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Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation

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Abstract

In this work, we studied the effect of fluoxetine on human T-lymphocyte proliferation using optimal and suboptimal concanavalin A concentrations. In particular, we analyzed the influence of fluoxetine on the kinases that are involved in intracellular signalling after stimulation with mitogens. We found that fluoxetine promoted the Ca^{2+}-mediated proteolysis of protein kinase C (PKC) and increased cyclic-AMP (cAMP) levels, thereby impairing lymphocyte proliferation, when optimal concanavalin A concentrations were used. In contrast, when suboptimal concanavalin A concentrations were used, fluoxetine only increased PKC translocation, without modifying cAMP levels, leading to T-cell proliferation. According to our results, fluoxetine has a dual effect on T-cell proliferation by modulating the PKC and protein kinase A pathways. This mechanism is thought to be mediated through Ca^{2+} mobilization.

Keywords: T-cell proliferation; Fluoxetine; Immune response; Protein kinase C; cAMP; Ca^{2+}

1. Introduction

There is considerable evidence that the brain and the immune system interact dynamically. Both stress and psychiatric illness are associated with impairments of the immune function. Major depressive disorder has been associated with a suppression of various aspects of the immune response, including a reduction of lymphocyte proliferation in response to mitogens, as well as a reduction of natural killer activity (Stein et al., 1991; Irwin, 1995). Such effects are particularly marked in depressed patients exhibiting melancholia (Kiecolt-Glaser et al., 1987; Irwin, 1995; Baldessarini, 1996), and at least, some of the immune changes (altered lymphocyte proliferation) were independent of age, sleep disturbances, menopausal status, or prior treatment with benzodiazepines (Kiecolt-Glaser et al., 1987; Schmidt et al., 1988; Levy et al., 1991; McDaniel, 1992; Kaplan et al., 1994; Irwin, 1995; Baldessarini, 1996).

Fluoxetine, an antidepressive drug that belongs to the selective serotonin reuptake inhibitor group, is the drug of first choice in the treatment of depression. Antidepressant therapy has been shown to be associated with immune dysfunction (Crowson and Magro, 1995). Fluoxetine was found to suppress lymphocyte proliferation in a dose-dependent manner (Berkeley et al., 1994). However, the immune effects of fluoxetine have been poorly studied. Previously, we demonstrated that fluoxetine exerts an immunomodulatory effect upon murine T-cell proliferation, depending on the cell proliferative state (Ayelli Edgar et al., 1996).

The purpose of the present study was to determine the effect of fluoxetine on human T-lymphocyte proliferation. We found that fluoxetine modulates the immune response by changing the activity of both the growth-promoting protein kinase C (PKC) and the inhibitory protein kinase A pathways. Ca^{2+} participation and the relationship between both pathways were also studied.

2. Materials and methods

2.1. Drugs

Fluoxetine hydrochloride {(±)-*N*-methyl-3-phenyl-[(α,α,α-trifluoro-*p*-tolyl)-oxy]-propyl-amine} was gener-

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ously provided by Bago. Ca^{2+} ionophore A 23187 (calcimycin), [^{3}H]thymidine (thymidine-methyl-^{3}H), concanavalin A, phorbol 12-myristate 13-acetate (4β,9α, 12β,13α,20-Penta-hydroxytiglia-1-6-dien-3 one 12β-acetate) (PMA), staurosporine (antibiotic AM-2282) and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma (St. Louis, MO). Human blood was obtained from normal volunteers. Other materials were from standard commercial sources.

2.2. Cell suspensions and culture conditions

Blood samples from normal human female volunteers (aged 30–40 years old) were drawn by venipuncture between 8:00 and 10:00 AM. Blood suspensions were depleted of non-lymphoid cells by centrifugation with Hysopaque. After three washes in RPMI 1640, the cells were resuspended in RPMI 1640 supplemented with 10% of batched–tested non-stimulatory fetal calf serum, 2 mM glutamine, antibiotics and 50 μM β-mercaptoethanol. Cell viability was estimated according to Trypan-blue exclusion criteria and was higher than 90%. T-lymphocyte-rich lymphoid cell suspensions were used in some experiments. T-cell-enriched populations were obtained by passage of the cell suspension through a nylon wool column according to the method of Julius et al. (1973). The cell suspension was more than 97% pure, as checked by direct immunofluorescence with fluorescein isothiocyanate isomer (FITC)-anti CD_3 antibody.

2.3. [^{3}H]thymidine incorporation

Proliferation was determined by culturing 10^5 cells per well in 96-well plates. [^{3}H]thymidine (1 μCi) was added for the last 18 h of culture (Genaro and Bosca, 1993). In some experiments, results are expressed in terms of the stimulation index (S.I.), calculated as the ratio between disintegrations per minute (dpm) obtained in stimulated cultures and those obtained in control cultures with unstimulated cells. T-cell mitogen-stimulated cultures displayed the proliferation kinetics expected for concanavalin A, with a peak of proliferation on the third day of culture.

2.4. PKC assay

After incubation with the appropriate ligands, lymphocyte suspensions (10^7 cells/ml) were immediately centrifuged ($5000 \times g$ for 30 s) and cell pellets were chilled on liquid N_2. The soluble (cytosolic fraction) and particulate fractions (pellet fraction) were obtained as described by Genaro and Bosca (1993). PKC enzyme was purified by filtration through a DE 52 column (3.5×0.5 cm). The enzyme was eluted in a buffer containing NaCl (120 mM), β-mercaptoethanol (10 mM), EGTA (ethylene glycol-bis(β-amino ethyl ether)N,N,N',N'-tetraacetic acid) (0.5 mM) and HEPES (*N*-[2-hydroxy-ethyl]piperazine-*N'*[2-ethanesulfone acid]) pH 7.4 (10 mM) (Diaz-Guerra and Bosca, 1990). The proteolytic fragment of PKC (termed protein kinase M, PKM) was eluted from a DE 52 column in buffer containing NaCl (300 mM). This kinase was characterized by its independence of Ca^{2+} and phospholipids and its ability to be inhibited by quercetin (3,3′,4′,5,7-Pentahydroxyflavone) (Junco et al., 1990). PKC was measured, by its histone kinase activity, at 30°C in an incubation volume of 170 μl as previously described (Diaz-Guerra and Bosca, 1990; Genaro and Bosca, 1993). The radioactivity retained on GF/C glass-fiber filters after filtration was determined by counting the oven-dried filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of Ca^{2+} and phospholipids. The activity of PKC in catalyzing ^{32}P incorporation into the substrate accounted for approximately 70–75% of the total kinase activity in the subcellular fractions. PKM was assayed in the absence of Ca^{2+} and phospholipids and in the presence of quercetin (10 μM) as specific inhibitor (Junco et al., 1990). The synthetic peptide myelin basic protein MBP-(4–14), a well-known specific PKC substrate (Jasuda et al., 1990) (Gibco BRL Life Technology), was also used in order to measure PKC activity purified from subcellular lymphocyte fractions, according to the instructions of the PKC assay system of Gibco. PKC specificity was confirmed by means of the PKC pseudosubstrate inhibitor peptide PKC-(19–36) provided by Gibco. PKC activity was 85–90% of the total kinase activity.

2.5. Cyclic-AMP (cAMP) production in intact cells

Cells (10^7 cells/ml) were incubated alone or with the appropriate ligands for the time indicated for each experiment. In some experiments, cells were pre-treated with IBMX (1 mM) at 30°C for 20 min in order to inhibit phosphodiesterase (PDE) activity. At the end of the incubation, 2 ml of chilled ethanol was added. Cells were homogenized, supernatants were evaporated at 55°C under nitrogen stream and cAMP contained in the residue was dissolved in 0.350 ml of assay buffer (Tris–HCl, pH: 7.4 (50 mM), theophylline (8 mM), 2-mercaptoethanol (6 mM), EDTA [ethylene-diaminetetraacetic acid] (1 mM). Aliquots of 100 μl samples were taken for nucleotide determination, using the protein kinase assay described by Brown et al. (1971).

2.6. Statistical analysis

The Student's *t*-test for unpaired values was used to determine the level of significance. When multiple comparison was necessary after analysis of variance, the Stu-

dent–Newman–Keuls test was applied. Differences between means were considered significant if $P \leq 0.05$.

3. Results

3.1. Effect of fluoxetine on human T-cell proliferation

Concanavalin A-stimulated T-lymphocyte cultures were used as a tool to study the effect of different fluoxetine concentrations on human T-cell proliferation. In order to determine whether the effect of fluoxetine is dependent on the basal mitogenic response, we evaluated proliferation in response to different concanavalin A concentrations. Fig. 1 shows a typical dose–response curve. As expected (Anderson et al., 1972), the response was markedly dose-dependent. Thus, concanavalin A, 4 μg/ml, gave an optimal response, whereas concanavalin A, 1 or 8 μg/ml, failed to stimulate the cells or gave a very weak response. In order to analyze the effect of fluoxetine on proliferation, we chose two concanavalin A concentrations: an optimal mitogenic (4 μg/ml) and a suboptimal submitogenic (2 μg/ml) concentration. Fluoxetine exerted an inhibitory effect at 4 μg/ml concanavalin A (Fig. 2a). In contrast, when 2 μg/ml concanavalin A was used, an increase in [^{3}H]thymidine incorporation was observed (Fig. 2b). The EC_{50} (concentration inducing 50% of the maximal response) values derived from the dose–response curve were

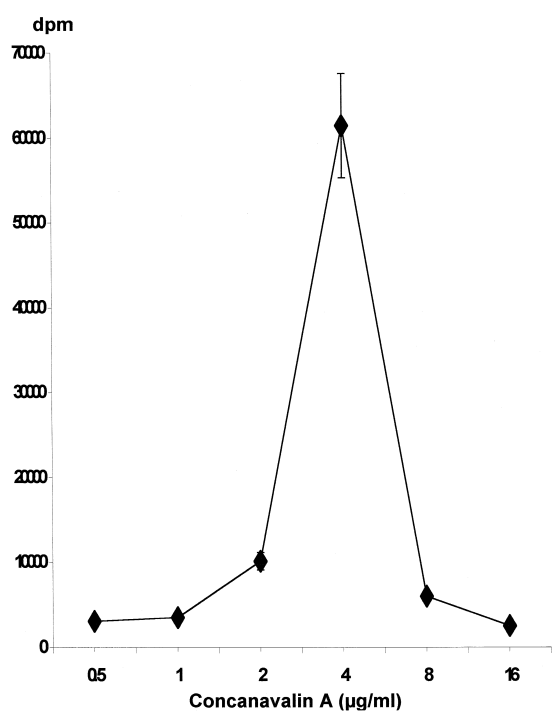


Fig. 1. T-cell proliferation in response to concanavalin A. T-lymphocytes were stimulated with concanavalin A at the indicated concentrations. Cells were cultured for 3 days and [^{3}H]thymidine incorporation was determined.

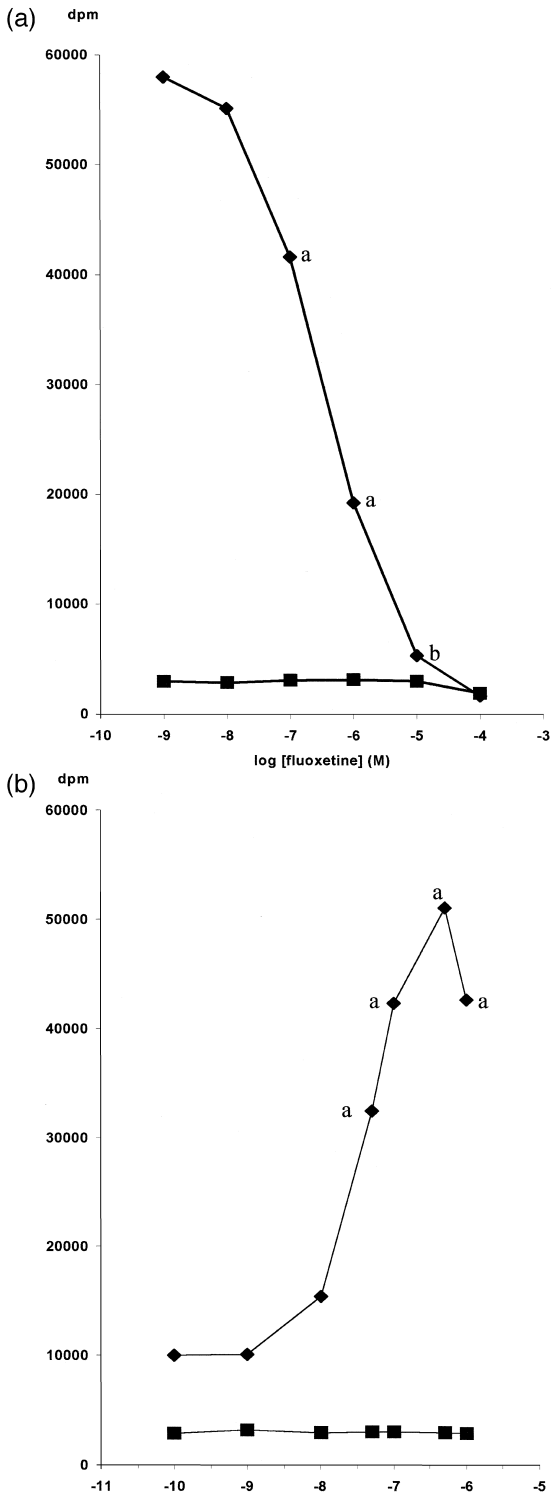
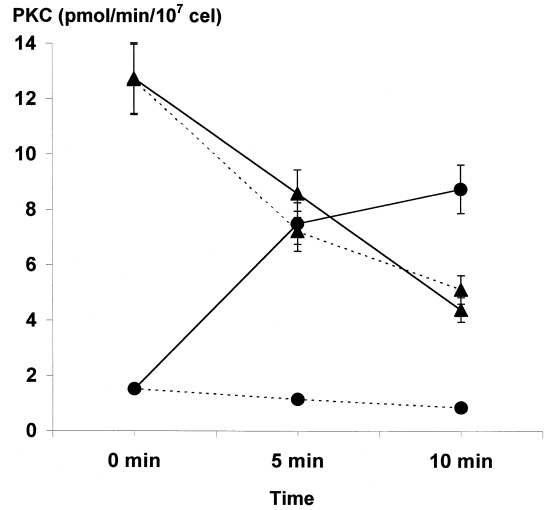


Fig. 2. Effect of fluoxetine on concanavalin A-stimulated T-cell proliferation (means ± S.E.M.; $n = 5$). Unstimulated (■) or concanavalin A-stimulated (♦) T-lymphocytes were cultured in the presence of different fluoxetine concentrations. Fluoxetine and concanavalin A were added at time 0 of the culture, then cells were cultured for 3 days and [^{3}H]thymidine incorporation was measured. Panel a shows the dose–response curve for T-lymphocytes stimulated with 4 μg/ml concanavalin A. Panel b shows the dose–response curve for T-lymphocytes stimulated with 2 μg/ml concanavalin A. $^aP \leq 0.05$. $^bP \leq 0.01$.

5×10^{-7} M for the inhibitory effect and 0.4×10^{-7} M for the stimulatory effect. It is worth noting that fluoxetine alone had no effect on T-cell proliferation (Fig. 2a and b).In order to analyze the temporal characteristics of the effect of fluoxetine on concanavalin A-stimulated T-lymphocyte proliferation, fluoxetine and concanavalin A were added to the cultures at different times. As shown in Table 1, the stimulatory effect of fluoxetine was evident when it was added either 180 min before or 120 min after the addition of concanavalin A. No effect of fluoxetine was observed when fluoxetine and concanavalin A were added with a time difference greater than that indicated above.The inhibitory effect was evident when fluoxetine and concanavalin A were added with a time difference of 60–90 min (Table 1).

3.2. Fluoxetine effect on mitogen to induced PKC activation

In order to analyze the effect of fluoxetine on concanavalin A-induced PKC activation, cells were incubated with concanavalin A in the presence or in the absence of fluoxetine for different times. Figs. 3 and 4 show the subcellular distribution of PKC activity at both optimal and suboptimal concanavalin A concentrations. At 4 μg/ml concanavalin A concentration (Fig. 3), PKC translocation toward the membrane was higher than that observed at 2 μg/ml concanavalin A (Fig. 4). Fluoxetine increased PKC translocation at 2 μg/ml concanavalin A (Fig. 4). In contrast, at 4 μg/ml concanavalin A, fluoxetine decreased the particulate PKC activity and no modification of the decrease of cytosolic PKC activity induced by concanavalin A was observed (Fig. 3). Possibly, proteolytic degradation of PKC occurred, which might explain the decrease in total PKC activity observed in this situation.There was a pronounced transient increase in PKM activity in the presence of fluoxetine and 4 μg/ml concanavalin A compared to 2 μg/ml concanavalin A (Figs. 3 and 4).



Treatment	Protein kinase M Activity (pmol/min/10^7 cel)	
	Basal	Concanavalin A 4 μg/ml
None	0.03 ± 0.01	0.21 ± 0.02
Fluoxetine 10^{-6} M	0.07 ± 0.02	2.31 ± 0.18[a]

Fig. 3. PKC distribution after T-cell stimulation with concanavalin A 4 μg/ml. T-lymphocytes were stimulated with 4 μg/ml concanavalin A. Soluble (▲) and particulate (●) PKC activity was measured in the absence (——) or in the presence (- - - -) of fluoxetine (10^{-6} M). Similar results were obtained when fluoxetine was added at 10^{-7} and 10^{-5} M (data not shown). PKM activity in the absence or in the presence of fluoxetine (10^{-6} M) is shown in the table below the figure. Data show PKC activity determined by using the specific peptide MBP-(4–14). Similar effects were observed when histone H1 was used for PKC activity determination. [a] $P \leq 0.01$.

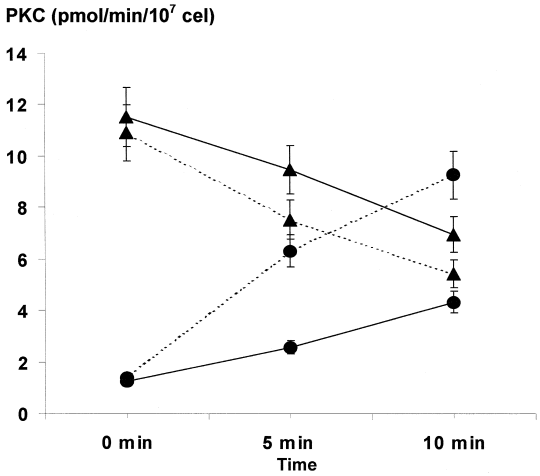
Table 1
Time-course of the effect of fluoxetine on concanavalin A-stimulated T-cell proliferation (means ± S.E.M.; $n = 3$)

Fluoxetine addition (min)[a]	Percentage (%) of control values[b] (concanavalin A)	
	4 μg/ml	2 μg/ml
−240	107 ± 8	109 ± 15
−180	103 ± 11	215 ± 23
−120	98 ± 13	319 ± 34
−90	104 ± 9	325 ± 45
−60	48 ± 5	442 ± 36
−30	35 ± 8	388 ± 52
0	38 ± 6	390 ± 40
+30	41 ± 9	410 ± 43
+60	52 ± 7	380 ± 36
+90	83 ± 9	358 ± 41
+120	111 ± 13	315 ± 42
+180	98 ± 9	99 ± 11

[a] T-lymphocytes were cultured in the presence of fluoxetine (10^{-6} M) and concanavalin A at the indicated concentrations. 'Time 0' indicates that fluoxetine and concanavalin A were added simultaneously at the start of the culture; '(−) time' indicates that fluoxetine was added before concanavalin A; '(+) time' indicates that fluoxetine was added after concanavalin A.
[b] Cell were cultured for 3 days and [^{3}H]thymidine incorporation was determined. The results are expressed as % of the control values (stimulated cells without fluoxetine).

3.3. Effect of fluoxetine on intracellular cAMP levels

In order to analyze the effect of fluoxetine on the cAMP-dependent protein kinase A pathway, we determined intracellular cAMP levels in concanavalin A-stimulated cells in the presence or in the absence of fluoxetine. As can be seen from Table 1, 4 μg/ml concanavalin A significantly decreased cAMP production. Under these experimental conditions, fluoxetine not only inhibited the decrease in cAMP production but also provoked an important increase in intracellular cAMP formation. At 2 μg/ml concanavalin A, alone or in the presence of fluoxetine, no significant changes in intracellular cAMP levels were observed. Fluoxetine alone elicited a non-significant increase



Treatment	Protein kinase M Activity (pmol/min/10^7 cel)	
	Basal	Concanavalin A 2 μg/ml
None	0.05 ± 0.01	0.15 ± 0.04
Fluoxetine 10^{-6} M	0.08 ± 0.01	0.26 ± 0.03

Fig. 4. PKC distribution after T-cell stimulation with concanavalin A 2 μg/ml. T-lymphocytes were stimulated with 2 μg/ml concanavalin A. Soluble (▲) and particulate (●) PKC activity was measured in the absence (——) or in the presence (- - - -) of fluoxetine 10^{-6} M. Similar results were obtained when fluoxetine was added at 10^{-7} and 10^{-5} M (data not shown). PKM activity in the absence or in the presence of fluoxetine (10^{-6} M) is shown in the table below the figure. Data show PKC activity determined by using the specific peptide MBP-(4–14). Similar effects were observed when histone H1 was used for PKC activity determination.

in cAMP levels. It is well known that cAMP synthesis depends on adenylate cyclase and that cAMP hydrolysis depends on cyclic nucleotide PDE. As an inhibitory influence of PKC on adenylate cyclase has been described (Beckner and Farrar, 1986), we studied the effect of the direct PKC activator PMA. Two PMA concentrations were used for different times in order to induce translocation or down-regulation of PKC. In order to induce translocation, lymphocytes were stimulated with PMA, 2×10^{-9} M for 5 min (Cazaux et al., 1995), while to down-regulate PKC, lymphocytes were incubated with PMA, 2×10^{-8} M for 180 min (Ozawa et al., 1993). As can be seen from Table 1, a significant decrease in cAMP levels was obtained when cells were incubated with PMA, 2×10^{-9} M for 5 min. In contrast, incubation with PMA, 2×10^{-8} M for 180 min, elicited a marked increase in cAMP levels. Fluoxetine did not induce significant changes under these conditions. The PKC inhibitor staurosporine (Schachtele et al., 1988) did not modify the decrease in intracellular cAMP levels induced by concanavalin A (Table 1). IBMX, an inhibitor of PDE activity (Beavo et al., 1970), reversed the ability of concanavalin A to reduce cAMP levels but not the ability of fluoxetine to increase cAMP levels in the presence of optimal concanavalin A concentrations. IBMX did not modify the changes in cAMP levels induced by PMA.

3.4. Ca^{2+} ionophore effects on proliferation, PKC activation and intracellular cAMP levels

Ca^{2+} is an essential signal needed to initiate T-cell proliferation (Gelfand et al., 1987) and also plays an important role in the activation of PKC (Kaibuchi et al., 1995) and in the proteolytic degradation of PKC (Young et al., 1987). The Ca^{2+} ionophore A 23187 (2 μM) was used in order to compare the effect of fluoxetine on both parameters. As can be seen from Tables 2 and 3, A 23187 induced T-cell proliferation at 2 μg/ml concanavalin A. It

Table 2
cAMP levels in stimulated T-cells in the presence or in the absence of a PDE inhibitor (means ± S.E.M.; $n = 4$)

Cell stimulant[a]	cAMP[b] (pmol/min/10^7 cell)	cAMP[c] with PDE inhibitor
None	2.11 ± 0.21	2.66 ± 0.30
Concanavalin A, 4 μg/ml	0.75 ± 0.13[d]	2.13 ± 0.21
Concanavalin A, 4 μg/ml + staurosporine, 1 nM	0.87 ± 0.11	Nd[f]
Concanavalin A, 4 μg/ml + fluoxetine, 10^{-6} M	7.42 ± 0.54[e]	5.53 ± 0.28[e]
Concanavalin A, 2 μg/ml	1.72 ± 0.26	2.23 ± 0.26
Concanavalin A, 2 μg/ml + fluoxetine, 10^{-6} M	2.48 ± 0.34	2.39 ± 0.25
Fluoxetine, 10^{-6} M	2.81 ± 0.31	2.69 ± 0.25
PMA, 2×10^{-9} M (5 min)	1.16 ± 0.21[d]	1.08 ± 0.17[d]
PMA, 2×10^{-9} M + fluoxetine, 10^{-6} M	1.09 ± 0.16[d]	0.97 ± 0.21[d]
PMA, 2×10^{-8} M (180 min)	6.95 ± 0.42[e]	6.03 ± 0.32[e]
PMA, 2×10^{-8} M + fluoxetine, 10^{-6} M	7.07 ± 0.44[e]	7.49 ± 0.38[e]

[a]Cells were cultured alone or in the presence of 2 or 4 μg/ml concanavalin A or PMA at the indicated concentrations. Fluoxetine 10^{-6} M was added to stimulated cells where indicated. Similar results were obtained when fluoxetine was added at 10^{-7} and 10^{-5} M (data not shown).
[b]cAMP levels were determined by radioimmunoassay.
[c]Cells were pretreated with IBMX 10^{-3} M and incubated with the appropiate ligands. cAMP levels were determined by radioimmunoassay.
[d]$P \leq 0.05$.
[e]$P \leq 0.01$.
[f]Nd = not determined.

Table 3
Comparison of the effects of fluoxetine and Ca^{2+} ionophore on T-cell proliferation, PKC degradation and cAMP levels (means ± S.E.M.; $n = 5$)

Cells stimulant[a]	S.I.[b]	PKM/PKC[c]	cAMP[d]
None	–	0.022 ± 0.008	2.03 ± 0.21
Fluoxetine, 10^{-6} M	1.5 ± 0.3	0.019 ± 0.008	2.77 ± 0.28
A 23187, 2 μM	1.4 ± 0.3	0.021 ± 0.003	2.93 ± 0.31
Concanavalin A, 2 μg/ml	11.3 ± 2.8	0.038 ± 0.009	1.89 ± 0.12
Concanavalin A, 2 μg/ml + fluoxetine, 10^{-6} M	40.7 ± 4.6[e]	0.076 ± 0.011	2.41 ± 0.18
Concanavalin A, 2 μg/ml + A 23187, 2 μM	33.8 ± 4.2[e]	0.089 ± 0.010	3.03 ± 0.32
Concanavalin A, 4 μg/ml	51.2 ± 5.3	0.022 ± 0.007	0.71 ± 0.11
Concanavalin A, 4 μg/ml + fluoxetine, 10^{-6} M	18.2 ± 1.6[e]	3.510 ± 0.412[f]	7.23 ± 0.65[f]
Concanavalin A, 4 μg/ml + A 23187, 2 μM	16.3 ± 3.2[e]	4.300 ± 0.384[f]	7.64 ± 0.71[f]

[a] Cells were cultured alone or in the presence of concanavalin A, 2 or 4 μg/ml. A 23187 (2 μM) or fluoxetine (10^{-6} M) was added to stimulated cells where indicated.
[b] S.I. values were obtained as the ratio between experimental and control dpm values.
[c] PKM formation and PKC activity were determined in the membrane fraction.
[d] cAMP levels were determined by radioimmunoassay.
[e] $P \leq 0.05$.
[f] $P \leq 0.01$.

did not alter the PKM/PKC ratio or cAMP production. Fluoxetine behaved in the same way. The Ca^{2+} ionophore, like fluoxetine, impaired the expected T-cell proliferation at 4 μg/ml concanavalin A. Similarly, both A 23187 and fluoxetine increased PKM levels and cAMP formation when cells were stimulated with 4 μg/ml of concanavalin A. However, neither A 23187 nor fluoxetine alone had any effect on the parameters studied.

4. Discussion

In a previous report, we described the effect of fluoxetine on mitogen-induced murine T-cell proliferation. We found that fluoxetine inhibited the proliferative response induced by optimal concentrations of the mitogen concanavalin A. When suboptimal concentrations were used, fluoxetine increased the basal proliferative response (Ayelli Edgar et al., 1996).

In this paper, we investigated the immunomodulatory effect of fluoxetine on mitogen-induced human T-cell proliferation. We found that fluoxetine had a dual effect on concanavalin A-stimulated human T-cell proliferation, as we previously described in a murine model (Ayelli Edgar et al., 1996). At optimal concanavalin A concentrations, fluoxetine inhibited proliferation while at suboptimal concanavalin A concentrations, the drug enhanced proliferation. The data obtained from the dose–response curve show that fluoxetine had an intermediate potency (in the 10^{-7}–10^{-8} M range) for both the stimulatory and the inhibitory effect. However, its stimulatory potency (EC_{50}: 0.4×10^{-7} M) was 12 times higher than its inhibitory potency (EC_{50}: 5×10^{-7} M). Berkeley et al. (1994) also found that fluoxetine in the micromolar range inhibited the maximal mitogen-induced proliferative response in both T and B rat lymphocytes. The time course studies revealed that, depending on the effect studied, fluoxetine exerted an immunomodulatory effect when it was added between 1 and 3 h before or after concanavalin A addition. These results could indicate that fluoxetine exerts immunomodulatory effects by affecting early events in the proliferative response. We therefore analyzed the influence of fluoxetine on the early signals triggered in T-lymphocytes by concanavalin A. These signals lead to the induction of two functionally well-characterized kinases, PKC and protein kinase A, which have opposite functions. PKC activation promotes cell proliferation (Gelfand et al., 1987; Altman et al., 1990; Isakov et al., 1992), whereas activation of the cAMP-dependent protein kinase A pathway leads to the inhibition of proliferation (Ledbetter et al., 1986; Kim et al., 1989). PKC is normally present in the cytoplasm and translocates to the plasma membrane after lectin binds to cell–surface receptors. This rapid PKC activation is critical for T-cells to enter to the proliferative phase of the cell cycle, as described by Berry et al. (1990). We observed that optimal mitogenic concanavalin A concentrations induced a rapid PKC translocation to the membrane. In the presence of fluoxetine, concanavalin A induced a decrease in cytosolic PKC activity, which was not recovered in the membrane. There is evidence that the membrane-associated (active) form of PKC is readily susceptible to proteolysis by a Ca^{2+}-activated neutral proteinase, which generates a catalytic fragment (PKM) that is no longer dependent on Ca^{2+} and phospholipids (Young et al., 1987). It is probable that fluoxetine induced the proteolytic degradation of PKC by a Ca^{2+}-mediated mechanism. In fact, we measured an increased PKM activity under these experimental conditions. With suboptimal concanavalin concentrations, there was little particulate PKC activity. Under these conditions, fluoxetine increased PKC translocation. Suboptimal concanavalin A concentrations are associated with a marked reduction in the stimulated increase in Ca^{2+} concentrations (Mills et al., 1985). It is possible that suboptimal concanavalin A concentrations induced lower

Ca^{2+} concentrations than those necessary to elicit maximal PKC translocation (Kaibuchi et al., 1995), which was increased by fluoxetine.

Concerning the cAMP-protein kinase A pathway, we found that optimal concanavalin A concentrations induced a decrease in intracellular cAMP levels. An inverse relationship exists between cAMP levels and lymphocyte proliferation (Coffey and Hadden, 1985). It is not clear if the decrease in cAMP levels triggers proliferation. In fact, some reports show that cAMP blocks the progression but not the initiation of T-cell proliferation. However, in most of the T-cell activation models, decreased cAMP levels accompany lymphocyte proliferation (Beckner and Farrar, 1986; Valette et al., 1990). Fluoxetine alone had little effect on cAMP levels. Interestingly, when optimal concanavalin A concentrations were used, fluoxetine elicited a significant increase in cAMP levels. This increase in cAMP levels probably activated the inhibitory cAMP-dependent protein kinase A pathway, impairing lymphocyte proliferation. When suboptimal concanavalin A concentrations were used, in the absence or in the presence of fluoxetine, no significant changes in cAMP levels were observed. The influence of fluoxetine on cAMP levels could reflect an effect on adenylate cyclase activity or on cAMP-PDE activity. Several reports have demonstrated that phorbol esters alter the hormone-responsive adenylate cyclase system, an effect presumably mediated by PKC (Kelleher et al., 1984; Rebois and Patel, 1985; Beckner and Farrar, 1986). We studied this possibility and found that PKC activation induced by incubation with PMA (2×10^{-9} M) for 5 min (Cazaux et al., 1995) led to a significant decrease in cAMP levels. These results indicate that PKC could be involved in the decrease in cAMP levels after concanavalin A stimulation. However, pretreatment with staurosporine did not reverse the ability of concanavalin A to reduce cAMP production. When down-regulation of the Ca^{2+}-dependent PKC was induced by treatment with PMA (2×10^{-8} M) for 180 min (Ozawa et al., 1993), a significant increase in cAMP production was observed. These findings suggest that Ca^{2+}-dependent PKC activity could play an important role in preventing the increase in cAMP levels, which in turn could lead to inhibition of the process that follows normal lymphocyte proliferation. Fluoxetine did not modify the effects produced by PMA. In order to study the role of cAMP-PDE activity in the effects of fluoxetine, we performed experiments using IBMX to inhibit this enzyme. We observed that this drug impaired the decrease in cAMP levels evoked by concanavalin A. Similar results were reported by Valette et al. (1990), who showed that concanavalin A stimulation increased cytosolic Rolipram-sensitive isoforms of cyclic nucleotide PDE activity. However, IBMX did not impair the increase in cAMP levels induced by fluoxetine when optimal concanavalin A concentrations were used. It is thus possible that the effect of fluoxetine on cAMP is essentially an indirect effect on adenylate cyclase activity exerted by modification of PKC activity. It seems that basal adenylate cyclase activity is up-regulated by PKC activity during mitogen-induced triggering of T-cells. When PKC activity is down-regulated, the negative influence on adenylate cyclase activity is abolished and there is increased cAMP production.

Concanavalin A-stimulated T-cell proliferation appears to be highly dependent on extracellular Ca^{2+} influx. The uptake, but not the release of Ca^{2+} from internal stores, was demonstrated to be sufficient and perhaps essential for T-cell proliferation (Gelfand et al., 1987). Helmeste et al. (1995) found that several antidepressants that are potent serotonin uptake inhibitors, including fluoxetine in the micromolar range, enhanced platelet intracellular Ca^{2+} mobilization. Our results seem to indicate that Ca^{2+} participates in the effect of fluoxetine. Given these facts, we studied the participation of Ca^{2+} in the immunomodulatory effect of fluoxetine. We observed that the Ca^{2+} ionophore and fluoxetine had similar effects on proliferation, PKC activity and cAMP levels. It is possible that fluoxetine could exert its effect by inducing extracellular Ca^{2+} influx. In fact, Helmeste et al. (1995) found that omission of extracellular Ca^{2+} inhibited but did not abolish the effect of the serotonin reuptake inhibitors. They concluded that the increased intracellular Ca^{2+} levels came from both intracellular and extracellular sources. However, in our experimental model, fluoxetine was not able to modify inositol phospholipid signalling (data not shown). It is important to note that we could not confirm the role of extracellular Ca^{2+} in the action of fluoxetine by studying the effect of Ca^{2+} channel blockers or the omission of external Ca^{2+}, because concanavalin A-induced T-cell proliferation is dependent on extracellular Ca^{2+} influx (Gelfand et al., 1987). In fact, under these conditions, concanavalin A-induced T-cell proliferation was completely prevented, so it was not possible to assess the effects of fluoxetine (data not shown).

Faraj et al. (1994a,b) described the existence of a high-affinity transport system for dopamine and serotonin in human lymphocytes and the inhibition of this system by fluoxetine. The possibility that the immunomodulatory effect of fluoxetine may reflect alterations in the serotonergic or dopaminergic uptake system in lymphocytes cannot be ruled out completely, but it is important to note that the potency of fluoxetine to inhibit the immune response (EC_{50}: 5×10^{-7} M) does not correlate with its potency to inhibit serotonin or dopamine uptake in lymphocytes (EC_{50}: 5.1 and 45 nM, respectively). However, its potency to stimulate the immune response (EC_{50}: 40 nM) correlates with its potency to affect monoamine uptake in lymphocytes. However, if monoamine uptake inhibitors lead to high extracellular concentrations of either amine under culture conditions, then proliferation may be suppressed because both norepinephrine and serotonin have been shown to inhibit lymphocyte proliferation (Werner et al., 1987). Given these facts, it does not seem possible that

these mechanisms participate in the effect of fluoxetine on T-cell proliferation.

Taking into account our results, it is reasonable to conclude that fluoxetine modulates the proliferative response by increasing Ca^{2+} influx, which, in turn, could influence growth-promoting PKC activity. The latter could modulate the inhibitory cAMP-protein kinase A pathway. When T-cells were stimulated with optimal concanavalin A concentrations, fluoxetine promoted Ca^{2+}-mediated PKC proteolysis, which led to an increase in cAMP levels. Both events, PKC proteolysis and the increase in cAMP levels, efficiently impaired mitogen-induced proliferation. In contrast, when a suboptimal concanavalin A concentration was used, fluoxetine increased PKC translocation but did not significantly change intracellular cAMP levels. Both these effects led to an increase in lymphocyte proliferation.

Finally, given the fact that the steady-state plasma concentration of fluoxetine achieved during treatment is between 0.15 to 1.5 μM (Orsulak et al., 1988; Kelly et al., 1989), 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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