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J Clin Invest. 1989;83(3):1061-1065. https://doi.org/10.1172/JCI113948.

Research Article

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Stretch-induced Atriopeptin Secretion in the Isolated Rat Myocyte and Its Negative Modulation by Calcium

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Abstract

Cellular mechanism(s) regulating atriopeptin secretion and processing by the atrial myocyte are currently unknown. Osmotic stretch of isolated atrial myocytes as well as potassium chloride depolarization were potent stimuli of atriopeptin secretion. Release was potentiated by buffering either extracellular calcium with EGTA or intracellular calcium with the intracellular chelator, BAPTA AM. Atrial release of atriopeptin was inhibited after administration of ionomycin which elevates intracellular calcium. Fetal or early neonatal ventricular myocytes actively synthesize atriopeptin. Atriopeptin secretion by ventricular myocytes was also markedly potentiated by osmotic stretch as well as KCl depolarization. Only the 126 amino acid prohormone was secreted by the stretch-stimulated atrial and ventricular myocyte. These data suggest that stretch of the myocyte plasma membrane is a major stimulus for atriopeptin secretion and that atriopeptin secretion is not stimulated by raising intracellular calcium and appears to be negatively modulated by this cation. Like the atrial myocyte, the ventricular myocyte possesses the cellular mechanism(s) necessary to secrete atriopeptin by a regulated mechanism.

Introduction

The heart has recently been demonstrated to play an important role in intravascular volume homeostasis. Mammalian atrial myocytes synthesize, store, and release a peptide hormone, atriopeptin (AP),[1] which has potent natriuretic, diuretic, and vasodilating activity (1–4). Thus, the heart is not only a pump but also an endocrine organ. Distension of the atrial chamber in vitro in isolated perfused hearts or in vivo stimulates release of AP (5–8); however, the precise cellular mechanism(s) coupling mechanical distension to hormonal release remains unresolved.

By analogy to other secretory systems (9) it has been postulated that a rise in intracellular calcium (Ca^{+2}) is a stimulus for AP release. This hypothesis is supported by the demonstration that AP release from the isolated rat heart is moderately enhanced by the Ca^{+2} ionophore A23187 (10) or the Ca^{+2} channel agonist BAY K8644 (11). The ventricular myocardium possesses trace amounts of AP immunoreactivity (APir) when compared with atrial tissue (12–15). Recently it has been demonstrated that mRNA coding for prepro-AP is present in both adult rat and human ventricle as well as newborn rat ventricular myocyte cell cultures, but at lower concentrations than atrial tissue or cultures (15). The abundance of AP mRNA and APir is increased in hypertrophied ventricles (12), ventricles from spontaneously hypertensive rats (13), and fetal and neonatal rat ventricles (14). In hypertrophied ventricles, the total mass of AP mRNA approaches one-third the amount present in the atrium (12). Pulse-chase experiments in cultured myocytes resulted in the suggestion that atrial myocytes secrete AP by a regulated process but that ventricular myocytes release the peptide constitutively (16). Unfortunately, these studies were restricted to the basal, i.e., unstimulated, release of AP. The physiological significance of ventricular AP and whether AP release in this tissue responds to physiological stimuli have yet to be determined.

The 126 amino acid AP prohormone is synthesized and stored in atrial-specific granules. In vivo, atrial stretch results in the rapid release and instant enzymatic cleavage of the prohormone to yield the 28 amino acid carboxy-terminal fragment (AP28), which has been demonstrated to be the circulating hormone (17). Cultured atrial myocytes release predominantly the 126 amino acid prohormone (18, 19) in the basal, nonstimulated state, whereas the isolated perfused heart releases only AP28 and no intact prohormone. It therefore appears that the myocardium (i.e., nonmyocytic cardiac cells) but not the myocyte has this enzymatic capacity for prohormone cleavage. It is possible, however, that pro-AP processing by cultured atrial myocytes has not been observed because the intracellular route of constitutively secreted pro-AP physically bypasses the processing enzyme and that stimulated (i.e., granular) secretion of AP may unmask myocytic, pro-AP processing.

To elucidate the cellular mechanism responsible for AP secretion and processing, we have examined stretch-induced AP release from suspensions of neonatal rat atrial myocytes. Furthermore, to evaluate whether ventricular AP secretion occurs via a regulated or constitutive pathway, we have also examined stretch-induced AP release from suspensions of neonatal rat ventricular myocytes. The use of myocyte suspensions rather than intact heart or isolated atrium offers a distinct advantage in that it is possible to study a nearly homoge-

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Received for publication 10 June 1988 and in revised form 9 November 1988.

1. *Abbreviations used in this paper:* AP, atriopeptin; APir, atriopeptin immunoreactivity; AP28, atriopeptin-28; LDH, lactate dehydrogenase; pro-AP, prohormone atriopeptin.

J. Clin. Invest.

0021-9738/89/03/1061/05 $2.00
Volume 83, March 1989, 1061–1065

neous population of cells in the absence of extrinsic neural or hormonal regulation.

Methods

Myocyte preparation and stimulation. Neonatal atrial and ventricular myocytes were prepared by the method of Porzig et al. (20). Myocytes were isolated from 4-d-old rat atrial appendages or from pieces of apical cardiac tissue, to ensure no atrial contamination from 1-d-old rat heart, by exposure to calcium-free HBSS and 0.06% trypsin. Single cell suspensions were enriched for myocytes by 1 h of preplating on tissue culture dishes (Falcon Plastics, Cockeysville, MD). Nonadherent myocytes were incubated for 15 min at 37° in an atmosphere of 5% CO_2, 95% air in the appropriate osmotic solutions. For stretch-stimulated experiments, osmotic solutions all contained equal concentrations of electrolytes (87 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose, 21.0 mM $NaHCO_3$, 1.0 mM $CaCl_2$, and 0.1% BSA). Osmolarity was altered with mannitol and measured using a vapor pressure osmometer (model 5500; Wescor Inc., Logan UT). AP was measured by RIA (21) and lactate dehydrogenase (LDH) activity was determined fluorometrically by the method of Lowry et al. (22). For depolarizing experiments, all samples were incubated in 310 mosM media replacing mannitol with equiosmolar KCl. Estimated membrane potential(s) was calculated according to the Nernst equation: $V = -58 \log K_i/K_0$, assuming that the intracellular potassium concentration = 140 mM. For experiments in which intracellular Ca^{+2} was manipulated, myocytes were preincubated in the presence or absence of BAPTA AM (Molecular Probes Inc., Junction City, OR) for 60 min before the addition of KCl or hypotonic solution. In other experiments, myocytes were stimulated with zero calcium solutions by adding 1 mM EGTA (Sigma Chemical Co., St. Louis, MO) to KCl or hypotonic solutions made in nominally calcium-free water. To study the effects of raising intracellular Ca^{2+}, 200 nM ionomycin (Calbiochem-Behring Corp., La Jolla, CA) was added to the hypotonic solution. To avoid changes in ionic composition, control and test solutions varied only with respect to the concentration of mannitol (an inert, osmotically active substance), which was used to adjust the osmolarity.

Intracellular calcium determinations. Approximately 1,000,000 neonatal rat atrial myocytes were incubated with INDO I AM (10 μM) for 30 min at 37°C. Extracellular INDO I AM was removed by repeated centrifugation and suspension of cells in standard 300 mosM solution. Intracellular INDO I was excited at 350 nm while fluorescence was continuously monitored at 405 and 480 nm. Time-dependent changes in intracellular Ca^{2+} were monitored during the addition of 3.0 ml of either 200 mosM or 87 mM KCl solution to the cuvette after basal measurements were made. This resulted in a final osmolarity of 225 mosM in stretch experiments and 65 mM KCl in depolarization experiments. Cellular dilution was not a problem since fluorescence was continously monitored at 405 and 480 nm and a ratiometric method was used for calculating intracellular Ca^{+2}. Furthermore, control experiments were performed that involved isotonic dilutions (as above) to evaluate whether dilutional factors could result in spurious calcium determinations.

Results

To develop an experimental model of stretch-induced AP release from myocyte suspensions, we reasoned that if the sarcolemma is the site of mechano-chemical transduction, then any manipulation that increases cell volume should "stretch" the cell membrane and thus result in AP release. Increases in cell volume (i.e., stretch) were produced by exchanging the isotonic suspension solution for hypotonic solutions. Roos (23) has demonstrated that the isolated myocyte undergoes ~ 50% of the predicted volume change upon reduction of extracellular osmolarity (300 to 200 mosM).

Rat neonatal atrial myocyte suspensions released AP as an inverse function of solution osmolarity (Fig. 1). Myocytes suspended in 200-mosM media for 15 min released fivefold the amount of AP released into isotonic (310 mosM) solution. Release produced by maximal stretch (200 mosM) represented ~ 20% of total AP intracellular stores, measured after extraction with acetic acid (1 N). Examination of AP release as a function of solution osmolarity demonstrated a nearly linear relationship between 200 and 300 mosM. Secretion was not potentiated by suspension of cells in hypertonic (350 mosM) media, although in several experiments slight reductions in AP release were observed. It was necessary, however, to confirm that peptide release was not a result of cell death or hypotonic lysis. The activity of LDH released into solutions during hypotonic stimulation was measured and taken to be an index of cell damage. As illustrated in Fig. 1, no significant relationship existed between LDH activity and solution osmolarity. The proportion of LDH in the cellular media as a function of total cellular LDH was determined to be < 5% in all experimental conditions, further demonstrating a > 95% viability of our suspended cells. We therefore conclude that hypotonic swelling (stretch) is a specific stimulus for AP release without cell lysis.

The molecular form of AP released by osmotic stretch was analyzed by HPLC. The immunoreactive AP released into 200 mosM solution comigrated at the position of the 126 amino acid, AP prohormone standard (data not shown), which suggests that the processing enzyme is not associated with the isolated atrial myocyte.

To understand the biochemical cellular processes involved in stretch-stimulated AP release, we evaluated the importance of cytosolic Ca^{+2} as a potential intracellular messenger. As cardiac myocytes have a large voltage-activated Ca^{+2} conductance, we evaluated whether cellular depolarization with KCl results in AP release as a direct result of Ca^{+2} influx through voltage-activated channels. KCl (25–65 mM) produced a dose-dependent release of AP (Fig. 2). Examination of the measured AP release as a function of the membrane potential, predicted from the Nernst equation for potassium, revealed a marked voltage dependence of the release process. Interestingly, the potential range over which AP release rises most rapidly is remarkably close to the potential at which the slow inward Ca^{+2} current begins to activate (24).

If, indeed, Ca^{+2} stimulates AP release, we hypothesized that both sarcolemmal stretch and depolarization with KCl should similarly produce increases in intracellular Ca^{+2}. Intra-

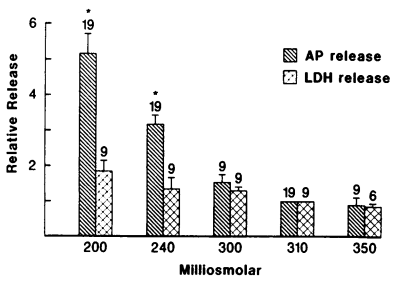

Figure 1. AP and lactate dehydrogenase release in response to osmotic stress from atrial myocytes. Numbers above the bars represent individual experiments on suspensions containing ~ 1,000,000 cells. All values are normalized to the amount of AP released into 310 mosM solution during parallel experiments. Data is reported as the mean±SEM. *$P < 0.01$.

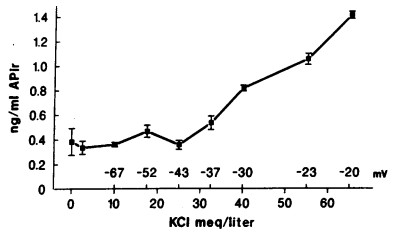

Figure 2. AP release in response to KCl depolarization from atrial myocytes. Data points represent the mean±SEM and determinations were performed in quadruplicate. The estimated membrane potential is exhibited on the abscissa above the corresponding KCl concentration.

Table I. Calcium Dependence of AP Release

Experiment	1 mM $[Ca^{++}]_o$	1 mM EGTA	BAPTA AM
			(10 μM)
300 mosM	1	1.11±0.10	1.34±0.07*
KCl (*65 meq/liter*)	2.24±0.04	3.02±0.35*	4.35±0.33*
200 mosM	7.69±0.74	12.78±0.74*	10.42±1.36

AP levels are normalized to the amount of AP released into the standard 300 mosM solution (containing 1 mM calcium). In these experiments basal AP values were 0.4 ng/ml. Data represents the mean±SEM from a minimum of three determinations.
* $P < 0.05$ vs. 1 mM $[Ca^{+2}]_o$.

cellular Ca^{+2} in atrial myocytes was monitored using the membrane permeable Ca^{+2} indicator, Indo I AM. Exposure of isolated myocytes to 225-mosM solutions produced an approximately twofold increase in intracellular Ca^{+2}. Depolarization of atrial myocytes with 50 mM KCl produced a similar but smaller increase in intracellular Ca^{+2}. The Ca^{+2} increase in response to both stretch and KCl depolarization was maximal within 20 s. Ca^{+2} increased from 82±7 nM to 187±24 nM (n = 7) and 121±22 (n = 4) in the presence of 225 mosM solution and isotonic solutions containing 50 mM KCl, respectively. As no increase in Ca^{+2} was observed when extracellular Ca^{+2} was buffered with 1 mM EGTA, it appeared that this increase in cytosolic Ca^{+2} was dependent on Ca^{+2} influx.

If KCl and stretch-stimulated AP release resulted from Ca^{+2} influx, then one would predict that AP release would be reduced or abolished in the absence of extracellular Ca^{+2}. AP secretion in response to 65 mM KCl or hypotonic (200 mosM) Ca^{+2}-free solution (buffered with 1 mM EGTA) was compared with peptide release evoked under similar conditions in the presence of 1 mM $CaCl_2$. Surprisingly, basal as well as stimulated AP release appeared to be enhanced rather than inhibited in the absence of extracellular Ca^{+2} (Table I). To further evaluate the role of Ca^{+2} and its ability to regulate AP release, we examined the effects of intracellular Ca^{+2} buffering and Ca^{+2} loading of myocytes on AP release. Intracellular Ca^{+2} was buffered by exposure of cells to the membrane permeant analogue of the chelator BAPTA. Within the cell, BAPTA AM is hydrolyzed to liberate the active chelator which is unable to cross hydrophobic membranes (25). BAPTA AM has been used effectively to block lectin-stimulated mast cell degranulation, which has been demonstrated to be a Ca^{+2}-dependent process (25). Atrial myocytes preincubated with BAPTA AM for 1 h exhibited a 34% increase in basal AP release and a greater response to both KCl depolarization and osmotic stretch when compared with cells not exposed to the chelator (Table I). Furthermore, Ca^{+2} loading of cells by exposure to the ionophore, ionomycin (200 nM), inhibited basal AP secretion by 19%. When cells were preincubated with 200 nM ionomycin and then subjected to maximal osmotic stretch (200 mosM), no stimulated secretion of AP was detected when compared with basal release. These data taken together strongly suggest that Ca^{+2} is not the intracellular stimulus for AP release. In contrast, increasing cytosolic Ca^{+2} may indeed be a negative modulator of the release process.

Because ventricular AP mRNA and APir is increased in ventricular tissue by aortic banding (12) and hypertension (13), manipulations that result in ventricular hypertrophy and elevated diastolic myocardial pressures, we hypothesized that stretch of the ventricular myocyte sarcolemma would stimulate AP secretion if regulated secretory mechanism(s) existed and were functional in these cells. Neonatal ventricular myocytes released AP as an inverse function of solution osmolarity (Fig. 3 *A*). Ventricular myocytes suspended in 200 mosM media released threefold the amount of AP released into isotonic (300 mosM) solution. This amount of AP release represented ~ 6% of the total cellular AP. To further evaluate AP secretion in response to a second potential ventricular AP secretagogue, we exposed neonatal ventricular myocytes to two concentrations of KCl that were effective in stimulating atrial AP release. As seen in Fig. 3 *B*, depolarization of ventricular myocytes with KCl produced a dose-dependent release of AP into the cellular media. We conclude from these data that neonatal ventricular myocytes, similar to atrial myocytes, release AP in response to sarcolemmal stretch and KCl depolarization, therefore demonstrating the potential of these cells to release AP in a regulated, nonconstitutive manner.

Discussion

The model of plasma membrane stretch used in these studies results from the initial swelling of the cells in hypotonic media. Therefore, plasma membrane stretch is a result of intracellular forces, different from the mechanism of stretch in the working heart. However, recently it has been demonstrated that hypotonic swelling of isolated cells results in the opening of cation-selective Ca^{+2}-permeable channels (26). These are the same channels that have been previously shown to respond to mechanical stretch (27). Therefore, hypotonic swelling of cells appears to modulate membrane events in a manner analogous

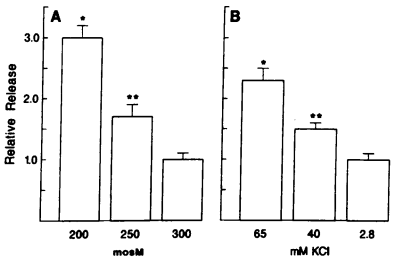

Figure 3. AP release in response to osmotic stress and KCl depolarization from ventricular myocytes. Each value represents the mean±SEM of four individual experiments. All values are normalized to the amount of AP released into 300 mosM solution during parallel experiments. *$P < 0.01$; **$P < 0.05$.

to extracellular stretch. AP release appears to be a Ca^{+2}-independent process. Osmotic swelling and KCl depolarization stimulate AP release (Figs. 1, 2) from atrial myocytes and produce increases in the cytosolic Ca^{+2} concentration. The removal of extracellular Ca^{+2} (Table I), however, prevents the observed changes in intracellular Ca^{+2} while potentiating AP release. Furthermore, AP release is potentiated after pretreatment of cells with the membrane-permeable Ca^{+2} chelator, BAPTA AM (Table I) either in the presence or absence of stimulated AP secretion. The small increase in basal AP secretion after pretreatment with BAPTA AM probably reflects the decrease in basal intracellular [Ca^{+2}] and does not represent an effect on cell viability since the agent has been successfully used in the identical concentration to inhibit mast cell degranulation, a Ca^{+2}-dependent process (25). AP secretion is inhibited significantly upon application of the Ca^{+2} ionophore, ionomycin. Recently, Hintze et al. (28) demonstrated that in the conscious dog, AP secretion best correlates with atrial wall stress measured during passive diastolic, not active systolic, atrial filling, a time when intracellular Ca^{+2} concentrations will be at a nadir. These data taken together suggest that AP release is temporally related to, but not contingent upon, a rise in intracellular Ca^{+2}. Ca^{+2} appears to negatively modulate AP release in a manner similar to that observed for renin (29) and parathyroid hormone (30) secretion. In contrast to this finding, BAY K8644 and A23187 stimulate AP release from isolated rat heart. It is possible that AP release under these circumstances reflects the Ca^{+2}-dependent release of catecholamines from residual neural elements or due to the increased inotropic and chronotropic effect and the resultant increased oxygen demand of these agonists on the intact myocardium. Hypoxia or pacing of isolated hearts or atria have been shown to increase AP release (31, 32).

The finding that the hypertrophied ventricle is associated with an increase in tissue AP mRNA, APir (12), and electron-dense granules that are morphologically similar to atrial AP–containing granules (33), demonstrates that the ventricle can be induced to store AP and suggests the existence of regulated secretory mechanisms in this tissue. Our finding that the neonatal ventricular myocyte can release AP as a function of solution osmolarity (i.e., stretch) and depolarization with KCl, secretagogues that also stimulate atrial AP release, demonstrates that the ventricle, like the atria, is able to synthesize then store AP for release in response to similar cellular signals. In contrast, Bloch (16) demonstrated that neonatal ventricular myocytes in culture do not store AP but immediately secrete the newly synthesized peptide by constitutive pathway(s). Because of the markedly different methods used in our study compared with that of Bloch et al. (16) such as cell suspensions vs. cultured cells, 1- vs. 4-d-old ventricular myocytes, and stimulated vs. basal AP secretion, the two studies cannot be readily compared. However, it is possible that as ventricular myocytes age and differentiate in culture, they lose the ability to initially store then synthesize AP as does the intact ventricle during its development to adult tissue (14).

Neonatal atrial myocytes secrete only the unprocessed AP prohormone during basal and stretch-stimulated AP secretion. These data suggest that the neonatal atrial myocyte under normal physiological conditions does not possess AP prohormone processing capabilities. However, a recent report by Shields et al. (34) demonstrated that AP28 can be extracted from the medium of neonatal rat atrial myocytes cultured in the presence of hydrocortisone. Differences in our results vs. those of Shields are probably a result of the markedly different treatment of the neonatal myocytes. First, the cells used in our protocol were studied immediately after enzymatic dissociation from neonatal atria, a tissue previously demonstrated to process the AP prohormone (35), while cells were maintained in tissue culture for 7–9 d in the study of Shields (34). Furthermore, it is indeed possible that glucocorticoids can pharmacologically induce the expression of a proteolytic enzyme that processes AP prohormone in cultured myocytes; however, the physiological significance of this has yet to be determined. We conclude that another cell type within the myocardium is required for the generation of the 28 amino acid peptide.

Acknowledgments

We thank S. Mills for help with the INDO I spectrofluorometry, O. Lowry and M. Chi for help in developing our LDH assay, and S. Sides for technical support.

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