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SERUM-MEDIATED ENHANCEMENT OF ANF ACCUMULATION IN THE CULTURE MEDIUM OF CARDIAC ATRIOCYTES

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Cultured cardiac myocytes provide a useful system for investigating ANF biosynthesis and regulation. It has previously been demonstrated that the predominant form of ANF stored in and released from this myocyte model is the 17kD prohormone, proANF. We report here that as quantitated both by radioimmunoassay and by SDS-PAGE of intrinsically labelled ANF released from these cardiocytes, the addition of serum promotes a 5-6 fold increase in ANF accumulation in the medium of these cells as compared to ANF accumulation in the presence of a defined chemical medium alone. The stimulating effect of serum is immediate and persists in a linear manner for at least 120 minutes. This effect of serum can be reproduced by the addition of albumin or other proteins to the medium but not by alterations in osmolality. Whether this phenomenon represents enhanced release of proANF or is secondary to inhibition of proANF degradation has yet to be determined.

Work from our laboratory (1) and others (2) using the cultured atrial myocyte as an in vitro model for studying ANF biosynthesis and release has demonstrated that proANF (1-126) is both stored and secreted from these cells in culture. Additional evidence suggests that cleavage to the smaller 28 (ANF 99-126) and 24 (ANF 103-126) amino acid forms may occur following release and possibly within the circulation (1,2,3). However, the factors and/or stimuli which ultimately promote its release are still poorly defined at the molecular level. While optimizing the conditions which promote ANF release in culture, we noted that addition of 20% fetal calf serum apparently increased ANF release 5-6 fold over that occurring when the cells were exposed to a defined chemical medium alone. Our further data, however, imply that the apparent enhancement of ANF release by fetal calf serum (FCS) may not be related to a specific factor(s). Rather, preliminary studies suggest that this stimulatory effect of FCS is,

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in some part, related to the effect of exogenous protein and can be recapitulated by the simple addition of albumin or globulins to the system. Whether this effect is secondary to inhibition of ANF degradation afforded by the addition of protein, or whether it is related to enhancement of ANF release as well, remains to be determined.

METHODS

1. Cell culture. Cultures of isolated cardiac myocytes were prepared from 1-2 day old neonatal rat hearts by trypsin dispersion (3). Cells were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% FCS, thymidine (600 ug/ml), penicillin (20 IU/ml) and gentamycin (30 ug/ml). Cells were plated in Falcon 6-well culture dishes at a density of 1-5 x 10^5 cells/well, and cells were used on the 4th-5th day after plating.

As previously reported, ANF-containing atrial myocytes comprise up to 10% of the entire population as defined by staining with the immunoperoxidase technique utilizing monospecific antiserum raised to ANF 103-126 and a second antibody (goat anti-rabbit) coupled to horseradish peroxidase (1). Greater than 90% of the cells are myocytes as defined by staining with anti-myosin antibody (3, 4). Viability is routinely greater than 95% as assessed by two different criteria, propidium iodide exclusion and lack of staining with anti-myosin antibody.

2. Anti-ANF Antiserum and Radioimmunoassay (RIA): High-affinity polyclonal antiserum to ANF was raised by immunizing rabbits with synthetic ANF 103-126 crosslinked to thyroglobulin with glutaraldehyde. Titers were obtained by determining the greatest serum dilution at which ^{125}I-ANF binding could still be reliably detected (30% binding at an ^{125}I-ANF concentration of 0.1nM). These were in the range of 1:20,000.

Rat ANF 103-126 (Peninsula) was iodinated by the chloramine T method and the iodinated species isolated by reverse phase HPLC as previously described (5). Radioimmunoassay of culture medium employed this probe and the above antiserum and was performed as described by Zisfein et al (5, 6). Separation of bound from free ligand was effected by a double antibody precipitation method utilizing goat anti-rabbit gamma globulin. This procedure quantitatively precipitated greater than 80% of all available rabbit antibody.

3. ANF Release. Stimulation of atriocytes was effected either by DMEM alone or by DMEM-based protein solutions. Wells of plated cells were assigned to a random order of stimulation to eliminate artifacts introduced by variability in cell density or viability, and to minimize artifactual differences in ANF release introduced by different sequences of stimulation. Each well was washed with unsupplemented DMEM prior to stimulation and was stimulated for an identical period of time.

RESULTS

Accumulation of ANF with time ranged from 42.0 $\pm$ 8.9 pg/ml/min in DMEM to 266 $\pm$ 30 pg/ml/min in FCS, an approximate 6-7 fold enhancement. A linear dose response relationship was found for serum concentrations

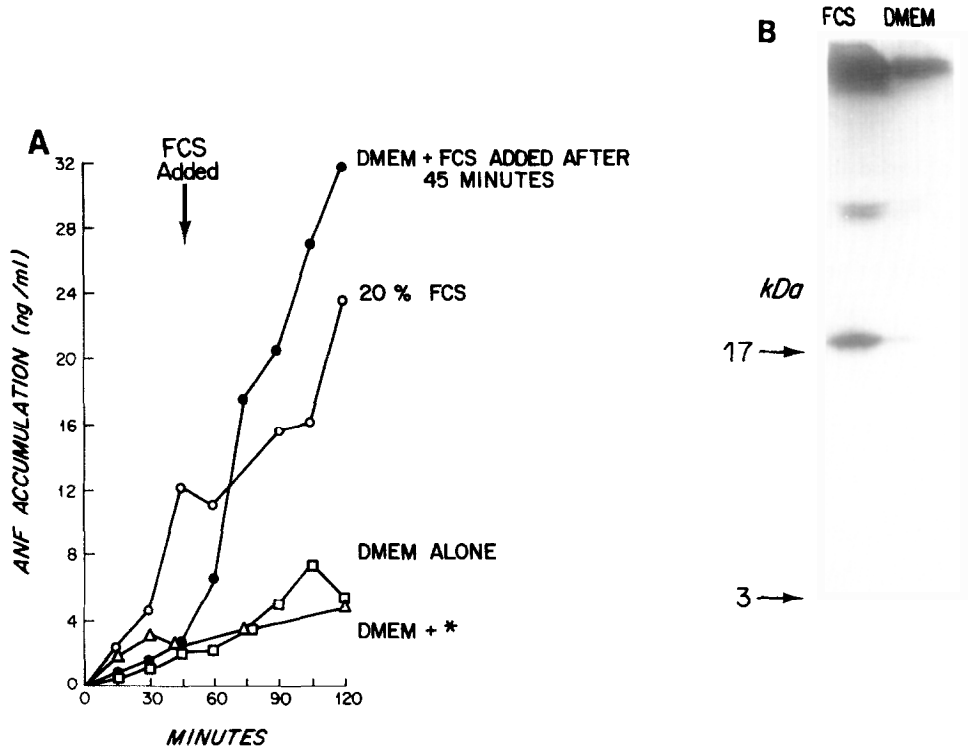

Figure 1. A. Time course for ANF release. Cells were plated as described in Methods and the medium removed at the indicated times. To insure that the foresence of FCS was not altering the RIA results, an aliquot of the medium obtained from the cells treated with DMEM alone was supplemented with 20% FCS before performing the RIA. No differences were noted between the values observed in these samples to those obtained without the addition of FCS. Definition of symbols: ○–○, 20% FCS present from zero time; ●–●, 20% FCS added at 45 minutes; □–□, DMEM alone; △–△, DMEM alone, plus FCS added to 20% following removal of the medium, and prior to the RIA.*

B. Effect of 20% FCS on intrinsically-labelled ANF accumulation. Accumulation of ^{35}S-methionine labelled ANF in DMEM vs. 20% FCS was compared. The predominant 17 kD proANF band was then excised and radioactivity (cpm) was measured by liquid scintillation counting: 20% FCS (cpm) = 1438 vs. DMEM (cpm) = 474. Background was 130 cpm.

ranging from 0.5 to 20%, the threshhold for this stimulatory effect occurring at a serum concentration as low as 0.5%. Horse and rat serum produced similar responses. The effect of serum was not altered following heat inactivation at 56° C for thirty minutes.

The time course for the serum-mediated enhancement of ANF accumulation was further investigated. ANF release was linear under either set of conditions, DMEM alone or supplemented with 20% FCS, for at least 120 minutes. Figure 1A demonstrates that serum exerts its effect within

minutes after its addition and that the enhancement of ANF accumulation remains linear with time when compared to control levels. Furthermore, upon the removal of serum and the reintroduction of DMEM alone, ANF accumulation declines almost immediately to basal levels (data not shown). ANF accumulation was also measured in serum treated with 0.1 mM PMSF to insure that cleavage of the prohormone to ANF 99-126 by serine protease activity was not occurring. We have previously shown that this agent completely prevents cleavage of proANF by serum (3). In the presence of serum treated with PMSF for at least one hour prior to exposure to cells, a similar increase in ANF release was observed by RIA. Furthermore, to demonstrate visually that proANF represented the predominant form of ANF released in the presence of fetal calf serum, intrinsically-labelled ANF was analyzed as previously described (1). Cells were first pre-labelled with ^{35}S-cysteine followed by a cold chase for one hour with DMEM and then exposed to either 20% fetal calf serum (pre-treated with 0.1 mM PMSF) or DMEM alone. The ANF was then quantitatively immunoprecipitated, the pellet dissolved in SDS and the constituents separated by SDS-PAGE. Following enhancement in Autofluor, the bands were visualized by autoradiography as seen in Figure 1B. The proANF (1-126) band was then cut out and ^{35}S-radioactivity measured in a Beckman liquid scintillation counter at an efficiency of 80%. It is apparent visually from the gel and it can be calculated from the radioactivity present in the excised bands that an approximate 4-5 fold enhancement in proANF levels was effected by exposure to 20% fetal calf serum (cf Figure 1B and legend).

The enhancing effect of serum on ANF release occurred almost immediately upon its addition and therefore suggested that new protein synthesis was not involved in this process. Nevertheless, experiments were performed in the presence of the translation inhibitor puromycin to confirm this impression. Puromycin(1 mM) completely inhibited the incorporation of ^{35}S-cysteine into ANF when it was added to the atriocytes 30 minutes before the radiolabelled amino acid. However, when the cells were prelabelled

with ^{35}S-cysteine prior to the addition of puromycin, radiolabelled ANF release over the next two hours remained comparable to that of control cells that had not been exposed to puromycin (data not shown). Furthermore, if serum was added to atriocytes pretreated with puromycin and then incubated for a further two hours, a marked enhancement of ANF release was still observed. This result underscores our prior impression that the rapidity of the serum effect in enhancing ANF release does not involve new protein synthesis.

In order to identify the species responsible for this effect, serum was fractionated by size exclusion gel filtration chromatography on an AcA 34 Ultrogel resin (5 ml applied to a 100 x 2.5 cm column). Fractions were assayed as described above for their ability to stimulate ANF release. A peak of stimulatory activity eluted within the included volume of the resin in a position coincident with the major protein peak, which consists principally of albumin. An additional region of stimulatory activity inconsistently appeared just prior to the albumin peak. Stimulatory activity was also found in other fractions and generally paralleled protein concentrations as assessed by OD_{280}. Stimulatory activity was not detected within the void or salt volumes of the column elution.

We therefore investigated directly the effects of BSA (bovine serum albumin, Sigma) at concentrations (6.7 mg/ml) equivalent to those present in 20% fetal calf serum. As can be seen in Figure 2A, an increase in the density of the 17 kilodalton prohormone band is apparent when cells are cultured in the presence of albumin. The increased concentration of proANF found in the presence of BSA was additionally confirmed by RIA quantitation of the ANF levels (cf Figure 2A, legend). A quantitatively similar increase in ANF levels was also seen when other proteins such as ovalbumin (Sigma) or bovine gamma globulins (Sigma) were added to the cultured myocytes at similar concentrations (Figure 2A and legend). As shown in Figure 2B, the ability of albumin to enhance ANF levels is dose-related, an 8-fold increase being observed at a concentration of albumin equivalent to

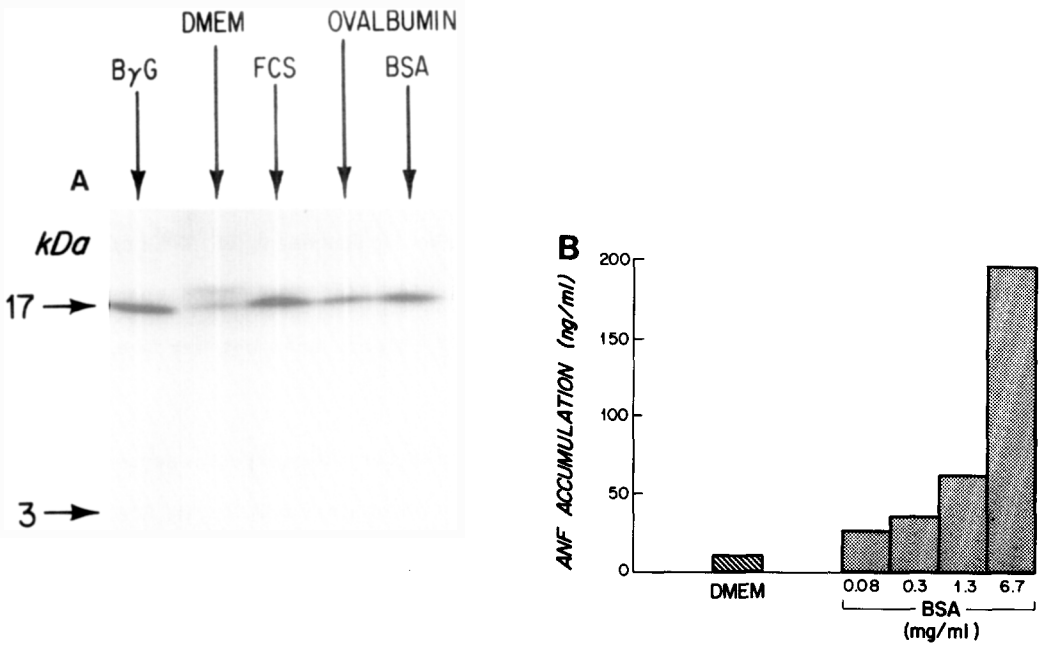

Figure 2. A. Effect of protein on accumulation of ANF from cultured myocytes as assessed by intrinsic labelling and simultaneous RIA. All proteins were present at a concentration of 6.7 mg/ml. Labelling and precipitation were carried out as described in Methods for one hour. Simultaneously, the medium was assayed in triplicate by RIA for ANF accumulation. The ANF concentrations (ng/ml) obtained with the different incubation conditions were as follows: DMEM, 9.4 ± 0.1; 20% FCS, 45.6 ± 0.4; Gamma globulin, 46.3 ± 0.9; BSA, 33.2 ± 0.5; Ovalbumin 28.4 ± 0.7.

The band running just before proANF (seen in DMEM lane)inconsistently coprecipitated with ANF 1-126 but its non-specific nature was determined by demonstrating that excess unlabelled ANF did not block its appearance in contrast to the complete disappearance of the 17kD prohormone band. This non-specific band is also apparent in several lanes in Figure 3.

B. Effect of bovine serum albumin on ANF accumulation. The dose response relationship was determined by assessing the effect of various concentrations of BSA on ANF accumulation over a one hour period. A concentration of 6.7 mg/ml represents the approximate concentration found in a solution containing 20% serum.

that found in 20% fetal calf serum. However, even at albumin concentrations as low as 0.3 mg/ml, an effect is apparent. In contrast to the above findings, neither the addition of 10 mM mannitol nor dextran (MW = 60,000) at 6.7 mg/ml, equivalent to the highest albumin concentration employed, produced an appreciable increase in ANF levels in the culture medium (Figure 3 and legend; for comparison, enhancement of ANF accumulation is again apparent in the presence of 20% FCS, albumin or ovalbumin). These findings indicate that the effect observed either with FCS or albumin is not due to an increase in the osmolality of the solution.

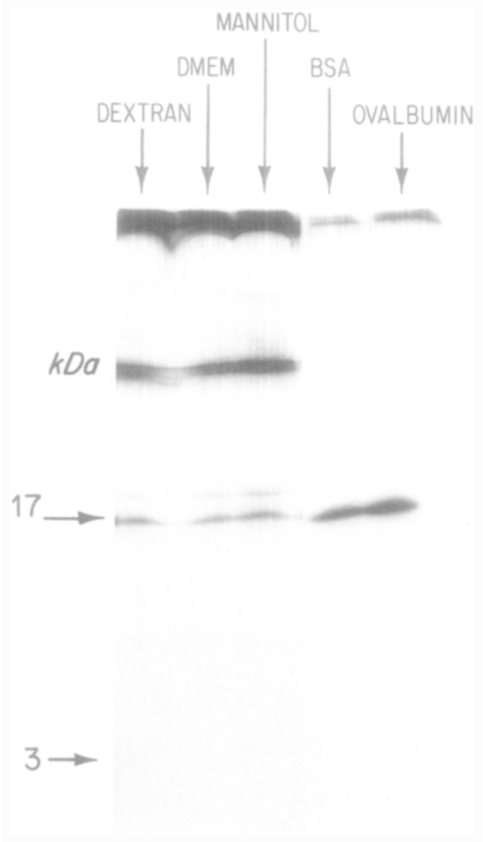

Figure 3. Effect of mannitol and large molecular weight dextran (60,000 daltons) on ANF accumulation as assessed by intrinsic labelling and RIA. Mannitol was present at a concentration of 10mM and dextran, BSA and ovalbumin at a concentration of 6.7 mg/ml. The incubation time was one hour. Simultaneously the medium was assayed for ANF accumulation. The ANF concentrations (ng/ml) obtained with the different incubation conditions were as follows: DMEM, 8.6 $\pm$ 0.2; Mannitol, 9.0 $\pm$ 0.6; Dextran; 10.8 $\pm$ 1.0; BSA 58.2 $\pm$ 5.0; Ovalbumin; 48.5 $\pm$ 1.2.

DISCUSSION

The cultured cardiac myocyte provides a system which can be readily manipulated so as to define environmental factors that may regulate ANF secretion. Our laboratory first employed this model to demonstrate that proANF rather than the smaller circulating forms of ANF are secreted under basal conditions (1). In the course of these studies on ANF release, it became apparent that serum could increase ANF levels by as much as 5-8-fold over that seen in the presence of unsupplemented DMEM. This response is dose-dependent, with concentrations of serum as low as 0.5% promoting this effect. Moreover, the serum-mediated increase in proANF levels remains

constant over time and does not plateau for at least 120 minutes. The effect is also apparent within minutes after the addition of serum suggesting that it is not mediated by the synthesis of new protein. The enhanced release observed in the presence of puromycin is also consistent with this conclusion. Furthermore, the process is readily reversible with ANF accumulation declining to basal levels within minutes following removal of serum-containing medium and its replacement by DMEM. Although initially we felt that these observations were reminiscent of the action of certain hormones whose end-organ effects are immediately reversible upon removal of the hormone, our later experiments led us to consider a second etiology. Following fractionation of fetal calf serum by gel filtration, it was apparent that the peak of the enhancing effect of serum eluted coincidentally with the major protein peak, which consists principally of albumin, and that stimulatory activity generally paralled protein concentration. Furthermore, ovalbumin, albumin, or bovine gamma globulins when included in culture medium at concentrations equivalent to that found in 20% FCS (6.7 mg/ml), all effected an increase in ANF levels equivalent to that obtained with 20% fetal calf serum, as detected either by RIA or by immunoprecipitation of intrinsically labelled ANF. The observed effect of fetal calf serum, at least in part, may be to inhibit the endogenous proteolysis of the 17 kilodalton proANF to smaller peptides not recognized by our antiserum. It is unlikely that this was degraded to a detectable smaller molecular weight form recognized by anti-ANF antiserum since such fragments were neither visualized in immunoprecipitation experiments nor detected by RIA.

Taken together, these results suggest the following conclusion. Although fetal calf serum may contain a factor(s) that stimulates ANF release, the serum-mediated enhancement of ANF accumulation in the medium of cultured myocytes is in part related to the simple addition of protein to the medium. Whether this addition of protein stabilizes the myocytes in some manner so as to promote ANF release, or inhibits its degradation in a

simple competitive manner, remains to be determined. Nevertheless, these observations are of importance in studying the mechanisms involved in ANF biosynthesis and processing when using the cultured myocyte as a model system. They may also be relevant to other models used to study ANF release, such as isolated perfused heart peparations, in which albumin or other proteins if added to the perfusate might exert a similar effect.

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