Transcriptional Mechanisms for Induction of 5-HT\textsubscript{1A} Receptor mRNA and Protein in Activated B and T Lymphocytes*  

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Mohamed Abdouh\textsuperscript{§}, John M. Storring\textsuperscript{§}, Mustapha Riad\textsuperscript{§}, Yves Paquette\textsuperscript{§}, Paul R. Albert\textsuperscript{**}, Elliot Drobetsky\textsuperscript{§}, and Edouard Kouassi\textsuperscript{††††}  

From the Human Health Research Center, INRS-Institut Armand-Frappier, Pointe-Claire, Quebec H9R 1G6, the Departments of Pharmacology and Pathology and Cellular Biology, University of Montreal, Montreal, Quebec H3C 3J7, the Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, and the Guy-Bernier Research Center, Maisonneuve-Rosemont Hospital, and the Neuroscience Research Institute, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada  

Serotonin (5-HT) up-regulates B and T lymphocyte proliferation by activating mitogen-induced cell surface 5-HT\textsubscript{1A} receptors. The mechanism of 5-HT\textsubscript{1A} receptor induction by B and T cell mitogens at the mRNA and protein levels in mouse splenocytes was addressed. Quantification by RNase protection assay showed maximal increases of 3.4-, 3.0-, 3.8-, and 4.9-fold in relative mRNA and protein levels in mouse splenocytes was addressed. Additionally, we used an affinity-purified anti-5-HT1A antibody to detect 5-HT1A receptor in activated B and T lymphocytes.  

5-HT1A receptor expression in B and T lymphocytes.  

Among the numerous 5-HT receptors, 5-HT\textsubscript{1A} belongs to G-protein-coupled receptor superfamily and is also widely distributed in brain and immune tissues (6, 7). The 5-HT\textsubscript{1A} gene has been cloned previously in human (8, 9), rat (10), and mouse (11), manifesting very high nucleotide and amino acid sequence homology in their respective putative transmembrane regions. 5-HT\textsubscript{1A} mRNA has been detected in various human tissues including lymph nodes, spleen, and thymus (8), as well as in human peripheral blood mononuclear cells (12) and activated T lymphocytes (13). In functional studies using selective agonists and antagonists, it has been shown that the 5-HT\textsubscript{1A} receptor is implicated in the regulation of T cell responses including human T-cell proliferation (13–16), production of Th1 cytokines such as interleukin-2 and interferon-\gamma both in mice (17) and in human (15, 16), and contact sensitivity reactions in mice (17). We have shown previously that mitogen-stimulated B lymphocyte proliferation in rodents is up-regulated by 5-HT via specific interaction with the 5-HT\textsubscript{1A} receptor (18). Thus, immune and inflammatory responses may be regulated in part through 5-HT\textsubscript{1A} receptor expression in B and T lymphocytes.  

A recent review of the role of 5-HT in the immune system and in neuroimmune interactions has underscored the necessity of characterizing the distribution of the various 5-HT receptors in different immune cell populations, preferably by using molecular biological methods (7). The previous studies cited above using essentially functional and radioligand binding criteria suggest that 5-HT\textsubscript{1A} receptor expression is increased following mitogen stimulation of both murine B cells (18) and human T cells (13), but little is known about the molecular mechanisms underlying this effect. Nuclear factor-\kappaB (NF-\kappaB) is a ubiquitous and inducible transcription factor involved in many immune and inflammatory responses, including activation and proliferation of B and T lymphocytes stimulated by mitogens such as LPS, PHA, and PMA (19–21). NF-\kappaB is mainly composed of p50 and p65 subunits, which are normally retained in the cytosol of nonstimulated cells by inhibitory molecules, I\kappaB. In response to stimuli, I\kappaB are rapidly phosphorylated and degraded, allowing translocation of NF-\kappaB complexes into the nucleus and activation of NF-\kappaB elements (22). In this report, we used RNase protection assay to quantitate the expression of 5-HT\textsubscript{1A} receptor mRNA in unstimulated versus mitogen-stimulated mouse splenocytes. In addition, we took advantage of the availability of pharmacological inhibitors of NF-\kappaB (23–25) to explore its role in regulation of 5-HT\textsubscript{1A} receptor mRNA expression following mitogenic stimulation. Additionally, we used an affinity-purified anti-5-HT\textsubscript{1A} anti-
serum (26) to evaluate the expression of the 5-HT<sub>1A</sub> receptor protein in the splenocytes. Our data demonstrate that 5-HT<sub>1A</sub> receptor mRNA and protein are markedly increased following mitogenic stimulation of B and T lymphocytes with similar quantitative variation in these lymphocyte populations. Furthermore, our data indicate that up-regulation of mitogen-stimulated B and T lymphocyte 5-HT<sub>1A</sub> receptor occurs at the transcriptional level, and that mitogen-induced nuclear translocation of NF-κB may be one of the important signaling mechanisms involved.

**EXPERIMENTAL PROCEDURES**

**Mice and Reagents**—Female BALB/c mice, 6–12 weeks of age, were purchased from Charles River (St-Constant, Canada) and maintained in our animal facilities until use. All culture media were purchased from Life Technologies, Inc. (Burlington, Canada). Fetal bovine serum was purchased from HyClone (Logan, UT), and diazoyed against PBS to remove molecules of molecular weight <12–14 kDa. Escherichia coli LPS (serotype 0111:B4), PHA, ConA, PMA, and 5-HT hydrochloride were from Sigma, R(+)-8-OH-DPAT hydrobromide (R-DPAT) and WAY100635 maleate from RBI (Natick, MA), and ionomycin from Calbiochem (La Jolla, CA). [3H]Thymidine (specific activity 2 Ci/mmol) was obtained from ICN (Santa Isabella, CA), and [3H]WAY100635 (specific activity 51 Ci/mmol) from Amersham Pharma Biotech (Little Chalfont, United Kingdom). Anti-5-HT<sub>1A</sub> anti-serum was produced as described previously (26), and all other Ab were from PharMingen (San Diego, CA).

**Isolation and Stimulation of Splenocytes**—BALB/c mice were killed by cervical dislocation. Spleens were then aseptically harvested and gently teased into a single-cell suspension in Hank’s balanced salt solution. Red blood cells were removed by osmotic shock with NH<sub>4</sub>Cl, and splenocytes were resuspended in a culture medium consisting of RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), and 10% decomplemented fetal bovine serum. Cells were cultured in flat-bottomed 96-well culture plates (Life Technologies, Inc.) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C at a density of 4 × 10<sup>4</sup> cells/well in a total volume of 200 μl. Cells were stimulated by incubation for different periods of time in the presence or absence of LPS (10 μg/ml), PHA (20 μg/ml), ConA (5 μg/ml), or a combination of PMA (1 ng/ml) and ionomycin (500 ng/ml).

In some experiments, splenocytes were incubated with 10 μg/ml actinomycin D (ICN, Saint-Laurent, Canada), to distinguish between existing and newly transcribed mRNA. The reactions were carried out using the RNase Protection Assay—Direct Protect System (Ambion) and 18 S ribosomal RNA as an internal standard. To prepare the template for 5-HT<sub>1A</sub> riboprobes, the first 860 bp of the mouse 5-HT<sub>1A</sub> cDNA were cut from the MA1-KS<sup>+</sup> vector (11) using the PstI enzyme. This cDNA fragment was subsequently inserted in the antisense orientation with respect to the T3 RNA polymerase promoter found in the pBluescript II KS<sup>+</sup> plasmid (Promega). To synthesize radiolabeled 5-HT<sub>1A</sub> antisense cRNA, the plasmid was linearized with the enzyme BssHII and transcribed with T3 RNA polymerase (Ambion) and 50 μCi of 800 Ci/mmol [α<sup>32</sup>P]UTP (Mandel, Guelph, Canada) using the MaxScript in vitro transcription kit (Ambion) at 37 °C for 1 h. The resulting transcripts were then treated with 2 units of RNase-free DNase I at 37 °C for 15 min. The 18 S ribosomal RNA antisense probe was generated using the RNA transcript kit (Ambion), which was transcribed with T3 RNA polymerase in the presence of 30 μC of [α<sup>32</sup>P]UTP. Total RNA was extracted from samples of 10<sup>6</sup> cells in 50 μl of Lysis/Denaturing solution (Ambion) and coprecipitated with the freshly radiolabeled 5-HT<sub>1A</sub> antisense cRNA, the plasmid was linearized with the enzyme BssHII and transcribed with T3 RNA polymerase in the presence of 30 μC of [α<sup>32</sup>P]UTP. Total RNA was extracted from samples of 10<sup>6</sup> cells in 50 μl of Lysis/Denaturing solution (Ambion) and coprecipitated with the freshly radiolabeled 5-HT<sub>1A</sub> antisense cRNA, the plasmid was linearized with the enzyme BssHII and transcribed with T3 RNA polymerase in the presence of 30 μC of [α<sup>32</sup>P]UTP. 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**B and T Lymphocyte Mitogenic 5-HT$\textsubscript{1A}$ Receptor**

Fig. 1. 5-HT$\textsubscript{1A}$-mediated up-regulation of mitogen-stimulated mouse T and B lymphocyte proliferation. Mouse splenocytes were pre-incubated for 30 min at 37°C in the presence or absence of the 5-HT$\textsubscript{1A}$ receptor antagonist WAY100635 (WAY; 5 x 10$^{-4}$ M), and then cells were stimulated with 20 μg/ml PHA (A) or 1 ng/ml PMA plus 500 ng/ml ionomycin (B) in the presence or absence of 10$^{-5}$ M 5-HT or 5 x 10$^{-5}$ M R-DPAT as indicated. Cells were incubated for 72 h, and proliferation was measured by $^{3}$H]thymidine uptake during the last 6 h of culture. Student’s t test was performed. For mitogen-stimulated splenocytes versus mitogen-stimulated splenocytes in the presence of WAY100635, $p$ value was not statistically significant; for mitogen-stimulated splenocytes versus mitogen-stimulated splenocytes in the presence of 5-HT or R-DPAT ($\ddagger$), $p < 0.05$; for mitogen-stimulated splenocytes + 5-HT or R-DPAT versus mitogen-stimulated splenocytes + WAY100635 + 5-HT or R-DPAT (*), $p < 0.05$.

**Immunocytochemistry Analysis of 5-HT$\textsubscript{1A}$ Receptor Protein—Cells (10$^6$) were layered 1 h at room temperature on microscope slides pre-treated with 50 μg/ml poly-lys-lysin. Slides were rinsed with PBS (50 mM, pH 7.4), fixed for 1 h at room temperature with 2% pararformaldehyde in PBS, and washed in PBS. Cells were then preincubated for 1 h in a blocking solution of PBS containing 5% normal goat serum, 0.2% Triton X-100, and 0.5% gelatin to saturate nonspecific sites, and incubated for 2 h with a 1/1000 dilution of rabbit anti-5-HT$\textsubscript{1A}$ antisera. After washes in PBS (three times for 10 min each time), the slides were incubated for 1 h with biotinylated goat anti-rabbit IgG or FITC-conjugated anti-Thy-1.2 Ab, and then with the anti-5-HT$\textsubscript{1A}$ receptor antiserum followed by goat anti-rabbit-Ig-PE as described above.

**RESULTS**

5-HT$\textsubscript{1A}$ Receptor-mediated Up-regulation of Mitogen-stimulated B and T Lymphocyte Proliferation—Previously, we demonstrated that 5-HT increases mitogen-stimulated murine B lymphocyte proliferation through a 5-HT$\textsubscript{1A}$ receptor-mediated mechanism (18). Here, we used mouse splenocytes stimulated by the T cell mitogen PHA to determine whether T lymphocyte proliferation is influenced by 5-HT$\textsubscript{1A}$ receptor ligands. Preliminary dose response studies indicated that 5-HT (10$^{-11}$ to 10$^{-4}$ M) and the selective 5-HT$\textsubscript{1A}$ receptor agonist R-DPAT (10$^{-11}$ to 10$^{-7}$ M) increased PHA-stimulated T lymphocyte proliferation in a dose-dependent manner with optimal concentrations of 10$^{-4}$ M and 5 x 10$^{-5}$ M, respectively. Those maximally effective concentrations were used in combination with the relatively selective 5-HT$\textsubscript{1A}$ receptor antagonist WAY100635 to evaluate receptor specificity of 5-HT and R-DPAT action. Fig. 1A shows that 5 x 10$^{-5}$ M WAY100635 effectively abrogated 5-HT- and R-DPAT-mediated enhancement of activated T lymphocyte proliferation, thus implicating the 5-HT$\textsubscript{1A}$ receptor in the control of T cell proliferation.

The combination of PMA plus ionomycin is known to bypass antigen receptor signaling in both B and T lymphocytes, engendering a potent activation and proliferation of these cells (29–31). To test whether 5-HT$\textsubscript{1A}$ ligands can influence B and T cell proliferation in this model, splenocytes were stimulated with a mitogenic combination of PMA (1 ng/ml) and ionomycin (500 ng/ml), in the presence of 5-HT or R-DPAT, with or without WAY100635. Fig. 1B shows that 5-HT and R-DPAT increased splenocyte proliferation induced by PMA plus ionomycin, and that WAY100635 reversed agonist-induced mitogenic potentiation, further indicating a role for 5-HT$\textsubscript{1A}$ receptor activation. Thus, we chose the model of mouse splenocytes incubated in the presence or absence of PMA plus ionomycin for most of the following experiments to further characterize the 5-HT$\textsubscript{1A}$ receptor mRNA and protein which are expressed in B and T lymphocytes.

5-HT$\textsubscript{1A}$ Receptor mRNA Expression in Mitogen-stimulated Splenocytes—The 5-HT$\textsubscript{1A}$ receptor belongs to the family of G protein-coupled receptors. These receptors are characterized by
Lane 1 was loaded with the RNA size marker, lymphocytes, and lanes 3 and 4 were sorted in the desired region by negative selection. The purity of the scatter-side scatter profiles in flow cytometry, and B and T lymphocytes, while cells treated with PMA plus ionomycin during 36–48 h were used for sorting of activated B and T lymphocytes. Freshly isolated mouse spleen cells were incubated for 48 h in the presence of culture medium or mitogen: LPS (10 μg/ml), PHA (20 μg/ml), ConA (5 μg/ml), or a combination of PMA (1 ng/ml) and ionomycin (500 ng/ml). All values represent the mean ± S.D. of at least four separate experiments.

| Mitogen         | No. of replicated experiments | Blast transformation | Increase in 5-HT1A mRNA expression |
|-----------------|-------------------------------|----------------------|-----------------------------------|
| No mitogen      | 7                             | 5 ± 2                | 0.98 ± 0.17                       |
| LPS             | 4                             | 41 ± 6               | 3.43 ± 1.41                       |
| PHA             | 4                             | 47 ± 13              | 3.03 ± 1.26                       |
| ConA            | 4                             | 83 ± 16              | 3.76 ± 1.42                       |
| PMA-ionomycin   | 7                             | 88 ± 11              | 4.90 ± 1.90                       |

*Resting and blast cells were distinguished by flow cytometry based on their forward scatter-side scatter profiles, and the values indicated represent the percentage of blast cells within the total cell population. Relative levels of 5-HT1A mRNA were determined by RNase protection assay using 18 S rRNA as an internal standard. Fold increase in 5-HT1A expression induced by mitogens was calculated by using the relative 5-HT1A level in freshly isolated splenocytes (incubation time, 0 h) as a reference.

**TABLE I**

Blast transformation and up-regulation of 5-HT1A mRNA expression in mitogen-stimulated mouse splenocytes

Freshly isolated mouse spleen cells were incubated for 48 h in the presence of culture medium or mitogen: LPS (10 μg/ml), PHA (20 μg/ml), ConA (5 μg/ml), or a combination of PMA (1 ng/ml) and ionomycin (500 ng/ml). All values represent the mean ± S.D. of at least four separate experiments.

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**FIG. 3.** Time-dependent up-regulation of 5-HT1A mRNA expression in mitogen-activated B and T lymphocytes. Total RNA from splenocytes incubated for different periods of time in the presence or absence of mitogens was hybridized with radiolabeled 5-HT1A and 18 S riboprobes. The protected RNA fragments were separated on a 5% polyacrylamide-urea gel and quantitated by PhosphorImager analysis. Mitogens used: medium control (lanes 4, 5, 10, and 15), LPS (lanes 6, 11, and 16), PHA (lanes 7, 12, and 17), ConA (lanes 8, 13, and 18), and PMA plus ionomycin (lanes 9, 14, and 19). The incubation times were: 0 h (lane 4), 24 h (lanes 5–9), 48 h (lanes 10–14), and 72 h (lanes 15–19), as indicated. Lane 1 was loaded with the RNA size marker, lane 2 with the undigested 5-HT1A and 18 S antisense probes, which migrate at 180 and 99 bp, respectively, and lane 3 with RNase-digested 5-HT1A and 18 S antisense probes. Relative 5-HT1A levels were calculated by normalizing the 5-HT1A mRNA band to that of the 18 S rRNA, and -fold increases in 5-HT1A expression induced by mitogens were calculated by using the relative 5-HT1A level in freshly isolated splenocytes (incubation time, 0 h) as a reference.

**FIG. 4.** 5-HT1A mRNA expression in purified B and T lymphocytes. Freshly isolated splenocytes were used for sorting of resting B and T lymphocytes, while cells treated with PMA plus ionomycin during 36–48 h were used for sorting of activated B and T lymphocytes. Resting and activated cells were gated on the basis of their forward scatter-side scatter profiles in flow cytometry, and B and T lymphocytes were sorted in the desired region by negative selection. The purity of the sorting was verified by immunophenotyping, and it was 93–97%. Total RNA isolated from resting lymphocytes (lanes 1–3) and activated lymphocytes (lanes 4–6) was analyzed by RNase protection assay. Lanes 1 and 4 represent unsorted lymphocytes, lanes 2 and 5 are purified B lymphocytes, and lanes 3 and 6 are purified T lymphocytes.

**FIG. 5.** Time course of 5-HT1A mRNA degradation in splenocytes. Freshly isolated splenocytes were let unstimulated (A) or were stimulated with PMA-ionomycin (PMA-Iono) for 36 h (B). Actinomycin D (Act. D, 10 μg/ml) was then added to stop all de novo RNA transcription. Cells were harvested at the indicated times after actinomycin D treatment and analyzed for 5-HT1A mRNA and 18 S rRNA by an RNase protection assay. Plots in C show the linear regression of the percentage of remaining 5-HT1A mRNA relative to time 0 and after normalization to the 18 S rRNA. The coefficient of regression (r²) is shown for unstimulated cells and for PMA-ionomycin-stimulated cells; the calculated half-life was the same (26 h).
NF-κB inhibitors

| NF-κB inhibitors | PMA-Ionomycin | SN50 (μg/ml) | Syxam (mM) | PDTC (μM) |
|------------------|------------|-------------|------------|-----------|
| -                | +          | -           | -          | -         |
| +                | +          | +           | +          | +         |
| +                | +          | +           | +          | +         |

5-HT₁A mRNA and 18S rRNA expression.

To determine the potential role of the transcription factor NF-κB in mitogen-stimulated 5-HT₁A mRNA expression, splenocytes were pretreated with SN50, a cell-permeable peptide that specifically inhibits nuclear translocation of NF-κB (23). Fig. 6 shows that SN50 dose-dependently blocked the increase in 5-HT₁A mRNA expression induced by PMA plus ionomycin. In contrast, SN50M (50 μg/ml), an inactive analogue of SN50, was devoid of any effect on 5-HT₁A mRNA expression (Fig. 6), indicating the specificity of the inhibitory action of SN50 on NF-κB activation. The effect of other NF-κB inhibitors acting through mechanisms different to SN50 were tested. These include PDTC that acts as both a radical scavenger and inhibitor of NF-κB activation (24). Results showed that PDTC (5–50 μM) caused a dose-dependent inhibition of

Fig. 6. Blockage of mitogen-induced 5-HT₁A mRNA up-regulation by NF-κB inhibitors. Total RNA was extracted from freshly isolated splenocytes or from splenocytes stimulated with PMA plus ionomycin for 48 h and analyzed by RNase protection assay for 5-HT₁A mRNA and 18S rRNA expression. PMA plus ionomycin stimulation was performed in the presence or absence of the indicated NF-κB inhibitors used at the indicated concentrations. Shown are the bands corresponding to the protected 5-HT₁A and 18S fragments, and the values of fold increase in the relative amount of 5-HT₁A expression in PMA-ionomycin-treated cells compared with freshly isolated cells.

the signal obtained in LM1A cells. Among a total of six experiments, 5-HT₁A mRNA was expressed in all splenocyte samples stimulated by PMA plus ionomycin. In marked contrast, 5-HT₁A mRNA was not detectable (n = 4) or only barely detectable (n = 2), in samples of unstimulated splenocytes, and in the latter case only if the amount of cDNA introduced in the PCR reaction was increased by a factor of at least 4-fold. Each of the RNA samples were also subjected to PCR assays without RT, and no DNA fragment was obtained, indicating that the product observed represented amplification of 5-HT₁A cDNA, and did not result from amplification of contaminating genomic DNA.

5-HT₁A Receptor mRNA Is Up-regulated in Activated B and T Lymphocytes—A quantitative analysis of 5-HT₁A up-regulation following treatment with various B and T cell mitogens was performed using the RNase protection assay. Splenocytes were incubated for different periods of time in the presence of culture medium (unstimulated control), LPS, PHA, ConA, or a combination of PMA plus ionomycin, and 5-HT₁A mRNA levels were determined and normalized to 18S ribosomal RNA expression. Fig. 3 shows that 5-HT₁A mRNA was expressed in unstimulated splenocytes and was increased by all four mitogens in a time-dependent manner. The level of 5-HT₁A receptor mRNA was significantly enhanced after 24 h of incubation, reached a maximum at 48 h, and declined toward the level in unstimulated cells after 72 h of culture. As shown in Table I, relative to 5-HT₁A mRNA level in freshly isolated splenocytes, the level of increase in 5-HT₁A mRNA in splenocytes treated for 48 h with mitogens was 3.4-, 3.0-, 3.8-, and 4.9-fold with LPS, PHA, ConA, or PMA plus ionomycin, respectively. There was no increase in 5-HT₁A expression in cells incubated for 48 h in the absence of mitogen. The level of 5-HT₁A expression correlated positively with the frequency of mitogen-induced blast transformation which averaged 41%, 47%, 83%, and 88% in splenocytes stimulated for 48 h with LPS, PHA, ConA, or PMA plus ionomycin, respectively (Table I).

Since mitogen-stimulated splenocytes contain mixtures of different cell types in different activation states, a more rigorous approach was required to distinguish between B and T lymphocytes, and between resting and activated lymphocytes. To this end, resting and activated cells were separated by flow cytometry on the basis of their light scatter properties, while CD19-positive B cells and Thy-1.2-positive T cells were sorted by negative selection to 93–97% purity. 5-HT₁A mRNA and 18S rRNA expressions were measured in unsorted as well as in sorted B and T lymphocyte populations by the RNase protection assay. As shown in Fig. 4, 5-HT₁A mRNA was detected in both resting B and T cells purified from freshly isolated splenocytes, and its level was increased in both activated B and activated T cells purified from PMA plus ionomycin-stimulated lymphocyte populations. Quantitation by PhosphorImager analysis or densitometry indicated that the increase in the relative level of 5-HT₁A mRNA after stimulation with PMA plus ionomycin was similar in RNA samples from unsorted lymphocytes, purified B lymphocytes, or purified T lymphocytes (Fig. 4), suggesting similar regulation of 5-HT₁A mRNA expression in the two cell types.

Transcriptional Mechanisms of Mitogen-induced 5-HT₁A Receptor mRNA Expression—Since 5-HT₁A receptor mRNA accumulation in activated lymphocytes could be attributed to enhanced stabilization of existing mRNA and/or to enhanced transcription of new mRNA, studies were performed to distinguish between these two possibilities. Splenocytes were incubated or not with a combination of PMA and ionomycin for 36 h prior to inhibition of de novo mRNA transcription by addition of 10 μg/ml actinomycin D. Total RNA was then extracted at fixed time intervals for quantitation by RNase protection assay. As shown in Fig. 5 (A–C), the profiles of mRNA degradation were superimposable in PMA-ionomycin-treated and untreated cells with a similar half-life of 26 h, indicating an absence of stabilization of 5-HT₁A transcripts upon mitogenic stimulation. Additional experiments using splenocytes pretreated for 15 min with actinomycin D (10 μg/ml) and subsequently stimulated with PMA-ionomycin for 36–48 h, showed that 5-HT₁A mRNA expression did not increase over the level in unstimulated cells (data not shown), indicating that induction of 5-HT₁A mRNA is dependent on enhanced RNA transcription in mitogen-stimulated cells.

To determine the potential role of the transcription factor NF-κB in mitogen-stimulated 5-HT₁A mRNA expression, splenocytes were pretreated with SN50, a cell-permeable peptide that specifically inhibits nuclear translocation of NF-κB (23).
mitogen-induced up-regulation of lymphocyte 5-HT<sub>1A</sub> mRNA (Fig. 6). The immunosuppressive fungal metabolite gliotoxin (0.01–10 μg/ml), which appears to prevent degradation of I<sub>B</sub>-α (25), also caused a significant dose-dependent inhibition of mitogen-induced 5-HT<sub>1A</sub> up-regulation, while its inactive derivative methylgliotoxin (1–10 μg/ml) had no significant effect (data not shown).

5-HT<sub>1A</sub> Receptor Protein Expression in Splenocytes and Up-regulation by B and T Cell Mitogens—To evaluate the expression of 5-HT<sub>1A</sub> receptor protein in unstimulated and mitogen-stimulated lymphocytes, cells were permeabilized, fixed, and subsequently analyzed by indirect immunofluorescence and flow cytometry using a specific anti-peptide antisera directed against the third intracellular loop of the 5-HT<sub>1A</sub> receptor (26). Unstimulated splenocytes constitutively expressed the 5-HT<sub>1A</sub> protein, since greater than 90% of the cells were positive (Fig. 7A). After stimulation with PMA plus ionomycin, the mean fluorescence intensity of 5-HT<sub>1A</sub> immunoreactivity was 4 times greater (Fig. 7B) as compared with unstimulated cells, indicating an increased expression of 5-HT<sub>1A</sub> receptor protein. Cell incubation with buffer or with preimmune serum yielded a much lower, nonspecific fluorescence signal compared with the anti-5-HT<sub>1A</sub> antiserum, without any variation between unstimulated (Fig. 7A) and mitogen-stimulated cells (Fig. 7B). Moreover, binding of the antisera to an intracellular epitope was revealed by the absence of any consistent signal above background, unless the cells were permeabilized (Fig. 7, C and D). Double staining with anti-CD19 or anti-Thy1.2 and the anti-5-HT<sub>1A</sub> receptor antiserum showed similar levels of 5-HT<sub>1A</sub> receptor protein expression in activated B and T cells (data not shown), consistent with the similar level of induction of 5-HT<sub>1A</sub> receptor RNA in the cells.

To visualize the localization of the 5-HT<sub>1A</sub> receptor immuno-reactivity, unstimulated and PMA plus ionomycin-stimulated cells were permeabilized and incubated with the anti-5-HT<sub>1A</sub> antisera whose binding was revealed by immunocytochemistry using the horseradish peroxidase system. Labeling with the anti-5-HT<sub>1A</sub> receptor antiserum yielded a little staining in the unstimulated cells (Fig. 7E), while labeling of mitogen-stimulated cells showed a marked and uniform staining of the cell membrane, without any consistent staining of the cytoplasm (Fig. 7F). Labeling with the preimmune serum manifested no detectable signal in unstimulated and mitogen-stimulated lymphocytes (data not shown).

**DISCUSSION**

We showed previously that rat and mouse B lymphocyte in vitro proliferation in response to mitogens is up-regulated by 5-HT and 5-HT<sub>1A</sub> agonists, and that selective 5-HT<sub>1A</sub> antagonists reverse the effect (18). Others have shown that exposure to 5-HT<sub>1A</sub> agonists potentiates mitogenic responses in human T cells, both in vivo (14) and in vitro (13, 15, 16). Conversely, exposure to inhibitors of 5-HT synthesis or to 5-HT<sub>1A</sub> antagonists, leads to inhibition of mouse T cell responses in vivo and human T cell responses in vitro (17). Additionally, previous radioligand binding studies using [3H]8-OH-DPAT, a relatively selective 5-HT1A agonist, have shown an increased level of specific binding sites on murine B lymphocytes (18), and human T lymphocytes (13) after mitogenic stimulation. To further characterize the mechanisms of 5-HT<sub>1A</sub> receptor regulation in lymphocytes, we used a quantitative RNAase protection assay to assess mRNA expression in mouse splenocytes. Our results demonstrate that unstimulated B and T lymphocytes express low levels of 5-HT<sub>1A</sub> receptor mRNA that is markedly increased after mitogenic stimulation in vitro, in accord with the previous operational studies cited above. The results also show that purified B and T lymphocytes behave similarly in their basal and mitogen-induced 5-HT<sub>1A</sub> mRNA expression. The increased expression of 5-HT<sub>1A</sub> in mitogen-stimulated B and T cells is detectable at 24 h, and reaches a maximum after 48 h. This delayed induction of 5-HT<sub>1A</sub> mRNA correlates with the delayed augmentation of mitogen-induced B and T lymphocyte proliferation, which peaks at 72 h of cell incubation in the presence of 5-HT<sub>1A</sub> agonists. The late induction of 5-HT<sub>1A</sub> mRNA by mitogens also suggests an indirect action involving, e.g., mitogen-induced cytokine synthesis that may in turn regulate expression of the mRNA for 5-HT<sub>1A</sub> in target B and T cells.

Our studies further elucidate the possible mechanism of mitogen-stimulated increase in 5-HT<sub>1A</sub> mRNA. In particular, 5-HT<sub>1A</sub> mRNA stability was not altered by mitogen treatment, indicating that increased RNA stabilization plays no detectable role in the induction. In contrast, the RNA synthesis inhibitor actinomycin D completely blocked the mitogen-induced overex-
pression of lymphocyte 5-HT1A mRNA, indicating that induction is due to transcriptional stimulation, as opposed to post-transcriptional mRNA stabilization. Moreover, we show that exposure to several NF-κB inhibitors, including SN50, PDTC, and glitoxin, prevents any increase in 5-HT1A mRNA expression in mitogen-treated cells, suggesting a role for nuclear translocation of NF-κB in the up-regulation of lymphocyte 5-HT1A mRNA. Treatment of transfected Chinese hamster ovary cells with 5-HT1A agonists has been shown previously to increase 5-HT1A receptor density via activation of the NF-κB pathway, by stimulating the degradation of the inhibitory subunit, I-κB (32). Two consensus NF-κB binding sites (at −64 and −365 bp upstream from the initiation ATG) are located in a region with strong enhancer activity that is highly conserved in rat and mouse (33–35). In addition, recent studies have shown that the p50/p65 subunits of NF-κB are positive regulators of the rat 5-HT1A receptor promoter activity (36). Both a proximal NF-κB site (at −64) and a distal NF-κB site (at −365) contribute to this activity, whereas corticosteroids can repress it via their glucocorticoid receptor. A variety of immune and inflammatory stimuli are well known activators of nuclear translocation of NF-κB in lymphocytes (19–21). Thus, we hypothesize that, like 5-HT1A agonists, immune stimulation may increase nuclear translocation of NF-κB to enhance transcription of the 5-HT1A receptor gene in B and T lymphocytes. Conversely, part of the immunosuppressive and anti-inflammatory action of drugs such as glucocorticoids may be explained by repression of NF-κB-mediated induction of 5-HT1A receptor gene transcription in immune cells.

Immunostaining with the anti-5-HT1A antiseraum followed by flow cytometry or by immunocytochemistry analysis demonstrates that the expression of the receptor is low in unstimulated lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation. This is consistent with previous binding studies directed lymphocytes, while it increased markedly upon mitogenic stimulation.
