

Irreversible antagonism of 5HT_{2c} receptors by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)

(channels/receptor binding/*Xenopus* oocytes/HeLa cells)

Y. G. NI*, N. CAMACHO, AND R. MILEDI†

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92697-4550

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ABSTRACT To determine if *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a carboxyl group activating agent, can inactivate 5HT_{2c} receptors, we have examined the effects of EEDQ on 5HT_{2c} receptor-mediated responses to 5-hydroxytryptamine (5HT) in *Xenopus* oocytes, and on the binding of [³H]5HT to 5HT_{2c} receptors in transfected HeLa cells. In oocytes expressing rat 5HT_{2c} receptors, EEDQ inhibited the 5HT_{2c} receptor-mediated Cl⁻ currents; and the response did not recover more than 24 h after removal of the EEDQ. To see if this effect of EEDQ was on the receptor itself, the binding of 5HT to 5HT_{2c} receptors was studied in transfected HeLa cells. EEDQ decreased the specific binding of [³H]5HT to 5HT_{2c} receptors. At ≈22°C, incubating the membranes with 2 × 10⁻⁴ M EEDQ for 1 h caused a 40% decrease in the B_{max}, without changing the K_d. At 37°C, the same treatment with EEDQ blocked [³H]5HT binding completely. Half-maximal inhibition occurred at 5 μM EEDQ at both temperatures, and washing for 1.5 h did not restore the binding, suggesting that the inactivation of 5HT_{2c} receptor binding was practically irreversible. Results from both systems showed clearly that EEDQ is an irreversible antagonist of 5HT_{2c} receptors and therefore can be used for many studies of this receptor.

The serotonin or 5-hydroxytryptamine (5HT)_{2c} receptor (previously designated 5HT_{1c} receptor) has been implicated in many important effects of 5HT, including pain, feeding, salt intake, and locomotion (1). In recent years, 5HT_{2c} receptors were shown to couple to several different second messenger pathways. For example, in choroid plexus, a tissue highly enriched in 5HT_{2c} receptors, activation of 5HT_{2c} receptors could increase both phosphoinositide turnover (2) and guanylate cyclase activity (3). In *Xenopus* oocytes injected with 5HT_{2c} receptor mRNA, application of 5HT elicited Ca²⁺-dependent Cl⁻ currents, which indicated a coupling between the 5HT_{2c} receptor and the oocyte endogenous phospholipase C pathway (4, 5). In addition, activation of 5HT_{2c} receptors expressed in oocytes also resulted in a closure of two different K⁺ channels (6, 7). In spite of all this knowledge, due to the paucity of specific 5HT_{2c} receptor ligands and to the complexity of signal transduction mechanisms, little is known about the functional significance of the coupling between 5HT_{2c} receptors and each effector system.

Compounds that bind to receptors irreversibly are very useful tools for studying receptor structure and function. EEDQ has been shown to inactivate irreversibly a number of neurotransmitter receptors, including 5HT_{1a}, 1b, 1f, and 2a receptors (8–12). Hence, EEDQ has been used frequently to study receptor

turnover rates and receptor reserves, both *in vivo* and *in vitro*. To determine if this reagent also can inactivate cloned 5HT_{2c} receptors, we have examined the effect of EEDQ on the 5HT_{2c} receptor-mediated oscillatory currents elicited by 5HT in *Xenopus* oocytes, and on the binding of [³H]5HT to 5HT_{2c} receptors in transfected HeLa cells.

MATERIALS AND METHODS

Drugs were purchased from the following companies: EEDQ, Aldrich; serotonin (5HT), Sigma; [³H]5HT (≈25 Ci/mmol; 1 Ci = 37 GBq), DuPont/NEN. EEDQ was dissolved in ethanol. Due to its low water solubility, the actual EEDQ concentrations in Ringer's solution or binding assay mixture are probably lower than stated.

RNA *In Vitro* Transcription and Translation in *Xenopus* Oocytes. *NotI*-linearized pSR1c (4) was transcribed using T7 RNA polymerase (Promega) in the presence of a cap analog 5'-me⁷Gppp5'G (Pharmacia) and expressed in *Xenopus* oocytes. Oocytes were injected with mRNA, kept in an incubator (15–17°C), and recordings were made 4–10 days afterwards as described previously (13, 14). Briefly, each oocyte was injected with 1 ng of 5HT_{2c} mRNA or 50 ng of rat cortex mRNA (6), and kept in Barth's medium containing 0.01 mg/ml gentamicin. Two days after the injection, the oocytes were treated with collagenase (Sigma, type I, 0.5 mg/ml) in frog Ringer's solution, at room temperature for 0.5–2 h, to remove the follicular and other enveloping cells (13, 15). Membrane currents were recorded, usually at -60 mV, using a two KCl microelectrode voltage-clamp, digitized, and stored for subsequent analyses. Drugs were applied by a continuous bath superfusion in Ringer's solution at about 5 ml per min (bath volume ca. 100 μl).

Transfection and Membrane Preparation. The *EcoRI* fragment of pSR1c, containing the entire coding region of the rat 5HT_{2c} receptor, was subcloned into a eukaryotic expression vector pcDNA3 (Invitrogen). The new plasmid, p5HT_{2c}/neo, was transfected into HeLa cells by electroporation (Bio-Rad Gene Pulser, 500 μF, 300 V). Two to three days after the transfection, cells were washed twice with PBS and harvested with a cell scraper. The collected cells were lysed in a hypotonic buffer (50 mM Tris-HCl, pH 7.4/1 mM MgCl₂) on ice for 10 min, and centrifuged at 25,000 × g for 30 min, at 4°C. The pellet was suspended in the buffer, incubated on ice for 15 min, and reprecipitated as above. The membranes obtained were resuspended in receptor binding buffer (50 mM Tris-HCl, pH 7.4/4 mM CaCl₂/0.1% ascorbic acid) and kept at -70°C until use. Membrane protein was measured according to Bradford's method (16).

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Abbreviations: 5HT, 5-hydroxytryptamine; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

*Present address: Yale University, School of Medicine, Department of Psychiatry, 34 Park Street, New Haven, CT 06508.

†To whom reprint requests should be addressed at: Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, 2205 Bio Sci II, Irvine, CA 92697-4550.

Rat cerebral cortex (including hippocampus) membranes were prepared with a similar method. Briefly, tissue was homogenized with a loose-fitting Polytron in 0.32 M sucrose and centrifuged at $800 \times g$ for 10 min. The supernatant was reprecipitated at $25,000 \times g$ for 30 min, and the resulting pellet was washed twice with 1 mM ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid and resuspended in the receptor binding buffer. During the entire process, samples were kept on ice, and the centrifugations were carried out at 4°C .

Receptor Binding Assay. Crude cell membranes (40–60 μg) were incubated with 10 nM [^3H]5HT in the binding buffer (200 μl), at room temperature (≈ 20 – 22°C) for 30 min. Nonspecific binding was determined with 10 μM unlabeled 5HT. Binding assays were terminated by rapid vacuum filtration through Whatman GF/B filters, followed by three washes with 5 ml of ice-cold 15 mM Tris-HCl (pH 7.4). The filters were presoaked with 0.1% polyethyleneimine to reduce nonspecific binding. Except in dose- or time-dependence experiments, the EEDQ treatment was done by incubating membranes with 2×10^{-4} M EEDQ, at room temperature or 37°C , for 1 h before adding [^3H]5HT.

RESULTS

When control, noninjected oocytes were exposed to 5×10^{-4} M EEDQ alone, there was a small membrane current change, which was accompanied by a decrease in membrane conductance. This indicated that K^+ channels were being closed, an effect that was seen more clearly for EEDQ acting on voltage-dependent channels induced by native rat cortex or spinal cord mRNAs (Fig. 1). This blockage of K^+ channels was rapid in onset and was also rapidly reversible. The inactivating effects of EEDQ on K^+ , Ca^{2+} , and Na^+ channels are being studied further and will be reported later. Here, we focus on the effects of EEDQ on 5HT receptors.

If EEDQ was briefly coapplied with 5HT to oocytes expressing 5HT receptors, the characteristic oscillatory currents elicited by 5HT (5) were greatly inhibited, and again the inhibition was reversible (Fig. 2). This reversibility disappeared when the oocytes were exposed to EEDQ for longer periods.

Effect of EEDQ on 5HT_{2c} Receptor-Mediated Currents. In oocytes injected with cloned 5HT_{2c} receptor mRNA, application of 5HT (10 nM) elicited large oscillatory currents, and

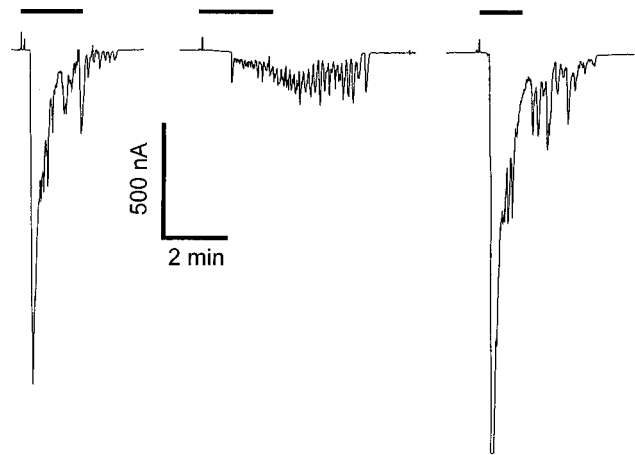


FIG. 2. Inhibition of 5HT current by EEDQ. The center record shows the current elicited by 5HT (10^{-7} M) coapplied with EEDQ (5×10^{-4} M), the other records show currents elicited by 5HT (10^{-7} M) alone. Intervals between traces were about 3 min. Oocyte injected with newborn rat spinal cord mRNA.

this current was greatly reduced, but not completely abolished, after treating the oocytes with EEDQ (2×10^{-4} M for 75 min). To determine whether the inhibitory effect of EEDQ on the responses to 5HT was irreversible, the same oocytes were tested again after being removed from EEDQ, rinsed several times, and then kept in Ringer's solution for different periods of time. There was no recovery of the 5HT-elicited membrane currents even after more than 24 h of washing (Fig. 3).

The oscillatory Cl^- currents elicited by 5HT result from receptor activation of an endogenous phosphatidylinositol pathway and activation of Ca^{2+} -gated Cl^- channels (see Discussion). Therefore, EEDQ could be inhibiting the 5HT-elicited currents by blocking the phosphatidylinositol pathway or the Cl^- channels; or it could be interfering with the binding of 5HT to the 5HT_{2c} receptors. It seems that the receptor-channel coupling pathway is not greatly affected by EEDQ, because the native receptor responses to angiotensin II or rabbit serum that involve the same pathway (17, 18), were not blocked by a 3-h exposure to 2×10^{-4} M EEDQ (data not shown). Therefore, we proceeded to study the effects of EEDQ

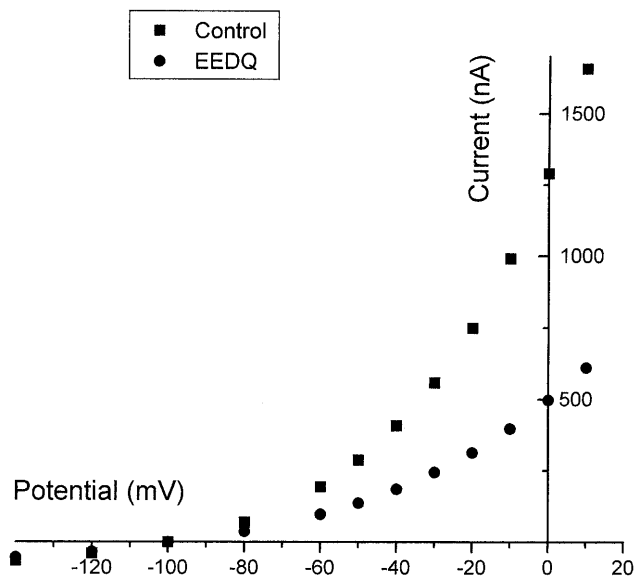


FIG. 1. Blockage of K^+ currents by EEDQ. Depolarizing pulses (3 sec) to -80 mV and beyond elicited a K^+ current that was appreciably reduced by 5×10^{-4} M EEDQ. Oocyte previously injected with newborn rat spinal cord mRNA to induce K^+ channels. Membrane potential held at -100 mV between pulses.

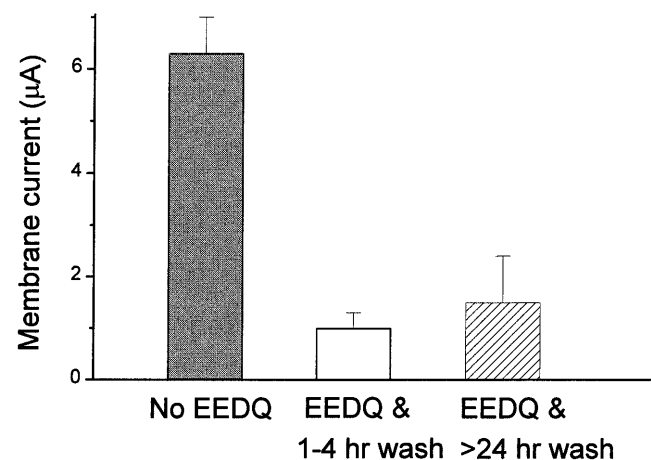


FIG. 3. Irreversible blockage of 5HT (10^{-8} M) currents by EEDQ in oocytes expressing 5HT_{2c} receptors. 5HT_{2c} receptor mediated oscillatory currents were measured in control oocytes and in oocytes pretreated with EEDQ (2×10^{-4} M, for 75 min). The EEDQ-treated oocytes were washed for 1–4 h, or for more than 24 h, in Ringer's solution before recording. Results are mean \pm SD ($n = 4$ oocytes). Recordings were made 6 and 7 days after cRNA injection.

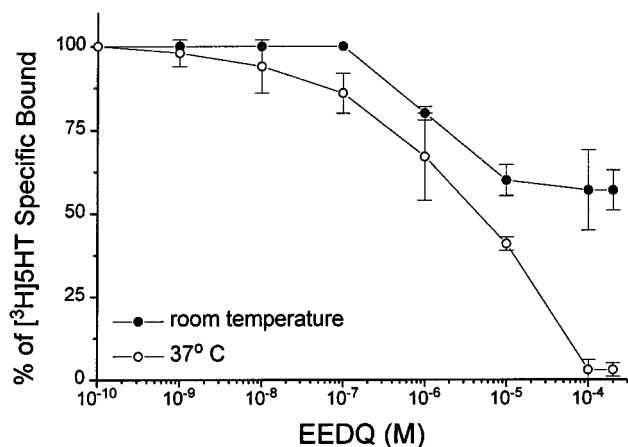


FIG. 4. Dose-response curve of EEDQ inhibition of binding of 5HT to 5HT_{2c} receptors at room temperature (●) or at 37°C (○). Ten nanomolar [³H]5HT was used for these experiments and those illustrated in Figs. 5 and 6. Results are mean ± SD (*n* = 3).

on the binding of [³H]5HT to membranes from HeLa cells transiently expressing 5HT_{2c} receptors.

Effect of EEDQ on the Binding of [³H]5HT to 5HT_{2c} Receptors. Functional rat 5HT_{2c} receptors were transiently expressed in HeLa cells, and there were no detectable native 5HT receptors in nontransfected HeLa cells. When the membranes were incubated at 37°C for 1 h without EEDQ, the binding of [³H]5HT to the expressed 5HT_{2c} receptors was not affected (data not shown). In contrast, treatment of the HeLa cell membranes with EEDQ reduced the binding of [³H]5HT to the 5HT_{2c} receptors. This inhibitory effect was clearly dose-dependent, with half-maximal inhibition (IC₅₀) occurring with ≈5 μM EEDQ, at both room temperature (≈22°C) and 37°C (Fig. 4). Incubating the membranes with 2 × 10⁻⁴ M EEDQ, for 1 h at room temperature, caused a 40% decrease in the binding *B*_{max}, without altering significantly the *K*_d (Fig. 5); while at 37°C the same EEDQ treatment blocked [³H]5HT binding completely (Fig. 4).

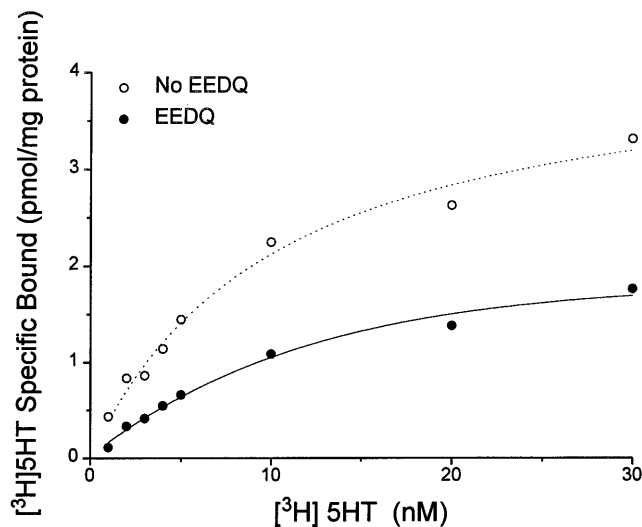


FIG. 5. Saturation assay of [³H]5HT binding to 5HT_{2c} receptors expressed in HeLa cells in the absence (○) or presence (●) of EEDQ. At room temperature (≈22°C), EEDQ pretreatment caused a 40% decrease in the binding *B*_{max}, without significantly altering the *K*_d: without EEDQ, the *K*_d and *B*_{max} were 10.1 nM and 4.2 pmol/mg protein; with EEDQ, 14.7 nM and 2.6 pmol/mg of protein, respectively. Similar results were obtained in two other experiments. In this and the following figures, unless otherwise stated, EEDQ treatment refers to incubating the membranes with 2 × 10⁻⁴ M EEDQ for 1 h.

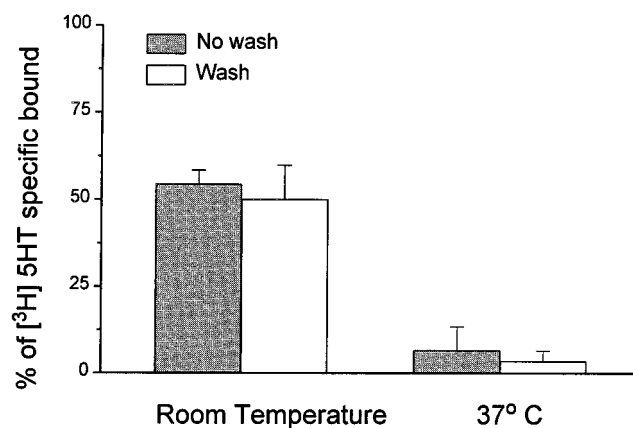


FIG. 6. Irreversible inhibition of 5HT binding to 5HT_{2c} receptors by EEDQ. Membranes were washed three times (≈1.5 h at 4°C) after EEDQ treatment, and exposed to 10 nM [³H]5HT. Results are mean ± SD (*n* = 3–4).

At room temperature, and also at 37°C, repeatedly washing the membranes treated with EEDQ did not restore the binding of [³H]5HT (Fig. 6). This indicates that, similar to the effect of EEDQ on the 5HT_{2c}-currents in oocytes, the EEDQ inhibition of binding to the receptors is practically irreversible. Therefore, it was puzzling that, at room temperature, the highest concentration of EEDQ used (2 × 10⁻⁴ M) blocked only 40% of the specific [³H]5HT (10 nM) binding (Fig. 4). We reasoned that if the inhibition was irreversible, it should achieve 100% inhibition with either a longer exposure, or with a higher concentration of EEDQ. Because higher concentrations of EEDQ could not be dissolved in the binding assay buffer (it seems that even at 2 × 10⁻⁴ M the EEDQ may not be fully in solution), we decided to test whether increasing the duration of EEDQ treatment enhanced the inhibition. Indeed, the binding of [³H]5HT to 5HT_{2c} receptors was increasingly inhibited with longer EEDQ incubations, and after an 8-h incubation with 10⁻⁵ M EEDQ at room temperature, the specific [³H]5HT binding was blocked completely (Fig. 7).

Similar to its inhibitory effect on the 5HT_{2c} receptors, EEDQ also blocked irreversibly the binding of [³H]5HT to 5HT receptors in rat cortex membranes (Fig. 8). Interestingly, at room temperature, EEDQ (2 × 10⁻⁴ M) blocked the binding to rat cortical 5HT receptors much more effectively than to the recombinantly expressed 5HT_{2c} receptors (80% compared with 40%).

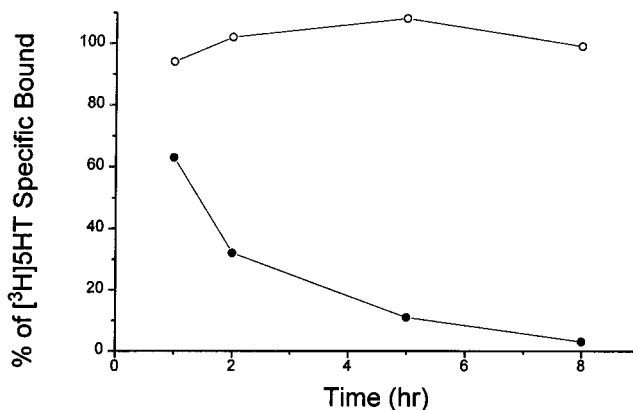


FIG. 7. Time course of EEDQ inhibition. After an 8-h incubation at room temperature, 10⁻⁵ M EEDQ blocked almost completely the [³H]5HT (10 nM) binding to 5HT_{2c} receptors (●). Without the EEDQ treatment, incubating the membranes at room temperature for 8 h did not change the [³H]5HT binding (○). This experiment was repeated twice with similar results.

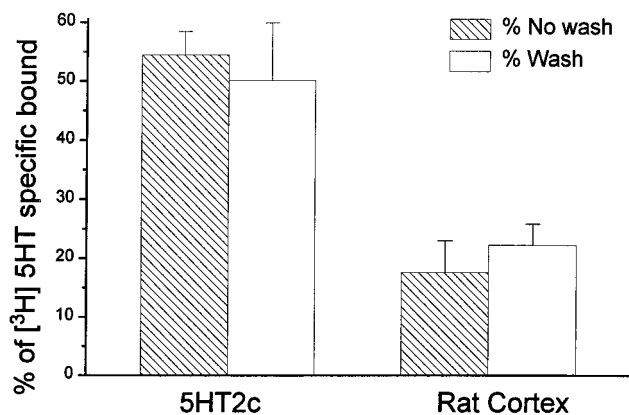


FIG. 8. Differential sensitivity of 5HT2c and rat cortex 5HT receptors to EEDQ. The binding of [³H]5HT to rat cortex 5HT receptors was blocked more than that to 5HT2c receptors. Results are mean \pm SD ($n = 2-4$).

DISCUSSION

EEDQ inactivation of receptors involves activation of a carboxyl group and crosslinking with a nucleophilic group near the receptor binding site and was first used to block irreversibly α -adrenergic receptors (19). Since then, EEDQ has been used as an irreversible blocker of muscarinic, dopamine, and H3 histamine receptors (20–22), and of several 5HT receptor subtypes including 5HT2a, 1a, 1b, and 1f receptors (9–12); although it seems to have little effect on 5HT3 receptors (23). Our results show clearly that EEDQ blocks the binding of 5HT to 5HT2c receptors and also blocks their electrophysiological responses. Thus, (i) in *Xenopus* oocytes, EEDQ blocks irreversibly the 5HT2c receptor-mediated oscillatory currents elicited by 5HT; (ii) in HeLa cell membranes, EEDQ inhibits irreversibly the binding of [³H]5HT to 5HT2c receptors by decreasing the B_{max} without changing the K_d ; (iii) the inhibition by EEDQ is dose- and time-dependent; (iv) the inhibition by EEDQ is more complete at 37°C than at room temperature. However, because there was virtually no difference in the apparent IC_{50} values ($\approx 5 \mu M$) at these two temperatures, the differences may be due, at least in part, to a higher solubility of EEDQ in the receptor binding mixture at 37°C.

The oscillatory currents elicited by activation of 5HT2c receptors in oocytes are generated by activation of a receptor-channel coupling pathway that involves phospholipase C hydrolysis of inositol phospholipids, release of intracellular Ca^{2+} by inositol trisphosphate, and activation of Ca^{2+} -dependent Cl^- channels in the oocyte membrane (14, 24–26). Therefore, the inhibition of the 5HT2c currents by EEDQ could have been due, in principle, to a blockage of binding of 5HT to the 5HT2c receptors, and/or blockage of any component of the phospholipase C receptor-channel coupling pathway. Although it is clear that EEDQ blocks some ionic channels, our results show clearly that EEDQ does have a direct inhibitory effect on agonist binding to 5HT2c receptors, which is mainly responsible for the inhibition of the 5HT2c currents. This is in agreement with a previous report suggesting that EEDQ treatment does not affect G-protein and phospholipase C activity (27).

We also compared the effect of EEDQ on 5HT receptors of rat cortex and hippocampus membranes with its effect on the cloned rat 5HT2c receptors. Many types of 5HT receptors are present in rat cortex and hippocampus including, at least, the 1a, 1b, 1e, 1f, 2a, 2c, 3, and 4 serotonin receptors, and mRNAs of the 5a, 5b, 6, and 7 receptor subtypes also have been detected in these brain areas (28). It is interesting to note that treatment with EEDQ caused a greater reduction in the binding of [³H]5HT to rat cortex 5HT receptors than to cloned 5HT2c receptors. This indicates that EEDQ is a more potent blocker of other subtypes of 5HT receptors present in rat

cortex than of the cloned 5HT2c receptors. This agrees with the results of a recent study that also demonstrated differential sensitivity of 5HT1a, 5HT1b, and 5HT2c/2a receptors to *in vivo* EEDQ treatment (29). In that study, EEDQ was shown to reduce ligand binding to 5HT receptor subtypes in rat cortical homogenates with a rank order: 5HT1a > 5HT1b > 5HT2a \approx 5HT2a/2c.

In many previous works, receptor antagonist binding was used to study the action of EEDQ, and it is not yet clear that the function of those receptors was similarly affected. More experiments, like those described here, will help to determine the potency for functional inactivation of the different receptors, and thus contribute to a better understanding of their structure/function relations.

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