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A Reduced Functionality of Gi Proteins as a Possible Cause of Fibromyalgia

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ABSTRACT. ***Objective.*** The etiopathogenesis of fibromyalgia (FM), a syndrome characterized by widespread pain and hyperalgesia, is still unknown. Since the involvement of Gi proteins in the modulation of pain perception has been widely established, the aim of the present study was to determine whether an altered functionality of the Gi proteins occurred in patients with FM.

Methods. Patients with FM and other painful diseases such as neuropathic pain, rheumatoid arthritis (RA), and osteoarthritis, used as reference painful pathologies, were included in the study. The functionality, evaluated as capability to inhibit forskolin-stimulated adenylyl cyclase activity, and the level of expression of Gi proteins were investigated in peripheral blood lymphocytes.

Results. Patients with FM showed a hypofunctionality of the Gi protein system. In contrast, unaltered Gi protein functionality was observed in patients with neuropathic pain, RA, and osteoarthritis. Patients with FM also showed basal cAMP levels higher than controls. The reduced activity of Gi proteins seems to be unrelated to a reduction of protein levels since only a slight reduction (about 20–30%) of the $Gi_{3\alpha}$ subunit was observed.

Conclusions: Gi protein hypofunctionality is the first biochemical alteration observed in FM that could be involved in the pathogenesis of this syndrome. In the complete absence of laboratory diagnostic tests, the determination of an increase in cAMP basal levels in lymphocytes, together with the assessment of a Gi protein hypofunctionality after adenylyl cyclase stimulation, may lead to the biochemical identification of patients with FM. (J Rheumatol 2001;28:2298–304)

Key Indexing Terms:
FIBROMYALGIA GI-PROTEIN HYPOFUNCTIONALITY DIAGNOSIS
REDUCED ADENYLATE CYCLASE ACTIVITY LYMPHOCYTES cAMP

Primary fibromyalgia (FM) (previously called fibrositis) is a clinically recognizable nonarticular rheumatic condition characterized by widespread pain and hyperalgesia, mainly located at the muscular level, associated with morning stiffness, diffuse aching and soreness, fatigue, and sleep disturbances (nonrestorative sleep)[1-3]. Affective disturbances, particularly high levels of anxiety, depression, and hypocondriasis, have also been noted among some patients[4,5]. However, perhaps the most salient feature of this syndrome is a predictable anatomical pattern of tender-points elicited by manual palpation[6]. In fact, the existence and number of such tender point sites constitutes one of the major defining characteristics of FM and helps differentiate this syndrome from other closely related musculoskeletal pain conditions, such as myofascial pain syndromes and articular diseases[7]. FM has a female prevalence usually occurring in middle-aged women[4]. To date, the pathophysiology of this syndrome is not completely elucidated. Even though a significant amount of research has been undertaken in this area, no unified hypothesis on its etiology has emerged and no single hypothesized mechanism can completely explain the origin of FM[8]. The main hypotheses fall into 4 categories: sleep disturbance[9], muscular alterations[10], altered neurotransmission of pain[11], and immunological factors[10]. Results from studies that suggested a possible pathologic basis for FM in muscle indicating the pain experienced by patients as the result of abnormalities in muscle blood flow, tissue hypoxia or energy metabolism defects are controversial[12]. At present, the most widely accepted model for the pathogenesis of this syndrome invokes central mechanisms rather than pathologically painful muscles[13,14]. Low serotonin levels and elevated substance P levels, mediators involved in the modulation of pain perception and in the regulation of sleep, were found in the cerebrospinal fluid[15,16], but not in muscles[17] of patients with primary FM.

A characteristic feature of this syndrome, in contrast to other chronic pain conditions, is a diffuse hypersensitivity to pain. The pain threshold is regulated by equilibrium between

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the endogenous system of pain amplification and inhibition. Pain indeed arises either when excitatory systems prevail or when the inhibitory systems are defective. A condition of hypofunctionality of the endogenous antinociceptive system can, therefore, be hypothesized as the main cause of the symptoms experienced by patients with FM. However, from the numerous studies conducted so far, no single receptor abnormality has been observed. These considerations led us to look beyond the receptor at the more universally distributed components of cell signalling, G-proteins.

G-proteins are heterotrimeric molecules with α, ß and γ subunits. The α subunits, which generally represent the functional subunit of these proteins, can be classified into families, depending on whether they are targets for cholera toxin (Gs), pertussis toxin (Gi and Go) or neither (Gq and G12)[18,19]. Activation of Gi proteins reduces cell excitability by inhibition of adenylyl cyclase activity and modulation of several K^+ and Ca^{++} channels[20-22]. The involvement of Gi proteins in the modulation of pain perception has long been known. The administration of pertussis toxin, which selectively inactivates Gi proteins[23], produced hyperalgesia and allodynia in laboratory animals[24-26], clearly indicating that a lack of functionality of Gi proteins enhances the sensitivity to pain. Hypofunctionality of Gi proteins also produces insensitivity to analgesic treatments. It has been observed that pertussis toxin prevents the enhancement of the pain threshold induced by potent analgesic drugs such as opioids, antihistamines, and tricyclic antidepressants[25,27,28].

Taking into account these considerations, our purpose was to determine whether an alteration in the Gi protein system occurred in the FM syndrome. The Gi protein functionality and expression were, therefore, investigated in peripheral blood lymphocytes from patients with clinically well-defined primary fibromyalgia.

MATERIALS AND METHODS

Subjects. Subjects were selected and classified into 3 groups: healthy volunteers (n = 17), patients with FM (n = 19) and patients with other types of pain such as neuropathic pain (sciatica, carpal tunnel and other entrapment syndromes, etc.), osteoarthritis, rheumatoid arthritis (n = 12). An informed consent was obtained. Patients were instructed to cease all medications at least 6 days prior to testing. They all had undergone laboratory and physical examination to exclude the presence of other concomitant affections. Patients with FM were classified, by attributing them an FM Score value, by following the criteria of the American College of Rheumatology[29]. The VAS (visual analog scale) value, which represents an arbitrary indication of the pain experienced, and the FM Score value were evaluated for all subjects. All experiments were performed blinded.

Isolation of human lymphocytes. Peripheral blood samples (15–20 ml) were immediately anticoagulated with heparin sodium (500 UI heparin/10 ml blood) and diluted 1:1 with saline solution. The lymphocytes were then isolated using Ficoll-isopaque density-gradient centrifugation as described by Böyum[30]. Mononuclear cells were counted.

Adenylyl cyclase activity assay in intact lymphocytes. Lymphocytes were first permeabilized with the detergent digitonin (10 μg/ml) under such conditions to maintain the biological functions intact[31]. The functionality of Gi proteins was assessed by investigating their ability to inhibit stimulated adenylyl cyclase activity in intact peripheral blood lymphocytes. Lymphocytes from healthy subjects were preincubated with or without pertussis toxin (PTX) at a concentration of 100 ng/ml at 37°C for 90 min as described[32], whereas lymphocytes from patients with FM and painful diseases were all preincubated without PTX. After preincubation, for the determination of adenosine 3′,5′ — cyclic monophosphate (cAMP) content, lymphocytes (1–2 × 10^6 intact cells/assay) were incubated either with dimethyl sulphoxide (DMSO) or forskolin 10^{-4} M to stimulate the cAMP formation[32]. To obtain the inhibition curve of cAMP production the non-hydrolysable analogue of GTP Gpp(NH)p (guanilyl 5′-imidophosphate) was added to the forskolin-containing samples in concentrations ranging from 10 nM to 100 μM, in a final volume of 300 μl. After a 15-minute incubation at 37°C, the cells were lysed and the sample centrifuged according to Brodde, *et al*[33]. The cAMP content was determined in 100 μl aliquot of the supernatant using an enzyme immunoassay kit (Amersham). Protein concentration was determined by the bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin as standard. All assays were performed in duplicate. The recovery of a known amount of cAMP (10 pmol) added to the incubation mixture after boiling was found to be 94.2 ± 7.8% (n = 6).

Lymphocyte lysates. Lymphocytes (5 × 10^6 cells) were lysed in 1 ml of 2 mM Tris buffer pH 7.5 containing 100 mM EDTA, 0.1 mM benzamidine, 2.6 mM leupeptin.

Western blot analysis. Lymphocyte Gi proteins were detected by Western blotting. Aliquots of lymphocyte lysates (100 ml), or recombinant $Gi_{2\alpha}$ and $Gi_{3\alpha}$, used as reference proteins, were boiled for 5 min with 30 ml of sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mMTris-HCl, 0.002% bromophenol blue at pH 8). The mixture (10 mg protein for each sample) was separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel according to Laemli[34] using 10% acrylamide in the running gel, then electroblotted to nitrocellulose membranes (Hybond-ECL 0.45 mm, Amersham). Blots were pre-blocked for 90 min at room temperature with Tris buffered saline (TBS) supplemented with 3% bovine serum albumin. Following 2 washes (10 min each) with TBS [containing 0.5% Tween 20 (TTBS)] at room temperature, blots were incubated overnight at 4°C in TBS supplemented with antisera suspension (1:2000 for anti $Gi_{2\alpha}$ and 1:10000 working dilutions for $Gi_{3\alpha}$). Following removal of antisera, blots were washed twice for 10 min with TTBS at room temperature. Thereafter each blot was incubated with TTBS supplemented with a second monoclonal goat anti-rabbit peroxide conjugate antibody (1:5000 working dilution) for 1 h at room temperature. Blots were then extensively washed (1 h) with several changes of TTBS. Specific bands were detected using ECL system (Amersham) according to the manufacturer's instructions. Densitometric analysis of the bands obtained was carried out using the Scion image program.

Statistical analysis. Results are given as the means ± SEM. An analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) procedure for *post-hoc* comparison, was used to verify the significance of differences between 2 means. Data were analysed with StatView software for the Macintosh (1992).

RESULTS

Clinical characteristics of subjects. The characteristics of the examined subjects are shown in Table 1. Patients with FM were selected following the criteria established by the American College of Rheumatology[29] and attributed the corresponding Score Value. Patients with a Score Value of tender points lower than 25 were not included in the study. As reported in Table 1, patients with FM had Score Values 3 to 5 times greater than patients with painful diseases, excluding the presence of a concomitant FM status in patients with neuropathic pain, osteoarthritis, and rheumatoid arthritis.

Patients with FM and painful diseases showed comparable

Table 1. Clinical variables in patients with FM and painful diseases.

Healthy				FM				Painful Diseases			
Sex	Age	Score	VAS	Sex	Age	Score	VAS	Sex	Age	Score	VAS
M	29	3	0	F	50	45	6.5	Neuropathic pain			
M	32	2	0	F	70	60	5.2	F	60	12	8.0
M	43	4	0	F	65	53	6.0	M	75	2	9.3
F	58	2	0	F	74	48	9.2	F	61	16	5.4
F	49	10	0	F	80	60	8.5	F	72	5	9.3
F	36	6	0	F	68	32	8.1	F	55	7	7.3
F	25	1	0	F	59	40	7.8	Osteoarthritis			
M	41	0	0	M	48	27	7.0	M	82	8	9.2
F	43	3	0	F	62	28	6.3	F	60	14	7.2
F	65	2	0	M	48	35	8.4	F	77	24	9.6
M	50	0	0	F	63	36	9.5	F	77	12	3.7
F	25	0	0	F	59	43	5.4	Rheumatoid arthritis			
M	48	0	0	F	40	39	6.4	F	73	9	4.6
M	44	0	0	F	65	33	6.1	M	61	8	2.6
M	62	0	0	F	43	36	8.1	F	75	8	9.2
F	25	0	0	F	51	51	9.3				
F	31	5	0	F	58	61	8.2				
				F	51	49	8.4				
				M	49	32	6.2				

Score: Classification of FM by the criteria of the American College of Rheumatology[28]; VAS: visual analog scale.

VAS values indicating homogeneity of intensity of pain experienced (Table 1).

After selection and inclusion in the study, no subject was then excluded.

Evaluation of Gi protein functionality. Adenylyl cyclase activity was stimulated by using the diterpene forskolin at a concentration of 10^{-4} M. The inhibition of cAMP production by Gi proteins was then initiated by administration of Gpp(NH)p, a non-hydrolyzable GTP analogue, in the concentration range of 10^{-8}–10^{-4} M. In lymphocytes from healthy subjects, Gpp(NH)p produced a dose-dependent inhibition of forskolin-stimulated adenylyl cyclase activity detectable by a reduction of cAMP levels of about 40% (Figure 1a). In contrast, Gpp(NH)p did not reduce cAMP levels after preincubation of control lymphocytes with pertussis toxin (PTX) (Figure 1a). A lack of inhibition of forskolin-stimulated adenylyl cyclase activity, comparable to that produced by PTX preincubation was observed in lymphocytes from patients with FM (Figure 1a). The distribution of single data points obtained from each patient, in comparison with healthy subjects, is reported in Figure 1b.

To exclude the possibility that the hypofunctionality of Gi proteins observed was secondary to a condition of chronic pain, we also investigated patients with painful syndromes other than FM. Gpp(NH)p reduced cAMP levels in lymphocytes from patients with neuropathic pain, rheumatoid arthritis, and osteoarthritis. In contrast to patients with FM, all these patients showed an ability to inhibit adenylyl cyclase activity similar to that of healthy subjects. The values reported in Figure 2 were detected after the administration of Gpp(NH)p 10^{-5} M, a concentration corresponding to its maximum effect. There was no statistically significant difference in cAMP val-

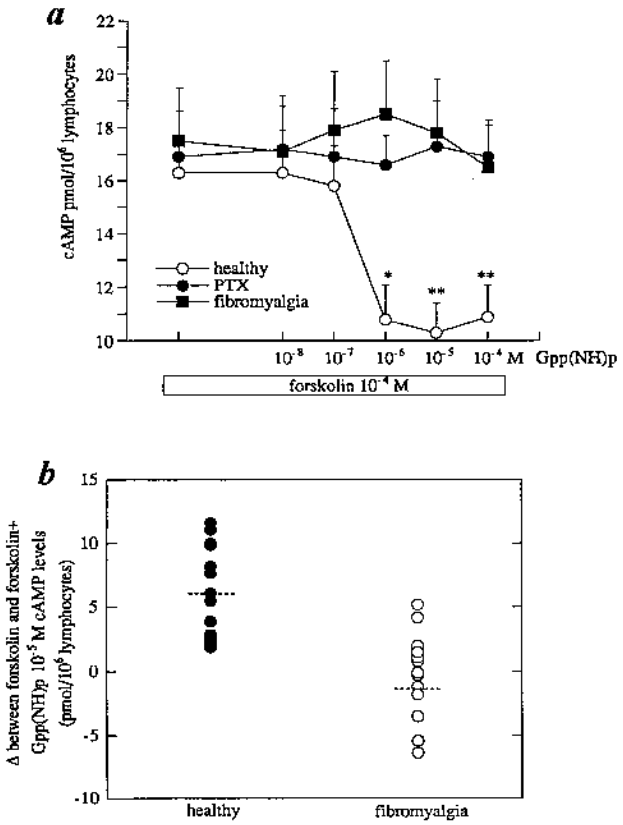

Figure 1. Evaluation of Gi protein functionality in human lymphocytes. a. Effect of increasing concentrations of Gpp(NH)p (10^{-8}–10^{-4} M) on forskolin-stimulated adenylyl cyclase activity in lymphocytes from healthy subjects preincubated with or without pertussis toxin (PTX) and in lymphocytes of patients with FM. Data are expressed as mean ± SEM ; *$p < 0.05$, **$p < 0.01$ in comparison with forskolin-stimulated lymphocytes. b. Distribution of single data points obtained from each patient and healthy subject.

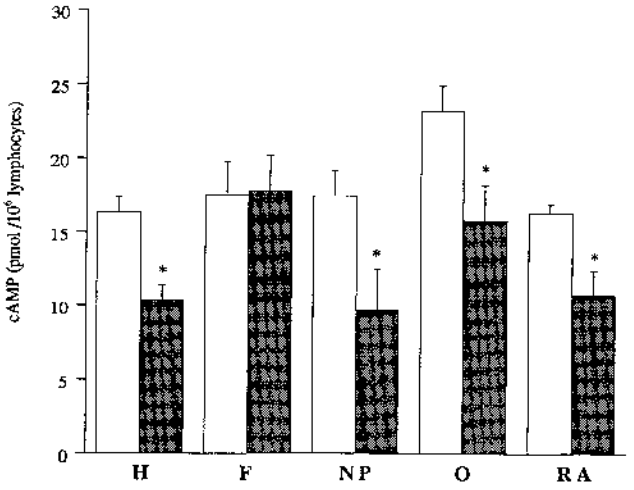

Figure 2. Hypofunctionality of Gi proteins in patients with FM. Effect of Gpp(NH)p (10^{-5} M) on forskolin-stimulated adenylyl cyclase activity in lymphocytes from patients with FM (F), neuropathic pain (NP), osteoarthritis (O), or rheumatoid arthritis (RA) in comparison with healthy (H) subjects. Data are expressed as mean ± SEM; *p < 0.05 in comparison with forskolin-stimulated lymphocytes. □: forskolin 10^{-4} M; ■: forskolin 10^{-4} M + Gpp(NH)p 10^{-5} M.

ues obtained after stimulation with forskolin despite the fact that in patients with osteoarthritis levels of cAMP were higher than in all other groups (Figure 2).

Evaluation of cAMP basal levels. Under standard assay conditions, basal cAMP levels in lymphocytes from healthy subjects were 2.1 ± 0.3 pmol/10^6 lymphocytes (Figure 3a). Patients with FM had basal levels of cAMP about 3 times higher than the healthy group (Figure 3a). In contrast, the basal cAMP levels of patients with neuropathic pain, osteoarthritis, and rheumatoid arthritis were not statistically different from controls (Figure 3a). Moreover, the percentage of enhancement of cAMP production from basal levels after stimulation of adenylyl cyclase activity with forskolin 10^{-4} M was similar in all groups (80–90%). In contrast, it was significantly lower in patients with FM (Figure 3b).

Quantitative determination of Gi_α levels. The expression of Gi_α and of the subtypes $Gi_{2\alpha}$ and $Gi_{3\alpha}$ proteins was investigated. As illustrated in Figure 4, levels of Gi_α, $Gi_{2\alpha}$ and $Gi_{3\alpha}$ quantified by optical densitometry were similar in lymphocytes from both healthy subjects and patients with FM. $Gi_{3\alpha}$ levels showed a slight reduction (about 25%), which did not reach statistical significance (Figure 4).

DISCUSSION

Lymphocytes from patients with FM showed a reduced capability to inhibit forskolin-stimulated adenylyl cyclase activity as well as higher cAMP basal levels, clearly indicating a hypofunctionality of Gi proteins in FM. The impaired capability to inhibit forskolin-stimulated adenylyl cyclase activity was similar to that produced by preincubation of lymphocytes from healthy subjects with pertussis toxin at a concentration

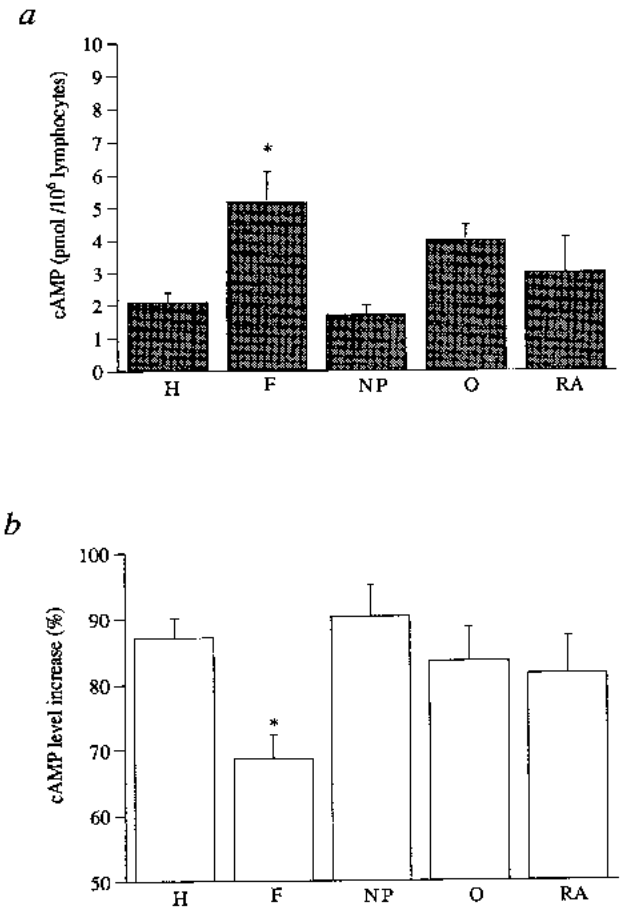

Figure 3. Evaluation of basal cAMP levels in patients with FM. The determination of basal cAMP levels (a) and increase of cAMP levels after stimulation with forskolin 10^{-4} M (b) in patients with FM (F), neuropathic pain (NP), osteoarthritis (O), or rheumatoid arthritis (RA) in comparison with healthy subjects. Data are expressed as mean ± SEM; *p < 0.05 in comparison with lymphocytes from healthy subjects.

consistent with a complete ADP-ribosilation of Gi proteins[35]. These considerations further support the hypothesis of a selective hypofunctionality of Gi proteins in this syndrome.

In addition, lymphocytes from patients with FM showed higher basal levels of cAMP, indicating an impaired equilibrium between the inhibitory and stimulatory system of adenylyl cyclase, mediated, respectively, by Gi and Gs proteins. These data further confirm the hypothesis of a hypofunctionality of Gi proteins, which is detectable both under basal conditions and after stimulation of the adenylyl cyclase system. An alteration at the level of adenylyl cyclase can be excluded since the direct stimulation of the enzyme by forskolin did not reveal any difference in the cAMP levels produced between patients and healthy subjects. However, it should be noted that subjects with osteoarthritis showed both basal and stimulated cAMP levels tendentially higher than all other subjects. This effect seems not to be related to an altered functionality of the Gi protein system since the percentage of increase of cAMP level after forskolin stimulation, as well as the amount of

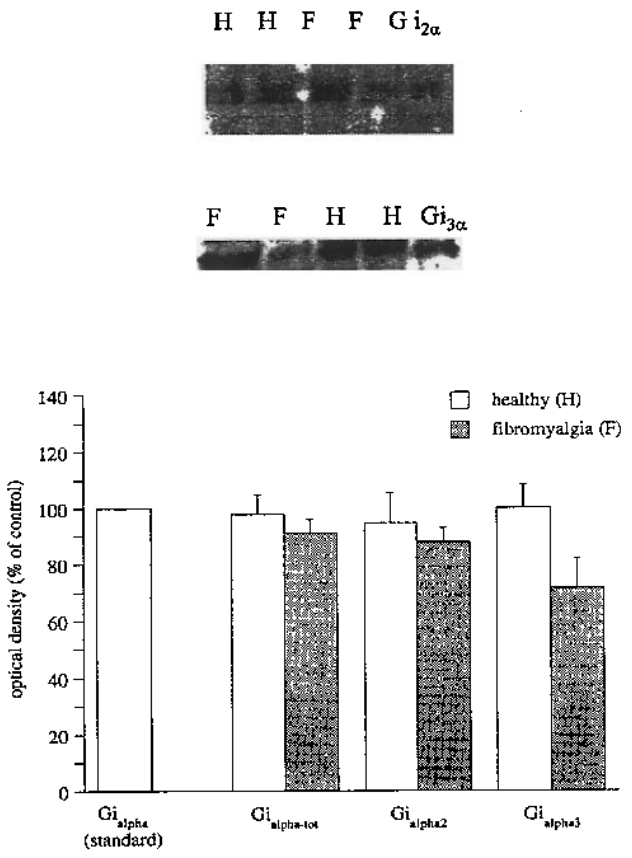

Figure 4. Evaluation of G_i protein levels in lymphocytes of patients with FM. Data are reported as a percentage of G_i levels in comparison with healthy subjects. Inset: bands obtained by Western blot analysis.

cAMP reduction after Gpp(NH)p administration, is comparable to those observed in healthy subjects. The presence of higher cAMP levels might be explained by taking into account that patients with osteoarthritis were slightly older. Indeed, in our experimental conditions, even though no statistically significant correlation between cAMP levels and age was detected, there was a tendency to an increase in cAMP production with the age (data not shown). However, other mechanisms cannot be excluded.

A reduced functionality of the endogenous antinociceptive system mediated by Gi proteins might be responsible for the widespread pain and allodynia typical of the FM syndrome. It is well known, indeed, that Gi protein activation inhibits the production of cyclic AMP, opens some potassium channels and closes some calcium channels, thus reducing cell excitability[22]. A deficiency of the Gi protein-mediated inhibitory system, therefore, produces cell hyperexcitability, which can lead to a hypersensitivity to pain. It has been reported that in laboratory animals, Gi protein inactivation produces hyperalgesia and allodynia[24-26]. Our results are also in agreement with the most recent hypothesis on the pathogenesis of FM, which considers an altered central mechanism of nociception as the biological basis of this syndrome[13,14]. Furthermore, the Gi protein hypofunctionality we have observed may well represent the biological mechanism responsible for the hypersensitivity to pain in FM.

To evaluate the functionality of Gi proteins, we detected the ability of the non-hydrolysable GTP analogue Gpp(NH)p to inhibit adenylyl cyclase activity. This enzymatic activity was previously amplified by forskolin, a diterpene that selectively activates adenylyl cyclase[36], at a concentration of 10^{-4} M, which is reported to be the most effective one in human lymphocytes[32].

Considering the multiplicity of GTP-binding proteins present in lymphocytes[37], it may seem difficult to detect a defect in a particular G-protein subtype with the use of a GTP analogue. However, not only a preferential affinity of Gpp(NH)p towards Gi proteins in the range of concentrations used has been evidenced[38,39], but also a widely different expression of each type of G protein is documented, indicating that Gi are commonly 10 times more abundant than Gs[40]. Therefore, Gpp(NH)p is a suitable tool for a selective investigation into Gi protein functionality.

Since the hypofunctionality of Gi proteins could underlie a reduction of the protein expression, we determined Gi protein levels by Western blot analysis. A slight reduction in Gi subunit levels was observed in lymphocytes from patients with FM. Considering that several Gi protein subtypes belong to the Gi family, in order to ascertain whether this reduction was related to a single subtype, we used specific antibody against $Gi_{2\alpha}$ and $Gi_{3\alpha}$. Gi_2 is the Gi protein subtype mainly involved in the inhibitory regulation of adenylyl cyclase activity *in vivo*[41] and it plays an important role in the modulation of pain perception. The administration of selective antibodies against $Gi_{2\alpha}$ as well as the inhibition of its expression by the use of specific antisense oligonucleotides prevented the analgesia induced by agonists of μ-opioid receptors[42,43]. Similarly to Gi_2, Gi_3 has been reported to be also involved in pain processing since antibodies against $Gi_{3\alpha}$ prevented δ-opioid analgesia[44]. Western blot analysis evidenced a slightly more marked reduction in $Gi_{3\alpha}$ levels rather than in $Gi_{2\alpha}$. However, considering that a difference in Gi_α levels between healthy and fibromyalgic subjects greater than 20–30% was never observed, we cannot establish from these data whether a reduction in protein levels contributes to the Gi protein hypofunctionality.

Gi proteins are coupled to many receptor types and subtypes. It is also well known that Gi protein levels can be down-regulated at several levels, such as turnover, transcription etc., by hormones and neurotransmitters. Considering that all subjects included in this study were drug-free for at least one week, we can assume that the observed Gi protein hypofunctionality represents an individual's predisposition to FM rather than a down-regulation produced by the pharmacological treatment.

It can also be excluded that the observed hypofunctionality of Gi proteins might be an adaptive response to a condition

of chronic pain. Patients with painful diseases such as neuropathic pain, osteoarthritis, or rheumatoid arthritis, who experienced pain sensations comparable to FM, as indicated by the VAS values, showed an unaltered Gi protein functionality.

The investigation into the Gi protein functionality was conducted in lymphocytes from peripheral blood taking into account that Gi proteins have an ubiquitous distribution[19] and that blood samples can be is easily obtained from patients in a painless manner. Lymphocytes represent an appropriate tool to detect Gi protein functionality since they have cell surface recognition sites for pertussis toxin, the selective inactivator of Gi. These recognition sites are lacking in other blood cell types such as erythrocytes[45]. Indeed, lymphocytes have been widely used to investigate the pathogenesis of various human diseases at a biochemical and molecular level[46-50].

Gi protein hypofunctionality is the first biochemical alteration observed in FM, and may well be involved in the pathogenesis of this syndrome. Furthermore, in the complete absence of laboratory diagnostic tests, the determination of an increase in cAMP basal levels in lymphocytes, together with the assessment of a Gi protein hypofunctionality after adenylyl cyclase stimulation, might lead to the biochemical identification of patients with FM.

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