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POST-TRANSLATIONAL PROCESSING OF ATRIAL NATRIURETIC FACTOR. A STUDY USING A NOVEL CELL CULTURE SYSTEM.

M.Sc. Thesis

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GLOSSARY

ACN ..... Acetonitrile

ACTH .... Adrenocorticotropic Hormone

ADH ..... Antidiuretic Hormone

ANF ..... Atrial Natriuretic Factor

ANOVA ... Analysis of Variance

ARA-C .... Arabinosylcytosine

ATP ..... Adenosine 5'-triphosphate

BNF ..... Brain Natriuretic Factor

BSA ..... Bovine Serum Albumin

cAMP .... Cyclic Adenosine 5'-monophosphate

cGMP .... Cyclic Guanosine 5'-monophosphate

CHF ..... Congestive Heart Failure

CMK ..... Chloromethylketone

ER ..... Endoplasmic Reticulum

EDTA .... Ethylenediaminetetraacetic acid

EGTA .... Ethylene Glycol-bis(ß-aminoethyl Ether)N-N-N'-N'-tetraacedic acid

ES ..... Enzyme Solution

FBS ..... Fetal Bovine Serum

FITC ..... Fluorescein Isothiocyanate

FS ..... Flanking Sequence

GFR..... Glomerular Filtration Rate

GTP ..... Guanosine 5'-trithosphate

hANP ..... Human Atrial Natriuretic Peptide

HEPES ... N-2-hydroxyethylpiperazine-N'-ethane-sulfonic acid

HT- ..... Heat Treated

IMCD .... Inner Medullary Collecting Duct

-if ..... Immunofluorescence

IP ..... Isoelectric Point

irANF .... Immunoreactive Atrial Natriuretic Factor

Kb ..... Kilobase

Kd ..... **Kilodalton**

LCM ..... **Low Calcium Medium**

MCA ..... **Methylcoumarinamide**

MW ..... **Molecular Weight**

PACE ..... **Pair basic Amino acid Cleaving Enzyme**

POMC .... **Proopiomelanocortin**

NGS ..... **Normal Goat Serum**

PBS ..... **Phosphate Buffer Saline**

RIA ..... **Radioimmunoassay**

RP-HPLC . **Reverse Phase-High Performance Liquid Chromatography**

SAG ..... **Specific Atrial Granules**

S.D. ..... **Standard Deviation**

SEM ..... **Standard Error of the Means**

TFA ..... **Trifluoroacetic Acid**

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ABSTRACT

Little is known regarding the details of ANF storage, processing and release. The definition of ANF post-translational processing has defied regular investigative efforts and the results obtained are highly controversial. The details of the maturation of ANF to its circulating form are not yet defined. In the present work, a reproducible cell culture system using adult rat atrial cardiocytes was developed to study ANF processing. Concomitantly, ANF storage and release were determined to characterize changes in the overall endocrine functions of the cultured cells. Atrial cardiocytes, in freshly isolated preparation and in culture for up to 15 days on micro-carrier beads, were superfused by recirculating serum-supplemented medium. During collection periods, serum-free medium was used. Fractions were assayed for ANF by radioimmunoassay and analyzed by reverse phase-high performance liquid chromatography. Freshly isolated atrial cardiocytes stored high molecular weight ANF (38.0 ± 4.5 pg/μg of DNA) and released almost exclusively (83.3% ± 6.7%) low molecular weight ANF, at an average rate of 12 pg/hour/μg of DNA. The cell content and the rate of release of ANF decreased over 15 days in culture to 3.9 ± 1.2 pg/μg of DNA and 0.32 pg/h/μg of DNA ± 0.08 respectively and 62.7% ± 6.3% of the released peptide was of a low molecular weight. Cultures of non-cardiocytes, superfused with exogenous proANF, did not process the peptide. To characterize the ratio of the number of cardiocytes to the total number of cells in culture, desmin immunofluorescence was used as a muscle specific marker together with Hoechst 33258 to stain all nuclei in the cultures. There was no correlation between the changes in cell population and the reduction in processing. Therefore, atrial non-cardiocytes are not involved in ANF processing. Microscopic analysis of atrial cardiocytes revealed that ANF immunofluorescence was diffuse during cell attachment but returned to the perinuclear region after 5 days in culture. The results presented in this work vary from other reports which found that ANF processing in cultures is absent. The discrepancies may be due to differences related to serum-free culture conditions versus serum supplemented cultures. This suggests that factors present in the serum may be responsible for maintaining ANF processing activity in culture.

INTRODUCTION

Atrial natriuretic factors are a group of peptides synthesised, stored and secreted by mammalian atrial cardiocytes. These peptides are involved in the homeostatic control of water and electrolyte balance (Review: A.J. de Bold, 1985). The predominant circulating form of ANF is a 28 amino acid peptide (ANF (99-126)), which results from the cleavage of its precursor, ANF (1-126). This cleavage occurs at a monobasic amino acid site rather than at a dibasic amino acid cleavage site (Arg^{101}-Arg^{102}), 2 amino acids away from the former. The proteolytic enzyme(s) involved in the maturation of ANF and the exact tissue location of the cleavage are still unknown. In the present work, a cell culture system designed to maintain adult rat atrial cardiocytes in culture under continuous superfusion was developed, characterized and used to study the events involved in this maturation process.

1) DISCOVERY, ISOLATION AND CHARACTERIZATION OF ATRIAL NATRIURETIC FACTOR

Although atrial natriuretic factor (ANF) was discovered in 1980, the concept of atrial cardiocytes having an endocrine function was postulated more than 30 years ago. In 1956, Kish reported electron dense granules in guinea pig atrial cardiocytes but not in ventricular cardiocytes. These observations were also reported by a number of investigators (Bencosme and Berger, 1971; Berger et al., 1971; Jamieson and Palade, 1964). Granules were shown to be distinct from other cell organelles such as lysosomes or lipofucin granules (Hibbs and Ferrans, 1969; Jamieson and Palade, 1964). Emphasizing the ultrastructural distribution of the granules at the nuclear pole and in association with the Golgi apparatus, Jamieson and

Palade (1964) proposed a possible endocrine role for the granules which they referred to as "Specific Atrial Granules" (SAG). Since SAG display morphological similarities to norepinephrine-containing granules (Berger et al., 1971), subcellular fractionation investigations were carried out to determine their catecholamine content, but no appreciable amount was detected (de Bold and Bencosme, 1973). In addition, histochemical analysis of atrial tissue did not support the view that the SAG contained catecholamine (Berger et al., 1971; Cantin et al., 1973). The association of SAG with an abundant Golgi complex in rat atrial tissue is also a characteristic of peptide hormone-producing cells (Jamieson and Palade, 1964), and based on this hypothesis, the proteinaceous nature of the granule content was investigated. Cytochemical analysis of the SAG strongly suggested the presence of a basic polypeptide containing sulphur amino acids (cysteine and/or methionine) and tryptophan (de Bold et al., 1978).

Significant progress regarding the possible function of SAG was made when it was demonstrated that the number and density of the SAG in atrial cells changed with alterations in sodium and water intake (de Bold, 1979; de Bold et al., 1978; Marie et al., 1976) suggesting a possible role for these granules in the homeostatic control of water and electrolytes. This potential endocrine role of the atria was demonstrated by de Bold et al. (1981) who showed that intravenous injection of crude homogenate of rat atrial tissue rapidly induced a short-lived and profound natriuretic and diuretic response in recipient rats, accompanied by a decrease in blood pressure and an increase in haematocrit. Such a response did not occur following injection of extracts obtained from ventricular tissue. The factor responsible for inducing these effects was named "Atrial Natriuretic Factor" or "ANF" (de Bold et al., 1981). The relationship between SAG and ANF was demonstrated when,

following atrial tissue fractionation, only the granule-enriched fraction displayed natriuretic and diuretic effects (Baines et al., 1983; de Bold, 1982; Garcia et al., 1982). Partially purified atrial extracts were subsequently shown to lose all biological activity after treatment with pronase (a low specificity protease) (de Bold, 1982) or trypsin (Trippodo et al., 1982). The extract was heat stable and acid soluble, both being characteristics of a small protein or a peptide (de Bold, 1981).

Isolation and purification of ANF was conducted by reverse phase high performance liquid chromatography (RP-HPLC) of acid extracts of rat atrial tissue (de Bold and Flynn, 1983). Four fractions containing diuretic, natriuretic, and vasorelaxant activity were isolated and named cardionatrin I, II, III, and IV, corresponding to the order of elution. Cardionatrin I, the lower molecular weight cardionatrin, was further purified to chemical homogeneity, submitted to amino acid sequencing by Edman degradation and characterized as a 28 amino acid long peptide, containing 2 cysteines but no tryptophan (Flynn et al., 1983).

Similar characterizations were carried out in other laboratories, but the different approaches used for peptide isolation resulted in reports of several peptides with lengths varying between 21 and 33 amino acids, though with approximately the same core sequence (Fig. 1). Currie and collaborators (1984a) and Geller et al. (1984b) reported the sequences of a series of shorter bioactive peptides isolated from rat atrial tissue, sharing a complete homology with cardionatrin I, but lacking several amino acids at the amino terminus. The 21, 23, and 24 amino acid long polypeptides were named atriopeptin I, II, and III respectively. Atlas and coworkers (1984) isolated 2 polypeptides from rat atrial extracts

referred to as auriculin A containing 24 amino acids, and auriculin B containing 25 amino acids that were also truncated at the amino terminus but differed from the atriopeptin family by an additional arginyl residue at the carboxyl terminus. Seidah et al. (1984) reported the isolation of a polypeptide 33 amino acids long and 3 shorter polypeptides, also extracted from rat atrial tissue. These were referred to as ANF 1-33, 2-33, 3-33 and 8-33. All four peptides were fully homologous to cardionatrin I, but contained additional amino acid residues at the amino terminal end.

Three distinct bioactive fractions were isolated from human atrial tissue (Kangawa et al., 1984) referred to as α, ß, and γ human atrial natriuretic peptides (hANP). Microsequencing of the α-hANP, the smaller peptide isolated, showed striking homology to rat cardionatrin I. Identical in length, the human peptide was found to differ from the rat peptide by the replacement of the isoleucine residue 12 by a methionine residue.

Cloning and sequencing of the complementary DNA (cDNA) derived from the messenger RNA (mRNA) encoding for rat ANF (Flynn et al., 1985a; Kennedy et al., 1984; Maki et al., 1984; Seidman et al., 1984; Yamanaka et al., 1984; Zivin et al., 1984) and the hANP (Greenberg et al., 1984; Kangawa et al., 1984; Oikawa et al., 1984; Zivin et al., 1984) was subsequently reported. All previously reported sequences were shown to be part of a single pre-prohormone with 152 and 151 amino acids for the rat and human sequences respectively (Fig. 2), with the typical amino terminal methionine, necessary for the initiation of protein translation in eukaryotes, followed by a sequence rich in hydrophobic residues, characteristic of a signal peptide. In the rat, the cDNA sequence revealed the presence of a diarginyl dipeptide at the carboxyl end of the pre-propeptide. This dipeptide was not

ANF 1-33

Leu Ala Gly Pro Arg Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

ANF 2-33

Ala Gly Pro Arg Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

ANF 3-33

Pro Arg Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

ANF 8-33

Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

CARDIONATRIN I

Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

ATRIOPEPTIN I

Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser

ATRIOPEPTIN II

Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg

ATRIOPEPTIN III

Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

AURICULIN A

Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg

AURICULIN B

Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

α-hANP

Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Met Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr

present in cardionatrin or atriopeptin peptides nor in the human pre-propeptide sequence.

Knowledge of the pre-propeptide sequence of ANF made possible the characterization of the high molecular weight peptides present in rat atrial extracts. Thus, cardionatrin IV, the high molecular weight form of cardionatrin, was characterized as a 126 amino acid long peptide (Flynn et al., 1985b), containing the full sequence of cardionatrin I at its carboxyl-terminus. Atriopeptigen was characterized as a 111 amino acids long polypeptide (Geller et al., 1984a) and was shown to contain the 3 atriopeptins previously described (Currie et al., 1984a; Geller et al., 1984b). The atriopeptigen sequence was also contained within the cardionatrin IV sequence, but truncated at the amino-terminus. It was latter determined that the cardionatrin IV or ANF (1-126) was the propeptide for ANF and that shorter high molecular weight peptides, such as atriopeptigen, were the result of artifactual peptides likely generated during isolation due to non-specific breakdown.

Beta and γ hANP were characterized as 56 and 126 amino acids long respectively. While γ-hANP, having the α-hANP sequence at its carboxyl terminus, was identified as the hANP precursor, ß-hANP was shown to be an antiparallel dimer of the α-hANP (Kangawa et al., 1985). Strong homology was found between rat and the human propeptides. The two were found to differ only in 17 of the 126 amino acid residues.

2) STRUCTURE AND REGULATION OF THE ANF GENE

In all mammalian species studied, ANF is coded by a single gene (Argentin et al., 1985; Greenberg et al., 1984; Mullins et al., 1987; Nemer et al., 1984; Seidman et al., 1984;

Met Gly Ser Phe Ser Ile Thr Lys Gly Phe Leu Phe Leu Ala Phe Trp Leu Pro
-24
Gly His Ile Gly Ala
-1
Asn Pro Val Tyr Ser Ala Val Ser Asn Thr Asp Leu Met Asp Phe Lys Asn Leu
1
Asp His Leu Glu Lys Met Pro Val Glu Asp Glu Val Met Pro Gln Ala Leu

Ser Glu Gln Thr Asp Glu Ala Gly Ala Leu Ser Leu Ser Glu Val Pro

Trp Thr Gly Glu Val Asn Pro Ser Gln Arg Asp Gly Ala Leu Gly Arg Gly Pro

Trp Asp Pro Ser Asp Arg Ser Ala Leu Lys Ser Lys Leu Arg Ala Leu Ala

Gly Pro Arg

Ser Leu Arg Ser Cys phe Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly 99

Leu Gly Cys Asn Ser Phe Arg Tyr Arg
126

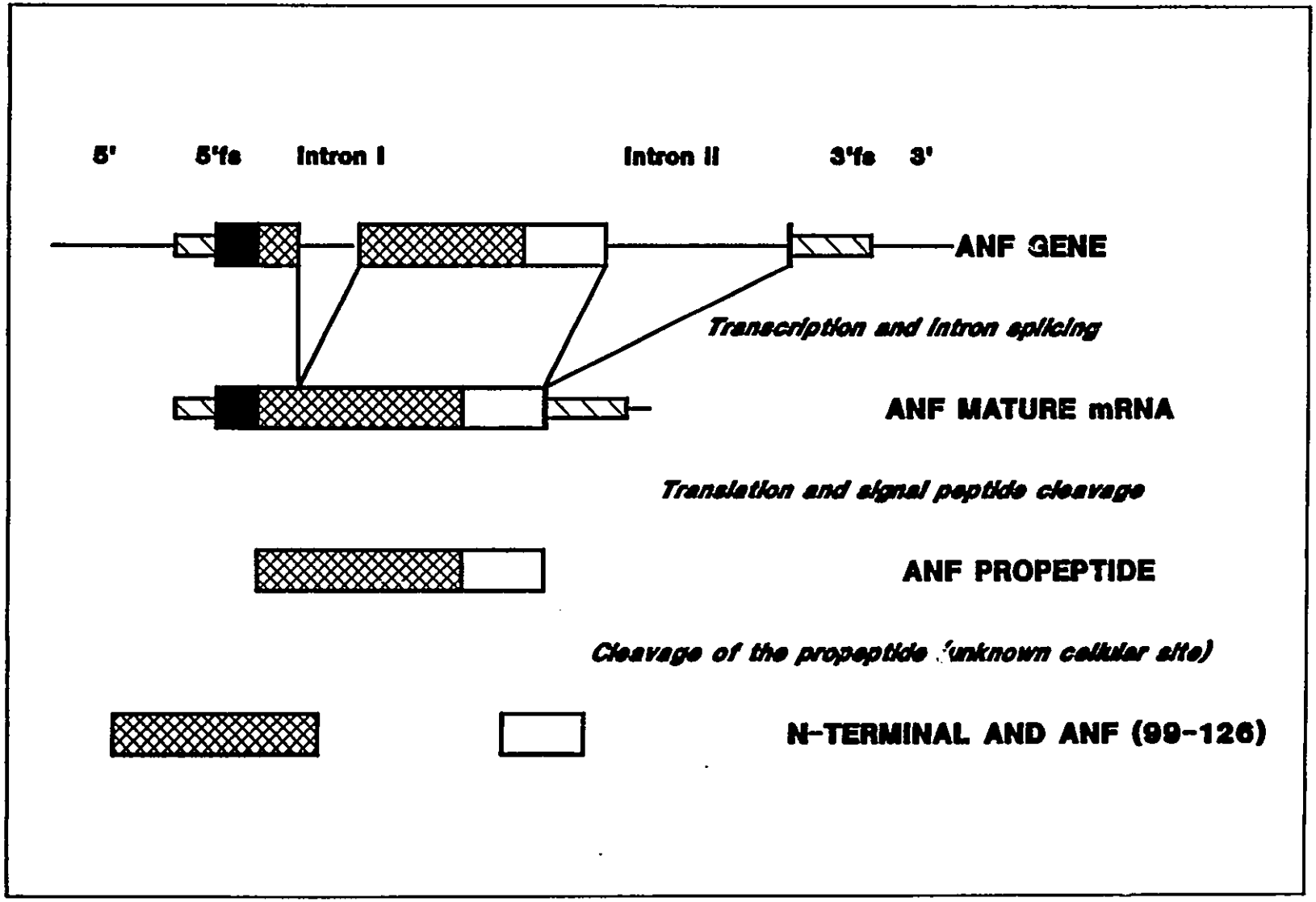
LEGEND:
SIGNAL PEPTIDE SEQUENCE
CIRCULATING ANF (99-126).

Vlasuk et al., 1986; Yang-Feng et al., 1985). A remarkable conservation in the nucleotide sequence and genomic organization has been observed between species. The ANF coding sequence is contained in 3 exons separated by 2 introns (Fig. 3). The first exon of the gene encodes for the hydrophobic signal peptide and the first 16 amino acids of the propeptide. The second exon encodes for the near totality of the propeptide, including the bioactive part of ANF, while the third exon, at the 3' end of the gene only encodes for 1 amino acid (Tyr^{126}) or 3 amino acids (Tyr^{126}-Arg^{127}-Arg^{128}) in human and rat ANF respectively. The absence of the di-Arginyl dipeptide in the human pre-propeptide is due to a single nucleotide mutation converting the Arginyl codon, CGA, to a stop codon, UGA. Using S1 nuclease and primer extension analysis, the transcription start site (the 5' terminus of the transcript) was determined to be 20 to 25 basepairs downstream from a genomic TATAAAA sequence (TATA box) (Gardner et al., 1986).

In the human gene, the second intron, separating the second and third exon by approximately 1050 basepairs, was shown to contain 2 complete 300 basepair regions with 70% to 86% homology to the previously characterized Alu family sequence (Deininger et al., 1981). No involvement of these sequences in gene expression or regulation has been reported so far.

The second intron in rat and human has been found to bear a region believed to be relevant with regard to the regulation of ANF gene expression (Greenberg et al., 1984; Gardner et al., 1986; Renkawitz et al., 1984; Seidman et al., 1984). Its sequence, TCTATTGTCCT, has a close homology with a putative glucocorticoid-receptor binding site (Karin et al., 1984; Payvar et al., 1983).

FIGURE 3: Schematic representation of the rat ANF gene, messenger RNA (mRNA) and peptide precursor (proANF). In the gene structure, the lines represent the 5' and 3' flanking regions (FS) and the intervening sequences (introns), which separate the three exons. The TATA box precedes the coding sequence at the 5' end and a polyadenylation signal is found at the 3' end. The striped boxes represent the untranslated region of the mRNA and the wide boxes represent the translated region of the mRNA. The filled box represents the portion of the mRNA or gene coding for the signal peptide which is cleaved during the translation. Post-translational processing of the ANF prohormone leads to the formation of the circulating form of ANF (open box) and a N-terminal cryptic peptide (hatched box).


5'
5'fs
Intron I
Intron II
3'fs
3'
ANF GENE
Transcription and intron splicing
ANF MATURE mRNA
Translation and signal peptide cleavage
ANF PROPEPTIDE
Cleavage of the propeptide (unknown cellular site)
N-TERMINAL AND ANF (99-126)

The putative regulatory sequence on the 5' end of the ANF gene has been partly characterized using the prokaryotic reporter gene chloramphenicol acetyltransferase (CAT) (Lapointe et al., 1988; Seidman et al., 1988; Wu et al.; 1989). A 2.5 kilobase (kb) fragment of the ANF 5' flanking sequence was enough to drive the CAT expression in transfected cultured neonatal rat atrial cardiocytes while no detectable levels were measured in ventricular cultures. These results suggest that tissue specific regulatory element(s) were included in this fragment (LaPointe et al., 1988; Seidman et al., 1988). Truncation of the flanking sequence (<700 basepairs) or deletion of the sequence between -409 to -332 produced a marked reduction in the expression of the reporter gene. To further investigate the regulation of ANF in rat, transgenic mice carrying a construct containing a 2.4 kb segment of the 5' flanking sequence driving the reporter gene CAT were created (Seidman et al., 1991). Results obtained from this *in vivo* model supported the earlier findings in neonatal cardiocytes. The 2.4 kb fragment was sufficient to induce high-level, tissue-specific and developmentally-regulated expression of the reporter gene.

"Gel shift" experiments, used to determine the ability of a DNA sequence to bind a specific protein, demonstrated the existence of a cardiac nuclear protein with a high affinity for the region contained between -400 and -333 of the 5' sequence of the ANF gene. These findings support the view that a sequence of this DNA segment, by providing a binding site for a specific nuclear protein, is involved in the regulation of the ANF gene expression (Wu et al., 1989). Rosenzweig and collaborators (1991) have recently identified a region lying within 609 basepairs of the transcription initiation site of the rat ANF gene with significant homology to the consensus AP-1 sequence. This conserved sequence is recognized by the AP-1 transcription factor, a heterodimer of the Fos/Jun proteins (2 protooncogene products) and may be involved in regulation of transcription of the ANF gene.

3) POST-TRANSLATIONAL PROCESSING OF ANF

ANF is synthesized as a 152 amino acid pre-propeptide in the rat, or a 151 amino acid in the human. The amino-terminal signal portion of the polypeptide, 24 amino acids in length in the rat (25 in the human), is then removed leaving a 128 amino acid propeptide (126 in human). The signal peptide contains several hydrophobic amino acids and is believed to be involved in the translocation of the nascent protein through the membrane of the rough endoplasmic reticulum. Its cleavage probably occurs early in the biosynthesis, possibly before completion of mRNA translation (Pfeffer and Rothman, 1987). In a cell specific manner, the signal peptide is involved in mediating the diversion of the newly synthesized polypeptide from the bulk transport pathway (constitutive pathway) to secretory storage vesicles or any other destinations (lysosome, membrane, retention in the ER or the Golgi) (Breitfeld et al., 1989; Pfeffer and Rothman, 1987).

In the rat, but not in humans, an additional processing step involves a carboxypeptidase proteolysis resulting in the loss of 2 Arg residues at the carboxyl-terminal end of the ANF propeptide. Lynch and coworkers (1988) reported the presence of carboxypeptidase-E (an enzyme capable of cleaving such a sequence) in rat atria which, by subcellular fractionation, was shown to be closely associated with the SAG. Since ANF 1-126-Arg-Arg is not found in extracts from atrial tissue or atrial cells in culture (Bloch et al., 1985; Flynn et al., 1985b), it appears that this molecular form of the peptide is quickly cleaved before or during packaging in SAG. The time course of carboxypeptidase-E expression in the ventricle parallels that of ANF, decreasing to low levels after birth (Lynch et al., 1988). These findings support the view that this enzyme is related to ANF processing. The exoproteolytic events described for ANF are similar to those characterized for other

peptide hormone systems (insulin, glucagon, gastrin, etc.) (W.A. Hsueh, 1984) although, contrary to ANF, these events are slower and occur later (Eipper et al., 1987).

Post-translational processing of polypeptide hormones comprises the cleavage of the prohormone (often biologically inactive) to low molecular weight, biologically active peptides. In some hormone producing systems, several different sets of end-product peptides with markedly different activity spectra can be generated from a single precursor by differential enzymatic processing. This is the case with pro-opiomelanocortin (POMC) from which up to 8 different peptides can be generated such as adrenocorticotropin (ACTH), lipotropin (LPH), or melanotrophic-stimulating hormones (α-, ß-, and γ-MSH). In other cases, similar active peptides can be generated from the same precursor molecule (Pro-enkephalin contains 7 similar enkephalin peptides) (Eipper et al., 1987). In other peptide hormone systems, as is the case for ANF, the cleavage of the prohormone yields the circulating form of the hormone and a "non-hormonal" domain often referred to as a cryptic peptide since no physiological function may be ascribed to this portion of the polypeptide. It is hypothesized that the cryptic peptide could play a role in the routing and intracellular trafficking of the hormone, or be involved in the regulation of the processing (Rholam et al., 1986). The most frequently encountered cleavage site of a propeptide is a sequence of two basic amino acids, as described for insulin (Steiner et al., 1967; Chance et al., 1968) and for glucagon (Tager and Steiner, 1973). Such dibasic cleavage sites have been characterized in almost all precursors so far described, but it has now become clear that in several peptide hormonal systems including ANF, processing occurs at a monobasic amino acid site (Schwartz, 1986). Some conclusions have been drawn about the amino acid sequence surrounding the single basic amino acid cleavage site. In one third of the cases, a proline

is found in the penultimate position to the single basic residue and is thought to influence the three dimensional orientation of the peptide backbone (Schwartz, 1986). Also, cleavage occurs virtually always at the carboxyl terminal end of an arginine. A leucine (isoleucine) or several alanines are present in the two amino acids preceding, and following, the cleavage site and a second arginine is present at three, five or seven amino acids before the cleavage site (Benoit et al., 1987). The ANF sequence is in full agreement with these postulates.

Little is known about the enzyme responsible for the post-transcriptional endoproteolysis of propeptides, but several recent findings have provided significant insight (Marx, 1991; Barr, 1991). A subtilisin- (a prokaryotic enzyme) like protease has been identified in lower eukaryotes such as yeast (Fuller et al., 1988). KEX2 is an enzyme involved in the dibasic amino acid cleavage of the α-factor mating pheromone and the killer toxin precursors. The availability of the KEX2 gene sequence led to the discovery of several homologous genes whose gene product was referred to as furin or PACE (Paired basic Amino acid residue Cleaving Enzyme) (Barr et al., 1991; Fuller et al., 1989; Roebroek et al., 1986; Wise et al., 1990), *PC1* (Seidah et al., 1991), *PC2*, (Smeekens and Steiner, 1990), and *PC3* (Smeekens et al., 1991). Initial cell culture studies have established that all the above proteases have the capacity and the specificity to cleave proproteins at a dibasic site when transfected in host cells. Enzymes capable of selectively cleaving at a single arginyl residue, as with ANF, have not yet been identified.

Cleavage of a propeptide is known to occur at various stages of its biosynthesis, including before, during or soon after packaging into secretory granules (Eipper et al., 1987; Mains et al., 1987; Loh, 1987). ANF biosynthesis differs from this scheme in that, in atria,

it is stored exclusively as ANF (1-126), the propeptide. Just before or during release of ANF from atrial cardiocytes, an endoproteolytic event occurs such that ANF (99-126) is the peptide released. This processing has been observed both *in vivo* and *in vitro* (de Bold et al., 1989). However, the exact tissue location of the endoproteolysis event, and the enzyme(s) involved remain to be characterized. Several proteases have been identified as potential ANF processing enzymes and are reviewed in this section and in Table 1 at the end of this section.

The molecular form of ANF released from the isolated perfused heart has been reported by Currie et al. (1984). The authors found that only the low molecular weight peptide was released in their preparation and concluded that the processing of proANF had to occur within the atrial tissue. This finding was subsequently confirmed by a number of laboratories using either isolated perfused heart preparations or isolated perifused atrial appendages from adult rats (de Bold et al., 1986; Lang et al., 1985; Thibault et al., 1986) and isolated perfused heart preparations from neonatal rats (Shields and Glembotski, 1987)

To further investigate the exact tissue location for ANF processing, studies have been performed using primary cultures of atrial cardiocytes. Gutkowska and collaborators (1985) examined the molecular form of ANF released from either neonatal or adult rat atrial cardiocytes in culture in Eagle's minimum essential medium containing 20% fetal bovine serum (FBS), vitamins, amino acids and antibiotics. ANF collection was performed in the same culture medium in which the FBS was replaced by 0.1% bovine serum albumin (BSA) ANF was extracted using activated Vycor glass beads. After amino acid analysis, it was determined that the cultures released exclusively a low molecular weight peptide, ANF

(101-126). However, in this study, the possibility of non-specific breakdown could not be excluded.

Unlike the findings of Gutkowska et al. (1985), Bloch and coworkers (1985) found that the molecular form of ANF stored in, and released from, serum-free primary cultures of neonatal rat atrial and ventricular cardiocytes was exclusively ANF (1-126). Similar results were obtained in several laboratories using neonatal atrial cardiocytes (Glembotski and Gibson, 1985; Bloch et al., 1986; Zisfein et al., 1986; Zisfein et al., 1987; Hassal et al., 1988) or adult rat atrial cardiocytes (Zisfein et al., 1986; Zisfein et al., 1987; Hassal et al., 1988), indicating that ANF processing was absent in cultured rat atrial cardiocytes. It was hypothesized that the activity of the ANF specific protease in culture was either deficient or markedly attenuated or, alternatively, that the ANF biosynthetic pathway might be altered in culture such that the prohormone escaped the protease cleavage.

The possibility of an extra-atrial site for ANF processing has been investigated by Currie and collaborators (1984). It was shown that mild digestion with trypsin (1U/mL) could cleave the ANF (1-126) to a low molecular weight peptide co-eluting on a gel filtration column with ANF (99-126), suggesting that the ANF precursor could be cleaved to a low molecular form by a seryl protease-like enzyme. Since the kidneys are target organs for ANF, the involvement of renal kallikreins in ANF processing was assessed. Urinary kallikreins were shown to cleave proANF to a low molecular weight peptide similar to ANF(99-126) (Currie et al., 1984). Tissue kallikreins have been identified, purified and localized in rat heart tissue (Xiong et al., 1990). The enzyme was shown to be similar to other tissue kallikreins in its inhibitor spectrum as well as in its immunoreactivity. The

mRNA for tissue kallikrein was observed in whole rat heart tissue, although at lower concentrations as compared to other tissues such as the kidney, spleen, and pancreas. Immunocytochemical electron microscopy of rat atrial tissue sections revealed that tissue kallikrein was associated with cytoplasmic elements between myofibrils as well as with granules. Although no specific functional role was found for the enzyme, a possible involvement in ANF processing was proposed.

Trippodo and coworkers (1985) found that incubation of the prohormone with rat blood at 37°C resulted in rapid conversion (2 minutes) of the high molecular weight ANF to a low molecular weight peptide, similar in size to the circulating form of ANF and with enhanced activity as compared to the high molecular weight peptide. After fractionation of the rat blood into plasma, erythrocytes, lymphocytes and platelets, conversion of the high molecular weight peptide was found to occur only after incubation with the platelet fraction. It was suggested that if a fraction of ANF was released in the circulation as an unprocessed peptide, platelets could convert it to a low molecular weight peptide. Rat serum and plasma were also shown to convert proANF released from neonatal rat atrial cardiocytes in culture into a low molecular weight peptide similar to the circulating form of ANF and a 14 Kd fragment (Bloch et al., 1985; Glembotski and Gibson, 1985). Bloch and coworkers (1987) further reported that a serum protease not present in plasma could cleave ANF (1-126) at the correct site (between Agr^{98} and Ser^{99}) to yield ANF (99-126). Only a portion of ANF (99-126) was further cleaved to a smaller peptide, ANF (103-126). Additional purification and characterization of this specific serum protease was carried out (Zisfein et al., 1987; Fischman et al., 1989). The isolated serine protease was different from plasma kallikrein, plasmin or vitamin K-dependent plasma protein. Characterization of the enzyme revealed

that it was a glycosylated protein, with a stoke radius of 45 Å (130 Kd) and an isoelectric point of 5.6 (Zisfein et al., 1987). Its specificity for ANF was compared to that of plasma kallikrein and thrombin (Fischman et al., 1989) using an antibody specific for ANF (99-126) and an antibody specific for ANF (103-126), which also recognized ANF (99-126). The results indicated that thrombin could cleave proANF to yield exclusively ANF (99-126), while kallikrein specifically cleaved at the Arg^{102} site to yield ANF (103-126). However, the serum protease was shown to cleave the prohormone at both sites with a preference for the Arg^{98} position. The specific proteolytic activity observed in rat serum was, therefore, distinct and broader than that of plasma kallikrein or thrombin. The participation of such a protease in ANF processing *in vivo* remains to be shown. Serum-induced proANF proteolysis was inhibited by D-Phe-Pro-Arg-Chloromethylketone (D-Phe-Pro-Arg-CMK) and D-Phe-Phe-Arg-CMK (Gibson et al., 1987). However, addition of these inhibitors to the isolated heart perfusate did not impede the processing of ANF. This suggests that the proANF cleavage occurs in the heart at a site not accessible to the inhibitors, or that the responsible enzymes are insensitive to these CMK analogues and is, therefore, not related to the serum-derived protease(s).

To determine whether cleavage of proANF occurs within atrial cardiocytes *in vivo*, investigations have been carried out by immunocryoultramicrotomy using specific antibodies directed against three fragments of the propeptide: the N-terminal (ANF (11-37)) portion, the C-terminal (ANF (101-126)) portion, and the putative cleavage site (ANF (94-103)) (Thibault et al., 1989). When applied to rat atrial tissue thin sections, the three antibodies produced an identical staining pattern: all secretory granules were heavily labelled and, to a lesser extent, the *cis* and *trans* Golgi saccules, and immature granules located close to the

Golgi. These results suggest that proANF is not processed to its circulating form during migration through the Golgi nor in the secretory granules. Similar results were obtained by Cantin et al. (1990) in normal and cardiomyopathic hamsters and by Gilloteaux et al. (1991) with fetal, neonatal and adult Syrian hamsters. Based on the morphology of atrial tissue, the latter authors suggested that the released ANF has to cross the endocardial and epicardial endothelial cells as well as the endothelial cells of the capillaries via a transcellular route before reaching the circulation and could therefore be processed upon crossing one of the cell linings (Gilloteaux et al., 1991).

The possibility that rat SAG could contain a processing enzyme has been examined using enriched atrial granule fractions lysed by detergent, osmotic shock or sonication (Corthorn et al., 1991). The lysate was then incubated at 37°C and processing was monitored by Western blotting using three specific antibodies: one directed against the N-terminal, one against the C-terminal and one against the cleavage site (98-99) of the proANF. No evidence of ANF processing was found, independently of the method used for lysis. Only slight degradation was observed, probably due to lysosomal enzyme contamination. These observations support the idea of an extra-cardiocyte site for ANF maturation.

Cathepsin B activity, a lysosomal cysteine protease, has been reported to be three times more concentrated in atrial than in the ventricular tissue (Watanabe et al., 1989). Double imuunoelectron microscopy using ANF and cathepsin B antisera, revealed that the two different sized gold particles were deposited in dense core secretory granules (SAG) in the paranuclear region of the atrial cells. Granules with small amounts of electron-dense

material, believed to be lysosomes, were densely stained with cathepsin B antiserum. Cathepsin B has been reported to generate bioactive products from precursor proteins such as prorenin (Taugner et al., 1985), proalbumin (Matsuda et al., 1986) and proinsulin (Docherty et al., 1982). A direct involvement of cathepsin B in ANF processing could not be demonstrated.

A kallikrein-like serine protease, arginine esterase A, was co-localized with ANF in SAG by immunoelectron microscopy (Simson et al., 1989). Incubation of proANF with this enzyme showed complete conversion of the precursor to a peptide of molecular size similar to the circulating form of ANF. However, interfibrillar regions of the sarcoplasm were also labelled for the enzyme. Furthermore, esterase A has an alkaline pH optimum (Chao, 1983) while SAG have been reported to be acidic (Somlyo et al., 1988). The authors hypothesized, nonetheless, that the enzyme could be activated upon secretion in the intracellular milieu where the pH is more alkaline (Simson et al., 1989).

Seidah et al. (1986, 1987) reported on the isolation and partial characterization of a seryl protease, the IRCM-sp1, from rat atrial extracts and, to a lesser extent, from ventricular extracts. However, the enzyme was found to have a greater affinity for the Arg-Arg sequence than for the Pro-Arg sequence of the propeptide.

Imada et al. (1987, 1988), using a synthetic fluorogenic substrate, BOC-Ala-Gly-Pro-Arg-MCA (methylcoumarinamide), isolated and purified a specific seryl peptidase, the "atrioactivase", from particulate (mainly microsomal membranes) fractions of rat and bovine atrial homogenates. Unlike the IRCM-sp1, the atrioactivase selectively cleaved the Arg^{98}-

Ser^{99} rather than other arginyl peptide bonds. The enzyme appeared to be a multimeric, 580 Kd, glycosylated protein. It is noteworthy that atrioactivase was not associated with SAG.

Wypij and Harris (1988) reported on the isolation of a specific seryl protease with the ability to correctly cleave the proANF to form ANF (99-126). Following fractionation of bovine atrial tissue homogenates, enzymatic activity characteristic to a seryl protease was associated with the SAG-enriched fractions homogenates. The protease, distinct from IRCM-sp1 and atrioactivase, was found to have a molecular weight of about 70 Kd and an optimal pH of 7.5. The authors proposed that the enzyme is packaged within the SAG and is only activated upon exocytosis, possibly by a change in pH.

Using an isolated heart preparation, Michener and collaborators (1987) found that recirculation of exogenous recombinant proANF-Arg-Arg resulted in the cleavage of the propeptide to a 3 Kd peptide similar to the circulating form of ANF. The absence of any circulating proteases in such a preparation suggested that the heart contains the necessary enzyme to cleave the endogenous and exogenous proANF. To further determine and characterize the presence and action of this endogenous heart protease, stretch-induced ANF release, a reproducible stimulus for increasing ANF release, was performed with the sequential withdrawal of calcium (plus 1 mM EGTA) from the perfusing medium, or by the addition of a protease inhibitor, aprotinin (a kallikrein inhibitor) (Ito et al., 1988). An increase in perfusion pressure resulted in an increase in ANF (99-126) release. However, both the withdrawal of calcium and the addition of aprotinin during stretch-induced release of ANF resulted in a reversible inhibition of the processing activity and an almost exclusive release of ANF (1-126). Both *p*-aminobenzamidine (2mM) or soybean trypsin inhibitor

(0.1%) failed to block ANF processing in identical circumstances (Toki et al., 1990). In addition, recirculation of exogenous proANF-Arg-Arg for 60 minutes was conducted in order to assess the effect of calcium withdrawal and aprotinin on its processing. Inhibition was only observed in the presence of aprotinin (Ito et al., 1988; Toki et al., 1990). Contrary to stretch-induced ANF release, processing activity during phenylephrine-induced release of ANF was not affected by the different inhibitors (aprotinin, *p*-aminobenzamidine or soybean trypsin inhibitor), suggesting a different pathway for the release of ANF with phenylephrine. The authors proposed a hypothetical model suggesting the concerted action of two types of cells: the atrial cardiocytes which contain and release proANF in the extracellular space, and the neighbouring cardiac mesenchymal cells, which process the prohormone to its circulating form. The results suggest either the involvement of different enzymes in the processing of phenylephrine- versus stretch-induced release ANF, or perhaps that the enzyme is not accessible to the inhibitors during α-adrenergic stimulation.

The spectrum of inhibition of ANF processing activity using perfused heart preparations differs from the results reported in studies characterizing partially purified ANF processing enzymes described above. The lack of inhibition of ANF processing by *p*-aminobenzamidine and soybean trypsin inhibitor is in contradiction with the atrioactivase inhibition spectrum (Imada et al., 1987, 1988), which was fully inhibited by *p*-aminobenzamidine, as well as with the IRCM-SP1 (Seidah et al., 1986) which was completely inhibited by the *p*-aminobenzamidine and soybean trypsin inhibitor. In addition, the protease isolated by Wypij and Harris (1988) was only inhibited by EGTA (cation chelator) but not by aprotinin. The discrepancies between the four enzymes characterized only suggest that the identification of the protease involved in ANF processing remains unsolved.

To verify the hypothesis that ANF processing deficiency in culture was due to the lack of proper conditions and/or factors in the culture medium, Shields and Glembotski (1988) directed their studies towards the development of a serum free culture medium which could support the processing of ANF in primary cultures of neonatal atrial cardiocytes. They found that ANF post-translational processing could be restored in culture upon the addition of glucocorticoids (hydrocortisone) to the serum-free culture medium. The activation of the processing activity was gradual, occurring over several days and no effect was seen on the molecular form of stored ANF. There was an increase in the immunoreactive ANF secreted by the cultures, in agreement with previous studies (Garcia et al., 1985; Matsubara et al., 1987; Gardner et al., 1986). Further characterizations of the effect of glucocorticoids on ANF post-translational processing were reported in subsequent reports (Shields et al., 1988; Glembotski et al., 1988). ANF processing activity in culture was shown to be reactivated by the simple addition of dexamethasone or the glucocorticoid receptor agonist, RU 28362. Activation of the processing activity was dose dependent (EC_{50} of 10nM) and reversible upon withdrawal of the glucocorticoids or by the addition of the receptor antagonist RU 38486. Glucocorticoids were shown to affect the atrial cardiocytes morphology, but only when added at early culture stages (first 7 days). Radiosequencing of the released peptide showed that the cleavage occurred at the correct sequence, Arg^{98}-Ser^{99}. Neonatal rat ventricular cells in culture were also shown to process ANF in the presence of glucocorticoids. Exogenous proANF was not efficiently processed by culture medium taken from the cultures nor by the cultured cells alone. These results suggested that the enzyme responsible for the ANF cleavage was not released in the culture medium, at least not in sufficient amounts to be detected. As well, it did not appear that the enzyme was on the surface of the cells (cardiocytes or others), nor was the ANF phagocytosed by

the cells to be processed. There is still a possibility that if the enzyme is a surface protein, it is a site not accessible to the exogenous propeptide. In summary, these studies showed that the majority of the stored proANF was converted to the circulating form of ANF, ANF (99-126), within or in close association with the cultured cardiocytes, and glucocorticoids were responsible for maintaining the activity of the cellular components involved in the post-translational processing of ANF.

4) REGULATION OF ANF RELEASE

Several stimuli have been reported to increase ANF release (Sonnenberg and Veress, 1984; Wong et al., 1988), however only mechanical distension of the atrial tissue and endothelin have been found to consistently increase this release (de Bold et al., 1986; Dietz, 1984; Ledsome, 1985). The notion of a relationship between the atrial stretch on diuresis and natriuresis (response effected by ANF) was suggested several decades ago by Henry et al. (1956) based on the finding that distension of the left atrium by inflation of a balloon induced a significant increase in urinary flow. Prevention of distension in volume overloaded animals precluded any renal response (Goetz et al., 1970). An increase in perfusion pressure in Langendorff preparations with rat hearts was reported to increase the release of immunoreactive ANF (Dietz, 1984), as did isolated perifused atrial appendages subjected to an increased preload (de Bold et al., 1986). Increased ANF release induced by atrial distension was also found *in vivo,* following mitral valve obstruction (Ledsome et al., 1985), by acute and volume loading in rat and human (Ballermann, 1988; Lang et al.,1985; Sagnella et al., 1985), and by hypervolemia induced by head-down tilt or head-out water immersion (Epstein et al., 1987; Hodsman et al., 1985). Signal transductions involved

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Table 1: Summary of the different protease candidates for ANF post-translational processing.

Enzyme	Reference	Cleavage site	Origin	Inhibition	Characteristics	Comments
Platelets	Trippodo et al. (85)	low molecular weight (?)	Rat blood	heparin		
Serum protein	Bloch et al. (85) Glembotski and Gibson (85) Zisfein et al. (87) Fischman et al. (89)	Arg^{98}-Ser^{99} and some Arg^{102}-Ser^{103}	Rat serum but not plasma	D-Phe-Phe-Arg-CMK D-Phe-Pro-Arg-CMK	- seryl protease - glycosilated - 45 Å - MW=130 Kd - IP= 5.6	Different from plasma kallikrein, Plasmin or Vitamin K dependent protease.
Cathepsin B	Watanabe et al. (89)	?	Rat atrial tissue		- Thiol protease	Co-localized with ANF in atrial granules by immunoelectron microscopy
Tissue Kallikrein	Xiong et al. (90)	Arg^{102}-Ser^{103}	Rat atrial tissue	Similar to other tissue kallikrein	- Seryl protease	mRNA detection and localization in atrial tissue by immuno-electron microscopy
Arginine Esterase A	Simson et al. (89)	?	Rat atrial tissue	-	- Seryl protease - high optimum pH	Co-localised with ANF in granules. The enzyme would not be active in granule because of low pH

Table 1: (continued)

Enzyme	Reference	Cleavage site	Origin	Inhibition	Characteristics	Comments
IRCM-sp1	Seidah et al. (86, 87)	Arg^{102}-Ser^{103} and some Arg^{98}-Ser^{99}	Rat atrial extract	Soybean trypsin inhibitor *p*-aminobenzamidine Aprotinin EDTA	Seryl protease	Originally isolated from pituitary gland
Atrioactivase	Imada et al. (87, 88)	Arg^{98}-Ser^{99}	Rat and bovine atrial extract	*p*-aminobenzamidine Aprotinin (partly)	- Seryl protease - Multimeric - MW=580 Kd - Glycosilated	Not associated with atrial granule fraction
-	Wypij and Harris (88)	Arg^{98}-Ser^{99}	Bovine atrial extract	EGTA	- Seryl protease - MW=70 Kd - optimal pH=7.5	Associated with atrial fraction. possibly activated upon exocytosis
-	Michener et al. (86) Ito et al. (88) Toki et al. (90)	Arg^{98}-Ser^{99}	Rat intact heart	EGTA Aprotinin	-	Only inhibited during stretch-induced ANF release but not during Phenylephrine-induced release

in stretch-induced release of ANF are still unknown but were shown to be independent of calcium (de Bold and de Bold, 1989).

Endothelin, a potent vasoconstrictant peptide synthesized by endothelial cells, has been recently shown to increase ANF release *in vitro* in cultured atrial cardiocytes (Fukuda et al., 1988; Sei and Glembotski, 1990), isolated perifused atria, isolated heart preparations (Stasch et al., 1989; Winquist et al., 1989), and *in vivo* by intravenous administration to rats (Stasch et al., 1989).

6) CLEARANCE OF ANF

The primary sites for the elimination of circulating ANF have not been established with certainty. Tang et al. (1984) compared and ranked the degree of degradative potency of different organs and observed that the kidney > liver > lung > plasma > heart. Similarly, Krieter and Trapani (1989) found that the kidney was responsible for up to one third of circulating ANF extraction. The proximal tubule of the nephron is rich in degradative enzymes including the neutral endopeptidase EC 24.11 (enkephalinase), which has been reported to cleave open the ring of ANF, between Cys^{106} and Phe^{107} therefore abolishing its activity (Erdös and Skidgel, 1989; Olins et al., 1987; Stephenson and Kenny, 1987). Inactivation of the endopeptidase EC 24.11 by a variety of agents (phosphoramidon, thiorphan and others) is accompanied by increases in urinary sodium output, circulating levels of ANF and urinary guanosine 3', 5'-cyclic monophosphate (cGMP) levels (Lafferty et al., 1989; Seymour et al., 1989, Sonnenberg et al., 1984; Ura et al., 1987). Besides

degradative enzymes, ANF is removed from the circulation by binding to a clearance receptor referred to as the C-receptor or ANF-R2 (section 7).

7) ANF RECEPTORS AND cGMP

ANF receptors have been located by autoradiographic techniques notably in the adrenal zona glomerulosa cells , in the kidney (mostly in glomeruli, renal medulla), and in the vasculature (arterial smooth muscle cells) all of which are known to be targets for ANF action (Bianchi et al., 1985; Mantyh et al., 1986; Mendelsohn et al., 1987). Three distinct types of receptors have been identified so far: ANF-R1, ANF-R2 and ANF-R3. Both ANF-R1 and R3 are coupled to a particulate guanylate cyclase which, upon binding of ANF, converts GTP to cGMP (Leitman and Murad, 1987; Takayanagi et al., 1987). Molecular characterization of the receptors has revealed a cytoplasmic domain in R1 and R3 containing two specific sub-domains sharing appreciable homology with protein kinases and the soluble guanylate cyclase respectively. ANF-stimulated guanylate cyclase activity increased in the presence of ATP (Chinkers and Garbers, 1989; Chinkers et al., 1989). The ANF-R1 and R3 affinities for ANF or BNF (a recently characterized natriuretic factor from the brain similar in structure and action to ANF) are different in that R1 was found to preferentially bind BNF whereas R3 responded to both natriuretic peptides with a similar affinity (Chang et al., 1989; Schultz et al., 1989).

The ANF-R2 receptor is a protein found at the membrane surface of cells, shown to be expressed at high levels by endothelial cells and smooth muscle cells and constitutes the overwhelming majority of the total ANF receptor population (Almeida et al., 1986;

Leitman et al., 1986; Maack et al., 1987). ANF-R2 was found to have an extracellular domain for ANF binding but a comparatively shorter intracellular domain than R1 or R3 receptors, characteristic of clearance receptors. No guanylate cyclase subunit is associated with the R2 receptor. This receptor was shown to be internalized when bound to its ligand and fused to lysosomes, resulting in ANF hydrolysis. Recycling of the receptor to the membrane has been observed (Nussenzveig et al., 1990).

cGMP has been characterized as the major second messenger for ANF action and was found to be responsible for the spasmolytic and natriuretic responses to ANF (Light et al., 1989; Murad, 1986; Murad et al., 1987; Zeidel et al., 1987). No effect of ANF on the soluble guanylate cyclase has been observed and desensitization of the latter had no effect on the ANF-coupled guanylate cyclase (Murad et al., 1987). In vascular smooth muscle cells, increase in cGMP results in the activation of a cGMP-dependent kinase (Leitman and Murad, 1987; Murad, 1986) along with a dephosphorylation of myosin light chain (Murad, 1986). In other types of cells in which ANF was shown to elevate levels of the cGMP, the mechanisms of action have not been fully characterized. In inner-medullary collecting duct epithelial cells, cGMP appears to block an amiloride-sensitive sodium channel (Light et al., 1989; Zeidel et al., 1988). ANF and cGMP have been reported to interfere with intracellular calcium levels by blocking phosphatidylinositol turnover and by lowering intracellular calcium possibly through the activation of Calcium-ATPase pumps (Appel et al., 1987; Chartier and Schiffrin, 1987; Hassid, 1987; Meisheri et al., 1986; Meyer-Lehnert et al., 1988; Rapoport, 1986).

8) BIOLOGICAL ACTIONS OF ANF

ANF induces increases in glomerular filtration rate (GFR), diuresis, natriuresis and excretion of phosphate, chloride, calcium, magnesium, and cGMP in the urine (Anderson et al., 1987; Burnett et al., 1984; Camargo et al., 1984; Mizelle et al., 1989; Wong et al., 1988). The renal response to physiological levels of ANF has been to strongly depend upon the activity of other neurohumoral and mechanical systems involved in body fluid homeostasis (Bie et al., 1988; Goetz et al., 1986, Greenwald et al., 1988). Manipulations aimed at blocking ANF degradation using enkephalinase inhibitors have been found to cause a modest increase in circulating ANF resulting in an increase in GFR, diuresis and natriuresis (Lafferty et al., 1989). The action of ANF on GFR has been observed even with a decrease in blood pressure and renal plasma flow (Atlas and Maack, 1987). ANF has been shown to dilate the renal afferent arterioles and constrict the efferent arterioles thereby increasing the hydrostatic pressure within the glomeruli (Dunn et al., 1986; Freid et al., 1986), and to relax the mesangial cells resulting in an increase in the filtration surface area (Appel, 1990; Appel et al., 1987; Singhal et al., 1989). ANF also induces redistribution of renal blood flow from the cortex to the medulla (Borenstein et al., 1983; Takezawa et al., 1987).

ANF-induced increases in sodium and water excretion have been observed without changes in GFR (Briggs et al., 1982; Murray et al., 1985; Sonnenberg et al., 1982). Studies have reported a decrease in solute transport at the proximal tubule and increased sodium delivery from this segment in the presence of ANF (Harris et al., 1987; Van de Stolpe et al., 1988a). ANF was reported to have little or no effect on the thin or thick limbs of the loop of Henle or on the distal convoluted tubule (Kondo et al., 1986; Peterson et al., 1987),

but many studies have shown that ANF acts on the collecting duct segment by inhibiting sodium reabsorption (Sonnenberg et al., 1982; Van de Stolpe et al., 1988b). In addition, a high concentration of ANF receptors has been reported in this segment (Charbardes et al., 1987; Nonoguchi, 1987; Tremblay et al., 1985). ANF has been found to inhibit vasopressin action on the inner-medullary collecting duct (IMCD) by preventing vasopressin induced production of cAMP (Dillingham and Anderson, 1986). Others have reported ANF inhibition of sodium transport in isolated medullary collecting ducts pretreated with mineralocorticoids (Nonoguchi et al., 1988), an effect which correlates with elevated cGMP production in this segment (Charbardes et al., 1987; Nonoguchi et al. 1987; Tremblay et al., 1985). Addition of cGMP analogues to the basolateral bathing solution was found to reduce sodium reabsorption in the cortical collecting duct *in vitro* (Nonoguchi et al., 1988).

In addition to its actions on the kidney, ANF produces a decrease in systemic blood pressure (de Bold et al., 1981). This decrease was later characterized as being due to a decrease in cardiac output, a reduction in peripheral vascular resistance and decreased intravascular volume. The reduction in cardiac output has been partly explained by the effect of the peptide on the sympathetic and parasympathetic nervous systems, possibly by stimulating vagal afferent (Ackermann et al., 1984; Allen and Gellai, 1987; Thoren et al., 1986). However, this effect of ANF on cardiac output was shown to depend on the autonomic state of the animal (Thoren et al., 1986). Part of the reduction of the cardiac output was also attributed to a decrease in preload as a result of a reduction in central venous and right atrial pressures (Breuhaus et al., 1985: Pegram et al., 1986; Shapiro et al., 1986) perhaps due to a decrease in circulating volume rather than alterations in venous compliance, since the administration of exogenous ANF resulted in an increase in

haematocrit and plasma protein concentration (de Bold et al., 1981; Flückiger et al., 1986; Trippodo and Barbee, 1987). Lastly, part of the effect of ANF on cardiac output was attributed to a decrease in coronary flow *in vitro*, which could hamper the contractility of the cardiac muscle (Wangler et al., 1985).

Investigation on the effects of ANF on vascular smooth muscle *in vivo* and *in vitro* have shown heterogeneous responses. Precontracted aortic rings, rabbit facial veins and portal veins were shown to relax in the presence of ANF (Winquist et al., 1984), but no effect was observed with precontracted mesenteric, femoral, coronary or cerebral arteries (Osol et al., 1986). Garcia et al. (1985), using *in vivo* microsphere analysis reported a marked increase in flow to the lung, heart, testes, spleen, kidney and mesentery in rats whereas others reported no effect (Fujioka et al., 1985; Lappe et al., 1985). The discrepancies between these reports have been partially attributed to the varying status of the autonomic activity of the animals in the different experiments.

ANF interacts with the renin-angiotensin-aldosterone system, and with vasopressin. ANF lowers renin secretion and plasma concentration *in vivo* and *in vitro* (Burnett et al., 1984; Henrich et al., 1986; Maack et al., 1984; Opgenorth et al., 1986). This effect is mediated by cGMP (Henrich et al., 1988; Kurtz et al., 1986) and is independent of solute delivery to the macula densa (Opgenorth et al., 1986). This ANF-mediated inhibition of renin release also may occur indirectly through the inhibition of neural stimuli (Thoren et al., 1986).

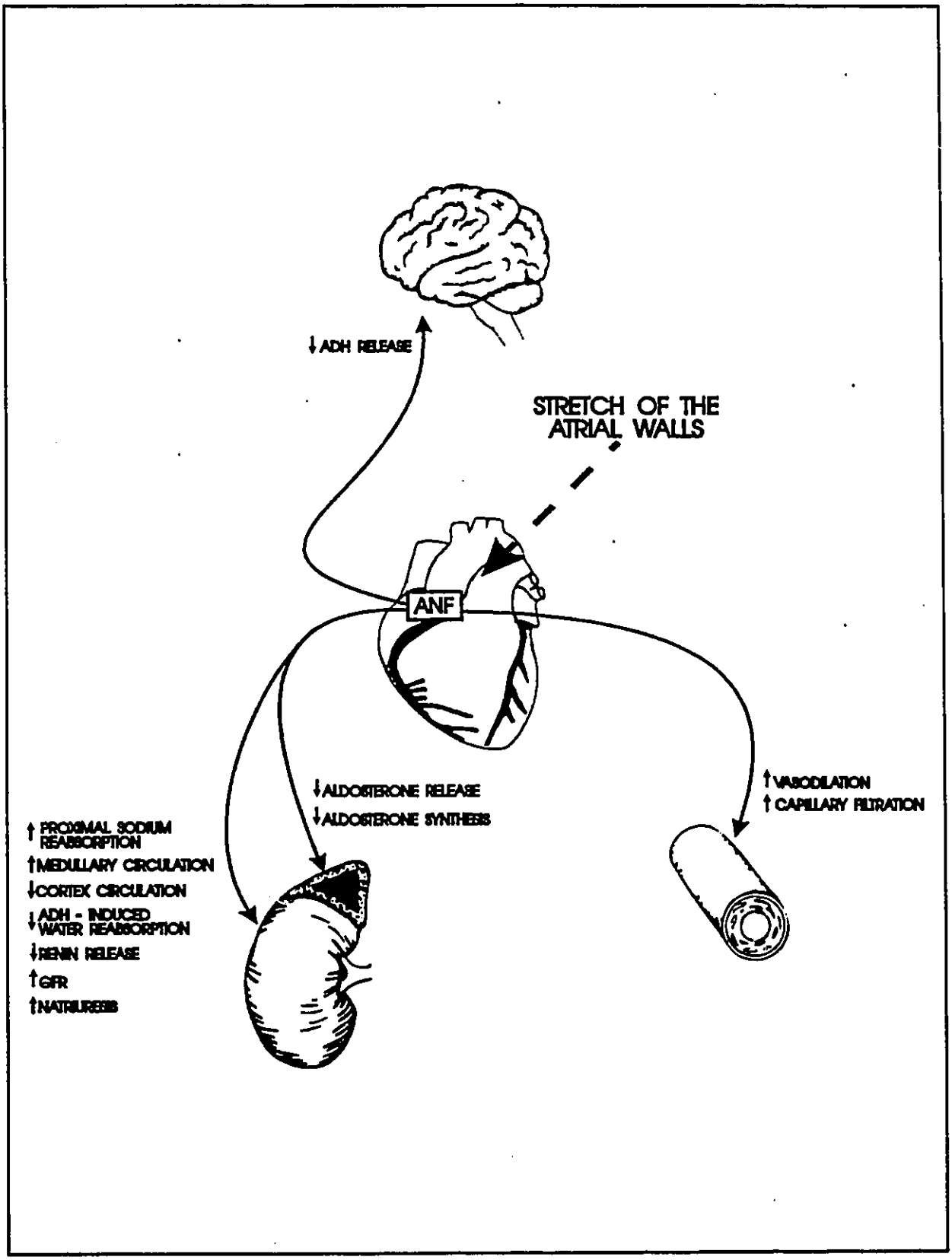
In addition to the decrease of aldosterone release resulting from the ANF-induced reduction in circulating levels of angiotensin, ANF directly inhibits aldosterone production and secretion. *In vitro*, ANF has been shown to inhibit both basal and angiotensin-, potassium-, and ACTH-stimulated release of aldosterone (Atarashi et al., 1985; Chartier and Schiffrin, 1987; Delean et al., 1984; Quinn and Williams, 1988; Schiebinger et al., 1988). Similar results have been reported *in vivo* (Maack et al., 1984; Metzler and Ramsay, 1989). This effect was independent of cGMP (Matsuoka et al., 1987). It has been speculated that ANF inhibition of aldosterone might be due to alterations in the calcium influx observed during stimulated release of aldosterone possibly by blocking directly calcium channels (Chartier and Schiffrin, 1987; Takagi et al., 1988). ANF has been reported to inhibit aldosterone synthesis by interfering with steps involved in the delivery of cholesterol to the mitochondrial cytochrome P-450 enzyme complex (Butlen et al., 1987; Chanderbhan et al., 1986) and, to a lesser extent, by altering the conversion of corticosterone to aldosterone (Campbell et al., 1985; Schiebinger et al., 1988).

Arginine-vasopressin producing-neurons were demonstrated to be a target for ANF actions; in situations where vasopressin release was increased, ANF was found to have a marked inhibition on the rate of firing of vasopressin neurons in the paraventricular region of the hypothalamus (Standaert et al., 1987). Furthermore, infusion of exogenous ANF in this region lowered plasma vasopressin levels (Lee et al., 1987).

9) PHYSIOLOGY AND PATHOPHYSIOLOGY OF ANF

ANF release is induced by acute and chronic elevations in atrial pressure resulting from manoeuvres such as volume expansion, salt loading, exercise, head down tilt or water immersion. Atrial distension resulting form increase in pressure appears to be the principal stimulus for ANF release and operates by an intrinsic stretch-secretion coupling mechanism. The combined actions of ANF on the kidneys, adrenals, and vasculature serve to decrease systemic blood pressure and intravascular volume. Physiological doses of ANF administered to normal subjects induces an increase in glomerular filtration rate, filtration fraction, urinary sodium excretion and free water clearance, along with a suppression of the renin-angiotensin-aldosterone system. In addition to these effects, supraphysiological doses of the peptide causes an acute fall in blood pressure. It is likely that ANF modulates the renin-angiotensin-aldosterone system on a continuing basis in regulating blood pressure, volume and sodium homeostasis.

The role of ANF in disorders of volume regulation such as congestive heart failure, hypertension, or acute and chronic renal failure is presently under intense investigation. In such disease states, chronic elevation of atrial pressure enhances both the synthesis and release of ANF, resulting in plasma levels that can be elevated several-fold above normal. In spite of this increase in its circulating levels, ANF fails to correct the situation suggesting a reduced sensitivity to the endogenous peptide. Therapeutically, attempts are being made to increase the circulating levels of ANF by several approaches including designing ANF analogues and agents that inhibit the endopeptidase 3.4.24.11 and/or block the clearance receptor. These approaches have been proven clinically useful (Cavero et al., 1990) and offers new hope for the treatment of congestive heart failure and hypertension.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↓ALDOSTERONE RELEASE
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↑CAPILLARY FILTRATION
↑PROXIMAL SODIUM REABSORPTION
↑MEDULLARY CIRCULATION
↓CORTEX CIRCULATION
↓ADH - INDUCED WATER REABSORPTION
↓RENIN RELEASE
↑GFR
↑NATRIURESIS

10) RATIONALE FOR THE STUDY

From the preceding review, it is clear that ANF plays an important role in cardiovascular homeostasis. It follows that studies on factors controlling its production may provide useful insight into the physiology and pathophysiology of the cardiovascular system. The biosynthesis and post-translational modifications of ANF in mammalian atrial cardiocytes have not been fully characterized, in particular the last step of its maturation which involves the cleavage of the ANF precursor, ANF (1-126) to its circulating form, ANF (99-126). ANF processing is present in isolated perfused heart preparations or isolated atrial appendages, but it is absent in atrial cardiocytes from either neonatal or adult hearts maintained in culture. The reason for this lack of processing activity could be explained by the fact that factor(s) or condition(s) present *in vivo*, which are necessary for processing activity, are not reproduced in a cell culture system. On the other hand, it could be that ANF post-translational processing requires or is achieved by another type of cell which is removed during the isolation and culture processes. Finally, it is possible that the isolation process itself is enough to destroy the enzymes responsible for ANF maturation. To test these hypotheses, a cell culture system was designed which provides means by which adult rat cardiocytes, freshly isolated and in primary cultures, are maintained under continuous superfusion. For the investigation of peptide processing, superfusion of the cell cultures is a justified approach over a static incubation method (in Petri dishes) because it minimizes any possible accumulation of non-specific proteolytic activity in the culture medium, a potential pitfall in peptide processing studies.

Experimental approach:

Adult rat atrial cardiocytes were cultured on micro-carrier beads and maintained in mini-chromatographic columns continuously superfused with heat treated fetal bovine serum (HT-FBS) supplemented medium. To investigate ANF processing before cell attachment (which occurs after 2-3 days in culture), BioGel P-2 chromatographic gel was used as a supporting matrix. Collection of the ANF peptides released from the cells was performed by changing the superfusing medium to a serum-free medium. The collected ANF was later quantified with a specific radioimmunoassay with no distinction for the molecular size of the peptide. The molecular species of ANF released from the cardiocytes on different days were then separated by reverse phase high performance liquid chromatography (RP-HPLC) and quantified by radioimmunoassay (RIA). In parallel, microscopical characterization of the primary cultures was carried out using ANF and desmin (a muscle specific marker) immunofluorescence in order to correlate findings in ANF processing and release to morphological changes brought about by the isolation and culture procedures. Since micro-carrier beads cultures is a 3 dimensional cell culture system, microcopical analysis of atrial cardiocytes cultured on beads was not possible. For this reason, atrial cardiocytes were grown onto laminin-coated cell culture chamber slides (flat cultures). Finally, *bis*-benzimidazole (Hoechst 33258), a fluorescent dye specific to DNA, was used together with desmin-like immunofluorescence to estimate the degree of heterogeneity of the cell population.

MATERIALS AND METHODS

1) ISOLATION AND CULTURE OF ADULT RAT CARDIOCYTES

Adult male Sprague-Dawley rats (300 to 350 g of body weight) had free access to food and water until sacrificed by decapitation. After washing with 70% ethanol, the thorax was opened and both atria or/and one half of the ventricles were excised and collected in a plastic petri dish (bacteriological grade; Fisher Sci.) containing low calcium medium (LCM; Iida et al., 1988; in mM: NaCl 120, KCl 5.4, $MgSO_4$ 5, Na pyruvate 5, glucose 20, HEPES (N-2-hydroxyethylpiperazine-N'-ethane-sulfonic acid) 10, taurine 20, pH=7.4.) and heparin (50 units/mL to prevent coagulation of blood) at room temperature. The tissue was rapidly minced with a clean razor blade (vertical strokes) into 1-3 mm pieces, rinsed 3 times in LCM and transferred to a siliconized 25 mL erlenmeyer flask containing 5 mL enzyme solution (ES; LCM with 0.5% heat treated-bovine serum albumin [HT-BSA; section 9 of materials and methods] and 0.1% collagenase type II [Worthington Biochemical Corp., Freehold, N.J.]). The flask was closed using a rubber stopper through which two 18G hypodermic needles were inserted to provide for an inlet and an outlet of oxygen. The flasks were incubated at 35°C in a shaking water bath at 150 strokes/min, 40 mm stroke amplitude and under O_2 atmosphere. After 10 min, the supernatant was discarded and the incubation was repeated with fresh ES for 15 min. The tissue pieces were gently triturated using a transfer pipet. The supernatant was decanted in a 50 mL polypropylene conical tube and centrifuged for 10 min at 60 g. The supernatant was discarded, the pellet was resuspended in 10 mL of modified KB medium (Isenberg and Klockner, 1982; in mM: KCl 25, KH_2PO_4 10, Na glutamate 70, EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] 0.5, glucose 11, HEPES 10, taurine 20, pH=7.4., 310 mOsmol/L) and left

at room temperature for a minimum of 45 min. The undissociated tissue was submitted to 3 more 15-min periods of digestion with a 0.05% collagenase solution, or until no tissue remained. Dissociated cells were handled as described above.

After 45 min incubation in the KB medium, calcium was gradually increased to 1.5 mM (the M-199 culture medium contains 1.8 mM calcium) calcium in sinstep of 100 μM every 5 minutes over 30 min using 100 mM $CaCl_2$. Cell viability was judged by the criterion of trypan blue exclusion. The percentage of cardiocytes able to exclude trypan blue (0.2% in phosphate buffer saline; PBS) was determined by microscopy using a haemocytometer chamber and a hand tally counter.

Except for experiments using acutely isolated cardiocytes, the cells were pelleted for 10 min at 60 g and the pellets were resuspended in 15 mL of M-199 culture medium (GIBCO, Grand Island, New York) supplemented with 20 % heat treated (30 min at 56°C) fetal bovine serum (FBS; GIBCO), penicillin (100 000 IU/L) and streptomycin (0.1 g/L) (Sigma Chemical Co., St Louis, MO). The cells were allowed to settle in a 150 cm^2 culture bottle in a CO_2 incubator at 37°C and 95% humidity for 24 hours in order for non-cardiocyte cells to attach. The unattached cells were then transferred to the proper substratum, and maintained in culture in the presence of an anti-mitotic agent, Cytosine-1-β-D-arabinofuranoside (ARA-C; 10μg/mL; Sigma) to restrict the proliferation of other types of cells such as fibroblasts. ARA-C was added again after 3 days of culture and the medium was changed to ARA-C free M-199 after 7 days in culture.

2) CULTURES OF ATRIAL NON-CARDIOCYTE CELLS

Atrial non-cardiocytes were obtained by trypsinization of the plates used for the pre-plating of the primary cultures of atrial cardiocytes as follows: after transferring the atrial cardiocytes to the proper culture substratum, flasks used for the pre-plating were incubated with fresh M-199 medium supplemented with 20% FBS until the cells reached about 75% confluence (as judged by phase contrast microscopy). The plates were washed with calcium and magnesium free PBS containing 1 mM EDTA and exposed to 0.025% trypsin in PBS/EDTA (GIBCO) for 10 min at 37°C. Harvested cells were centrifuged at 100 X g for 10 min, counted using a haemocytometer chamber and a hand tally counter. The cells were resuspended in fresh culture medium at a concentration of 250 x 10^3 cells/mL. Cells were plated on culture slides (0.5 mL/chamber) or on beads (10 mL/30 mg of Cytodex III™) and incubated until confluence.

3) SUPERFUSION OF CELL SUSPENSIONS

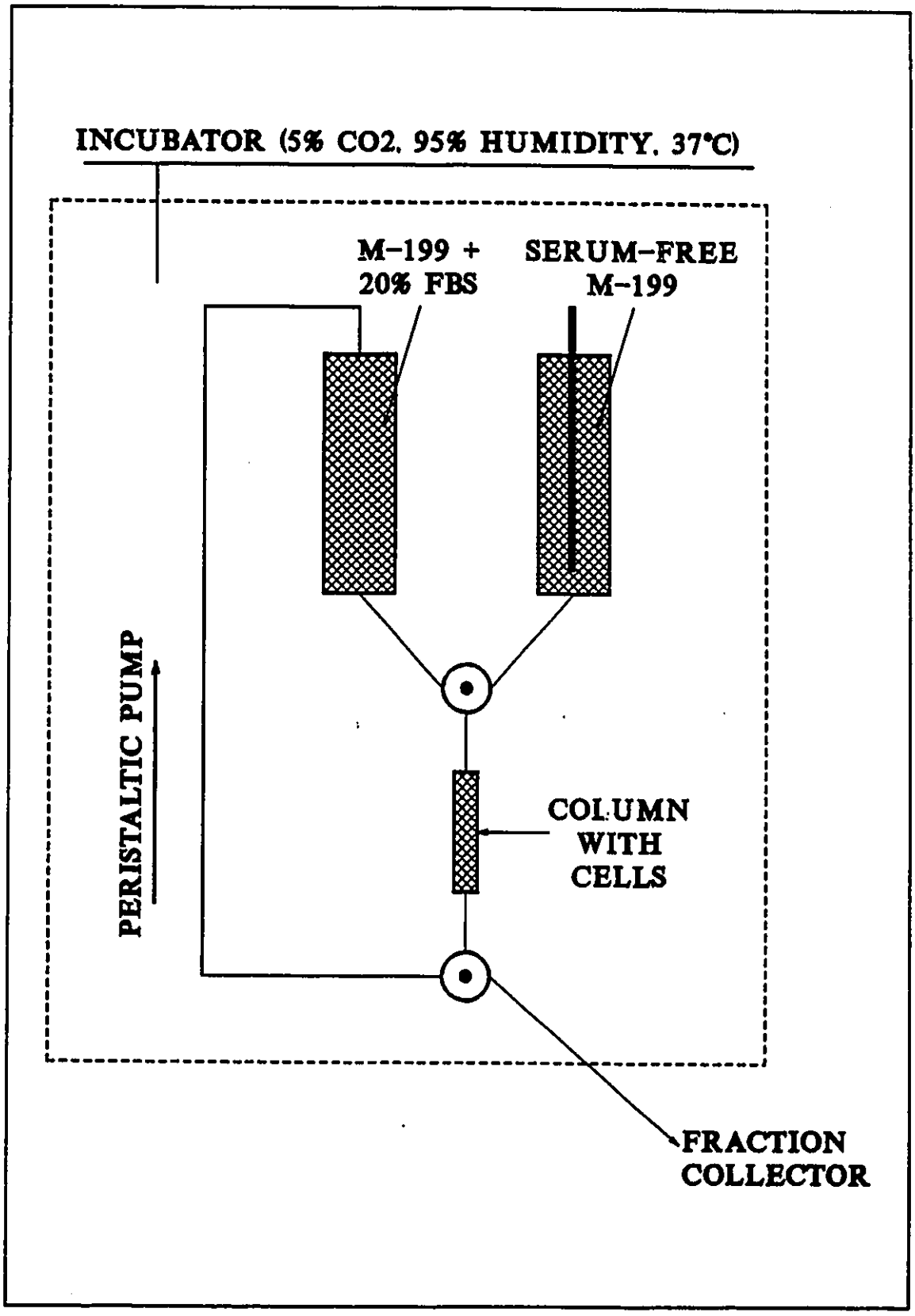
Superfusion of non attached cell preparation (freshly isolated cells or cardiocytes in culture for 1 or 2 days), was performed using an adaptation of a method originally described by Gillies and Lowry (1978), used with acutely isolated atrial cardiocytes by Gibbs (1987). Approximately 0.5 g of Bio-Gel P2 (Bio-Rad Laboratories, Mississauga, On.) was hydrated in 0.9% NaCl overnight at room temperature and washed 3 times with M-199 buffered with 24 mM sodium bicarbonate and 50 mM HEPES (pH=7.4) with a measured osmolality of 330 mOs/kg. Cell suspensions were mixed with the Bio-Gel, transferred into mini chromatographic columns (Isolab, Akron, OH), mounted on the superfusion set-up (Fig. 5) and superfused with M-199 at a flow rate of 0.5 mL/min (gravity fed) at 37°C and 5% CO_2. Following a 60 min equilibration period, fractions were collected at 1 min intervals for 1 to

2 hours. These collected fractions were stored at -20°C until radioimmunoassay was performed (section 9 of materials and methods). An additional 20 mL fraction was collected and frozen for reverse phase-high performance liquid chromatography (RP-HPLC; section 8 of materials and methods).

4) SUPERFUSION OF CELLS CULTURED ON MICRO-CARRIER BEADS

Superfusion of cardiocytes cultured on micro-carrier beads was carried out using a protocol adapted from that described by Smith and Vale (1980). Five hundred milligrams of Cytodex III™ micro-carrier beads (Pharmacia) were rehydrated and sterilized by autoclaving as per product instructions. Between 1.5 and 2 mL of the Cytodex III™ suspension (30-50 mg) were then incubated with 2.8 $\mu g/cm^2$ (125 μg/30 mg of Cytodex III™) of laminin (Sigma) for 1 hour at 37°C (Lungren et al., 1985) and rinsed 3 times with M-199. The beads were then mixed with the cell suspensions (after 24 hours of pre-plating), transferred to bacteriological grade Petri dishes and maintained in culture for 3 days. At the fourth day in culture, viable cells had attached to the beads and the dead cells, in suspension, were eliminated by differential sedimentation. The beads were transferred to mini chromatographic columns and superfused as described above. After the collection period (1 to 2 hours), the superfusion medium was replaced by M-199 supplemented with 20% FBS and, using a small peristaltic pump to return the media back to the reservoir, the columns were left under continuous superfusion until the next day (Fig. 5).

FIGURE 5: Schematic representation of the superfusion apparatus. Superfusion medium is contained in the right reservoir while the serum containing medium is contained in the left and is recirculated by a peristaltic pump. The cells are contained in a mini-chromatographic column. The apparatus is placed in a CO_2 incubator.


INCUBATOR (5% CO2. 95% HUMIDITY. 37°C)
M-199 + 20% FBS
SERUM-FREE M-199
PERISTALTIC PUMP
COLUMN WITH CELLS
FRACTION COLLECTOR

5) SUPERFUSION OF ATRIAL NON-CARDIOCYTES CULTURED ON MICRO-CARRIER BEADS WITH proANF-CONTAINING MEDIUM

Atrial non-cardiocytes cultured on micro-carrier beads were superfused as described above using the same superfusion medium but with the addition of exogenous proANF (previously extracted from rat atrial tissue and purified by RP-HPLC; section 8). Three different concentrations of proANF were used (3500 pg/mL, 550 pg/mL, and 125 pg/mL) representing concentrations of ANF released in early, intermediate and late stages of culture (as characterized in preliminary experiments). The superfusion rate used, 0.5 mL/min, was the same as with previously described experiments. Artifactual break-down of ANF was monitored simultaneously by superfusing exogenous propeptide through a column containing only Cytodex beads.

6) DNA DETERMINATION

DNA determination was carried out using the DNA binding fluorochrome H33258 (*bis*-benzimidazole; Sigma) (Labarca and Paigen, 1980). Cell aliquots (one third of the column content) were resuspended in 1 mL of 0.05 M sodium phosphate buffer containing 2 M NaCl and 2 mM EDTA, pH 7.4, homogenized for 15 sec on ice using a polytron (50% of maximum power with a PTA 10s probe; Brickmann instruments inc.) and were left to sediment for 30 min. on ice. The supernatants were saved and the pellets rehomogenized. The supernatants were pooled and their DNA concentration was measured against a standard curve made with calf thymus DNA (Sigma) from 10 ng/mL to 250 ng/mL and H33258 (Sigma) at a final concentration of 0.1µg/mL. Fluorescence was measured with a TKO 100 dedicated mini fluorometer (Hoefer Scientific Instruments San Fransisco, Ca) using 365/460 nm excitation/emission wavelength.

7) ANF EXTRACTION

ANF extraction was performed following a method previously described by de Bold and Flynn (1983). Cell aliquots (one third of the column contents) were transferred to 3 mL of cold extractant containing 1.0 M acetic acid, 0.1 N HCl and 1% NaCl, and homogenized for 15 sec using a Polytron (50% of maximum power with a PTA 10s probe). Homogenates were left on ice for 30 min and then centrifuged for 30 min at 8000 x g at 4°C. The supernatants were saved and the pellets were homogenized in 3 mL of extractant for 15 sec, left on ice for 15 min and centrifuged for 30 min at 8000 x g. The supernatants were pooled, passed 3 times through pre-wetted Sep-Pak™ Cartridges (Waters), washed with 20 mL of 0.1% trifluoroacetic acid (TFA; Sigma), eluted with 3 mL of 80% of HPLC grade acetonitrile (ACN; Burdick & Jackson) in 0.1% TFA in siliconized Vacutainer™ glass tubes (100 x 16 mm; Becton Dickinson) and lyophilized. For pre-wetting, 5 mL of 80% ACN in 0.1% TFA was passed through the cartridges followed by 5 mL of 0.1% TFA to rinse.

8) REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ANF polypeptides from either superfusate or cell extracts were separated using a method previously developed by de Bold and Flynn (1983). In this puplication, a clear separation of the ANF 99-126 and the proANF was achieved following a single RP-HPLC run through a C-18 column.

Superfusates were filtered through a 0.45µm syringe filter, loaded through the aqueous pump and submitted to reverse phase high performance liquid chromatography (RP-HPLC) using a Vydac™ C-18 column (The Separation Group, Hesperia, Ca; Cat# 218

tp54, sn# 58 851010) equilibrated with 15% of 80% ACN in 0.1% TFA. Elution was carried out over 80 min at 1.5 mL/min with a linear gradient of 15 to 55% of 80 % ACN in 0.1% TFA and fractions were collected every 2 min in 10 mL siliconized Vacutainer™ glass tubes containing 0.5% heat treated bovine serum albumin (section 9 of materials and methods) and lyophilized. The elution profile for proteins was monitored at 280 nm. The elution profile of the ANF peptides was determined by radioimmunoassay on the reconstituted fractions (1 mL of RIA Buffer per fraction; section 9 of materials and methods)

RP-HPLC of cell extracts was carried out as described above except that the lyophilized samples were reconstituted with 1 mL of 0.1% TFA and loaded into the column through the injector.

9) RADIOIMMUNOASSAY FOR ANF

Heat treated bovine serum albumin (HT-BSA):

RIA grade bovine serum albumin (fraction V; Sigma) was heat treated as described by Sarda et al. (1989): BSA (200 g/L of 0.05 M Na_2HPO_4 and 0.15 M NaCl) was incubated in a water bath at 56°C for 2 h with stirring and cooled on ice. Five thousand Units of penicillin and 5000 mg of streptomycin (Gibco, Grand Island, New York) were added and the BSA was dialysed against distilled water at 4°C for 2 days. The dialysed BSA was filtered through a 0.45 μ filter and lyophilized.

The RIA buffer used consisted of 0.1 M sodium phosphate containing 0.05 M NaCl, 0.01% sodium azide, 0.1% triton X-100 and 0.1% HT-BSA. Polystyrene tubes (12 X 75 mm; Sarsted Canada Inc. St. Laurent, Can.) were used for the reaction. All reagents were prepared in the RIA buffer. The standard curve was made with ANF (99-126) (Peninsula Laboratories, Belmont, CA) at the following concentration: 31.25, 62.50, 125.0, 250.0, 500.0 and 1000 pg/mL. Two different protocols were used:

Protocol 1

One hundred µL of the ANF standard or samples, were added to 100 µL of 1:1500 dilution of the antiserum (Peninsula Laboratories, Belmont, CA) and 100 µL (10 000 CPM) of radio-iodinated ANF (99-126) (New England Nuclear, Mississauga, On) and was incubated for 24 h at 4°C. This protocol was used on the RP-HPLC eluate fractions and is referred to as the equilibrium method.

Protocol 2

One hundred µL of the ANF standard or samples was added to 100 µL of 1:1500 dilution of the antiserum (Peninsula Laboratories, Belmont, CA) and incubated for 24 h at 4°C. After 24 h, 100 µL (10 000 CPM) of radio-iodinated ANF (99-126) (New England Nuclear) was added and the tubes were incubated for a further 24h at 4°C. This method, more sensitive than the first, was used to measure ANF in superfusates collected from columns.

In both cases, free and bound fractions were separated by adding goat anti-rabbit gamma globulin (Bio-Rad Laboratories, Mississauga, On; Cat# 170-6010) and 100 µL of 5%

normal rabbit serum. The tubes were incubated for 2 h at room temperature followed by the addition of 1.5 mL of RIA buffer. Tubes were centrifuged at 2000 x g at 4°C for 45 min, the supernatants were discarded and the pellets counted for radioactivity on a γ-counter.

10) IMMUNOFLUORESCENCE MICROSCOPY

Zero to 2 day old cultures of atrial cells were attached to 0.5% poly-L-lysine-(Sigma) coated slides by centrifugation (70-100 x g) using cyto-buckets™ (Fisher Scientific). Four to 15 day old cultures were grown on laminin-coated (2.8 $\mu g/cm^2$) glass chamber slides for cell culture (Lab-Tek, Nunc Inc., Naperville, Il) containing 8 separated fields. In all cases, cells were fixed in 8% paraformaldehyde in 0.05M phosphate buffer pH 7.4 for 10 min at room temperature, washed and stored in a washing solution (0.1 M phosphate buffer containing 10% sucrose) at 4°C until use. For cells attached to Cytodex III (collagen Coated beads), following the superfusion protocol, 1/3 of the column content was washed in calcium-free PBS containing 1mM EGTA and submitted to collagenase digestion for 30 minutes at 37°C. The released cell were separated from the beads by differential sedimentation, fixed in 8% paraformaldehyde and centrifuged on poly-L-lysine-coated slides as described above. The slides were processed immediately after fixation in order to minimize the loss of cells.

For the staining protocol, all incubation steps were conducted at room temperature in a humidified chamber. Samples were incubated in 1% hydrogen peroxide in methanol for 1 hour to inhibit endogenous peroxidase, and washed in PBS. Non-specific binding was blocked with 10% normal goat serum (NGS) for 10 min followed incubation with the

primary antibody for 60 min. The primary antibodies used were a) 1:1000 dilution of rabbit anti-rat ANF (99-126) antiserum (R5:B8, developed in our laboratory), b) 1:30 dilution of rabbit anti-chicken smooth muscle desmin antiserum (Sigma Chemical Co.; Cat#: D-8281) or c) mouse anti-myosin antiserum (MF-20; Boder et al., 1982; kindly supplied by Dr McBurney, Dept. of Biochemistry, University of Ottawa). The slides were washed in PBS and incubated for 60 min in a) goat anti rabbit biotinylated IgG for ANF (Vector Laboratories, Burlingame, Ca; Cat# BA-1000), b) 1:50 dilution goat anti rabbit FITC conjugated IgG for desmin (Dimension Laboratories Inc., Mississauga, On; Cat# FI-1000) or c) a rhodamine conjugated goat anti-mouse IgG for myosin. Finally, for ANF slides were incubated with a 1:1000 dilution of streptavidin-texas red conjugate for 60 min (Bethesda Research Laboratories (BRL) Life Technologies, Inc; Cat# 9540 SA). *Bis*-benzimidazole (5 ng/mL; Hoechst 33258; Sigma), a fluorescence dye which specifically binds to DNA in nuclei was used to reveal the presence of cell types not stained by either of the 3 antibodies. Slide were rinsed in PBS followed by a rinse in double distilled water and mounted with Immuno-Mount (Lerner Laboratories, Sewickley, PA). Controls consisted of a) omission of the primary antiserum, b) pre-absorption of the primary antiserum with 1 μg/mL of synthetic ANF (99-126) (Peninsula Laboratories, Belmont, CA), c) omission of conjugate second antibody, and d) omission of the streptavidin-texas red. In addition, heart tissue sections and cultured atrial non-cardiocytes were processed in parallel and used as controls.

Specimens were viewed with a Leitz Orthoplan microscope equipped with a Leitz Pleomopak fluorescence vertical illuminator filters for FITC (excitation:450/490 nm; emission: 515 nm), Texas red (excitation: 530/560 nm; emission: 580 nm) and H33258 (excitation: 355/425 nm; emission: 460 nm), and Leitz Vario Orthomat 2 automatic

microscope camera. The photographs were taken using Kodak Ektachrome P800/1600 film processed for an equivalent of 1600 ASA.

Quantization of the different cell types present in culture was carried using low magnification pictures (16x objective) of 5 to 10 different areas randomly selected. Desmin positive cells were taken as cardiocytes and desmin negative cells (revealed by nuclear staining) as non-cardiocytes. Cardiocytes containing more than one nucleus were counted as single cells since the whole cell was easily visible at the emission wavelength used for H33258.

11) STATISTICAL ANALYSIS

All results are expressed as a mean ± SEM. Statistical comparison between the ANF processing activity at different days was performed by analysis of variance (ANOVA) using the Systat® statistical package (Systat inc., Evanston, IL) on an IBM computer.

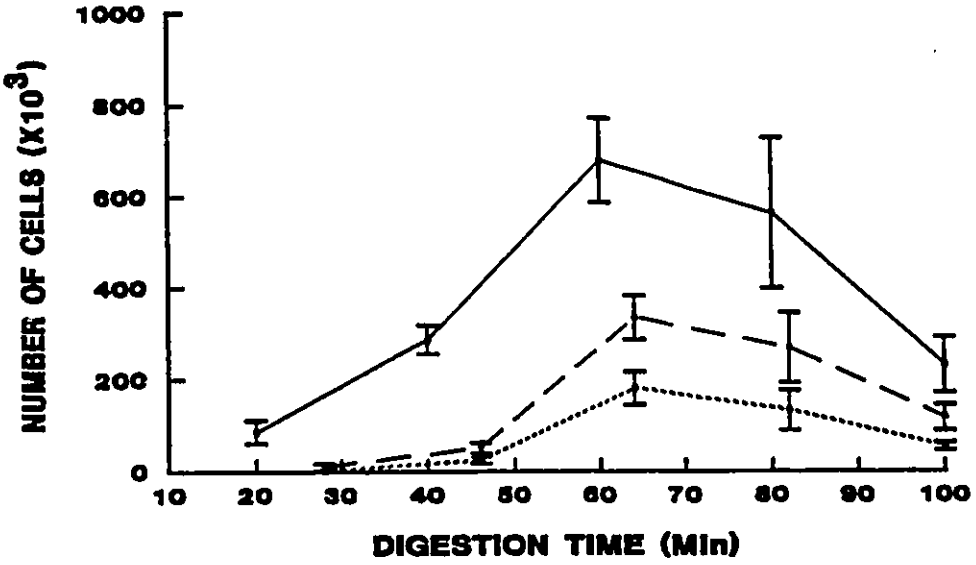
RESULTS

1) ISOLATION AND CULTURE OF ADULT RAT ATRIAL CARDIOCYTES

The isolation procedure produced a consistent yield of $1.33 \pm 0.08 \times 10^6$ cells/rat (2 atria) with $0.88 \pm 0.06 \times 10^6$ viable cells (n=23) as measured by trypan blue exclusion, corresponding to a viability index of 66%. The kinetics of isolation of atrial and/or ventricular cardiocytes from adult rat tissue was determined in some experiments (Fig. 6). Approximately half the dye excluding atrial cardiocytes were found to remain elongated after calcium repletion with binucleation and cross-striations visible in the phase-contrast microscope (Fig. 7a). After a few hours in culture, these cells gradually rounded-up. Viable round cardiocytes were discriminated from hypercontracted cells by their smooth-membrane appearance as compared to others displaying blebs on the membrane (Fig. 7a). Freshly isolated preparations contained spontaneously contracting cells, but atrial cardiocytes were observed to be quiescent 24 hours after the isolation.

Figure 6: Kinetics of isolation of adult rat atrial (A) and ventricular (B) cardiocytes by multiple collagenase digestion at 37°C. Total number of cells isolated (——), number of trypan blue excluding cells (---) and number of cylindrical cells (···). Mean ± SEM.

(A)


NUMBER OF CELLS (X10^3)
1000
800
600
400
200
0
10
20
30
40
50
60
70
80
90
100
DIGESTION TIME (Min)

(B)

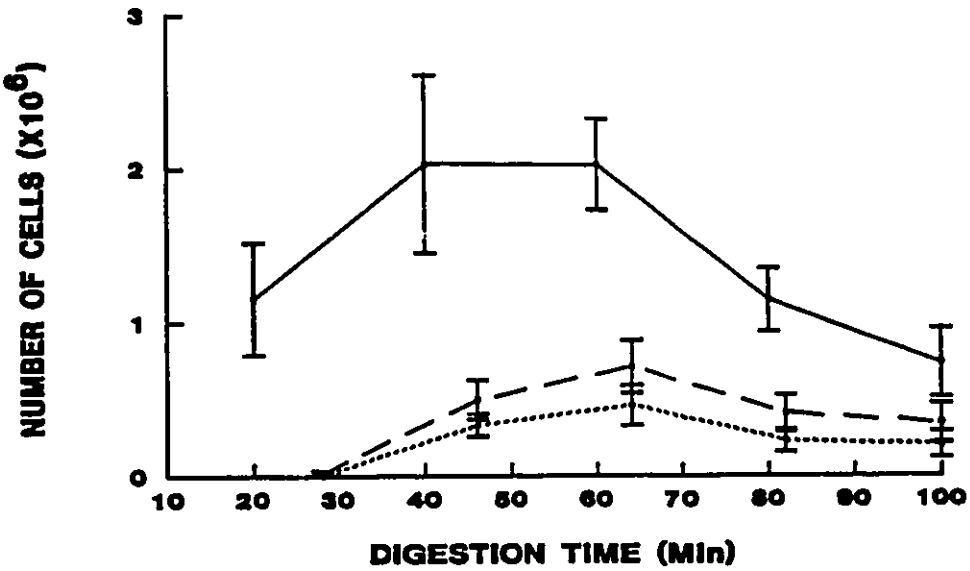
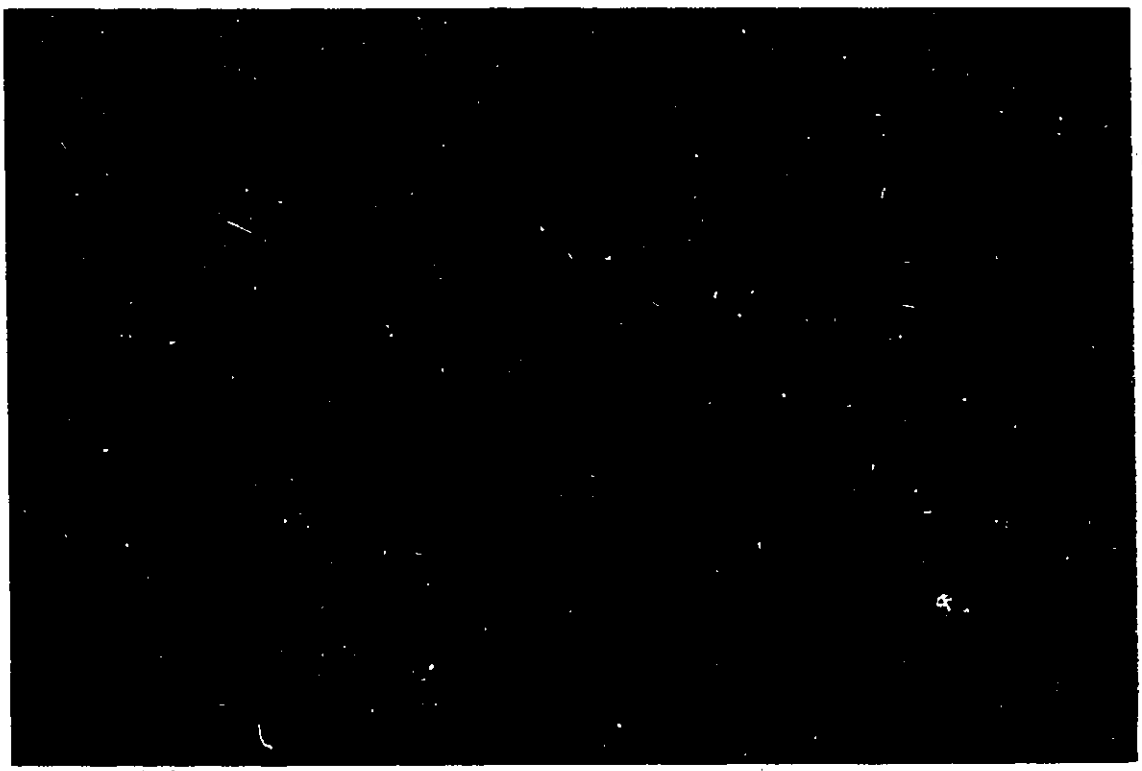

NUMBER OF CELLS (X10^6)
3
2
1
0
10
20
30
40
50
60
70
80
90
100
DIGESTION TIME (Min)

FIGURE 7: Phase contrast micrograph of a primary cultures of adult rat atrial cardiocytes. (A) Freshly isolated cell preparation after calcium repletion, showing typical cylindrical cardiocytes, cells in the process of loosing their cylindrical shape and round cardiocytes with smooth cytoplasmic membranes. (B) Micrograph of atrial cardiocytes after 7 days in culture. Some cells still have the original cell body on top. (C) Micrograph of an atrial cardiocyte after 7 days in culture attached to a Cytodex III® micro-carrier bead.

Atrial cardiocytes remained unattached for 2 to 3 days after isolation during which time the cells remained spherical and quiescent. Subsequently, the viable atrial cardiocytes were attached to the culture substratum and, upon flattening, the emergence of cytoplasmic elongations was observed. Beating activity was noticeable after 4 to 5 days in culture and the number of beating cells increased with increasing time in culture. After 5-6 days in culture, all atrial cardiocytes had flattened and spreaded-out (Fig. 7b, c). The morphology of cultured atrial cardiocytes was not found to change substantially after 6 to 7 days in culture and virtually all cardiocytes were beating spontaneously. Beyong 10 days in culture, a general diminution of the beating activity (reflected by both a decrease in the number of beating cells and in the beating frequency) was observed.

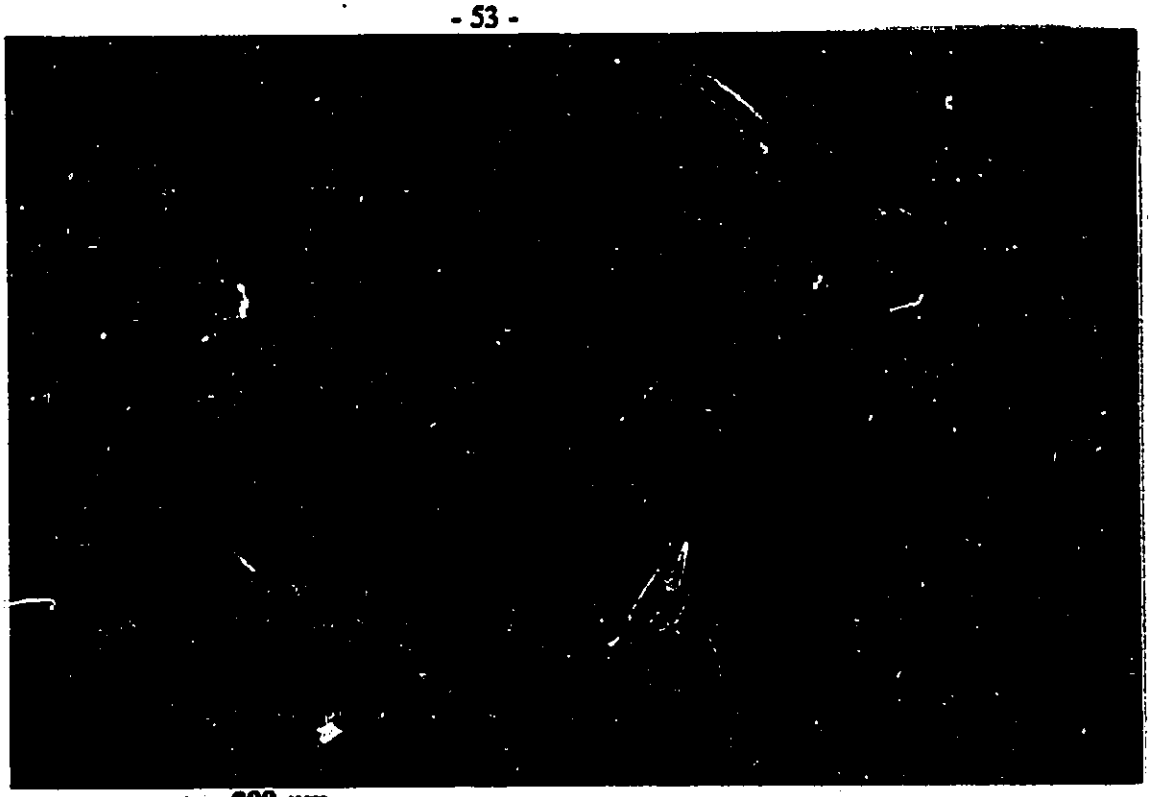
(7-A)



—— =125 µm

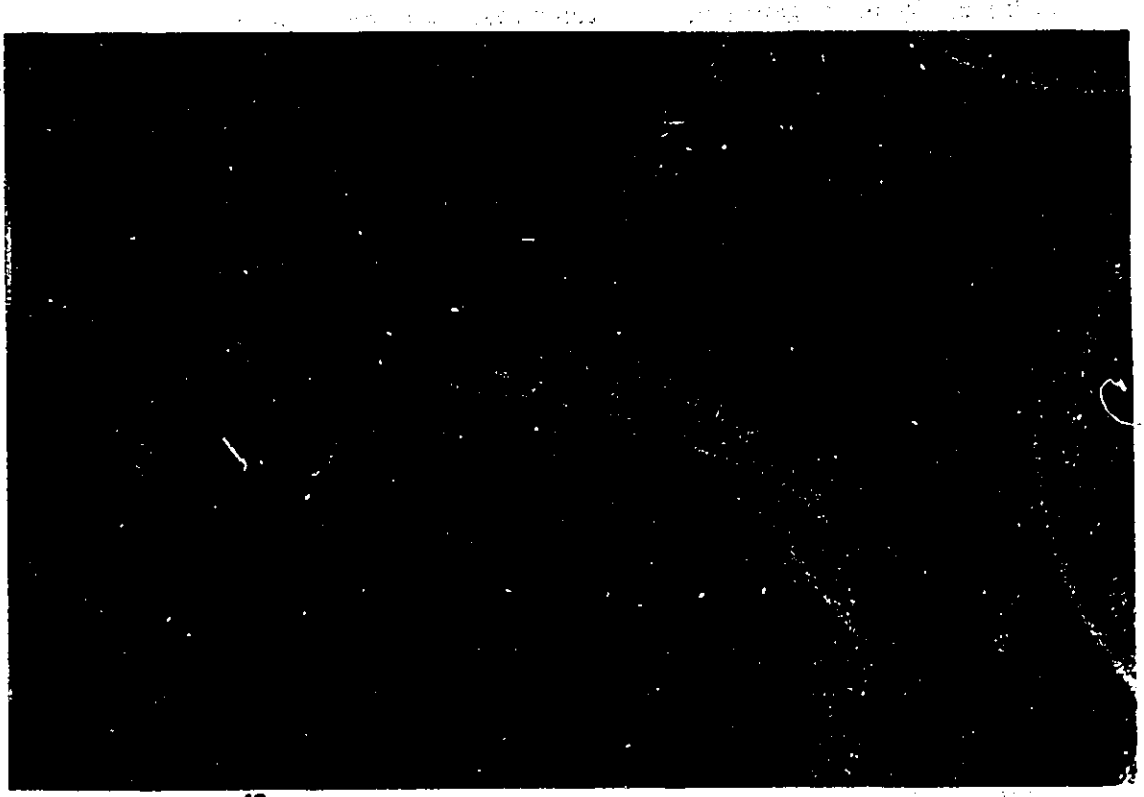
FIGURE 7: Phase contrast micrograph of a primary cultures of adult rat atrial cardiocytes. (A) Freshly isolated cell preparation after calcium repletion, showing typical cylindrical cardiocytes, cells in the process of loosing their cylindrical shape and round cardiocytes with smooth cytoplasmic membranes. (B) Micrograph of atrial cardiocytes after 7 days in culture. Some cells still have the original cell body on top. (C) Micrograph of an atrial cardiocyte after 7 days in culture attached to a Cytodex III® micro-carrier bead.

(7-B)



——— =200 μm

(7-C)



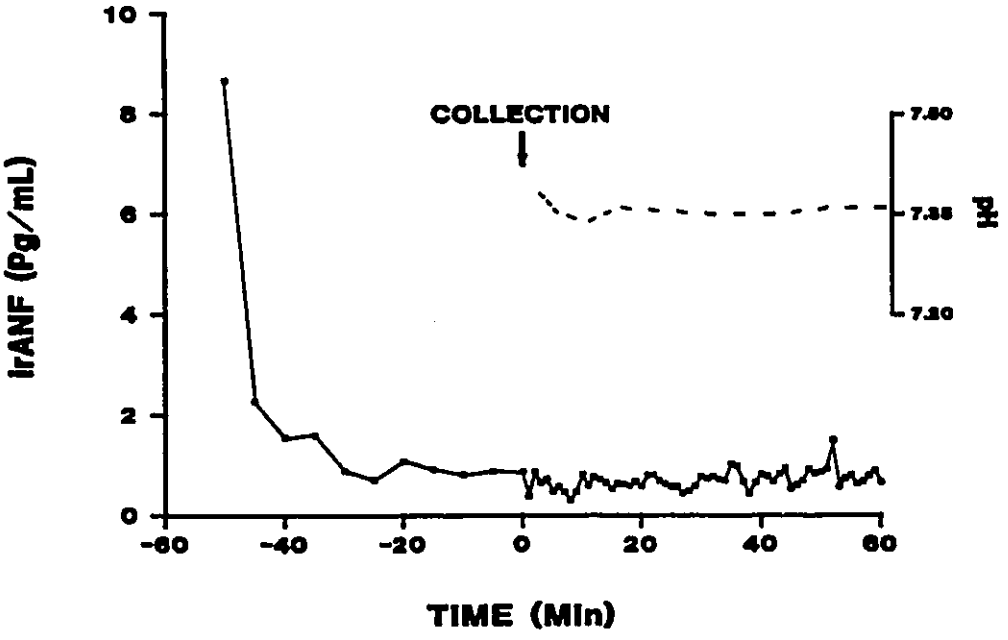
——— =40 μm

FIGURE 8: Release of irANF by primary cultures of adult rat atrial cardiocytes after 3 days in culture. Cells were contained in a mini-chromatographic column with Bio-Gel P2 beads as a supporting matrix and superfused with serum M-199 culture medium at a rate of 0.5 mL/min. Negative time indicates the equilibration period during which fractions were collected every 5 minutes. In the post-equilibration period, fractions were collected every minute for up to 60 min.

2) ANF STORAGE, POST-TRANSLATIONAL PROCESSING AND RELEASE

2a. ANF storage and release from isolated adult rat atrial cardiocytes at different times in culture:

Experiments using either freshly isolated atrial cardiocytes or cells in culture demonstrated that a one hour equilibration period was sufficient to achieve steady basal immunoreactive ANF (irANF) release. This release remained stable for up to 2 hours after the equilibration period (Fig. 8). No changes in pH were observed during the collection period.

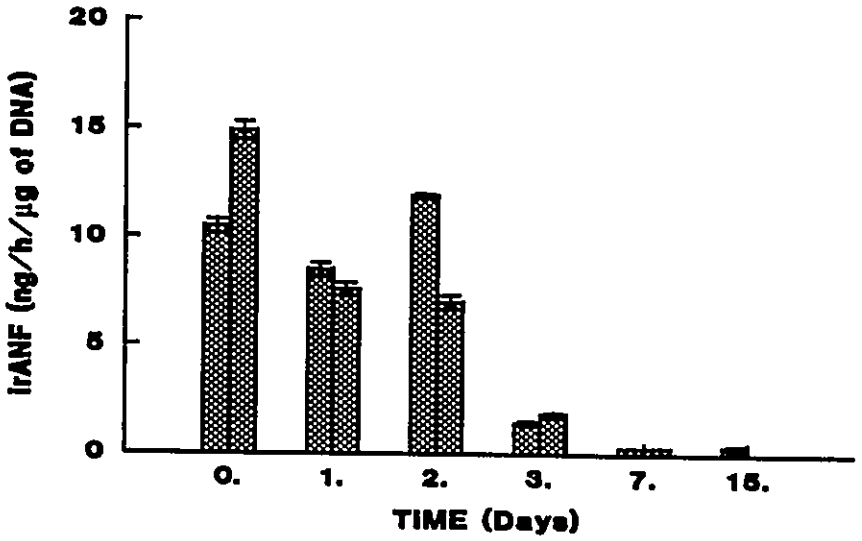


The release of irANF from isolated atrial cardiocytes was determined in freshly isolated preparations and in culture for up to 15 days. ANF release was calculated and expressed as a cumulative irANF release in ng per hour per μg of DNA (Fig. 9a). Freshly (day 0) isolated atrial cardiocytes released irANF at a rate of 10.5 ± 0.3 and 14.9 ± 0.4 ng/h/μg of DNA. The irANF release was found to decrease with increasing time in culture and after 15 days in culture the rate of release was 0.32 ± 0.08 ng/h/μg of DNA (n=6). Most of the reduction was observed after 3 days in culture (10 fold decrease) (Fig. 9a).

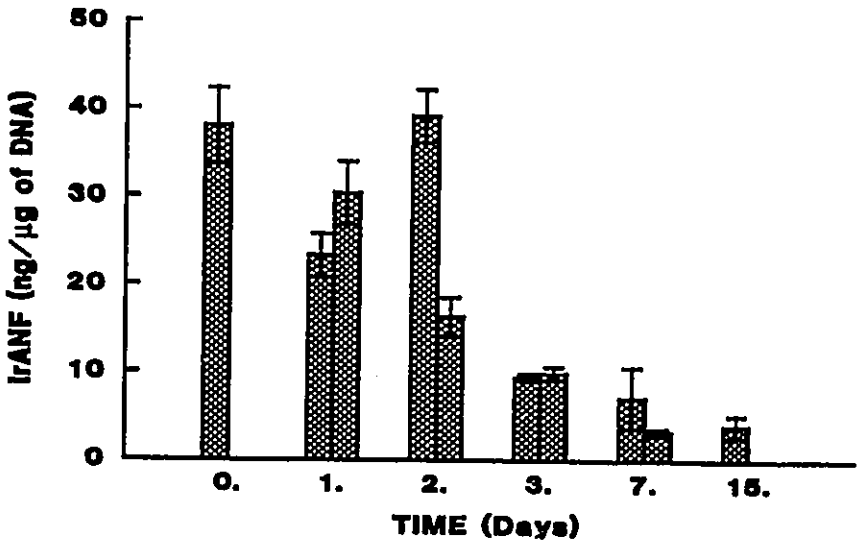
Immunoreactive cell content of ANF was also measured at different times in culture and was found to follow a trend similar to that observed for irANF release. In the freshly isolated cell preparation, irANF content was 38.0 ± 4.5 ng/μg of DNA and decreased about 10 fold over 15 days in culture to 3.9 ± 1.2 ng/μg of DNA (n=6) (Fig. 9b).

FIGURE 9: Effect of time in culture on irANF rate of release (A) and cellular content (B). Cells were superfused as described in Figure 8. The rate of release is the average release for 1 hour. Results are expressed as an average ± SEM of individual experiments, except for day 15 which is an average of 6 experiments ± SEM.

(A)


20
15
10
5
0
IrANF (ng/h/µg of DNA)
0.
1.
2.
3.
7.
15.
TIME (Days)

(B)


50
40
30
20
10
0
IrANF (ng/µg of DNA)
0.
1.
2.
3.
7.
15.
TIME (Days)

2b. ANF post-translational processing by atrial cardiocytes at different times in culture:

The percentage of low molecular weight irANF peptide to the total of the low and high molecular weight irANF, as characterized by RP-HPLC, was used as an index for ANF post-translational processing activity. Superfusates collected from freshly isolated preparations contained mostly irANF eluting in the same region as ANF (99-126) peptide (Fig. 10b) indicating that freshly isolated atrial cardiocytes released irANF with a molecular form chromatographically indistinguishable from the circulating form of ANF. Freshly isolated cardiocytes were also found to store irANF eluting in the same region as ANF (1-126) (Fig. 10a), indicating that, as in intact tissue, ANF is stored as a high molecular weight peptide. With increasing time in culture, the amount of high molecular weight peptide released was found to increase (in percentage) (Fig. 10), but the percentage of low molecular weight peptide to the total irANF remained at levels greater than 59% of the activity measured with freshly isolated cardiocytes. After 15 days in culture, ANF post-translational processing was greater than 60% (Fig. 10d). The complete time course for changes in ANF post-translational processing with increasing time in culture is summarized in Figure 11.

FIGURE 10: Reverse phase elution profile of irANF on Vydac C-18 column (gradient from 15% to 55% of 80% ACN in 0.1% TFA). (A), Cell extract from freshly isolated atrial cardiocytes, showing that ANF is stored exclusively as a low molecular weight peptide. (B) irANF released from freshly isolated atrial cardiocytes, showing that ANF is released from the cells as a low molecular weight peptide. (C) irANF released from atrial cardiocytes after 7 days in culture. (D) irANF released from 15 atrial cardiocytes after 15 days in culture, and shows that, in culture, ANF is released mainly as a low molecular weight peptide. Column was calibrated with synthetic ANF (99-126) (fraction 15-18) and acetic acid/ HCl atrial tissue extract (fraction 29-33).

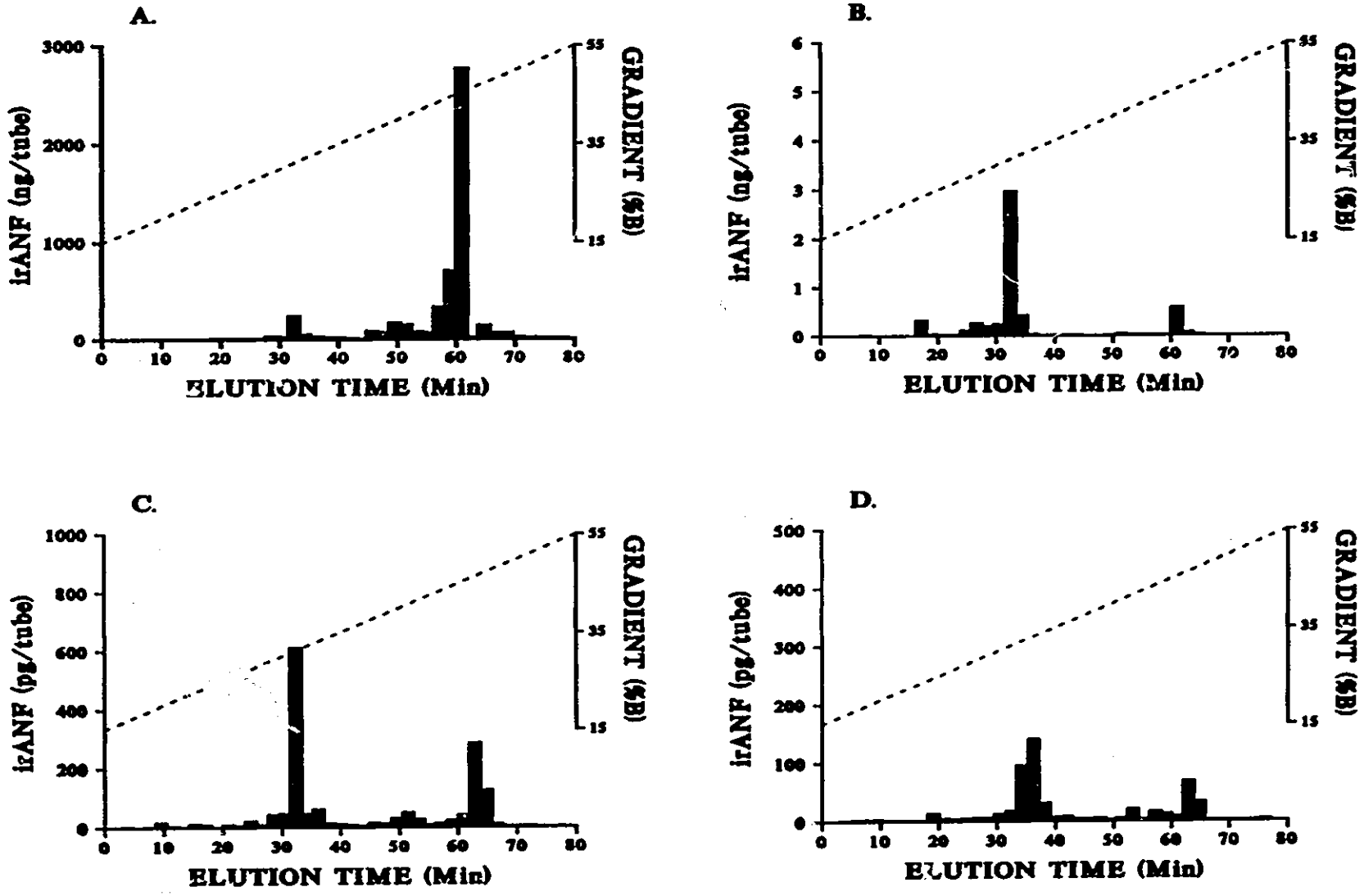
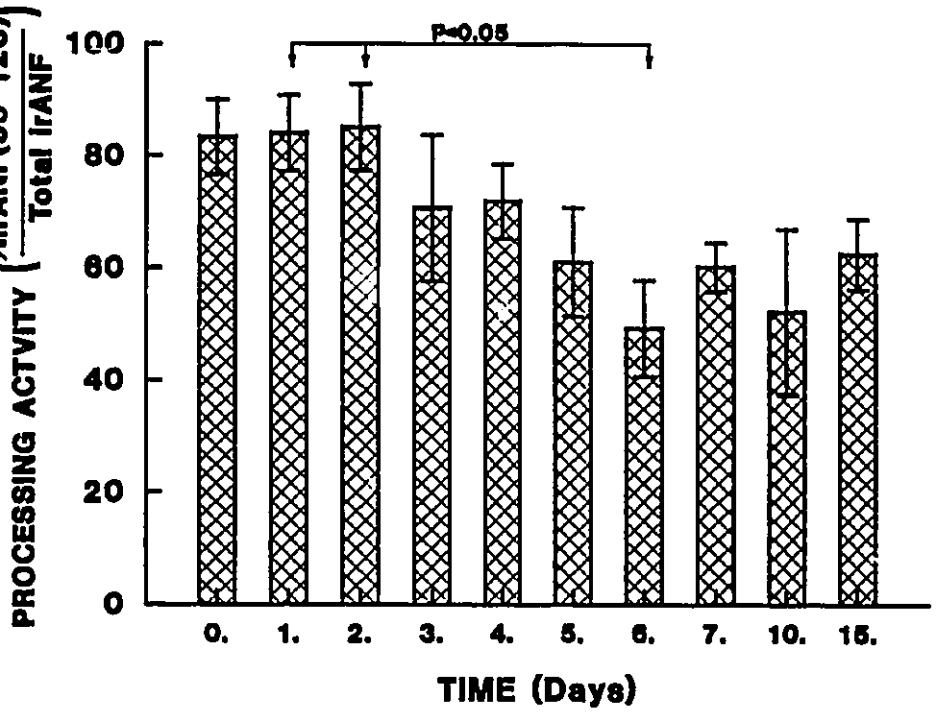

A.
irANF (ng/tube)
3000
2000
1000
0
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
55
35
15
GRADIENT (%B)
B.
irANF (ng/tube)
6
5
4
3
2
1
0
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
55
35
15
GRADIENT (%B)
C.
irANF (pg/tube)
1000
800
600
400
200
0
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
55
35
15
GRADIENT (%B)
D.
irANF (pg/tube)
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
55
35
15
GRADIENT (%B)

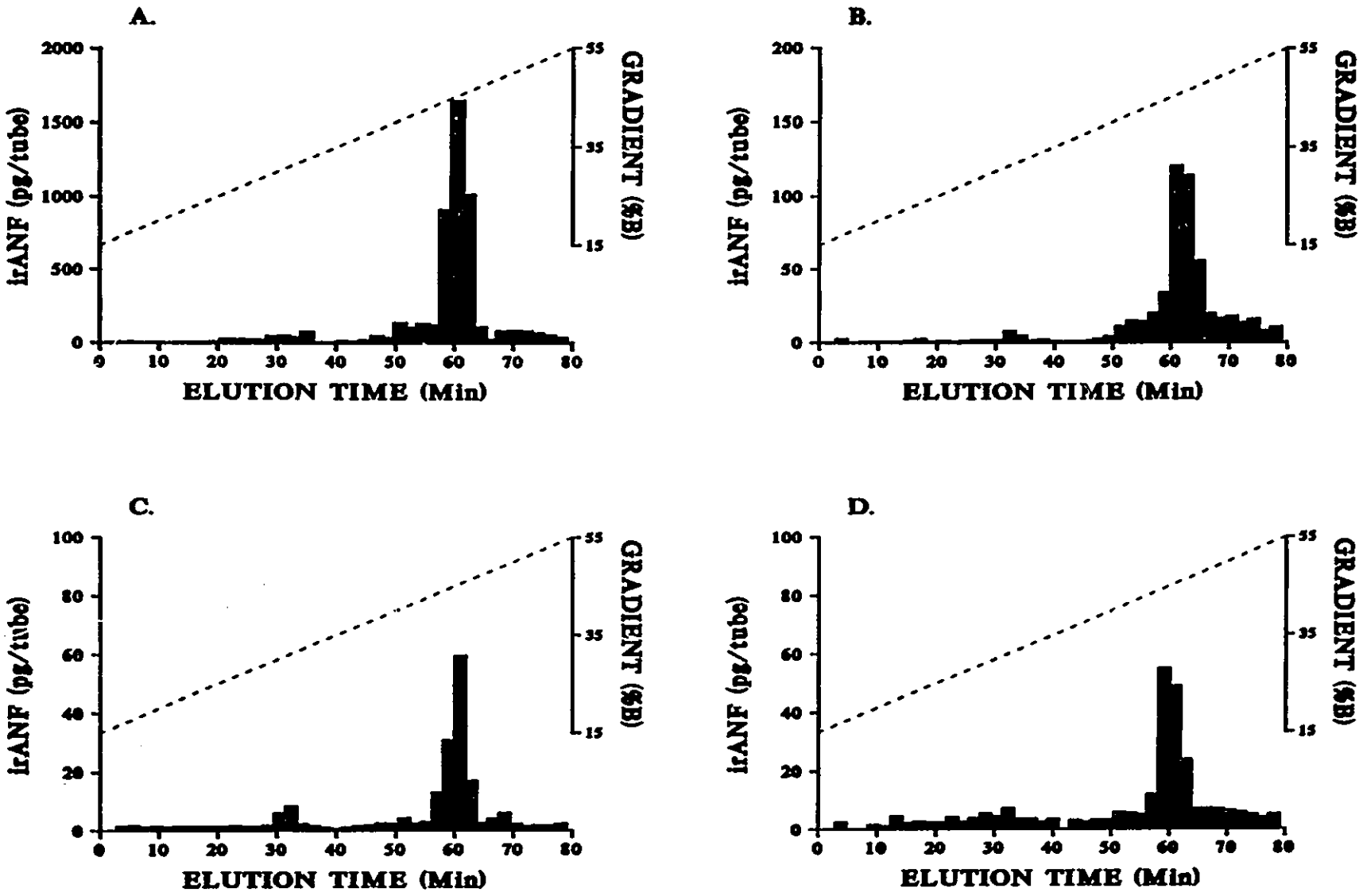
FIGURE 11: Effect of time in culture upon irANF post-translational processing by primary cultures of adult rat atrial cardiocytes. The processing activity is expressed as a percentage of the low molecular weight peptide to the total low and high molecular weight irANF (mean ± SEM; statistical analysis by ANOVA).


PROCESSING ACTVITY (%irANF(99-126) / Total irANF)
100
80
60
40
20
0
P<0.05
0.
1.
2.
3.
4.
5.
6.
7.
10.
15.
TIME (Days)

2c. Processing of exogenous proANF by adult rat atrial non-cardiocytes in cultures on micro-carrier beads:

Direct assessment of the ability of non-cardiocytes to cleave the ANF precursor to its circulating form was carried out using atrial non-cardiocytes, cultured on micro-carrier beads and superfused with exogenous proANF (extracted from a rat atrium and purified by RP-HPLC as described in material and methods). The RP-HPLC elution profiles of superfusates after passage through the cultured non-cardiocytes, for any of the 3 concentrations used (Fig. 12a, b, c), were identical to the superfusate after passage through the column containing only micro-carrier beads (Fig 12d), that is, the entire irANF was found to elute in the region of proANF.

FIGURE 12: Immunoreactive ANF reverse phase elution profiles of different concentrations of exogenous proANF using a Vydac C-18 column (gradient from 15% to 55% of 80% ACN in 0.1% TFA). (A) exogenous proANF (3500 pg/mL), (B) exogenous proANF (550 pg/mL), (C) exogenous proANF (125 pg/mL) after passage through a column containing cultured non-cardiocytes at a rate of 0.5 mL/min, and (D) control exogenous proANF (125 pg/mL) after passage in a column containing only Cytodex III™. The four graphs show that, except for artifactual degradation, proANF remained intact after its passage through the different columns.


A.
irANF (pg/tube)
2000
1500
1000
500
0
55
35
15
GRADIENT (%B)
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
B.
irANF (pg/tube)
200
150
100
50
0
55
35
15
GRADIENT (%B)
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
C.
irANF (pg/tube)
100
80
60
40
20
0
55
35
15
GRADIENT (%B)
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)
D.
irANF (pg/tube)
100
80
60
40
20
0
55
35
15
GRADIENT (%B)
0 10 20 30 40 50 60 70 80
ELUTION TIME (Min)

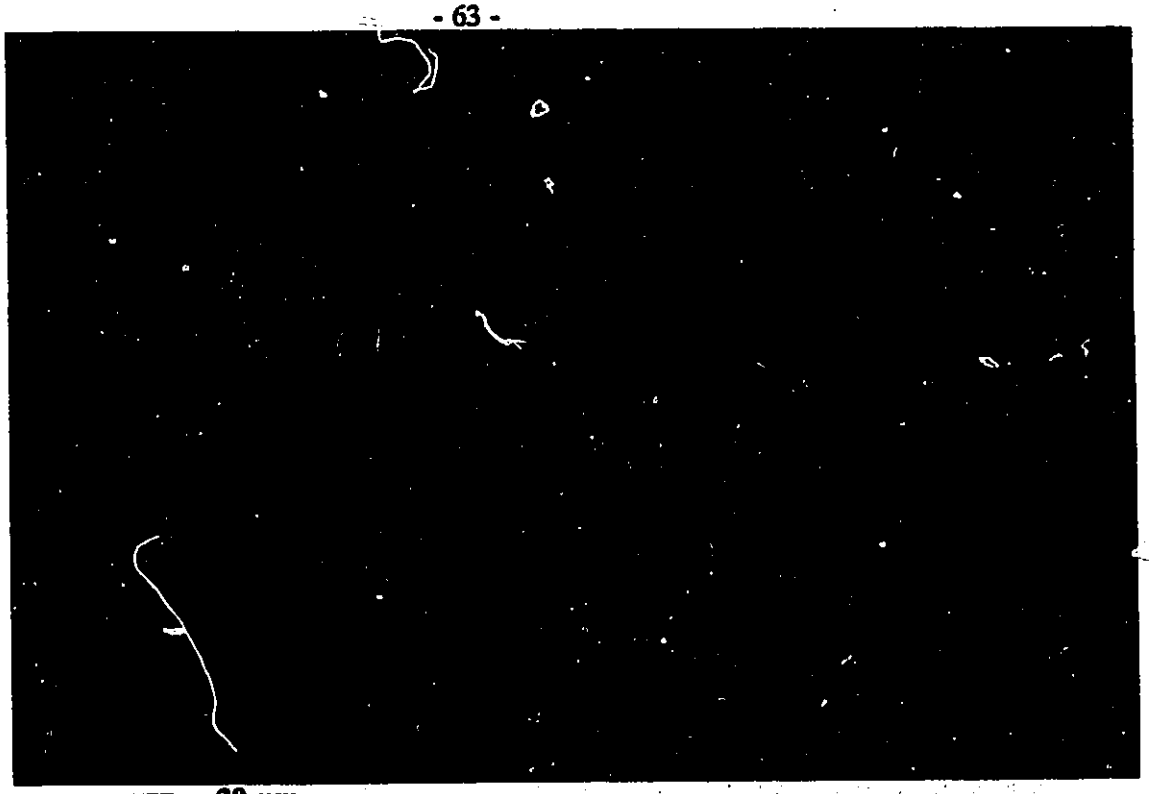
3) IMMUNOFLUORESCENCE MICROSCOPICAL ANALYSIS OF ADULT RAT ATRIAL CARDIOCYTES IN CULTURE:

3a. Distribution of desmin immunofluorescence:

Freshly isolated cylindrical cardiocytes displayed desmin immunofluorescence (desmin-if) closely associated with the Z-band as observed in intact tissue (Fig. 13a, b). This pattern was gradually lost as the cells assumed a spherical shape. No specific arrangement could be observed in rounded-up cells. Spherical cardiocytes displayed, nonetheless, desmin-if which when observed at different focal planes, appeared to be distributed randomly, although a clear assessment of the intracellular structure was not possible due to an overwhelming background fluorescence. During attachment, which occurred between the third and fourth days of culture, desmin-if exhibited a meshwork distribution observed within the cytoplasmic elongations while the middle of the cells was often overwhelmingly fluorescent (Fig. 13c). The atrophying original cell body, still attached to the flattening cell, remained overwhelmingly fluorescent and no specific arrangement could be observed. The distribution of desmin-if remained the same with increasing time in culture (Fig. 13d, e and Fig. 14c). Controls with omission of the first antibody (Fig. 13f) or omission of the second antibody displayed no specific staining. The specificity of the desmin antibody was also assessed on confluent cultures of non-cardiocyte atrial cells obtained after 1 passage of the cells resulting from the pre-plating. No specific immunofluorescence was observed (Fig. 13g, h).

FIGURE 13: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for desmin. (A) Atrial tissue section (stained with texas red). (B) Freshly isolated atrial cardiocytes (stained with texas red) showing that desmin appears associated with the Z-bands as in tissue. (C), (D), (E), Atrial cardiocytes in culture for 4, 7 and 15 days respectively showing that desmin is distributed all over the cells as a meshwork and does not form a striated patern as observed with freshly isolated cells. (F), Atrial cardiocytes: control without the primary antibody showing no staining. (G), (H), Non-cardiocyte cells in confluent culture, showing no specific staining.

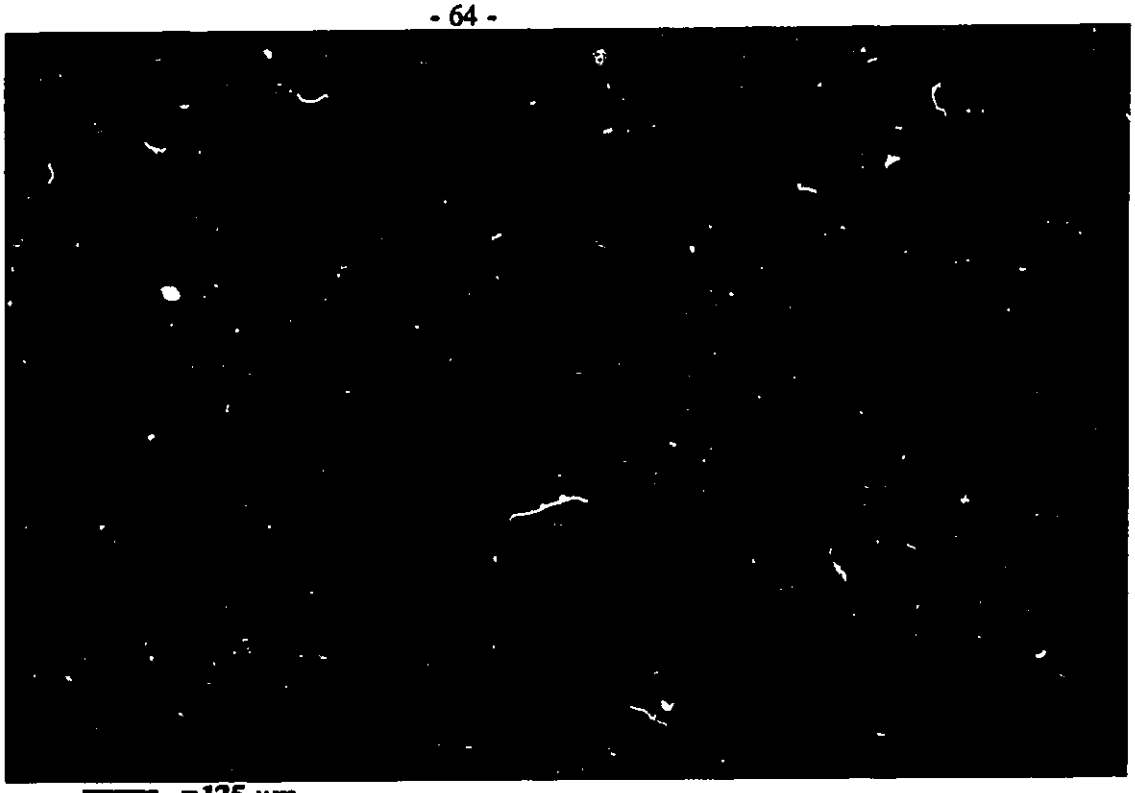
(13-A)



(13-B)

FIGURE 13: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for desmin. (A) Atrial tissue section (stained with texas red). (B) Freshly isolated atrial cardiocytes (stained with texas red) showing that desmin appears associated with the Z-bands as in tissue. (C), (D), (E), Atrial cardiocytes in culture for 4, 7 and 15 days respectively showing that desmin is distributed all over the cells as a meshwork and does not form a striated patern as observed with freshly isolated cells. (F), Atrial cardiocytes: control without the primary antibody showing no staining. (G), (H), Non-cardiocyte cells in confluent culture, showing no specific staining.

(13-C)



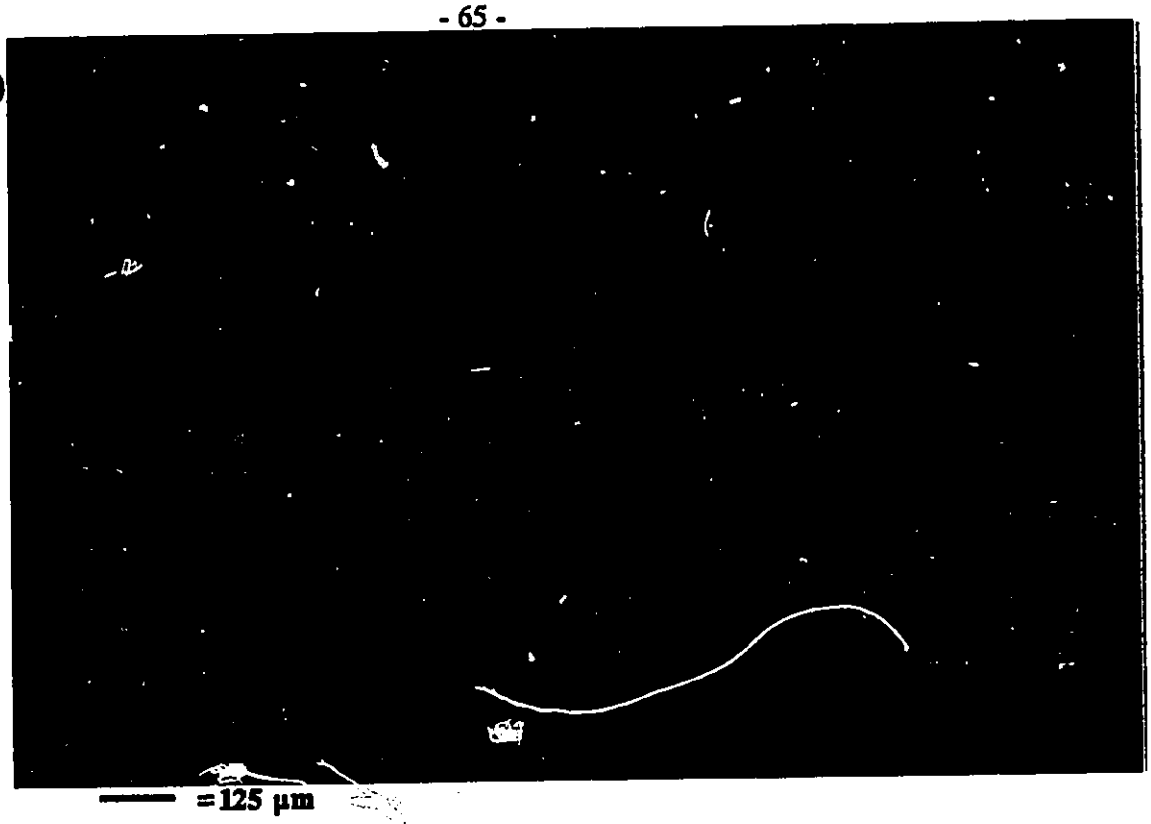
=125 µm

(13-D)

=125 µm

FIGURE 13: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for desmin. (A) Atrial tissue section (stained with texas red). (B) Freshly isolated atrial cardiocytes (stained with texas red) showing that desmin appears associated with the Z-bands as in tissue. (C), (D), (E), Atrial cardiocytes in culture for 4, 7 and 15 days respectively showing that desmin is distributed all over the cells as a meshwork and does not form a striated patern as observed with freshly isolated cells. (F), Atrial cardiocytes: control without the primary antibody showing no staining. (G), (H), Non-cardiocyte cells in confluent culture, showing no specific staining.

(13-E)



(13-F)

(13-G)

(13-H)

FIGURE 14: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for myosin. (A), (B), Atrial cardiocytes in culture for 7 and 15 days respectively showing well developed sarcomeres. (C) Same cell as (A) stained with desmin and (D) doubled stained with desmin and myosin showing different distributions. Desmin was not associated with Z-bands, even after 15 days in culture.

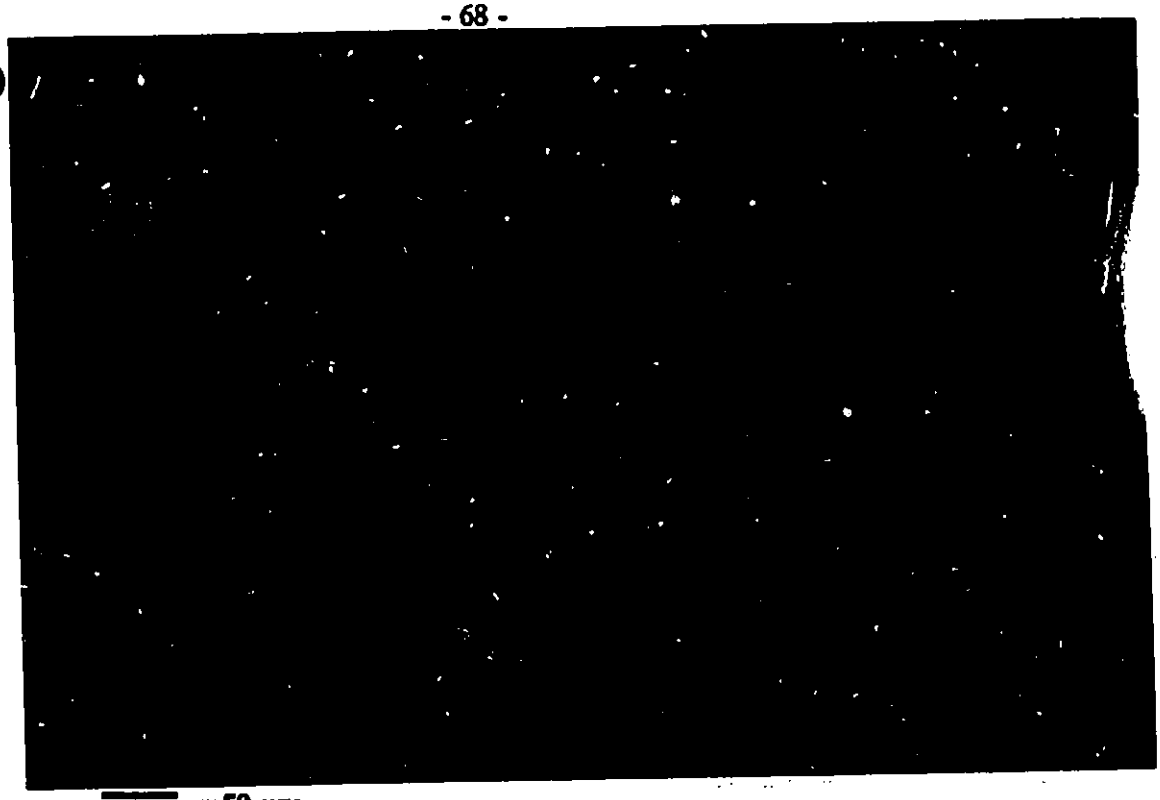
The presence of well developed sarcomeric structures in cultured atrial cardiocytes was examined by the distribution of myosin-if. While no specific staining was observed in 4 day old cultures (data not shown), abundant sarcomeric structures were seen in 7 and 15 day old cultures (Fig. 14a, b), but dual immunofluorescence staining with desmin and myosin in these cultures showed no apparent association of desmin-if with Z-bands (Fig. 14c, d).

(14-A)

—— =50 μm

FIGURE 14: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for myosin. (A), (B), Atrial cardiocytes in culture for 7 and 15 days respectively showing well developed sarcomeres. (C) Same cell as (A) stained with desmin and (D) doubled stained with desmin and myosin showing different distributions. Desmin was not associated with Z-bands, even after 15 days in culture.

(14-B)



=50 μm

(14-C)

=50 μm

FIGURE 14: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for myosin. (A), (B), Atrial cardiocytes in culture for 7 and 15 days respectively showing well developed sarcomeres. (C) Same cell as (A) stained with desmin and (D) doubled stained with desmin and myosin showing different distributions. Desmin was not associated with Z-bands, even after 15 days in culture.

(14-D)

=50 µm

3b. Distribution of ANF immunofluorescence:

Freshly isolated, cylindrical atrial cardiocytes displayed ANF-if mostly perinuclearly as observed in atrial tissue sections (Fig. 15a, b) although not as well defined as the latter. With the loss of the cylindrical morphology, ANF was found randomly distributed within the cell. In spherical cardiocytes, as observed in 1 day old cultures, the ANF-if was uniform throughout the cells even when observed at different focal planes (Fig. 15c, d). Again, the overwhelming background fluorescence restricted a clear assessment of the ANF distribution at this stage in culture. During cell attachment (4 days after isolation) ANF-if was localized mainly in the atrophying cell oody (overlying the cytoplasmic elongations) (Fig 15e, f). In cells where nuclei were visible, ANF appeared to be localized mainly perinuclearly. The difference in distribution of ANF-if between the perinuclear zone and the periphery appeared to increase with increasing time in culture. After 7 days in culture, cardiocytes were fully spread-out and ANF-if was distributed in the perinuclear zone clearly outlining the nuclei (Fig. 15g, h). This distribution remained unchanged in older cultures but the intensity of the fluorescence declined with time in culture (Fig. 15i, j). Controls with pre-absorbed primary antiserum (Fig. 15k) or omission of first or second antibodies, or omission of the texas red showed no specific labelling of the cardiocytes in culture. Occasionally, nuclei appeared non-specifically stained in tests and in controls with pre-absorbed first antibody, but not with other controls. Cardiac non-myocytic cells in confluent cultures also displayed some labelling of the nuclei but no staining was observed in the cytoplasm of the cells (Fig. 15l, m).

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-A)

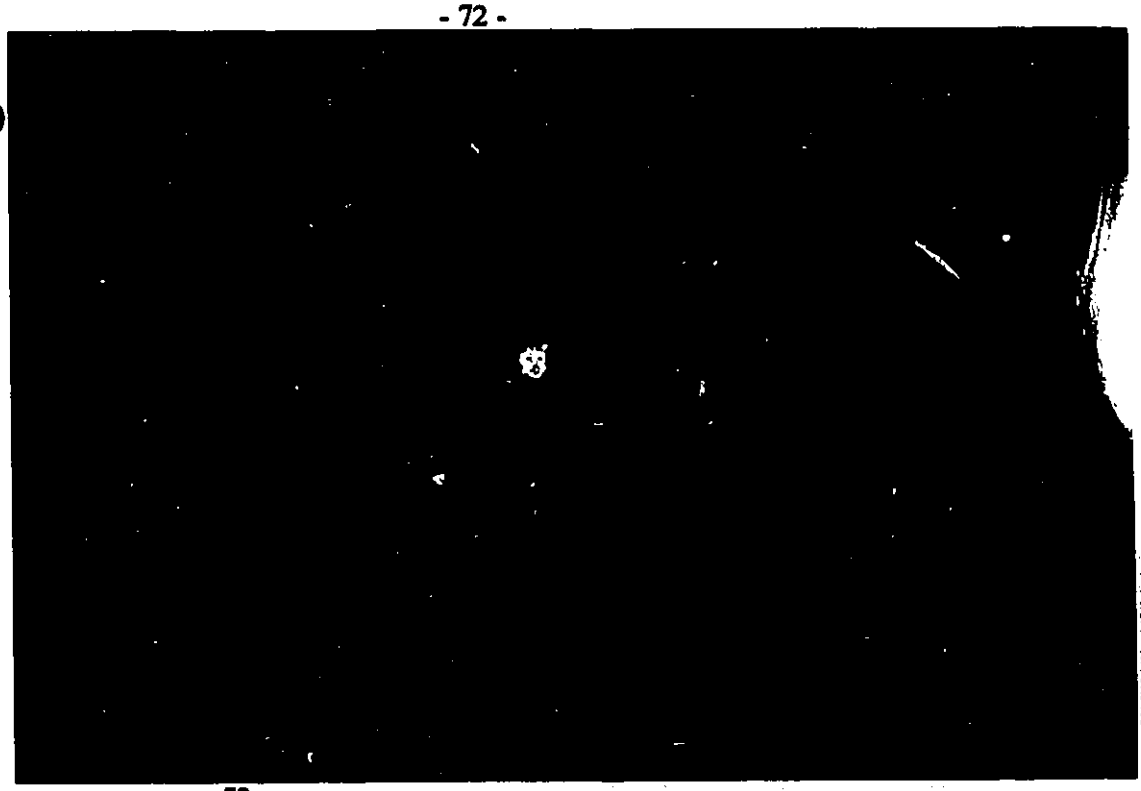
=50 μm

(15-B)

=50 μm

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-C)



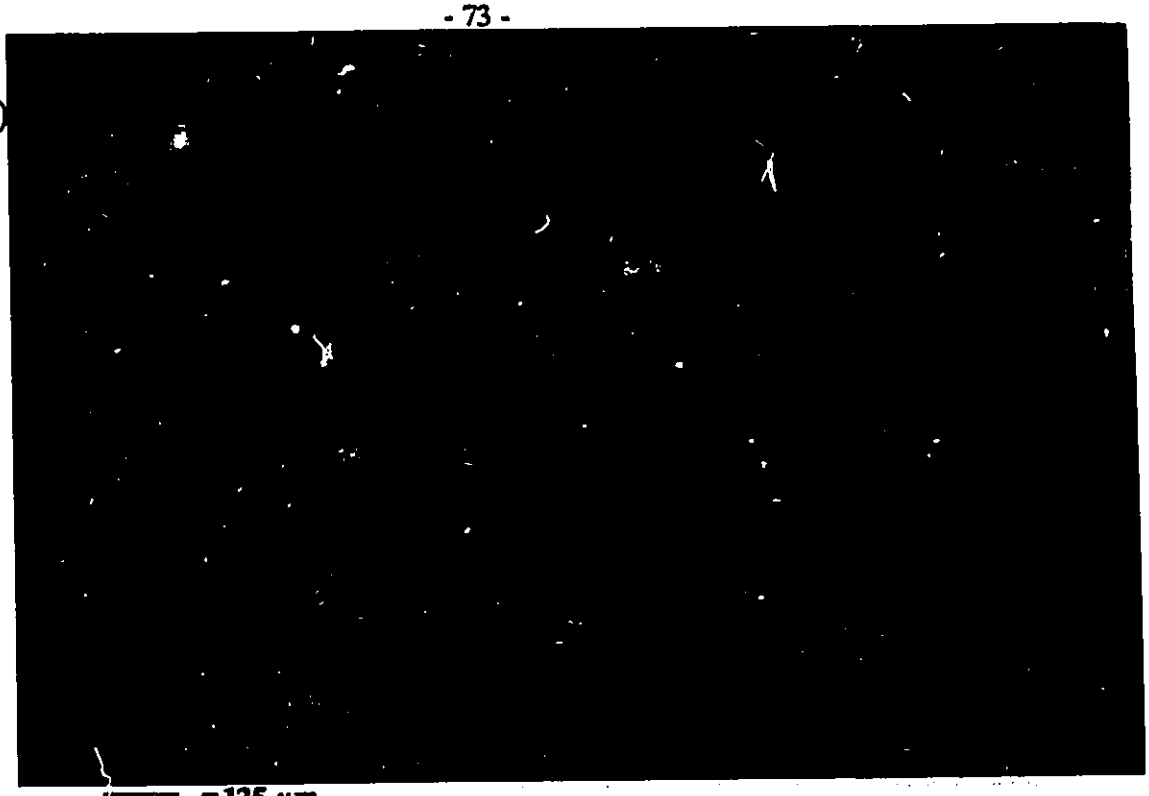
——— =50 μm

(15-D)

——— =50 μm

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-E)



=125 µm

(15-F)

=50 µm

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-G)

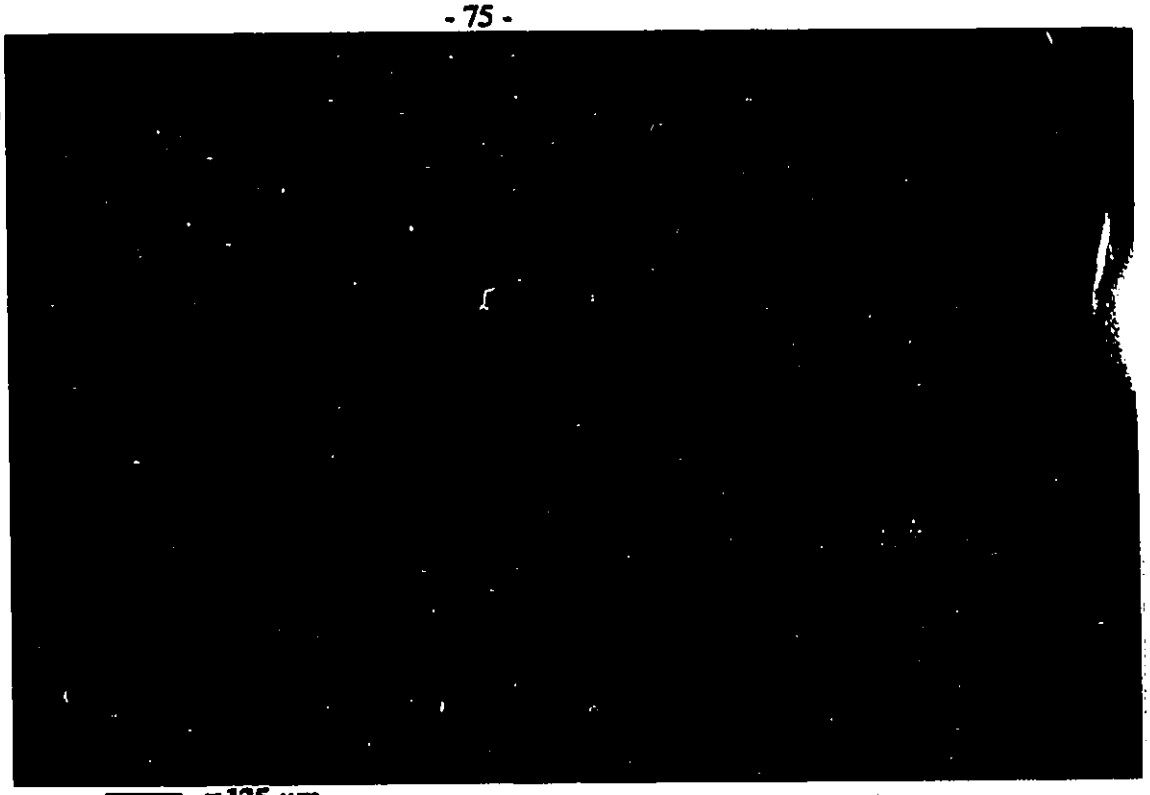
——— =125 µm

(15-H)

——— =20 µm

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-I)



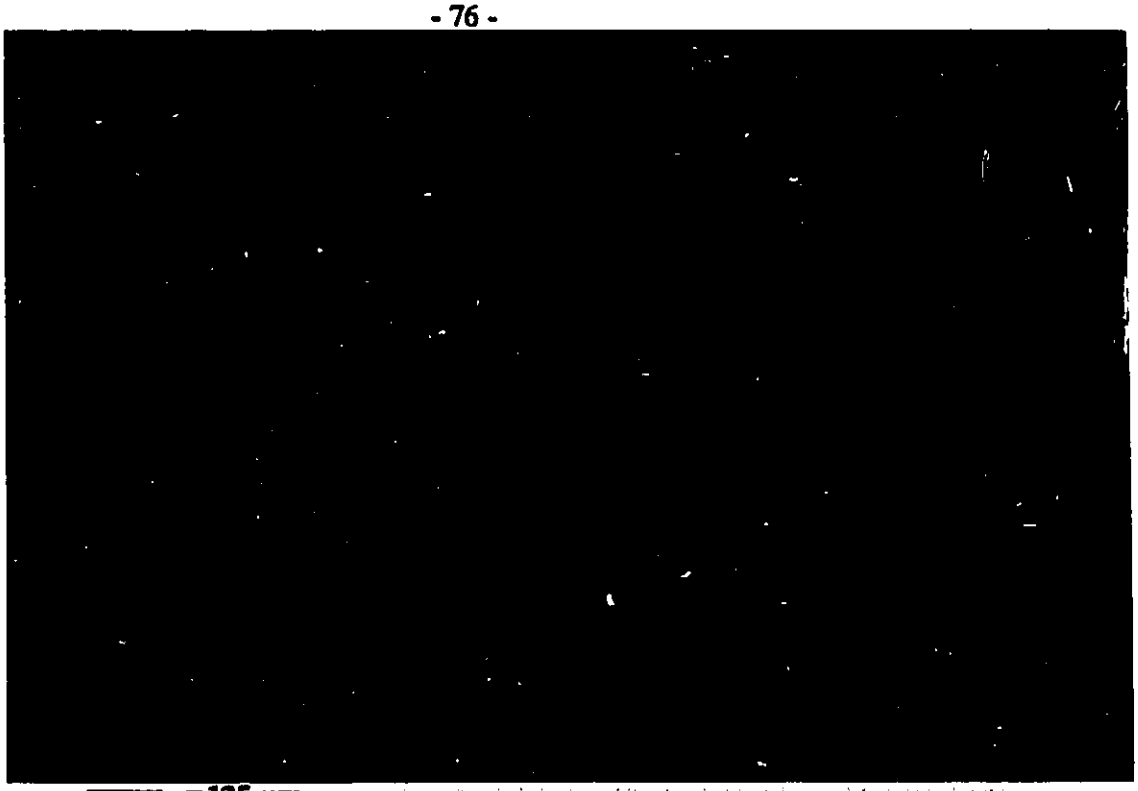
——— =125 µm

(15-J)

——— =50 µm

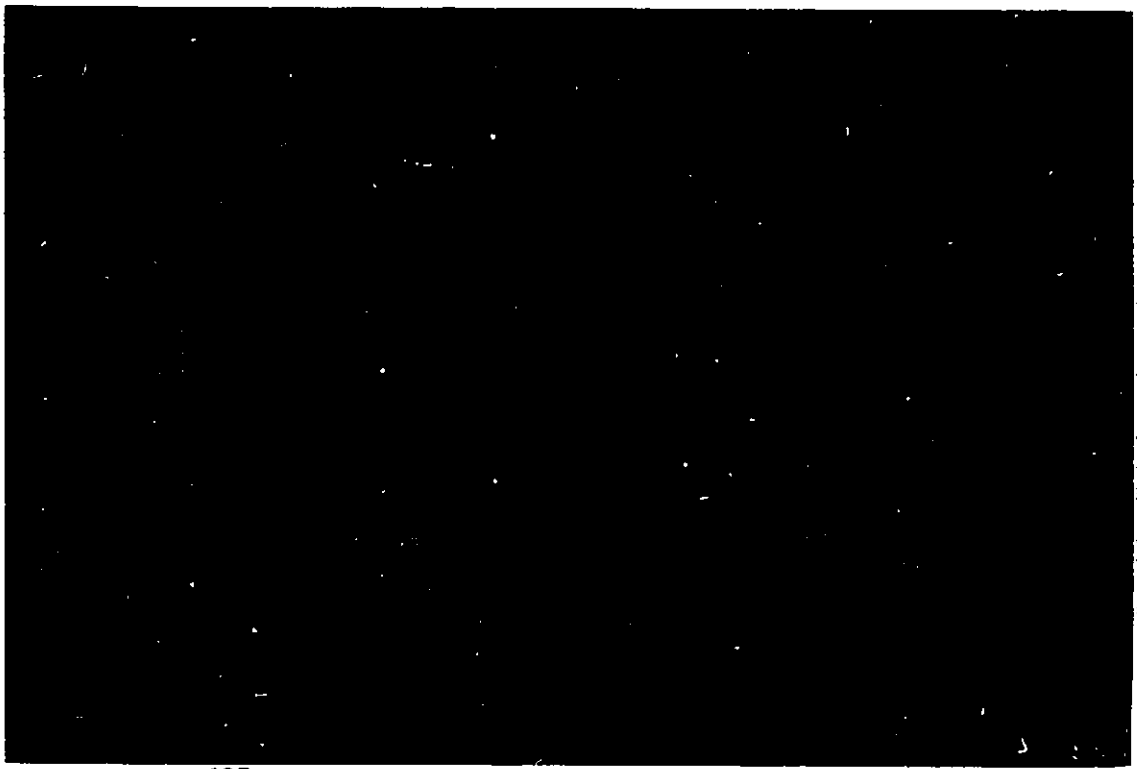
FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-K)



——— =125 µm

(15-L)



——— =125 µm

FIGURE 15: Immunofluorescence micrograph of primary cultures of adult rat atrial cardiocytes stained for ANF. (A) Atrial tissue section. (B) Freshly isolated atrial cardiocytes. (C), (D), Different foci of an atrial cardiocytes after 1 day in culture (E), (F) Atrial cardiocytes in culture for 4 days in culture at different magnification and showing the cell body on top of the spreading cardiocytes (F). (G), (H), Atrial cardiocytes after 7 days in culture at different magnification and showing the perinuclear distribution of ANF (H). (I), (J), Atrial cardiocytes after 15 days in culture at different magnification. (K) atrial cardiocytes after 4 days in culture: control with preabsorbed first antiserum. (L), (M) Non-cardiocyte cells in confluent cultures at different magnification.

(15-M)

=50 µm

FIGURE 16: Fluorescent micrograph of primary cultures of atrial cardiocytes after 7 days in flat culture, stained for desmin (A) and nuclei (B). (C) High magnification micrograph of atrial cardiocytes stained for DNA and showing cells of different sizes.

4) ESTIMATION OF HETEROGENEITY OF THE CELL POPULATION IN CULTURE

4a. Estimation of cell population in flat cultures of atrial cardiocytes:

Evaluation of the cell population heterogeneity was carried-out on 5, 6, 7, 10 and 15 day old cultures of atrial cardiocytes. Five days after dissociation of the cardiocytes, cultures were found to bear many non-cardiocytes (Fig. 16). The majority of these cells appeared to be clustered in colonies in close proximity to cardiocytes often between their cell processes. The number of non-cardiocyte cells appeared to decrease with increased time in culture such that at 15 days their numbers were significantly reduced. This decrease resulted from a decline in the size of the colonies. To better characterize the effect of time in culture on the cell population, the average content of cardiocytes and non-cardiocytes in culture at different days was determined (Fig. 16a, b).

(16-A)

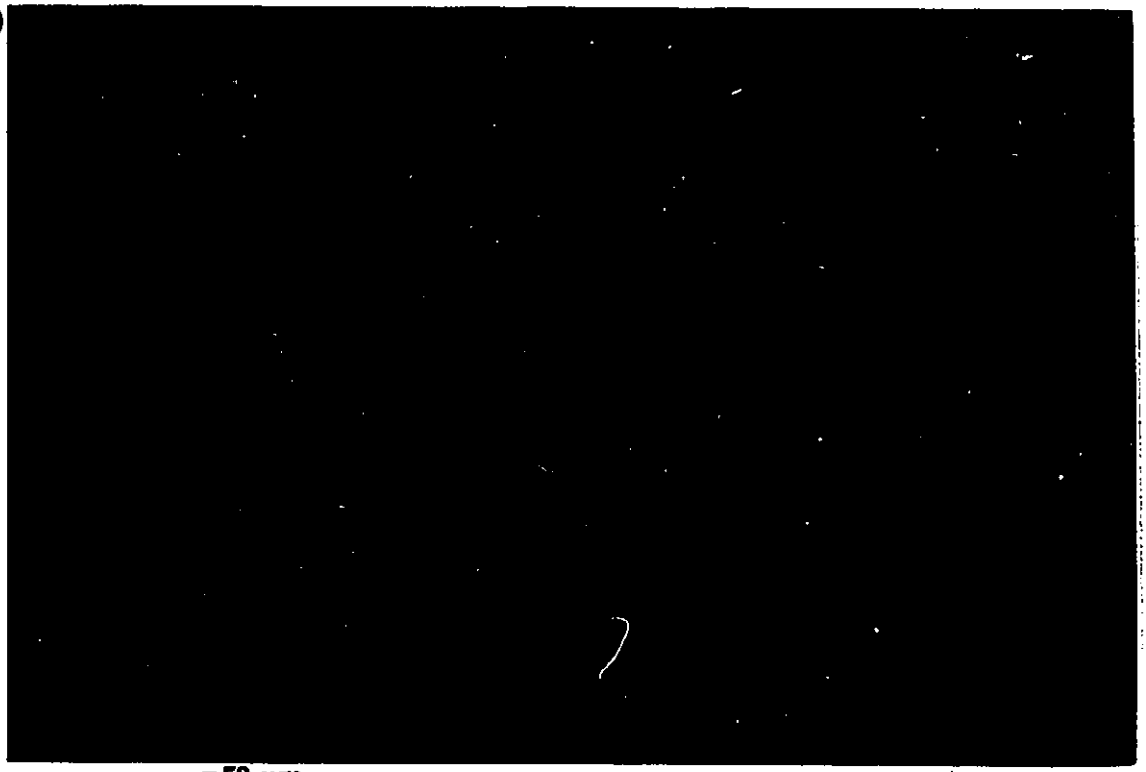
=125 µm

FIGURE 16: Fluorescent micrograph of primary cultures of atrial cardiocytes after 7 days in flat culture, stained for desmin (A) and nuclei (B). (C) High magnification micrograph of atrial cardiocytes stained for DNA and showing cells of different sizes.

(16-B)

——— =125 µm

(16-C)



——— =50 µm

Results, compiled in Table 2, show that the number of contaminating non-cardiocytes was effectively reduced between the 5th and 15th day in culture and that the ratio of the number of cardiocytes to the total number of cells increased 4 fold during that period (Fig. 17a). However, the ratio of cardiocytes to the total number of colonies (Fig. 17b) remained constant between the fifth and tenth and increased only between day 10 and 15. There was no statistical difference between the ratio of the number of cardiocytes to the total number of cells or to the number of colonies at 15 days.

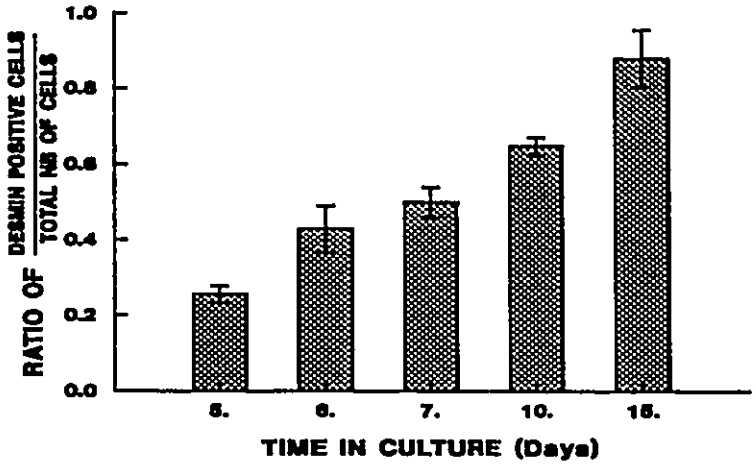
TABLE 2: Effect of time on cell population heterogeneity in primary cultures of adult rat atrial cardiocytes maintained in 20% HI-FBS serum after a 24 h pre-plating and a 7 day treatment with the anti-mitotic agent ARA-C (10 µg/mL).

days in culture	n	total # of cells	# of cardiocytes	ratio # of cardiocytes/ total # of cells	# of colonies	ratio # of cardiocytes/ # of colonies
5	7	124 ± 14	31 ± 4	0.26 ± 0.02	44 ± 5	0.69 ± 0.03
6	8	71 ± 11	27 ± 2	0.43 ± 0.06	39 ± 3	0.69 ± 0.03
7	6	35 ± 3	17 ± 1	0.50 ± 0.04	27 ± 2	0.67 ± 0.03
10	5	30 ± 2	20 ± 1	0.65 ± 0.02	26 ± 2	0.75 ± 0.02
15	5	10 ± 2	8 ± 1	0.88 ± 0.08	9 ± 2	0.91 ± 0.05

(mean ± SEM)
(n=number of fields counted pooled from two separated experiments)

FIGURE 17: **Heterogeneity of the cell population in primary cultures of adult rat atrial cardiocytes maintained in 20% HI-FBS supplemented medium after a 24 h pre-plating period and ARA-C treatment for the first 7 days in culture. A) Ratio of desmin positive cells to the total number of cells revealed with H-33258, and B) Ratio of desmin positive cells to the total number of colonies.**

(A)


RATIO OF DESMIN POSITIVE CELLS / TOTAL NB OF CELLS
1.0
0.8
0.6
0.4
0.2
0.0
5.
6.
7.
10.
15.
TIME IN CULTURE (Days)

(B)

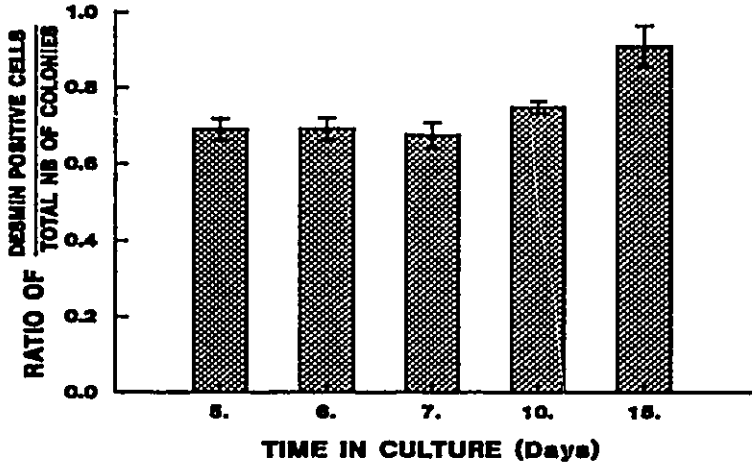
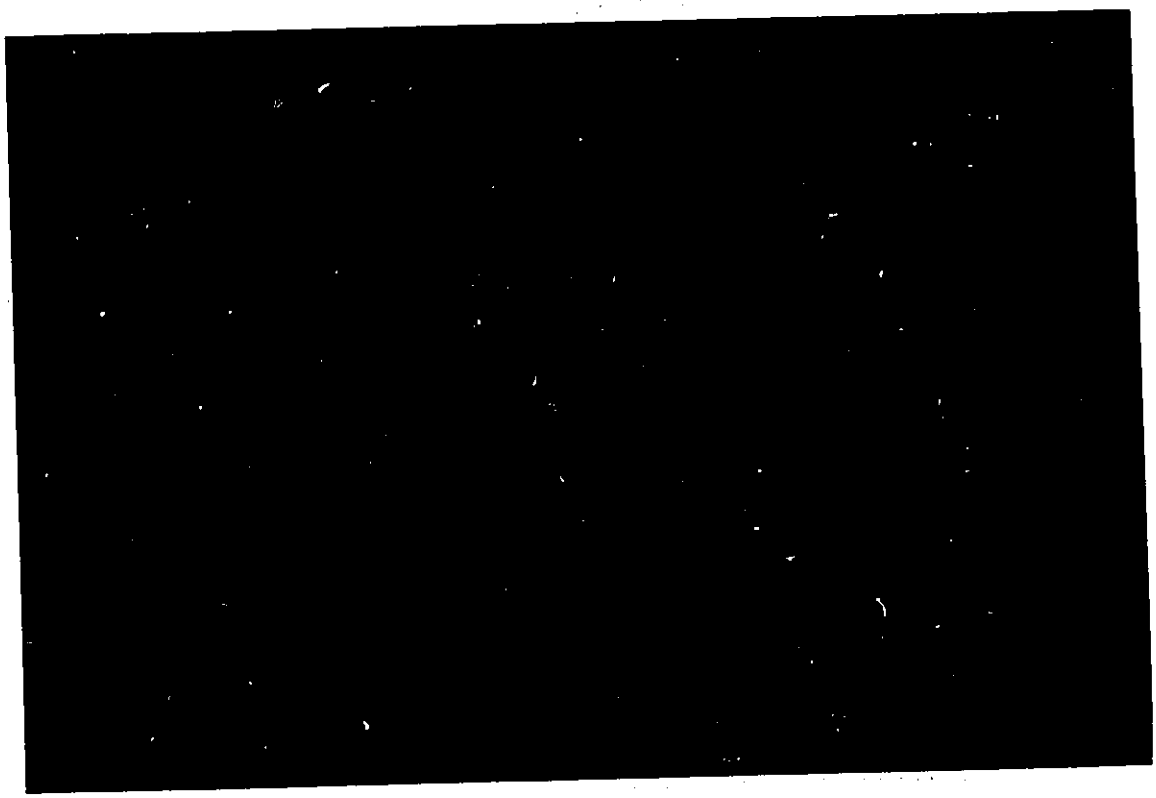

RATIO OF DESMIN POSITIVE CELLS / TOTAL NB OF COLONIES
1.0
0.8
0.6
0.4
0.2
0.0
5.
6.
7.
10.
15.
TIME IN CULTURE (Days)

FIGURE 18: Fluorescence micrograph of atrial cardiocytes after 7 days in cultures on micro-carrier beads, detached by collagenase digestion and stained for desmin (A) and nuclei (B).

4b. Estimation of the cell population in cultures of atrial cardiocytes attached to micro-carrier beads:

Heterogeneity of the cell population, and ANF release and processing activity in micro-carrier cultures was evaluated after 7 and 15 days in culture. Table 5 shows the results on ANF processing activity obtained, which are similar to those previously described in Figure 11. Dissociated cardiocytes appeared damaged by the collagenase digestion and no specific desmin arrangement was observed (Fig. 18a, b). Nuclei were often found at the surface of the cells and some cardiocytes did not display any DNA staining.

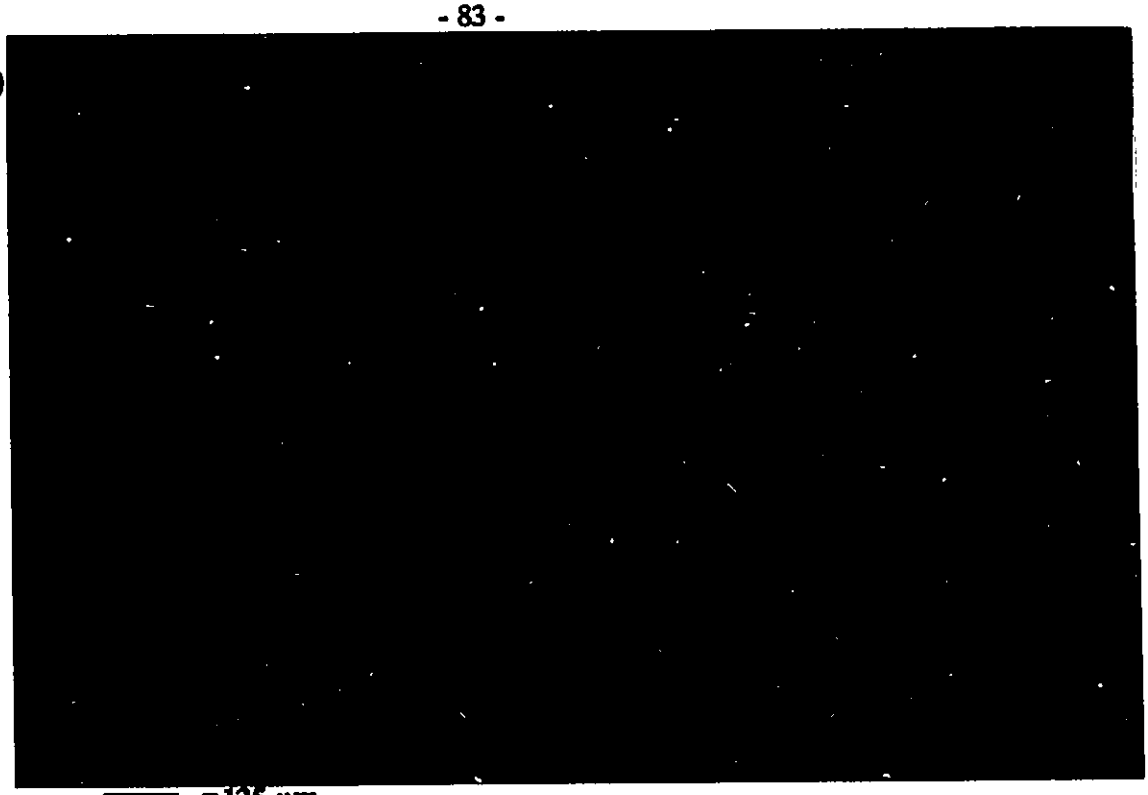
(18-A)



—— =125 µm

FIGURE 18: Fluorescence micrograph of atrial cardiocytes after 7 days in cultures on micro-carrier beads, detached by collagenase digestion and stained for desmin (A) and nuclei (B).

(18-B)



—— =125 µm

The cell population in these preparations was analyzed as in the previous section, and expressed as a percentage of the number of cardiocytes to the total number of cells (nuclei). The results of 4 individual experiments are presented along with their respective processing activities (Table 5). The percentages of cardiocytes in each preparation from the same day were similar and an increase in their number, from 54% (average of the two 7 days experiments) to 82% (average of the two 15 days experiments), was observed reflecting the measured percentages found in flat culture experiments (50% to 88%; Table 2, Section 4a.). This increase in the percentage of atrial cardiocytes to the total number of cells was not accompanied by similar changes in ANF post-translational processing and no correlation was found between the 2 set of results.

TABLE 5: Effect of time on cell population heterogeneity in primary cultures of adult rat atrial cardiocytes cultured on micro-carrier beads and maintained in 20% HI-FBS after 24h of pre-plating and treated for 7 days with the anti-mitotic agent ARA-C (10 µg/mL; to restrict cell division).

Days in culture	# of cardiocytes/ total # of cells (%)	processing activity (% irANF(99-126)/total irANF)
7	53 ± 4 (n=12)	70.5
7	55 ± 4 (n=12)	42.1
15	82 ± 4 (n=9)	47.3
15	82 ± 6 (n=7)	70.8

(mean ± SEM)
(n=number of fields counted)

DISCUSSION:

1) ESTABLISHMENT OF A CELL CULTURE PROCEDURE FOR ADULT RAT ATRIAL CARDIOCYTES

The aim of this study was to obtain insights into the mechanism of ANF post-translational processing. For this purpose, the molecular forms of ANF released by adult rat atrial cardiocytes from the moment of isolation and in culture for up to 15 days was characterized. The ratio of the low molecular weight peptide over the total ANF immunoreactivity released was used as an index for the endogenous post-translational processing activity. A microscopical characterization of the cultures was also carried out in an attempt to correlate changes in cell morphology and changes in cell population to the degree of post-translational processing activity measured. Cardiocytes were isolated from adult rather than neonatal rat atrial tissue since the adult cells are believed to be a better *in vitro* representation of the mature mammalian heart (Bugaisky and Zak, 1989). Cantin and collaborators (1985) found substantial differences between newborn and adult rat atrial cardiocytes in terms of ANF content and release. In cultures of neonatal rat atrial cardiocytes, the amount of ANF stored and released was found to be significantly different between cells derived from either right or left atria. Cardiocytes derived from right or left adult rat atria were found to store and secrete equal amounts of ANF, although after 12 days in culture some differences in the amount of stored ANF were found. Therefore, by using adult rat atrial tissue to establish primary cultures of cardiocytes, both right and left atria can be used together. In addition, restriction of non-cardiocyte growth is easier in primary cultures of adult rat cardiocytes, which do not undergo cell division and do not attach as rapidly as cardiocytes derived from neonatal rats do. Cantin et al. (1985) also

reported changes in the kinetics of ANF release correlating with the degree of cell confluency in cultures of newborn rat cardiocytes, a problem which is avoided by using cells derived from adult rats, since the adult cardiocytes are unable of hyperplasia.

Numerous procedures for isolating adult rat cardiocytes from atrial or ventricular tissue have been published over the past 10 years. Most of these procedures use enzymes in a retrograde perfusion system (for review of techniques see: S.L. Jacobson, 1989; Jacobson and Piper, 1986). Retrograde perfusion, however, was not the most efficient method in this study for several reasons: a) Investigation of ANF release and processing requires the isolation of a large number of atrial cells (20 atria for a set of 2 experiments), since release decreases profoundly with increasing time in culture, and b) with such a system, atrial tissue is not as well perfused as the ventricular tissue. Some procedures have been published in which adult rat cardiocytes were isolated by mechanical dispersion in the presence of enzymes. Most of these protocols have been difficult to reproduce and several modifications had to be made before a reproducible protocol was established. The present protocol was based on a procedure published by Isenberg and Klockner (1982), which was later modified by Iida et al. (1988) and further modified in the present study.

Several factors in the isolation procedure appear critical for the success of the isolation. The use of nominally calcium-free solutions throughout the isolation procedure was necessary for several reasons. Low concentrations of calcium help cell dissociation by disrupting cell-to-cell bonds such as desmosomal and intermediate junctions (Jacobson, 1989). Finally, since calcium is a cofactor to several proteolytic enzymes such as trypsin or collagenase its presence in the dissociation medium results in the disruption of the isolated

cells (Glick et al., 1974). It is noteworthy that calcium is still required for collagenase activity but the concentration of contaminating calcium ($\approx 10^{-5}$M) is sufficient to support this activity (Glick et al., 1974).

The choice of the correct enzyme mixture for isolating adult rat cardiocytes is more critical than for cardiocytes isolated from immature mammalian hearts. Neonatal cardiocytes can be readily isolated following exposure to trypsin. In contrast, in initial experiments in this study and others (Masson-Prevest et al., 1976), isolation of cardiocytes from adult tissue by exposure to trypsin yielded preparations with low viability (about 10%; data not shown). In the present work, the utilisation of collagenase, without the addition of trypsin to the dissociation medium resulted in an increase in cell viability from 10% to over 60% for atrial cardiocytes. This can be explained by the apparent ability of trypsin to penetrate heart cells, thereby causing severe damage to the cellular components (Masson-Prevest et al., 1976). Some studies, using minced adult rat ventricular tissue, have shown beneficial effects of sequential trypsin exposure in digesting collagenase-resistant components of the extracellular matrix during isolation (Jacobson, 1989) but this is not the case for atrial cardiocytes which have been successfully isolated by exposure to both trypsin (Cantin et al., 1981) or collagenase (Nathan, 1986). Finally, the viability of the cell preparation was enhanced by simply coating all glassware with silicone which reduces the interaction of the charges on the glass and the isolated cardiocytes. For the same reason, polypropylene tubes were chosen over polystyrene tubes (Dr J. Kako, personal communication).

Two indexes were chosen to determine the cell viability: trypan blue exclusion and morphological appearance (elongated cells versus round and hypercontracted). Morphological characterization of isolated cardiocytes has been reported to be a more accurate method than dye exclusion, since, in isolated ventricular cell preparations, trypan blue sometimes fails to stain hypercontracted cells, and stains some cylindrical cells (Cheung et al., 1985; Jacobson, 1989; Nag et al., 1983). It was observed in the present study that, unlike freshly isolated ventricular cardiocytes, viable atrial cardiocytes rapidly (minutes to hours) lose their cylindrical appearance to assume a spherical shape, even prior to calcium repletion. Therefore, the application of the morphological viability test on freshly isolated atrial cardiocyte preparations underestimate the number of viable cells. Noteworthy is the absence of high molecular weight ANF in the superfusate from experiments in this study using freshly isolated cells. The presence of proANF in the collected superfusate would have suggested leakage of the stored prohormone from the cardiocytes. This observation provides a good indication of the viability of the cells.

2) CHARACTERIZATION OF CULTURED ATRIAL CARDIOCYTES

Important changes in morphology of adult rat atrial cardiocytes were observed in this study with increasing time in culture. Characterization of these changes was carried out in several ways. Phase contrast microscopy clearly demonstrated important morphological changes induced by isolation and culture of adult rat atrial cardiocytes. As described above, freshly isolated atrial cardiocytes were found to rapidly lose their cylindrical arrangement and typical cross-striations to assume a spherical shape with a concomitant loss of beating activity. These observations are in line with that described by others for adult rat ventricular cardiocytes (Claycomb and Palazzo, 1980; Guo et al., 1986; Jacobson, 1977; Nag et al., 1983)

and for adult rat atrial cardiocytes (Bechem et al., 1983; Cantin et al., 1981; Cantin et al., 1985; Moses and Claycomb, 1984). Moses and Claycomb (1984) reported that, unlike ventricular cardiocytes, all atrial cells which eventually attach and spread out, proceed through a transient spherical stage. This process was reported to be accompanied by a disorganization of the myofilaments as observed by phase contrast, immunofluorescence and electron microscopy (Moses and Claycomb, 1984). This was also observed with ventricular cells in some preparation (Guo et al., 1986, Nag et al., 1983). This phenomenon is believed to be due to the activation of calcium-sensitive endogenous proteases and to the exposure to exogenous proteases during the isolation process (Cantin et al. 1985; Moses and Claycomb, 1984).

Phase-contrast observations were substantiated with immunofluorescence microscopical distribution analysis of ANF and desmin immunofluorescence (desmin-if) at different times in culture to correlate changes in ANF-if cellular distribution to changes observed in the content, post-translational processing and release of the peptide. Desmin-if was chosen as a muscle-specific marker. Desmin is a structural protein, localized in Z-bands of sarcomeres *in vivo* and, unlike other sarcomeric proteins, desmin has only been identified in muscle cells, making it an excellent muscle marker (D.J. Evans, 1983). In the present work, the specificity of the desmin antibody was assessed on enriched cultures of atrial non-cardiocyte cells (cells obtained after 2 to 3 passages of cells attached during the pre-plating) where no staining was observed.

Desmin and ANF-if distributions did not escape from the general disorganization of the atrial cardiocytes. Desmin-if, which was associated with Z-bands in freshly isolated

cylindrical cells, showed a rapid disorganisation in its distribution as the cardiocytes assumed a spherical shape. Similarly, ANF-if was observed to be randomly distributed within the cell while in freshly isolated cylindrical cells, ANF-if was found mostly restricted to the perinuclar zone. A clear assessment of ANF-if and desmin-if distributions in round cells was not possible because of the thickness of the cells and overwhelming fluorescence (also observed by Guo et al., 1986 with antibodsies against tubulin or actin). Cell sectioning should be considered for a better appreciation of ANF- and desmin-if at this stage in culture.

Upon attachment, desmin-if in the cytoplasmic elongations developed into a meshwork pattern comparable to stress fibre structures (Dlugosz et al., 1984). All sarcoplasmic elongations were desmin positive. Except in freshly isolated preparations, desmin was not associated with Z-bands of sarcomeres, even though the attached cardiocytes were observed to be spontaneously beating after 5 to 7 days in culture and contained immunoreactive myosin assembled as sarcomeric structures. Similar observations have been reported by several authors (Dlugosz et al., 1984; Isobe et al., 1989; Vitadello et al., 1990). Dlugosz et al. (1984) demonstrated that only long after formation of the Z-bands and the onset of the contracting activity in culture does desmin localize to the I-Z bands, raising questions about the role of this protein in the contractile functions of the cardiocyte.

Unlike desmin-if distribution in cultured atrial cardiocytes after attachment, ANF-if was concentrated in the perinuclear region, in a manner comparable to the distribution observed in freshly isolated cylindrical cells. A similar distribution has been reported by several investigators (Cantin et al., 1980; Cantin et al., 1985; Iida and Page, 1988). The

amount of ANF-if decreased with increasing time in culture. This is in agreement with the data presented here regarding ANF cellular content as measured by radioimmunoassay. Noteworthy is that all desmin positive cardiocytes were found to display ANF-if. Taken together, the above results suggest that adult rat atrial cardiocytes in culture retain specialized endocrine characteristics.

3) CHARACTERIZATION OF ANF STORAGE, POST-TRANSLATIONAL PROCESSING AND RELEASE IN CULTURES OF ADULT RAT ATRIAL CARDIOCYTES

In the present work, ANF storage, post-translational processing and release were all found to decrease with increasing time in culture as compared to levels measured with freshly isolated cardiocyte preparations. The quantity of ANF stored and released by atrial cardiocytes after 15 days in culture was found to be 10 and 40 times lower respectively as compared to experiments using freshly isolated cells. These results are in agreement with previous studies reported by Cantin et al. (1985), although their results showed a greater decrease in ANF storage and release. The amount of ANF stored and released in freshly isolated atrial cardiocyte preparations and whole tissue preparation appears to be in the same range or higher (by two folds in average), but more experiment are necessary to correlate the results from the two system. Interestingly, the onset of the decrease in levels of ANF observed in the present study occurred on the third day in culture, which coincides with the attachment of the cardiocytes to the culture substratum. In light of these results, it would be tempting to speculate that during structural rearrangement occurring during cell attachment, there may be an increase in the rate of synthesis of a number of proteins at the

expense of synthesis of other proteins such as ANF. Alternatively, the effect of time in culture on ANF might simply be due to the absence of stimulatory factor(s) or conditions that are present *in vivo* and are not reproduced by the culture conditions.

In freshly isolated atrial cardiocyte preparations, more than 80% of the ANF released was processed to a chromatographically identical molecular form of ANF (99-126) as has been previously found in isolated heart preparations (Currie et al., 1984; Lang et al., 1985; de Bold et al., 1986). Therefore, it is apparent that the collagenase digestion did not impair the processing ability of atrial cardiocytes. This processing activity was maintained at constant levels for the first 2 days in culture and decreased thereafter. This reduction in ANF processing activity with increasing time in culture was not as marked as that measured for ANF storage and release, and remained at levels where about 60% of the released ANF was of a low molecular weight. This reduced processing activity was only statistically significantly lower at the sixth day in culture at which time the processing activity was found to be 40% of that measured with freshly isolated cell preparations or with cells in culture for 1 to 2 days. Therefore, in this study, cultures of adult rat atrial cardiocytes maintained their ability to cleave the ANF precursor to a low molecular weight peptide.

The results reported here on ANF post-translational processing in culture are in disagreement with most other reports in the field (Bloch et al., 1985; Glembotski and Gibson, 1985; Hassal et al., 1988), in which total loss of processing activity in culture was observed. The discrepancy could arise from a fundamental technical difference; most investigations on ANF processing in culture have used primary cultures of neonatal rat atrial cardiocytes maintained in a serum-free culture medium. Serum in culture provides an

undetermined number of factors which cannot be reproduced by a serum-free culture medium. Perhaps the presence of fetal bovine serum in our culture system provides stimulatory factors which maintain ANF post-translational processing active. A study by Zisfein and collaborators (1987) reported some processing activity (25-30% of ANF immunoreactivity was of low molecular weight) in 7 day old cultures of adult rat atrial cardiocytes maintained in 20% NU-serum®. This commercial synthetic serum contains 25% bovine serum (Jacobson and Piper, 1986). In an attempt to verify the effect of serum on ANF processing, experiments were performed using cultures of adult rat atrial cardiocytes maintained in regular cell culture medium in absence of serum. This manipulation resulted in an abrupt decrease in ANF release to levels not detectable by radioimmunoassay after only 6 or 7 days in culture (data not shown). Therefore, post-translational processing could not be measured in these conditions.

It is not evident what factors in fetal bovine serum may be responsible for maintaining significant levels of ANF processing and release in long-term cultures of adult rat atrial cardiocytes. However, glucocorticoids have been shown to maintain and/or restore processing activity in long term serum-free cultures of newborn rat cardiocytes (Shields and Glembotski, 1988; Shields et al., 1988). In these studies, hydrocortisone and dexamethasone were able to reversibly modulate, in a dose-responsive fashion, the processing activity of ANF in culture. Interestingly, the average concentration of cortisol in the human fetal circulation and amniotic fluid is between 35-45 µg/100 mL (Goldkrand et al., 1976). Therefore, assuming comparable levels for the bovine fetus, medium containing 20% fetal bovine serum would contain approximately 200 nM of cortisol, whereas in the aforementioned study, dexamethasone was used at a concentration of 191 nM. In addition,

several studies have shown a stimulatory effect of glucocorticoids on ANF gene expression and mRNA levels (Gardner at al., 1986, 1988).

4) QUALITATIVE AND QUANTITATIVE CHARACTERIZATION OF THE CELL POPULATION OF PRIMARY CULTURES OF ADULT RAT ATRIAL CARDIOCYTES: CORRELATION WITH ANF POST-TRANSLATIONAL PROCESSING

In intact adult rat hearts, the number of cardiocytes constitutes about 20% of the total number of cells (Jacobson, 1989). Special efforts must be made to restrict the presence and proliferation of non-cardiocyte cells to prevent them from taking over the cultures. It has been reported that non-cardiocyte cells, as many as one per cardiocyte, are attached to the latter ones and cannot be eliminated by differential attachment (pre-plating) (Cutilletta et al., 1977). As suggested by some investigators (Ito et al., 1988; Toki et al., 1990; Gilloteaux et al., 1991), these non-cardiocyte cells (fibroblasts or endothelial cells, and/or others) could be essential participants in the ANF post-translational processing. Results obtained in the present work with cardiocytes in flat cultures, show that non-cardiocytes were clustered in colonies and most of them were localized in proximity to the cardiocytes, often between the cell processes. These colonies were probably the result of multiplication of a single non-cardiocyte. This observation supports the idea that, following cell dissociation, non-cardiocyte cells might be attached to cardiocytes and, upon flattening of the latter, the non-cardiocytes remain closely associated with the cardiocytes (Cutilletta et al., 1977).

The cell population in flat cultures was characterized by counting the number of cardiocytes, the number of colonies and the total number of cells. The ratio of the cardiocytes to the total number of colonies remained constant through the fifth and seventh days, while the ratio of the cardiocytes to the total number of cells increased by 2 fold. The decrease in the number of non-cardiocytes in the cultures is a consequence of a decrease in the number of cells in each colony, not a decrease in the number of colonies. Therefore, the number of cardiocytes in proximity to a non-cardiocyte cell remained the same. If there is an involvement of the non-cardiocytes in ANF post-translational processing, we can speculate that the proximity of non-cardiocytes is a key factor, where ANF would be released in the vicinity of the non-cardiocyte cells and cleaved to its circulating form. However, the number of colonies and, therefore, the number of cardiocytes at proximity to non-cardiocytes decreased significantly after 10 and 15 days in culture without a significant change in post-translational processing activity. These results suggest a lack of participation of the non-cardiocytes in ANF maturation. The results obtained with cultures on micro-carrier beads also support this view since, in those preparations, no correlation was found between the number of non-cardiocytes and the processing activity measured from the same cultures. Noteworthy is the finding that the ratios of cardiocytes to the total number of cells in culture obtained with micro-carrier beads at 7 and 15 days in culture correlated with those obtained with flat cultures.

To directly test the hypothesis that non-cardiocytes are responsible for the cleavage of the ANF precursor, columns containing micro-carrier bead cultures of atrial non-cardiocytes were superfused with different concentrations of exogenous proANF. Non-cardiocytes were unable to cleave the prohormone to smaller molecular weight ANF

peptides. This is also in agreement with the results from Shields and collaborators (1988) showing that primary cultures of neonatal rat atrial cardiocytes were unable to cleave exogenous recombinant proANF, even after 1 hour in contact with the cells in a static incubation system. Together, these results support the concept that processing of proANF is an intrinsic property of the atrial cardiocytes and is independent of any other type of cells. Cleavage of the propeptide must therefore take place during release or very soon thereafter.

The observed changes in cell population do not appear to correlate with the decrease observed in the rate of release or the amount of stored ANF, since the largest decrease in the number of non-cardiocytes occurs between the seventh and fifteenth days, while most of the reduction in ANF release and processing was found after 3 days in culture, when, as described earlier, cardiocytes undergo attachment.

CONCLUSIONS:

In conclusion, this study shows that adult rat atrial cardiocytes, maintained in culture in a serum supplemented medium for up to 15 days, store atrial natriuretic factor (ANF) as a prohormone and secrete an ANF (99-126)-like peptide, as found *in vivo*, suggesting that the post translational processing of ANF remains functional in these cultures. The cellular distribution of ANF immunofluorescence after 5 to 7 days in culture was found to correspond with that observed *in vivo*, indicating that the cardiocytes retained the phenotype characteristic of peptide hormone secreting cells. It seems likely that fetal bovine serum is partly or entirely responsible for the maintenance of the ANF processing activity in the

cultures. The reduction in the rate of ANF release, the amount of stored ANF, as well as the apparent decrease in post-translational processing activity might reflect a lack of factor(s) or condition(s) in culture which are present *in vivo*. The results reported here support the view that the post-translational processing of ANF in an intrinsic function of atrial cardiocytes and does not depend upon the participation of other cell types.

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APPENDIX 1: Data on cell isolation and viability.

TABLE 1: Number of atrial and ventricular cells isolated from 1 rat (2 atria or ½ of a ventricle) by multiple collagenase digestions at 37°C after repletion of calcium to 1.5 mM. Viability was determined by trypan blue exclusion (viable) and by cell morphology (rod)

digestion period (min)	atrial total	atrial viable ($X 10^3$)	atrial rod	ventricle total	ventricle viable ($X 10^6$)	ventricle rod
20	65.000	0.000	0.000	1.600	0.010	0.000
20	65.000	2.500	0.000	0.200	0.000	0.000
20	207.500	7.500	0.000	2.310	0.000	0.000
20	35.000	0.000	0.000	0.770	0.000	0.000
20	90.000	42.500	7.500	0.890	0.080	0.020
20	58.000	12.000	3.750			
40	350.000	50.000	27.500	0.250	0.500	0.360
40	192.500	30.000	7.500	3.500	0.560	0.450
40	332.500	17.500	5.000	1.400	0.060	0.060
40	375.000	42.500	22.500	2.000	0.490	0.310
40	250.000	72.500	30.000	3.000	0.860	0.460
40	215.500	93.500	54.250			
60	750.000	350.000	230.000	1.400	0.440	0.290
60	960.000	480.000	307.500	2.900	0.830	0.680
60	382.500	135.000	47.500	1.330	0.220	0.110
60	875.000	400.000	157.500	2.300	0.870	0.380
60	623.500	361.500	192.250			
60	475.000	275.000	147.500	2.200	1.200	0.830
80	292.500	120.000	45.000	1.070	0.340	0.180
80	775.000	375.000	127.500	1.500	0.470	0.200
80	1275.000	595.000	332.500	1.120	0.250	0.210
80	300.000	132.500	60.000	1.600	0.830	0.490
80	222.500	140.000	77.500	0.430	0.140	0.050
80	511.500	246.250	153.750			
100	197.500	97.500	50.000	0.680	0.340	0.290
100	500.000	245.000	60.000	0.630	0.220	0.040
100	127.500	97.500	85.000	0.400	0.120	0.080
100	300.000	132.500	60.000	1.600	0.830	0.490
100	125.000	80.000	42.500	0.360	0.190	0.100
100	135.500	47.500	24.250			
120	155.000	82.500	45.000	0.720	0.380	0.240
120	140.000	55.000	25.000	0.490	0.200	0.100

TABLE 2: Average number of atrial and ventricular cells isolated from 1 rat (2 atria or ½ of a ventricle) by multiple collagenase digestions at 37°C after repletion of calcium to 1.5 mM. Viability was determined by trypan blue exclusion (viable) and by cell morphology (rod). (mean ± sem).

digestion period (min)	atrial total	atrial viable	atrial rod	ventricle total	ventricle viable	ventricle rod
		(X 10^3)			(X 10^6)	
20	86.7 ± 25.2	10.8 ± 6.6	1.8 ± 1.3	289 ± 91	4.5 ± 3.9	1.0 ± 1.0
40	285 ± 31	51.0 ± 11.4	24.5 ± 7.3	508 ± 144	124 ± 32	82.0 ± 18.2
60	678 ± 92	334 ± 48	180 ± 36	507 ± 74	178 ± 43	115 ± 33
80	563 ± 165	268 ± 76	133 ± 43	286 ± 55	102 ± 30	56.5 ± 18.0
100	231 ± 60	117 ± 28	53.6 ± 8.3	184 ± 56	85.0 ± 31.9	50.0 ± 21.1

TABLE 3: Data on total number of isolated atrial cells and total number of viable cell as measured by trypan blue exclusion after calcium repletion and resuspension in M-199 supplemented with 20% HI-FBS. (n=23 separate experiments)

total	viable	total	viable
(X 10^6)			
0.950	0.700	1.280	1.088
1.550	0.800	1.075	0.675
1.175	0.600	0.800	0.400
1.740	1.078	0.770	0.560
1.580	1.153	1.560	0.970
1.720	1.180	1.540	1.020
1.540	1.120	2.200	1.518
1.780	1.330	1.340	0.536
1.220	0.647	1.150	0.800
0.940	0.720	0.920	0.599
1.100	0.830	1.130	0.824
1.450	1.100		

	TOTAL	VIABLE
N OF CASES	23	23
MEAN	1.327	0.880
STANDARD DEV.	0.359	0.284
STD. ERROR	0.075	0.059
C.V.	0.271	0.322

APPENDIX 2: Data on ANF release

TABLE 4: Release of ANF from freshly isolated adult rat atrial cardiocytes.

TIME (MIN)	A* (pg/mL)	B (pg/mL)
1	59.706	24.021
2	42.696	18.780
3	39.462	19.972
4	31.402	20.537
5	34.597	20.797
6	32.063	23.315
7	27.865	23.326
8	31.334	18.502
9	54.967	19.231
10	17.210	21.127
11	33.552	15.808
12	29.346	18.473
13	33.671	22.395
14	23.653	17.891
15	25.532	21.210
16	26.000	25.000
17	28.383	21.795
18	26.380	18.094
19	33.142	22.544
20	26.509	15.415
21	30.697	12.994
22	36.778	19.927
23	42.593	19.016
24	33.552	17.272
25	32.894	19.463
26	28.202	19.287
27	29.935	20.382
28	28.807	17.127
29	32.471	16.584
30	39.504	16.952
31	30.493	16.681
32	32.586	16.584
33	54.802	21.491
34	33.449	17.280
35	29.561	20.444
36	39.468	19.152
37	32.416	21.686
38	33.413	19.692
39	27.978	17.307
40	35.115	29.979
41	34.613	16.758
42	30.587	20.910
43	33.413	19.903
44	29.360	21.566
45	30.173	16.376
46	35.984	16.301
47	34.884	11.851
48	32.944	3.708
49	44.299	26.382
50	36.890	29.750
51	32.321	15.214
52	32.621	13.458
53	38.808	17.106
54	28.132	17.531
55	14.413	21.654
56	32.381	20.115
57	30.413	22.746
58	31.040	22.237
59	34.613	18.791
60	50.398	18.714

* The letters are used to distinguish the different experiment in appendix section.

TABLE 5: Release of ANF from 1 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	7.625	4.978
2	6.419	6.476
3	6.134	5.984
4	4.072	5.318
5	4.649	5.356
6	4.944	5.851
7	5.637	5.636
8	4.962	6.619
9	4.466	7.267
10	4.747	5.497
11	5.327	6.378
12	4.479	5.857
13	4.884	8.431
14	5.410	5.607
15	7.359	4.145
16	4.811	4.870
17	6.737	4.515
18	7.115	4.399
19	6.561	6.399
20	5.952	3.896
21	12.507	4.351
22	9.093	4.025
23	7.799	5.159
24	7.013	5.666
25	6.301	5.643
26	6.000	8.912
27	5.900	4.833
28	6.000	5.518
29	6.662	4.666
30	4.179	5.278
31	6.723	6.981
32	6.363	6.333
33	7.061	6.896
34	6.987	6.797
35	15.652	6.397
36	6.247	7.286
37	5.728	5.804
38	7.938	5.261
39	6.518	5.075
40	7.729	5.023
41	6.215	7.003
42	5.865	6.118
43	9.299	6.163
44	8.945	5.221
45	11.229	4.512
46	7.950	5.491
47	7.241	16.071
48	6.984	6.270
49	6.625	6.444
50	4.636	4.967
51	6.867	5.759
52	7.271	5.099
53	7.397	6.163
54	7.146	6.212
55	7.820	4.307
56	5.994	6.012
57	7.283	4.409
58	7.503	7.891
59	7.831	5.958
60	6.024	11.460

TABLE 6: Release of ANF from 2 day old cultures adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	57.215	19.318
2	24.332	12.210
3	25.932	16.659
4	34.204	15.438
5	17.458	17.818
6	12.551	12.887
7	16.427	12.069
8	25.870	12.626
9	14.688	10.566
10	14.163	12.161
11	24.894	17.677
12	14.977	13.359
13	10.326	16.616
14	11.422	11.587
15	13.389	14.242
16	11.769	12.351
17	17.434	24.709
18	16.044	11.191
19	20.172	9.780
20	14.779	10.875
21	11.143	13.419
22	6.159	14.828
23	7.371	17.192
24	13.282	14.232
25	10.134	10.772
26	7.416	10.311
27	7.387	11.693
28	9.185	12.052
29	7.120	13.051
30	8.143	9.797

TABLE 7: Release of ANF from 3 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	1787.500	1787.500
2	1455.900	1455.900
3	2126.100	2126.100
4	1993.400	1993.400
5	1346.200	1346.200
6	1926.200	1926.200
7	2663.400	2663.400
8	2508.400	2508.400
9	2550.500	2550.500
10	2217.600	2217.600
11	2480.400	2480.400
12	2691.000	2691.000
13	2197.000	2197.000
14	2134.800	2134.800
15	1885.700	1885.700

TABLE 8: Release of ANF from 7 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	1811.900	3667.700
2	1937.900	2445.100
3	1373.300	1306.000
4	2075.600	2247.100
5	1759.600	2158.700
6	1555.600	1407.500
7	2074.800	
8	2151.600	2492.100
9	2262.500	3020.500
10	1957.500	2837.500
11	2924.800	2516.200
12	3732.900	2856.900
13	2629.000	2425.300
14	4818.200	2251.600
15	4557.900	2279.500
16	3549.900	2831.300
17	3855.400	3576.500
18	2372.000	2162.100
19	3928.600	2051.100
20	3640.900	2316.100
21	5259.100	1615.900
22	3485.600	1943.100
23	4445.300	2085.400
24	4174.200	2553.800
25	2377.500	2240.800
26	2493.700	2138.700
27	2804.000	2944.700
28	3022.000	2737.300
29	1521.100	2024.600
30	3514.400	2426.800
31	3971.500	2558.900
32	4188.700	2077.500
33	3894.200	1688.900
34	3435.300	1808.500
35	4287.100	2185.900
36	2990.500	274.200
37	4027.400	687.900
38	2957.300	2145.400
39	4890.400	2888.700
40	3415.600	2800.600
41	3997.900	2613.500
42	2406.300	2847.900
43	3212.200	2301.900
44	4207.700	1652.400
45	2264.800	2425.300
46	3122.200	1758.400
47	874.700	2048.500
48	3350.200	2905.200
49	2843.000	3064.500
50	3597.000	2754.200
51	3227.100	3268.000
52	3898.000	2908.100
53	4604.800	2710.400
54	4528.800	2470.000
55	3310.900	2749.600
56	4740.500	2480.700
57	1533.900	2816.900
58	4008.600	3040.800
59	4456.800	2846.500
60	3486.600	2340.100

TABLE 9: Release of ANF from 15 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	0.406	0.545
2	0.480	0.457
3	0.527	0.407
4	0.563	0.401
5	0.496	0.416
6	0.431	0.451
7	0.365	0.493
8	0.493	0.447
9	0.419	0.451
10	0.486	0.414
11	0.436	0.438
12	0.514	0.422
13	0.521	0.471
14	0.506	0.436
15	0.468	0.431
16	0.481	0.362
17	0.406	0.464
18	0.475	0.385
19	0.448	0.398
20	0.503	0.418
21	0.430	0.480
22	0.519	0.459
23	0.555	0.404
24	0.482	0.397
25	0.470	0.316
26	0.464	0.305
27	0.402	0.441
28	0.446	0.415
29	0.388	0.363
30	.	0.359

TABLE 10: Release of ANF from 15 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	53.540	46.055
2	51.560	55.835
3	45.885	48.220
4	77.060	49.925
5	49.610	47.825
6	48.400	45.965
7	45.015	47.730
8	49.550	48.615
9	45.745	52.920
10	47.015	49.810
11	41.715	38.100
12	40.735	40.900
13	43.310	44.160
14	47.040	45.835
15	46.360	50.160

TABLE 11: Release of ANF from 15 day old cultures of adult rat atrial cardiocytes.

TIME (MIN)	A (pg/mL)	B (pg/mL)
1	198.870	251.6 [illegible]
2	161.660	307.470
3	245.130	378.850
4	.	249.270
5	.	165.810
6	.	186.360
7	215.490	222.050
8	233.180	184.500
9	150.320	290.500
10	189.080	310.130
11	193.410	412.370
12	236.740	315.280
13	199.150	427.300
14	236.570	632.850
15	188.390	443.480
16	210.950	356.810
17	208.240	348.960
18	217.570	381.780
19	197.280	199.830
20	209.920	270.310
21	179.870	276.550
22	189.690	178.040
23	351.530	286.420
24	225.950	293.650
25	264.640	341.830
26	235.000	360.880
27	216.100	399.230
28	109.770	346.420
29	200.800	343.920
30	284.090	419.500
31	283.180	369.500
32	325.260	294.270
33	271.430	338.570
34	268.840	270.830
35	220.670	299.700
36	207.240	279.150
37	204.880	285.660
38	230.410	376.720
39	277.880	143.680
40	261.340	357.480
41	216.710	459.440
42	228.710	536.730
43	161.850	547.220
44	263.440	544.810
45	223.000	624.790
46	318.080	.
47	.	490.080
48	276.010	758.910
49	373.730	577.760
50	303.440	330.290
51	307.960	488.470
52	306.700	520.410
53	328.150	493.900
54	377.630	457.110
55	304.390	707.120
56	130.640	530.520
57	276.550	599.380
58	243.860	700.980
59	282.770	552.820
60	357.080	408.730

TABLE 12: Effect of time in culture on the rate of release and cell content of irANF in primary cultures of adult rat atrial cardiocytes

DAYS IN CULTURE	irANF RELEASE ng/h/µg of DNA	irANF CONTENT ng/µg of DNA
0	10.5 ± 0.3	
	14.9 ± 0.4	38.0 ± 4.5
1	8.5 ± 0.3	23.3 ± 2.4
	7.6 ± 0.3	30.4 ± 3.6
2	11.9 ± 0.1	39.2 ± 3.0
	7.0 ± 0.3	16.3 ± 2.2
3	1.4 ± 0.1	9.4 ± 0.4
	1.8 ± 0.1	9.9 ± 0.7
7	0.29 ± 0.01	7.1 ± 3.5
	0.28 ± 0.01	3.2 ± 0.4
15[c]	0.32 ± 0.08	3.9 ± 1.2

* average of 6 experiments (mean ± sem)

APPENDIX 3: DNA DETERMINATION

TABLE 13: Determination of DNA concentration by fluorometry using a DNA-specific dye, Hoechst 33258 for days 0a, 0b, 1a, 1b, 2a, 2b, 15a and 15b. (spectrofluorometer:Aminco Bowman; excitation=352nm, emission=492nm).

Standard curve		Sample			
DNA conc. (ng/mL)	Average fluorescence (FU)	Sample	Average fluorescence (FU)	Dilution	DNA conc. (µg/mL)
0	0.082 ± 0.001	Day 0a (3)	0.190 ± 0.000	1500	95.6 ± 0.0
10	0.073 ± 0.006	Day 0b (3)	0.150 ± 0.005	1500	38.8 ± 3.2
20	0.188 ± 0.008	Day 1a (3)	0.165 ± 0.005	1500	48.1 ± 3.2
40	0.157 ± 0.003	Day 1b (3)	0.343 ± 0.006	300	47.6 ± 0.5
60	0.187 ± 0.006	Day 2a (3)	0.132 ± 0.008	1500	41.0 ± 7.2
80	0.235 ± 0.009	Day 2b (3)	0.182 ± 0.003	1500	58.5 ± 1.8
100	0.253 ± 0.015	Day 15a (3)	0.323 ± 0.006	300	29.4 ± 0.7
200	0.423 ± 0.006	Day 15b (3)	0.247 ± 0.003	300	29.7 ± 0.6
500	0.872 ± 0.003				

(mean ± s.d.)

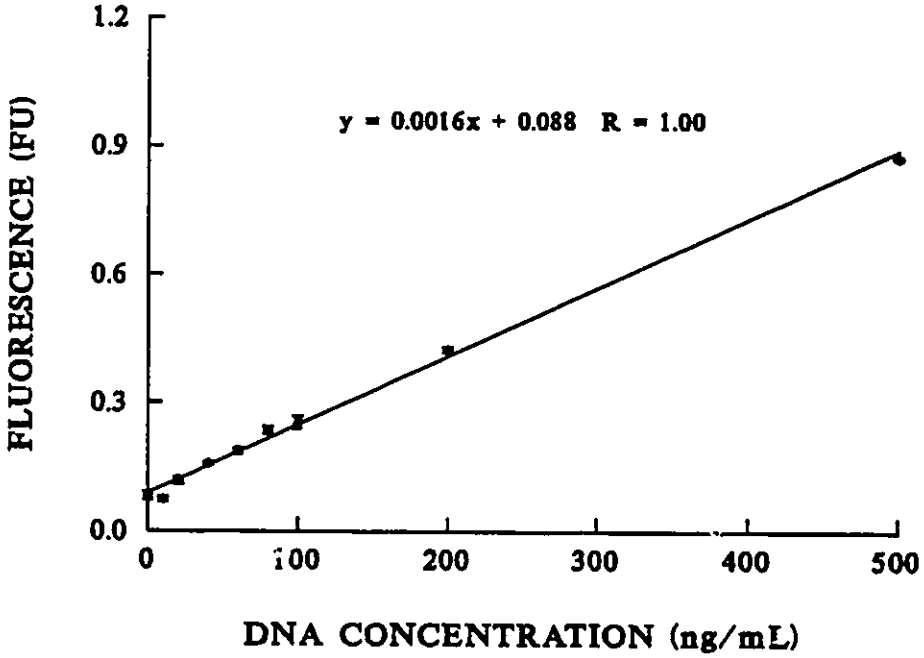

FIGURE 1: Standard curve for DNA measurement by fluorometry using a DNA-specific dye (Hoechst 33258) and measured on an Aminco Bowman Spectrofluorometer (excitation=353nm, emission=492nm). Slope determined by method of least squares.

TABLE 14: Determination of DNA concentration by fluorometry using a DNA-specific dye, Hoechst 33258 for days 3a and 3b. (Dedicated fluorometer: Hoefer TKO 100; excitation=365nm, emission=460nm).

Standard curve		Sample			
DNA conc.	Average fluorescence	Sample	Average fluorescence	Dilution	DNA conc.
(ng/mL)	(FU)		(FU)		(µg/mL)
10	108 ± 11	Day 3a (7)	495 ± 87	160	19.72 ± 2.90
20	91 ± 17	Day 3b (5)	264 ± 23	160	9.34 ± 0.87
40	143 ± 4				
60	225 ± 0				
80	354 ± 6				
100	496 ± 6				
250	1075 ± 35				

(mean ± s.d.)

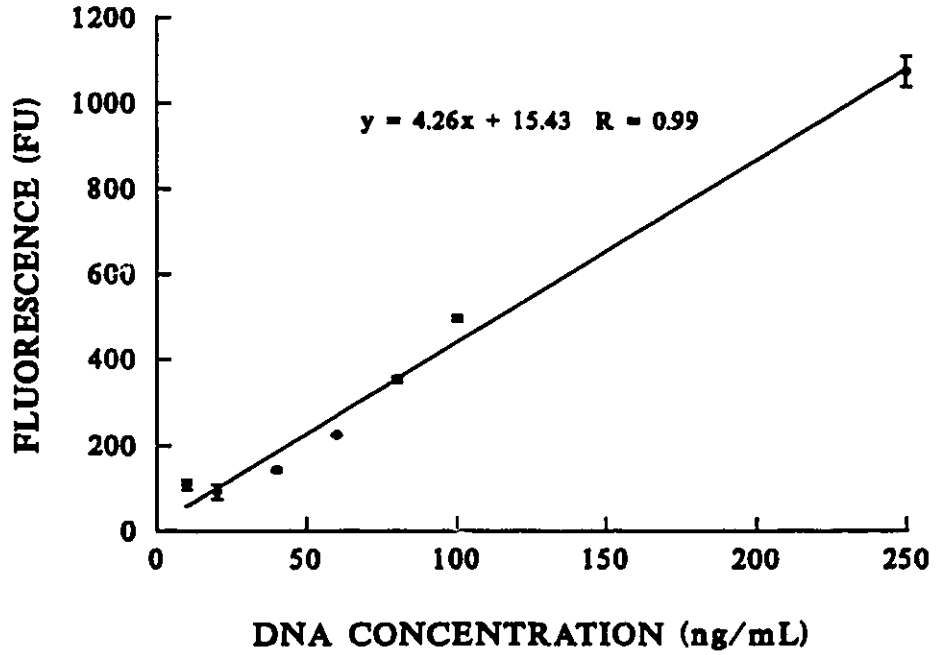

FIGURE 2: Standard curve for DNA measurement by fluorometry using a DNA-specific dye (Hoechst 33258) and measured on an Hoefer TKO1 100 dedicated fluorometer (excitation=365nm, emission=460nm). Slope determined by method of least squares.

TABLE 15: Determination of DNA concentration by fluorometry using a DNA-specific dye, Hoechst 33258 for days 15c and 15d. (Dedicated fluorometer: Hoefer TKO 100; excitation=365nm, emission=460nm).

Standard curve		Sample			
DNA conc.	Average fluorescence	Sample	Average fluorescence	Dilution	DNA conc.
(ng/mL)	(FU)		(FU)		(µg/mL)
10	58 ± 4	Day 15c (3)	94.3 ± 0.5	3000	49.7 ± 0.3
20	107 ± 6	Day 15b (3)	87.6 ± 5.1	1800	27.6 ± 1.5
50	268 ± 8				
100	542 ± 21				
250	1305 ± 50				

(mean ± s.d.)

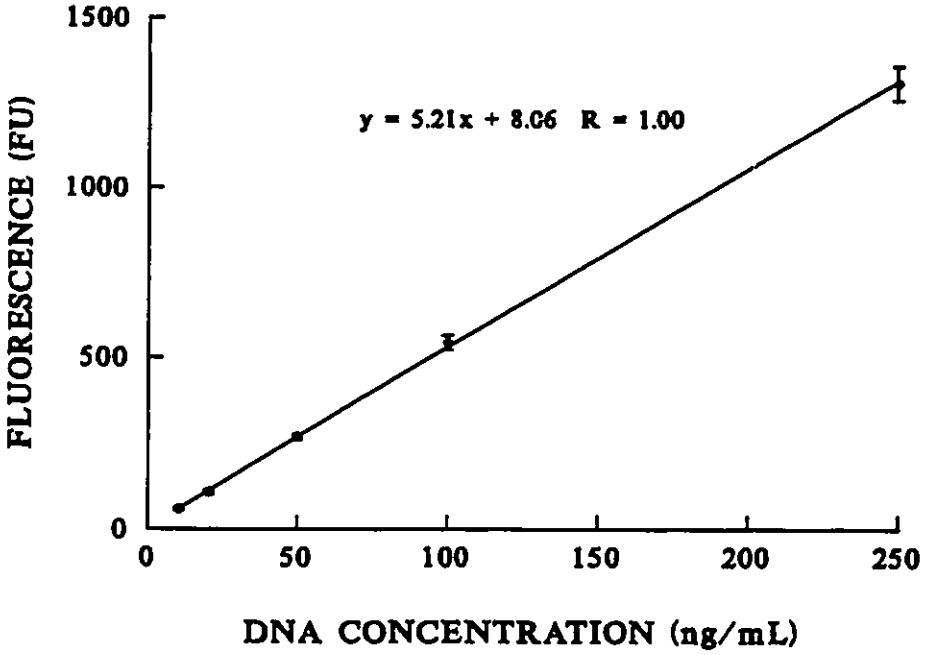

FIGURE 3: Standard curve for DNA measurement by fluorometry using a DNA-specific dye (Hoechst 33258) and measured on an Hoefer TKO1 100 dedicated fluorometer (excitation=365nm, emission=460nm). Slope determined by method of least squares.

TABLE 16: Determination of DNA concentration by fluorometry using a DNA-specific dye, Hoechst 33258 for days 7a, 7b, 15e, 15f. (Dedicated fluorometer: Hoefer TKO 100; excitation=365nm, emission=460nm).

Standard curve		Sample			
DNA conc.	Average fluorescence	Sample	Average fluorescence	Dilution	DNA conc.
(ng/mL)	(FU)		(FU)		(µg/mL)
10	20 ± 2	Day 7a (3)	47 ± 6	6000	135 ± 23
50	95 ± 68	Day 7b (3)	38 ± 3	6000	101 ± 10
100	182 ± 11	Day 15e (4)	260 ± 8	101	16 ± 4
250	411 ± 7	Day 15f (3)	73 ± 14	400	31 ± 1

(mean ± s.d.)

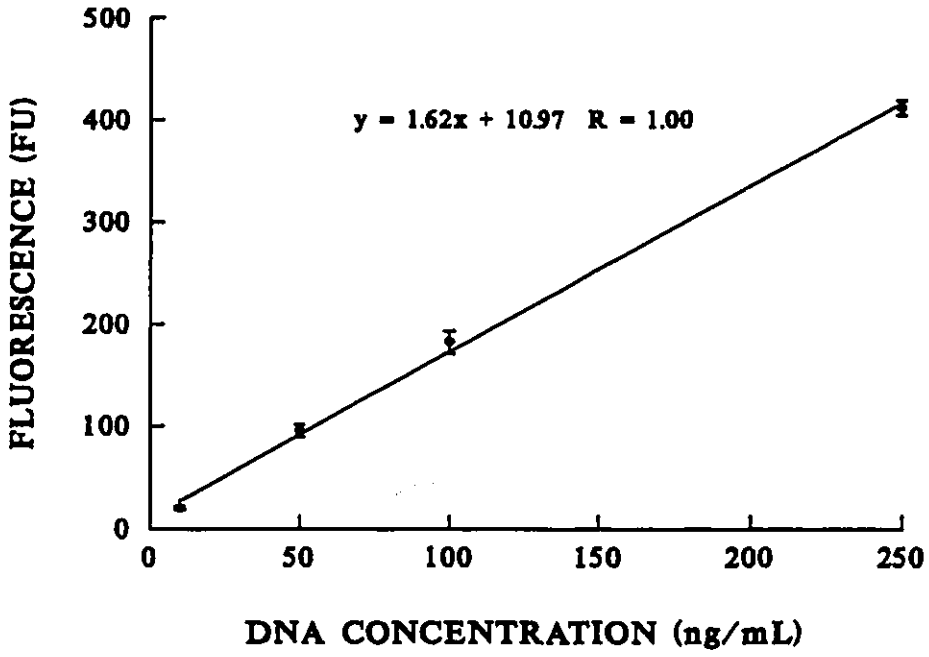

FIGURE 4: Standard curve for DNA measurement by fluorometry using a DNA-specific dye (Hoechst 33258) and measured on an Hoefer TKO1 100 dedicated fluorometer (excitation=365nm, emission=460nm). Slope determined by method of least squares.

APPENDIX 4: Data for RP-HPLC immunoreactive ANF elution profiles.

TABLE 17: Freshly isolated adult rat atrial cardiocytes

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	0.001	0.122	3.800	4.561
2	17	0.003	0.089	6.099	4.392
3	18	0.004	0.089	5.778	5.296
4	19	0.002	0.056	0.000	6.211
5	20	0.003	0.056	24.859	21.851
6	21	0.004	0.056	24.535	21.104
7	22	0.004	0.056	22.242	0.000
8	23	0.311	0.211	25.656	32.612
9	24	0.030	0.078	0.000	35.522
10	25	0.018	0.111	43.005	30.639
11	26	0.025	0.133	47.686	12.014
12	27	0.100	0.144	75.559	57.936
13	28	0.256	0.122	69.476	90.778
14	29	0.189	0.156	55.378	90.937
15	30	0.233	0.267	79.476	334.700
16	31	2.961	5.489	975.780	95.482
17	32	0.411	0.278	519.270	91.498
18	33	0.032	0.089	30.716	69.332
19	34	0.011	0.056	89.217	71.628
20	35	0.008	0.056	53.177	39.981
21	36	0.006	0.067	23.845	0.000
22	37	0.008	0.067	23.496	5.120
23	38	0.002	0.067	21.176	18.653
24	39	0.004	0.067	0.000	27.628
25	40	0.010	0.067	47.515	5.844
26	41	0.032	0.089	67.417	80.381
27	42	0.014	0.056	80.098	88.080
28	43	0.014	0.067	57.015	71.807
29	44	0.014	0.067	41.743	51.588
30	45	0.010	0.067	46.686	87.855
31	46	0.574	0.111	99.811	38.416
32	47	0.067	0.067	89.371	133.770
33	48	0.023	0.067	95.351	91.498
34	49	0.000	0.067	33.944	73.531
35	50	0.000	0.056	23.248	55.442
36	51	0.001	0.067	16.701	30.034
37	52	0.001	0.056	13.501	19.161
38	53	0.001	0.044	8.849	11.520
39	54	0.001	0.056	7.993	4.530
40	55	0.001	0.056	8.814	9.634

TABLE 18: Adult rat atrial cardiocytes after 1 day in culture

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	68.966	86.207	0.000	0.966
2	17	34.483	86.207	0.000	0.896
3	18	86.207	68.966	0.000	1.567
4	19	68.966	51.724	0.350	2.357
5	20	51.724	51.724	19.700	20.269
6	21	68.966	68.966	12.435	15.695
7	22	51.724	34.483	7.178	14.130
8	23	86.207	120.690	9.704	14.474
9	24	86.207	86.207	9.217	24.197
10	25	68.966	68.966	24.028	34.027
11	26	86.207	103.448	31.459	42.770
12	27	137.931	155.172	23.845	58.336
13	28	155.172	103.448	72.922	115.645
14	29	137.931	172.414	51.168	425.870
15	30	275.862	206.897	96.230	159.167
16	31	3172.414	5801.724	74.069	155.583
17	32	568.966	431.034	456.420	88.343
18	33	103.448	68.966	111.972	23.105
19	34	103.448	68.966	58.336	54.435
20	35	103.448	0.000	41.616	38.802
21	36	86.207	86.207	37.565	27.220
22	37	86.207	68.966	24.984	20.090
23	38	103.448	86.207	16.519	16.961
24	39	103.448	86.207	17.268	0.000
25	40	103.448	86.207	24.777	49.432
26	41	6810.345	86.207	50.240	76.814
27	42	120.690	86.207	95.351	104.222
28	43	103.448	51.724	113.780	62.371
29	44	120.690	51.724	47.563	52.113
30	45	120.690	120.690	7.324	144.070
31	46	120.690	103.448	63.967	86.714
32	47	172.414	86.207	113.658	89.758
33	48	189.655	86.207	39.250	114.954
34	49	120.690	103.448	94.061	34.629
35	50	86.207	68.966	40.757	39.151
36	51	103.448	103.448	22.722	31.983
37	52	103.448	86.207	17.307	21.152
38	53	103.448	86.207	8.359	13.702
39	54	86.207	68.966	6.420	9.497
40	55	86.207	0.000	4.367	9.146

TABLE 19: Adult rat atrial cardiocytes after 2 days in culture.

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	35.294	30.769	0.445	0.859
2	17	29.412	25.641	0.000	0.630
3	18	29.412	30.769	0.052	1.171
4	19	29.412	25.641	0.622	2.632
5	20	29.412	25.641	8.478	1.894
6	21	29.412	25.641	8.428	1.742
7	22	29.412	51.282	4.705	2.688
8	23	29.412	51.282	4.831	0.000
9	24	47.059	41.026	4.894	10.439
10	25	29.412	61.538	14.804	8.957
11	26	47.059	56.410	17.618	13.174
12	27	52.941	20.513	29.302	32.253
13	28	88.235	82.051	54.839	50.095
14	29	76.471	112.821	41.433	57.871
15	30	170.588	143.590	97.654	59.271
16	31	1205.882	1256.410	470.910	80.442
17	32	182.353	415.385	490.820	444.410
18	33	64.706	66.667	70.982	99.280
19	34	29.412	46.154	29.927	43.094
20	35	35.294	41.026	43.908	39.430
21	36	47.059	35.897	13.769	11.582
22	37	41.176	35.897	10.455	15.939
23	38	29.412	30.769	6.928	8.229
24	39	47.059	30.769	7.310	12.613
25	40	47.059	25.641	17.531	36.355
26	41	35.294	41.026	39.848	59.460
27	42	29.412	0.000	53.349	64.205
28	43	23.529	25.641	17.854	26.595
29	44	17.647	35.897	27.063	33.035
30	45	23.529	35.897	47.065	75.925
31	46	35.294	35.897	42.043	58.076
32	47	35.294	66.667	88.834	221.480
33	48	23.529	25.641	284.410	33.668
34	49	35.294	25.641	21.909	34.993
35	50	23.529	25.641	5.324	23.779
36	51	23.529	20.513	9.388	15.593
37	52	23.529	25.641	7.350	11.002
38	53	23.529	20.513	5.441	7.935
39	54	23.529	25.641	3.201	6.084
40	55	17.647	30.769	2.874	5.469

TABLE 20: Adult rat atrial cardiocytes after 3 days in culture

tube	gradient %B	Immunoreactive ANF A pg/ml of medium	Immunoreactive ANF B pg/ml of medium
0	15	0	0
1	16	0.000	1.963
2	17	0.000	1.016
3	18	0.262	1.596
4	19	0.736	1.424
5	20	3.271	1.453
6	21	0.618	1.967
7	22	1.269	16.480
8	23	0.742	11.309
9	24	0.870	4.767
10	25	6.180	8.561
11	26	4.193	23.004
12	27	4.162	15.796
13	28	16.186	57.809
14	29	9.807	43.647
15	30	21.175	77.676
16	31	18.695	49.628
17	32	227.155	111.711
18	33	46.699	121.451
19	34	25.082	55.127
20	35	12.577	26.905
21	36	6.394	29.181
22	37	3.810	10.783
23	38	3.886	8.003
24	39	6.214	7.223
25	40	17.701	14.319
26	41	11.653	34.703
27	42	17.938	53.072
28	43	5.036	31.323
29	44	4.939	18.971
30	45	8.195	31.289
31	46	16.874	31.513
32	47	28.091	84.469
33	48	42.775	105.755
34	49	4.359	78.549
35	50	6.563	19.076
36	51	3.469	13.197
37	52	2.563	19.985
38	53	1.934	5.116
39	54	1.406	4.692
40	55	0.948	4.377

TABLE 21: Adult rat atrial cardiocytes after 4 days in culture

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	1.879	0.445	3.639	1.740
2	17	1.141	0.000	3.299	1.830
3	18	1.035	3.971	2.265	1.895
4	19	1.172	0.919	1.590	2.332
5	20	0.774	4.374	3.205	2.076
6	21	1.333	6.179	13.849	2.050
7	22	0.986	3.510	6.731	3.420
8	23	48.846	2.865	4.448	3.941
9	24	11.007	5.684	8.594	3.777
10	25	18.390	8.725	22.918	6.316
11	26	14.362	9.516	22.384	5.472
12	27	30.544	18.133	59.735	13.100
13	28	14.148	11.418	120.194	34.133
14	29	22.852	19.859	38.191	28.525
15	30	42.780	21.882	227.490	51.589
16	31	942.400	21.531	86.425	101.115
17	32	118.012	191.715	659.780	186.870
18	33	54.576	27.614	97.285	25.146
19	34	22.900	11.592	47.140	9.937
20	35	28.349	10.556	22.485	8.936
21	36	7.632	4.774	14.346	4.128
22	37	4.737	3.470	6.253	3.195
23	38	4.196	3.177	4.673	1.900
24	39	5.907	2.478	3.270	6.827
25	40	1.521	9.962	7.507	21.253
26	41	26.910	12.215	20.181	33.781
27	42	20.483	7.997	34.283	44.450
28	43	5.744	4.501	29.564	11.667
29	44	6.402	10.321	16.555	29.686
30	45	9.574	7.407	22.595	29.269
31	46	23.830	20.100	11.063	83.499
32	47	215.272	49.656	37.960	213.070
33	48	58.004	22.669	347.040	13.215
34	49	14.069	10.430	37.662	10.167
35	50	6.413	5.782	11.117	5.729
36	51	4.151	3.566	6.735	3.569
37	52	3.563	2.111	4.952	2.856
38	53	0.000	1.518	2.750	1.617
39	54	2.593	1.092	3.017	2.069
40	55	3.086	1.697	1.499	2.814

TABLE 22: Adult rat atrial cardiocytes after 5 days in culture.

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	2.645	0.000	1.313	0.916
2	17	2.574	0.157	0.869	0.610
3	18	1.702	0.171	1.603	1.316
4	19	3.213	0.507	1.386	1.358
5	20	4.397	4.476	1.973	1.044
6	21	3.773	7.717	1.612	1.463
7	22	5.113	2.663	1.688	0.402
8	23	64.735	3.377	1.165	1.675
9	24	18.788	7.025	0.919	0.567
10	25	17.693	9.331	1.314	1.987
11	26	11.537	10.006	1.427	2.444
12	27	41.908	22.036	5.876	2.884
13	28	18.219	24.342	4.913	4.281
14	29	3.123	55.021	10.927	2.764
15	30	102.793	34.566	6.526	5.234
16	31	348.480	897.850	39.419	20.714
17	32	111.622	224.100	13.304	13.665
18	33	134.987	41.254	4.637	3.801
19	34	64.997	30.061	3.516	1.452
20	35	40.101	15.355	4.971	3.630
21	36	0.000	0.000	6.355	2.871
22	37	16.915	0.000	1.467	0.000
23	38	14.916	0.000	1.721	2.355
24	39	15.900	0.000	2.388	3.773
25	40	33.730	0.000	4.824	3.826
26	41	57.985	0.000	6.932	5.649
27	42	61.183	0.000	5.963	4.622
28	43	3.812	0.000	4.069	5.657
29	44	9.060	0.000	5.646	5.197
30	45	18.191	0.000	11.651	14.130
31	46	37.575	21.106	9.975	12.153
32	47	137.527	155.460	21.500	24.524
33	48	244.493	94.502	1.987	4.439
34	49	11.671	20.163	1.976	4.672
35	50	7.587	11.878	2.782	4.080
36	51	4.819	6.332	2.289	3.186
37	52	6.887	9.075	2.547	4.998
38	53	4.193	5.590	2.696	3.504
39	54	3.658	2.910	2.388	3.941
40	55	2.045	2.498	0.116	3.108

TABLE 23: Adult rat atrial cardiocytes after 6 days in culture.

tube	gradient %B	Immunoreactive ANF			
		A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	2.502	0.383	12.760	6.130
2	17	1.863	0.445	16.420	0.200
3	18	2.208	1.214	13.730	10.180
4	19	2.312	8.603	9.250	3.990
5	20	1.033	1.217	16.170	11.980
6	21	2.613	0.616	5.320	0.000
7	22	30.424	2.244	8.030	0.000
8	23	5.378	2.636	7.770	4.840
9	24	7.574	7.795	0.530	0.000
10	25	9.505	9.695	7.420	0.000
11	26	9.481	9.263	18.650	0.000
12	27	31.232	30.873	28.150	1.310
13	28	16.241	18.580	16.610	2.130
14	29	54.663	46.635	28.460	13.210
15	30	474.721	45.489	31.710	20.910
16	31	269.342	342.553	50.670	2.020
17	32	28.568	158.747	55.240	20.890
18	33	21.019	39.137	39.050	7.280
19	34	15.568	19.535	16.050	8.640
20	35	5.754	13.133	7.850	6.100
21	36	7.496	6.251	0.900	10.360
22	37	10.507	5.334	8.930	1.730
23	38	15.348	4.420	7.180	6.880
24	39	35.516	3.808	13.280	12.850
25	40	48.579	14.637	19.170	7.740
26	41	56.059	16.727	29.720	11.850
27	42	11.886	27.349	30.430	34.410
28	43	9.028	9.197	28.220	4.010
29	44	24.490	6.971	54.610	24.290
30	45	46.544	15.606	180.230	37.550
31	46	84.332	28.793	66.290	12.760
32	47	323.947	47.893	71.320	18.100
33	48	10.598	290.400	5.370	17.310
34	49	6.706	12.539	27.670	11.660
35	50	2.484	0.000	24.350	11.270
36	51	2.519	0.000	19.570	32.030
37	52	1.497	1.979	23.550	13.910
38	53	2.377	1.510	12.820	0.000
39	54	1.249	1.313	45.020	0.000
40	55	0.000	0.000	35.380	1.470

TABLE 24: Adult rat atrial cardiocytes after 7 days in culture.

tube	gradient %B	Immunoreactive ANF A	B	C	D	E	F
		pg/ml of medium					
0	15	0	0	0	0	0	0
1	16	1.670	0.000	5.193	0.000	1.249	2.617
2	17	0.886	0.000	1.247	0.000	3.391	0.739
3	18	1.574	0.000	1.960	0.000	2.261	1.063
4	19	15.998	0.000	3.191	0.000	0.000	1.733
5	20	4.199	0.000	2.697	0.725	6.881	4.700
6	21	2.577	2.162	2.605	0.171	28.037	4.671
7	22	10.043	2.443	5.310	0.000	11.021	6.085
8	23	9.336	1.083	1.137	10.294	7.833	5.701
9	24	0.000	9.111	4.738	1.656	18.567	23.844
10	25	8.457	8.001	6.014	2.505	41.449	14.499
11	26	5.084	10.745	7.211	0.371	30.242	16.851
12	27	20.540	22.819	5.141	5.513	40.261	101.494
13	28	9.550	24.588	12.411	0.407	151.336	47.865
14	29	42.966	21.419	9.462	4.080	1244.867	104.908
15	30	46.111	79.167	20.259	9.225	461.220	133.387
16	31	613.115	314.200	103.560	68.115	477.300	375.173
17	32	46.646	93.220	37.481	49.149	227.420	167.133
18	33	59.925	40.550	8.844	16.791	63.272	60.791
19	34	15.034	8.629	3.725	6.082	24.469	28.385
20	35	11.058	11.893	3.631	2.765	16.213	17.253
21	36	7.943	3.650	1.916	1.073	8.979	9.513
22	37	6.123	5.000	2.412	1.831	7.982	6.638
23	38	13.957	5.128	0.000	0.000	7.213	5.433
24	39	11.824	8.165	3.509	2.521	26.818	6.813
25	40	30.749	12.819	4.166	2.173	11.477	29.357
26	41	49.443	28.261	9.848	2.151	69.224	65.480
27	42	26.741	41.133	6.084	12.232	71.196	368.465
28	43	8.693	15.375	1.083	5.962	21.370	32.292
29	44	14.618	13.743	1.644	0.000	19.769	17.547
30	45	23.320	28.149	8.389	4.272	24.602	25.043
31	46	41.636	58.307	9.529	6.355	34.285	34.162
32	47	285.540	296.487	37.963	8.730	90.177	92.567
33	48	127.475	66.643	14.829	41.362	864.233	963.227
34	49	10.894	17.114	2.107	16.415	22.617	68.373
35	50	4.298	6.175	0.000	2.570	13.969	15.769
36	51	4.469	1.901	0.000	0.479	9.581	11.119
37	52	3.587	4.285	1.005	1.156	7.032	6.990
38	53	2.266	2.457	0.000	0.000	3.933	5.371
39	54	2.089	1.335	0.000	1.321	3.018	4.603
40	55	1.754	1.657	0.930	1.724	3.456	3.546

TABLE 25: Adult rat atrial cardiocytes after 10 days in culture

tube	gradient %B	Immunoreactive ANF A	B	C	D
		pg/ml of medium			
0	15	0	0	0	0
1	16	3.626	0.227	0.101	0.000
2	17	2.139	0.266	0.000	0.000
3	18	1.988	0.000	0.321	0.000
4	19	2.375	0.401	0.000	0.000
5	20	1.602	1.765	0.000	18.040
6	21	0.279	0.397	0.000	0.000
7	22	2.833	0.319	0.000	0.000
8	23	5.945	0.746	20.487	0.000
9	24	5.083	1.558	2.383	4.200
10	25	5.895	1.854	0.543	0.000
11	26	6.298	2.149	3.432	1.690
12	27	3.491	11.038	1.575	3.760
13	28	2.180	4.891	5.895	15.140
14	29	5.343	16.406	10.817	0.000
15	30	9.546	15.509	20.857	18.810
16	31	69.255	200.613	104.760	0.000
17	32	151.228	25.597	61.644	14.330
18	33	22.548	22.476	13.260	0.000
19	34	4.443	5.685	6.780	0.000
20	35	2.430	5.065	2.176	0.000
21	36	2.573	2.806	0.463	0.000
22	37	2.292	2.724	0.658	0.000
23	38	2.830	2.868	3.013	67.930
24	39	4.494	5.223	0.000	2.000
25	40	8.729	11.320	4.725	16.590
26	41	10.863	0.000	9.177	0.820
27	42	11.757	6.112	12.081	63.630
28	43	3.385	6.917	4.098	49.890
29	44	6.724	17.529	3.963	32.810
30	45	22.192	41.119	2.613	33.500
31	46	8.405	73.033	1.269	20.580
32	47	90.465	182.007	13.533	92.790
33	48	39.185	14.524	18.311	21.290
34	49	5.624	9.287	2.348	20.020
35	50	5.420	4.235	3.101	0.000
36	51	2.009	3.940	0.440	14.500
37	52	1.667	2.717	0.839	11.140
38	53	1.169	2.469	0.000	2.660
39	54	1.794	1.164	0.000	10.870
40	55	1.456	0.000	1.293	40.820

TABLE 26: Adult rat atrial cardiocytes after 15 days in culture.

tube	gradient %B	Immunoreactive ANF A	B	C	D	E
		pg/ml of medium				
0	15	0	0	0	0	0
1	16	1.194	0.401	2.287	0.000	0.000
2	17	1.811	0.269	1.724	0.373	0.000
3	18	2.622	0.634	0.000	0.300	0.000
4	19	2.693	0.691	0.000	0.000	0.000
5	20	1.898	0.220	0.000	0.000	0.689
6	21	1.638	0.083	0.000	0.000	0.186
7	22	2.266	0.922	0.000	0.000	0.000
8	23	1.351	0.821	0.260	0.000	0.000
9	24	13.123	3.455	0.000	0.000	0.000
10	25	4.069	1.443	0.000	0.763	0.000
11	26	3.846	3.579	0.000	0.173	0.325
12	27	4.260	13.441	0.300	0.501	5.491
13	28	5.675	8.839	0.854	0.832	1.685
14	29	5.045	17.258	2.447	2.767	6.276
15	30	12.906	49.007	0.813	1.363	5.113
16	31	17.322	205.880	13.693	3.621	35.324
17	32	95.385	35.624	10.787	2.901	2.919
18	33	140.449	21.416	3.552	17.584	3.793
19	34	31.219	8.209	0.000	2.300	0.597
20	35	6.855	6.957	0.344	3.943	0.739
21	36	7.675	6.221	0.000	1.596	0.357
22	37	4.179	5.353	0.000	1.505	0.072
23	38	3.611	4.405	1.099	0.681	0.000
24	39	5.437	4.413	0.000	0.000	0.000
25	40	2.979	11.449	0.000	0.600	1.323
26	41	5.365	21.991	0.607	0.141	0.867
27	42	20.561	7.200	1.941	0.313	0.471
28	43	3.858	6.648	6.123	1.625	0.000
29	44	15.578	17.461	0.315	1.087	1.546
30	45	12.680	11.519	10.085	1.249	0.709
31	46	8.390	26.993	2.570	1.119	3.709
32	47	68.925	85.887	9.867	1.029	17.132
33	48	33.462	10.043	5.327	4.494	1.188
34	49	2.036	9.990	5.380	20.315	0.691
35	50	2.350	6.023	1.631	2.099	0.532
36	51	1.745	3.813	0.000	0.000	0.225
37	52	1.043	2.411	0.227	0.257	0.141
38	53	1.374	1.791	0.282	0.079	1.205
39	54	3.523	1.835	0.475	0.404	0.000
40	55	1.223	1.659	0.000	0.000	0.570

TABLE 27: Effect of time in culture on released irANF post-translational processing by primary cultures of adult rat atrial cardiocytes

DAYS IN CULTURE	n	irANF(99-126)/ TOTAL irANF (%)
0	4	83.3 ± 6.7
1	4	84.1 ± 6.7
2	4	85.1 ± 7.7
3	2	71 ± 13
4	4	71.9 ± 6.7
5	4	61.05 ± 9.7
6	4	49.25 ± 8.6
7	6	60.3 ± 4.4
10	4	52.3 ± 14.8
15	5	62.7 ± 6.3

(mean ± sem)