FMS*CALCIUMFLUOR INCREASES ALKALINE PHOSPHATASE EXPRESSION DURING OSTEOGENESIS IN VITRO OF TIBIA-DERIVED RAT OSTEOBLASTS BY ACTIVATION OF $G\alpha_0/G\alpha_i$ PROTEINS

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INTRODUCTION

FMS*Calciumfluor is a preparation developed with the rationale of resonance homeopathy according to the protocols of the German homeopathic pharmacopoea (1,2).

We previously reported that treatment with FMS*Calciumfluor of cultured rat tibia derived osteoblasts (ROB) in differentiating conditions stimulates Alkaline Phosphatase (AP) expression via increase of AP mRNA level and increases the incorporation of radiolabeled Ca^{+2} in the matux while the drug has no effect on cell viability or proliferation (3).

Fluoride stimulation of the proliferation and differentiation of osteoprogenitor cells and of the differentiation of osteoblasts was previously reported (4-6) and the concentrations of NaF that induced osteogenic effects in vitro were usually between 10 and 100 μM. Protein-tyrosyl phosphorilation and signal transduction via MAP kinases has been shown to be required for the response of the osteoblasts to induction by NaF but conflicting evidence exists on the role of G-proteins (7-10). FMS*Calciumfluor is effective in stimulating osteogenesis at $8.4x10^{-8}$ M fluoride and the modalities of signal transduction are not known.

Stimulation of membrane receptors activates a group of GTP binding, coupling proteins (G-proteins) regulating many cellular responses. The targets of G-proteins (effectors) cause changes in second messengers which ultimately lead to the cellular response. G-proteins are made up

of three polypeptides: an α subunit that binds and hydrolyzes GTP, and the β and the γ subunits forming a dimer that can dissociate only if it is denatured becoming, therefore, a functional monomer. Eukaryotic cells contain many receptors acting via different types of G-proteins and many effectors, and mammalian cells were shown to have over 20 different α subunits of G-proteins (11).

Toxins which function as activators or inhibitors of specific subfamilies of G-proteins were utilized in this study in order to illustrate the pathway of signal transduction in osteoblast cultures which were induced to the enhancement of the osteogenic phenotype upon treatment with FMS*Calciumfluor. Cholera Toxin (CTX), which is an ADP-ribosil transferase, is an activator of $G\alpha_s$ protein. Pertussis Toxin (PTX), which functions in uncoupling receptors from $G\alpha_0/G\alpha_i$ trimers and Mastoparan 7 are activators of $G\alpha_0/G\alpha_i$. Mastoparan 17 (an inactive analog of Mastoparan 7) was also utilized as an experimental control.

We here show that activity of the $G\alpha_0/G\alpha_i$ proteins is required for the enhancement of osteogenic differentiation suggesting that specific membrane receptors can now be sought for the components of FMS*Calciumfluor.

MATERIALS AND METHODS

Cell Culture treatments - Cells obtained from 7-day-old rat tibiae, were cultured in Coon's modified F12 medium supplemented with 10% fetal calf serum (FCS) and expanded to 120 cumulative population doubling (CDP). When the cells reached the confluence the medium was added with 100 μg/ml ascorbic acid and 10 mM β-Glycerol Phosphate (differentiating conditions).

24 h before the maximal expression of AP, the culture medium was replaced with F12 containing 1% FCS and the cells were treated for the following 24 h with either Pertussis Toxin (0,1 μg/ml) or with Mastoparan 7 and 17 (0,1 μg/ml). Alternatively they were treated for 1 h with Cholera Toxin (1 μg/ml). Immediately after removal of these drugs from the medium, FMS*Calciumfluor was added at inducing concentration (1/25 dilution) for

the following 24 hours and AP was tested in the cultures thereafter. All chemicals were from Sigma, unless otherwise stated. FMS*Calciumfluor (D6/D12) (FM-Pharma GMBH D-76135, Karisruhe, Germany), was obtained from Omeopiacenza srl (Italy). The concentration of each component in the D6/D12 starting preparation was 2.15×10^{-6} M CaF_2, 9.7×10^{-7}M $MgHPO_3$, 2.81×10^{-7} M SiO_2 and 0.5% lactose in 40% Ethanol (3).

Alkaline Phosphatase Determination - Quantitative determination of the enzyme was performed on duplicate aliquots of cell lysates in PBS, 0.01% SDS, using Sigma Aldrich 104-LL kit according to the manufacturer recommendations (3,12). Duplicate determinations were done for each experimental point.

Fluorimetric Determination of DNA - DNA content was determined in aliquots of cell lysates by fluorimetry using Hoechst 33258 (Sigma), according to published protocol (3,12). Duplicate determinations were done for each experimental point.

RESULTS

Effect of the transient activation of $G\alpha_s$ protein on the induction of AP by FMS*Calciumfluor - We tested the possible involvement in the response to FMS*Calciumfluor of signal transduction via $G\alpha_s$ protein by activating this protein by treatment with Cholera Toxin. Osteoblasts were preincubated with or without 1 μg/ml Cholera Toxin for 1 h prior to the treatment with FMS*Calciumfluor for the following 24 h (fig. 1). No effect was observed in the level of the expression of AP in the control, which did not receive FMS*Calciumfluor and in the culture induced by FMS*Calciumfluor, suggesting that the endogenous expression of AP as well as the increased expression induced by treatment with FMS*Calciumfluor are unaffected by activation of $G\alpha_s$ protein. Higher concentration (5 μg/ml) were also tested and found uneffective (data not show).

Effect of the transient inhibition of $G\alpha_0/G\alpha_i$ proteins on the induction of AP by FMS*Calciumfluor - We tested the possible involvement in the response to FMS*Calciumfluor of signal transduction via $G\alpha_0/G\alpha_i$ proteins by inhibiting them by treatment with Pertussis toxin or activating them

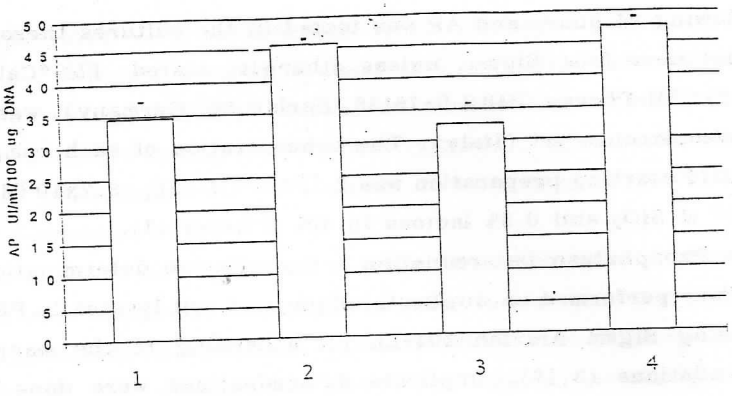

Fig. 1 - Effect of a pre-treatment with Cholera Toxin (CTX). ROB of 120 CPD were cultured for 5 days in differentiation condition with 10% FCS. At 5 days the culture medium was changed to 1% FCS for 24 h and then the cultures were additioned in the following different ways:
1 - no additions, 24 h
2 - FMS*Calciumfluor (1/25), 24 h
3 - CTX (1 μg/ml) for 1 h followed by medium, 23 h
4 - CTX (1 μg/ml) for 1 h followed by FMS*Calciumfluor (1/25), 23 h.
AP was measured in duplicates at the end of the treatment.

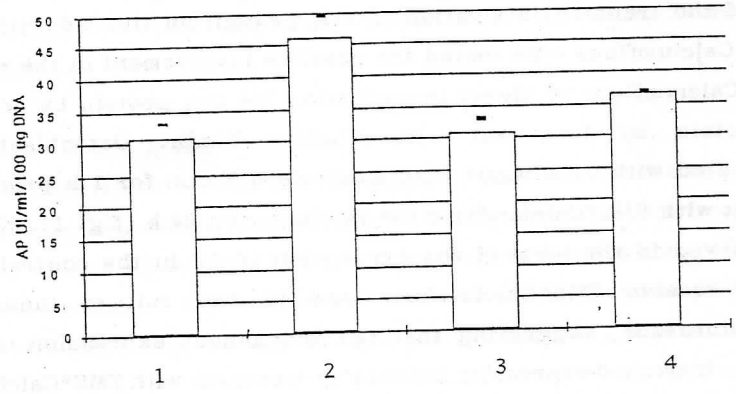

Fig. 2 - Effect of a pre-treatment with Pertussis toxin (PTX). ROB of 120 CPD were cultured for 5 days in differentiation condition with 10% FCS. At 5 days the culture medium was changed to 1% FCS for 24 h and then the cultures were additioned in the following 4 different ways:
1 - no additions, 48 h
2 - no additions, 24 h, followed by FMS*Calciumfluor (1/25), 24 h
3 - PTX (0.1 μg/ml), 24 h, followed by no additions, 24 h
4 - PTX (0.1 μg/ml), 24 h, followed by FMS*Calciumfluor (1/25), 24 h.
AP was measured in duplicates at the end of the treatment.

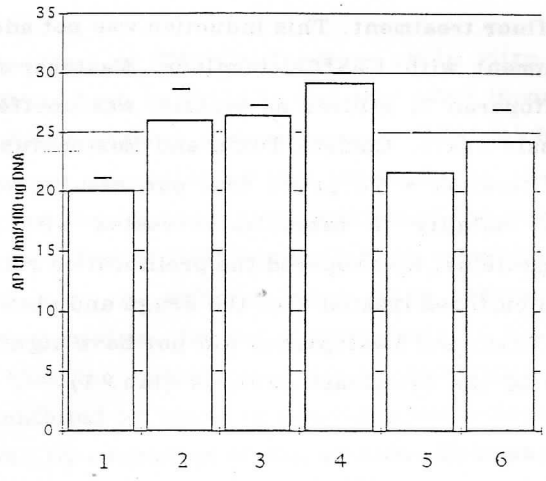

Fig. 3 - Effect of a pre-treatment with Mastoparan.
ROB of 120 CPD were cultured for 5 days in differentiation condition with 10% FCS. At 5 days the culture was treated in the following 6 ways:
1 - no additions, 48 h
2 - no additions, 24 h, followed by FMS*Calciumfluor (1/25), 24 h
3 - Mastoparan 7 (0.1 μg/ml), 24 h, followed by no additions, 24 h
4 - Mastoparan 7 (0.1 μg/ml), 24 h, followed by FMS*Calciumfluor (1/25), 24 h
5 - Mastoparan 17 (0.1 μg/ml), 24 h, followed by no additions, 24 h
6 - Mastoparan 17 (0.1 μg/ml), 24 h, followed by FMS*Calciumfluor (1/25), 24 h.
AP was measured in duplicates at the end of the treatment.

by treatment with Mastoparan 7. Osteoblasts were preincubated with or without 0.1 μg/ml Pertussis Toxin for 24 h prior to the exposition to FMS*Calciumfluor for the following 24 h. Induction of AP by FMS*Calciumfluor was inhibited by pretreatment with Pertussis Toxin, suggesting that the response pathway to FMS*Calciumfluor involves $G\alpha_0/G\alpha_i$ proteins coupled receptors (fig. 2). The inhibition was observed also at higher concentrations of PTX, while 1/10 less concentrated PTX was uneffective (data not shown). Endogenous levels of AP expression were unaffected by treatment. Incubation with Mastoparan 7 for 24 h caused by itself an increase of AP induction not significatively different than that induced

by FMS*Calciumfluor treatment. This induction was not additive with that caused by treatment with FMS*Calciumfluor. Mastoparan 17, inherent analogue of Mastoparan 7, utilized as control, was uneffective (fig. 3).

Effect of Pertussis Toxin, Cholera Toxin and Mastoparan -7 and -17 on osteoblasts proliferation - To prove that our results were not due to differential cell viability in osteoblasts treated with inhibitors and activators of G-proteins, we compared the proliferation rate of osteoblasts in control and in cultures treated with the drugs and show that Pertussis Toxin, Cholera Toxin and Mastoparans did not have significative effects on DNA content of the osteoblast cultures (tab. 1).

Tab. 1 - Effect of Cholera Toxin (a), Pertussis Toxin (b), Mastoparan 7 and Mastoparan 17 (c) on osteoblasts proliferation.
ROB of 120 CPD were cultured for 5 days in differentiation condition with 10% FCS and then at 5 days the cultures were treated as described in Fig. 1, Fig. 2, and Fig. 3. DNA content of osteoblasts in control and in cultures treated with the drugs were measured at day 6 (a) and 7 (b,c).

TREATMENT	DNA mg/ml
a)	
untreated	72.82 ± 5
FMS*Calciumfluor	76.42 ± 3.3
CTX	79.26 ± 4.6
FMS*Calciumfluor + CTX	68.12 ± 8.8
b)	
untreated	78.82 ± 6.9
FMS*Calciumfluor	86.42 ± 1
PTX	80.33 ± 7.6
FMS*Calciumfluor + PTX	88.09 ± 2
c)	
untreated	70.75 ± 8
FMS*Calciumfluor	74.5 ± 2.2
Mastoparan 7	78.1 ± 0
FMS*Calciumfluor + Mastoparan 7	76.9 ± 4.7
Mastoparan 17	76.0 ± 7.6
FMS*Calciumfluor + Mastoparan 7	74.1 ± 3

DISCUSSION

The stimulatory effect of FMS*Calciumfluor on in vitro osteogenesis is complex and affects both early (AP) and late (Ca^{+2} incorporation in the extracellular matrix) markers of osteogenesis. FMS*Calciumfluor induces both increase in the amount of AP and shortening of the time course of its transient expression through up-regulation of the amount of its mRNA (3). Moreover, the effect of FMS*Calciumfluor on osteoblast differentiation changes according to the differentiation stage previously reached by osteoblasts, being maximal for early osteoblasts (5,6).

We here show that the induction of AP by FMS*Calciumfluor in early osteoblasts is inhibited by blocking $G\alpha_0/G\alpha_i$ protein-dependent pathways and is unaffected by activation of $G\alpha_s$ protein. Mastoparan 7 mimics the activity of FMS*Calciumfluor, by acting via the $G\alpha_0/G\alpha_i$ protein-dependent pathway. We also show that the pathway of endogenous activation of AP expression and the one induced by FMS*Calciumfluor treatment differ in their response to drugs interfering with G proteins, since endogenous activation is not affected by inhibition of $G\alpha_0/G\alpha_i$.

We are presently trying to identify the proteins differentially phosphorilated downstream of $G\alpha_0/G\alpha_i$ proteins and which might be involved in the specific differentiative response to FMS*Calciumfluor of early osteoblasts.

We studied the involvement of G-proteins in transducing the inductive signal generated by treatment of tibial-derived neonatal rat osteoblasts (ROB) cultured in vitro with FMS*Calciumfluor, a homeopathic preparation utilized in the therapy of osteoporosis. We previously reported that FMS*Calciumfluor acts as inducer and potentiator of osteogenic differentiation in vitro, among other effects, by increasing the expression of Alkaline phosphatase (AP). We utilized Pertussis Toxin (PTX), an inhibitor of $G\alpha_0/G\alpha_i$ proteins, Mastoparan 7, an activator of $G\alpha_0/G\alpha_i$ proteins and Cholera Toxin (CTX), a stimulator of $G\alpha_s$ protein to show involvement of specific G proteins in the inductive effect on AP of FMS*Calciumfluor. We here show that the increase in AP expression

induced by FMS*Calciumfluor is dependent on the activation of $G\alpha_0/G\alpha_i$ proteins, while it is unaffected by the activation stage of the $G\alpha_s$ protein. Moreover, we show that the expression of endogenous AP during osteogenesis in vitro is regulated independently from G proteins, and unaffected by their activation stage and therefore that treatment with FMS*Calciumfluor activates a new pathway of cellular response.

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