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Effect of atrial natriuretic factor on cytosolic free calcium in rat glomerular mesangial cells

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Since atrial natriuretic factor (ANF) inhibits angiotensin II (ANGII)-induced mesangial cell contraction, we studied its effect on cytosolic free calcium in fura-2-loaded monolayers. ANF lowered basal calcium from 117 ± 5 to 89 ± 2 nM, and also reduced the ANGII-induced calcium transient. The absolute level of cytosolic calcium after ANGII was lower in cells preincubated with ANF compared to buffer. In a few cell lines, ANF had no effect on cytosolic calcium, while in all cell lines ANF had inhibited contraction. Thus, while ANF has effects on cytosolic calcium in mesangial cells, the vasorelaxant effects of ANF may require additional physiologic interactions.

Atrial natriuretic factor; cytosolic free Ca^{2+}; Tissue culture; Fura-2; (Mesangial cell)

1. INTRODUCTION

Atrial natriuretic factor (ANF) has well-known vasorelaxant properties [1]. In particular, we have shown that atriopeptin III (APIII) inhibits angiotensin II (ANGII)-induced contraction of cultured rat glomerular mesangial cells while the shorter fragment atriopeptin I (API) has no such effect [2]. Recent evidence in vascular smooth muscle suggests that ANF-induced vasorelaxation may be related to effects on cytosolic free calcium [3]. Therefore, we studied the effect of ANF on cytosolic free calcium in cultured rat glomerular mesangial cells, which have properties in common with vascular smooth muscle [4].

2. MATERIALS AND METHODS

The synthetic rat atrial natriuretic peptides atriopeptin I (API) and atriopeptin III (APIII) were purchased from Bachem (Torrance, CA). ANGII and arginine-vasopressin (AVP) were purchased from Calbiochem (San Diego, CA).

Glomerular mesangial cells were started from isolated glomeruli using the method of Lovett et al. [5]. Primary cultures were incubated for approx. 1 week until the cells approached confluence. The cells were subcultured, and experiments were performed on monolayers from passages 2–4.

In order to measure cytosolic free calcium, monolayers were grown on plastic coverslips (Aclar type 33c, Allied Engineered Plastics, Pottsville, PA). Prior to experiments, the cells were loaded with 1 μM fluorescent Ca^{2+} indicator fura-2 acetoxy methyl ester (Molecular Probes, Eugene, OR). The loaded coverslips were placed diagonally in a quartz cuvette containing Krebs-Henseleit-Hepes (KHH) buffer. Fluorescence was monitored continuously before and after addition of agonists using a single excitation wavelength of 339 nm, and an emission wavelength of 500 nm. These studies were performed at 37°C with constant mixing utilizing a fluorometer designed by the Johnson Foundation Biomedical Instrumentation Group. Cytosolic free calcium was calculated from the fura-2 signal using previously described

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formulae [6] after calibration of the signal by addition of 40 μM ionomycin (maximal fluorescence) followed by 7.5 mM EGTA/60 mM Tris-HCl, pH 10.5 (minimal fluorescence).

Experimental incubations designed to measure mesangial cell cyclic GMP (cGMP) responses were performed on mesangial cell monolayers. Triplicate determinations of cGMP were made of each experimental condition in each cell line. Dose-response data for the effect of APIII on intracellular cGMP were generated by the addition of the agonist at time zero, followed by a 3 min incubation that was ended by the removal of the experimental media followed by the addition of 0.1 N HCl, which extracts intracellular cyclic nucleotides from cell monolayers. cGMP was measured by radioimmunoassay, utilizing ^{125}I-tyrosine methyl ester cGMP (New England Nuclear) and rabbit antisera to cGMP (Kew Scientific). All samples were performed in duplicate after acetylation. There was no cross-reactivity of the cGMP antisera with APIII, 0.1 N HCl, or incubation media. Cellular protein was measured in each culture dish after an overnight digestion with 1 N NaOH according to Lowry et al. [7].

Results are expressed as means ± SE. Statistical significance was determined by use of Student's *t*-test for unpaired data. Data were considered statistically significant if $p < 0.05$.

3. RESULTS

Fig.1 portrays actual calcium fluorescence tracings under a variety of circumstances. In panels A–C, ANGII caused a dose-dependent increase in cytosolic free calcium with threshold at less than 1 nM. Basal calcium was approx. 125 nM. Panel D indicates that AVP was a somewhat more potent agonist than ANGII. Panel E points out that preincubation with EGTA lowered basal cytosolic calcium, but there was no change in the initial calcium transient induced by AVP. However, there

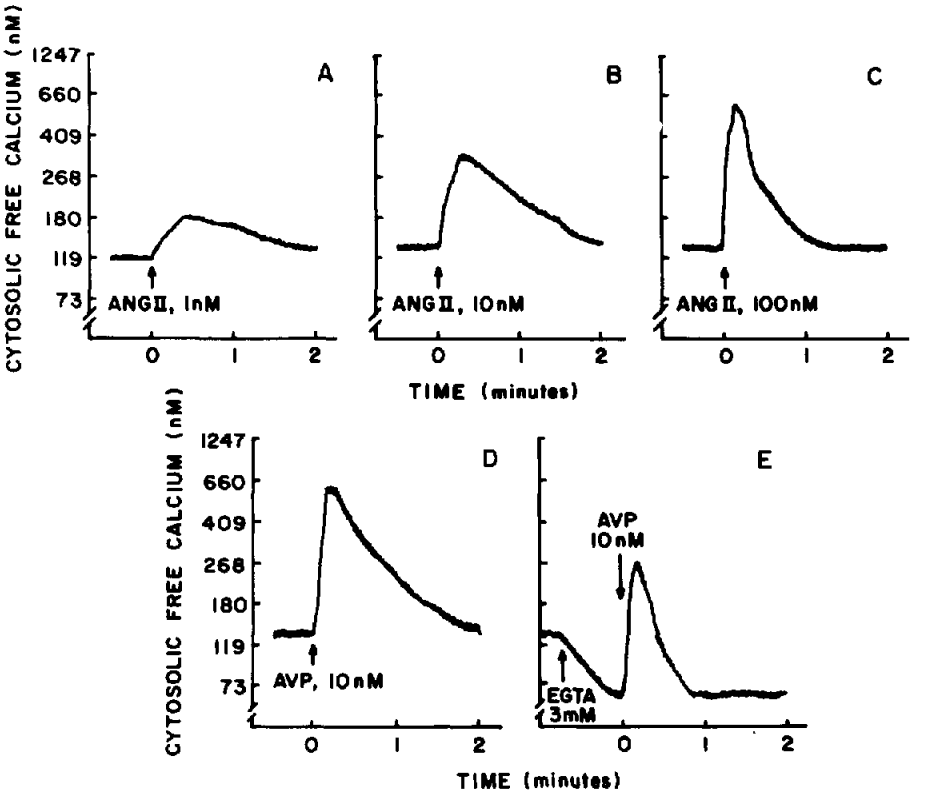

Fig.1. Continuous calcium fluorescence tracings in fura-2-loaded monolayers of cultured rat mesangial cells. (A–C) Dose response to ANGII. (D) Effect of AVP. (E) Effect of EGTA followed by AVP.

was a marked increase in the rate of return to basal. This indicates that there are two phases to the agonist-induced calcium increase; an initial transient based mainly on release from intracellular stores; and a sustained phase that is dependent to some extent on extracellular calcium.

In fig.2, panel A shows that a 3 min incubation with KHH buffer containing 1.25 mM calcium had no effect on basal cytosolic free calcium. In panel B, APIII (100 nM) added at time zero promptly resulted in a significant decrease in basal cytosolic free calcium. In this case, calcium dropped from 119 to 86 nM, a 28% decrease. The effect of APIII was prolonged, with only a very slight recovery after 3 min. The addition of 10 nM ANGII promptly increased cytosolic free calcium to 200 nM. The magnitude of this transient was slightly reduced compared to that seen in cells preincubated with buffer alone. In panel C, 1 μM API reduced basal calcium by 15% from 119 to 101 nM; there was no inhibitory effect seen on the ANGII-induced transient. Panel D superimposes the tracings from panels A–C. It can be seen that the absolute level of calcium reached after ANGII was significantly less in cells preincubated with APIII compared to either API or KHH.

There was no effect of APIII or API on cytosolic free calcium in 5 of 11 cell lines tested. In

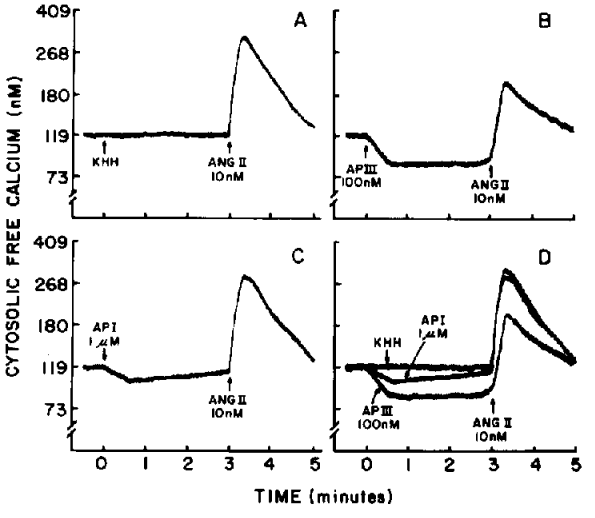

Fig.2. Continuous calcium fluorescence tracings in fura-2-loaded monolayers of cultured rat mesangial cells. Effects of preincubation with KHH buffer (A), APIII (B), and API (C). Panel D compares tracings.

Table 1

Mesangial cell cytosolic free calcium (nM)

Condition	R	NR	*n*
Basal	117 ± 5	113 ± 3	10
Post ANGII	304 ± 16	289 ± 19	10

Studies were performed in fura-2-loaded monolayers. Values represent means ± SE. R, responding cells; NR, non-responding cells. See text for details. ANGII concentration, 10 nM

the 6 cell lines that did respond, the results were very reproducible, with all monolayers showing similar findings. The cell lines that were nonresponsive to ANF in terms of cytosolic free calcium did respond appropriately to ANGII and AVP. As shown in table 1, there were no differences in cytosolic free calcium seen in the basal state, or post ANGII, in responding and nonresponding cells. In addition, as seen in table 2, APIII stimulated intracellular cGMP accumulation in nonresponding cells to an extent similar to that seen in cells responding to ANF with decrements in cytosolic free calcium. Thus, the nonresponsive cell lines probably still retain ANF receptors.

Table 3 expands on fig.2D by summarizing all the data on those cell lines which did show changes in cytosolic free calcium in response to ANF. Note that APIII, and to a lesser extent API, lowered basal cytosolic free calcium while KHH buffer had no such effect. The column labelled Δ represents the magnitude of the ANGII-induced calcium tran-

Table 2

Mesangial cell cGMP responses

Concentration	R	NR	*n*
Basal	4.2 ± 1.0	3.9 ± 1.9	4
APIII (10^{-8} M)	142 ± 48	163 ± 69	4
APIII (10^{-7} M)	1488 ± 158	2031 ± 198[a]	4

[a] $p = 0.07$ vs R, independent *t*-test

Values represent means ± SE of triplicate wells from 4 different cell lines. Intracellular cGMP expressed as fmol/μg protein per 3 min. R, responding cells; NR, nonresponding cells. See text for details

Table 3

Mesangial cell cytosolic free calcium (nM)

Agonist	n	Basal	Post agonist	Post ANGII	Δ
KHH	10	117 ± 5	117 ± 5	304 ± 16	188 ± 11
APIII	16	114 ± 3	89 ± 2[a]	213 ± 10[a]	124 ± 10[a]
API	12	115 ± 4	99 ± 6[b]	281 ± 20	181 ± 15

[a] $p < 0.001$ vs KHH, independent t-test
[b] $p = 0.0504$ vs KHH, independent t-test

Studies were performed in fura-2-loaded monolayers. Δ, cytosolic free calcium post ANGII minus cytosolic free calcium post agonist (the magnitude of the calcium transient). Values represent means ± SE. APIII concentration, 100 nM; API concentration, 1 μM

sient. This was depressed only by APIII. The net result of the decrease in basal and slight decrease in the transient was that the absolute level of cytosolic free calcium was lower in cells preincubated with APIII compared to API or buffer.

4. DISCUSSION

The explanation for the fact that not all cell lines respond to ANF with inhibitory effects on cytosolic free calcium remains unclear. The data in tables 1 and 2 indicate that the nonresponding cell lines are healthy and probably retain ANF receptors. Tissue culture itself certainly can alter in vivo cellular physiologic parameters and may be partly responsible for this dilemma. In addition, the presence of responding and nonresponding cell lines may indicate that there are subpopulations of mesangial cells subserving different functions.

The effect of ANF on cytosolic free calcium in vascular smooth muscle has been studied by others. Most investigators have found inhibitory effects although there has not been a uniformity of findings. Capponi et al. [8] found no effect of APIII on cytosolic free calcium in quin2-loaded rat aortic smooth muscle cells. On the other hand, Hassid [3] obtained inhibitory results very similar to those described herein in fura-2-loaded rat aortic smooth muscle cells. Specifically, APII-decreased basal and vasoconstrictor-elevated cytosolic free calcium. Others have found no effect of ANF on basal calcium, but inhibitory effects on vasopressor-induced ^{45}Ca efflux and/or influx [9–12]. In view of this lack of uniformity, it is difficult to pinpoint whether ANF interferes with release of calcium from intracellular stores, or inhibits calcium translocation through receptor-operated calcium channels, or enhances calcium extrusion, or some combination of these mechanisms.

What is the physiologic role of these ANF-induced calcium changes in ANF-induced vasorelaxation? Our previous work [2] demonstrated that APIII inhibited ANGII-induced contraction of cultured rat glomerular mesangial cells. This effect was not seen with the shorter fragment API. In the present work, fig.2D indicates that the absolute level of calcium reached after ANGII was significantly less in cells preincubated with APIII compared to either API or buffer. Thus, there is a correlation between the functional response and the physiologic parameter suggesting the possibility of a causal relationship. On the other hand, the inhibitory effect of APIII on ANGII-induced mesangial cell contraction [2] was seen in all cell lines tested, while, as described above, the inhibitory effects on cytosolic free calcium occurred in only 6 of 11 cell lines tested. This is a strong argument against the concept that ANF is vasorelaxant directly because of its effects on cytosolic free calcium. In this regard, a recent report studied the effect of ANF on calcium in rabbit aortic strips utilizing aequorin as a calcium indicator [13]. In this study, ANF inhibited both the norepinephrine-induced calcium transient as well as the norepinephrine-induced contraction. However, ANF had no effect on the ANGII-induced calcium transient while still significantly inhibiting the ANGII-induced contraction. Again, there is a dissociation between the physiologic parameter and the functional response.

In summary, we believe that while ANF has effects on cytosolic free calcium in vascular smooth muscle and glomerular mesangial cells, the vasorelaxant effects of ANF require additional, possibly more distal physiologic interactions.

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