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SHORT COMMUNICATION

Regulation of Expression and Function of m2 and m4 Muscarinic Receptors in Cultured Embryonic Chick Heart Cells by Transforming Growth Factor-β_1

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ABSTRACT. Incubation of cultured embryonic chicken heart cells with transforming growth factor β_1 (TGF-β_1) resulted in a concentration- and time-dependent decrease in the number of muscarinic acetylcholine receptors (mAChR), which reached a maximum by 24 hr. Twenty-four hours following TGF-β_1 treatment, cm2 and cm4 mAChR protein levels were decreased 59 and 41%, respectively, and cm2 mRNA and cm4 mRNA levels were both decreased by approximately 40%. Chick heart cells treated with TGF-β_1 exhibited a decreased sensitivity for carbachol-mediated inhibition of adenylyl cyclase activity compared with control cells. Thus, TGF-β_1 stimulation of chick heart cells results in decreases in the expression of both cm2 and cm4 mAChR and in muscarinic responsiveness. BIOCHEM PHARMACOL **54**;4:525–527, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. muscarinic acetylcholine receptor; cardiac cell culture; growth factor; mRNA; cAMP; signal transduction

The TGF-βs‡ have been implicated in several different aspects of cardiac development and function. TGF-β_1, -β_2, and -β_3 stimulate cardiac muscle development in embryonic stem cells and other model systems [1], and the TGF-βs are expressed in a spatial and temporal pattern that suggests that they are involved in the induction of cardiac muscle, septation, and formation of the cardiac valves [2–4]. Treatment of neonatal rat myocytes with TGF-β_1 has been reported to induce expression of contractile protein genes, such as β-myosin heavy chain (β-MHC), and skeletal and smooth muscle α-actin, which are up-regulated by mechanical stress [5]. In the late phase of myocardial infarction, the levels of TGF-β_1 mRNA and protein are increased [6, 7]. Furthermore, TGF-β treatment of heart cells has been shown to maintain the beating rate of cells treated with interleukin-1 [8]. Treatment of mouse heart cell cultures with TGF-β_1 decreases β-adrenergic receptors [9], but it is not known if TGF-β_1 affects muscarinic receptor expression in the heart. We report here that treatment of primary chick embryonic heart cell cultures with TGF-β_1 decreased muscarinic receptor expression and function.

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‡ *Abbreviations:* cAMP, cyclic 3′,5′-monophosphate; mAChR, muscarinic acetylcholine receptor(s); QNB, quinuclidinyl benzilate; TFA, trifluroacetic acid; and TGF-β_1, transforming growth factor-β_1.

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MATERIALS AND METHODS

Materials

White leghorn chicken eggs were obtained from H & N International (Redmond, WA) and maintained in a humidified 38° incubator until day 9 of incubation. TGF-β_1 was obtained from Upstate Biotechnology, Inc. All other materials were purchased as described previously [10, 11].

Cell Culture

Heart cells from 9-day embryonic chickens were prepared in serum-free defined medium as described by Subers and Nathanson [11]. Cardiac cells were plated at a density of 1.0×10^6 cells/cm^2 on 100-mm plastic tissue culture dishes and maintained in a 5% CO_2 environment at 37°. TGF-β_1, dissolved in 36% acetonitrile/0.1% TFA containing 40 μg BSA, or vehicle (36% acetonitrile/0.1% TFA containing 40 μg BSA) was added 72 hr after culture preparation or as otherwise noted. The medium was changed on day 3, and assays were performed on day 4 of culture.

Binding Assays in Membrane Homogenates

The number of mAChR binding sites was determined by the binding of [^{3}H]QNB as described by Habecker and Nathanson [12].

cAMP Accumulation Assays

Chick heart cells were cultured on 60-mm tissue culture plates, and carbachol-mediated inhibition of forskolin-

stimulated cAMP accumulation was measured as described by Subers and Nathanson [11].

Solution Hybridization

The absolute number of molecules of mRNA encoding cm2 and cm4 mRNA was measured by solution hybridization of RNA samples with subtype specific antisense riboprobes as described by Habecker and Nathanson [12].

Immunoprecipitation Assay

The levels of cm2 and cm4 mAChR proteins were measured by immunoprecipitation using subtype-specific antibodies as described by McKinnon and Nathanson [10]. Crude membrane homogenates obtained from pooled samples of control or treated chick heart cell cultures were frozen immediately in dry ice–acetone and stored at −70°. Immunoprecipitation assays were carried out with 1.42 mg of solubilized protein per assay. Antibodies were used at a final concentration of 6 μg/mL.

RESULTS AND DISCUSSION

TGF-β_1-Mediated Decrease in mAChR Numbers

Treatment of embryonic chick heart cells cultured in defined medium with TGF-β_1 for 24 hr resulted in a concentration-dependent decrease in the number of mAChRs as measured with the muscarinic antagonist [^{3}H]QNB (Fig. 1). Muscarinic receptor number began to decrease by 12 hr and was reduced maximally by 24 hr after TGF-β_1 stimulation (Fig. 2). The decrease in muscarinic receptor numbers at 48 hr of TGF-β_1 treatment was similar to that observed at 24 hr (data not shown).

Immunoprecipitation Assay of cm2 and cm4 from TGF-β_1-Treated Cells

The main subtypes of mAChR expressed in chick heart are the cm2 and cm4 receptors [10]. We used immunoprecipitation with subtype-specific antibodies to determine the effects of TGF-β_1 treatment on the expression of cm2 and cm4 mAChR proteins. The level of expression of cm2 mAChR in cultured chick heart cells stimulated by TGF-β_1 for 24 hr was reduced from 17.6 ± 2.1 to 7.3 ± 2.5 fmol/mg protein, and the levels of cm4 mAChR protein levels were reduced from 6.8 ± 1.0 to 4.0 ± 1.1 fmol/mg protein (mean ± SD, N = 3). Thus, the decrease in mAChR expression in TGF-β_1-treated cells is due to a reduction in both cm2 and cm4 mAChR protein levels.

cAMP Accumulation Assay

To determine if the decrease in mAChR number following TGF-β_1 stimulation resulted in a decreased functional responsiveness to muscarinic agonists, we examined the ability of the mAChR to inhibit adenylyl cyclase activity.

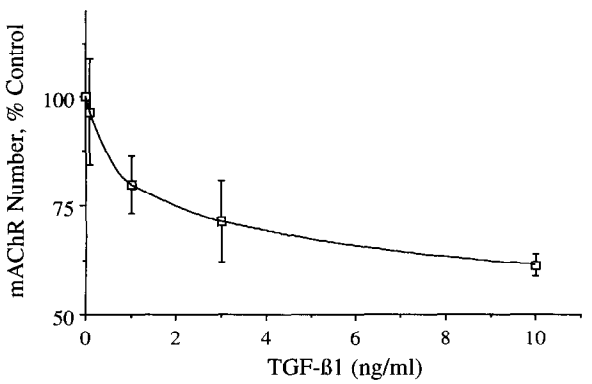

FIG. 1. Concentration–response curve for TGF-β_1-mediated decrease in [^{3}H]QNB binding to chick heart cell cultures. Embryonic chick heart cells were cultured in serum-free defined medium as described in Materials and Methods. The medium was changed, and TGF-β_1 was added on day 3, and the binding of [^{3}H]QNB to membrane homogenates was measured on day 4. Data are presented as the percent of control ± SD from three separate experiments, each of which had four independent determinations. The mean value of [^{3}H]QNB binding to control heart cells was 165 ± 24 fmol/mg cellular protein. There were significant differences between control and TGF-β_1-treated heart cells at 1, 3, and 10 ng/mL (Student's *t*-test for unpaired observations, $P = 0.03$, $P = 0.0002$, and $P = 0.0001$, respectively).

The concentration–response curves for carbachol-mediated inhibition of forskolin stimulation of cAMP formation demonstrated that chick heart cells treated with 10 ng/mL TGF-β_1 exhibited a decreased physiological sensitivity to muscarinic agonists (Fig. 3). At agonist concentrations of 10^{-3} to 10^{-6} M, carbachol-mediated inhibition of forskolin-stimulated adenylyl cyclase activity was reduced significantly in TGF-β_1 treated cells (Fig. 3), with a decrease in both the extent of maximal inhibition and nearly a 10-fold increase in the EC_{50} for carbachol. Thus, the decrease in mAChR number induced by TGF-β_1 treatment is accom-

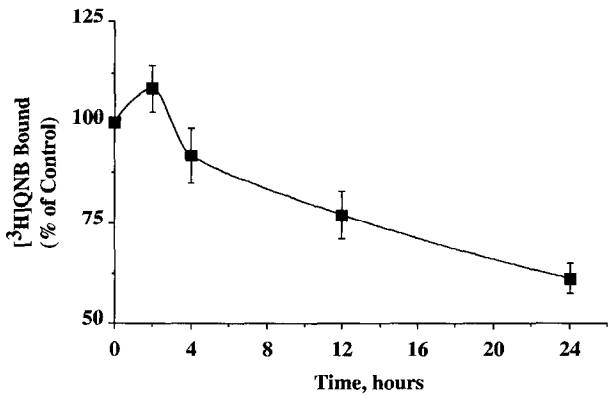

FIG. 2. Time–course of TGF-β_1-mediated decrease in mAChR number. Heart cells were treated at the indicated times with 10 ng/mL TGF-β_1 prior to determination of [^{3}H]QNB binding as described in Fig. 1. Data are presented as the means ± SD from three separate experiments, each of which had four independent determinations.

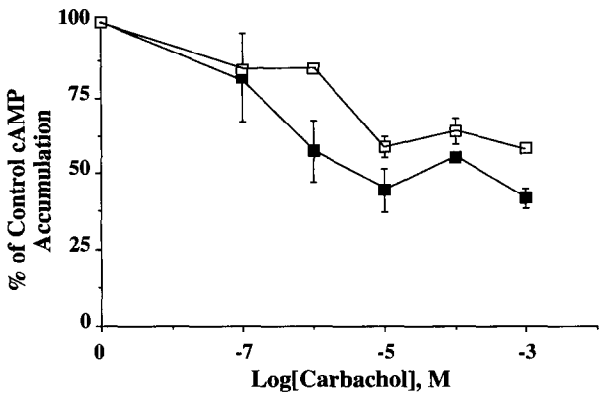

FIG. 3. Decreased sensitivity to carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation in TGF-β_1 cells. Cultures were incubated with either vehicle (■) or 10 ng/mL TGF-β_1 (□) for 24 hr. Heart cells were incubated with 100 μM forskolin and the indicated concentrations of carbachol for 5 min, and then cellular cAMP levels were determined. Values are presented as percent of control cAMP (±SD). The forskolin-stimulated levels of cAMP in the absence of carbachol were 4950 ± 930 pmol cAMP/mg protein in untreated cells and 3790 ± 550 in TGF-β_1-treated cells. Each experiment was performed in triplicate, and the results shown are the averages of two to three separate experiments. At carbachol concentrations of 10^{-3} to 10^{-6} M, TGF-β_1-treated cells were significantly different from control treated cells, $P < 0.05$.

panied by a decreased functional sensitivity to muscarinic agonists.

Solution Hybridization Analysis of mAChR mRNA Levels

We examined if the decrease in mAChR number was due to a decreased level of mRNA encoding the two main mAChR subtypes, cm2 and cm4. The levels of cm2 and cm4 mRNA were measured by solution hybridization using subtype-specific riboprobes [11]. Incubation with 10 ng/mL TGF-β_1 for 24 hr decreased cm2 from $1.32 \pm 0.15 \times 10^6$ to $0.75 \pm 0.15 \times 10^6$ molecules/μg mRNA, and cm4 mRNA from $5.8 \pm 0.06 \times 10^5$ to $3.4 \pm 0.04 \times 10^6$ molecules/μg mRNA (mean ± SD, N = 3). Thus, down-regulation of mAChR numbers in cultured embryonic chick heart cells by TGF-β_1 is due to reduced expression of both cm2 and cm4 muscarinic receptor subtypes.

In conclusion, TGF-β_1 has been implicated in the development of the heart and in the regulation of cardiac contractile gene expression [1]. The expression of TGF-β_1 increases in several types of cardiac disease, and infusion of TGF-β_1 reduces myocardial necrosis of reprofusion injury. We have shown that TGF-β_1 stimulation causes a decrease in mAChR expression and function in cardiac cells. Thus, the regulation of cardiac mAChR expression by TGF-β_1 may not only represent an interesting example of the regulation of neurotransmitter receptors by peptide growth factors but also have important clinical and pathological implications as well.

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