Proteinase-Activated Receptor Agonists Stimulate the Increase in Intracellular Ca^{2+} in Cardiomyocytes and Proliferation of Cardiac Fibroblasts from Chick Embryos

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We studied activation of cultured cardiomyocytes and cardiac fibroblasts from chick embryos induced by agonists of PAR1 (thrombin and PAR1 peptide agonist) and PAR2 (trypsin, factor Xa, and peptide SLIGRL) by analyzing changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cardiac fibroblast proliferation. Exposure of cardiomyocytes with thrombin induced immediate permanent dose-dependent increase in $[Ca^{2+}]_i$. Ca^{2+} response decreased in a calcium-free medium. Peptide agonists of PAR1 and PAR2 also stimulated the increase in $[Ca^{2+}]_i$ in cardiomyocytes. Thrombin induced a short-term increase in $[Ca^{2+}]_i$ in cardiac fibroblasts and potentiated cell proliferation. PAR2 agonists trypsin and peptide SLIGRL stimulated proliferation of cardiac fibroblasts. Our results indicate that cardiomyocytes and cardiac fibroblasts from chick embryos have at least two types of PAR (types 1 and 2).

Key Words: *cardiomyocytes; cardiac fibroblasts; PAR agonists*

Thrombin, a key serine proteinase of hemostasis, acts as cellular regulator involved in inflammatory processes, tissue reparation after damage, embryogenesis, *etc*. [1,2]. Thrombin modulates cell functions through specific proteinase-activated receptors (PAR) [1,4,6,9]. PAR proteins belong to a family of seven-domain G protein-coupled transmembrane receptors. Proteinases regulate cell activity via cleavage of one peptide bond in the extracellular domain of PAR and opening of a new N-terminal end in the receptor (bound ligand), which activates this receptor [4]. There are 4 members of the PAR family: PAR1, PAR3, and PAR4 are thrombin receptors and PAR2 is the receptor for trypsin, mast cell tryptase, factor VIIa, factor Xa, *etc*. [1,4,6,9]. Synthetic peptide agonists of PAR (PAR-AP) are structurally similar to bound ligands. They serve as agonists of specific receptors. PAR1 proteolysis by thrombin is a critical event in embryogenesis. Organogenesis in mice is accompanied by the expression of prothrombin and thrombin receptor. Previous studies revealed partial embryonic mortality and early neonatal death of mice that are deficient in prothrombin and PAR1 [13,14].

PAR agonists regulate the response to inflammation and tissue injury [1,6,9,12]. For example, thrombin stimulates proliferation and mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and prevents apoptosis in myoblasts of skeletal muscles [5]. Experiments on $PAR1^{-/-}$ knockout mice showed that thrombin-mediated regulation of myoblast apoptosis is not realized via PAR1. However, PAR1 is the only receptor for thrombin that induces proliferation and Ca^{2+} mobilization in neonatal mouse myoblasts.

Hence, thrombin and other agonists of PAR are involved in various biological responses of cells.

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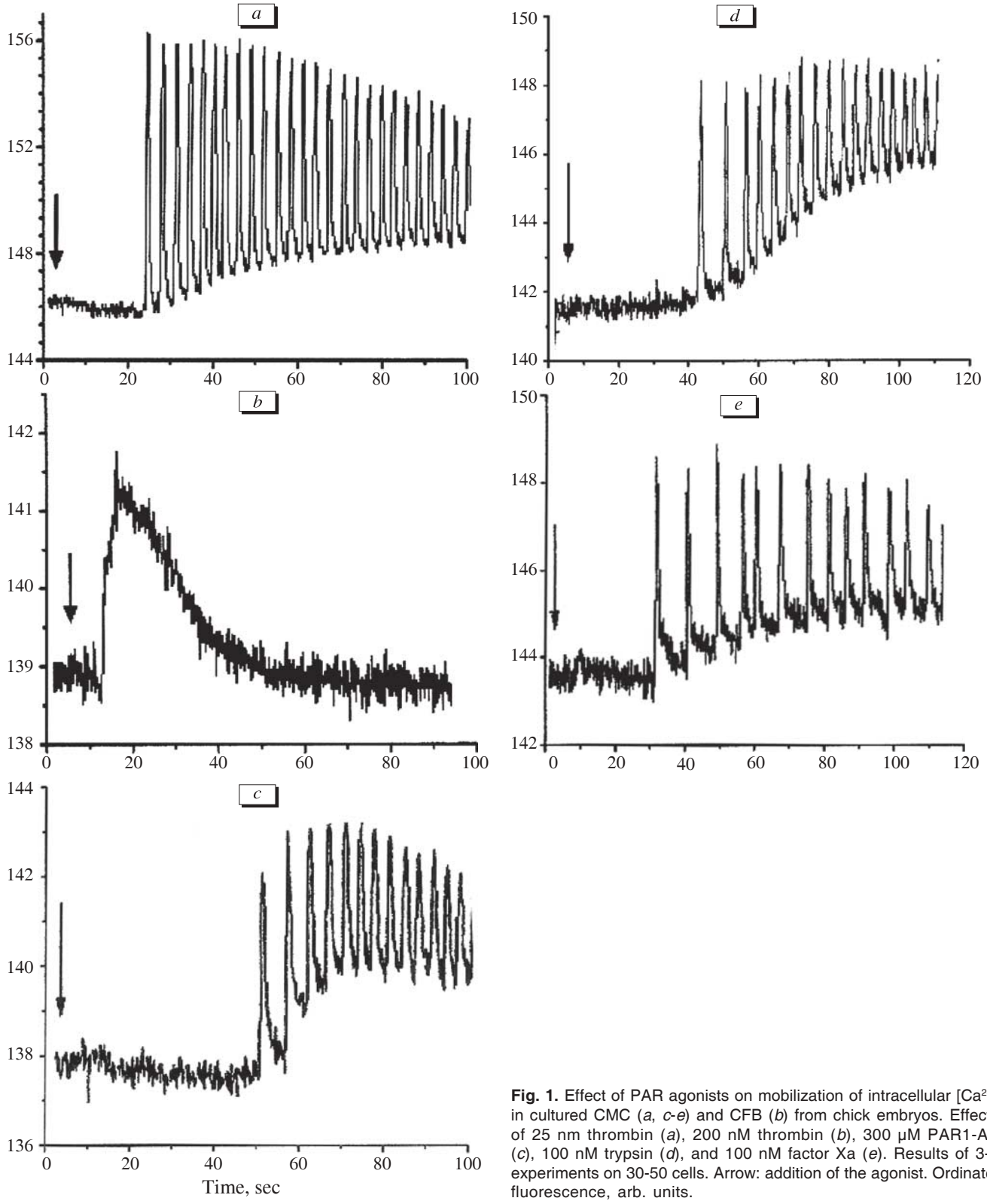

Fig. 1. Effect of PAR agonists on mobilization of intracellular $[Ca^{2+}]_i$ in cultured CMC (*a*, *c*-*e*) and CFB (*b*) from chick embryos. Effects of 25 nm thrombin (*a*), 200 nM thrombin (*b*), 300 μM PAR1-AP (*c*), 100 nM trypsin (*d*), and 100 nM factor Xa (*e*). Results of 3-5 experiments on 30-50 cells. Arrow: addition of the agonist. Ordinate: fluorescence, arb. units.

However, little is known about the role of these substances in the function of embryonic cardiomyocytes (CMC) and cardiac fibroblasts (CFB). Previous studies showed that activation of thrombin receptor in ventricular CMC of adult rats induced an increase in $[Ca^{2+}]_i$ [7]. PAR1 and PAR2 are co-expressed in neonatal CMC and CFB of rats. Their activation increases CMC excitability [10]. The

PAR-mediated effects in CMC and CFB of chick embryos remain unknown.

In this work, the effects of PAR1 agonists (thrombin and PAR1-AP) and PAR2 agonists (trypsin, factor Xa, and PAR2-AP) on cultured CMC and CFB from chick embryos were evaluated from changes in $[Ca^{2+}]_i$ and CFB proliferation.

MATERIALS AND METHODS

CMC and CFB were isolated from heart ventricles of chick embryos (9 days of gestation, n=50) and cultured in DMEM containing 10% fetal bovine serum and penicillin-streptomycin at 37°C and 5% CO_2 [8]. CMC and CFB were separated by the ability of CFB to adhere to Petri dishes. Cell concentration was 1.5-2.0×10⁴/ml. In some experiments, serum-free medium was used.

Isolated CMC and CFB were loaded with a Fluo-3 AM Ca^{2+}-sensitive fluorescent probe (Molecular Probe, 5 μM) for 30 min. $[Ca^{2+}]_i$ was measured by fluorescent microscopy (IX70, Olympus) [2]. The fluorescence image was analyzed by means of Silicon Graphics (Lab VIEW) and NIH images software. The cells were treated with bovine thrombin (Sigma, 25-200 nM), PAR1-AP (SFLLRN, Biosyntan, 30-300 μM), trypsin (Sigma, 25-200 nM), PAR2-AP (SLIGRL, Biosyntan, 30-300 μM), or factor Xa (Sigma, 25-1000 nM).

Proliferation of cultured CFB was studied as described elsewhere [3]. Some cells were subjected to serum deprivation and incubated in the presence or absence of thrombin, PAR1-AP, trypsin, PAR2-AP, or factor Xa. After 24 h, these cells were treated with trypsin, washed, and stained with trypan blue. Viable cells were counted in a hemocytometer. The results are expressed as the ratio of agonist-treated cells to agonist-untreated cells.

The data on cell count are expressed as the mean value and mean square deviation (5 independent experiments). The results were analyzed by paired Student's t test.

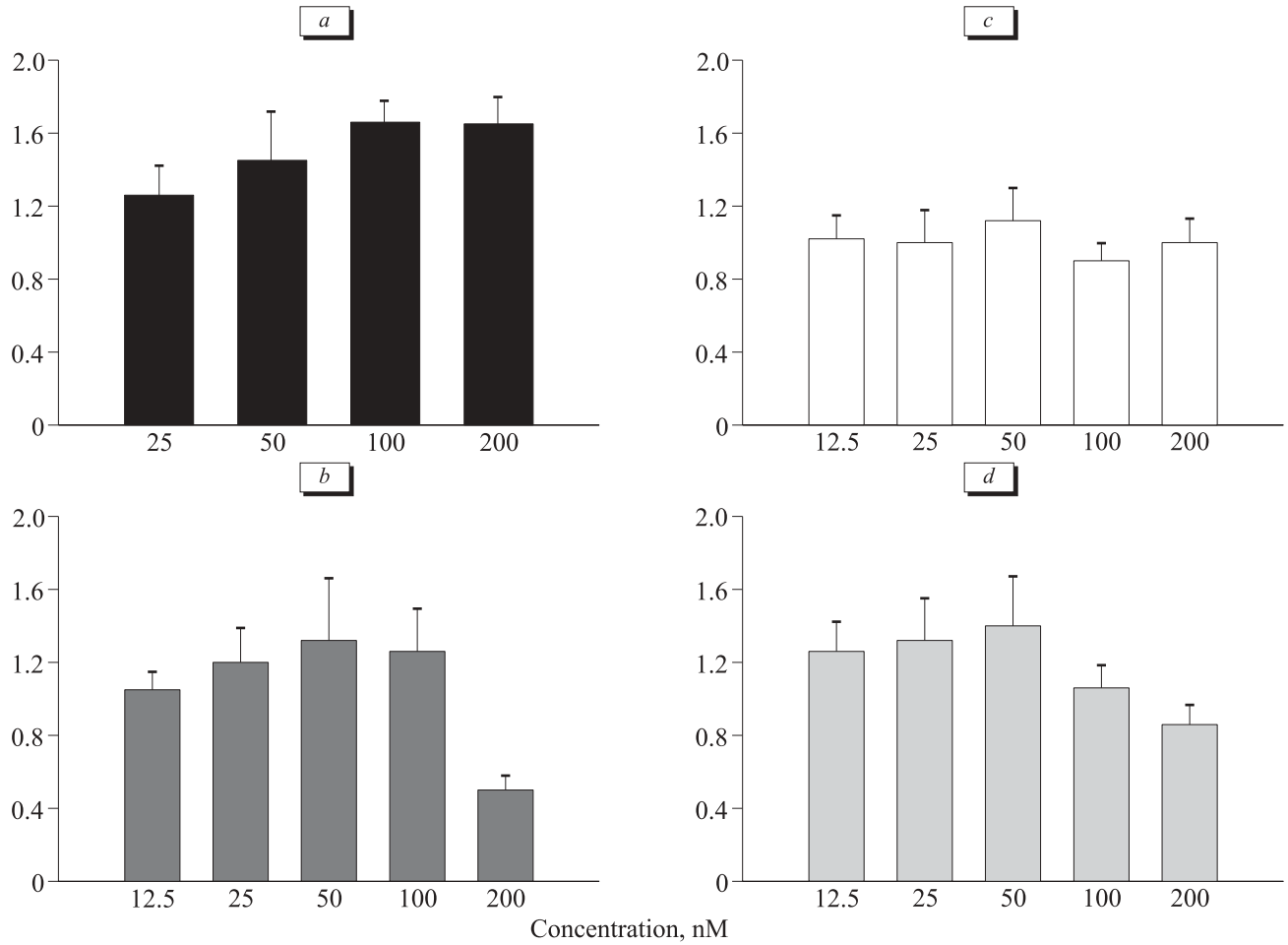

Fig. 2. Changes in CFB proliferation after exposure to thrombin (*a*), trypsin (*b*), factor Xa (*c*), and PAR2-AP (*d*) in different concentrations. Ordinate: ratio of cell numbers in agonist-treated and agonist-untreated samples.

RESULTS

Thrombin (25 nM) induced a permanent increase in $[Ca^{2+}]_i$ in CMC and rhythmic oscillations of Ca^{2+} (Fig. 1, *a*). Thrombin in concentrations of 25-200 nM stimulated the increase in $[Ca^{2+}]_i$ in CMC in a concentration-dependent manner. These changes were most pronounced at a thrombin concentration of 100 nM (data not shown). Similarly to thrombin, PAR1-AP activated CMC. However, the concentration of PAR1-AP was much higher compared to that of thrombin (by several orders of magnitude, Fig. 1, *c*). In a calcium-free medium, thrombin increased $[Ca^{2+}]_i$ only in high concentration (200 nM). The amplitude of $[Ca^{2+}]_i$ oscillations under these conditions was much lower than under the influence of 25 nM thrombin in a calcium-containing medium (data not shown). The PAR1 agonist-induced increase in $[Ca^{2+}]_i$ and rhythmic oscillations of Ca^{2+} are probably associated with Ca^{2+} efflux from the intracellular pool and Ca^{2+} influx through receptor-activated Ca^{2+} channels. Similarly to PAR1 agonists, PAR2 agonists trypsin and factor Xa (100 nM) stimulated the increase in $[Ca^{2+}]_i$ in CMC (Fig. 1).

Thrombin activated embryonic CFB by inducing a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1, *b*). The increase in the rate of cell proliferation depended on thrombin concentration (Fig. 2, *a*). Thrombin in a concentration of 100 nM had most pronounced effect on cell proliferation. Not only PAR1 agonists, but also PAR2 agonists trypsin and PAR2-AP induced a dose-dependent increase in cell proliferation (Fig. 2, *b*, *d*). The proliferative effect was maximum after treatment with trypsin in a concentration of 50 nM. Increasing the concentration of trypsin was accompanied by a decrease in proliferation rate. The rate of cell proliferation did not differ from the basal level at a trypsin concentration of 200 nM. PAR2-AP in a narrow range of concentrations (25-50 μM) produced a proliferative effect on CFB. Factor Xa in concentrations of 25-200 nM did not modulate CFB proliferation (Fig. 2, *c*).

We showed for the first time that CMC and CFB from chick embryos express at least two types of PAR (PAR1 and PAR2). Our data support the results of studies with neonatal rat CMC and CFB, which show that PAR1 agonists are involved in tissue remodeling during cardiac injury and/or inflammation [11].

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