

## Chapter 12

# Early Activation of Intracellular Signals after Myocardial Stretch: Anrep Effect, Myocardial Hypertrophy and Heart Failure

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### 12.1 Introduction

The link between the Anrep effect and myocardial hypertrophy and failure, although obvious—since this effect is the result of myocardial strain—was not appreciated until we proposed it in the 2005 edition of the book “Mechanosensitivity in Cells and Tissues”. Previous experiments by Izumo and Sadoshima (Sadoshima et al. 1993), Ito et al. (1993) and Yamazaki et al. (1996) performed in neonatal cardiomyocytes showed that mechanical stretch induces the release of preformed angiotensin II (A2) to the surrounding media, yet these findings were never linked to the Anrep effect, a phenomenon originally described in the open chest dog model (von Anrep 1912). Furthermore, Ito et al. (1993) showed that in stretched-conditioned medium where A2 was released, the hypertrophic signals were abolished by interfering with the endothelin (ET) action, finding that is in agreement with our own results demonstrating that stretching adult myocardium releases A2 (or activates the AT1 receptor by deformation) triggering the release and/or formation of ET (for review see Cingolani et al. 2011a).

### 12.2 The Anrep Effect

Although the contractile performance of the heart is under continuous neurohormonal and electrophysiological influence, the heart possesses intrinsic mechanisms, adapting to different hemodynamic conditions by changing its cardiac output. An

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increase in ventricular end diastolic volume, induced by increasing aortic resistance to ejection or venous return, leads to a more powerful contraction. This occurs immediately and is the well-known Frank-Starling mechanism that allows the heart to increase its output after an increase in venous return or to eject the same stroke volume against a greater afterload. However, over the next 10 or 15 min after the sudden stretch, there is a further increase in myocardial performance and the end diastolic volume returns towards its original value. The time constant of this phenomenon will depend on several factors such as species differences, temperature, coronary blood flow, etc. In 1912, von Anrep showed that after clamping the ascending aorta in a dog (acutely decreasing outflow and increasing intraventricular pressures), its heart initially dilated. This was followed by a progressive decline in heart volumes towards initial values over the next minutes (von Anrep 1912). Von Anrep interpreted these findings as secondary to a positive inotropic effect mediated by the release of catecholamines by the adrenal glands, which were receiving low blood flow. In 1959 Rosenblueth et al. (1959) called attention to the fact that both, an increase in heart rate (Bowditch effect) and in afterload augmented the contractility in the isolated canine right ventricle, “the two staircase phenomenon”. Sarnoff, in 1960, coined the term “homeometric autoregulation” to define the decrease in left ventricular end diastolic volume after the initial increase in diastolic volume that occurs after an increase in afterload (Sarnoff et al. 1960). Since both reports (Rosenblueth et al. 1959; Sarnoff et al. 1960) were based on experiments performed in isolated hearts, the possibility of a positive inotropic effect due to the release of catecholamines by the adrenal glands was ruled out.

In 1973, Parmley and Chuck reproduced this phenomenon in isolated strips of ventricular myocardium (Parmley and Chuck 1973). They showed that if the length of the muscle was increased, there were corresponding rapid and slow increases in developed force. The rapid change in force is thought to be the basis of the Frank-Starling mechanism and occurs secondary to an increase in myofilament  $\text{Ca}^{2+}$  sensitivity (Hofmann and Fuchs 1988). The slow force response (SFR) after a change in length is due to a progressive increase in the  $\text{Ca}^{2+}$  transient, as demonstrated by Allen and Kurihara in 1982 (Allen and Kurihara 1982) and later on confirmed by other authors, including us (Kentish and Wrzosek 1998; Alvarez et al. 1999). Parmley and Chuck also ruled out the possible role played by catecholamines at the nerve endings in the development of the SFR, since the response was also present in isolated muscles from reserpinized animals (Parmley and Chuck 1973).

Although the cellular and molecular bases of the Frank-Starling mechanism (or “heterometric autoregulation”) are well-known and involve mainly an increase in the response of cardiac myofilaments to calcium (Hofmann and Fuchs 1988), the mechanism of the Anrep effect (homeometric autoregulation) is less understood. It is accepted that the increase in cardiac contractility that develops during the 10–15 min following muscle stretch can be quantitatively explained by a progressive increase in calcium transients (Allen and Kurihara 1982; Kentish and Wrzosek 1998; Alvarez et al. 1999). However, the source for this increase in calcium was less understood. It could not be explained by a hyperactive sarcoplasmic reticulum (SR) (Kentish and Wrzosek 1998) nor by an increased transarcolemmal calcium current (Hongo et al.

1996). The mechanism leading to the increase in the calcium transient was clarified by experiments performed in our laboratory that demonstrated a link between calcium influx and an autocrine/paracrine response to muscle stretch (Cingolani et al. 1998, 2003a; Alvarez et al. 1999; Perez et al. 2001, 2011; Caldiz et al. 2007; Villa-Abrille et al. 2010).

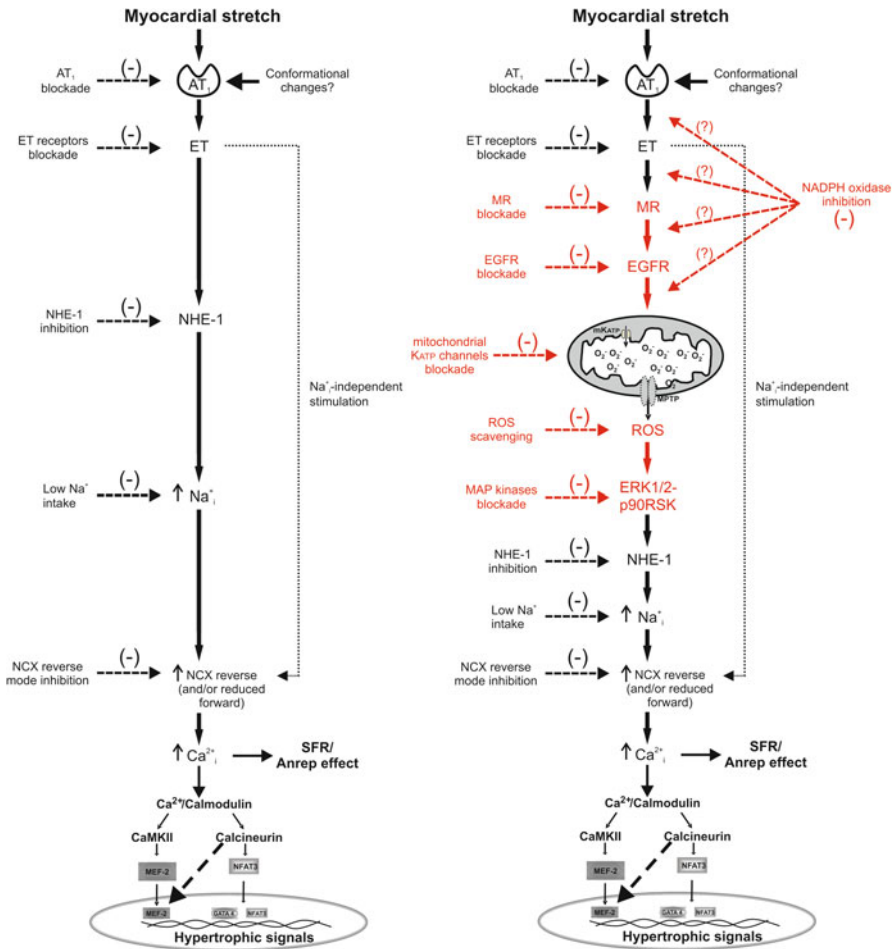
### 12.3 The Autocrine/Paracrine Loop Triggered by Myocardial Stretch

As stated before, the stretch of cardiac muscle increases developed force in two phases. The first phase, which occurs rapidly, is generally attributed to enhanced myofilament responsiveness to calcium and is probably not affected by the autocrine/paracrine mechanism. The second phase (SFR) occurs gradually and is due to an increase in the calcium transient amplitude as a result of the autocrine/paracrine mechanism. The SFR was proposed to be the *in vitro* equivalent of the Anrep phenomenon and its genesis was unknown until we proposed that  $\text{Na}^+/\text{H}^+$  exchanger (NHE-1) activation was the main step in the autocrine/paracrine mechanism leading to the increase in contractility by increasing intracellular sodium and calcium (Alvarez et al. 1999).

Most intracellular pathways leading to cardiac hypertrophy and failure are triggered by increases in intracellular calcium levels. Actually, the rise in cardiac muscle calcium causing the SFR or Anrep effect occurs as fast as 10–15 min after stretch. It is surprising that most investigators working in the field of excitation-contraction coupling and cardiac mechanics have not established a link between the Anrep effect and cardiac hypertrophy and failure. Interestingly, while several years ago we proposed the crucial role of the NHE-1 in the SFR development, more recently, elegant experiments by Wakabayashi's group demonstrated that NHE-1 activation is sufficient to generate calcium signals causing cardiac hypertrophy and failure (Nakamura et al. 2008).

An attractive idea, albeit speculative, will be that the fate of the myocardium may be determined during the first few minutes after stretch (i.e. it is possible that a pharmacological intervention that prevents the development of the Anrep effect might blunt the subsequent hypertrophy and failure). Approximately 23 million people are affected with heart failure, and 2 million new cases of heart failure are diagnosed each year worldwide. All the basic studies mentioned in this chapter need to be considered when designing new therapeutic strategies in the treatment of cardiac hypertrophy and failure. A clear understanding of the early triggering mechanisms that stretch imposes to the myocardium will allow us to design novel weapons to win the battle against this major disease.

In the next sections, we will present the experimental evidence that led us to propose the autocrine/paracrine mechanism underlying the SFR, as well as its resemblance to signals that have been described for cardiac hypertrophy development and heart failure.

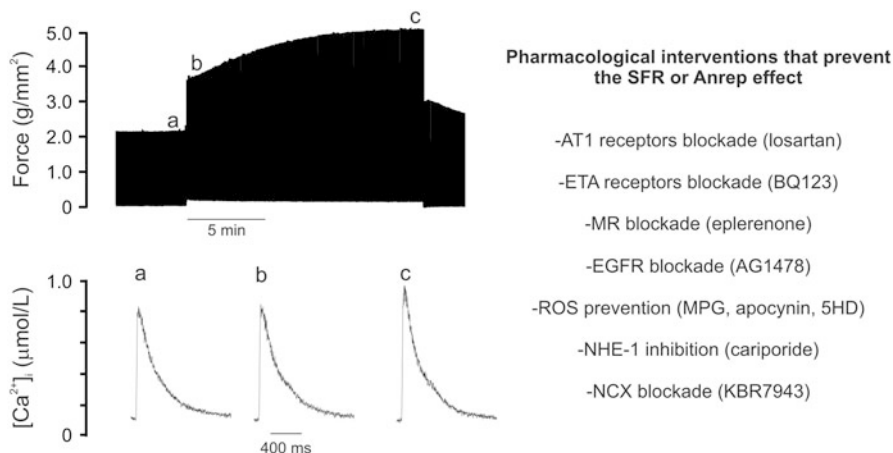


**Fig. 12.1** State of knowledge of the chain of events triggered by myocardial stretch at 2005 (*left* panel) and the updated sequence at 2011 (*right* panel)

### 12.4 Recent Advances in the Anrep Effect and Myocardial Hypertrophy and Failure

Figure 12.1 (left panel) depicts the state of knowledge on this subject when we wrote the chapter in the 2005 edition of the present book. In summary, the chain of events hypothesized at that time comprised the following: (1) AT<sub>1</sub> receptor activation, (2) release/formation of ET, (3) NHE-1 hyperactivity, (4) increase in intracellular Na<sup>+</sup> concentration, and (5) increase in Ca<sup>2+</sup> transient amplitude through the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX).

On the right panel of Fig. 12.1 we present the recent advances in this particular field, stressing the idea that myocardial hypertrophy and failure begins with cardiac



**Fig. 12.2** Left: Contractile response to stretch of an isolated papillary muscle. The first increase in force (from “a” to “b”, *top*) occurs without changes in the  $\text{Ca}^{2+}$  transient (“a” to “b”, *bottom*) while the SFR (from “b” to “c”, *top*) is due to an increase in the  $\text{Ca}^{2+}$  transient (“b” to “c”, *bottom*). Right: Pharmacological interventions proved to inhibit the SFR. (Modified with permission from Cingolani et al. (2001))

strain-triggered intracellular pathways that are in part common to hypertrophy and failure development and the mechanical counterpart, the so called SFR. Our updated proposal is that the chain of events triggered by myocardial stretch is as follows: (1) release of A2, (2) release/formation of ET, (3) MR activation, (4) transactivation of the EGFR, (5) NADPH oxidase activation, (6) mitochondrial reactive oxygen species (ROS) production, (7) activation of redox-sensitive kinases, (8) NHE-1 hyperactivity, (9) increase in intracellular  $\text{Na}^+$  concentration, and (10) increase in  $\text{Ca}^{2+}$  transient amplitude through the NCX.

The left panel of Fig. 12.2 shows the typical behavior of a control papillary muscle before and after stretch. The first increase in force occurs immediately after stretch without changes in the  $\text{Ca}^{2+}$  transient and is attributed to the Frank-Starling mechanism. The SFR is the mechanical result of a chain of intracellular signals triggered by the stretch that ends with an increase in the  $\text{Ca}^{2+}$  transient. The pharmacological interventions that abolished the SFR or Anrep effect are summarized in the right panel of Fig. 12.2. Note the relationships between these interventions and the therapeutic treatments used to regress myocardial hypertrophy or to treat heart failure.

During the 5–6 years after the 2005 chapter was written, the followings steps were added to the sequence of the SFR generation:

#### **12.4.1 The Critical Role of Mitochondrial ROS in the Activation of Redox Sensitive Kinases Leading to the Anrep Effect**

The participation of ROS as intracellular signalling markers of A2/ET-1 in the myocardium is a well accepted fact (Sugden and Clerk 2006). In fact, we have

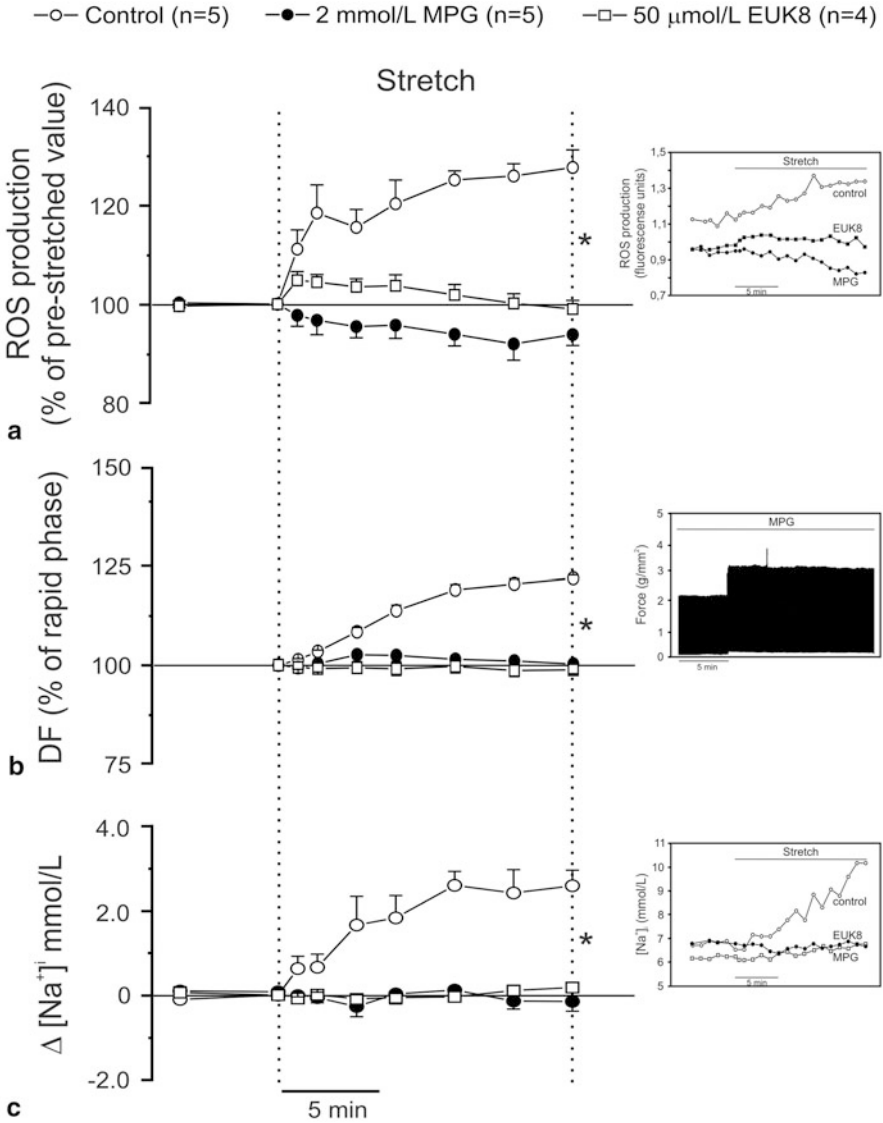
demonstrated that a low dose of A2 (1 nmol/L) increases sarcomere shortening of isolated cat cardiomyocytes through an autocrine crosstalk with endogenous ET-1 (Cingolani et al. 2006), being this effect dependent on ROS production. Both peptides, A2 and ET-1, are well known activators of the NADPH oxidase (Giordano 2005; Kimura et al. 2005b) and through this action it has been reported a phenomenon called “ROS-induced ROS-release”, by which a small amount of ROS triggers a greater ROS production from the mitochondria (Zorov et al. 2000; Brandes 2005; Kimura et al. 2005a). The fact that ROS were implicated in myocardial strain-triggered hypertrophy (Pimentel et al. 2006), lead us to explore the possibility that the ROS-induced ROS-release mechanism would underlie the SFR. Figure 12.3a shows that stretch -in addition to its mechanical effect- induces an increase in intracellular ROS formation of approximately 30 % above baseline levels. Furthermore, scavenging ROS with N-(2-mercapto-propionyl)-glycine (MPG) or EUK8 inhibited both stretch-induced increase in ROS (Fig. 12.3a) and the SFR (Fig. 12.3b). We also found that ROS scavenging inhibited the increase in  $[Na^+]_i$  that occurs in response to stretch (Fig. 12.3c).

These results allow us to hypothesize that activation of NADPH oxidase after stretch would produce a small amount of  $O_2^-$ , which may open the ATP-sensitive mitochondrial potassium (mKATP) channels and produce a larger amount of  $O_2^-$  enough to generate the SFR. Experimental evidence supports these assumptions since the SFR was abolished after NADPH oxidase inhibition (apocynin or diphenyleneiodonium chloride, DPI) or after blockade of mKATP channels (5-hydroxydecanoate, 5HD, or glibenclamide) (Fig. 12.4a). Furthermore, the NHE-1-induced increase in  $[Na^+]_i$  underlying the SFR was also abolished by these interventions (Fig. 12.4b).

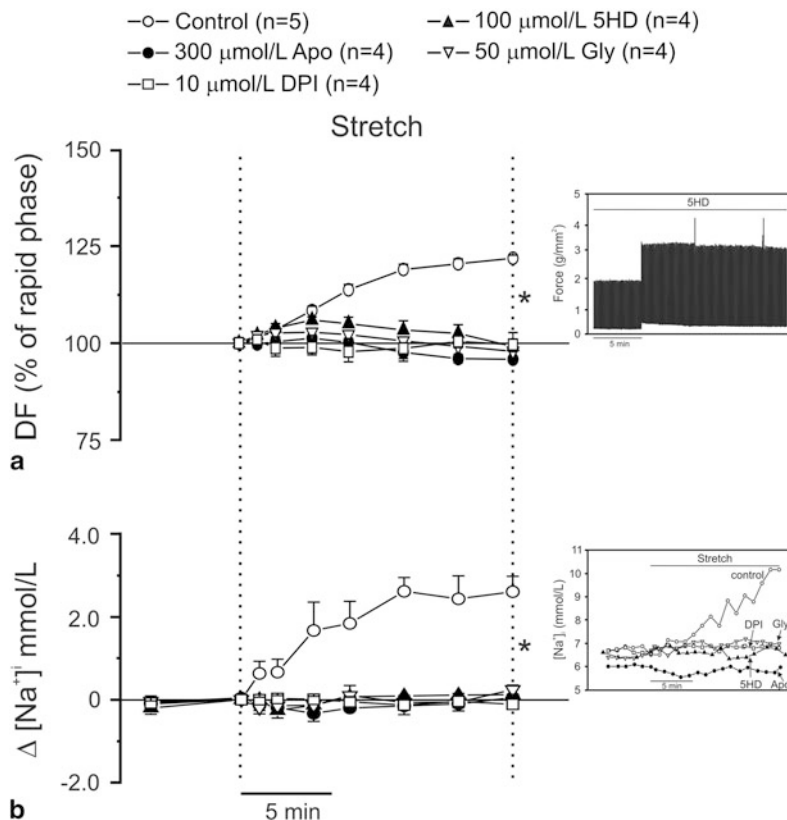
In this context, it appears reasonable to speculate that stretch-mediated mitochondrial ROS production leads to phosphorylation and activation of the NHE-1. Actually, ROS-mediated activation of NHE-1 has been reported to be due to redox sensitive kinase-mediated phosphorylation of the exchanger cytosolic tail, being MEK, ERK1/2 and p90rsk kinases the favourite candidates (Rothstein et al. 2002). In this regard: (1) RAS-dependent activation of these kinases has been reported after stretch in neonatal cardiomyocytes (Pimentel et al. 2006); (2) we have detected significant increases in ERK1/2 and p90rsk phosphorylation after stretch (Fig. 12.5) that were abolished with 1  $\mu$ mol/L losartan (Fig. 12.5); and (3) inhibition of MEK (a kinase upstream ERK1/2) also blunted the SFR (Fig. 12.5).

#### ***12.4.2 The Role of Epidermal Growth Factor Receptor (EGFR) in the SFR Development***

It has been recently established that transactivation of the EGFR is the primary mechanism underlying G-protein coupled receptor (GPCR) agonist activation of ERK1/2 and its downstream intracellular pathways (Lemarie et al. 2008). Furthermore, myocardial stretch (Anderson et al. 2004; Duquesnes et al. 2009), myocardial hypertrophy (Kagiya et al. 2002), and ET-1 signaling (Asakura et al. 2002) have



**Fig. 12.3** Myocardial stretch induced an intracellular ROS increase of ~30% above the baseline levels that was cancelled by the ROS scavengers MPG and EUK8 (Panel a). MPG and EUK8 also cancelled the slow force response (expressed as percent of initial rapid phase) (Panel b). Furthermore, ROS scavenging also blunted stretch-induced increase in (Na<sup>+</sup>); (Panel c). Insets show original raw data. \* indicates P < 0.05 control vs. MPG and EUK8. DF = developed force. (Modified with permission from Caldiz et al. (2007))

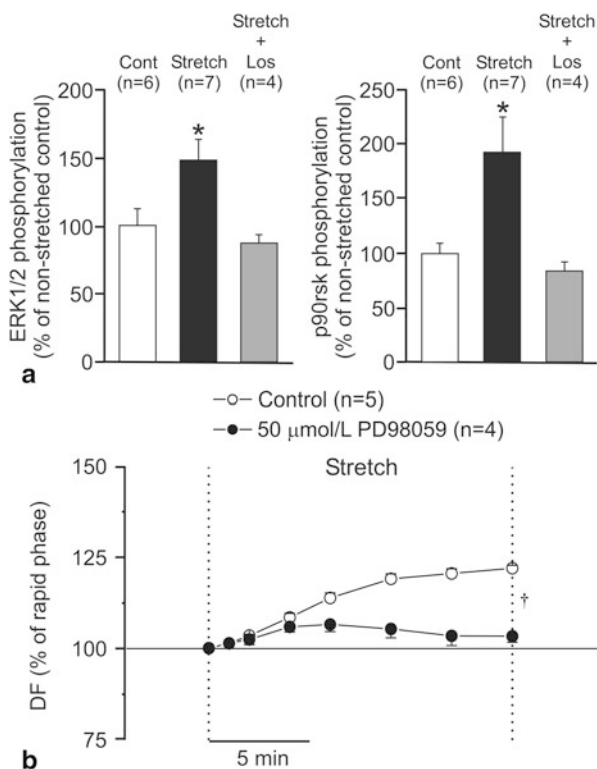


**Fig. 12.4** NADPH oxidase inhibition by apocynin (*Apo*) or diphenyleneiodonium chloride (*DPI*) as well as  $mK_{ATP}$  channels blockade with 5-hydroxydecanoate (*5HD*) or glybenclamide (*Gly*) abolished slow force response (expressed as percent of initial rapid phase) (Panel **a**). All these interventions also cancelled NHE-1-mediated increase in  $[Na^+]_i$  that accompanied the slow force response (Panel **b**). Insets show original raw data. \* indicates  $P < 0.05$  control vs. all other groups. *DF* = developed force. (Modified with permission from Caldiz et al. (2007))

been shown to involve EGFR transactivation. The possibility that this receptor was playing a role in the chain of events following myocardial stretch was examined. To this aim we explored whether inhibiting EGFR transactivation would impact on the SFR. Several mediators are known to be involved in the transactivation process, but the precise communication between GPCR and EGFR remains not entirely understood (Wetzker and Bohmer 2003). One proposed mediator is Src tyrosine kinase (Wetzker and Bohmer 2003); thus, as a first step in probing our hypothesis, we inhibited Src kinase with the specific tyrosine kinase inhibitor PP1. Figure 12.6b shows that PP1 (1 μmol/l) completely abolished the SFR. Another proposed mediator (Krieg et al. 2004; Szokodi et al. 2008) of EGFR transactivation is heparin-binding EGF (HB-EGF). HB-EGF is generated through extracellular proteolytic cleavage of proHB-EGF by the action of a matrix metalloproteinase (MMP). To test the



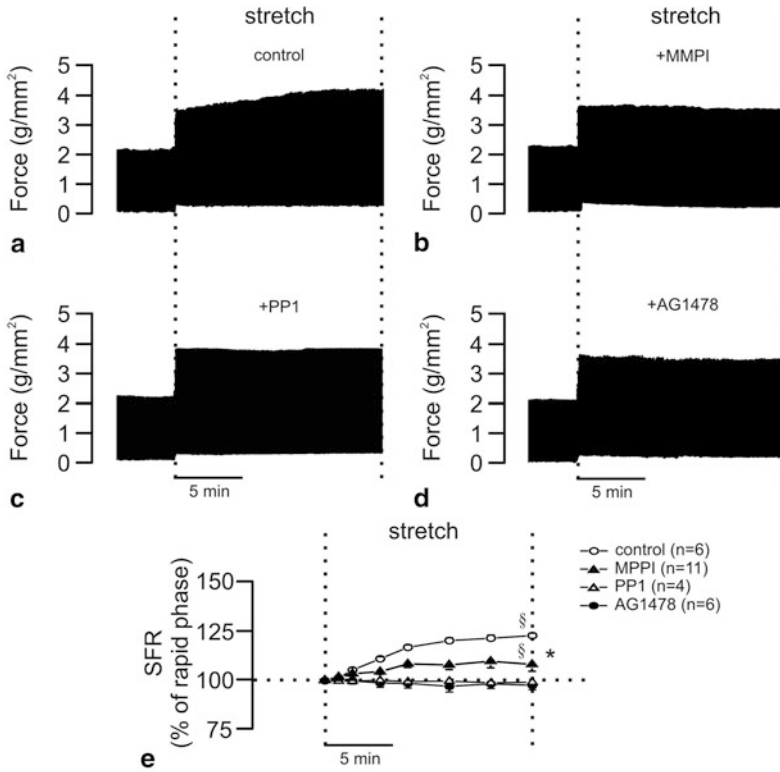
**Fig. 12.5 a** Myocardial stretch significantly increased ERK1/2 and p90rsk phosphorylation, effect that was cancelled by losartan (Los). **b** Inhibition of MEK (a kinase upstream ERK1/2 and downstream RAS) by PD98059 cancelled the SFR (expressed as percent of the initial rapid phase). *DF* = developed force. \* indicates  $P < 0.05$  vs. non-stretched control (cont); † indicates  $P < 0.05$  control vs. PD98059. (Modified with permission from Caldiz et al. (2007))



contribution of this signaling pathway to the SFR, we inhibited MMP with MMP inhibitor III (MMPI), which specifically targeted MMPs 1, 2, 3, 7 and 13. MMPI (3 μmol/l) did not completely eliminate the SFR, but significantly reduced its magnitude by <60 % (Fig. 12.6c) providing further support to the notion that EGFR transactivation was required for a fully developed SFR. Finally, we specifically inhibited the EGFR with AG1478 (1 μmol/l), which is known to prevent receptor phosphorylation, and consequently its activation. Under these conditions, the SFR was completely abolished (Fig. 12.6d). Thus, these three interventions that interfered with the mechanism of EGFR transactivation significantly decreased the SFR confirming that EGFR transactivation plays an essential role in the development of the SFR in cat myocardium.

### 12.4.3 Activation of Redox Sensitive Kinases and NHE Phosphorylation after Myocardial Stretch. Role of the EGFR Transactivation

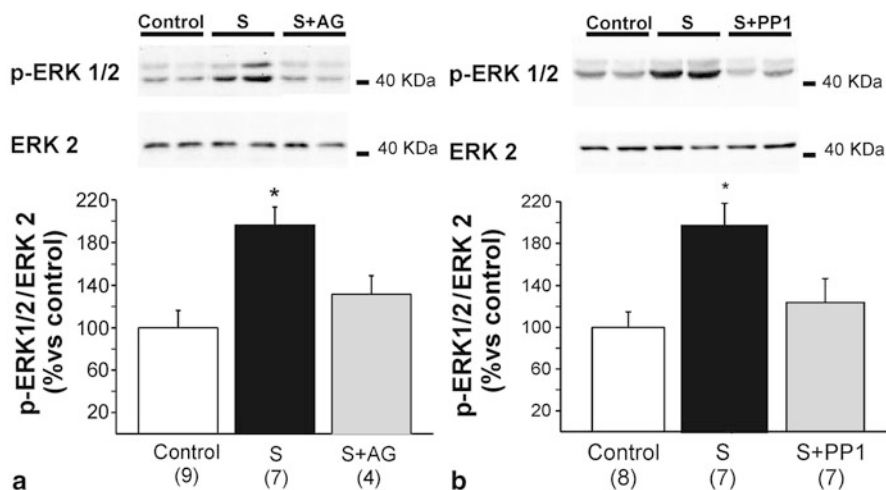
We (Cingolani et al. 2005; Caldiz et al. 2007) and others (Zhang et al. 2009) previously showed that the SFR depends on the activation of NHE-1, which is a target



**Fig. 12.6** SFR and EGFR transactivation. **(a)** a typical force record from a cat papillary muscle subjected to an increase in length from 92 to 98 % of Lmax; the biphasic response to stretch can be seen (vertical dotted lines indicate stretching interval). **(b–d)**, same as **a** but from muscles pretreated with matrix metalloproteinase inhibitor (MMPI, **b**), the Src kinase inhibitor PP1 (**c**) or the EGFR blocker AG1478 (**d**), interventions that cancel EGFR transactivation. As can be seen, all these pharmacological interventions prevented the development of the SFR to stretch. **(e)**, the averaged results obtained under the different experimental conditions expressed as a percentage of the initial rapid phase. \*P < 0.05 control curve vs. others (2-way ANOVA). §P < 0.05 vs. initial rapid phase (for the sake of clarity, significance is indicated only for 15 min of stretch. (Modified with permission from Villa-Abrille et al. (2010))

of the redox sensitive kinases, ERK1/2. Others showed that ROS stimulated NHE-1 through MAPK (Rothstein et al. 2002; Haworth et al. 2003; Fliegel and Karmazyn 2004; Akram et al. 2006), and we recently proposed that stretch induced the mitochondrial production of ROS (Caldiz et al. 2007). We detected a significant increase in ERK1/2 phosphorylation after myocardial stretch. This effect was cancelled by pre-treatment with either AG1478 or PP1 (Fig. 12.7), two inhibitors of EGFR transactivation that also blocked the mechanical response. These findings showed that the prevention of EGFR activation is able to cancel both the increase in ERK1/2 phosphorylation and the mechanical response to stretch.

Additionally, we estimated the levels of NHE-1 phosphorylation at Ser703 with a phospho-Ser 14-3-3 binding motif antibody. Figure 12.8 shows that phosphory-



**Fig. 12.7** ERK1/2 phosphorylation after stretch. Myocardial stretch (S) significantly increased ERK1/2 phosphorylation. This effect was blunted either by EGFR blockade with AG1478 (AG) (a) or by Src kinase inhibition with PP1 (b), demonstrating that EGFR transactivation after stretch is necessary for ERK1/2 phosphorylation. AG1478 and PP1 alone did not modify basal ERK1/2 phosphorylation ( $92 \pm 4\%$ ,  $n = 4$ , and  $107 \pm 8\%$ ,  $n = 4$ , of control respectively). \* $P < 0.05$  vs. non-stretched control (control). (Modified with permission from Villa-Abrille et al. (2010))

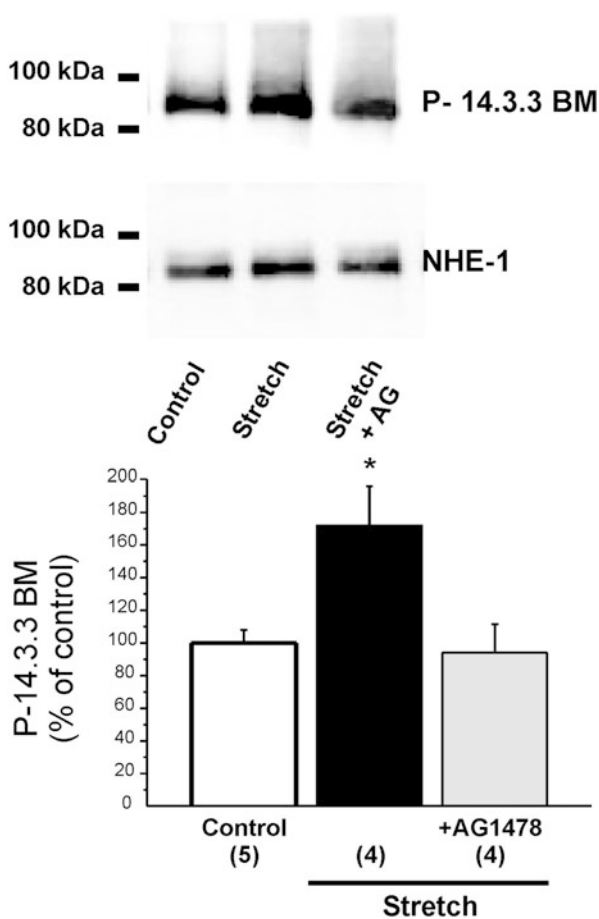
lation at the 14-3-3 binding motif was increased after myocardial stretch, and this increase was prevented with AG1478. Our results support the concept that GPCR induced-EGFR transactivation plays a role in the chain of events that lead to NHE-1 phosphorylation and SFR development.

Recently, it has been demonstrated that the Anrep effect was absent in a transgenic mouse lacking thrombospondin-4 (Cingolani et al. 2011b), a matricellular protein that is normally expressed at modest levels in the heart under normal conditions, but has been shown to be elevated in animals as well as humans with heart failure. Interestingly, the thrombospondin-4 molecule carries an EGF-like repeat, and as mentioned before, mice not expressing this protein not only had the Anrep effect blunted, but also failed to phosphorylate ERK1/2, as controls did. Surprisingly, these mice showed a phenotype of dilated cardiomyopathy after their aortas were banded, suggesting that a complex- not yet completely understood- cross-talk between the extracellular matrix and myocytes takes place after stretch. Further studies will continue to address the mechanistic role these matricellular proteins have in the development of heart failure.

#### 12.4.4 Activation of the MR as a Consequence of Muscle Stretch

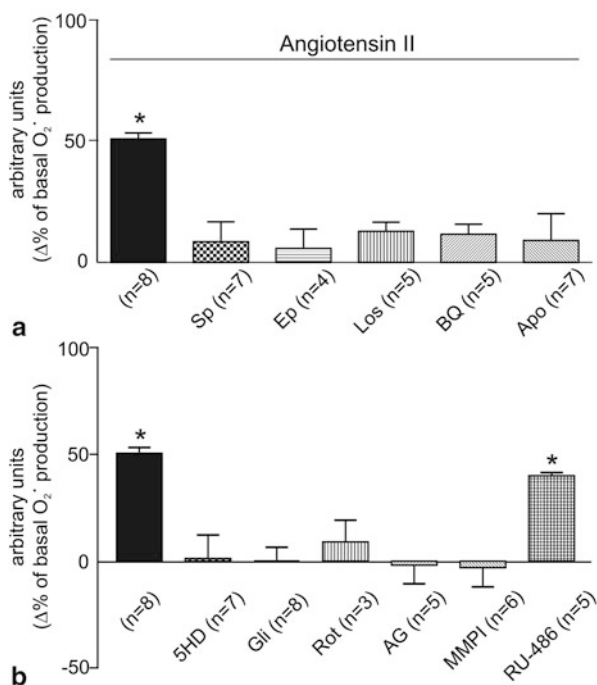
The link between A2 or its AT1 receptor and the mineralocorticoid receptor (MR) is an accepted fact (Lemarie et al. 2008, 2009; Grossmann and Gekle 2009). Although

**Fig. 12.8** Stretch-induced NHE-1 phosphorylation. Myocardial stretch significantly increased NHE-1 phosphorylation at Ser703 estimated by a phospho-Ser 14-3-3 binding motif antibody. This effect was cancelled when the EGFR was blocked by AG1478 (AG). These results support a role of the EGFR transactivation in the chain of events leading to NHE-1 phosphorylation and SFR development. AG1478 alone did not modify basal NHE-1 phosphorylation ( $93 \pm 4$  % of control,  $n = 4$ ). \* $P < 0.05$  vs. control. (Modified with permission from Villa-Abrille et al. (2010))



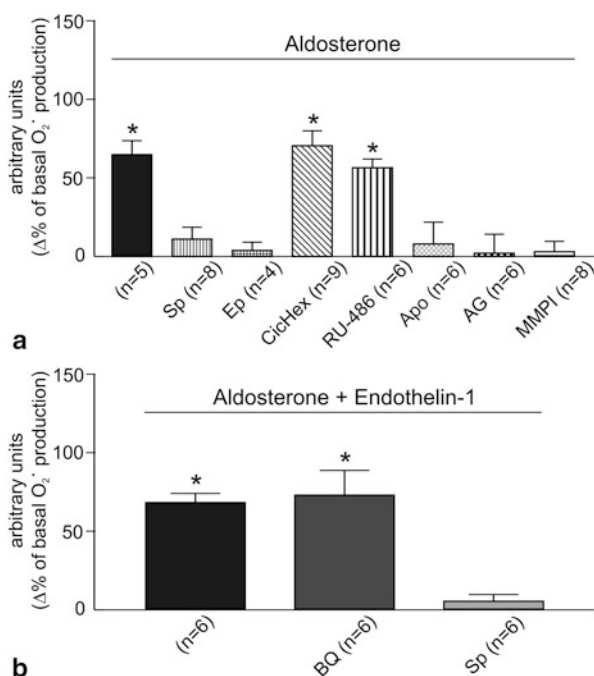
still somewhat controversial, aldosterone (ALD), which is known to be regulated by A2, appears to be synthesized and/or released by cardiac muscle (Gomez-Sanchez et al. 2004; Chai and Danser 2006); (Silvestre et al. 1998, 1999; Takeda et al. 2000). We have recently hypothesized that if a crosstalk between A2 and the MR occurs during ROS production, and at the same time A2 and ROS are crucial for SFR development, therefore MR inhibition would blunt the SFR. In this section we will present evidence that MR activation is involved in the signalling pathway leading to the Anrep effect.

Figure 12.9 shows that MR activation is necessary to promote ROS formation by a physiological concentration of A2 (1 nmol/L), since the increase in superoxide anion formation of  $\sim 50$  % was suppressed after blocking MR with spironolactone or eplerenone. This effect was also suppressed by blocking AT1, ET1 (type A) receptor or EGFRs, by inhibiting NADPH oxidase, or by targeting mitochondria; and it was unaffected by glucocorticoid receptor inhibition.



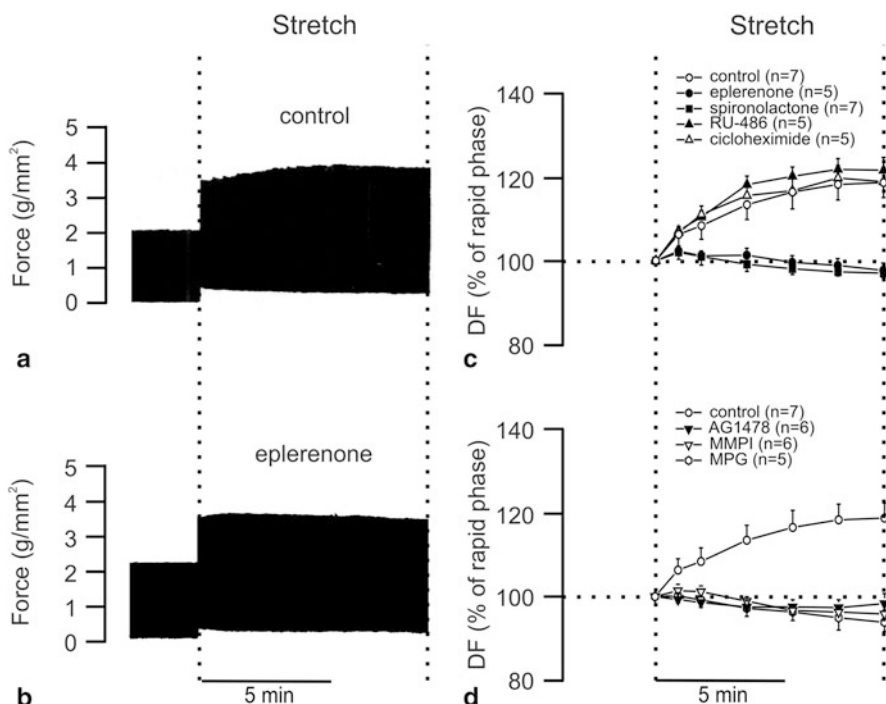
**Fig. 12.9** Superoxide anion production induced by angiotensin II. **a** MR blockade with spiro lactone (*Sp*, 10  $\mu\text{mol/L}$ ) or eplerenone (*Ep*, 10  $\mu\text{mol/L}$ ) abrogated the effect of 1 nmol/L A2 on the basal rate of  $O_2^-$  production. This effect was also blunted by the AT<sub>1</sub> and ET<sub>A</sub> receptor antagonists losartan (*Los*, 1  $\mu\text{mol/L}$ ) and BQ123 (*BQ*, 10  $\mu\text{mol/L}$ ), respectively, and by NADPH oxidase inhibition with apocynin (*Apo*, 300  $\mu\text{mol/L}$ ). **b** A2-induced  $O_2^-$  formation was also blunted by targeting mitochondria with 5HD (100  $\mu\text{mol/L}$ ), glibenclamide (*Gli*, 50  $\mu\text{mol/L}$ ), or rotenone (*Rot*, 10  $\mu\text{mol/L}$ ), and by preventing EGFR activation either by EGFR blockade with AG1478 (*AG*, 1  $\mu\text{mol/L}$ ) or by inhibiting the metalloproteinase involved in EGFR transactivation with MMPI (3  $\mu\text{mol/L}$ ). Glucocorticoid receptor inhibition with Ru-486 (10  $\mu\text{mol/L}$ ) did not influence the effect of A2. \* $P < 0.05$  vs. basal  $O_2^-$  production. (Modified with permission from Caldiz et al. (2011))

All interventions except AT<sub>1</sub> receptor blockade blunted the increase in superoxide anion promoted by an equipotent dose of ET-1 (1 nmol/L), confirming that ET receptor activation is downstream of AT<sub>1</sub> receptor (not shown, (Caldiz et al. 2011)). Similarly, an increase in superoxide anion promoted by an equipotent dose of ALD (10 nmol/L) was blocked by spironolactone or eplerenone, by preventing EGFR transactivation, but not after inhibiting glucocorticoid receptors or protein synthesis, suggesting a non-genomic MR effect (Fig. 12.10a). Combination of ALD and ET-1 did not further increase superoxide anion formation (Fig. 12.10b). ALD increased phosphorylation of the redox-sensitive kinases ERK1/2, p90RSK, and the NHE-1, effects that were eliminated by eplerenone or by preventing EGFR transactivation (not shown, see ref. Caldiz et al. 2011).



**Fig. 12.10** Superoxide anion production induced by aldosterone. **a** The effect of ALD at a concentration (10 nmol/L) that mimicked the effect of A2 and ET on the basal rate of O<sub>2</sub><sup>-</sup> production was suppressed by spiro lactone (*Sp*) and eplerenone (*Ep*), but not by the glucocorticoid receptor inhibitor Ru-486 or by preventing protein synthesis with cycloheximide (*CicHex*, 7 mmol/L). This demonstrates that MR activation has nongenomic consequences and excludes the possibility of glucocorticoid receptor activation. On the other hand, as shown for A2 and ET, the ALD-mediated increase in ROS formation was prevented by NADPH oxidase inhibition (*Apo*) and by preventing EGFR activation (*AG* and *MMPI*). This suggests that transactivation occurs in the direction of activated MR to EGFR, and that metalloproteinase activation downstream of MR is crucial for EGFR transactivation. **b** The combination of ALD and ET did not promote any further increase in O<sub>2</sub><sup>-</sup> production. Under this condition, mitochondrial O<sub>2</sub><sup>-</sup> production was abrogated by spironolactone (*Sp*), but unaffected by ET<sub>A</sub> receptor blockade with BQ123 (*BQ*), indicating that the only possible sequence of events is from ET<sub>A</sub> to MR. \*P < 0.05 vs. basal O<sub>2</sub><sup>-</sup> production. (Modified with permission from Caldiz et al. (2011))

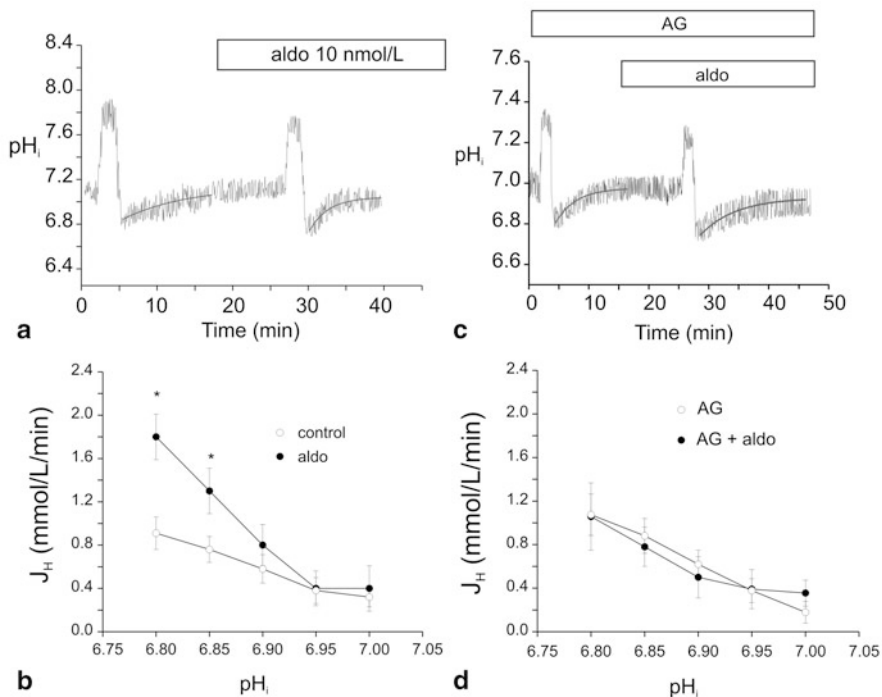
Finally, the SFR was suppressed by MR blockade, by preventing EGFR transactivation or by scavenging ROS, but it was unaffected by glucocorticoid receptor blockade or protein synthesis inhibition as shown in Fig. 12.11. These results clearly suggest that MR activation is a necessary step in stretch-triggered mitochondrial ROS that mediates the activation of redox-sensitive kinases upstream NHE-1, leading to de Anrep effect.



**Fig. 12.11** SFR and MR activation. **a** Typical force record from rat papillary muscle subjected to an increase in length from 92 to 98 % of Lmax. The biphasic force response to stretch can be observed. **b** Same as **(a)** but from a muscle pre-treated with the MR blocker eplerenone, demonstrating that prevention of MR activation after stretch eliminated the SFR. **c** Averaged results of the SFR expressed as percentages of the initial rapid phase. MR blockade, not only by eplerenone but also by spironolactone, completely suppressed the SFR. However, the SFR was unaffected by the glucocorticoid receptor inhibitor Ru-486 or the protein synthesis inhibitor cycloheximide. **d** As reported previously in cat myocardium (Villa-Abrille et al. 2010) the SFR required EGFR transactivation, since it was blunted either by direct EGFR inhibition (AG1478) or by blocking transactivation with MMPI. Furthermore, the SFR was suppressed by the ROS scavenger MPG, supporting the notion that ROS formation is a key factor in the chain of events leading to the Anrep effect. (Modified with permission from Caldiz et al. (2011))

## 12.5 Direct Measurements of NHE-1 Stimulation by Aldosterone: Transactivation of the EGFR

Fujisawa et al. (2003) demonstrated that mineralocorticoid/salt-induced rat cardiac fibrosis and hypertrophy was prevented by the selective NHE-1 blocker cariporide. It has also been reported that ALD upregulates the expression and function of NHE-1 (Ebata et al. 1999; Karmazyn et al. 2003; Barbato et al. 2004; Matsui et al. 2007) and that selective blockade of this transporter prevents and/or reverts left ventricular hypertrophy in various animal models (Cingolani and Ennis 2007). According to

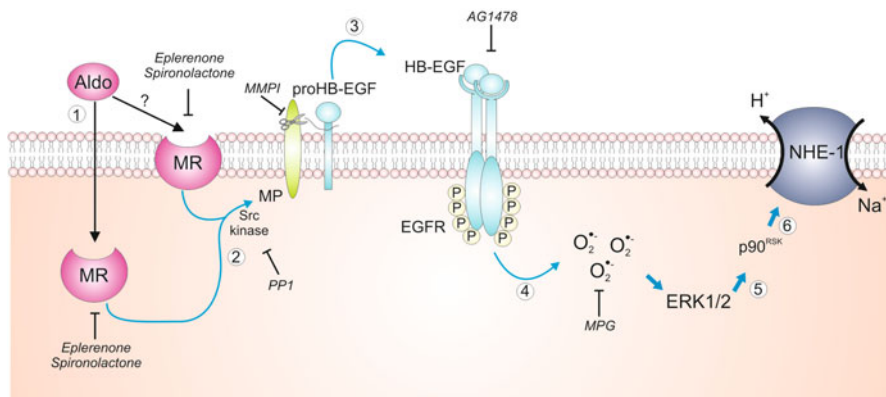


**Fig. 12.12** Aldosterone induced activation of the NHE-1 and its blockade by inhibiting the EGFR. Panel **a**: representative traces of  $pH_i$  during the application of two consecutive ammonium pulses (20 mmol/L  $NH_4Cl$ ), in the absence (*first* pulse) and presence of 10 nmol/L ALD (*aldo*, *second* pulse). ALD was applied 10 min before the second pulse. Panel **b**: average proton efflux  $J_H$ , carried by the NHE-1, before (*first* pulses, closed circles,  $n = 5$ ) and after application of 10 nmol/L ALD (*second* pulses, open circles,  $n = 5$ ).  $J_H$  is significantly enhanced by ALD. \* $P < 0.05$  vs. control. Panel **c**: representative traces of  $pH_i$  during the application of two consecutive ammonium pulses (20 mmol/L  $NH_4Cl$ ), in the absence (*first* pulse) and presence of 10 nmol/L ALD (*second* pulse). The EGFR blocker AG1478 (AG, 1  $\mu$ mol/L) was applied 10 min before the first pulse and maintained throughout the experiment. ALD was applied 10 min before the second pulse. Panel **d**: average proton efflux  $J_H$ , carried by the NHE-1, before (*first* pulses, open circles,  $n = 4$ ) and after application of 10 nmol/L ALD (*second* pulses, closed circles,  $n = 4$ ) in the continuous presence of 1  $\mu$ mol/L AG1478. The transactivation of the EGFR by ALD leads to the activation of the NHE-1. (Modified with permission from De Giusti et al. (2011))

these data and in agreement with our previous results on the SFR described above, we have recently shown that ALD increases NHE-1 activity in rat ventricular myocytes through a non-genomic pathway (Fig. 12.12a, b) (De Giusti et al. 2011).

As commented above, EGFR activation represents one of the signaling pathways involving ALD (Grossmann and Gekle 2007; Grossmann et al. 2007). It has been shown that the MR blocker spironolactone reduces the EGFR mRNA synthesis after cerebral ischemia (Dorrance et al. 2001). Accordingly, Grossmann et al. (2007) reported that MR activation by ALD enhanced EGFR expression via an interaction





**Fig. 12.13** Signaling cascade involved in the aldosterone-induced NHE-1 stimulation in rat cardiomyocytes. The activation of the MR by ALD (*Aldo*) (1) leads to EGFR transactivation. This mechanism is mediated by the activation of the src-kinase (2) and the metalloproteinases. HB-EGF is released from the cell surface following shedding of the extracellular domain (ectodomain shedding) by this zinc-dependent metalloproteinases (3). HB-EGF binds to the EGFR and increases the production of superoxide anion ( $O_2^-$ ) (4). These reactive oxygen species (ROS) stimulates the redox-sensitive kinase ERK1/2 (5), which phosphorylates the kinase p90<sup>RSK</sup>. This kinase phosphorylates the NHE-1 (6), stimulating its activity. Eplerenone (*eple*) and spironolactone (*spiro*) (MR blockers), PP1 (src-kinase inhibitor), MMPI (metalloproteinases blocker) and MPG (ROS scavenger) were employed to investigate pathways 1–4 (Ref. (De Giusti et al. 2011)). The inhibitor of the EGFR kinase AG1478 (AG) was used to evaluate the transactivation of this receptor by aldosterone. (Modified with permission from De Giusti et al. (2011))

with the EGFR promoter of vascular smooth muscle. In addition to these genomic effects, non-genomic actions of ALD involving EGFR transactivation have also been reported (Grossmann and Gekle 2008, 2009). Consistent with this evidence, we have recently shown that ALD enhances NHE-1 activity via transactivation of EGFR (Fig. 12.12c, d) (De Giusti et al. 2011). The stimulatory effect of this hormone on NHE-1 was blocked by the inhibitor of the Src-kinase PP1 and the blocker of metalloproteinases MMPI (De Giusti et al. 2011).

As commented above, these proteases release HB-EGF from its precursor, proHB-EGF. Figure 12.13 depicts that activation of EGFR by HB-EGF increases the production of intracellular ROS and triggers the ERK1/2 pathway, which phosphorylates p90RSK (De Giusti et al. 2011).

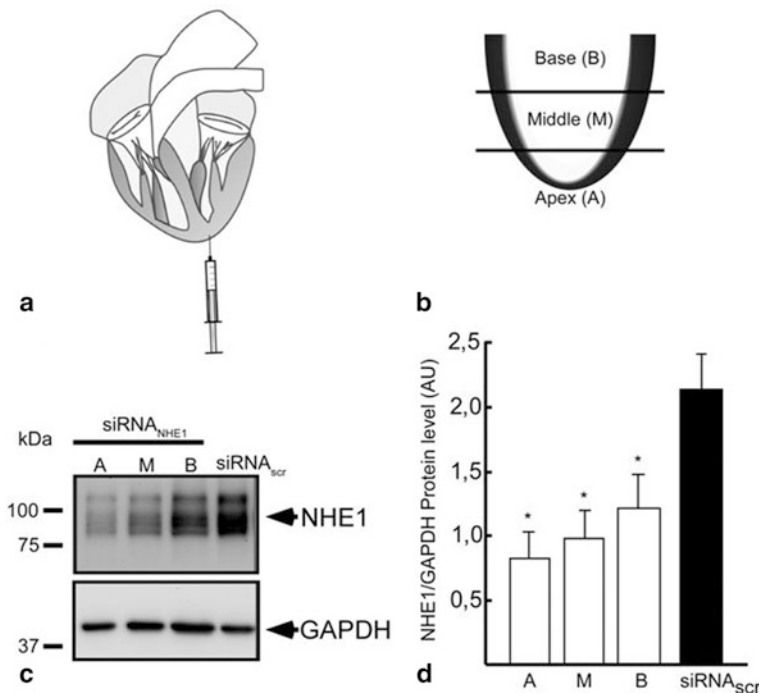
This kinase, in turn, phosphorylates Serine703 of the NHE-1, leading to the activation of the transporter. As noted in Fig. 12.13, at least a fraction of the total amount of MR appears to be linked to the sarcolemma, likely co-localized to the EGFR (Grossmann et al. 2010) and/or associated to caveolin-1 (Krug et al. 2011). This data would explain the binding of ALD to the sarcolemmal fraction reported by Le Moellie et al. (2004). In addition, non genomic effects of ALD altering stimulation of a GPCR (GPR30) has been recently reported in vascular smooth muscle and endothelial cells (Gros et al. 2011).

## 12.6 Molecular Approach Targeting the Anrep Effect: Silencing NHE-1 Expression by Interference RNA

Gene silencing by RNA interference is a natural process occurring in cells by which a specific mRNA is degraded and therefore the expression of the encoded protein prevented. This mechanism is mediated by a double-stranded RNA (dsRNA) of approximately 18–23 nucleotides of length present inside the cell, known as siRNA (small interfering RNA) (Mello and Conte 2004; Kim and Rossi 2007). Briefly, through a multiple step pathway, one of the RNA strands of the siRNA is matched to a complementary mRNA which in turn is cleaved and ultimately degraded. In the research arena, *in vivo* or *in vitro* delivery of siRNA molecules to cells provides a powerful tool to specifically silence a single type of protein (Akhtar and Benter 2007; Kim and Rossi 2007). This technology has several advantages: (1) It is highly specific and can differentiate between members of the same family and even between isoforms of the same protein; (2) It has a lasting effect whose extension varies according to the strategy used to deliver the siRNA or modifications of the siRNA molecule; (3) It can be reversed; (4) It is relatively easy to obtain a siRNA. Moreover, siRNA technology has the potential to be used in the therapeutic field, to validate a protein as a suitable target whose inhibition would mediate the cure or alleviation of a disease. After finding the target protein, a synthetic drug could be designed to treat the disease. However, the RNA interference methodology also allows consideration of the siRNA molecule itself as a possible therapeutic tool (Kim and Rossi 2007). Delivery of siRNA molecules into the cell portends several challenges, starting with penetration of the plasma membrane, stability of siRNA inside and outside the cell, toxicity, and triggering of immune responses (Akhtar and Benter 2007; Kim and Rossi 2007; Manjunath et al. 2009).

Classic pharmacological techniques to inhibit a desired protein *in vivo* have several disadvantages compared to interference RNA: (1) The drug distributes broad-wide in the organism, condition that may affect undesired targets or generate side effects; (2) It is very difficult to make a drug that can differentiate between members of the family or isoforms; (3) Drug concentration change along time therefore requiring frequent administration.

Pharmacological inhibition of NHE-1 was beneficial in different experimental models of cardiac pathologies (Ennis et al. 1998, 2003; Karmazyn 1999; Avkiran and Marber 2002; Camilion de Hurtado et al. 2002; Engelhardt et al. 2002; Cingolani et al. 2003b). However, clinical trials with NHE-1 inhibitors like GUARDIAN (Theroux et al. 2000), ESCAMI (Zeymer et al. 2001) and EXPEDITION (Mentzer et al. 2008) failed to provide such benefits, and one of these studies was suspended due to undesired cerebrovascular side effects (Mentzer et al. 2008). Although the mechanism for this negative effect is still not clear, it could be related to blockade of NHE-1 activity in the brain where NHE-1 function seems to be essential, since animals lacking NHE-1 showed aberrant phenotype that included ataxia and epileptic-like seizures (Cox et al. 1997; Bell et al. 1999). It is possible then, that pharmacological inhibition of NHE-1 affects the exchanger in tissues other than the myocardium where its role is crucial or, even inhibits an unrelated protein required for normal function (Villafuerte et al. 2007). Activation of the RNA interference

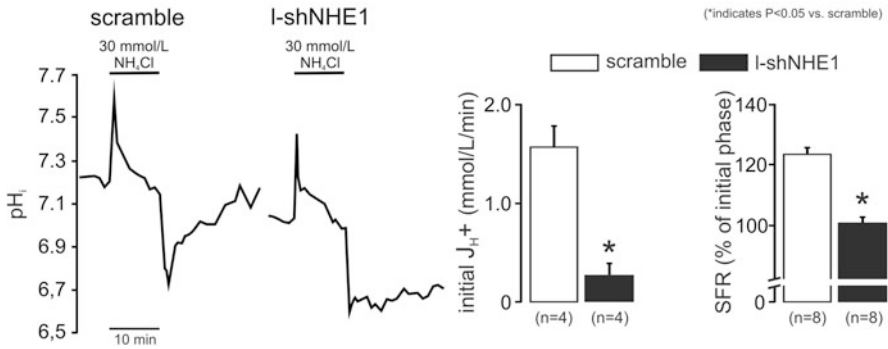


**Fig. 12.14** NHE-1 expression reduction distally from the injection site. **a** Mice were injected once in the apex of the left ventricle with naked siRNA<sub>NHE-1</sub> or siRNA<sub>scramble</sub> (siRNA<sub>SCR</sub>) as shown in the heart scheme, and sacrificed after 72 h. **b** Left ventricle was divided into three parts (apex, middle, and base) to evaluate NHE-1 expression. **c** Representative immunoblots of lysates of the different fractions of the left ventricle. **d** Average expression of protein, quantified by densitometry and normalized to the amount of GAPDH (n = 7, \*P < 0.05 vs. siRNA<sub>SCR</sub>). (Modified with permission from Morgan et al. (2011))

pathway appeared to be a suitable method to specifically block the NHE-1 because it spares the other nine members of the protein family. In our laboratory, we designed two different approaches to target the NHE-1 protein exclusively in the heart: (a) the delivery of naked siRNA, and (b) the delivery of siRNA using the lentivirus backbone. The use of viral vectors for delivering siRNA inside the cells facilitates its entry and provides a long lasting inhibition of a protein. Disadvantages of this strategy are potential changes of the gene expression after integration of viral DNA in the host genome and generation of immune responses. The use of naked siRNA has the advantage of preventing a massive immune response, but carries the difficult task of crossing the plasma membranes, allowing optimal propagation, mainly due to its size and electrostatic charge. Furthermore, it is more susceptible to be degraded by nucleases producing a less lasting silencing effect.

We have demonstrated that after a week of a single injection of naked siRNA<sub>NHE-1</sub> to the left ventricle, an extensive reduction of the NHE-1 expression and function took place (Morgan et al. 2011) (Fig. 12.14).

These results, suggests the ability of siRNA molecules to spread through the myocardium and reduce NHE-1 expression and activity faraway from the injection



**Fig. 12.15** Functional activity of the NHE-1 was evaluated by the recovery of  $pH_i$  after an acidic load ( $NH_4^+$  prepulse) in isolated papillary muscles where the SFR was also tested. The original  $pH_i$  recordings (*left*) as well as the averaged initial  $H^+$  fluxes ( $J_{H^+}$ , *middle*) clearly demonstrate the significant depression of NHE-1 activity in l-shNHE1 injected muscles compared to scramble. Furthermore, silencing the NHE-1 blunted the SFR (*right*). \* $P < 0.05$  vs. scramble

site. In agreement with our findings, Kizana et al. (2009) demonstrated that siRNA molecules can move through cultured neighbour neonatal rat ventricle myocytes when coupled by gap junctions. Accordingly, it was found in different cultured cells that siRNA molecules can travel from one cell to another through gap junctions (Valiunas et al. 2005; Wolvetang et al. 2007) if connexin 43 (the predominant connexin isoform in adult hearts (Dhein 1998) is expressed (Valiunas et al. 2005). This local injection of siRNA in the left ventricle did not produce any effect on other organs, which allowed concluding that the procedure was successful in limiting the effects of siRNA to the heart (Morgan et al. 2011).

We have also recently incorporated the siRNA sequence able to mediate specific NHE-1 knockdown into a lentiviral vector (l-shNHE1) and injected into the left ventricular wall of Wistar rats (Pérez et al. 2011). A separated group of rats injected with a vector expressing a non-silencing sequence (scramble) served as control. Confocal microscopy analysis of heart tissue revealed spreading of l-shNHE1 (DsRed tagged) from the sites of injection throughout the myocardium. Hearts with l-shNHE1 showed reduced NHE-1 protein expression ( $44 \pm 8$  % of controls,  $n = 4$ ,  $P < 0.05$ ) that correlated with depressed  $pH_i$  recovery after acidosis and abolishment of the SFR (Fig. 12.15), despite preserved ERK1/2 activation (in % of control: stretch  $241 \pm 10$   $n = 5$ ; stretch l-shNHE1  $285 \pm 36$   $n = 6$ ). These data provide unequivocal support to our proposal that NHE-1 activation is crucial to the Anrep effect.

## 12.7 Potential Link Between NHE-1 Activation and Cardiac Hypertrophy and Failure

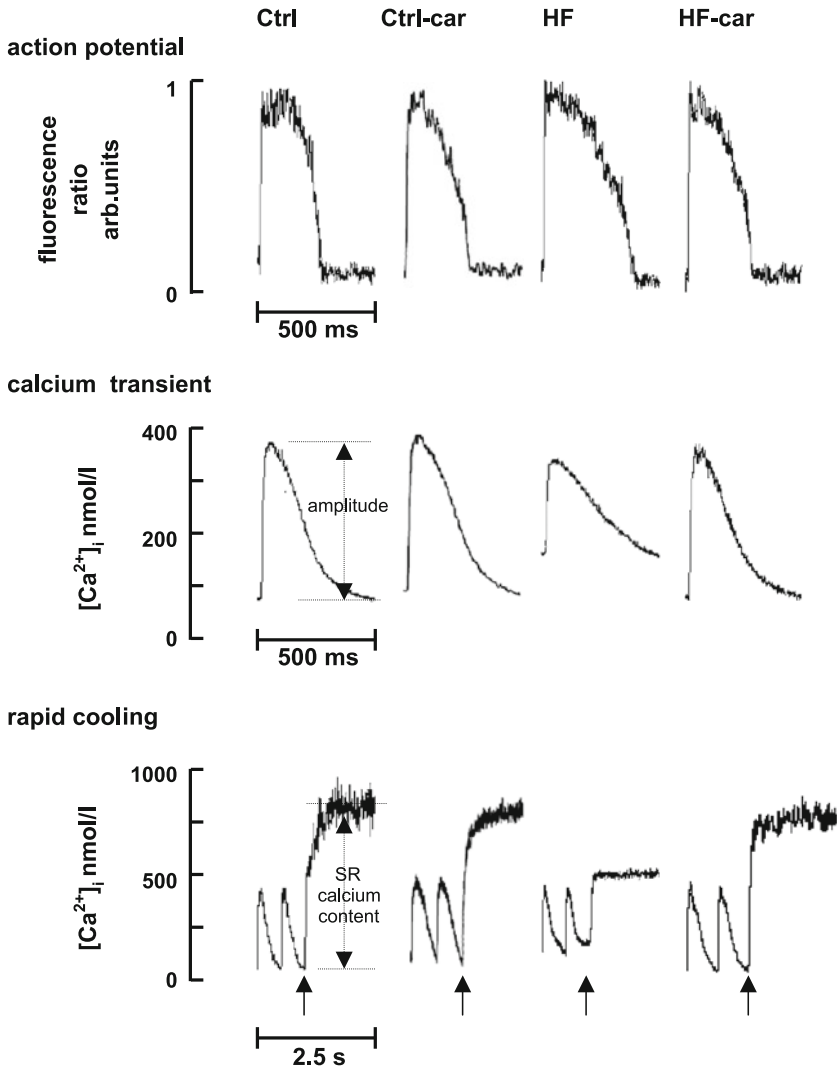
Cardiac hypertrophy is known to be one of the main cardiovascular risk factors and a poor prognostic sign associated with nearly all forms of heart failure (Koren et al. 1991; Lloyd-Jones et al. 2002). Most intracellular pathways leading to pathological

cardiac hypertrophy and failure converge at the increase in intracellular calcium levels and downstream activation of the calcineurin-dependent transcriptional pathway. The rise in calcium may occur through different mechanisms. One of them is an increase in intracellular  $\text{Na}^+$  resulting from enhanced function of the NHE-1, which drives the NCX to increase cytosolic calcium. As stated before, after cardiac muscle is stretched, an autocrine/paracrine chain of steps occur in which AT1 receptor activation is an early event (Sadoshima et al. 1993). This pathway also involves NADPH oxidase-dependent mitochondrial reactive oxygen species release, which itself activates the NHE-1 redox-sensitive kinase  $\text{p90}^{\text{RSK}}$ , among others.

Enhanced NHE-1 activity as a possible mechanism involved in cardiac hypertrophy and failure was previously reported in the hypertrophic myocardium of adult spontaneously hypertensive rats (SHR) (Perez et al. 1995), in human ventricular myocytes from hearts with chronic end-stage heart failure (Yokoyama et al. 2000), in a pressure-volume overload model of cardiac hypertrophy and failure in rabbits (Baartscheer et al. 2008), in the hypertrophied heart of a type 2 diabetic rat model (Darmellah et al. 2007) and in neonatal rats (Dulce et al. 2006).

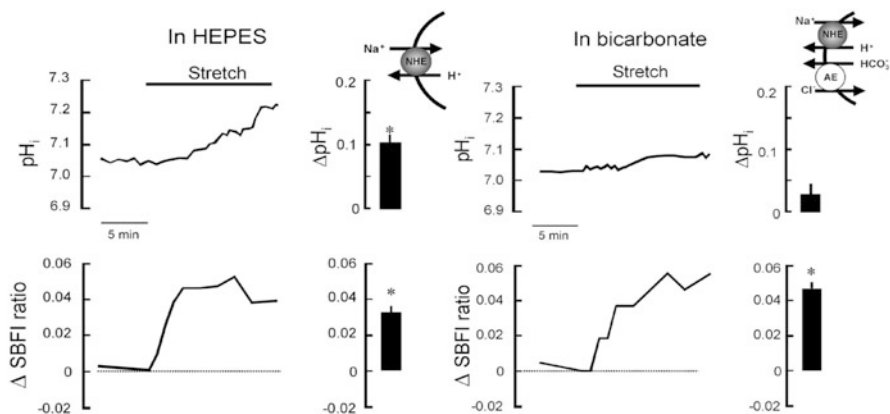
Interestingly, Nakamura et al. (2008) have recently demonstrated *in vitro* that NHE-1 hyperactivity is sufficient to generate calcium signals required for cardiac hypertrophy to take place. Although *in vivo* physiological data supporting the involvement of this mechanism in the transition to chronic cardiac hypertrophy and its consequences is scant, Baartscheer et al. (2005) have shown in elegant experiments that long-term NHE-1 inhibition with cariporide in rabbits with combined pressure and volume overload cardiac hypertrophy and failure attenuated hypertrophy and decreased the previously augmented diastolic calcium without significant alteration of systolic calcium (Fig. 12.16).

An increased activity of calcineurin in the myocardium of the SHR, and its suppression by the antihypertrophic treatment has been reported previously (Zou et al. 2002; Ennis et al. 2007). Similarly, in the hypertrophied myocardium of rats with salt-sensitive hypertension, an increase in the activity of calcineurin and its prevention by treatment with an AT1 blocker has been reported (Nagata et al. 2002). We were the first to report that the regression of cardiac hypertrophy caused by NHE-1 inhibition was accompanied by normalization of the activity of the calcineurin pathway and preservation or even improvement of cardiac function. (Ennis et al. 2007). NHE-1 inhibition by decreasing  $[\text{Na}^+]_i$  diminishes calcium either by decreasing calcium entry (reverse mode) or by increasing calcium efflux (forward mode) through the NCX. At first glance, it may appear difficult to understand how a decrease in cytosolic calcium induced by NHE-1 inhibition can improve myocardial contractility in the long term. However, the preservation of cardiac function after regression of cardiac hypertrophy seems not to be unique to the regression of cardiac hypertrophy induced by NHE-1 inhibition (Esposito et al. 2002). In the myocardium, intracellular calcium is compartmentalized in such way that the contractile pool is different from the pool that regulates reactive signaling. In agreement with this, it has been suggested that calcineurin, as well as CaMKII are preferentially activated by specific sub-cellular calcium pools (Frey et al. 2004; Wu et al. 2010). Therefore we can speculate that the decrease in diastolic calcium might be sensed by the calcium calmodulin-calcineurin



**Fig. 12.16** Prevention of cardiac hypertrophy and normalization of the previously augmented diastolic  $Ca^{2+}$  observed in rabbits with cardiac hypertrophy and failure (caused by combined pressure and volume overload) treated during 3 months with the NHE-1 inhibitor cariporide. Action potentials (*top*) and  $Ca^{2+}$  transients (*bottom*) in isolated myocytes from control (*Ctrl*), control plus cariporide (*Ctrl-car*), heart failure (*HF*), and heart failure plus cariporide (*HF-car*) groups. (Modified with permission from Baartscheer et al. (2005))

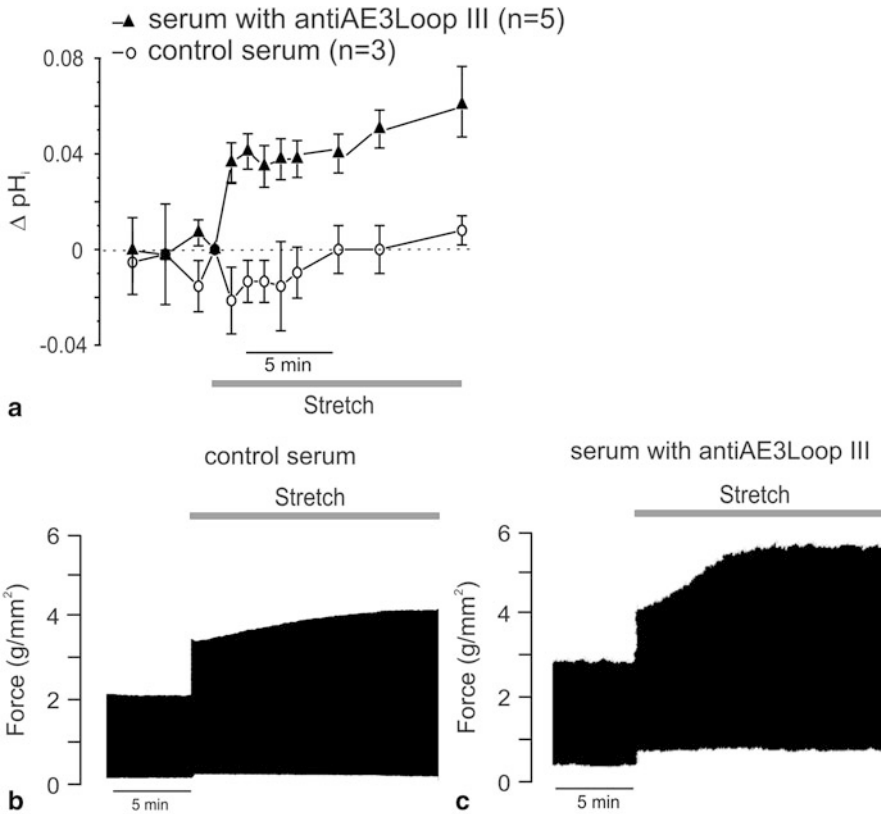
pathway, but not by the contractile machinery. Moreover, a negative inotropic effect of calcineurin through different mechanisms has been described (Sah et al. 2002; Li et al. 2003), and a positive inotropic effect could therefore be expected with the phosphatase deactivation.



**Fig. 12.17** Representative experiments showing that in the presence of bicarbonate, NHE-1 activation by stretch causes elevation of  $[Na^+]_i$  (assessed by SBF1 340/380 fluorescence ratio) but not of  $pH_i$ , due to the simultaneous activation of the AE. (Modified with permission from Cingolani et al. (2003))

In our scheme, stretch-triggered NHE-1 activation is the main step leading to cardiac hypertrophy and failure. The experiments that originally induced us to consider the activation of NHE-1 after myocardial stretch were performed in the absence of bicarbonate in the medium, where the only active  $pH_i$  regulating mechanism was this exchanger (Cingolani et al. 1998). Therefore, the increase in  $pH_i$  served as a “marker” for NHE-1 activation by A2-ET. This activation was protein kinase C-dependent since chelerythrine prevented it (Cingolani et al. 1998). It is interesting to emphasize that the NHE-1 exchanges one intracellular  $H^+$  for one extracellular  $Na^+$ , therefore, its activation would be followed by an increase in both  $[Na^+]_i$  and  $pH_i$ . However, during our experiments it became evident that the increase in  $pH_i$  occurred only in the absence of bicarbonate in the medium. In contrast, when bicarbonate was present in the media, the simultaneous activation by the stretch of the NHE-1 and the  $Na^+$  independent  $Cl^-/HCO_3^-$  exchanger (AE) precluded significant changes in  $pH_i$  but not in  $[Na^+]_i$  (Fig. 12.17). Regarding the intracellular signals leading to activation of NHE-1 by ET, they are not fully understood. If we consider that part of the positive inotropic effect of ET-1 is the result of endogenously generated ROS (Sand et al. 2003) and that ROS, through MAPK pathways, phosphorylate the cytosolic tail of the NHE-1 increasing its activity (Rothstein et al. 2002), we could suggest that ROS may be involved in the activation of the NHE-1 after stretch.

As pointed out before, the increase in  $[Na^+]_i$  induced by NHE-1 activation is the most important step in the chain of events leading to its mechanical counterpart, the SFR, and perhaps portending implications in the mechanism(s) that lead to myocardial hypertrophy and failure (Gray et al. 2001). In connection with this, we have demonstrated that exogenous applied A2 stimulates AE activity through endogenous ET (Camili3n de Hurtado et al. 2000). Therefore, under physiological conditions, stretch will be followed by a sequential release of A2 and ET leading



**Fig. 12.18** When the AE activation is prevented by a functional antibody (antiAE3Loop III) an increase in  $\text{pH}_i$  takes place (panel a). Thus, the SFR in this condition results from the increase in the calcium transient plus an increase in myofilament calcium responsiveness due to cytosolic alkalization and, therefore, the SFR to stretch is greater (compare panels b and c). (Modified with permission from Cingolani et al. (2003a))

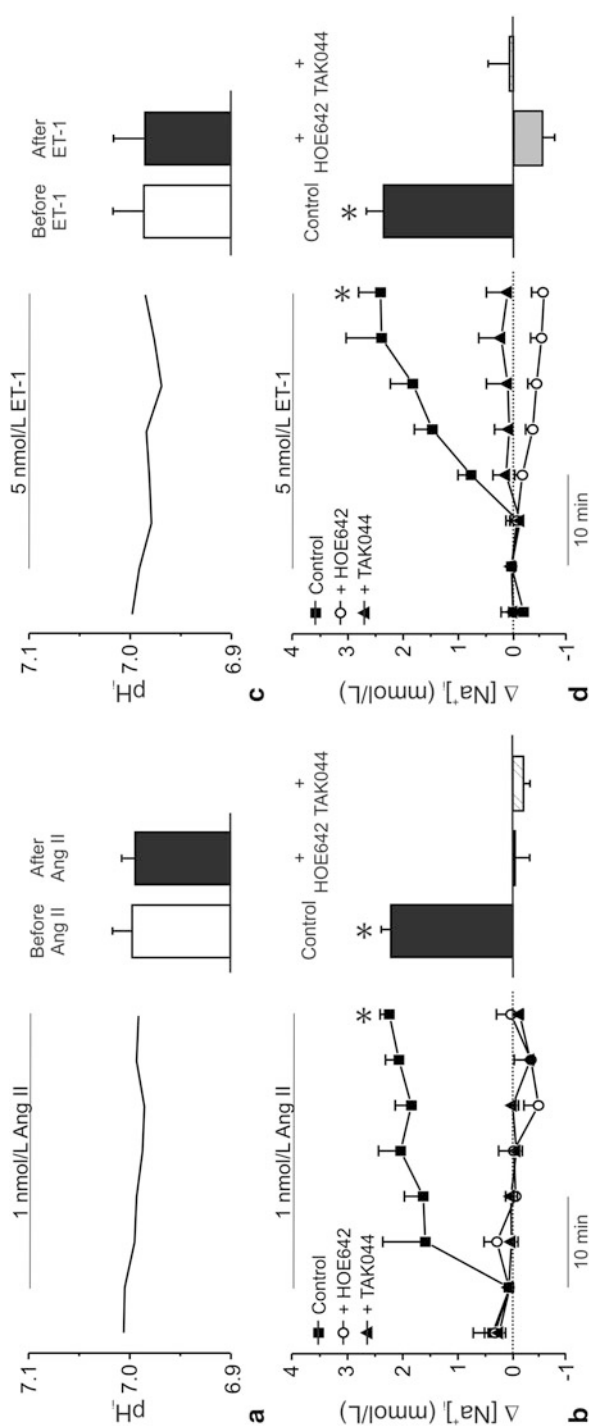
to the simultaneous activation of NHE and AE. The rise in  $\text{pH}_i$  induced by NHE-1 activation might be prevented by AE, but this is not the case for the increase in  $[\text{Na}^+]_i$ , due to its  $\text{Na}^+$  independency. This increase in  $[\text{Na}^+]_i$  will trigger an increase in calcium influx (and consequently the calcium transient) by reducing the NCX forward mode and/or favoring its reverse mode of operation. Instead, when the activation of the AE is prevented by a functional antibody, an increase in  $[\text{Na}^+]_i$  takes place (Fig. 12.18a) together with an increase in  $\text{pH}_i$ . The mechanical counterpart may therefore result from the increase in calcium transient and also from the increase in myofilament calcium responsiveness due to cytosolic alkalization (Fabiato and Fabiato 1978; Mattiazzi et al. 1979; Orchard and Kentish 1990). In agreement with the latter, Fig. 12.18b, c shows that in the presence of the AE antibody, the stretch of a cat papillary muscle produces a greater SFR.



It may be argued that the  $\text{Na}^+/\text{K}^+\text{ATPase}$  should prevent the increase in  $[\text{Na}^+]_i$  elicited by NHE-1 hyperactivity, however, Bers et al. (2003) have shown that the changes in  $[\text{Na}^+]_i$  necessary to alter the  $\text{Na}^+$  pump activity should be greater than those detected by us after the stretch. Furthermore, we should consider that an enhanced activity of the pump would be probably detected due to the increase in  $[\text{Na}^+]_i$  after stretch, but not enough to normalize the  $[\text{Na}^+]_i$ . In other words, the rise in  $[\text{Na}^+]_i$  detected during the SFR should be higher if the pump was inhibited. We may also speculate that, similarly to the  $\text{Na}^+$  pump lag hypothesis for the force-frequency relationship, the greater  $\text{Na}^+$  entry is balanced by an increased  $\text{Na}^+$  pump activity, but only at the cost of elevated  $[\text{Na}^+]_i$  and hence increased calcium entry. In contrast, the changes in  $[\text{Na}^+]_i$  detected after stretch may suffice to alter the activity of the NCX, specially if the NHE and the NCX are co-localized (Petrecca et al. 1999; Brette et al. 2002). Accordingly, we recently showed that the positive inotropic effect of exogenous A2 or ET-1 is accompanied by a cariporide-sensitive increase in  $[\text{Na}^+]_i$  (Fig. 12.19) (Perez et al. 2003). Additionally, the fact that ET receptors blockade with TAK044 canceled the A2-induced rise in  $[\text{Na}^+]_i$  reinforces the role of ET as mediator of A2 effects (Perez et al. 2003).

Although the role of NHE-1 activation early after stretch leading to the SFR development (and possibly to cardiac hypertrophy and failure) has been detected in different species including cat (Perez et al. 2001; Caldiz et al. 2007), human (von Lewinski et al. 2004), rabbit (Luers et al. 2005), and rat (Alvarez et al. 1999; Calaghan and White 2004) myocardium, involvement of stretch-operated channels in this response was recently proposed in mouse ventricular muscle by Ward et al. (2008), who showed that canonical transient receptor-operated channels (TRPC) are sensitive to stretch in mice myocardium. Furthermore, Takahashi et al. (2007) showed TRPC sensitivity to A2 in human coronary artery smooth muscle cells. Interestingly, it was proposed that TRPC channels were necessary mediators of pathological cardiac hypertrophy in mice, in part through calcineurin-NFAT signaling (Wu et al. 2010), a pathway that we showed to be sensitive to NHE-1 inhibition in rats (Ennis et al. 2007). This discrepancy may be explained by two alternative hypotheses: (1) the TRPC channels were involved in one or some of the steps in the chain of events described previously; i.e., to induce A2 release after stretch or (2) by species differences.

Regarding whether some early intracellular signals triggered by the autocrine/paracrine mechanism, (i.e; NHE-1 activation) persists over time, we recently explore this in a mouse model of cardiac hypertrophy and failure by transverse aortic constriction (TAC). After 7 weeks of TAC, cardiac hypertrophy and decreased myocardial performance was detected, along with enhanced oxidative stress, as well as increased activity of redox-sensitive  $\text{p90}^{\text{RSK}}$  kinase and NHE-1 phosphorylation. Selective AT1 receptors blockade with losartan prevented  $\text{p90}^{\text{RSK}}$  and NHE-1 activation and decreased hypertrophy development, preserving contractility in spite of a higher workload (Cingolani et al. 2010, 2011c). It is important to highlight that losartan treatment did not restore wall thickness to control values (it remained  $\sim 24\%$  higher than controls), but certainly reduced it to levels “necessary” to counteract for the increase in pressure induced by the aortic constriction. In other words, an excessive



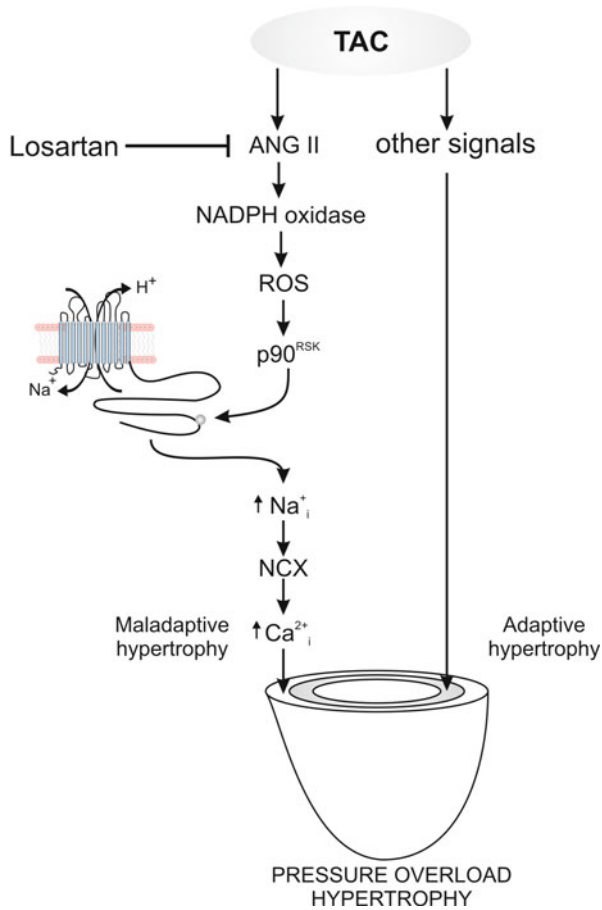
**Fig. 12.19** The positive inotropic effect of exogenous Ang II or ET-1 is accompanied by a cariporide-sensitive rise in  $[Na^+]_i$ . Panel **a**: Typical experiment and averaged results of the effect of a low dose of A2 on pH; Panel **b**: Time course of  $[Na^+]_i$  changes induced by A2 and averaged values after 30 min, in control and after NHE-1 inhibition with cariporide (HOE642) or ET receptors blockade (TAK044). Panels **c** and **d**: Same as A and B respectively, but after addition of an equipotent dose of exogenous ET-1. Note that despite the lack of pH change, there is an A2-induced NHE-1 stimulation detected by the increase in  $[Na^+]_i$  that requires available ET receptors and that this effect can be mimicked by an equipotent dose of exogenous ET-1. \* $P < 0.05$  vs pre-peptide control value. (Modified with permission from Perez et al. (2003))

cardiac hypertrophy was eliminated. Interestingly, in spite of the increase in wall stress seen with losartan in the present study, the reduction in cardiac hypertrophy was accompanied by an increased cardiac performance. These findings suggest that the degree of cardiac hypertrophy prevented by losartan was maladaptive or “inappropriate”, a concept previously coined by others (Mureddu et al. 2009). In this regard, it seems that pressure overload may trigger multiple intracellular signaling pathways in addition to enhanced AT1 receptor stimulation. Whereas some of these may be deleterious, others may benefit the heart allowing it to adapt to different stressors. The hypothetical proposal to explain these striking findings is schematized in Fig. 12.20.

### 12.7.1 Hypertrophic Signals Triggered by NHE-1

The possible link between the SFR and myocardial hypertrophy and failure is supported by the fact that an enhanced activity of NHE-1 is detected in several models of cardiac hypertrophy (Kusumoto et al. 2001; Engelhardt et al. 2002). In the hypertrophied myocardium of SHR (Wang et al. 2003), an increased activity of NHE-1 has been detected (Perez et al. 1995; Schussheim and Radda 1995) due to a kinase-dependent posttranslational phosphorylation of its cytosolic tail (Siczkowski et al. 1995; Ennis et al. 1998). The regression of myocardial hypertrophy produced by several pharmacological interventions was accompanied by normalization of the NHE-1 activity (Ennis et al. 1998; Alvarez et al. 2002). Moreover, chronic treatment of SHR rats with NHE-1 inhibitors caused load-independent regression of cardiomyocyte hypertrophy and fibrosis (Camilion de Hurtado et al. 2002; Cingolani et al. 2003b), although the latter effect took longer than the regression of myocyte size (Cingolani et al. 2003b), possibly as a reflection of the slower turnover rate of collagen metabolism (Weber and Brilla 1991).

Based on our previous results in adult multicellular cardiac preparations, hypertensive cardiac hypertrophy and failure are caused by an autocrine/paracrine chain of events triggered by myocardial stretch that begins with the activation of the AT1 receptors followed by the release/formation of endothelin-1 (ET-1), MR activation, EGFR transactivation and stimulation of the NHE-1 (Cingolani et al. 2005; Villa-Abrille et al. 2010; Caldiz et al. 2011). The increased production of ROS that results from A2/ET-1 stimulation of the NADPH oxidase may be responsible for ERK1/2-p90<sup>RSK</sup> activation and NHE-1 stimulation (Caldiz et al. 2007; Garcarena et al. 2008). NHE-1 hyperactivity leads to an increase in intracellular Na<sup>+</sup> concentration that promotes cytoplasmic calcium overload through the NCX (Cingolani et al. 2005). Calcium is widely recognized as one of the main pro-hypertrophic intracellular signal. It activates several intracellular pathways like calcineurin, nuclear factor of activated T cells (NFAT), calcium/calmodulin-dependent kinase II, protein kinase C and possibly other intracellular signaling pathways. Calcineurin is a pro-hypertrophic serine-threonine protein phosphatase that is activated in response to sustained elevations of intracellular levels of calcium. Once activated, calcineurin



**Fig. 12.20** Schematic representation of the proposed signaling pathway involved in the prevention of cardiac hypertrophy by AT1 receptors blockade. In our scheme, the AT1 receptors-sensitive part of the TAC-induced CH is maladaptive and related to redox-sensitive p90<sup>RSK</sup> activation, NHE-1 phosphorylation/activation, increase in intracellular Na<sup>+</sup> and the consequent increase in intracellular calcium through the NCX. The increased calcium concentration would then activate the calcineurin-NFAT signaling pathway responsible for triggering an abnormal cardiac growth. On the other hand, the same mechanical stimulus (*stretch* of cardiac muscle) may trigger other prohypertrophic signals intended to compensate for the increased wall stress (“adaptive hypertrophy”) (Catalucci et al. 2008). The reason for an improvement in cardiac performance accompanying the regression in cardiac hypertrophy due to AT1 receptors blockade is not apparent to us at present. However, we could speculate about cancellation of the negative inotropic effect assigned to calcineurin phosphatase activation (Sah et al. 2002; Li et al. 2003). (Modified with permission from Cingolani et al. (2011c))

directly dephosphorylates NFATs within the cytoplasm and promotes their translocation into the nucleus to induce the transcription of several genes. In SHR, Ennis et al. (2007) described that NHE-1 blockade regressed cardiac hypertrophy, decreased myocardial BNP, calcineurin A $\beta$  and nuclear NFAT expression. Additionally, they demonstrated by echocardiography, a reduction in left ventricular wall thickness

without changes in cavity dimensions or a significant decrease in blood pressure (Ennis et al. 2007).

Emerging evidence indicates that NHE-1 can be activated by ROS (Sabri et al. 1998; Snabaitis et al. 2002). Less well explored is the possibility that the increased ROS production, in addition to its role played upstream to NHE-1, may be induced by a rise in  $[Na^+]_i$  secondary to NHE-1 hyperactivity. Javadov et al. (2005, 2006) showed in rats with myocardial infarction that NHE-1 inhibition was able to prevent cardiac hypertrophy and decreased the vulnerability of mitochondria to calcium. In addition, they attributed the anti-hypertrophic effect of NHE-1 inhibition to the decreased generation of mitochondrial-derived ROS. In relation to this, we recently reported that the cardiac superoxide production induced by A2 was reduced under NHE-1 inhibition (Javadov et al. 2006; Garcarena et al. 2008). Moreover, the decrease in infarct size and level of tissue lipoperoxidation, induced by ROS scavengers administered during the reperfusion, can be mimicked by specific blockade of NHE-1 (Fantinelli et al. 2006). Therefore, NHE-1 inhibition may exert its beneficial effects by decreasing  $[Na^+]_i$  and/or ROS production. Both  $[Na^+]_i$  and ROS target the NCX to modify its activity, and therefore target calcium either at the bulk of the cytosol, or to more restricted spaces.

## 12.8 Conclusion and Perspectives. Possible Applications in the Clinical Arena

The RALES trial in 1999, the EPHESUS in 2003 and the EMPHASIS-HF in 2010, called attention to the beneficial effects of ALD antagonism in the treatment of heart failure. Cardiovascular disease and specially heart failure is one of the most important health problems in the world. Cardiac hypertrophy is known to be the main entrance door to the failing heart. As described above, cardiac hypertrophy and failure are triggered by intracellular signals that occur following myocardial stretch. Surprisingly, investigators working in the area of cardiac mechanics did not often extrapolate their early findings seen after stretch to the development of cardiac hypertrophy and/or failure. The reason for this could be that time frames in which these two phenomena occur are quite different. However, the long journey toward myocardial hypertrophy and failure begins with one step, and this first step may well be the autocrine/paracrine intracellular signaling pathway triggered by myocardial stretch as was proposed by Izumo and Sadoshima in neonatal cardiac myocytes (Sadoshima et al. 1993) and by us (Cingolani et al. 1998; Alvarez et al. 1999; Perez et al. 2001; Caldiz et al. 2007; Villa-Abrille et al. 2010) in adult multicellular preparations.

Current treatment against cardiac failure is mainly based on inhibition of hormones (A2, ALD, catecholamines). Despite the term “ALD inhibition” has been widespread used, this is often misleading and should be replaced by MR antagonism, mainly because ALD is not the only agonist binding to and activating MR (Mihailidou et al. 2009). Although several studies have demonstrated the important benefits of MR

antagonists in heart failure, their clinical use remains lower than expected and the exact mechanism of the beneficial effect is still unknown.

In 1990 Swedberg et al. (1990) established a relationship between plasma levels of ALD and mortality in patients with heart failure. This finding called attention to the possibility of predicting indexes of morbidity and mortality in patients suffering from this disease with excessive plasma levels of ALD. On the other hand, Karl Weber's laboratory carried out several investigations demonstrating that ALD itself is able to increase myocardial fibrosis independently of blood pressure level (for review see (Gandhi et al. 2011)). Furthermore, it was also shown that spironolactone, an MR blocker with diuretic properties developed by Searle laboratories, abolished these effects.

Contemporarily, several research labs reported that MR are not only expressed in classical ALD target tissues, but also in many others, including smooth and cardiac muscle.

The most potent stimulator of ALD synthesis is A2. Consequently, interfering with A2 actions should decrease systemic ALD levels. However, despite complete vascular angiotensin converting enzyme inhibition plasma ALD levels were elevated in patients with heart failure (Jorde et al. 2002). Even the combination of angiotensin converting enzyme inhibition and A2 antagonism only transiently reduces ALD plasma levels in patients with heart failure (McKelvie et al. 1999) suggesting A2 independent ALD production. This phenomenon known as ALD escape and whose underlying mechanism has not been completely clarified yet constitutes a strong proof to directly inhibit MR activation on top of angiotensin converting enzyme inhibition or AT1 blockers in the treatment of heart failure.

Among MR inhibitors, spironolactone was the first marketed compound in the early 1960s, and although proved to be clinically useful, it also showed tolerability problems due to painful gynecomastia or menstrual disturbances due to its androgenic and progesteronergic effects. Nevertheless, it was the only compound approved to be used in the RALES in 1663 patients with severe heart failure (Class III-IV NYHA). The trial was discontinued after a mean follow-up period of 24 months, because interim analysis determined that spironolactone reduced the risk of death by 30 %. Later on, more specific compounds that inhibit MR were developed, and after several years of delay Searle patented eplerenone in 1984. Eplerenone was tested in a clinical study called EPHEsus performed on 6642 patients with acute myocardial infarction complicated with left ventricular systolic dysfunction (ejection fraction less than 40 %). Treatment started 3–14 days after myocardial infarction and was maintained during 16 months. The results were positive, favoring the active treatment arm, and the main difference with RALES was that most of the patients in EPHEsus were receiving beta blockers (75 % vs. 11 % in RALES). All cause mortality decreased by ~15 % and sudden cardiac death by ~21 %. Interestingly, a post hoc analysis of the EPHEsus (Pitt et al. 2005) showed a reduction of all cause mortality by ~31 % as early as 30 days after eplerenone treatment. One important fact to emphasize, specially after the widely spread concept that high levels of ALD characterizes heart failure, is that plasma levels of ALD and Na<sup>+</sup> were in the normal range in both RALES and EPHEsus trials at randomization, and the beneficial effects were seen

early, probably before the “ALD escape” took place. These findings may suggest that: (1) MRs are activated by ligands other than ALD, or (2) cytosolic MRs are activated by increased intracellular levels of ALD independently of its plasma levels (Silvestre et al. 1998).

In contrast with the two mentioned clinical trials, the recently published EM-PHASIC (Zannad et al. 2010, 2011) was carried out on patients with less severe heart failure. This study enrolled 2737 patients with heart failure class II and III of the NYHA and left ventricular ejection fraction of no more than 35 %. The trial was stopped prematurely according to their rules, after a median follow-up of 21 months, due to the excess of benefit in reducing the risk of cardiovascular death or hospitalization for heart failure, obtained by anti-aldosteronic therapy with eplerenone, which was then extended to both arms of the trial. Another clinical trial which is currently running is the TOPCAT. This study will test the effects of MR inhibition in patients suffering from heart failure with left ventricular ejection fraction of at least 45 %. TOPCAT will probably end in 2013.

Finally, ALBATROS is a clinical trial designed to assess the potential superiority of MR inhibition early after myocardial infarction. The study will evaluate the intravenous bolus of potassium camreonate followed by a daily dose of 25 mg of spironolactone for 6 months on top of standard therapy in 1600 patients with myocardial infarction.

Although clinical evidence undoubtedly showed beneficial effects of treating heart failure patients with MR blockers, the mechanisms by which MR antagonism provide cardiovascular protection are not completely understood. In this regard, our own results assigning a crucial role for MR activation as an early hypertrophic signal triggered by myocardial stretch (presented before in this chapter) encouraged us to suggest that prevention of oxidative stress and NHE-1 activation should be considered as a potential key factor for the salutary effects of ALD antagonism in humans.

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