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Differential Effects of Fluoxetine on Murine B-cell Proliferation Depending on the Biochemical Pathways Triggered by Distinct Mitogens

Ana María Genaro, Valeria Ayelli Edgar and Leonor Sterin-Borda*

Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET), and Cátedra de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

ABSTRACT. The effect of fluoxetine on mitogen-induced B-cell proliferation was studied. In particular, we analyzed the influence of fluoxetine on the signal transduction pathways triggered after stimulation with lipopolysaccharide (LPS) and anti-immunoglobulin M antibodies (anti-IgM). We showed that fluoxetine had a dual effect on anti-IgM-stimulated B-cell proliferation: at optimal anti-IgM concentration, fluoxetine inhibited proliferation, whereas at suboptimal anti-IgM concentration, the drug enhanced proliferation. Fluoxetine exerted only an inhibitory effect on LPS-induced B-cell proliferation. Calcium influx seemed to be involved in these effects. BIOCHEM PHARMACOL **60**;9:1279–1283, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. B-cell proliferation; fluoxetine; LPS; anti-IgM; Ca^{2+}; inositol phosphates

Considerable evidence has accumulated over the last years that shows dynamic interactions occurring between the brain and the immune system [1]. Both stress and psychiatric illness are associated with impairment of immune function [1, 2]. Such alterations are particularly marked in depressed patients exhibiting melancholia [3]. Thus, depressed patients and people with depressive symptoms who are undergoing severe life stressors show alterations in cellular and humoral immunity including reduction of lymphocyte proliferation in response to mitogen stimulation [4, 5].

Fluoxetine, an antidepressive drug that belongs to the selective serotonin reuptake inhibitor group, is the drug of first choice in the treatment of depression [6]. Antidepressant therapy has been shown to be associated with immune dysfunction [7]. Berkeley *et al.* [8] found that fluoxetine inhibits the mitogen-induced maximal proliferative response in both T and B rat lymphocytes. In a previous report [9], we demonstrated that fluoxetine exerts an immunomodulatory effect upon murine T-cell proliferation, potentiating the proliferative effects of submitogenic doses of Con A† and diminishing the proliferation obtained with mitogenic Con A concentrations through a mechanism that involves Ca^{2+} mobilization. The purpose of the present study was to determine the effect of fluoxetine on B-cell proliferation. B-cells can be activated by mitogens via different biochemical pathways that either do or do not involve Ca^{2+} participation. Given these facts, we were interested in determining if Ca^{2+} mobilization participated in the effects of fluoxetine on the different pathways of mitogen-induced B-cell proliferation by using anti-IgM as a Ca^{2+}-dependent mitogen and LPS as a Ca^{2+}-independent mitogen [10].

MATERIALS AND METHODS

Drugs

Fluoxetine hydrochloride was provided by Bago. Verapamil, EGTA, Ca^{2+} ionophore A-23187 (calcimycin), LPS, and anti-IgM were purchased from the Sigma Chemical Co. [^{3}H]TdR was purchased from Amersham. Other materials were from standard commercial sources.

Cell Suspensions and Culture Conditions

Lymphoid cell suspensions from female BALB/c inbred mouse spleens were removed and disrupted through a 1-mm metal mesh, and the cell suspension was filtered through a 10-μm nylon mesh as described [11]. The suspension was depleted of non-lymphoid cells after centrifugation over Ficoll/Hypaque. After three washes in RPMI 1640, the cells were resuspended in RPMI 1640 supplemented with 10% of batch-tested non-stimulatory FBS, 2 mM glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 50 μM β-mercaptoethanol. B-cell enriched populations were obtained by treatment with anti-Thy-1 antibody plus complement as described elsewhere [12]. To remove adherent cells, the lymphocyte suspension was incubated twice in plastic

* Corresponding author: Dr. Ana María Genaro, CEFYBO-CONICET, Serrano 669, (1414) Buenos Aires, Argentina. Tel. (54) 11 4857 1434; FAX (54) 11 4856 2751; E-mail: vedgar@cefybo.edu.ar

† *Abbreviations*: Con A, concanavalin A; anti-IgM, anti-immunoglobulin M antibodies; LPS, lypopolysaccharide; [^{3}H]TdR, [*methyl*-^{3}H]thymidine; FBS, fetal bovine serum; S.I., stimulation index; PBS, phosphate-buffered saline; IP_3, inositol 1,4,5-triphosphate; IP, inositol phosphate; DAG, diacyl glycerol; PLC, phospholipase C; and PKC, protein kinase C.

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tissue culture dishes in the presence of RPMI 1640 supplemented with 5% FBS. The purity of the B-cell population was assessed by direct immunofluorescence and resulted in the expression of more than 95% IgG-bearing cells. Cell viability was estimated according to trypan blue exclusion criteria and was higher than 90%.

[^{3}H]TdR Incorporation

Proliferation was determined by culturing 10^5 cells/well in 96-well plates. [^{3}H]TdR (1 μCi) was added for the last 18 hr of culture [11]. [^{3}H]TdR incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters of the acid-insoluble macromolecular fraction. Results are expressed in terms of the S.I., calculated as the ratio between disintegrations per minute (dpm) values obtained in experimental cultures and those obtained in control cultures with unstimulated cells. B-cell mitogen-stimulated cultures displayed proliferation kinetics expected for LPS and for anti-IgM, with a peak of proliferation on day 3 of culture for both mitogens [10, 13].

Phosphoinositide Assays

Phosphoinositide hydrolysis was monitored as previously described [14]. Briefly, 10^6 cells were labeled overnight at 37° with 2.5 μCi *myo*-[^{3}H]inositol/mL. At the time of the assay, cells were washed with PBS, viability was checked by the trypan blue exclusion test (≥85%), and cells were incubated at 37° with 10 mM LiCl for 10 min. Then cells were incubated for 20 min alone or in the presence of the indicated drugs. The reaction was stopped by the addition of 0.1 mL HCl (0.22 M) in 1 mL chloroform:methanol (1:2). IPs were extracted by the addition of chloroform and water (1:1). Water-soluble [^{3}H]inositol phosphates were collected by anion exchange chromatography as described by Berridge *et al.* [15]. Results corresponding to the IP peak were expressed as absolute values of area units under the curve per 10^6 cells, following the criteria of Simpson's equation.

Statistical Analysis

Student's *t*-test for unpaired values was used to determine the level of significance. When multiple comparison was necessary after ANOVA, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P \leq 0.05$.

RESULTS

Effects of Fluoxetine on LPS and Anti-IgM-Induced B-cell Proliferation

Figure 1A shows that the maximal proliferative response to LPS was obtained at 15–60 μg/mL. Fluoxetine exerted an inhibitory effect at all LPS concentrations tested, independent of the basal LPS-proliferative response. Figure 1B shows that the maximal proliferative response to anti-IgM was obtained at 20 μg/mL. Fluoxetine exerted a dual effect on this response, depending on the basal anti-IgM proliferative response: an inhibitory effect at the optimal mitogenic anti-IgM concentration (20 μg/mL) and a stimulatory effect at a suboptimal mitogenic anti-IgM concentration (5 μg/mL). Fluoxetine exerted these effects in a range of concentrations between 10^{-7} and 10^{-5} M (data not shown).

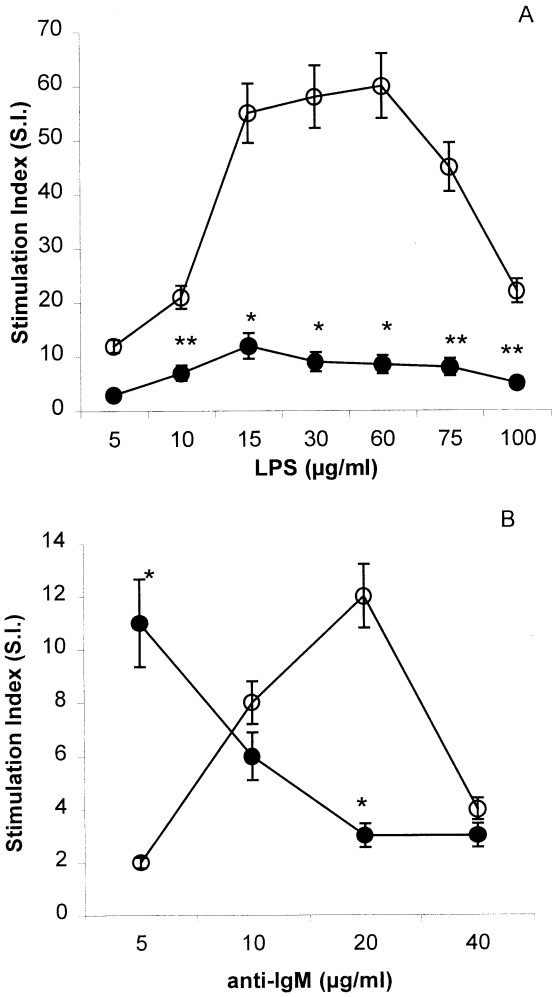

FIG. 1. (A) Effect of fluoxetine on LPS-induced B-cell proliferation. B lymphocytes were stimulated with LPS at the indicated concentrations in the absence (○) or in the presence (●) of fluoxetine (10^{-6} M). Basal (unstimulated) [^{3}H]TdR incorporation was 2800 ± 288 dpm in the absence of fluoxetine, and 3300 ± 372 dpm in the presence of fluoxetine. Results show the S.I. obtained as the ratio between stimulated and unstimulated dpm values. Data are the means ± SEM from five independent experiments of triplicate cultures. Key: (*) $P \leq 0.01$; and (**) $P \leq 0.05$. (B) Effect of fluoxetine on anti-IgM-induced B-cell proliferation. B lymphocytes were stimulated with anti-IgM at the indicated concentrations. Open circles (○) show the S.I. obtained in the absence of fluoxetine, and closed circles (●) show the S.I. obtained in the presence of 10^{-6} M fluoxetine. [^{3}H]TdR incorporation values for unstimulated B cells were similar to those shown in panel A. Data are the means ± SEM from five independent experiments of triplicate cultures. Key: (*) $P \leq 0.01$.

TABLE 1. Effect of fluoxetine on IP formation

Mitogen	Drug	IP formation (AUC/10^6 cells)
None	None	1395 ± 102
	Fluoxetine 10^{-7} M	1583 ± 124
	Fluoxetine 10^{-6} M	1533 ± 119
	Fluoxetine 10^{-5} M	1476 ± 126
Anti-IgM, 5 μg/mL	None	1782 ± 114
	Fluoxetine 10^{-7} M	2146 ± 198
	Fluoxetine 10^{-6} M	2274 ± 201
	Fluoxetine 10^{-5} M	2323 ± 203
Anti-IgM, 20 μg/mL	None	3156 ± 158
	Fluoxetine 10^{-7} M	3539 ± 241
	Fluoxetine 10^{-6} M	3651 ± 237
	Fluoxetine 10^{-5} M	3704 ± 296
LPS, 5 μg/mL	None	1402 ± 106
	Fluoxetine 10^{-7} M	1356 ± 103
	Fluoxetine 10^{-6} M	1433 ± 111
	Fluoxetine 10^{-5} M	1489 ± 118
LPS, 30 μg/mL	None	1399 ± 114
	Fluoxetine 10^{-7} M	1435 ± 123
	Fluoxetine 10^{-6} M	1456 ± 131
	Fluoxetine 10^{-5} M	1501 ± 146

Cells (10^6) were incubated with myo[^{3}H]-inositol precursor, and the water-soluble IPs obtained were determined as described in Materials and Methods. Data represent the means ± SEM from 3 independent experiments of duplicate cultures.

Participation of Ca^{2+} in the Effects of Fluoxetine

To analyze the influence of fluoxetine on intracellular Ca^{2+} released from IP_3-sensitive Ca^{2+} stores, we measured the formation of IPs on both LPS- and anti-IgM-induced B-cell proliferation. As expected, anti-IgM increased IP formation, whereas LPS did not induce phosphoinositide hydrolysis (Table 1). Fluoxetine neither exerted a significant effect on basal IP formation nor modified the IP formation obtained in the presence of anti-IgM or LPS (Table 1), indicating that intracellular Ca^{2+} release does not seem to participate in the effect of fluoxetine on LPS- or anti-IgM-induced lymphocyte proliferation.

To study the effect of fluoxetine on extracellular Ca^{2+} influx, we performed experiments in the absence of external Ca^{2+} by using Ca^{2+} chelators, and in the presence of Ca^{2+} channel blockers at concentrations that do not affect basal proliferation. Table 2 shows that both the Ca^{2+} channel blocker verapamil and the Ca^{2+} chelator EGTA reversed the inhibitory effect of fluoxetine on the proliferation induced by LPS. The results obtained from Ca^{2+} blockers on anti-IgM-induced B-cell proliferation are difficult to interpret because both drugs (verapamil and EGTA) affected the proliferation *per se* at all the concentrations tested (Table 2). On the other hand, the effects of the Ca^{2+} ionophore A-23187 resembled those of fluoxetine on B-cell proliferation (Table 2).

DISCUSSION

Previously, we showed that fluoxetine exerts an immunomodulatory effect on Con A-induced T-cell proliferation, inhibiting the maximal proliferative response and increasing the suboptimal proliferative response. The participation of extracellular Ca^{2+} mobilization could be involved in these mechanisms [9]. In the present paper, we describe the effect of fluoxetine on B-cell proliferation. Early signals elicited after B-cell triggering have been elucidated in the last few years [16]. Activation via cross-linking of membrane immunoglobulins with anti-IgM antibodies or antigens elicits the generation of two second messengers after phosphatidyl inositol-specific PLC (lecithinase C; phosphatidylcholine–phosphohydrolase; EC 3.1.4.3) stimulation: IP_3, which in turn produces a transient increase in the cytosolic free Ca^{2+} levels, and DAG, which activates PKC (EC 2.7.1.37). In contrast, LPS does not induce activation of PLC but is able to activate lymphocytes by mechanisms that are completely independent from Ca^{2+} changes or DAG generation, although the activation of PKC has been proposed in the LPS-activated pathway [17]. Given the fact that anti-IgM and LPS use different pathways to stimulate B cells to proliferate, we used these mitogens to induce proliferation and to study the effect of fluoxetine. We found

TABLE 2. Participation of Ca^{2+} influx in the effects of fluoxetine

	[^{3}H]TdR incorporation (dpm)			
	LPS (μg/mL)		Anti-IgM (μg/mL)	
Treatment	5	30	5	20
None	33,515 ± 3,115	145,628 ± 15,429	5,119 ± 613	34,535 ± 3,603
Fluoxetine, 10^{-6} M	7,929 ± 529*	25,219 ± 2,145*	30,839 ± 3,213*	8,129 ± 915*
Verapamil, 10^{-6} M	27,649 ± 2,981	121,415 ± 15,340	3,246 ± 228	7,428 ± 792
Fluoxetine, 10^{-6} M + verapamil, 10^{-6} M	25,678 ± 2,619†	132,420 ± 11,112†	ND‡	ND‡
EGTA, 6 mM	26,708 ± 3,045	132,115 ± 14,230	2,245 ± 213	4,129 ± 15
Fluoxetine, 10^{-6} M + EGTA, 6 mM	29,327 ± 2,817†	139,428 ± 12,629†	ND‡	ND‡
A-23187, 2×10^{-6} M	8,599 ± 726*	21,122 ± 2,090*	31,129 ± 4,152*	9,223 ± 834*

Cells (10^6) were stimulated with LPS or anti-IgM alone (none) or in the presence of the drugs at the indicated concentrations and cultured for 3 days. [^{3}H]TdR incorporation was determined as indicated in Materials and Methods. Data represent the means ± SEM from 3 independent experiments of triplicate cultures.

* $P \leq 0.01$ versus basal proliferation (none).

† $P \leq 0.01$ versus fluoxetine alone.

‡ Not determined.

that fluoxetine exerts a differential effect depending on the type of mitogen used to induce the proliferation of B-cells. In fact, fluoxetine showed a dual effect on anti-IgM-stimulated B-cell proliferation, as we previously described for Con A-stimulated T-cell proliferation [9]: at optimal anti-IgM concentration, fluoxetine inhibited proliferation, whereas at suboptimal anti-IgM concentrations, the drug enhanced the proliferative response. On the other hand, fluoxetine showed only an inhibitory effect on LPS-induced B-cell proliferation at all concentrations tested.

As previously reported, modulation of Ca^{2+} influx can lead to either suppression or potentiation of the response of stimulated B lymphocytes [18]. On the other hand, Helmeste *et al*. [19] demonstrated that several antidepressants that are potent serotonin reuptake inhibitors, including fluoxetine, enhance platelet intracellular Ca^{2+} mobilization. Moreover, we previously found that intracellular Ca^{2+} elevation could be involved in the immunomodulatory effect exerted by fluoxetine on Con A-induced T-cell proliferation [9]. Given these facts, we studied Ca^{2+} participation in the effect of fluoxetine on B-cell proliferation.

The effect of fluoxetine is not due to the release of Ca^{2+} induced by the formation of IPs because the drug was not able to modify IP signaling obtained in the absence or in the presence of anti-IgM or LPS. On the other hand, the effect of fluoxetine on LPS-induced proliferation was inhibited by Ca^{2+} channel blockers and by Ca^{2+} chelators. It is important to note that we could not study the effect of these drugs on anti-IgM-induced proliferation because this response was affected by the absence of Ca^{2+} influx. These findings are in accord with those reported by Rawson *et al*. [20], who found that the bulk of the initial rise in intracellular Ca^{2+} following treatment with anti-IgM appears to come from outside the cell, since it is inhibited by Ca^{2+} chelators, resulting in an inhibition of B-cell stimulation. Our results could indicate that fluoxetine exerted its effect by inducing an intracellular Ca^{2+} increase, mainly from extracellular origin. This suggestion is also reinforced by the fact that Ca^{2+} ionophores and fluoxetine had similar effects on both anti-IgM- and LPS-induced B-cell proliferation. According to our results, fluoxetine could be affecting the proliferative response by increasing Ca^{2+} influx. When B cells were stimulated with anti-IgM, which is dependent on Ca^{2+} increase, a dual effect was observed. Probably fluoxetine could potentiate the proliferative response triggered by submitogenic anti-IgM concentrations, inducing an optimal Ca^{2+} increase. When an optimal response is obtained, fluoxetine could promote an excess of Ca^{2+} influx that negatively regulates the signaling pathway of anti-IgM. When B cells were stimulated with LPS, which is not dependent upon an increase in intracellular Ca^{2+}, only the inhibitory effect of fluoxetine was observed, probably by an excess of Ca^{2+} influx. Kolb *et al*. [18] suggested that an excess of Ca^{2+} influx could lead to the induction of apoptosis in B cells activated by lymphokines. The action of fluoxetine on the intracellular messengers that are involved in Ca^{2+}-dependent and Ca^{2+}-independent pathways that accomplish polyclonal B-cell triggering is the subject of ongoing investigations. It is worth noting that fluoxetine steady-state plasma concentrations achieved during treatment range between 0.15 and 1.5 μM [21, 22]. The effects of fluoxetine described in the present report were obtained using concentrations of the antidepressant that ranged between 10^{-7} and 10^{-5} M. Finally, our results could be of interest for understanding the contradictory reports about the effects of antidepressant therapy on immunity. Further experiments are needed to address these hypotheses.

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