ELSEVIER

European Journal of Pharmacology 318 (1996) 497–504

ejp

Evidence for receptor subtype cross-talk in the mitogenic action of serotonin on human small-cell lung carcinoma cells

Lucia M. Vicentini [a], Maria G. Cattaneo [a], Riccardo Fesce [a,b,*]

[a] *Department of Medical Pharmacology, Via Vanvitelli, 32-20129 Milano, Italy*

[b] *CNR Center Cellular / Molecular Pharmacology and DIBIT, San Raffaele Scientific Institute, Via Olgettina 58, I-20132 Milano, Italy*

Received 23 July 1996; accepted 8 October 1996

Abstract

We previously reported a significant mitogenic effect of serotonin (5-hydroxytryptamine, 5-HT) on human small-cell lung carcinoma cells (SCLC, GLC-8), mediated by both 5-HT_{1D} and 5-HT_{1A} receptors. Here we investigate possible interactions between the two receptor subtypes. Dose-effect curves obtained by simultaneously applying equipotent concentrations of the selective 5-HT_{1A} agonist 8-OH-DPAT and the selective 5-HT_{1D} receptor agonist sumatriptan are shifted to the right, although maximal effects are additive. The nonselective 5-HT antagonist metergoline displays higher potency when both receptor subtypes are activated. The 5-HT_{1D} receptor antagonist GR127935 is markedly more potent against sumatriptan than against the sensitive portion of 5-HT effect. Indeed, both GR127935 and the 5-HT_{1A} antagonist spiperone shift the EC_{50} for the residual effect of 5-HT from $\approx$ 300 to 120–150 nM, suggesting that blocking one receptor subtype may facilitate activation of the other. Preincubation with either 8-OH-DPAT or sumatriptan suppresses the mitogenic response to the other specific receptor agonist; suppression is complete within 10 min at 37°C, and is not observed when the preincubation is done at 4°C. Measurements of adenylate cyclase activity do not help in interpreting the results. Conversely, measurements of MAP kinase activity reveals biphasic activation with a delayed activation at 1 h, and reproduce the suppression of the effect of the second drug by 15 min preincubation. These findings constitute the first evidence of a reciprocal negative interference between human 5-HT_{1A} and 5-HT_{1D} receptors, and indicate that SCLC GLC-8 cells simultaneously express both receptor subtypes. Mere reciprocal antagonism of the drugs employed cannot account for these data. We suggest that in this cell system cross-talk occurs in the transduction pathways of the two receptor subtypes.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); Small-cell lung carcinoma; Receptor cross-talk; 5-HT receptor subtype

1. Introduction

Small-cell lung carcinoma (SCLC) is a particularly aggressive and metastatic tumour often associated with tobacco smoking. SCLC cells produce and release peptides like bombesin and insulin-like growth factor 1 which may establish an autocrine mitogenic loop (Cuttitta et al., 1985; Nakanishi et al., 1988; Moody and Cuttitta, 1993). It was recently shown that serotonin (5-hydroxytryptamine, 5-HT) is also contained and released by SCLC cell lines NCI-N-592 and GLC8 (Cattaneo et al., 1993), and stimulates mitosis via activation of both 5-HT_{1A} and 5-HT_{1D} receptor subtypes (Cattaneo et al., 1995). Such activation appears to be additive and to account for 100% of the mitogenic effect of serotonin. However spiperone and SDZ 216-525 which were shown to behave as pure and specific 5-HT_{1A} receptor antagonists in this system were 10 times more potent against the selective 5-HT_{1A} receptor agonist 8-OH-DPAT than on the sensitive portion of serotonin effect (Cattaneo et al., 1994, 1995). This suggested that some kind of interaction might occur between the two receptor subtypes.

We therefore decided to investigate more thoroughly such possible interaction. A series of further experiments are presented and discussed here in the search for a possible pharmacodynamic model of the receptor subtype interaction.

* Corresponding author at address b. Tel.: (39-2) 2643-4809; Fax: (39-2) 2643-4813; e-mail: fesced@dibit.hsr.it

PII S0014-2999(96)00812-6

2. Materials and methods

2.1. Cell culture

GLC8 cells (kindly provided by E. Sher, CNR, Center of Cytopharmacology, University of Milan, Milan, Italy) were routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated foetal bovine serum (GIBCO, Grand Island, NY, USA). The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. [^{3}H]thymidine incorporation assay

GLC8 cells were plated in RPMI-1640 medium without serum in 96-well microtiter plates at a density of $5-10 \times 10^3$ cells/well, treated with the various substances and incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2. [methyl-^{3}H]Thymidine (1 μCi/well; specific activity 2 Ci/mmol, Amersham, UK) was added during the last 6 h of incubation. The cells were then washed, lysed with distilled water and collected on filters with an automatic cell harvester (Titerteck, Flow Laboratories, Rockville, MD, USA). The filters were placed in Filter Counter Scintillation fluid (Packard, Downers Grove, IL, USA) and counted using standard procedures.

2.3. Receptor binding

GLC8 cells were washed once with RPMI, homogenized in ice cold 50 mM Tris HCl, 5 mM EDTA, pH 7.4 using a blade homogenizer (Ultraturrax) and centrifuged at $350 \times g$ at 4°C for 10 min. After 15 min at 37°C to remove endogenous 5-HT, the homogenate was centrifuged at $40\,000 \times g$ at 4°C for 30 min. The pellet was resuspended in assay buffer (50 mM Tris HCl pH 7.4 containing ascorbic acid 0.1%, pargyline 10 μM and $CaCl_2$ 4 mM) to give a final protein content of 200 μg/sample. Incubation was carried out at 25°C for 35 min in a final volume of 0.5 ml in the presence of various concentrations of [^{3}H]5-HT (from 0.5 to 100 nM, 25.4 Ci/mmol, NEN-Dupont, Wilmington, DE, USA). Specific binding was defined by using 10 μM 5-HT. After the incubation, the samples were rapidly filtered over Whatman GF/B filters (presoaked in 50 mM Tris HCl, pH 7.8, containing 1% bovine serum albumin) and rinsed three times with 5 ml ice-cold 50 mM Tris HCl, pH 7.8. The filters were placed in Filter Count scintillation fluid (Packard) and counted using standard procedures.

2.4. Determination of mytogen-activated protein kinase (MAP kinase) activity

After 48-h serum deprivation, GLC-8 cells ($1.5-2.0 \times 10^6$) kept in RPMI were treated with the various stimuli for the times indicated in the figure. After the incubation, the medium was removed and the cells were washed and resuspended in 0.5 ml of homogenization buffer, according to Seger et al. (1994). After sonication (2×7 s) and centrifugation at $15\,000 \times g$ for 10 min at 4°C, the supernatants were fractionated on DEAE-cellulose minicolumns. MAP kinase activity was determined by phosphate incorporation into myelin basic protein (MBP, Sigma, St. Louis, MO, USA) in the presence of [γ^{32}P]ATP (2 μCi/sample; specific activity 3000 Ci/mmol; Amersham, UK), according to Seger et al. (1994).

2.5. Drugs

The drugs used were the following: 5-hydroxytryptamine creatine-sulphate (Sigma Chemicals, St. Louis, MO, USA); *R*(+)-8-hydroxy-dipropylaminotetralin (8-OH-DPAT) and spiperone (RBI, Natick, MA, USA); sumatriptan for medical use (Permicran, Ellem, Milano, Italy); metergoline (Farmitalia Carlo Erba, Milano, Italy); 2′-methyl-4-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-metoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide (GR127935, kind gift from Glaxo, Stevenage, UK); methyl 4-{4-[4-(1,1,3-trioxo-2*H*-1,2-benzoisothiazol-2-yl)-butyl]-1-piperazinyl}-1*H*-indole-2-carboxilate (SDZ 216-525, Sandoz Italia, Milan, Italy).

2.6. Fitting the dose-response curves

In order to compare dose-effects curves obtained under the different conditions it was convenient to employ a standard analytical representation, thus Hill functions of the form $E = [D]^n/([D]^n + EC_{50}^n) \cdot E_M + B$ were fit to the data, where E is the effect ([^{3}H]thymidine incorporation measured as cpm), $[D]$ is drug concentration, EC_{50} is the drug concentration producing 50% of the maximum effect, n (an integer number) is the estimated cooperativity order, E_M is the estimated maximum effect (cpm) and B is the estimated background cpm value. Fits were obtained by standard Gaussian approaches of minimisation of the sum of square errors ('least square errors'). Best fitting values of n from 12 combined dose-effect curves (each point in quadruplicate) of each agonist in isolation were rounded to the nearest integer and kept fixed in fitting all responses to the same agonist both in isolation and in the presence of other drugs (i.e., the free parameters were EC_{50}, E_M and B). Two parameters fully described the potency (EC_{50}) and efficacy (E_M) of the drug under each specific condition.

Possible interactions among different drugs were examined based on the null hypothesis of simple antagonism/partial agonism on the receptors (either one-way or reciprocal) between agonists for different receptor subtypes. The predictions of such null hypothesis are easily computed from standard equations; in particular in this case the presence of a partial agonist or antagonist is

expected to shift the apparent receptor affinity and therefore the EC_{50}.

In all figures, continuous lines are direct fits to the data, obtained from standard Hill functions as explained above. Dotted lines represent instead the mathematical predictions for the null hypothesis of receptor antagonism/partial agonism, based on the curves obtained with each drug in isolation. The statistical significance of the departure of experimental data from the predictions of the null hypothesis has been evaluated from the cumulative distribution of the Fisher F statistic, where F is the variance ratio: [mean square departure from null hypothesis predictions] to [mean square standard error of the experimental points], and the numbers of degrees of freedom, $n_1 = n_2 = n$, equal the number of experimental points.

The qualitative predictions of more complex models – interactions of downstream processes on receptor availability/function, like down-regulation and/or cross-talk – may be quite different, and may account for features which cannot be explained by simple competition for binding sites. In particular, it is conceivable that (i) the relation be not reciprocal, i.e., a drug applied first might affect the response to the second drug more than vice versa, (ii) the E_M values might be affected as well as, or rather than, the apparent EC_{50} values, and (iii) the magnitude of the interactions might change with time.

3. Results

3.1. Binding studies

Serotonin specific binding was not measurable in GLC-8 cells, presumably due to the scarce number of receptors expressed and/or to cell heterogeneity. Therefore the possible interactions between $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptors could only be investigated by functional studies.

3.2. Imperfect additivity of $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptor activation

The application of either the $5\text{-}HT_{1A}$ receptor agonist 8-OH-DPAT or the $5\text{-}HT_{1D}$ receptor agonist sumatriptan to SCLC-GLC8 cells produces a concentration-dependent increase in [^{3}H]thymidine incorporation. The top and middle panel in Fig. 1 illustrate the dose-effect curves obtained with either receptor agonist in isolation. The data are fit by Hill functions (quite arbitrarily, in fact) in order to obtain analytical representations of the data (see Materials and methods). It can be seen that the maximum effect of sumatriptan is about 50% higher than the maximum effect of 8-OH-DPAT. The sum of the maximum effects of the two drugs was previously reported to amount to the full effect of serotonin, suggesting additivity (Cattaneo et al., 1995).

The additivity of the two receptor pathways was tested

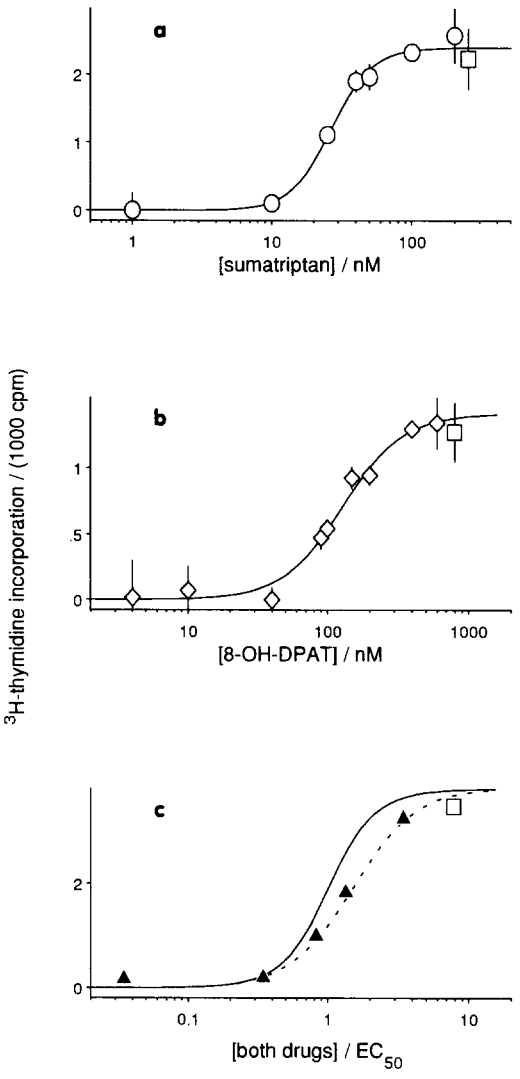

Fig. 1. The effect of the $5\text{-}HT_{1D}$ receptor agonist sumatriptan (○), the $5\text{-}HT_{1A}$ receptor agonist 8-OH-DPAT (◇) and of equipotent concentrations of both drugs applied in association (▲) on [^{3}H]thymidine incorporation by GLC-8 SCLC cells. (a) Pooled results from 12 control experiments (each point in quadruplicate), fit by a Hill equation with $n = 3$ and $EC_{50} = 26.5$ nM; (b) pooled results from 12 experiments, fit by a Hill equation with $n = 2$ and $EC_{50} = 128$ nM; (c) each filled triangle is the average of 3 experiments in quadruplicate; the continous line is the sum of the data in panels a and b; the dashed line is the predicted effect of the association of the two drugs in the presence of cross-antagonism or partial agonism (see Methods). In all panels, the open square is the result of a further experiment (in quadruplicate), aimed at checking saturation and additivity of the combined application of maximal doses of the two drugs (250 nM sumatriptan + 800 nM 8-OH-DPAT); counts for this experiment at lower concentrations of the agonists (not shown) fell within a standard deviation from the mean result of the other experiments.

by simultaneously applying equipotent concentrations of the two agonists. The results are shown in the bottom panel of Fig. 1, superimposed onto the sum of the two curves obtained with each agonist in isolation (solid line). A right-shift of the EC_{50} by about 50% is apparent (data significantly depart from the solid line: variance ratio $F = 22.95$, $n = 5$, $P = 0.0019$). This kind of shift is well

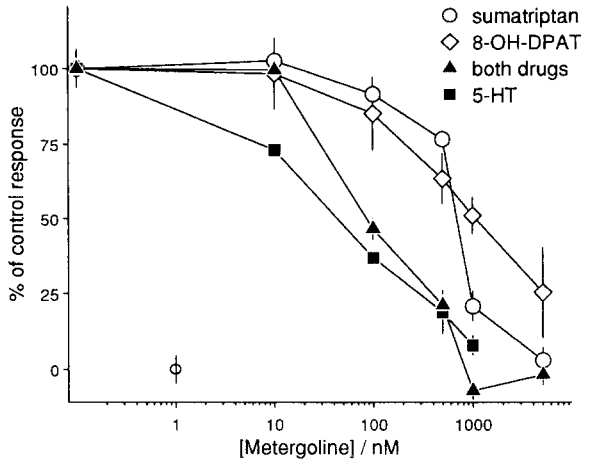

Fig. 2. Antagonistic action of metergoline on [^{3}H]thymidine incorporation increase produced by the 5-HT$_{1D}$ receptor agonist sumatriptan (○, 100 nM), the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (◇, 400 nM), both drugs in association (▲) or 5-HT (■, 1 μM). Note that metergoline, which was administered 5 min before the agonists, is several-fold more potent against treatments simultaneously activating both receptor subtypes. Each point is the average of 3 experiments in quadruplicate.

reproduced by the dotted curve, which was drawn using only the data of either receptor agonist in isolation and assuming reciprocal antagonism of the two drugs (see Materials and methods), either at the receptor level or further on in the transduction pathway.

3.3. The action of antagonists

As illustrated in Fig. 2, the nonselective 5-HT receptor antagonist metergoline inhibits the action of both 8-OH-DPAT and sumatriptan applied together with a 50 nM IC$_{50}$ (the same as against 5-HT; Cattaneo et al., 1994), but is much less potent against either receptor agonist in isolation (IC$_{50}$ = 0.5–1 μM). We previously reported that the selective 5-HT$_{1A}$ receptor antagonists spiperone and SDZ216-525 (Lum and Piercey, 1988; Boddeke et al., 1992; Schoeffter et al., 1993) are much less potent against the sensitive portion of 5-HT effect than against 8-OH-DPAT (IC$_{50}$ 400 nM vs. 30 nM; Cattaneo et al., 1995). Similarly, Fig. 3 shows that the selective 5-HT$_{1D}$ receptor antagonist GR127935 is much less potent against the sensitive portion of 5-HT effect than against sumatriptan. None of the receptor antagonists affected the basal rate of [^{3}H]thymidine incorporation.

The above described features of the effects of receptor antagonists are consistent with the idea that the actions of the two receptor subtypes are not simply and fully additive, and a form of negative interaction is instead present. If the action of 5-HT is affected by such negative interaction, as data in Fig. 1 suggest for the association of 5-HT$_{1A}$

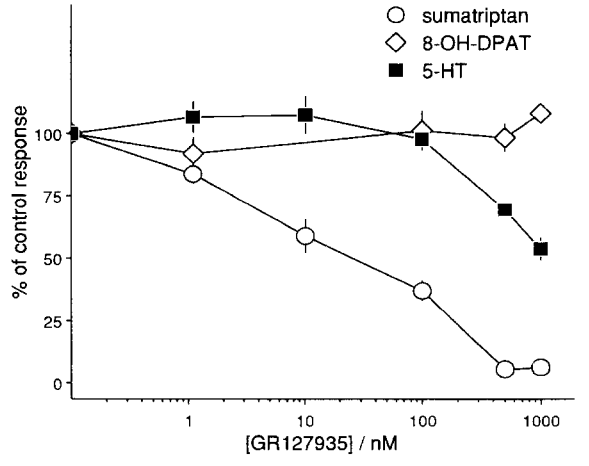

Fig. 3. Effect of the 5-HT$_{1D}$ receptor antagonist GR127935 on [^{3}H]thymidine incorporation increase produced by sumatriptan (○, 100 nM), 8-OH-DPAT (◇, 400 nM) or 5-HT (■, 1 μM). Notice that the effect of 8-OH-DPAT is unchanged, thus confirming the specificity of GR127935, and that the drug is much more potent against sumatriptan than against the sensitive portion of 5-HT effect. Each point is the average of 2 experiments in quadruplicate.

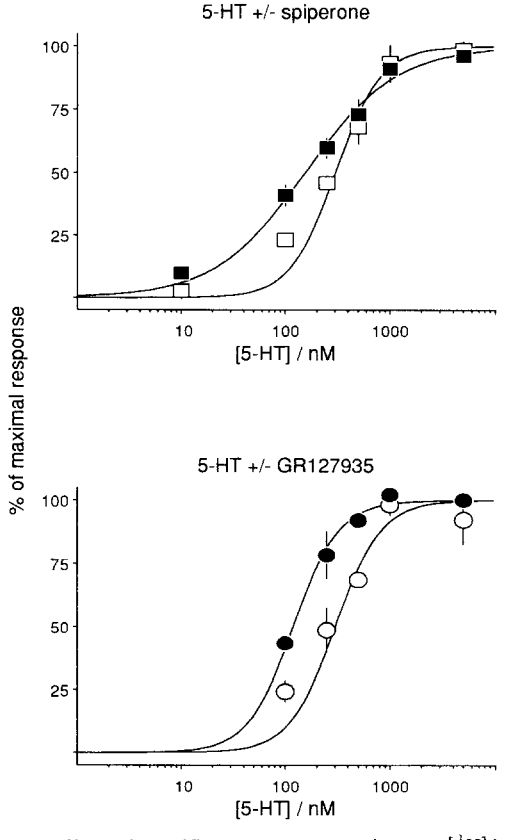

Fig. 4. The effect of specific receptor antagonism on [^{3}H]thymidine incorporation dose-effect curves in response to 5-HT. The curves are normalised to the maximal responses. Notice the shift to the left in the presence of spiperone (500 nM, ■ vs. □) and GR127935 (500 nM, ● vs. ○). Data are fit by Hill equations, with the following parameters: control (□ and ○), EC$_{50}$ = 301 nM, order n = 2, E_M = 5334 cpm or 4449 cpm; in the presence of spiperone (■), EC$_{50}$ = 154 nM, n = 1, E_M = 4042 cpm; in the presence of GR127935 (●), EC$_{50}$ = 119 nM, n = 2, E_M = 3449 c.p.m. Each point is the average of 2 experiments in quadruplicate.

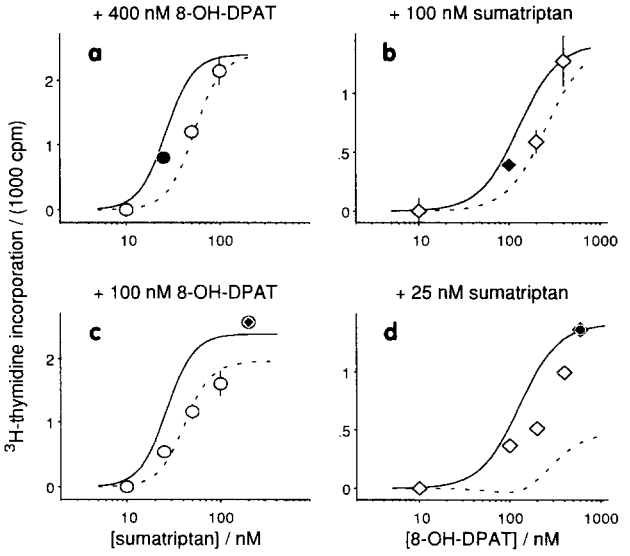

Fig. 5. Dose-effect curves of the 5-HT_{1D} receptor agonist sumatriptan (○) on [^{3}H]thymidine incorporation in the presence of maximal concentrations (a) or EC_{50} (c), of the 5-HT_{1A} receptor agonist 8-OH-DPAT, and dose effect curves of 8-OH-DPAT (◇) on [^{3}H]thymidine incorporation in the presence of maximal concentrations (b) or EC_{50} (d), of sumatriptan. Continuous lines are fits to control dose-effect curves of Fig. 1, and illustrate that all dose-effect curves are shifted towards the right by pre-exposure to a different agonist. Dashed curves illustrate the predictions of a cross-antagonism/partial agonism model (see Methods). Whereas such predictions well describe the results obtained with one drug in the presence of maximal concentrations of the other (a and b), they cannot describe the results obtained after pre-exposure to EC_{50} concentrations of a different agonist (c and d). The filled symbols indicate two pairs of points in a and d and b and c which were obtained with the same concentrations of the two agonists, although applied in reverse order; they illustrate that whereas the action of intermediate concentrations of the second drug are depressed (producing the right-shifts in a and b), the action of half-maximal concentrations of the drug applied first are not depressed by the second drug (last points to the right in c and d). All experiments performed in quadruplicate.

and 5-HT_{1D} receptor agonists, then the blockade of one receptor subtype should induce a shift to the left of the EC_{50} for the residual 5-HT effect. Fig. 4 shows that this is indeed the case: although both the 5-HT_{1A} receptor antagonist spiperone and the 5-HT_{1D} receptor antagonist GR127935 reduced the maximal response to 5-HT (by 24.2 and 22.5%, respectively), both drugs proved able to markedly shift the EC_{50} of the remaining effect of 5-HT to the left, as if blockade of one receptor subtype would relief some sort of inhibition of the other receptor pathway. The lines in Fig. 4 are Hill functions fit to the data in the presence of spiperone (■, 500 nM) or GR127935 (●, 500 nM), or in the absence of antagonists (empty symbols). The data in Fig. 4 are normalized to the respective maximal responses: data obtained in the presence of antagonists (the dose response for the residual effect) significantly depart from the dose responses in the absence of antagonists ($F = 12.9$, $n = 6$, $P = 0.0033$ for spiperone; $F = 30.29$, $n = 5$, $P = 0.00096$ for GR127935).

3.4. Interactions between selective agonists

Fig. 5 illustrates the changes produced in the dose effect curves for either selective receptor agonist by the previous (1–2 min) application of maximal doses (top panels) or EC_{50} (bottom panels) of the other agonist.

In all cases a right-shift in EC_{50} is observed, with no apparent action on E_M. The shift is statistically significant for data in panels A ($F = 13.77$, $n = 4$, $P = 0.013$), C ($F = 28.12$, $n = 5$, $P = 0.0011$) and D ($F = 30.80$, $n = 5$, $P = 0.00092$), but not for panel B ($F = 3.018$, $n = 4$, $P = 0.155$). The expectations for cross antagonism are also displayed (dotted curves) and illustrate that this simple interpretation of the data is not tenable. In fact, if the inhibition were reciprocal, in the presence of drug *A* at half-maximal concentration, the curve for drug *B* should display a shift to the right as well as a reduced apparent E_M, because increasing concentrations of drug *B* should depress the effect of lower-than-maximal concentrations of drug *A*. The departure from mere antagonism is quite clear if one compares the two pairs of results obtained with the half-maximal concentration of one drug and the maximal concentration of the other (filled circles) applied in reverse order, which fall short of additivity in the top panels but not in the bottom panels.

In these experiments, a short time interval (1–2 min)

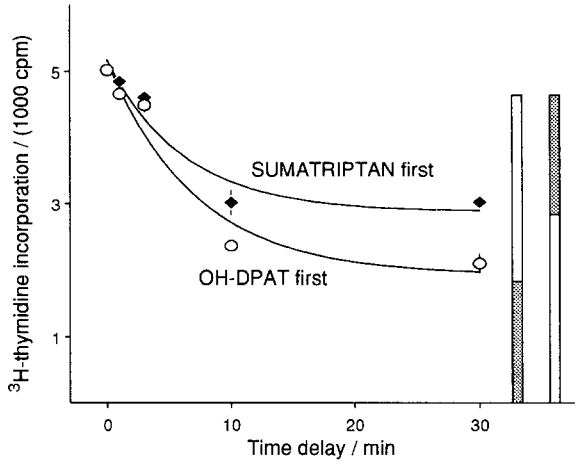

Fig. 6. Time-course of 5-HT receptor-subtype cross-inhibition at 37°C. [^{3}H]Thymidine incorporation by GLC-8 SCLC cells exposed to 100 nM sumatriptan is displayed (○) as a function of the time elapsed since exposure to 400 nM 8-OH-DPAT. The bars on the right illustrate [^{3}H]thymidine incorporation produced by sumatriptan alone (empty portion) and by 8-OH-DPAT alone (filled portion) and show that sumatriptan effect is fully additive at time 0 but vanishes as the interval between the treatments approaches 30 min. Also displayed is 8-OH-DPAT effect (◆, 400 nM) as a function of the time elapsed since exposure to 100 nM sumatriptan. Here, too, initial additivity and slow suppression are observed. The lines represent first-order decays best-fitting the data, with a 7.1 min time constant for 8-OH-DPAT first, and 6.2 min time constant for sumatriptan first. Each point is the average of 2 experiments in quadruplicate.

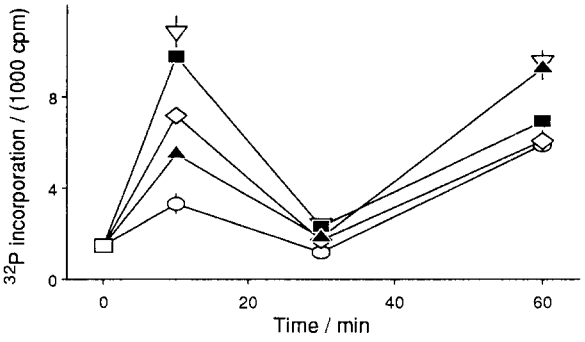

Fig. 7. Time-course of the activation of MAP kinase. [^{32}P]Phosphate incorporation into myelin basic protein induced by cell extracts from GLC-8 SCLC cells exposed to the 5-HT_{1D} receptor agonist sumatriptan (○, 100 nM), the 5-HT_{1A} receptor agonist 8-OH-DPAT (◇, 400 nM), both drugs in association (▲), 5-HT (■, 1 μM) or foetal bovine serum (▽). Notice that a first, variable-height peak at 8 min is followed by a silent phase and a further, late activation of MAP kinase at 1 h. All measurements performed in triplicate. Consistent results were obtained in a similar experiment at 5, 10 and 15 min.

elapsed between the applications of the two drugs, because the first one was added at the same concentration to all wells and the second drug was applied thereafter, at different concentrations. It was therefore possible that the first treatment was more efficient at inhibiting the binding/action of the second drug than vice versa.

3.5. Time and temperature dependence of the interaction

A time-course experiment was therefore performed, by applying maximal concentrations of the 5-HT_{1A} receptor agonist 8-OH-DPAT and the 5-HT_{1D} receptor agonist sumatriptan at different time intervals. Fig. 6 shows the results of this series of experiments.

The response to maximal concentrations of 8-OH-DPAT applied after maximal concentrations of sumatriptan declined from a value comparable to the E_M of 8-OH-DPAT (on top of sumatriptan's effect) down to zero (only sumatriptan's effect remaining) by increasing the time interval between the two treatments, with an apparent time constant of 6.2 min (actually the effect was little affected for short intervals but it virtually disappeared by 10 min). The same was true for sumatriptan application following 8-OH-DPAT; in this case the estimated time constant was 7.1 min, and again little inhibition was observed for short intervals and almost complete inhibition occurred for times $\geq$ 10 min.

Once again, simple explanations like partial agonism or

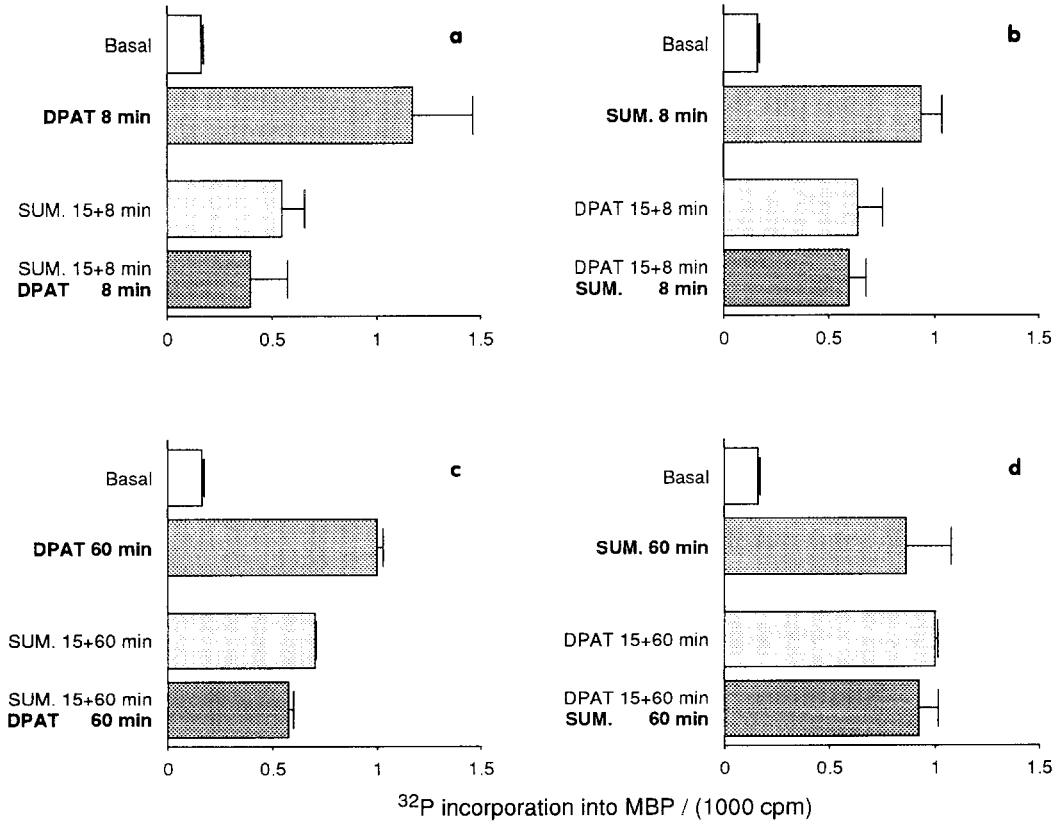

Fig. 8. Effect of 15-min pretreatment with one receptor subtype agonist on MAP kinase activation by the other receptor subtype agonist. (a,c) [^{32}P]Phosphate incorporation into myelin basic protein induced by cell extracts from GLC-8 SCLC cells exposed to the 5-HT_{1A} receptor agonist 8-OH-DPAT (400 nM) for 8 min (a) or 60 min (c), with or without previous exposure to the 5-HT_{1D} receptor agonist sumatriptan (100 nM) for 15 min. Notice the evident effects of 8-OH-DPAT without pretreatment, as opposed to the total lack of its effect in cell preincubated with sumatriptan for 15 min. (b,d) The effect of exposure to 100 nM sumatriptan for 8 min (b) or 60 min (d), with or without previous exposure to 400 nM 8-OH-DPAT for 15 min. Notice the obliteration of sumatriptan effects by pretreatment with 8-OH-DPAT. All measurements in triplicate.

cross-antagonism cannot account for this observation. Rather, it appears that slow processes like receptor down-regulation or interference in the transduction pathways might be involved. The experiment of Fig. 6 was repeated by performing the preincubation with the first drug at 4°C and then warming up the medium back to 37°C. In these conditions no decrease in the effect of the second treatment was observed, following 30–60 min preincubation (not shown), strongly suggesting that some highly temperature-sensitive cellular process mediates this interaction between the two receptor subtypes.

3.6. Investigation of transduction pathways

5-HT was previously shown to inhibit adenylate cyclase activity in SCLC cells (Cattaneo et al., 1994). When this aspect was investigated by using the previously reported procedures, both 8-OH-DPAT and sumatriptan turned out to produce about 20% inhibition of adenylate cyclase; however, no additivity could be shown, and the degree of cyclase inhibition could not be correlated with the mitogenic activity as measured by [^{3}H]thymidine incorporation (data not shown).

Conversely, the 5-HT_{1A} receptor agonist 8-OH-DPAT, the 5-HT_{1D} receptor agonist sumatriptan – applied either in isolation or together – and 5-HT, all were able to produce activation of MAP kinase, peaking at about 10 min. Furthermore, all these treatments as well as foetal bovine serum produced a second, delayed activation of MAP kinase activity (Fig. 7), as it was previously reported to occur for several mitogenic stimuli in other cell types (Kahan et al., 1992). Whereas the height of the first activation peak appears to be variable and not to correlate well with the mitogenic effect of the various treatments, the effects of 8-OH-DPAT and sumatriptan on the late activation of MAP kinase seem to be additive.

MAP kinase activity produced at 8 min by either 8-OH-DPAT or sumatriptan is abolished by a 15-min pretreatment with the other drug (Fig. 8a,b). The delayed MAP kinase activation at 60 min is also abolished by a 15-min pretreatment with the other drug, leaving the response to the drug applied first (Fig. 8c,d) unchanged.

4. Discussion

Our previous findings indicate that serotonin exerts a mitogenic action on small-cell lung carcinoma cell lines in culture, by acting on both 5-HT_{1A} and 5-HT_{1D} receptors (Cattaneo et al., 1993, 1994, 1995). As these cells produce and secrete 5-HT, among other substances, it is likely that the amine plays an autocrine mitogenic role, and it is therefore of interest to understand the mechanism and regulation of the 5-HT effect.

In previous studies, the activation of the two receptor subtypes was shown to be additive, in terms of maximum effects. However, 5-HT_{1A} receptor antagonists curiously proved more potent against the 5-HT_{1A} receptor agonist 8-OH-DPAT than against serotonin, as if the blockade of 5-HT_{1A} receptors made the activation of 5-HT_{1D} receptors somehow more effective. This suggested that the two receptor subtypes were present on the same cell and interacted in some way. The problem seemed very intriguing and worth pursuing. A direct approach at investigating receptor interactions by binding studies did not succeed, possibly due to the scarce number of receptors and/or cell heterogeneity. We therefore decided to further characterise this interaction by means of functional studies.

The results obtained by simultaneously applying both receptor-subtype agonists, 8-OH-DPAT and sumatriptan, indicate that the interaction is reciprocal. Most kinds of interactions could explain the dose response curve obtained with equipotent concentrations of the two drugs (Fig. 1), as the data are well fit even by the simple assumption of mild reciprocal antagonism between the drugs, either at the receptor level or at some steps in the transduction pathway. However, we found a clear asymmetry in the interaction, when the two agonists were not applied simultaneously. The effect of the drug applied later was inhibited (see the shifts to the right of the curves in Fig. 5) whereas no inhibition of the effect of the drug applied first was apparent (compare the rightmost points in the lower panels of Fig. 5 with the predictions for a reciprocal inhibition, i.e., the dotted curves). In particular, the inhibition – by the activation of one receptor subtype – on subsequent activation of the other receptor pathway proved to be time and temperature dependent, thus ruling out simple antagonism at the receptor level.

One puzzling aspect of these results is the following: in experiments where the two receptor subtypes were activated simultaneously or at short intervals (Fig. 1 and Fig. 4 and Fig. 5 and leftmost points in Fig. 6), marked changes were observed in the EC_{50} values but little if any changes occurred in the E_M values. Conversely, longer time intervals resulted in a decrease (down to complete inhibition) in the response to maximal doses of the second drug. It appears therefore that the interference between the two receptor pathways involves both a rapid change in sensitivity and a time and temperature dependent change in efficacy. A possible mechanism for such complex interaction is as follows: (i) a molecular interaction and/or association might occur between receptors of two different subtypes; (ii) ligand binding to one of them might produce a conformational change in the other receptor thereby reducing its affinity for its ligands (rapid change in EC_{50}); (iii) subsequently, down regulation might involve both receptors simultaneously, thus reducing any response to a second agonist, in a time and temperature-dependent manner.

Measurements of MAP kinase activity yielded further interesting clues as to the mechanism of the complex interaction between the two receptor pathways. A biphasic activation is observed in response to serotonin, foetal serum and either or both 5-HT_{1A}/5-HT_{1D} receptor ago-

nists. Furthermore, after a 15-min preincubation with an agonist, the pattern of MAP kinase activation appears to become insensitive to the activation of the other receptor pathway. In hamster fibroblasts, the mitogenic effect of growth factors was reported to correlate well with the extent of the delayed activation ($\geq$ 1 h) of MAP kinase rather than with the height of the first activation peak (Kahan et al., 1992); our results are consistent with this finding. It appears therefore that stimulation of serotonin receptors triggers in SCLC cells the arousal of a cellular programme characterised by a complex time course, whereby the mitogenic response is correlated not only with the intensity, but also with the duration and temporal pattern of receptor stimulation. Such a cellular programme, once initiated, might also switch off other cellular responses. This would provide a possible explanation for the complex interactions between receptor subtypes highlighted by the experiments reported here.

Based on behavioural, biochemical and electrophysiological studies in rats, reciprocal interactions between 5-HT_{1A} and 5-HT_2 receptors have been reported (Lakoski and Aghajanian, 1985; Weiss et al., 1986; Backus et al., 1990). However, to our knowledge this is the first report on the existence of molecular interactions between the 5-HT_{1A} and 5-HT_{1D} receptors in human cells. In the human brain, 5-HT_{1A} receptors are predominantly located in the limbic system, i.e., in regions concerned with mood and anxiety, and in the dorsal and medial raphe nuclei (Zifa and Fillion, 1992; Saudou and Hen, 1994); clinical studies suggest that the 5-HT_{1A} receptor subtype may be involved in the pathogenesis of anxiety and depression (Goldberg and Finnerty, 1979; Blier and de Montigny, 1994). The 5-HT_{1D} receptor subtype is probably the most common 5-HT receptor type in human brain. Its distribution partially overlaps that of 5-HT_{1A} receptors, at least in some areas of the brain like the hippocampus, cortex and raphe (Zifa and Fillion, 1992; Saudou and Hen, 1994). If the described interactions between the 5-HT_{1A} and 5-HT_{1D} receptors occur in neurones, in those brain areas where the two receptor subtypes colocalise, this might have important physiological and pharmacological consequences on the function of the serotoninergic system and on the response to serotoninergic drugs.

Acknowledgements

We thank F. D'Atri and I. Forza for technical assistance. This work was supported by grants from CNR ('ACRO') and from the Italian Association for Cancer Research (AIRC) to L.M.V. M.G.C. is the recipient of a fellowship from AIRC.

References

Backus, L.I., T. Sharp and D.G. Grahame-Smith, 1990, Behavioral evidence for a functional interaction between central 5-HT_2 and 5-HT_{1A} receptors, Br. J. Pharmacol. 100, 793.

Blier, P. and C. De Montigny, 1994, Current advances and trends in the treatment of depression, Trends Pharmacol. Sci. 15, 220.

Boddeke, H.W.G.M., A. Fargin, J.R. Raymond, P. Schoeffter and D. Hoyer, 1992, Agonist/antagonist interactions with cloned 5-HT_{1A} receptors: variations in intrinsic activity studied in transfected HeLa cells, Naunyn-Schmiedeberg's Arch. Pharmacol. 345, 257.

Cattaneo, M.G., A. Codignola, L.M. Vicentini, F. Clementi and E. Sher, 1993, Nicotine stimulates a serotoninergic autocrine loop in human small-cell lung carcinoma, Cancer Res. 53, 5566.

Cattaneo, M.G., E. Palazzi, G. Bondiolotti and L.M. Vicentini, 1994, 5-HT_{1D} receptor type is involved in stimulation of cell proliferation by serotonin in human small cell lung carcinoma, Eur. J. Pharmacol. 268, 425.

Cattaneo, M.G., R. Fesce and L.M. Vicentini, 1995, Mitogenic effect of serotonin in human small cell lung carcinoma cells: role of 5-HT_{1A} and 5-HT_{1D} receptors, Eur. J. Pharmacol. 291, 209.

Cuttitta, F., D.N. Carney, J. Mulshine, T.W. Moody, J. Fedorko, A. Fishler and J.D. Minna, 1985, Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer, Nature 316, 823.

Goldberg, H. and R. Finnerty, 1979, The comparative efficacy of buspirone and diazepam in the treatment of anxiety, Am. J. Psychiatry 136, 1184.

Kahan, C., K. Seuwen, S. Meloche and J. Pouysségur, 1992, Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts, J. Biol. Chem. 267, 13369.

Lakoski, J.M. and G.K. Aghajanian, 1985, Effects of ketanserin on neuronal responses to serotonin in the prefrontal cortex, lateral geniculate and dorsal raphe nucleus, Neuropharmacology 24, 265.

Lum, T.J. and M.F. Piercey, 1988, Electrophysiological evidence that spiperone is an antagonist of 5-HT_{1A} receptors in the dorsal raphe nucleus, Eur. J. Pharmacol. 149, 9.

Moody, T.W. and F. Cuttitta, 1993, Growth factor and peptide receptors in small cell lung cancer, Life Sci. 52, 1161.

Nakanishi, J., J.L. Mulshine, P.G. Kasprzyk, R.B. Natale, R. Maneckjie, I. Avis, A.M. Treston, A.F. Gazdar, J.D. Minna and F. Cuttitta, 1988, Insulin-like growth factor I can mediate autocrine proliferation of human small cell lung cancer cell lines in vitro, J. Clin. Invest. 82, 354.

Saudou, F. and R. Hen, 1994, 5-Hydroxytryptamine receptor subtypes in vertebrates and invertebrates, Neurochem. Int. 25, 503.

Schoeffter, P., J.R. Fozard, A. Stoll, H. Siegl, M.P. Seiler and D. Hoyer, 1993, SDZ 216-525, a selective and potent 5-HT_{1A} receptor antagonist, Eur. J. Pharmacol. 244, 251.

Seger, R., D. Seger, A.A. Reszka, E.S. Munar, H. Eldar-Finkelman, G. Dobrowolska, A.M. James, J.S. Campbell, E.H. Fisher and E.G. Krebs, 1994, Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells, J. Biol. Chem. 269, 25699.

Weiss, S., M. Sebben, D. Kemp and J. Bockaert, 1986, Serotonin 5-HT_1 receptors mediate inhibition of cAMP production in neurones, Eur. J. Pharmacol. 120, 227.

Zifa, E. and G. Fillion, 1992, 5-Hydroxytryptamine receptors, Pharmacol. Rev. 44, 401.