, earlier studies have not included agents in the incubation medium which prevent agonist uptake or degradation, both of which have been shown to occur in cultured astrocytes and glial-derived cell lines and could contribute to rapid agonist inactivation (Sudditu *et al.*, 1978; Pelton *et al.*, 1981; Whitaker *et al.*, 1983), and subsequent apparent reductions in affinity.

At a concentration which was maximally effective in potentiating noradrenaline-mediated cAMP production (1 μM), somatostatin reversed the effect of Gpp(NH)p on high affinity agonist binding. That we were able to demonstrate an effect of Gpp(NH)p on agonist affinity in the absence of somatostatin in intact cells suggests that it did indeed enter the cells, as this effect has been attributed to the action of guanine nucleotides on the internal, but not external face of the plasma membrane (Rodbell, 1980; Gilman, 1984). The protective effect of somatostatin could involve a change in either the receptor or N_s, resulting in an increased rate of formation of the ternary complex. The original observation that somatostatin potentiates the formation of cAMP by noradrenaline (Rougon *et al.*, 1983) could therefore be explained by a peptide-induced increase in the rate of receptor-N_s association which leads to an increase in N^*_{GTP} capable of activating adenylate cyclase. We are currently investigating this mechanism in more detail. Our data not not exclude other mechanisms such as an alteration in the relative affinity of N_s for particular guanine nucleotides, thereby enhancing the exchange of GDP for GTP. However, our results emphasize the importance of indirect receptor regulation as an alternative way in which neuropeptides in particular can influence the action of 'classical' neurotransmitters.

Materials and methods

Culture of rat cortical astrocytes

Purified cultures of rat cortical astrocytes were prepared by a modification of the method of McCarthy and deVellis (1980), which has been described previously (Rougon *et al.*, 1983). Cells from such cultures were replated into 16 mm polylysine-coated multiwell plates (Costar) at a density of 2 x 10^4 cells/well, and grown for an additional 3 – 6 days. During this period, they were fed every 2 – 3 days. Cells from the same preparation were grown on polylysine-coated coverslips rather than multiwell plates and screened for purity by indirect immunofluorescence using a rabbit antiserum to glial fibrillary acidic protein (GFAP), a specific marker for astrocytes (Raff *et al.*, 1979). Only cultures which contained 95% $GFAP^+$ cells and which were devoid of neurones and macrophages were utilized in receptor binding assays.

[^{125}I]CYP binding assay

β-adrenergic receptor binding was assessed in intact astrocytes using the highly specific β-antagonist [^{125}I]CYP (>2000 Ci/mmol, Amersham, Engel *et al.*, 1981). Medium was removed from astrocyte cultures (3 – 6 days post-plating in polylysine coated multiwell plates) and the wells were rinsed with a modified salt buffer (131 mM NaCl, 3 mM KCl, 3.7 mM $CaCl_2$, and 25 mM Hepes adjusted to pH 7.4 with 12.5 mM NaOH). Mg^{2+} was omitted to enhance membrane permeability to nucleotides. The buffer also contained 10 μM catechol, 10 μM desipramine, 10 μM pargyline, and 1 mM ascorbate to inhibit uptake and degradation. Cells were subsequently incubated for 1 h at 37°C in 500 μl of this buffer containing [^{125}I]CYP and various drugs. In experiments examining the effects of somatostatin, the peptide was diluted in Mg^{2+}-free incubation buffer containing, in addition to the protective agents described above, 0.2% bovine serum albumin and 10 μM phenyl methylsulfonylfluoride, neither of which affected basal binding parameters. Analysis of somatostatin by h.p.l.c. and radioimmunoassay subsequent to incubation at 37°C for 1 h in the presence or absence of cells indicated that the material recovered in both cases did not differ from native somatostatin. Hence, no significant degradation of the peptide occurred during the incubation period. The order of addition of components to the wells was: Gpp(NH)p, somatostatin, isoproterenol and [^{125}I]CYP. Blanks were co-incubated with 5 x 10^{-7} M ($\pm$)-propranolol and routinely constituted 35 – 40% of the total binding. Cells remained viable throughout the assay procedure, and continued to adhere strongly to the substrate. Astrocytes which were subjected to the incubation protocol (without [^{125}I]CYP), then washed and fed, were >90% viable when assessed by trypan blue exclusion 24 h later (data not shown). At the end of the incubation period the plate was rapidly immersed sequentially in four washes of ice-cold 0.17 M NaCl; protein was subsequently solubilized by the addition to each well of 500 μl of 0.1 N NaOH and shaking for 1 h. Aliquots from each well were counted at 90% efficiency in a Nuclear Enterprises gamma counter. Total binding at all concentrations comprised 2 – 4% of the added radioactivity.

Computer analysis of binding data

Computer analysis of the data was performed using a non-linear least-square regression program written by W.Piotrowski at University College. One-site and two-site models were statistically compared for goodness-of-fit using an F ratio test based on the 'extra sum of squares' principle (Munson and Rodbard, 1980). The more complex model was accepted only if the calculated F value was significant at the 1% probability level. Because our curve analysis program can only fit a single curve at a time, and has no provision for the determination of correction factors to simultaneously analyze multiple experiments, in experiments examining the effects of Gpp(NH)p and somatostatin on the displacement of the agonist, isoproterenol, data from four individual experiments were pooled prior to analysis. Individual values at a given isoproterenol concentration within each conditions were expressed as fmol bound/well and, on the whole, varied by <15% between the four experiments. Analyses of data from individual experiments always yielded the same conclusion, i.e., data in the control, somatostatin, and somatostatin + Gpp(NH)p conditions fit a 2-site model, while in the presence of Gpp(NH)p alone a single, low-affinity site was observed.

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