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β-Adrenoceptor-Linked Protein Kinase A (PKA) Activity in Human Fibroblasts from Normal Subjects and from Patients with Major Depression

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Human fibroblasts from normal subjects and from patients with major depression are cultured and their β-adrenoreceptor-cyclic AMP-protein kinase A (PKA) system characterized. The results indicate that the β-adrenoreceptor-mediated activation of PKA in the 900 g supernatant fraction of human fibroblasts is mediated via β-adrenoreceptors. The activation of PKA by isoproterenol is very rapid with maximal stimulation occurring at 5 seconds. The time course of PKA activation by isoproterenol in fibroblasts from patients with major depression is identical to that in fibroblasts from normal subjects but the magnitude of activation is significantly reduced in fibroblasts from patients with major depression. Dose-response curves on cyclic AMP mediated activation of PKA confirmed the previously reported reduction in activation of PKA in patients with major depression but demonstrated that this reduction occurs without a change in the EC_{50} values of cyclic AMP (approximately 20 nmol/L). The blunted β-adrenoceptor-linked PKA responses in patients with major depression occur without a change in the expression of the PKA catalytic subunit Cα. The studies suggest that the β-adrenoceptor-coupled adenylate cyclase PKA system in human fibroblasts may represent a valid model to explore possible abnormalities in the fine tuning of the β-adrenergic transduction cascade in patients with affective disorders. **© 1996 American College of Neuropsychopharmacology**
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KEY WORDS: *Human fibroblasts; Major depression; Cyclic AMP; Isoproterenol; Protein kinase A (PKA); β-adrenoceptor subtype*

The evolution of the monoamine hypotheses of affective disorders reflects by and large the evolution of our understanding of the mode of action of clinically effective antidepressant drugs (Pryor and Sulser 1991). The discovery that chronic antidepressant treatments cause subsensitivity of the β-adrenoceptor-coupled adenylate cyclase system in brain shifted research on the mode of action of antidepressants and on the pathophysiology of affective disorders from acute presynaptic to delayed postsynaptic receptor-mediated events. Clinically, many attempts have been made to assess β-adrenoceptor sensitivity and function in platelets and lymphocytes of patients with affective disorders. However, such studies have yielded conflicting results with decreases, increases, and no differences in β-adrenoceptor activity being reported in patients with depression (Pandy et al. 1979, 1985; Extein et al. 1979; Healy et al. 1983; Mann et al. 1985, Jeanningros et al. 1991). The reasons for these discrepancies are many and may include—besides dif-

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ferent assay methodologies—changing environmental factors such as fluctuations in circulating catecholamines and glucocorticoids due to stressful life events, drug effects, and diurnal and seasonal rhythms. To avoid such influences, Berettini et al. (1987) studied β-adrenoceptor function in subcultured human fibroblasts taken from normal subjects and from patients with bipolar disorder. Because no evidence was found in these studies for abnormal β-adrenoceptor function (β-adrenoceptor density, formation of cyclic AMP), Shelton et al. (1996) studied the β-adrenergic cyclic AMP–generating system in fibroblasts at the next level of the signal transduction cascade, i.e., the activation of protein kinase A (PKA). These studies revealed that cyclic AMP stimulated protein kinase activity is significantly reduced in the 900 *g* supernatant fraction of fibroblasts from patients with major depression. Because PKA-mediated phosphorylation represents one of the major mechanisms of signal integration in eukaryotic cells and a final common pathway in the regulation of neuronal function (Nestler and Greengard 1984), a more rigorous characterization of the β-adrenoceptor—cyclic AMP-PKA system in human fibroblasts from normal subjects and from patients with major depression seemed warranted. The present studies focus on this characterization.

MATERIALS AND METHODS

Subjects, Diagnostic Criteria, and Skin Biopsy

Fibroblasts from subjects of this study (five subjects with major depression; four women, one man; mean age ± SEM: 44 ± 5; five control subjects; five women; mean age ± SEM: 37 ± 6) were selected at random from our cell repository, which contained fibroblast samples subcultured from skin biopsies of 12 patients with major depression (11 women, one man; mean age ± SEM: 42 ± 3) and from 10 normal subjects (seven women, three men; mean age ± SEM: 39 ± 4). As previously described in detail by Shelton et al. (1996), depressed patients were recruited from persons who requested treatment or participated in research studies in the Psychopharmacology Clinic of the Department of Psychiatry, Vanderbilt University Medical Center. Normal volunteers were recruited by advertisement flyers. No medical center personnel were included. Written consent was obtained from all subjects after a full explanation of all procedures and before participation in any research activities. All subjects were evaluated by the Structured Clinical Interview for DSM-III-R, Patient version (SCID-P[42]) that was administered by a trained and experienced senior psychology graduate student or research assistant. A second, confirmatory diagnostic evaluation was performed by an experienced senior research psychiatrist (RCS). A consensus diagnosis was obtained for each subject. Consensus agreement for depressed persons included the presence of major depression, presence or absence of coexisting dysthymia, and absence of any excluded Axis I disorder. Consensus agreement for normal volunteers included the absence of any current or past history of Axis I disorder. Reliability evaluations for the classification of depression using the SCID-P have yielded kappa > 0.90.

The participants were physically healthy and free from contraindication for participation in the study, including bleeding diathesis, sensitivity to lidocaine anesthesia, pregnancy, or lactation. No depressed subject was included who met current DSM-III-R criteria for any other Axis I disorder except dysthymia. Further, subjects were excluded if they met criteria for any substance abuse or dependence disorder within the last 6 months, or a past history of bipolar disorder, psychotic disorder, or obsessive compulsive disorder.

All subjects were prescreened by telephone and then scheduled for interview and biopsy. The skin biopsy was performed within 2 weeks after the diagnostic interview. The skin biopsy for fibroblast sampling was obtained from the lateral aspect of the left arm. Skin was scrubbed with iodine solution and surgical soap and cleaned with an alcohol prep. Local anesthesia was induced by injection of 1% lidocaine, using a 25-gauge needle. A sterile, disposable 2-mm punch was used to remove a sample of skin to 5 mm depth. The sample was transferred to a culture flask containing Dulbecco's modified Eagle medium (high glucose) containing 10% fetal calf serum, 30 mg/L streptomycin, 30,000 U/L penicillin, and 75 μg/L amphortericin B1. The wound was bandaged with a butterfly closure, clean gauze pad, and triple antibiotic ointment (neomycin, bacitracin zinc, polymyxin B sulfate). Instructions were given to rebandage the wound daily using triple antibiotic ointment for seven days or until healing. Subjects were paid $20 for participation in the skin biopsy procedure.

Tissue Culture of Human Fibroblasts

Human skin fibroblasts from normal subjects and from patients with major depression were cultured from the skin biopsies. Explant cultures were established under conditions that select for fibroblast growth (Edelstein and Breakefield 1980). The ATCC cell line #CRL 1467 was obtained from American Type Culture Collection, Bethesda, MD. The fibroblast cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2. The Dulbecco's modified Eagle medium (high glucose containing 10% fetal calf serum, 30 mg/L streptomycin, 30,000 units/L penicillin and 75 μg/L amphotericin B_1) was changed three times per week. The cultures were inspected daily by microscope and subcultured after reaching confluency (4×10^6 cells/75 cm^2). Confluent

cultures were split at a ratio 1:4. There were at least five growth passages in culture before any assay.

Biochemical and Pharmacologic Studies

Cyclic AMP–dependent protein kinase (PKA) activity was determined in soluble fractions obtained from confluent human fibroblast cultures. The cells were disrupted with a glass teflon homogenizer (six strokes) after scraping them into 1 ml of homogenization buffer (sucrose, 0.32 mol/L; KH_2PO_4, 10 mmol/L; $MgCl_2$, 5 mmol/L NaCl, 150 mmol/L; pH 6.5). Supernatant and particulate fractions were separated by centrifugation (900 *g*, 10 minutes). Four assay conditions were used; namely, total PKA activity (+100 μmol/L cyclic AMP) with and without PKA inhibitor and endogenous activity (no cyclic AMP added) with and without inhibitor. Total incubation volume was 80 μl in Tris-$MgCl_2$ buffer. After an incubation time of 5 minutes (30°C), 20-μl aliquots were spotted onto phosphocellulose paper (2 × 2 cm, Whatman P81). The filter squares were washed twice for 5 minutes in 75 mmol/L H_3PO_4 and twice for 5 minutes in H_2O, and afterward counted by liquid scintillation spectrometry. PKA activity is defined as the transfer of PO_4 from ATP (100 μmol/L; [^{32}P]-tracer, 300 cpm/pmol) to the heptapeptide Kemptide (LRRASLG; 50 μmol/L) and normalized per units protein and time. Proteins were quantitated according to Lowry et al. (1951).

Cyclic AMP was determined in whole cell preparations using a radioimmunoassay kit (NEN Research Products, Boston, MA).

Molecular Biological Studies

Total cellular RNA was isolated, and Northern blots were analyzed as previously described (Eiring et al. 1992). Then 10 μg each of total cellular RNA from control fibroblasts (ATCC #CRL 1467), normal control subjects and depressed subjects was separated on a 1.2% agarose gel and run under glyoxal denaturing conditions and then transferred to nitrocellulose blotting membrane in order to establish PKA expression and hybridization patterns in the various groups of fibroblasts. The blot was then probed with a cDNA clone of the Cα subunit of PKA. The probe was obtained as a plasmid pSKG4 from Dr. Steven Hanks, Department of Cell Biology, Vanderbilt University, and was subsequently used to transform DH5α strain of E. coli and propagated for isolation of the EcoRI–EcoRV fragment used in the hybridization of the Northern blot. Dot blot analysis was used for statistical comparison of the steady-state Cα mRNA in fibroblasts from control subjects and from patients with major depression as previously described (Eiring et al. 1992). Briefly, total cellular RNA was isolated from confluent cell cultures of patients and normal subjects. RNA was quantitated by spectrophotometric analysis. Purity of RNA was also checked by spectrophotometric methods; all OD260/280 ratios were between 1.9 and 2.0. Five micrograms of each sample were assayed in triplicate and averaged for statistical comparison of the two groups.

RESULTS

Characterization of the β-adrenoceptor Subtype Mediating PKA Activation in Human Fibroblasts

We determined the β-adrenoceptor subtype that mediates the activation of PKA in the 900 *g* supernatant fraction of fibroblasts from normal subjects (ATCC cells). Isoproterenol (10 μmol/L) caused within 30 minutes a significant increase in the activity of PKA. The β-selective antagonist metoprolol (20 μmol/L) failed to block the action of isoproterenol, whereas the β_2-selective antagonist ICI 118 551 (20 μmol/L) completely blocked the activation of PKA by isoproterenol (Figure 1). Simultaneous incubation with both ICI 118551 and metoprolol did not alter the extent of the blockade. The data indicate that the β-adrenoceptor-mediated activation of PKA in human fibroblasts occurs via β_2 adrenoceptors.

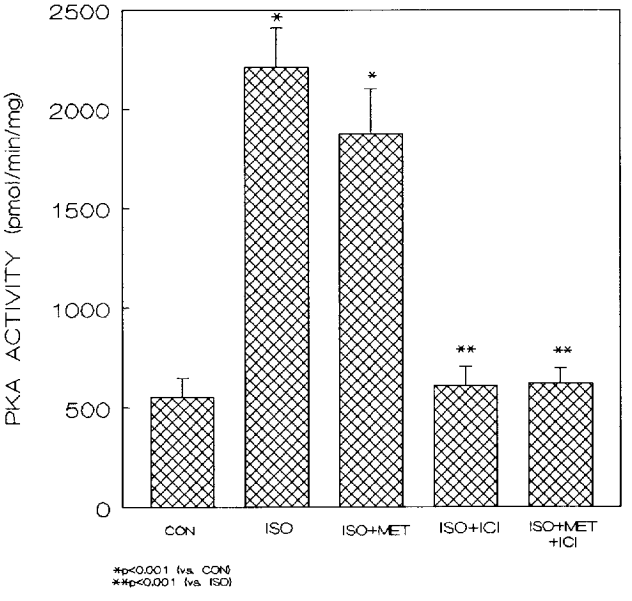

Figure 1. Effect of metoprolol and ICI 118551 on PKA activity in human fibroblasts. Human fibroblast cell cultures (ATCC #CRL 1467) were incubated for 30 minutes with isoproterenol (10 μmol/L), isoproterenol plus metoprolol (20 μmol/L), isoproterenol plus ICI 118551 (20 μmol/L), or isoproterenol plus metoprolol and ICI 118551. PKA activity in the 900 *g* supernatant fraction was determined as described in detail in Materials and Methods. The values represent mean values for PKA activity in pmol/min/mg protein ± SEM. n = 5. CON = control; ISO = isoproterenol; MET = metoprolol; ICI = ICI 118551.

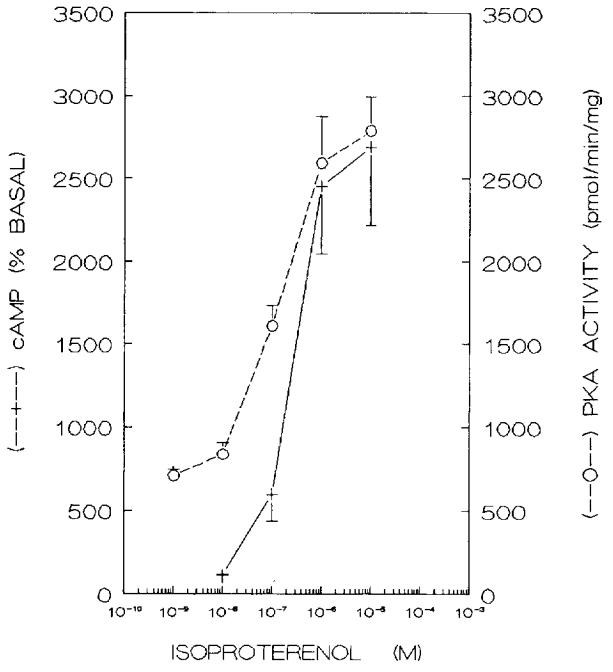

Figure 2. Effect of isoproterenol on cyclic AMP levels and PKA activity in human fibroblasts—dose-response curves. Human fibroblast cell cultures (ATCC #CRL 1467) were incubated for 30 minutes with increasing concentrations of isoproterenol. Cyclic AMP was measured in whole-cell preparations, and PKA activity in the 900 *g* supernatant fraction was determined as described in Materials and Methods. Cyclic AMP levels are expressed as a percentage of basal levels, which were 24 ± 3 pmol/mg protein ± SEM. $n = 5$. PKA activity is expressed in pmol/min/mg protein ± SEM ($n = 3$).

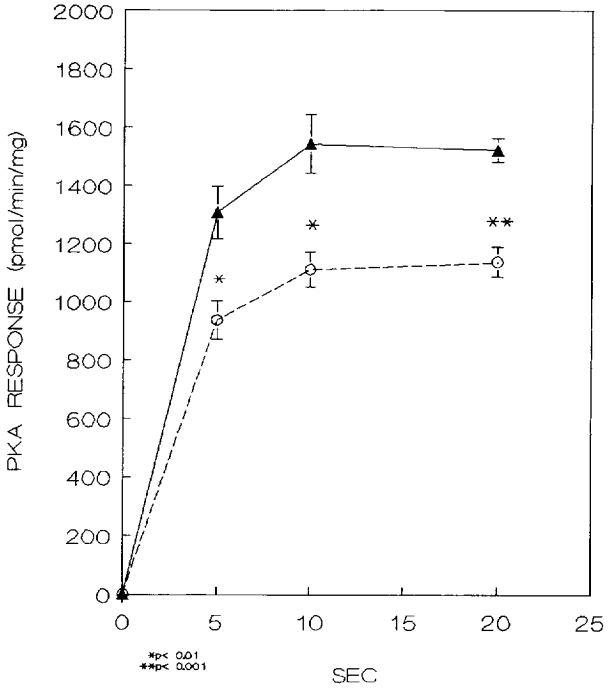

Figure 3. Time course of isoproterenol-mediated PKA response in human fibroblasts. Fibroblasts from normal subjects ▲ - - - - ▲ and from patients with major depression ○ - - - - ○ were incubated with 10 μmol/L isoproterenol. After incubation with isoproterenol for the times indicated, the cell monolayer was washed two times with phosphate-buffered saline, and cells were harvested into homogenization buffer. PKA activity was determined in the 900 *g* supernatant fraction as described in Materials and Methods. PKA response represents the isoproterenol-stimulated PKA activity minus the basal activity. The values represent the mean responses in pmol/min/mg protein ± SEM. $n = 4$ (normal subjects) and $n = 5$ (major depression).

Effect of Isoproterenol on Cyclic AMP Levels and PKA Activity in Human Fibroblasts

In fibroblasts subcultured from normal subjects, isoproterenol increased cyclic AMP responses in a dose-dependent manner with an EC_{50} value of approximately 0.2 μmol/L (Figure 2), which is in good agreement with values reported by Berettini et al. (1986). Maximal cyclic AMP stimulation occurred with 1 μmol/L isoproterenol, which is also the concentration that maximally activates PKA in the 900 *g* supernatant fraction, with half maximal stimulation occurring at 0.1 μmol/L isoproterenol. Data in Figure 3 show the time-response curve of activation of PKA by isoproterenol. The activation by isoproterenol is very rapid, with maximal stimulation occurring at 5 seconds. The time course of PKA activation by isoproterenol in fibroblast preparations from patients with major depression is identical to that of fibroblasts from normal subjects but the magnitude of activation is significantly reduced in fibroblast cultures from patients with major depression (Figure 3).

Effect of Cyclic AMP and Isoproterenol on PKA Activity in Human Fibroblasts from Normal Subjects and from Patients with Major Depression

Because previous studies have shown that the cyclic AMP–stimulated PKA activity is significantly reduced in the 900 *g* supernatant fraction of fibroblasts from patients with major depression (Shelton et al. 1996), we constructed dose-response curves with cyclic AMP in fibroblasts from normal individuals and from patients with major depression. The results demonstrate that the cyclic AMP–mediated PKA activation is significantly decreased in fibroblasts from patients with major depression without a significant change in the EC_{50} values (Figure 4). To further ascertain that the reduced PKA response to cyclic AMP in fibroblasts from the patient population is β-adrenoceptor linked, endogenous and cyclic AMP–stimulated PKA activities were determined in fibroblasts from normal subjects and from patients

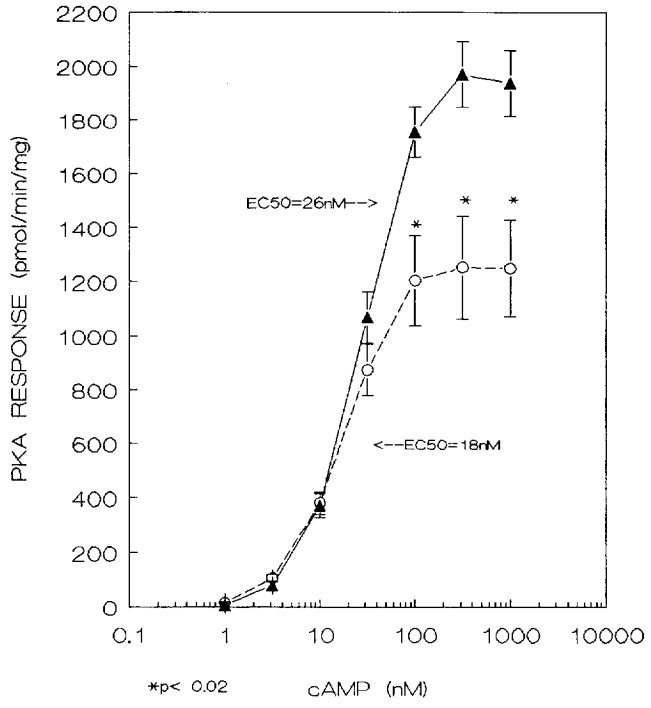

Figure 4. Effect of cyclic AMP on PKA responses in human fibroblasts from normal subjects and from patients with major depression—dose-response curves. Fibroblasts preparations (900 *g* supernatant) from normal subjects (▲ - - - - ▲) and from patients with major depression (○ - - - - ○) were assayed using various concentrations of cyclic AMP. PKA response represents the cyclic AMP stimulated PKA activity minus the basal PKA activity. The values represent mean values in pmol/min/mg protein ± SEM. $n = 4$.

with major depression in the presence and absence of the β-adrenoceptor agonist isoproterenol. The results in Table 1 indicate that—like the cyclic AMP–stimulated PKA activation—the β-adrenoceptor-mediated PKA activation is significantly reduced in fibroblasts from the patient population.

Expression in Human Fibroblasts of PKA Catalytic Subunit Cα

Data in Figure 5 show that the catalytic subunit Cα of PKA is expressed in human fibroblasts. The hybridization pattern was the same for ATCC cells (shown) and normal and depressed subjects. Statistical analysis did not, however, reveal significant differences between steady-state Cα mRNA levels in fibroblasts of the two populations (controls vs. major depressives). The relative Cα mRNA levels from laser densitometer analysis of autoradiographs in arbitrary absorption units were: controls, 0.68 ± 0.09; major depression, 0.73 ± 0.12 ($n = 5$).

DISCUSSION

Fibroblast cultures have proven useful in investigating inherited biochemical lesions in man (Edelstein and Breakefield 1980). Concerning psychiatric disorders, the study of β-adrenoceptor function in cultured human skin fibroblasts is one approach to probe the monoamine pathophysiology in affective disorders. Though humoral rather than neuronal factors influence receptor number and function, fibroblasts possess a β-adrenoceptor-coupled adenylate cyclase system, which is responsive to catecholamines (Berettini et al. 1986, 1987).

Results of the present investigations indicate that in human fibroblasts the activation of PKA by isoproterenol is mediated via β-adrenoceptors. This receptor-mediated activation of PKA is rapid, with the maximal activation occurring in less than 5 seconds. Although most clinically effective antidepressants preferentially

Table 1. β-Adrenoceptor-Mediated Activation of PKA[a]

	Supernatant pmol/min/mg ± SEM			
	Without Isoproterenol		With Isoproterenol (10 μmol/L)	
	+ cAMP	Endogenous	+ cAMP	Endogenous
Normal subjects (5)	2041 ± 89	462 ± 52	1882 ± 61	1819 ± 162
Major depression (5)	1385 ± 52[b]	426 ± 68	1302 ± 67[b]	1191 ± 165[c]

[a] PKA activity was determined in the 900 × g supernatant fraction in the presence or absence of 100 μmol/ L cyclic AMP as described in Materials and Methods. In the experiments with isoproterenol, confluent fibroblast cultures were incubated for 30 min. with 10 μ mol/L isoproterenol and then homogenized and PKA activity determined as described in Materials and Methods. Numbers in parentheses indicate the number of subjects.

[b] $p < .001$.

[c] $p < .025$.

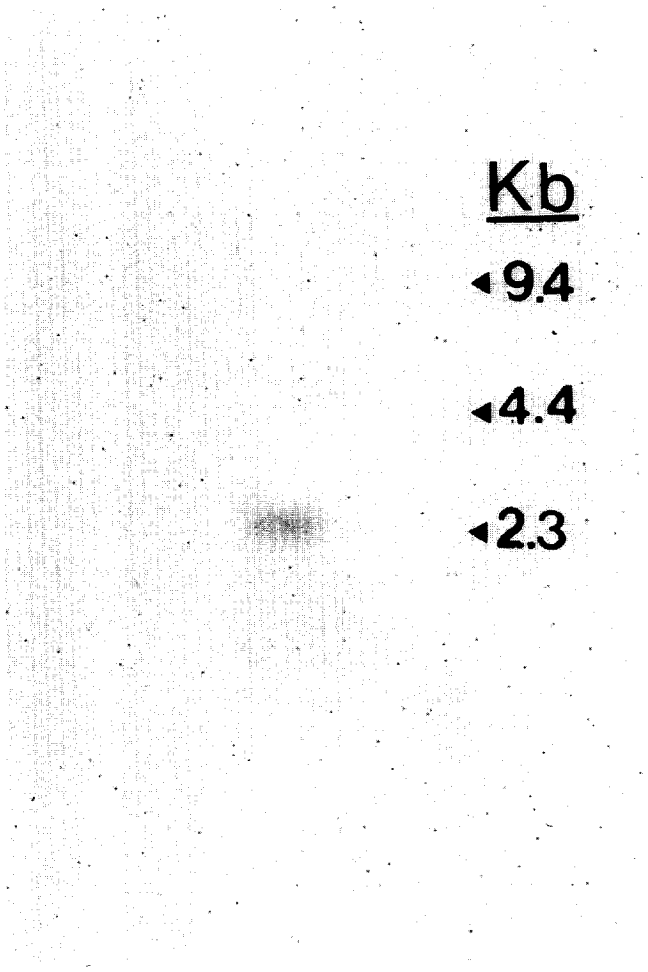

Figure 5. Expression of protein kinase A catalytic subunit (Cα) in human fibroblasts (ATCC cell line CRL 1467). RNA was isolated according to Materials and Methods and 10 μg separated on a glyoxal agarose gel. After transfer to nitrocellulose membrane, the blot was hybridized with [^{32}P]-labeled cDNA. A single band of mRNA was detected, approximately 2.3 kilobases in size.

regulate β-adrenoceptors in brain (Ordway et al. 1991), β_2-adrenoceptors in fibroblasts, like β_1-adrenoceptors in brain, are positively linked to adenylate cyclase with an EC_{50} value for isoproterenol of approximately 0.2 μmol/L, a concentration that also represents the EC_{50} value for the activation of PKA. The regulation of PKA and β-adrenoceptor kinase of the density and sensitivity of β_2-adrenoceptors is well understood (Benovic et al. 1988; Lohse et al. 1990; Strader et al. 1989; Cheung et al. 1990). Consequently, the β-adrenoceptor-coupled adenylate cyclase–PKA system in human fibroblasts represents a heuristic model to explore possible abnormalities in the fine tuning of the β-adrenergic transduction cascade in patients with affective disorders. Previous studies from our laboratory have demonstrated that both basal and cyclic AMP stimulated PKA activities are significantly reduced in the 900 *g* supernatant fraction of fibroblasts from patients with major depression compared with the activities determined in fibroblasts of normal subjects (Shelton et al. 1996). The present results demonstrate that the attenuated PKA response is accompanied by a blunted β-adrenergic receptor response, and that it is the maximal PKA response to cyclic AMP that is significantly reduced in fibroblasts from patients with major depression with no change in the corresponding EC_{50} values. This suggests that "abnormalities" exist in the ability of cyclic AMP to stimulate PKA-mediated phosphorylation. Whether or not such "abnormalities" reside in the regulatory subunits that serve as receptors for cyclic AMP or in the catalytic subunits that dissociate from the holoenzyme after binding of cyclic AMP to the regulatory subunits (Taylor et al. 1990) is not known. In the present experiments, no differences were found in the steady-state levels of the catalytic subunit Cα mRNA of PKA in fibroblasts taken from control subjects or from patients with major depression. However, this does not preclude a difference in the actual catalytic protein subunit of PKA. Moreover, because the standard PKA assay used in the present studies does not include a protein phosphatase inhibitor, potential differences in the activity of protein phosphatases cannot be ruled out. Because the C subunit of PKA can be inhibited by the protein kinase inhibitor (PKI) peptide (Glass et al. 1989), future studies on function and expression of the PKI isoforms may provide a clue to the attenuated PKA responsiveness in fibroblasts from patients with major depression.

Because one of the most common covalent means of regulating β_2-adrenoceptor function is via phosphorylation by PKA and/or β-adrenoceptor kinase (BARK) (Benovic et al. 1988; Strader et al. 1989; Lohse et al. 1990; Cheung et al. 1990), it seems conceivable that a blunted β-agonist-mediated PKA response could result in a reduced desensitization and/or down-regulation of β_2-adrenoceptors by the β-agonist isoproterenol. It is noteworthy in this regard that Berettini et al. (1987) noted a trend of attenuation of β-adrenergic subsensitivity in fibroblasts from bipolar patients after incubation with isoproterenol though this trend did not reach statistical significance. However, Kay et al. (1993, 1994) reported an attenuated down-regulation of β-adrenoceptors by isoproterenol in cultured lymphoblasts from severely depressed patients compared with that in normal subjects. These data could be interpreted as being the consequence of a reduction in β_2-adrenoceptor-mediated phosphorylation by PKA. Because PKA is a "pleiotropic" enzyme that through phosphorylation affects the activity of transcription factors, the exploration of the possible consequences of the blunted β-adrenoceptor-mediated PKA activation in depression at the level of gene expression remains a future challenge.

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