Serotonin regulates cytosolic Ca\(^{2+}\) activity and membrane potential in a neuronal and in a glial cell line via 5-HT\(_3\) and 5-HT\(_2\) receptors by different mechanisms

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Summary

The mechanisms of action of two different serotonin receptors, found in a neuronal cell line (neuroblastoma \(\times\) glioma hybrid cells) and in a non-excitable glioma cell line, were explored. In both cell lines, serotonin induced a dose-dependent, transient rise of cytosolic Ca\(^{2+}\) activity (measured by fura-2 or indo-1 fluorescence). Ca\(^{2+}\) channel blockers (Ni\(^{2+}\) and La\(^{3+}\), not nifedipine) suppressed the Ca\(^{2+}\) response to serotonin in the hybrid cells but not in the glioma cells. After application of Ca\(^{2+}\) ionophores (ionomycin and A23187) in order to short-circuit internal Ca\(^{2+}\) stores, serotonin was still able to induce a Ca\(^{2+}\) response in the hybrid cells but not in the glioma cells. Serotonin dose-dependently stimulated the rate of \(^{45}\)Ca\(^{2+}\) uptake several-fold in the hybrid cells, but hardly at all in the glioma cells. Thus, in the neuronal cell line cytosolic Ca\(^{2+}\) activity is raised through enhancement of Ca\(^{2+}\) entry into the cells from the extracellular environment via 5-HT\(_3\) receptors (blocked by ICS 205-930, MDL 72222 and GR 38032 F). The depolarization response caused by serotonin in the hybrid cells is due to activation of cation conductance(s), obviously allowing entry of extracellular Ca\(^{2+}\). In contrast to the neuronal cell line, in the glioma cell line the rise of Ca\(^{2+}\) activity is mediated by ketanserin-susceptible 5-HT\(_2\) receptors (not affected by treatment with pertussis toxin) mainly liberating Ca\(^{2+}\) from internal stores. In the glioma cells the release of Ca\(^{2+}\) from internal stores leads to opening of Ca\(^{2+}\)-dependent K\(^+\) channels, responsible for the hyperpolarizing response. Thus, the neuronal and the glial cell lines might provide suitable systems in which to study the diverse cellular functions triggered by the rise of cytosolic Ca\(^{2+}\) activity, which is caused by different serotonin receptors.

Key words: neuroblastoma \(\times\) glioma hybrid cell, glioma cell, 5-HT\(_2\) receptor, 5-HT\(_3\) receptor.

Introduction

Evidence for the existence of multiple types of receptors for serotonin (5-HT) in the mammalian central nervous system has been derived from electrophysiological and radioligand binding studies (Peroutka & Snyder, 1979). The distinct binding sites for serotonin were designated 5-HT\(_{1A}\), 5-HT\(_{1B}\), 5-HT\(_{1C}\) and 5-HT\(_{2}\) (5-HT, 5-hydroxytryptamine) receptors (see references quoted by Bradley et al. 1986; Peroutka, 1988). Serotonin has been found to induce various electrophysiological responses, such as activation of a K\(^+\) conductance leading to a hyperpolarizing response (Colino & Halliwell, 1987; Davies et al. 1987), or suppression of K\(^+\) conductances, thus enhancing neuronal excitatio (Colino & Halliwell, 1987; Davies et al. 1987). The hyperpolarizing response has been attributed to 5-HT\(_{1A}\) receptors. However, the mechanisms by which serotonin regulates the ion channels are not understood. In peripheral neurons fast and slow depolarizing responses (Wallis & Dun, 1988) or hyperpolarizing responses (Ireland & Tyers, 1987) have been observed and attempts have been made to classify the underlying 5-HT receptors pharmacologically (Wallis & Dun, 1988).

Highly potent antagonists of serotonin, MDL 72222 (Fozard, 1984) and ICS 205-930 (Richardson & Engel, 1986), revealed the existence of a further class, designated 5-HT\(_3\) receptors. 5-HT\(_3\) receptors have been demonstrated to be located on peripheral autonomic, sensory and enteric neurons and to mediate excitation (Richardson & Engel, 1986). Recently, 5-HT\(_3\) receptors have also been claimed to be present in the central nervous system (Kilpatrick et al. 1987).

A major focus of research in serotonin receptors is the
elucidation of the cellular effector systems coupled to the receptor subtypes. Subtypes of 5-HT<sub>1</sub> receptors are proposed to activate adenylate cyclase (Peroutka, 1988) and phospholipase C (Jankowsky et al. 1984) in rat hippocampus and to mediate presynaptic inhibition of evoked transmitter (5-HT) release (Richardson & Engel, 1986) or phosphatidylinositol turnover (Conn & Sanders-Bush, 1985). Xenopus oocytes injected with rat brain mRNA synthesize serotonin receptors (Gunderson et al. 1984). Stimulation of these receptors leads to an increased membrane conductance for Cl<sup>-</sup>, which seems to be mediated by a rise in the cytosolic Ca<sup>2+</sup> activity caused by inositol-1,4,5-trisphosphate (Ins<sub>1,4,5</sub>) formation. This serotonin receptor has been classified as 5-HT<sub>1c</sub> receptor (Lübbert et al. 1987).

5-HT<sub>2</sub> receptors (Bradley et al. 1986) are linked to neuronal depolarization and can be blocked selectively by ketanserin (Leysen et al. 1982). 5-HT<sub>2</sub> receptors are suggested to be associated with a phosphoinositide-specific phospholipase C in platelets (DeChaffoy de Courcelles et al. 1985) and in rat frontal cortex (Conn & Sanders-Bush, 1985).

In the present study we have used two neural cell lines to investigate further the mechanisms of action of serotonin at different types of 5-HT receptors. First, we studied a polyploid rat glioma cell line (Heumann et al. 1982) derived from C6 glioma cells. The latter cells have been reported to respond to serotonin biochemically (Ogura et al. 1986; Ananth et al. 1987) and electrophysiology (Ogura & Amano, 1984). Second, as a neuronal cell line we employed mouse neuroblastoma x rat glioma hybrid cells (Hamprecht, 1977), which show a depolarization response to serotonin (Christian et al. 1978), which is sensitive to 5-HT<sub>3</sub> receptor antagonists (Reiser et al. 1988). In the hybrid cells serotonin activates a transient rise of cyclic GMP level via 5-HT<sub>3</sub> receptors (Reiser & Hamprecht, 1989). Moreover, on the hybrid cells 5-HT<sub>3</sub> recognition sites have been identified by radioisotope binding studies (Hoyer & Nejít, 1987). In our experiments the influence of serotonin on cytosolic Ca<sup>2+</sup> activity, 45Ca<sup>2+</sup> uptake and membrane potential in both cell lines is compared.

**Materials and methods**

**Cell cultures**

Mouse neuroblastoma x rat glioma hybrid cells, clone 108CC15 and clone 108CC25 (for review, see Hamprecht, 1977), and polyplloid rat glioma cells, clone C6-4-2 (Heumann et al. 1982) of passage numbers between 14 and 32 were cultured as described (Hamprecht et al. 1985). Cells were grown to confluency in plastic flasks (175 cm<sup>2</sup>; Nunc, Wiesbaden, FRG) before use in experiments.

**Determination of cytosolic Ca<sup>2+</sup> activity**

Cells were detached from their substratum by incubating the glioma/hybrid cells with trypsin (0.05% / 0.005%) for 3–5 min in Puck’s D<sub>1</sub> buffer (see Hamprecht et al. 1985). Cells had a viability of more than 96% and were suspended in Dulbecco’s Modified Eagle’s Medium without bicarbonate, but buffered with 20 mM-N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes) adjusted to pH 7.4 with tris(hydroxymethyl)-aminomethane. The cell suspension (density 3×10<sup>6</sup> to 4×10<sup>6</sup> cells ml<sup>−1</sup>) was continuously stirred at 37°C and supplemented with fura-2 pentacacetoxymethylster at concentrations from 3 to 4 µM. This dye was added in a sample of a concentrated stock solution (2.5 mM) in dimethylsulfoxide. The medium thus contained a maximum of 0.15% dimethylsulfoxide. The ester of the dye freely permeates the cell membrane, whereas the membrane-impermeant anion fura-2, which is formed by intracellular esterases, is trapped inside the cells. The fluorescence of fura-2 depends on the Ca<sup>2+</sup> activity (Gryniewicz et al. 1985).

After 15 min of incubation the suspension was diluted fourfold with the same medium and incubated for a further 30 min at 37°C. Before being used for fluorescence measurements the cell suspension (in some cases diluted with the same medium by 10–20%) was stirred at 4°C, when the cells produced similar responses to serotonin and bradykinin for up to 3 h.

After the loading period, between 5×10<sup>6</sup> and 10×10<sup>6</sup> cells were centrifuged, washed once with 2 ml of incubation medium and resuspended in 2 ml of incubation medium containing 145 mM-NaCl, 5.4 mM-KCl, 1.8 mM-CaCl<sub>2</sub>, 1.0 mM-MgCl<sub>2</sub>, 20 mM-glucose, 20 mM-Hepes, adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane. All incubation media used had osmolarities ranging from 320 to 350 mmol l<sup>−1</sup>.

The cell suspension was transferred to a quartz cuvette in a thermostatically controlled holder (37°C) in an Aminco SPF-500 spectrofluorimeter (Silver Spring, Maryland, USA). The suspension could be stirred by means of a magnetic stir bar. Fura-2 fluorescence, with excitation at 340 nm (2 nm slit width) and emission at 500 nm (5 nm slit width) was recorded on a chart recorder with the time constant of the amplifying system set at 0.5 s. Fura-2 fluorescence was calibrated in terms of Ca<sup>2+</sup> activity using a dissociation constant (K<sub>D</sub>) of 225 nM for the Ca<sup>2+</sup>-dye complex, as described by Gryniewicz et al. (1985). Maximum fluorescence (F<sub>max</sub>) was obtained by adding either 0.1% (v/v) Triton X-100 or digitonin (50 µM) to the suspension, thus permeabilizing the cells and saturating the dye with Ca<sup>2+</sup>. Minimum fluorescence (F<sub>min</sub>) was obtained after adding 2 mM-Mn<sup>2+</sup>, which binds much more avidly than Ca<sup>2+</sup> to fura-2 and completely quenches the Ca<sup>2+</sup>-dependent fura-2 fluorescence at all wavelengths. Cytosolic Ca<sup>2+</sup> activity was calculated from the equation [Ca<sup>2+</sup>]<sub>Ca<sup>2+</sup></sub> = K<sub>D</sub>[(F<sub>max</sub>−F)/F<sub>max</sub>−F].

In some experiments the Ca<sup>2+</sup> indicator indo-1 was used instead of fura-2. The procedures were identical, except that 1 µM-indo-1 acetoxymethylster was added for loading the cells. Fluorescence of indo-1 was measured with excitation at 331 nm (2 nm slit) and emission at 410 nm (8 nm slit). For converting the fluorescence signal into Ca<sup>2+</sup> activity the K<sub>D</sub> was taken as 250 nM (Gryniewicz et al. 1985).

**Measurement of 45Ca<sup>2+</sup> uptake**

Cells were subcultured and seeded at a density of 2–5×10<sup>5</sup> hybrid cells 108CC15 or 3×10<sup>5</sup> polyplloid glioma cells C6-4-2 in plastic Petri dishes (60 mm diameter) in 8 ml growth medium and grown for 2 days. For the experiment the medium was aspirated and the cells were washed with 3 ml prewarmed (37°C) medium containing 145 mM-NaCl, 5.4 mM-KCl, 0.5 mM-CaCl<sub>2</sub>, 1.0 mM-MgCl<sub>2</sub>, 20 mM-glucose, 20 mM-Hepes, adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane (37°C). The 1.5-ml uptake medium added immediately to the cells had the same composition and in addition contained bovine serum albumin (1 mg ml<sup>−1</sup>). After preincubating the cells for 10 min with uptake medium at 37°C, another 0.5 ml of uptake medium supplemented with 45Ca<sup>2+</sup> (1 µCi ml<sup>−1</sup> at 37 kBq ml<sup>−1</sup>) and, if indicated, test agent was added to the culture dish to start the uptake period. The uptake was terminated by remov-
Electrophysiology

(1951) method using bovine serum albumin as standard. Wards incubated with 1 ml of 0-4M-NaOH to lyse the cells. The four times with 3 ml each of an ice-cold solution containing the uptake medium and washing the cell layer within 35 s, cell lysate plus 0.4 ml of 1 M-HCl and 1 ml of water used to wash adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane.

Cellular protein was determined according to the Lowry et al. (1951) method using bovine serum albumin as standard.

Electrophysiology

For electrophysiological experiments, neuroblastoma X glioma hybrid cells (cell line 108CC25) differentiated morphologically or polyoid rat glioma cells were used. The cells grown on glass coverslips were transferred into a perfusion chamber and superfused constantly with recording medium containing 145 mM-NaCl, 5.4 mM-KCl, 1.8 mM-CaCl₂, 1.0 mM-MgCl₂, 20 mM-glucose and 20 mM-Hepes, adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane. Membrane potential was recorded with glass microelectrodes filled with 3 M-KCl using conventional electrophysiological techniques (Reiser & Hamer, 1982). Serotonin was applied iontophoretically to the plasma membrane from electrodes filled with a 37 mM solution of serotonin in 75 mM-HCl.

Materials

Serotonin (5-hydroxytryptamine creatinine sulfate), cyproheptadine hydrochloride-(1-methyl-4-(5H-dibenzo[a,d]carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-aminophenylidene)piperidine), EGTA (ethylene-bis(oxyethylene-nitrilo)-tetraacetic acid) and digitonin were from Sigma (München, FRG). Ketanserin tartrate ((+)-3-[2-[(5H-dibenzo[a,d]carboxyoxazol-2-yl)-6-aminobenzo[d][5-oxo]-2-(2'-aminophenylidene)ethane-N,N',N''-tetraacetic acid) and fura-2/AM (fura-2/pentaacetylbenzyl ester), ionomycin and 4,2-Ar-Br23187 were from Calbiochem (Frankfurt, FRG).

The following substances were generously provided: ICS 205-930 (3α-tropanyl-1H-indole-3-carboxylic acid ester), methysergide (9,10-didehydro-6-N-[1-(hydroxymethyl)propyl]-1,6-dimethyl-ergoline-8-carboxamide) and 2-methyl-5-hydroxytryptamine from Sandoz A.G. (Basel, Switzerland), MDL 72222 (10H, 3α,5α-tropan-3-yl-3,5-dichlorobenzoxaze) from Merrell Dow Research Institute (Strasbourg, France), GR 38032 F (1,2,3,9-tetrahydro-9-methyl-3-[2-(methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one hydrochloride dihydrate) from Glaxo Research (Ware, Hertfordshire, UK). CaCl₂ (specific activity 10-40 mCi/mg⁻¹ Ca) was from Amersham (Braunschweig, FRG). All other chemicals, of analytical grade, were from E. Merck (Darmstadt, FRG) or from Sigma (München, FRG).

Results

Cytosolic Ca²⁺ activity

Addition of serotonin to suspensions of hybrid cells loaded with fura-2 caused a transient increase in fluorescence intensity (Fig. 1A), which can be attributed to a rise in cytosolic Ca²⁺ activity (Gryniewicz et al. 1985). Ca²⁺ activity returned to its basal level after about 40 s. The magnitude of the stimulation by serotonin depended on the concentration of the monoamine (Fig. 1A and C). The maximum was reached at about 2 μM-serotonin, which was, however, smaller than the signal obtained by adding the peptide bradykinin. Analogous experiments were carried out with glioma cells (Fig. 1B). At micromolar concentrations of serotonin a concentration-dependent increase in the cytosolic Ca²⁺ activity was observed. However, in the glioma cells the response to serotonin was more variable in amplitude than in the hybrid cells. A concentration response curve established with the hybrid cells (Fig. 1C) revealed an EC₅₀ of around 0-6 μM for serotonin and 4.5 μM for 2-methyl-serotonin. In the glioma cells, 2-methyl-serotonin had no effect in concentrations up to 50 μM (not illustrated).

Various serotonin antagonists were tested. Fig. 2A demonstrates that in the glioma cells the Ca²⁺ response was blocked by ketanserin, a 5-HT₂ receptor antagonist (Leyson et al. 1982), whereas the response of the glioma cells to bradykinin was not affected by ketanserin. In the glioma cells, ketanserin at concentrations between 1 and 10 nM suppressed the response to serotonin. In the hybrid cells, however, ketanserin did not reduce the effect of serotonin on cytosolic Ca²⁺ activity (Fig. 2B). Methysergide, a blocker of 5-HT₁ and 5-HT₂ receptors, could not be used because the fluorescence signal caused by adding methysergide completely obscured the fluorescence of fura-2.

Antagonists of 5-HT₁ receptors (see references quoted by Richardson & Engel, 1986) were also employed. ICS 205-930 at nanomolar concentration inhibited the Ca²⁺ response to serotonin in the hybrid cells (Fig. 3A). MDL 72222, at higher concentrations, also suppressed the rise in cytosolic Ca²⁺ activity caused by serotonin. Both ICS 205-930 and MDL 72222 did not alter the effect of bradykinin (Fig. 3A), proving the specificity of the effect of the serotonin antagonists. Another antagonist of 5-HT₁ receptors, GR 38032 F (Kilpatrick et al. 1987), was similarly effective on the hybrid cells, also leaving the response to bradykinin unchanged (Fig. 3B and C). In the glioma cells, however, ICS 205-930 in concentrations up to 0-2 μM had no effect on the rise in Ca²⁺ activity induced by serotonin (data not shown). Cyproheptadine, a serotonin antagonist with limited specificity, suppressed the Ca²⁺ response to serotonin both in the hybrid cells and in the glioma cells at concentrations from 2 to 20 μM (not shown).

In the following experiments employing some blockers of Ca²⁺ channels we tried to explore the mechanisms by which serotonin brings about the rise in cytosolic Ca²⁺ activity. Fig. 4A shows the response to serotonin and to bradykinin of fura-2-loaded hybrid cells under control conditions. Upon addition of 5 mM-Ni²⁺ the fluorescence fell to a lower level (Fig. 4B). This drop could be explained by a quenching effect of Ni²⁺ on the fura-2 fluorescence signal dependent on extracellular Ca²⁺ and dye leakage. This interpretation is consistent with our observation that addition of Triton X-100 (see Materials and methods), which is used to obtain maximal fluorescence for calibration, resulted not in an increase but in a decrease of fluorescence intensity in the presence of

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5 mM-Ni²⁺ (not shown). In the presence of Ni²⁺, Ca²⁺ activity in the hybrid cells remained constant after addition of serotonin, whereas the cells still responded to bradykinin as seen before under control conditions (Fig. 4B).

With the glioma cells, addition of 5 mM-Ni²⁺ caused a decrease in the fluorescence intensity similar to that observed in the hybrid cells. On the other hand, the response to serotonin did not disappear. In the experiment shown in Fig. 4C and D, in the glioma cells the rise in Ca²⁺ activity was even somewhat larger in the presence of Ni²⁺ than under control conditions. This effect of Ni²⁺ still has to be explored in detail.

In another series of experiments La³⁺, which also blocks Ca²⁺ channels (see references quoted by Tsien, 1983), was used. Hybrid cells, which had produced a rise in Ca²⁺ activity upon addition of serotonin in control medium, did not respond to serotonin but to bradykinin in the presence of 0.5 mM-La³⁺. In glioma cells, however, La³⁺ did not suppress the response to serotonin (data not shown). The results of the experiments with Ni²⁺ and La³⁺ point to different mechanisms of Ca²⁺ activation in the hybrid and the glioma cells by serotonin.

Addition of the organic Ca²⁺ channel blocking agent nifedipine (see references quoted by Tsien, 1983) at concentrations of 0.1 or 0.2 µM reduced the baseline fluorescence of hybrid cells and glioma cells loaded with fura-2. The responses to serotonin, however, were not or only slightly affected by nifedipine in both cell lines (data not shown).

Ca²⁺ ionophores served to elucidate further the role of intracellular Ca²⁺ stores in generating the Ca²⁺ signal upon serotonin application to the hybrid cells or to the glioma cells. Fig. 5A and C shows the Ca²⁺ responses of hybrid cells to serotonin and to bradykinin under control conditions. Ionomycin, a calcium ionophore (Liu & Hermann, 1978), at 0.1 µM caused a fast rise in cytosolic Ca²⁺ activity, comparable in size to that elicited by bradykinin (Fig. 5B and D), but with a longer duration. After the ionomycin-induced rise, Ca²⁺ activity returned to baseline within 50–60 s. After addition of ionomycin, the size of the Ca²⁺ signal caused by serotonin was not significantly different from the control value, whereas addition of bradykinin led to a rise in Ca²⁺ activity amounting only to a small fraction of the rise previously observed in control medium. The Ca²⁺ response to serotonin remained the same after application of ionomycin, irrespective of whether bradykinin had been applied to the cells or not (cf. Fig. 5B and D). Another Ca²⁺ ionophore, A23187, was used in a similar way. We employed the 4-Br-substituted derivative of A23187, because of its lower intrinsic fluorescence (Deber et al. 1985), which makes this compound applicable for the Ca²⁺ determination using fura-2. 4-Br-A23187 (4 µM) caused a transient rise in Ca²⁺ activity. Thereafter the hybrid cells responded barely or not at all to bradykinin,
Fig. 2. Effect of ketanserin (Ket) on serotonin-induced rise in cytosolic Ca\textsuperscript{2+} activity in the glioma (A) and the hybrid cells (B). Final concentrations of serotonin were 10 \mu M in A, and 1 \mu M in B; and of ketanserin were 100 nM in A, and 5 \mu M in B. For comparison, bradykinin was applied at final concentrations of 100 nM in A and 20 nM in B. In two similar experiments ketanserin (10 nM–5 \mu M) blocked the response to serotonin (3–10 \mu M) in the glioma cells, whereas in two further samples of the hybrid cells ketanserin (5 \mu M) did not change the effect of serotonin (1 and 2 \mu M).

but the effect of serotonin was hardly affected (data not shown).

Also in the glioma cells, ionomycin caused a transient rise in cellular Ca\textsuperscript{2+} activity (Fig. 5E and F). Subsequently, the response of the cells to both serotonin and bradykinin was reduced substantially as compared to the effect seen in control cells (Fig. 5E).

In glioma cells pretreated with pertussis toxin (130 ng ml\textsuperscript{-1} for 6 h) the Ca\textsuperscript{2+} response to application of serotonin (10 \mu M) or of bradykinin (100 nM) was virtually the same as in untreated cells (data not shown).

The experiment shown in Fig. 6A and B was designed to shed light on the mechanism of Ca\textsuperscript{2+} removal after the increase induced by serotonin. In the hybrid cells, the Ca\textsuperscript{2+} response to ionomycin under control conditions (Fig. 6A) was smaller than the one observed when a serotonin response had been elicited before adding ionomycin (Fig. 6B). At the concentrations used, ionomycin supposedly acted primarily on internal Ca\textsuperscript{2+} stores. Therefore the higher amplitude of the response to ionomycin after having added serotonin could be due to an extra load of Ca\textsuperscript{2+} in the stores, which accumulate the excess cytosolic Ca\textsuperscript{2+} after the serotonin stimulus.

To clarify the route of Ca\textsuperscript{2+} activation by serotonin more distinctly, the uptake of \textsuperscript{45}Ca\textsuperscript{2+} was determined. With 2–5 \mu M-serotonin, the rate of \textsuperscript{45}Ca\textsuperscript{2+} uptake of the hybrid cells was enhanced by a factor of 2–7 (Fig. 6C). A concentration–response curve obtained with the hybrid cells yielded an EC\textsubscript{50} of 1 \mu M for serotonin (not shown). In the glioma cells, however, 5 \mu M-serotonin caused an increase only maximally 20% above control (not shown).

Electrophysiological response to serotonin

The effect of serotonin on membrane potential was tested in both the hybrid cells and the polyploid glioma cells. As described (Reiser et al., 1988), iontophoretic application of serotonin elicited a fast depolarization response in the hybrid cells (Fig. 7A). The depolarization was associated with an increase in conductance. The dependence of the amplitude on the extracellular Na\textsuperscript{+} concentration has been attributed to activation of a cation conductance in the hybrid cells (Reiser et al., 1988). In some cells the depolarization response to serotonin was superimposed by one or several action potentials (cf. Reiser et al., 1988), as shown in the example in Fig. 7A. The response to serotonin in the hybrid cells was blocked reversibly by ICS 205-930 (Fig. 7A), by MDL 72222 and by GR 38032 F (not illustrated), but was not sensitive to ketanserin (up to 2 \mu M; not shown).

In the glioma cells, however, iontophoretic pulses of serotonin caused a transient hyperpolarization (Fig. 7B).
Fig. 3. Effect of 5-HT3 receptor antagonists on the rise of cytosolic Ca2+ activity induced by serotonin in the hybrid cells. A. Upper trace gives control responses, lower trace responses of two samples of cells from the same suspension to serotonin (1 μM) and bradykinin (20 nM) after adding either ICS 205-930 (10 nM) or MDL 72222 (1 μM). B. Response to serotonin (4 μM) and bradykinin (20 nM) before and after adding 100 nM-GR 38032 F. C. Ca2+ activity in hybrid cells (clone 108CC25) upon addition of serotonin (5 μM) and bradykinin (100 nM) before and after application of 100 nM-GR 38032 F. ICS 205-930, GR 38032 F and MDL 72222 had comparable effects in four, two and two further experiments, respectively. In another experiment testing different concentrations of GR 38032 F, the antagonist blocked, at 10 nM by about 50% and at 50 nM completely, the response to 2 μM-serotonin; 10 nM-MDL 72222 inhibited the response by 50%.

and C). The response to serotonin, appearing after a delay of a few seconds, was characterized pharmacologically. Ketanserin or methysergid blocked the effect of serotonin, which reappeared after washing out the antagonist (Fig. 7). Ketanserin was able to block completely the membrane potential response to serotonin at concentrations ranging from 5 to 100 nM in different cells. In one cell, the efficacies of ketanserin and methysergid were compared. In this case 10 nM-methysergid was without any influence, but 100 nM-methysergid or 10 nM-ketanserin entirely blocked the response (data not shown). ICS 205-930 and GR 38032 F, however, even at 10 μM concentration, had no effect on the responsiveness of the same cells to serotonin (not shown).

Discussion

To understand the actions of the diverse 5-HT receptors, it is important to know which molecular mechanisms are coupled to a certain type of receptor. In the present study we investigated the effects of serotonin in: (1) an excitable cell line with neuronal properties, neuroblastoma × glioma hybrid cells; and (2) a non-excitable cell line with glial properties.

The 5-HT receptors mediating the rise of cytosolic Ca2+ activity in the two cell lines were characterized pharmacologically. In the neuronal cells ketanserin, a 5-HT2 receptor antagonist, was ineffective. However, ICS 205-930, MDL 72222 and GR 38032 F, inhibitors of 5-HT3 receptors, blocked the response even at nanomolar concentrations. This indicates the presence of 5-HT3 receptors on the hybrid cells and is consistent with earlier observations that in the hybrid cells serotonin activates membrane depolarization (Reiser et al. 1988), cyclic GMP synthesis (Reiser & Hamprecht, 1989) and guanidinium (substitute for Na+) uptake (Reiser & Hamprecht, 1988) via a 5-HT3 receptor. Binding studies
Fig. 4. Influence of Ni\textsuperscript{2+} on the rise in cytosolic Ca\textsuperscript{2+} activity induced by serotonin. A,B. Responses of the hybrid cells to serotonin (1 \textmu M) and bradykinin (20 nM). C,D. Responses of glioma cells to serotonin (10 nM) and bradykinin (20 nM).

B,D. Responses after adding 5 mM-Ni\textsuperscript{2+}. Traces (B and D) were shifted upwards to the extent that the plateaux reached after the addition of Ni\textsuperscript{2+} corresponded to baseline fluorescence under control conditions. The Ca\textsuperscript{2+} activity before and after adding Ni\textsuperscript{2+} are assumed to be the same. The reason for the drop in fluorescence intensity is discussed in the text. Results similar to the ones shown in A and B were observed in three further experiments, and in C and D in two more.

using radiolabelled ICS 205-930 have demonstrated the existence of 5-HT\textsubscript{3} receptors on plasma membranes of the hybrid cells (Hoyer & Neijt, 1987).

In the glioma cells 5-HT\textsubscript{3} receptor antagonists, even at micromolar concentrations, had no effect on the rise in Ca\textsuperscript{2+} activity induced by serotonin. Ketanserin blocked the response in the nanomolar range, suggesting the presence of 5-HT\textsubscript{3} receptors on the glioma cells.

The serotonin-induced, concentration-dependent, transient rise in cytosolic Ca\textsuperscript{2+} activity in both the hybrid and the glioma cells appeared to be similar. However, the mechanisms of action of serotonin are obviously different in the two cell lines. Ni\textsuperscript{2+} and La\textsuperscript{3+}, known to block Ca\textsuperscript{2+} channels (Tsien, 1983), suppressed the response to serotonin in the neuronal cells but not in the glioma cells. This indicates that serotonin causes the entry of extracellular Ca\textsuperscript{2+} through the plasma membrane of the hybrid cells, whereas in the glioma cells serotonin mainly releases Ca\textsuperscript{2+} from intracellular stores. Upon removal of extracellular Ca\textsuperscript{2+} the response to serotonin was abolished in the hybrid cells and in the glioma cells (data not shown). It still has to be elucidated whether the entry of a small amount of external Ca\textsuperscript{2+} is required for activation of internal Ca\textsuperscript{2+} release by serotonin in the glioma cells. Similarly, the Ca\textsuperscript{2+} response elicited by the neuropeptide bradykinin in the hybrid cells (e.g. see Figs 1 and 2) seems to be due largely to release of Ca\textsuperscript{2+} from intracellular stores (Jackson et al. 1988), but is also reduced by lowering extracellular Ca\textsuperscript{2+} concentration (Reiser & Hamprecht, 1985).

To establish the route of Ca\textsuperscript{2+} activation by serotonin more clearly, experiments using the Ca\textsuperscript{2+} ionophores ionomycin and A23187 (Liu & Hermann, 1978) were carried out. Previously, Kurzungere\textsuperscript{2+}al. (1980) suggested that A23187 at micromolar concentrations functionally eliminated intracellular Ca\textsuperscript{2+} stores in the hybrid cells. Furthermore, Pollock et al. (1987) have shown that resequestration of internally released Ca\textsuperscript{2+} did not occur in platelets treated with 1 \textmu M-ionomycin. Thus, in the continued presence of the ionophores, the internal stores are short-circuited, and recovery of an elevated cytosolic Ca\textsuperscript{2+} activity is presumably almost entirely due to Ca\textsuperscript{2+} extrusion (Pollock et al. 1987). When applied at low concentrations, 4-Br-A23187 and ionomycin induced a transient increase in intracellular Ca\textsuperscript{2+} activity, but with a duration longer than the signals induced by bradykinin.
or by serotonin. The longer duration might be explained by the fact that the ionophores had reduced the capacity of the internal stores to re-accumulate the cytosolic \( \text{Ca}^{2+} \).

The glioma cells, after being challenged with \( 1 \text{ \mu M} \) ionomycin, no longer responded to serotonin. This is consistent with the interpretation that serotonin in the polyploid glioma cells induced the release of \( \text{Ca}^{2+} \) from internal stores. In the hybrid cells, however, even after treatment with ionomycin, serotonin still caused a transient rise in \( \text{Ca}^{2+} \) activity that was not much different from the signal seen in cells without previous exposure to \( \text{Ca}^{2+} \) ionophores. Furthermore, in the hybrid cells serotonin largely enhanced the uptake of \( \text{Ca}^{2+} \), but only slightly in the glioma cells. These observations corroborate the conclusion that in the hybrid cells, upon addition of serotonin, \( \text{Ca}^{2+} \) mainly enters the cells from the extracellular space.

Experiments using \( \text{Ca}^{2+} \) ionophores can also give a clue as to the mechanism that restores the cytosolic \( \text{Ca}^{2+} \) level back to the baseline after a serotonin-induced rise. Since in the hybrid cells after the addition of serotonin the amount of \( \text{Ca}^{2+} \) releasable by ionophores from internal \( \text{Ca}^{2+} \) stores is increased, we conclude that a sizeable portion of the \( \text{Ca}^{2+} \) entering after serotonin receptor activation is taken up into intracellular stores.

Electrophysiological recordings showed that in the polyploid glioma cells, iontophoretic application of serotonin caused a hyperpolarization response, as already reported for rat glioma cells, clone C6-BU-1 (Ogura & Amano, 1984). The electrophysiological effect of serotonin in the polyploid glioma cells could be blocked by nanomolar concentrations of ketanserin or methysergid, but was not affected by 5-HT3 receptor antagonists at concentrations up to \( 10 \text{ \mu M} \). Similarly, methysergid has been found to inhibit the influence of serotonin on membrane potential in C6-BU-1 cells (Ogura & Amano, 1984), indicating the presence of the same type of serotonin receptor in the C6-BU-1 glioma cells and in the polyploid glioma cells C6-4-2.

The hyperpolarizing response in the polyploid glioma cells appearing with a time lag after the serotonin pulse resembles very much the response to bradykinin (Reiser & Hamprecht, 1982). The response to bradykinin has been shown to involve activation of \( \text{Ca}^{2+} \)-dependent \( K^+ \) channels (Reiser et al. 1986). In the hybrid cells, however, the membrane is depolarized by serotonin,
Fig. 6. A, B. Influence of serotonin on the rise in cytosolic Ca\(^{2+}\) activity induced by ionomycin in the hybrid cells. Ca\(^{2+}\) activity was determined by fluorescence of indo-1. In A 0.1 \(\mu\)M-ionomycin; in B 2 \(\mu\)M-serotonin and 0.1 \(\mu\)M-ionomycin were applied. Comparable results were seen in one and three other experiments using indo-1 and fura-2, respectively. C. Stimulation of \(^{45}\)Ca\(^{2+}\) uptake by serotonin in hybrid cells. Cells were preincubated for 10 min at 37°C in incubation medium containing only 0.5 mM-CaCl\(_2\) and the uptake was started by supplementing the medium with 1 \(\mu\)Ci ml\(^{-1}\) \((37\,\text{kBq}\,\text{ml}^{-1})\) \(^{45}\)Ca\(^{2+}\), without (○) or with (●) serotonin (2.5 \(\mu\)M). After the uptake period cellular content of \(^{45}\)Ca\(^{2+}\) was determined as described in Materials and methods. Data represent mean values ± S.D. of triplicate incubations carried out in parallel. Similar results were obtained in two further experiments.

Fig. 7. Membrane potential response to serotonin of hybrid cells (A) and glioma cells (B, C). A. Pen recorder diagram of membrane potential. At times marked on the zero potential line iontophoretic current pulses of serotonin (7 nA, 1 s) were given. Cells were superfused for the time indicated with medium without (control) or with 30 nm-ICS 205-930. B, C. Membrane potential response to serotonin of polyploid glioma cells and the inhibitory action of methysergid and ketanserin in two different cells. Traces 1–4 show membrane potential, the upper line marks the iontophoretic current pulses of 48 nA (B) and 50 nA (C), respectively. Traces 2 and 3 in B are responses to serotonin, 2 min and 4 min, respectively, after addition of 100 nM-ketanserin. Traces 2 and 3 in C give responses to serotonin 1 min and 2 min, respectively, after admission of 100 nM-methysergide. Traces 1 and 4 are control responses before adding and after washing out the antagonist. Similar effects were seen with ketanserin and methysergide in eight and two other cells, respectively. Resting membrane potential was -57 mV in B and -53 mV in C.
presumably through activation of a cation conductance with limited selectivity. It is unclear whether this same channel might also be permeable to Ca$^{2+}$ or whether a different Ca$^{2+}$-specific pathway is triggered by a separate mechanism.

Thus, serotonin activates receptors with different pharmacological properties in neuroblastoma × glioma hybrid cells (5-HT$_3$ receptors) and in polyplody glioma cells (5-HT$_7$ receptors). The 5-HT$_3$ receptor enhances without delay, most likely directly, a cation conductance leading to depolarization of the cell. Concomitantly, the entry of extracellular Ca$^{2+}$ is augmented. The resulting rise in cytosolic Ca$^{2+}$ activity seems to be necessary for a transient stimulation of cyclic GMP synthesis (Reiser, unpublished results).

The 5-HT$_2$ receptor in the glioma cells described here induces a rise in cytosolic Ca$^{2+}$ activity mainly by release of Ca$^{2+}$ from intracellular stores through a mechanism not involving a pertussis toxin-sensitive G-protein. A mediating role for inositol-1,4,5-trisphosphate is still a possibility. In the related rat glioma cell line C6 a link between serotonin receptors and inositol phosphate metabolism has already been demonstrated (Ananth et al. 1987). The rise in cytosolic Ca$^{2+}$ activity leads to the opening of Ca$^{2+}$-dependent K$^+$ channels, causing a hyperpolarization of the plasma membrane.

This work was supported by a project grant from the Deutsche Forschungsgemeinschaft (Re 563/2-1 and 2). We thank Professor B. Hamprecht for his support.

References


(Received 13 February 1989 – Accepted 14 April 1989)