

# Nongenomic Effects of Aldosterone on Phosphocreatine Levels in Human Calf Muscle during Recovery from Exercise

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## ABSTRACT

Nongenomic *in vitro* effects of aldosterone on the sodium-proton antiport and intracellular second messengers have been described in human mononuclear leukocytes, vascular smooth muscle cells, and endothelial cells. To test the potential physiological relevance of these effects, an *in vivo*  $^{31}\text{P}$  magnetic resonance spectroscopy study on the human calf at rest and during exercise was performed in 10 healthy volunteers receiving either 1 mg aldosterone or placebo iv in a double blind, randomized, cross-over trial. Spectra were analyzed for phosphocreatine, ATP, phosphomonoesters, inorganic intracellular phosphate, and intracellular pH. Resting values remained unchanged by

aldosterone. After isometric contraction of the calf (50% body weight for 3 min), phosphocreatine recovered to significantly higher levels after application of aldosterone compared with placebo. Other parameters were not significantly changed by aldosterone. Effects appeared immediately after isometric contraction and, thus, occurred within 8 min of aldosterone administration. They are, therefore, likely to represent the first contemporary evidence of nongenomic *in vivo* effects of aldosterone in man. These findings also point to an involvement of aldosterone in the acute stress adaptation of cellular oxidative metabolism in human muscle physiology. (*J Clin Endocrinol Metab* 81: 4296–4300, 1996)

RECENTLY, rapid *in vitro* effects of aldosterone on the sodium, potassium, and calcium concentrations and cell volume of human mononuclear leukocytes (HML) (1–3) and on the activity of the sodium-proton exchanger of the cell membrane in HML and vascular smooth muscle cells (4–6) have been demonstrated. These effects are not compatible with an action via classical type I mineralocorticoid receptors and suggest the existence of distinct receptors subsequently described in plasma membranes from HML and pig kidney and liver (7–9). The phosphoinositide pathway, protein kinase C, and free intracellular calcium appear to be involved in intracellular signaling in HML and vascular smooth muscle cells (4, 10–12). For example, the second messenger, intracellular  $\text{Ca}^{2+}$ , is consistently increased by aldosterone within 1–2 min. In vascular smooth muscle cells, calcium is released from perinuclear stores, whereas in endothelial cells, a predominant increase in subplasmalemmal calcium is seen. Effects are half-maximal at physiological concentrations of free aldosterone (0.1 nmol/L), whereas cortisol is inactive up to 0.1  $\mu\text{mol/L}$ ; the classical mineralocorticoid antagonist canrenone is ineffective in blocking this action of aldosterone (13).

Nongenomic effects of mineralocorticoids are very rapid,

as opposed to genomic effects, which are characterized by a substantial delay. The latency of genomic steroid effects corresponds to a long series of subcellular processes, including messenger ribonucleic acid production, messenger ribonucleic acid modification, translation into proteins, protein translocation, and/or insertion into membranes. Among the earliest genomic effects known in the action of mineralocorticoids is the increased rate of mouse mammary tumor virus long terminal repeat transcription, which begins within 30 min and peaks after 3 h in a feline renal cell line (14). This process is insensitive to cycloheximide, as it does not require new protein synthesis. Although even more rapid effects of steroids on transcriptional processes are known (glucocorticoid effects on mouse mammary tumor virus long terminal repeat in  $\text{L tk}^- \text{aprt}^-$  cells were first seen after 7.5 min (15)), no actinomycin D- or cycloheximide-sensitive effects are known to occur within less than 30 min of mineralocorticoid application. This additional delay at the effector levels is probably due to the time-consuming translation and processing of newly synthesized protein molecules from their site of origin to their final location in the cell membrane.

The physiological and clinical relevance of the rapid nongenomic effects is not yet clear. The extent of their response to standard stimuli is relatively modest, and they, thus, may have been overlooked in related clinical studies. To our knowledge, the only *in vivo* evidence for rapid aldosterone action is a study on cardiovascular parameters in man (16) and a report on the baroreceptor reflex in the dog (17), which demonstrated effects of aldosterone after 5–15 min on pe-

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ripheral resistance, cardiac index, and spiking activity of baroreceptor neurons.

In the present report, rapid effects of aldosterone were assessed by the use of a 40-cm horizontal bore  $^{31}\text{P}$  magnetic resonance spectrometer, which allows for continuous *in vivo* monitoring of markers of aerobic and anaerobic energy metabolism and cellular acid-base regulation in a human limb. The latter parameter was thought to be affected by aldosterone, as those effects on sodium-proton exchange described above may be reflected in intracellular pH. The calf was chosen as it complies with the dimensions of the instrument, and a relatively large muscle can be monitored in which a constant force can be generated.

## Materials and Methods

### Materials

Aldosterone for human administration was a gift from Parke-Davis (Lainate, Italy); it was dissolved in isotonic NaCl (0.5 mg/mL) under sterile conditions and verified as pyrogen free.

### Study volunteers

Ten healthy volunteers (seven men and three women; mean age,  $34 \pm 5$  yr) were recruited from the German Aerospace Research Establishment (Cologne, Germany). A history was taken to exclude chronic and acute diseases such as hypertension, heart failure, diabetes mellitus, drug allergies, infections, or psychiatric disorders. Special care was taken to exclude carriers of metal implants or prostheses. Physical examination and routine electrocardiogram (12 lead) revealed no abnormalities. The study design was approved by the ethical committee of the University of Aachen (Aachen, Germany).

### Laboratory methods

Venous blood samples were obtained after a minimum of 30 min of rest in the supine position in a quiet environment. Blood was collected via an indwelling catheter into heparinized containers on ice and immediately processed. Routine laboratory tests on serum and urine were performed by standard methods in a BM/Hitachi 717 (Boehringer Mannheim, Mannheim, Germany). Plasma renin and aldosterone were determined by RIA kits (Serono Diagnostika, Freiburg, Germany; ERIA Diagnostics, Pasteur, France).

### $^{31}\text{P}$ magnetic resonance spectroscopy ( $^{31}\text{P}$ -MRS)

MR spectra of the right calf were obtained in a 4.7 Tesla 40-cm horizontal bore spectrometer (Bruker-Biospec 47/40, Bruker-Medizintechnik, Karlsruhe, Germany). The  $^1\text{H}/^{31}\text{P}$  double tuned surface coil (5-cm diameter) was placed under the right calf at its largest diameter. A specially designed pedal ergometer allows the performance of isometric foot plantarflexion. The contraction force at the ball of the foot was measured continuously and displayed to the subject. Spectra were integrated from 64 scans at 20-s intervals. The resonance frequency of the pulse was 81 megahertz for  $^{31}\text{P}$ . The flip angle was  $60^\circ$  at the center of the coil. Spectra were corrected for saturation artifacts. Spectrum analysis used a Bruker X32 computer and Bruker software UXNMR. Relative changes in the levels of phosphocreatine (PCr), phosphomonoesters (PME), inorganic phosphate (Pi), and ATP were monitored, with initial values set at 100%. Intracellular pH was determined by the chemical shift of Pi relative to that of PCr.

### Study design

The study was designed as a double blind, placebo-controlled, randomized, cross-over trial. All 10 volunteers were randomly subjected to 4 tests; 2 (for aldosterone and placebo) were performed at rest, and 2 during exercise. The examinations at rest and after exercise were performed on 2 separate days, with a minimum interval of 5 h for washout of aldosterone, which has a plasma half-life of less than 30 min (18), after

iv administration. The 2 test sets were 4 days apart. Before the study, a 1-mm diameter catheter was placed in the left antecubital vein for blood sampling and aldosterone/placebo injection. A two-lead electrocardiogram was continuously monitored throughout the tests, and blood pressure measurements were performed at 3-min intervals. For the resting tests, MR spectra of the right calf were recorded for 3 min to establish baseline conditions, followed by aldosterone/placebo injection and an observation period of 17 min.

For exercise tests, the design was the same, except for the period from 4–7 min after injection, during which the volunteer was instructed to press the pedal isometrically at a force of 50% of the body weight. Force measurements were communicated to the subject through an electronic display, and the volunteer was advised to adjust the efforts to match closely the preset tension level.

Blood sampling was performed at the beginning of the experiment.

### Statistical methods

The effects of aldosterone were evaluated by the area under the curve (AUC) method, with integration of values for the periods indicated. Comparisons between the two groups were made using the nonparametric Wilcoxon test for paired data. For all tests,  $P < 0.05$  was considered statistically significant. Primary end points (defined prospectively) were AUCs and steady state levels of PCr, PME, Pi, pH, and ATP.

## Results

Basic clinical characteristics and laboratory parameters are shown in Table 1. No subject had pathological values for any parameter tested. The increased basal aldosterone level in one volunteer (normal upper limit for recumbent position, 200 pg/mL) may reflect an insufficient time of rest before blood sampling.

Aldosterone applied at rest did not induce any change in PCr (Fig. 1) and other phosphate metabolites (not shown). Effects of borderline significance were observed for intracellular pH. The initial resting intracellular pH was  $7.03 \pm 0.03$  in the placebo group and  $7.02 \pm 0.03$  in the aldosterone group. The measurement in the prestudy period was unstable in two subjects, probably reflecting ischemia of the calf due to inappropriate positioning in the ergometer; thus, these two subjects were excluded from pH analysis. Within 3 min of application of aldosterone, a tendency toward acidification was observed, which continued after a brief recovery up to the end of the experiment (Fig. 1). This change was

**TABLE 1.** Clinical characteristics and laboratory parameters of the study volunteers

Parameter	Mean $\pm$ SD
Female/male	3/7
Age (yr)	$34 \pm 5$
Wt (kg)	$72.6 \pm 12.8$
Heart rate (beats/min)	$65.1 \pm 9.7$
Systolic BP (mm Hg)	$120.9 \pm 13.7$
Diastolic BP (mm Hg)	$68.2 \pm 13.3$
Hematocrit (L/L)	$0.429 \pm 0.037$
$\text{Na}^+$ (mmol/L)	$143 \pm 0.5$
$\text{K}^+$ (mmol/L)	$4.15 \pm 0.09$
Protein (mg/dL)	$7.46 \pm 0.16$
Hemoglobin (g/dL)	$14.5 \pm 1.4$
GOT (U/L)	$10 \pm 2$
Creatinine (mg/dL)	$0.93 \pm 0.19$
Basal aldosterone (pg/mL), upper normal limit	$192.0 \pm 58.0$ 200
Basal renin (pg/mL), upper normal limit	$12.5 \pm 1.8$ 26

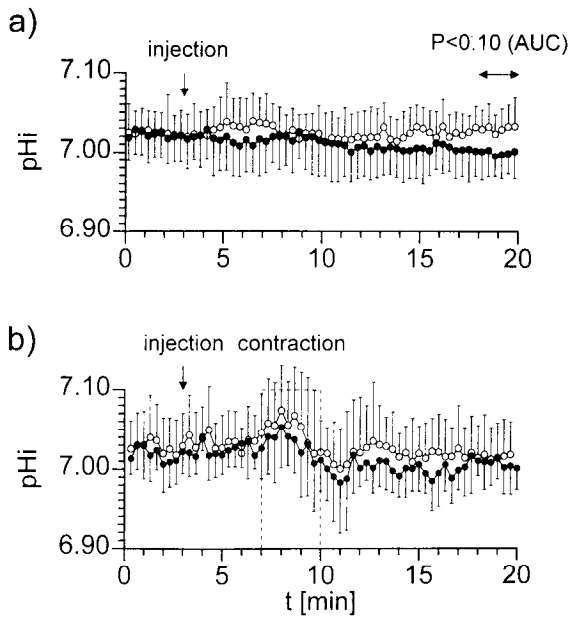


FIG. 1. Mean intracellular pH in calf muscles of normal volunteers determined by  $^{31}\text{P}$ -MRS. At the time indicated, 1 mg aldosterone (●) or placebo (○) was given iv. Spectra were recorded at rest (a;  $n = 8$ ) or during isometric contraction (b;  $n = 10$ ) at 50% of body weight for 3 min. When analyzed by the AUC method, differences in pH at rest (a) were of borderline significance ( $P < 0.10$ ) for 15–17 min after injection.

of borderline significance for 15–17 min postinjection when analyzed by the AUC method ( $P < 0.10$ ).

During exercise no difference in the time course of changes in intracellular pH was seen with aldosterone (Fig. 1). Furthermore, PCr remained unaffected by aldosterone at rest and during isometric contraction of the calf (Fig. 2). However, oxidative PCr recovery was significantly facilitated by aldosterone. The first separation of the curves was seen 8 min after injection, with a significant difference between AUCs from 7–17 min after drug application (Table 2 and Fig. 2;  $P < 0.05$ ). When average values from 14–17 min after aldosterone application (steady state levels) were compared, aldosterone significantly increased PCr levels by 3% above initial resting and recovery values in the placebo group (Table 2 and Fig. 2;  $P < 0.01$ ).

This small effect of aldosterone on oxidative PCr recovery was even more pronounced in subjects in whom PCr decreased by more than 20% ( $n = 6$ ) during exercise. In these subjects, PCr reached 5% higher steady state levels at the end of recovery compared with their initial values and recovery levels in the placebo experiment. All females responded by PCr decreases of more than 20%. There were no differences in PCr levels if compared with men ( $4.0 \pm 3.3\%$  in women and  $3.3 \pm 2.3\%$  in men, aldosterone vs. placebo levels).

In those subjects who maintained exercise almost exclusively through the aerobic pathway ( $n = 4$ ), the PCr at the end of contraction was not different from the initial resting and recovery levels in the placebo group (Table 2 and Fig. 2). There were no significant differences for the other primary end points tested (ATP, Pi, PME, and Pi, PME, plus ATP), both at rest and under stress. The increase in PCr during recovery from anaerobic or mixed contraction was, therefore,

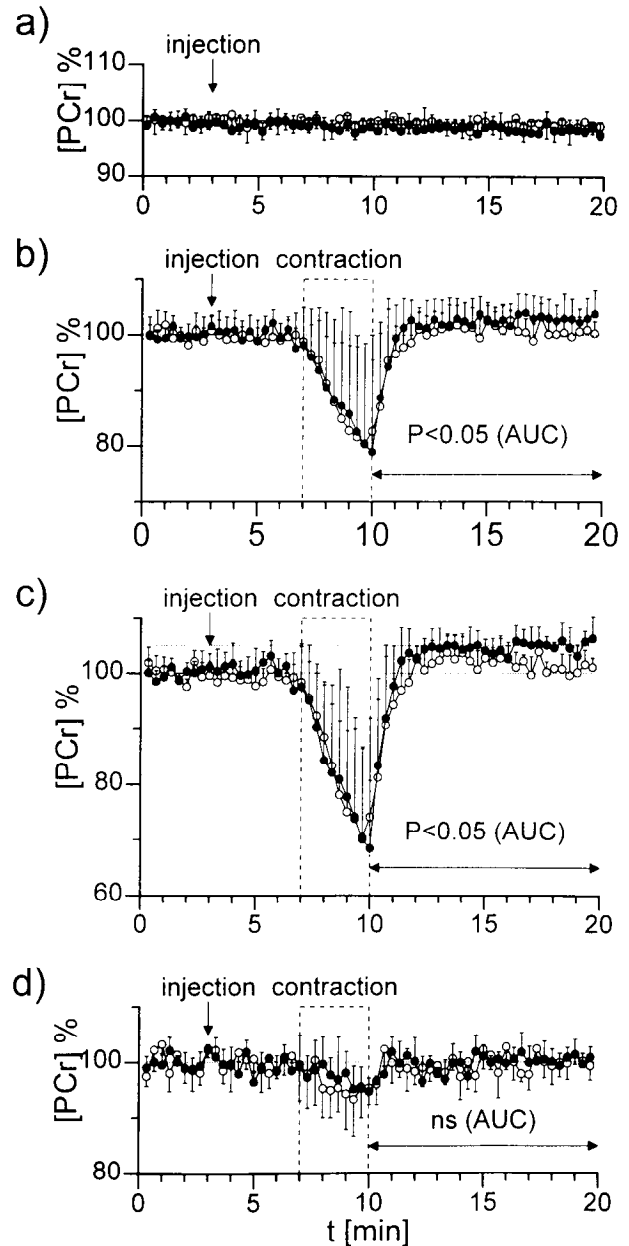


FIG. 2. Mean phosphocreatine levels (percentage of initial values averaged for 3 min before aldosterone injection) in calf muscles of normal volunteers determined by  $^{31}\text{P}$ -MRS. At the time indicated, 1 mg aldosterone (●) or placebo (○) was given iv. Spectra were recorded at rest (a;  $n = 8$ ) or during isometric contraction (b;  $n = 10$ ) at 50% of body weight for 3 min. Results from b were assigned to two groups according to the decrease in PCr during contraction (c:  $\Delta\text{PCr} > 20\%$ ;  $n = 6$ ; d:  $\Delta\text{PCr} < 20\%$ ;  $n = 4$ ). For both the total number of volunteers and volunteers with  $\Delta\text{PCr}$  greater than 20%, AUCs were significantly different between aldosterone and placebo tests from 7–17 min after drug administration (b and c;  $P < 0.05$ ). Dotted lines are drawn at 100% (initial level) and additionally in c at the mean level during recovery from stress after aldosterone administration.

not compensated by a decrease in visible internal stores of phosphates. Arterial pressure and heart rate remained essentially constant throughout the tests, although both hemodynamic indexes tended to increase during physical stress.

**TABLE 2.** Comparison of PCr recovery in normal subjects after injection of 1 mg aldosterone or placebo and isometric exercise (50% body weight for 3 min) by the area under the curve method (AUC; 7–17 min after injection) and steady state PCr at the end of recovery (14–17 min after injection)

Subject no.	AUC from 7–17 min after injection (sum of % of initial values; % × min)				Average of steady state levels from 14–17 min after injection (% of initial values)			
	Aldosterone	Placebo	Difference	Rank no. (±)	Aldosterone	Placebo	Difference	Rank no. (±)
1 <sup>a</sup>	3247	3105	142	10 (+)	106.1	99.9	6.2	10 (+)
2	3084	3018	66	6 (+)	101.2	97.6	3.6	7 (+)
3	3089	3078	11	2 (+)	100.6	100.5	0.1	1 (+)
4 <sup>a</sup>	3140	3070	70	7 (+)	107.0	101.3	5.7	9 (+)
5	3099	3107	-8	1 (-)	101.0	100.6	0.4	4 (+)
6 <sup>a</sup>	3167	3089	78	8 (+)	103.7	100.6	3.1	6 (+)
7 <sup>a</sup>	3180	3086	94	9 (+)	105.2	99.7	5.5	8 (+)
8 <sup>a</sup>	3144	3110	34	4 (+)	103.5	103.3	0.2	2 (+)
9	3099	3130	-31	3 (-)	100.3	100.6	-0.3	3 (-)
10 <sup>a</sup>	3150	3094	56	5 (+)	103.1	102.0	1.1	5 (+)
Average ± SD	3140 ± 50	3089 ± 30	51 ± 51	<i>P</i> < 0.05	103.2 ± 2.4	100.6 ± 1.5	2.6 ± 2.6	<i>P</i> < 0.01

<sup>a</sup> PCr decreased by more than 20% under resting levels during contraction.

### Discussion

The main finding of this paper is the demonstration of a rapid effect of 1 mg aldosterone, iv, on the recovery of PCr after exercise in the calf of healthy volunteers. Increased ATP turnover induced by workload alone was insufficient to precipitate the changes observed, and utilization of the additional anaerobic pathway was required. Given a circulation time of 2 min after injection, this effect was observed within 6 min after the time aldosterone had potentially reached peripheral effector sites. Earlier effects of aldosterone on PCr could not be tested in this study, as the substantial variability in PCr breakdown during exercise would have covered minor steroid effects. Whether aldosterone affected muscles at rest remains questionable. In the experiments performed exclusively at rest, the levels of phosphate metabolites did not change; the minute extent of acidification observed after aldosterone was of only borderline significance.

The time frame of the aldosterone effect on PCr after recovery from exercise certainly is consistent with that of nongenomic steroid action. As previously mentioned, genomic steroid action is characterized by a typical delay at the effector level by 1–2 h. The earliest genomic effects known in the action of mineralocorticoids on *in vitro* systems have required at least 30 min after hormone application (16, 17). In this context, the findings presented here strongly support the existence of nongenomic aldosterone effects *in vivo*. Such effects can be observed in the presence of endogenous hormone and indicate that the rapid effector is sensitive to changes in the aldosterone concentration and is not completely and invariably turned on. The comparably high dose of aldosterone (8- to 10-fold the daily production of the hormone) was chosen to result in maximal effects, as small effects were expected, and to minimize delays from hormone transport to potential receptors. With regard to the exact mechanisms involved, it should be mentioned as a note of caution that the rapid actions of aldosterone observed *in vitro* (see above) and those observed here do not necessarily depend on the same mechanisms.

Unlike those *in vitro* studies cited above, in the present *in vivo* investigation, the lack of an antagonist activity of classical antimineralocorticoid compounds (e.g. spironolactone)

was not shown, which, as in those *in vitro* studies cited above, would clearly indicate an involvement of receptors other than type I receptors. Therefore, there is still the possibility that protein-protein interactions of classical steroid receptors that do not require time-consuming protein synthesis may be involved in those effects reported here.

The significance of a very early paper (16) describing *in vivo* effects of aldosterone after 5 min in humans may be limited, as the methods used at that time are perhaps open to question. The effects in the more recent study on the dog (17) were obtained after 15 min, a time at which the separation of nongenomic and genomic effects merely on time criteria might not be as convincing as that for 6 min effects. In addition, spironolactone blocked this effect, although the data presented did not unequivocally support this conclusion. This finding is in conflict with a previous report on the nongenomic aldosterone effects mentioned above (13).

There are few reports on a relation between exercise and plasma aldosterone levels. Increased plasma aldosterone levels have been found immediately after exhaustive exercise (e.g. treadmill exercise at levels >50% maximum oxygen uptake). Levels were positively correlated to the extent of the workload, thus possibly reflecting increased volume demand (19–22). Physical endurance training, however, does not affect plasma aldosterone levels at rest or after exercise stress. These findings have been discussed in context of the highly significant effects of endurance exercise training on plasma fluid volume regulation during rest and acute exercise stress. Hespel *et al.* (22) concluded that physical training, leading to a substantial gain of physical working capacity, may suppress the plasma renin-angiotensin-aldosterone system in man, thereby counteracting stress-induced increases.

As the former studies focused on the role of aldosterone in stress-related volume regulation, it seems remarkable that an independent effect of aldosterone on cellular energy metabolism in working skeletal muscle was demonstrated here. Most key enzymes of energy metabolism are controlled by ADP and the ADP/ATP ratio. In muscle fibers, ADP is determined by the phosphocreatine kinase reaction. After stress, aldosterone application increases PCr, whereas Pi, ATP, and intracellular pH remain constant. This may reflect

an increased potential to activate oxidative and/or glycolytic ATP formation during additional periods of work. Consequently, plasma aldosterone may be involved in an adaptation mechanism that improves the ATP supply and prevents muscle from early metabolic fatigue during endurance exercise. However, this speculation must be confirmed by further studies.

Small changes in PCr can largely affect phosphorylation potential and free energy of ATP through the creatine kinase reaction. Therefore, even changes that small may have considerable implications for oxidative phosphorylation and energy fueling (23).

Increased PCr after aldosterone indicates either an uptake of external phosphate or a dissociation of phosphate bound to macromolecules in the cell because the levels of the other free intracellular phosphates (Pi, PME, ATP, and their sum, Pi + PME + ATP), did not change. A change opposite that in PCr would have been expected if PCr generation would have been fueled from the free intracellular phosphate pool.

The cellular mechanisms of the effect observed here need to be explored further on the basis of the *in vitro* data discussed above. A rapid aldosterone receptor/effector may be present in skeletal muscle fibers, which has not yet been shown in this tissue *in vitro*. Cellular activation may include a rise in the levels of intracellular calcium, sodium, and probably protons, leading to metabolic activation and resulting in an accelerated regeneration of phosphocreatine stores. This hypothesis is consistent with the sparse *in vivo* observations, all of which indicate that aldosterone is a stress hormone evoking acute circulatory and muscular effects. In addition, the method used here appears to be a suitable tool for a more extensive *in vivo* exploration of hormonal regulation of skeletal muscle energy metabolism through various hormones, including other steroids and drugs in humans, which is still widely lacking.

Taking the findings of this study and those few reports on the rapid *in vivo* action of aldosterone together, a patchy sketch of the physiology of aldosterone as a stress hormone may be drawn. Postural changes or exercise, which are known conditions of a rapid stimulation of aldosterone secretion, result in increases in vascular tone, especially of veins, and/or increases in cardiac output by elevated preload and heart rate. These rapid cardiovascular effects of aldosterone probably occur *in vivo* through the action on intracellular second messengers, such as calcium in vascular smooth muscle and endothelial cells, resulting in increased vascular tone and/or reduced baroreceptor depressor activity. Stimulatory effects on energy metabolism in skeletal muscle would facilitate the generation of adequate fueling, e.g. for prolonged aerobic contraction of calf muscles, which is important for venous blood return in the upright position and, possibly, for support of ventilatory muscles.

In conclusion, rapid *in vivo* changes in intracellular phosphocreatine by aldosterone are fully compatible with earlier

observations of nongenomic *in vitro* effects on intracellular second messengers and the sodium-proton exchanger. These observations underline the potential physiological and, perhaps, the clinical relevance of nongenomic steroid effects.

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