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Possible mechanism of the negative inotropic effect of α_1-adrenoceptor agonists in rat isolated left atria after exposure to free radicals

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1 This study was designed to investigate the mechanism(s) of the negative inotropic effects of α_1-adrenoceptor agonists observed in rat isolated left atria after exposure to free radicals.

2 Ouabain and calphostin C were used in contraction experiments to block the sodium pump and protein kinase C. Methoxamine-induced phospholipase C and Na^+/K^+ ATPase activities were measured.

3 Methoxamine (300 μM) increased contractile force by 1.6 ± 0.2 mN in control atria but decreased contractile force in electrolysis-treated atria by 2.0 ± 0.1 mN ($P<0.05$), as determined 10 min after methoxamine addition. In contrast, the positive inotropic effects of endothelin-1 (30 nM) and isoprenaline (10 μM) were reduced from 2.6 ± 0.3 to 1.3 ± 0.1 mN and from 2.6 ± 0.3 to 1.7 ± 0.2 mN, respectively, by electrolysis treatment ($P<0.05$), but not converted into a negative inotropic action.

4 In an inositol phosphate assay we observed that the stimulation of phospholipase C by methoxamine was attenuated by electrolysis when the (electrolyzed) medium from the organ bath was used, but the phospholipase C responses were restored by the use of fresh medium. However, fresh medium did not counteract the negative inotropic effect of methoxamine. Accordingly, the negative inotropic effect of methoxamine is not directly related to the impaired phospholipase C responses seen in atria subjected to electrolysis.

5 Ouabain (10 μM) and the protein kinase C inhibitor calphostin C (50 nM), completely prevented the negative inotropic effect of 300 μM methoxamine in electrolysis-treated atria.

6 Measurement of the Na^+/K^+ ATPase activity, revealed that in control atria, α_1-adrenoceptor stimulation with 300 μM methoxamine, decreased the Na^+/K^+ ATPase activity by $14.4 \pm 7.7\%$. In contrast, methoxamine increased the Na^+/K^+ ATPase activity by $48.8 \pm 8.9\%$ ($P<0.05$) in electrolysis-treated atria. Interestingly, this increase in Na^+/K^+ ATPase activity was completely counteracted by calphostin C ($1.4 \pm 0.1\%$ over basal).

7 These results indicate that the negative inotropic effects of α_1-adrenoceptor agonists, observed in rat isolated left atria exposed to free radicals, are likely to be caused by protein kinase C-mediated phosphorylation and subsequent activation of the Na^+/K^+ ATPase.

Keywords: Free radicals; α_1-adrenoceptor; negative inotropy; phospholipase C; protein kinase C; Na^+/K^+ ATPase

Introduction

Under most experimental conditions, the stimulation of myocardial α_1-adrenoceptors causes a positive inotropic response (for review see Li *et al*., 1997), but negative inotropic actions of α_1-adrenoceptor agonists have also been observed. For example, negative inotropic effects have been described at high electrical stimulation frequencies in rabbit papillary muscle (Endoh & Schumann, 1975), upon simultaneous activation of α- and β-adrenoceptors (Danziger *et al*., 1990) and upon simultaneous activation of α_1-adrenoceptors and endothelin receptors (Yang *et al*., 1996).

We have previously shown that free radicals, generated by electrolysis of the organ bath solution, reduce the inotropic responses to various inotropic stimuli in rat isolated left atria. These inotropic stimuli include lowering of stimulation frequency, sodium withdrawal, extracellular Ca^{2+} addition, the adenylyl cyclase activator forskolin, the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) analogue dibutyryl-cyclic AMP, and agonists at β-adrenoceptors (Peters *et al*., 1997). Thus, electrolysis and concomitant free radical generation appear to impair cardiac contractility in general. However, the alterations of the responses to the α_1-adrenoceptor agonists, methoxamine, cirazoline and ST 587 were affected in a different manner, since these agonists surprisingly caused negative inotropic actions in electrolysis-treated atria (Peters *et al*., 1997). Since negative inotropic effects in electrolysis-treated atria were not observed with any other inotropic manoeuvre, the present study was designed to investigate the underlying mechanism(s) in more detail.

The signal transduction mechanism of the α_1-adrenoceptor is complex and not understood in detail. Stimulation of the α_1-adrenoceptor leads to a rapid breakdown of phosphoinositide by phospholipase C, which results in the formation of inositol 1,4,5 trisphosphate (IP_3) and 1,2 diacylglycerol. IP_3 mobilizes Ca^{2+} from intracellular stores in various tissues including myocardial sarcoplasmic reticulum (Fabiato, 1986; Nosek *et al*., 1986), although contradictory findings have also been obtained (Movsesian *et al*., 1985). Diacylglycerol is the endogenous activator of protein kinase C (PKC) which can target several substrates in the myocardial cell such as

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phospholamban (Movsesian *et al*., 1984), C-protein (Venema & Kuo, 1993), troponin I and T (Kato *et al*., 1983), the Na^+/H^+ exchanger (Moolenaar *et al*., 1984), and possibly the Na^+/K^+ ATPase (Feschenko & Sweadner, 1994). α_1-Adrenoceptor stimulation enhances the Na^+/H^+ exchange via a PKC-mediated mechanism, resulting in an intracellular alkalinization and the accumulation of Na^+. Subsequent activation of the Na^+/Ca^{2+} exchange might result in an enhanced Ca^{2+} influx and hence a rise in contractile force (Iwakura *et al*., 1990). It has been shown that intracellular alkalinization induces sensitization of myofilaments (Fabiato & Fabiato, 1978) and this mechanism could be responsible for the sustained positive inotropic effect. However, because these hypotheses are based mainly on experiments with rather unspecific amiloride derivatives, the results may only be interpreted with great caution (Pucéat, 1995). Phosphorylation of tropinin I and T by PKC decreases the rate of crossbridge cycling and reduces the myofibrillar actomyosin Mg ATPase activity, and will thus decrease the force of contraction (Venema & Kuo, 1993; Strang & Moss, 1995). Several studies have shown that the Na^+/K^+ ATPase is involved in the α_1-adrenoceptor signal transduction cascade. Shah *et al*. (1988) have shown that the activation of the sodium pump after α_1-adrenoceptor stimulation is sensitive to pertussis toxin. However, other groups have provided evidence that, at least *in vitro*, the Na^+/K^+ ATPase is a good substrate for PKC. Whether phosphorylation of the sodium pump leads to an increase or decrease of the enzyme activity is still unclear (Carranza *et al*., 1996; Logvinenko *et al*., 1996). Among the listed components of α_1-adrenergic signal transduction in the heart, phosphorylation of the troponins and activation of the Na^+/K^+ ATPase will reduce inotropy while the other processes will enhance it (for overview see Figure 6).

Based on these findings we have hypothesized that oxidative stress leads to biochemical changes which result in an altered ratio of the negative and positive inotropic components of the α_1-adrenoceptor signal transduction pathway. To test this hypothesis we performed direct measurements of methoxamine-induced phospholipase C and Na^+/K^+ ATPase activity changes, and also used ouabain to block the Na^+/K^+ ATPase, and calphostin C to inhibit the PKC-mediated responses.

Methods

Electrolysis treatment and contraction studies

Male Wistar rats (Iffa Credo, Les Oncins, France) weighing 240–300 g were killed by stunning and decapitation. Electrolysis treatment and contraction measurements were performed as previously described in detail (Peters *et al*., 1997). Briefly, the hearts were removed quickly and placed in a Tyrode solution of the following composition (in mM): NaCl 119, KCl 4.5, $MgCl_2$ 0.5, $CaCl_2$ 2.5, glucose 11 and Tris 30 at pH 7.5 at room temperature; and were bubbled with 100% oxygen. The isolated left atria were suspended in water jacketed organ baths (kept at 37°C and gassed with 100% oxygen) filled with 5 ml Tyrode solution (pH adjusted to 7.5 at 37°C), and connected with a silk thread to an isometric force transducer. The atria were paced with a field stimulator (Hugo Sachs Electronic, Germany) at a frequency of 3 Hz (0.5 V, 5 ms). The isometric force of contraction was recorded on a Maclab/8e data acquisition system (ADInstruments, Australia). The resting tension was adjusted to 5 mN and the atria were allowed to equilibrate for at least 45 min. At 20 min intervals the medium was replaced with fresh buffer.

After the equilibration period, free radicals were generated by electrolysis of the medium with two additional circular platinum wire electrodes (each 0.75 cm in length) at the bottom of the organ baths (∅ 1.4 cm). A constant current of 30 mA, generated by a 6 channel constant current device (Dept. of Electronics, Academic Medical Centre, Amsterdam), was applied for 75 s. Agonists were added 30 min after the electrolysis procedure, to ensure that the tissue was damaged significantly. Inhibitory drugs were added 15 min before the addition of agonist. Unless indicated otherwise the atria remained in the electrolysis-treated buffer throughout the experiment. The concentrations of agonists used in this study gave a maximum response in control atria based on concentration-response curves (Peters *et al*., 1997).

[^{3}H]-inositol phosphate assay

Basal and methoxamine induced inositol phosphate formation were measured in atrial slices according to the method of Eid & De Champlain (1988). For this purpose the atria were removed from the organ bath 30 min after electrolysis and cut into small slices of approximately 2–3 mg. Individual slices were labelled with 10 μCi ml^{-1} *myo*-[^{3}H]-inositol for 60 min at 37°C in 3 ml vials containing 300 μl Tyrode solution from the organ bath. Thereafter, 10 μl LiCl solution (final concentration 10 mM) was added to prevent degradation of inositol monophosphates by inositol monophosphatase. Twenty minutes later 20 μl methoxamine solution (final concentration 300 μM) or saline was added to the vials, and the incubation continued for another 60 min. Thereafter, the slices were washed twice with 1 ml ice-cold buffer, and the reaction was stopped by the addition of 330 μl ice-cold methanol and 660 μl chloroform. The vials were vortexed for 30 s and centrifuged 15 min at 750 *g* at 4°C. Aliquots of the upper phase (450 μl) were placed on columns containing 1 ml of AG 1-X8 resin (200–400 mesh, formate form). The columns were washed twice with 5 ml distilled water. Glycerophosphoinositides were eluted with 2×5 ml 60 mM ammonium formate solution. [^{3}H]-IPs were eluted with 2×1 ml of 0.1 M formic acid/1 M ammonium formate. The latter fraction was added to 8 ml scintillation fluid, and radioactivity was measured in a liquid scintillation counter at 42% efficiency.

In experiments with doxazosin (1 μM) and phentolamine (1 μM), the α-adrenoceptor antagonists were added to the medium in the organ bath 10 min after electrolysis. Experiments were performed in quadruplicate.

Na^+/K^+ ATPase activity measurement

Na^+/K^+ ATPase activities were measured by determination of the K^+-dependent *p*-nitrophenyl phosphatase activity (Larsen & Kjeldsen, 1995) in crude atrial homogenates. Briefly, atria were removed from the organ bath 10 min after addition of methoxamine (300 μM) or saline. The atria (10 mg wet weight tissue ml^{-1}) were washed and homogenized with an Ultra-turrax (15 s full speed, 0°C) in buffer containing 30 mM histidine, 2 mM EDTA and 250 mM sucrose (pH 7.2).

For the assay, 100 μl of tissue homogenate was added to 800 μl reaction buffer (25 mM histidine at pH 7.4, 15 mM $MgCl_2$ and 50 mM KCl or 100 mM NaCl). After 10 min preincubation at 37°C, the reaction was initiated by addition of 100 μl 100 mM *p*-nitrophenylphosphate to the reaction mixture and incubated for 30 min at 37°C. The reaction was stopped by the addition of 2 ml ice-cold buffer containing 500 mM Tris and 55 mM EDTA. The formation of *p*-nitrophenol was quantified spectrophotometrically at a wavelength of 410 nm

with a Zeiss Specord u.v./Vis S10 spectrophotometer. The K^+-dependent *p*-nitrophenyl phosphatase activity was measured as the difference in activity in the presence or absence of K^+. Enzyme activity was calculated by use of the molar absorption coefficient of *p*-nitrophenol (1.81×10^4) and expressed as μmol min^{-1} g^{-1} wet weight tissue.

Data analysis

Data are expressed as means ± s.e.mean Student's *t* test (two tailed, unpaired) and ANOVA (Dunnett's) were used, and *P* values <0.05 were considered to be statistically significant.

Chemicals

Endothelin-1, isoprenaline bitartrate, methoxamine HCl and phentolamine HCl were purchased from Sigma Chemicals (St Louis, MO, U.S.A.). *p*-Nitrophenyl phosphate and calphostin C from ICN Biomedicals (Zoetermeer, The Netherlands), *myo*-[^{3}H]-inositol from Amersham (Buckinghamshire, U.K.). Resin AG 1-x8 was purchased from BIO RAD (Hercules CA, U.S.A.). Doxazosin mesylate and ouabain were obtained from OPG (Utrecht, The Netherlands). All drugs were dissolved in distilled water. Calphostin was dissolved in 99% dimethylsulphoxide (DMSO).

Results

Contraction experiments

The initial force of contraction before the application of electrolysis was 9.4 ± 0.3 mN ($n=10$). Whereas the force of contraction remained stable in control atria, a continuous deterioration was observed in atria subjected to electrolysis. Accordingly, the basal force of contraction determined 30 min after electrolysis was only 3.6 ± 0.5 mN (i.e. approximately 40% of initial force of contraction) while it was 8.6 ± 0.4 mN (i.e. approximately 90% of initial force of contraction) in control preparations not subjected to electrolysis. Methoxamine (300 μM) increased contractile force by 1.6 ± 0.2 mN ($n=4$) in control atria but decreased contractile force in electrolysis-treated atria by 2.0 ± 0.1 mN ($n=5$, $P<0.05$, Figure 1), as determined 10 min after methoxamine addition. In contrast, the positive inotropic effects of endothelin-1 (30 nM) and isoprenaline (10 μM) were reduced from 2.6 ± 0.3 to 1.3 ± 0.1 mN and from 2.6 ± 0.3 to 1.7 ± 0.2 mN, respectively, by electrolysis treatment ($n=6$, $P<0.05$; Figure 1) but these agents did not cause any reduction of contractile force.

We tested the antagonism of the α_1-adrenoceptor antagonist doxazosin under two conditions; in electrolysis-subjected medium and in fresh medium. Doxazosin (10 μM) did not block the negative inotropic effect of methoxamine in electrolysis-treated atria when the buffer was not changed before antagonist addition (Figure 2a). However, doxazosin significantly inhibited the negative inotropic effect of methoxamine when the medium was changed before antagonist addition (Figure 2b). Doxazosin had no effect on the gradual decrease in contractile force normally seen (i.e. without the addition of methoxamine) after electrolysis.

Ouabain (10 μM, added 15 min after electrolysis) had no significant effect on the contractile force of control and electrolysis-treated atria; neither did it change the inotropic response to 300 μM methoxamine in control atria. However, in the presence of ouabain, 300 μM methoxamine had no negative inotropic effect in electrolysis-treated atria. Accordingly, the force of contraction 10 min after methoxamine addition (that is 40 min after electrolysis) was 0.8 ± 0.2 mN in the absence and 4.3 ± 0.5 mN in the presence of ouabain (and 2.5 ± 0.3 mN for electrolysis without ouabain; $n=5$ each, $P<0.05$; Figure 3).

The protein kinase C inhibitor calphostin C (50 nM, added 15 min after electrolysis) did not significantly affect contractile

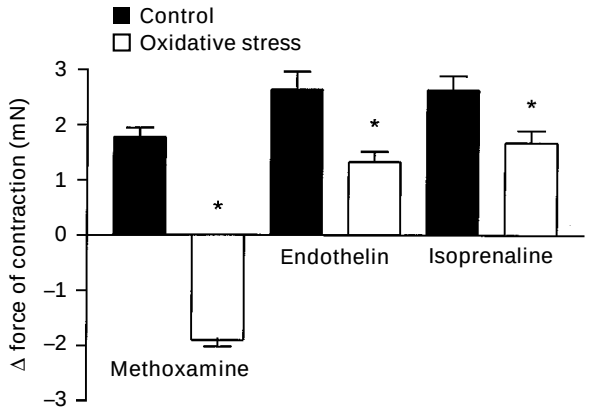

Figure 1 Comparison of the inotropic effects of 300 μM methoxamine, 30 nM endothelin and 10 μM isoprenaline in control atria and in atria subjected to oxidative stress, measured 10 min after their addition. *$P<0.05$ compared to their controls (two-tailed Student's *t* test; $n=4-8$).

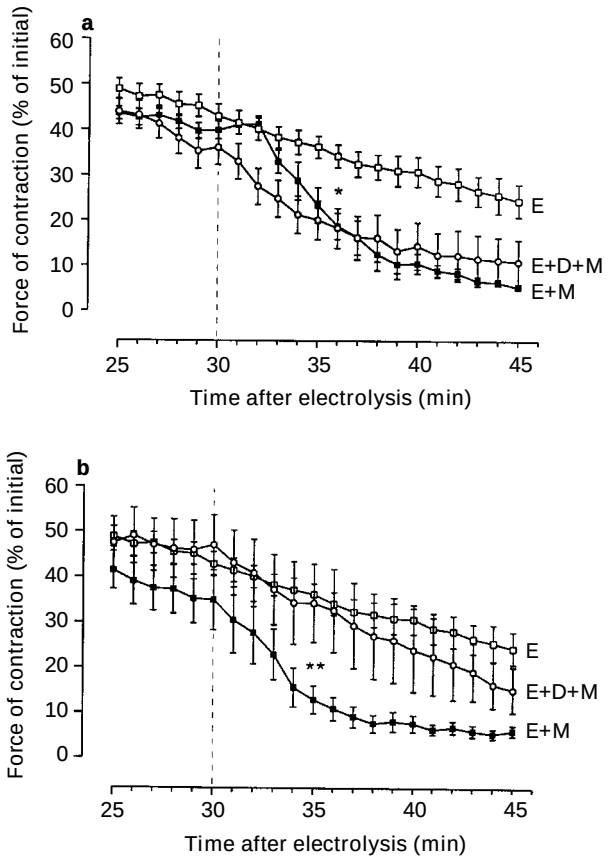

Figure 2 Effect of changing the medium on the antagonism of the negative inotropic effect of 300 μM methoxamine (M) by 1 μM doxazosine (D) in electrolysis (E)-treated atria. (a) Results in electrolysis-treated medium; (b) results in fresh medium after electrolysis. Data are expressed as % of initial values (9.4 ± 0.3 mN). *$P<0.05$ compared to electrolysis only. **$P<0.05$ compared to electrolysis with methoxamine ($n=6-8$). Vertical lines show s.e.mean.

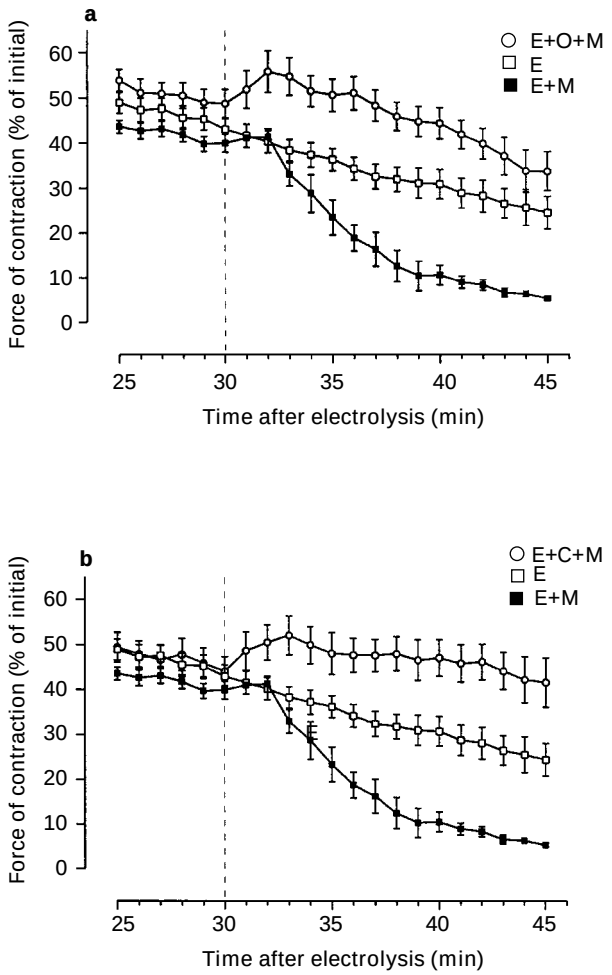

Figure 3 Effect of (a) 10 μM ouabain (O) and (b) 50 nM calphostin C (C) on the negative inotropic effect of 300 μM methoxamine (M) in rat isolated left atria exposed to electrolysis (E). Methoxamine was added to the medium 30 min after electrolysis. Ouabain or calphostin C was added to the medium 15 min before methoxamine. The curve representing the normal gradual decrease in contractile force due to electrolysis is also shown (E). Data are expressed as % of initial values (9.4$\pm$0.3 mN). $P<0.05$ compared to curves without ouabain or calphostin C ($n=6-7$).

force in control and electrolysis-treated atria, and neither did it alter the methoxamine (300 μM)-induced positive inotropic effect in control atria. Calphostin C completely blocked the negative inotropic effect of methoxamine in electrolysis-treated atria and partially restored the positive inotropic effect (Figure 3). Accordingly, the force of contraction 10 min after methoxamine addition amounted to 0.8$\pm$0.2 mN in the absence and to 4.4$\pm$0.4 mN in the presence of calphostin C (and 2.5$\pm$0.3 mN for electrolysis only; $n=5$ each, $P<0.05$; Figure 3).

[^{3}H]-inositol phosphate assay

Basal inositol phosphate formation was not significantly different between the control (352$\pm$46 c.p.m.) and the electrolysis-treated organs (258$\pm$17 c.p.m., $n=5$). Methoxamine enhanced inositol phosphate formation in control atria by 136$\pm$19% over basal ($n=3$), while the corresponding increase in electrolysis-treated atria was only 68$\pm$15% over basal ($n=5$, $P<0.05$ vs control in an unpaired t test). Doxazosin (1 μM) and phentolamine (1 μM) almost completely

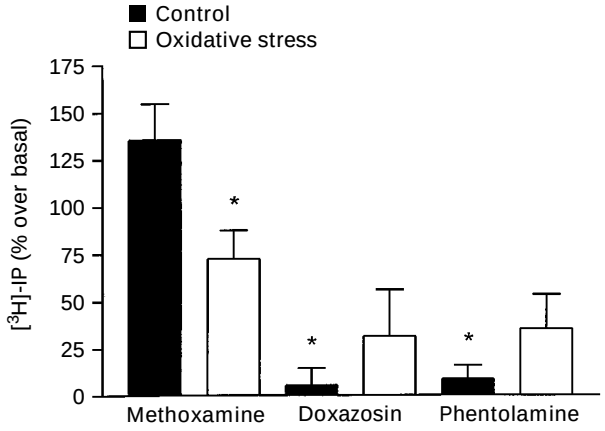

Figure 4 Inositol phosphate ([^{3}H]-IP) formation induced by α_1-adrenoceptor stimulation with 300 μM methoxamine in slices of control atria and of atria subjected to oxidative stress without medium refreshment. Phentolamine (1 μM) and doxazosin (1 μM) were added to the organ bath 10 min after electrolysis. Slices were prepared and incubated with *myo*-[^{3}H]-inositol 30 min after electrolysis. Note the incomplete inhibition by the antagonists in the electrolysis group. Data are expressed as % over basal (control 352$\pm$46 c.p.m. electrolysis 258$\pm$17 c.p.m.). *$P<0.05$ compared to methoxamine control ($n=4-5$).

inhibited methoxamine-stimulated inositol phosphate formation in control organs (Figure 4), but the inhibition was considerably weaker and no longer statistically significant in electrolysis-treated atria (Figure 4).

When these experiments were repeated with fresh buffer for labelling, somewhat different results were obtained. The enhancements of inositol phosphate formation by methoxamine in control atria (146$\pm$36% over basal, $n=5$) were similar to those seen in control atria without buffer refreshment. A quantitatively similar enhancement was also seen in electrolysis-treated atria (126$\pm$25% over basal, $n=5$).

Na^+/K^+ ATPase activity

The Na^+/K^+ ATPase activity was measured in crude homogenates of control or electrolysis-treated atria by determination of the K^+-dependent *p*-nitrophenyl phosphatase activity. Basal activity was significantly lower in electrolysis-treated compared to control atria (1.01$\pm$0.12 vs 1.72$\pm$0.16 μmol min^{-1} g^{-1}, $n=5$, $P<0.05$). When calphostin C (50 nM) or vehicle (DMSO<0.01%) was added to the organ bath 15 min after electrolysis, the electrolysis did not significantly decrease basal Na^+/K^+ ATPase activity (1.43$\pm$0.05 μmol min^{-1} g^{-1} and 1.28$\pm$0.09 for vehicle only, $n=5$). Addition of ouabain (10 μM) directly to the reaction mixture reduced the basal activities by approximately 56%, thus confirming the ability of our assay to detect alterations in sodium-pump activity (Larsen & Kjeldsen, 1995).

Methoxamine treatment of the atria decreased the Na^+/K^+ ATPase activity in control atria by 14.4$\pm$7.7% ($n=5$, Figure 5). In contrast, methoxamine increased the Na^+/K^+ ATPase activity by 48.8$\pm$8.9% in electrolysis-treated atria ($n=6$; $P<0.05$, Figure 5). When calphostin C (50 nM) was added to the organ bath 15 min after electrolysis, methoxamine (added 30 min after electrolysis to the organ bath) no longer stimulated Na^+/K^+ ATPase activity (1.4$\pm$0.1% over basal, $n=6$, Figure 5). Addition of vehicle (DMSO, <0.01%) alone to the organ bath had no influence on the methoxamine-induced increase in Na^+/K^+ ATPase activity in electrolysis-treated atria.

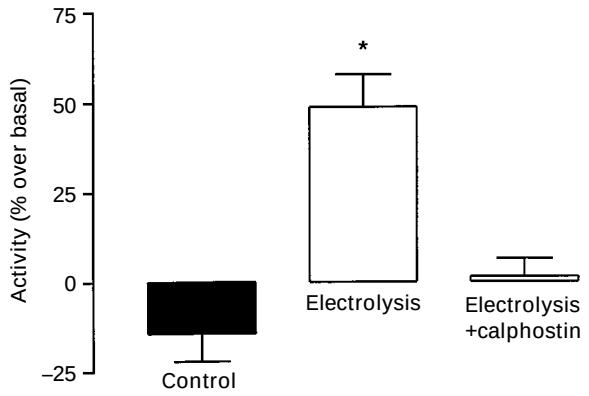

Figure 5 Na^+/K^+ ATPase activity, measured as K^+-dependent *p*-nitrophenyl phosphatase activity, after α_1-adrenoceptor stimulation with 300 μM methoxamine in crude homogenates of control atria and of atria exposed to electrolysis. Calphostin C (50 nM) and methoxamine were administered to the medium in the organ bath 15 min and 30 min after electrolysis, respectively. Homogenates were prepared 10 min after addition of methoxamine or saline. Data are expressed as % over basal (control: 1.72 ± 0.16, electrolysis: 1.01 ± 0.12 and calphostin C: 1.43 ± 0.05 μmol *p*-nitrophenol min^{-1} g^{-1}). *$P<0.05$ compared to control, $n=5-7$.

Discussion

We have previously shown that electrolysis treatment attenuates the inotropic responses to reduced stimulation frequency, the elevation of extracellular Ca^{2+}, withdrawal of extracellular Na^+, the β-adrenoceptor agonist, isoprenaline, and to forskolin and db-cyclic AMP. On the other hand, electrolysis treatment appears to reverse the positive inotropic effects of the α_1-adrenoceptor agonists, methoxamine, cirazoline and ST 587 into opposite, negative inotropic actions (Peters *et al*., 1997). Accordingly, electrolysis appears to involve a general impairment of inotropic responses to almost all stimuli and, unexpectedly, a reversal into negative responses which is limited to α_1-adrenoceptor agonists. The present study was designed to investigate the mechanisms underlying the latter surprising phenomenon, that is the reversal into negative inotropic responses for the α_1-adrenoceptor agonists in electrolysis-treated, isolated left atria of the rat.

Electrolysis of the bath fluid generates hydroxyl radicals, superoxide anions, hydrogen peroxide, hypochlorite and singlet oxygen in a current- and time-dependent manner (Jackson *et al*., 1986; Chahine *et al*., 1991; Niu *et al*., 1995; de Keulenaer *et al*., 1995). While most of these free radicals are believed to be short-lived, some of them (or lipid peroxidation products like malondialdehyde and hydroxynonenal) may survive sufficiently long enough to oxidize some of the pharmacological agents being used. Therefore, we performed our functional and inositol phosphate experiments in both electrolysis-treated and fresh bath fluids. This procedure allows us to distinguish between biological free radical actions on the atria and physico-chemical effects on the molecules of the agonists and antagonists. Our data suggest that both types of effect may indeed occur: firstly, the antagonists doxazosin and phentolamine failed to inhibit the methoxamine effects in electrolysis-treated atria in both, the present and our previous study (Peters *et al*., 1997) when added directly to the electrolysis-treated medium. However, they inhibited the methoxamine effects under control conditions and after addition to electrolysis-treated atria in the presence of fresh buffer. Consequently, electrolysis-generated free radicals, or lipid peroxidation products, appear to damage, or interfere chemically with the action of the antagonists. Secondly. the negative inotropic effect of methoxamine was observed when the agonist was added to both, electrolysis-treated and fresh medium. For this reason, the negative inotropic effects appear to result from changes within the atria rather than from chemical alterations of methoxamine (present study) or the α_1-adrenoceptor agonists cirazoline and ST 587 (Peters *et al*., 1997).

α_1-Adrenoceptor agonists clearly differ from the other inotropic stimuli investigated in our previous study (Peters *et al*., 1997), since they couple to phospholipase C with the subsequent elevation of intracellular Ca^{2+} and activation of PKC. This raised the possibility that the negative inotropic effects of the α_1-adrenoceptor agonists following electrolysis may be related to alterations of the PLC signalling pathway. On the other hand, the inotropic responses to another PLC-coupled receptor agonist, endothelin-1 (Vigne *et al*., 1989; Vogelsang *et al*., 1994) proved impaired but not reversed. The experiments with electrolysis-treated and fresh medium have shed additional light on a possible alteration of PLC as the cause for the negative inotropic effects of α_1-adrenoceptor agonists. Accordingly, we observed that the stimulation of PLC by methoxamine was indeed attenuated by electrolysis when the medium from the organ bath was used. However, the PLC responses were restored by the use of fresh medium. This is in contrast to the functional data which showed negative inotropic responses to methoxamine with electrolysis-treated and fresh bath media. It may therefore be possible that free radicals impair PLC responses and hence contribute to the attenuated inotropic responses, for instance those for endothelin-1. On the other hand, this phenomenon does not explain why the positive inotropic responses to α_1-adrenoceptor agonists (which occur with fresh and old buffer) were reversed into negative ones. Possible alternative candidates include signal transduction steps which occur distally or in parallel to PLC, for instance PKC or Na^+/K^+ ATPase.

Cardiac α_1-adrenoceptor stimulation activates PKC and, under some circumstances, may stimulate Na^+/K^+ ATPase (Endoh, 1991). This latter effect may be partially mediated by PKC, but there is still some debate as to whether phosphorylation of the Na^+/K^+ ATPase will increase or decrease the activity of this enzyme (Carranza *et al*., 1996; Logvinenko *et al*., 1996). In the present study the PKC inhibitor, calphostin C, and the Na^+/K^+ ATPase inhibitor, ouabain, both prevented the negative inotropic effects of methoxamine by electrolysis treatment. These results indicate the involvement of the Na^+/K^+ ATPase and possibly PKC in the negative inotropic effects of methoxamine after oxidative stress. Our previous observation that the PKC inhibitor chelerythrine (2 μM) did not prevent the negative inotropic activity of methoxamine (Peters *et al*., 1997), may be related to the fact that calphostin C and chelerythrine are structurally distinct inhibitors of PKC which may interfere with its inhibitory activity by different mechanisms (Gordge & Ryves, 1994).

For this reason, we investigated the regulation of cardiac Na^+/K^+ ATPase activity by electrolysis and α_1-adrenoceptor stimulation in a direct manner. Our assay only measured changes in activity due to modifications of the enzyme itself (phosphorylation and/or conformational changes due to free radical exposure) but not those due to differences in the electrochemical Na^+ or K^+ gradients, because the activitites were measured in tissue homogenates. Our data confirm previous observations that Na^+/K^+ ATPase activity is reduced by oxidative stress (Huang *et al*., 1994). The latter effect was blocked by the PKC inhibitor, calphostin C, but also by its vehicle, DMSO (<0.01%). As DMSO is known to have

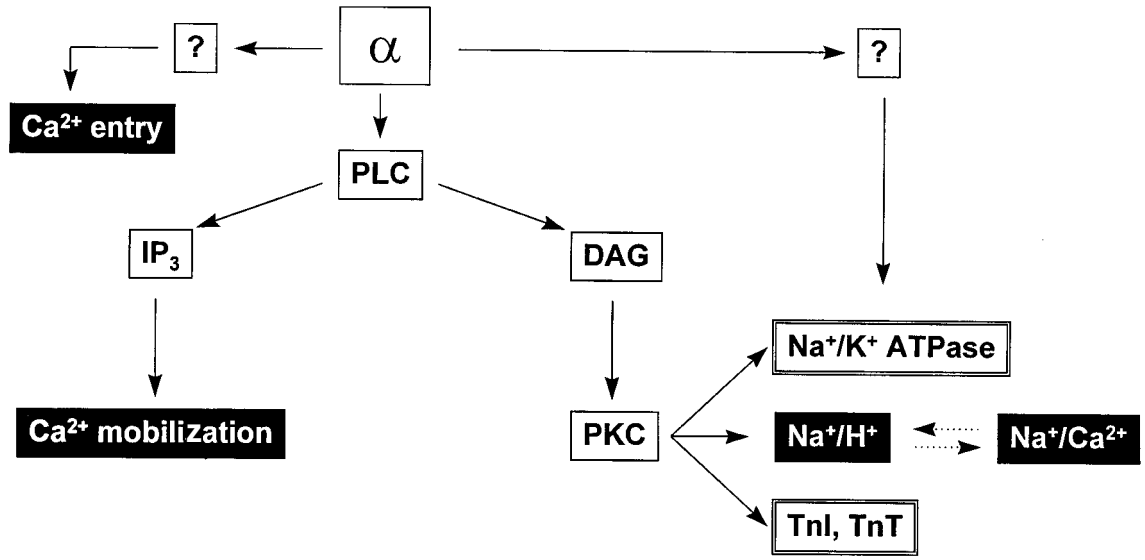

Figure 6 Schematic representation of the α-adrenoceptor signal transduction. PLC = phospholipase C, IP_3 = inositol 1,4,5 trisphosphate, DAG = 1,2 diacylglycerol, PKC = protein kinase C, TnI and TnT = troponins I and T. Solid boxes represent positive inotropic components and double-lined boxes negative inotropic components. For details see text.

hydroxyl radical scavenger activity, these data suggest that the observed effect of calphostin C on basal Na^+/K^+ ATPase activity may be related to the free radical scavenging by its vehicle rather than to PKC inhibition.

The inhibition of Na^+/K^+ ATPase by methoxamine by approximately 15% in control atria was reversed into an approximately 50% stimulation in electrolysis-treated atria. Interestingly, this stimulation of Na^+/K^+ ATPase activity by methoxamine in electrolysis-treated atria was prevented by the PKC inhibitor calphostin C but not by its vehicle DMSO, indeed suggesting that this effect was mediated via PKC. The finding that calphostin C completely inhibited the methoxamine-induced increase of Na^+/K^+ ATPase activity in electrolysis-treated atria also indicates that, under our experimental conditions, phosphorylation by PKC may increase Na^+/K^+ ATPase activity.

On the basis of these data we propose the following model to explain the negative inotropic effects of α_1-adrenoceptor agonists by electrolysis (Figure 6). Cardiac α_1-adrenoceptors can couple to a multitude of signalling pathways which may either enhance or reduce cardiac contractility (Endoh, 1991). Electrolysis treatment may affect these signalling pathways in multiple ways to alter the balance between positive and negative inotropic events. Consequently, chemical destruction of the agonists, impairments of PLC and adenylyl cyclase (Peters *et al*., 1997) activities and of the general contractile machinery of the cardiomyocyte may occur. These alterations may explain the reduced inotropic effects of all the stimuli tested in electrolysis-treated atria. However, they do not explain the reversal into negative inotropic effects which appear to be specific for α_1-adrenoceptor agonists. Hence, we propose that electrolysis alters the coupling between α_1-adrenoceptors, PKC and Na^+/K^+ ATPase to yield activation instead of inhibition of Na^+/K^+ ATPase. It has been shown that phosphorylation of the Na^+/K^+ ATPase by PKC is conformation-dependent (Feschenko & Sweadner, 1994). A possible explanation for the increased PKC-mediated Na^+/K^+ ATPase activity after methoxamine stimulation in atria subjected to electrolysis could be that free radicals induce a conformational change of the Na^+/K^+ ATPase, and thereby make the enzyme more susceptible to phosphorylation by PKC.

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