ATP concentrations and muscle tension increase linearly with muscle contraction

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DURING EXERCISE, THE SYMPATHETIC nervous system is engaged via activation of a muscle-based reflex. This leads to increases in arterial blood pressure and heart rate (HR) (9, 26, 31, 32, 34). Mechanically and metabolically sensitive group III and IV afferent nerve endings (receptors) located in the muscle interstitium are engaged by contraction and initiate a reflex (21, 22, 32, 34). This autonomic response to muscle contraction has been termed the "exercise pressor reflex" (34, 35). Although a number of substances in the muscle interstitial space, such as potassium and phosphate, have been reported to contribute to the activation of the exercise pressor reflex (13, 29, 40, 43, 44), the precise interstitial metabolites that stimulate the nerve-ending receptors and evoke the exercise pressor reflex remain unclear.

It has been shown that ATP and analogs excite sensory afferent nerves. For example, ATP depolarizes isolated vagus nerve trunks (47, 48), and it excites cutaneous, visceral, and knee joint afferent nerves (4, 11, 23) and carotid chemoreceptors (33). Furthermore, it has also been reported that ATP is released in vitro mouse bladder-pelvic nerve preparation by urinary bladder distention and that an ATP agonist activates pelvic afferents and potentiates their response to bladder distention (50).

Our recent study (25) has shown that 1) activation of ATP-sensitive P2X receptors evokes a skeletal afferent-mediated pressor response and 2) ATP enhances the muscle pressor response to muscle stretch via P2X receptors. Additionally, a recent report also supports the concept that ATP mediates the exercise pressor reflex via P2X receptors (17). This recent report demonstrates that the pressor response seen with static hindlimb contraction in cats is attenuated when P2X-receptor blockers are administered. Accordingly, these studies suggest that activation of ATP receptors may play an important role in mediating the autonomic adjustments to exercise.

In the present report, we examined whether the muscle interstitial ATP concentration increases with muscle contraction. We found that muscle dialysate ATP increases with contraction and that the rise in ATP is linked to the tension generated during contraction. Additional studies demonstrated that the sympathetic or motor nerves are not the major source of ATP release in skeletal muscle and that muscle contraction per se is a necessary and sufficient stimulus to raise dialysate ATP.

METHODS

All experimental procedures were approved by the Animal Care Committee of the Pennsylvania State University College of Medicine and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on 12 adult male cats (4.0–5.0 kg). Animals were housed in standard cat cages and regu-
lated on a 12:12-h light-dark schedule. Food and water were made available ad libitum.

General Methods

Animal surgical preparation. The cats were anesthetized initially with ketamine (25 mg/kg im). An endotracheal tube was inserted into the trachea and attached to a ventilator (model 683, Harvard, South Natick, MA). The cats were then anesthetized by inhalation of an isoflurane-oxygen mixture. Polyethylene catheters (PE-90) were inserted into an external jugular vein and a common carotid artery for drug administration and measurements of arterial blood pressure, respectively. The triceps surae muscles of both limbs were isolated, and the Achilles (calcaneal) tendons were cut. The ventilator was set with a tidal volume of 20 ml/stroke and a rate of 20–30 strokes/min. Arterial blood gases and pH were periodically checked (ABL 510 pH blood gas analyzer, Radiometer, Copenhagen, Denmark). pH was maintained at ~7.35–7.45, Pco2 at ~30–40 Torr, and HC03 at ~20–25 mmol/l by adjusting the ventilator or by intravenous injection of a 1 M sodium bicarbonate solution. Body temperature was continuously monitored with a rectal thermometer (series 400, Yellow Springs Instruments) and maintained between 37.0 and 38.5°C by a water-perfused heating pad and external heat lamps.

Decerebration was performed because it allowed an examination of autonomic reflex responses without having to consider the confounding effects of anesthesia (20). Before the decerebration procedure, the cats were given a 4-mg intravenous injection of dexamethasone to help prevent decerebration-induced brain stem edema. The head was fixed into a Kopf stereotaxic instrument, and decerebration was performed as anesthesia was continued. The majority of the temporal and parietal plates were removed. The two cortical hemispheres were also removed. A transverse section was made anterior to the superior colliculus and extending ventrally to the mamillary bodies. The brain rostral to the inhaled mixture. The general procedures employed for decerebration were performed as reported previously (25, 30).

A laminectomy was performed to expose the lower lumbar and upper sacral portions of the spinal cord.

The dura was then opened, allowing visual identification of the L7 and S1 spinal roots. The dorsal and ventral roots of L7 and S1 were carefully separated, and the ventral roots were cut close to the spinal cord. The peripheral ends of the transected L7 and S1 ventral roots were then placed on platinum bipolar stimulating electrodes. A pool was formed around the exposed neural and muscular tissue by using skin flaps sutured to brass bars, and the exposed spinal cord region was immersed in a pool of warm (37°C) mineral oil.

Insertion of microdialysis probes. The semipermeable fibers with a molecular weight cutoff of 30,000 (0.20 mm ID, 0.22 mm OD; Spectrum Laboratories, Laguna, CA) were used to construct the microdialysis probes. Each end of a single fiber was inserted ~1 cm into a hollow polycarbonate tube (0.25 mm ID, 0.36 mm OD) and glued. The length of the probe was 4 cm (between 2 polycarbonate tubes). The skin directly over the triceps surae muscle on both hindlimbs was dissected away, and four microdialysis probes were inserted into the gastrocnemius muscle of each hindlimb. The probes were inserted into the muscle parallel to fiber orientation via a cannula. The microdialysis probes were then attached to a perfusion pump (model 102, CMA) and perfused at a rate of 5 μl/min with Ringer solution. The dialysate was collected in 250-μl microcentrifuge tubes and immediately sealed (to prevent evaporation) and stored at −80°C freezer until analyzed.

In all experiments, “control dialysate” was also collected from the probes in the leg contralateral to the contracting hindlimb. We reasoned that if the ATP concentrations seen during contraction rose to the same degree in the contracting and control limbs, then we could conclude that muscle reflex engagement and the resultant engagement of sympathetic efferent nerves were responsible for the increase in dialysate ATP.

Microdialysis probe recovery. The percent recovery rate of microdialysis probes for ATP was examined in vitro. Briefly, four probes were inserted into a dish containing varying concentrations of ATP (2.5, 5, 7.5, and 10 μM, dissolved in artificial extracellular fluid). Each of the probes was then connected to a perfusion pump (model 102, CMA) and perfused with a Ringer solution at the same perfusion rate that was used for the animal experiment (5 μl/min). A minimum of three timed collections were taken from each dish to ensure that stabilization of the concentration gradient occurred. Dual 50-μl samples were collected over 20 min. The dialysate ATP concentrations were divided by the ATP concentration within the collection dish to obtain percent recovery values at the different concentrations. The probe recovery rates for 2.5, 5, 7.5, and 10 μM were 33.1 ± 1.9, 30.8 ± 2.9, 26.8 ± 2.3, and 26.5 ± 1.2%, respectively. The relationship between dish and dialysate concentration was linear (r = 0.952, P < 0.001; Fig. 1), indicating that the dialysate concentration was directly proportional to the dish ATP concentration. From these results, we concluded that dialysate ATP would be linearly related to the interstitial ATP obtained from the cat hindlimb.

Determination of Adenine Nucleotides Concentrations

In this study, we measured dialysate ATP as well as the concentrations of ADP, AMP, and adenosine by using HPLC methods (49). This approach allowed the determination of purine nucleotides in the picomolar range on very small sample volumes (40–50 μl). Because the dialysate samples were ultraclean, purification was not required before analysis. All metabolites were determined in each of the dialysis probes. In this report, we present dialysate concentrations without considering the recovery rate of the probes.

![Fig. 1. Relationship between “standard” ATP concentrations and dialysate ATP concentrations. A regression analysis was used for this relationship. Each point represents 1 sample.](http://jap.physiology.org/Downloadedfrom/10.2203/jap.03.08.08)
ADENINE NUCLEOTIDES AND CONTRACTION

Fig. 2. Muscle tension (A) and mean arterial pressure (MAP; B) response during 10-min electrical stimulation of the ventral roots. 1) The 3- and 5-Hz stimulations significantly increased MAP responses during the first 3 min, 2) the pressor response evoked by ventral root stimulation of 5 Hz was eliminated after section of the L7 and S1 dorsal roots, and 3) ventral root stimulation of 5 Hz evoked no change in muscle tension or blood pressure after muscle paralysis. Values are means ± SE. *P < 0.05 vs. control before the stimulation.

Experimental Protocols

After the microdialysis probes were inserted, a 2-h equilibration period was observed. This allowed resting ATP concentrations to reach a stabilized state (5). Contractions induced by electrical stimulation of the L7 and S1 ventral roots were then performed. In eight cats, two bouts of rhythmic contraction were conducted at frequencies of 3 and 5 Hz (3 times motor threshold and 0.1-ms duration). Each of the two frequencies was sustained for 10 min. There was a 60-min rest period between each bout of contraction. The previous work has shown that this time period is sufficient to allow high-energy phosphate stores to replete (24, 41). Additionally, prior work has also shown that interstitial ATP falls to values that are not different from the baseline within the first 10 min of recovery after muscle contraction (36). The samples for baseline, contraction, and recovery data were collected for 10 min before, during, and after each workload.

In separate experiments (n = 6), the dorsal roots of L7 and S1 were cut and a bout of contraction at 5 Hz was performed. If interstitial ATP failed to rise under these circumstances, it would suggest that engagement of the muscle reflex was necessary to evoke the response.

In a final group of studies (n = 4), the cats were paralyzed by intravenous injection of pancuronium bromide (200 µg/kg wt). The ventral roots were then stimulated at 5 Hz. This experiment was performed to determine whether muscle contraction per se was a necessary contributor to the ATP response.

Experimental Data Analysis

Experimental data acquisition. Arterial blood pressure was measured by connecting the carotid arterial catheter to a pressure transducer (model P23ID, Statham). Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was derived from the arterial pressure pulse. Ties were placed around the tendons, and they were attached to a tension transducer (model F10, Grass Instruments) for measurement of developed tension. All measured variables were continuously recorded on an eight-channel chart recorder (model TA 4000, Gould, Valley View, OH) and a computer (Dell, Dimension P751) that used PowerLab system software (ADInstruments, Castle Hill, Australia). Computer-acquired data were used in the post hoc analyses. Control values were determined by analyzing at least 30 s of the data immediately before electrical stimulation. The peak response of each variable was determined by the peak change from the control value.

Experimental data (MAP, HR, muscle peak tension, and adenine nucleotides concentrations) were analyzed by using one-way repeated-measures ANOVA. As appropriate, Tukey's post hoc analyses were utilized. All values are expressed as means ± SE. For all analyses, differences were considered significant if P < 0.05. All statistical analyses were performed by using Sigma Stat for Windows version 2.03 (SPSS, Chicago, IL).

RESULTS

3- and 5-Hz Stimulations

Muscle tensions and cardiovascular responses. Electrical stimulation of the ventral roots at 3 and 5 Hz was performed to evoke twitch muscle contractions (n = 8). The evoked maximal tensions were 3.9 ± 0.4 and 4.3 ± 0.3 kg (P < 0.05), respectively. The 3-Hz stimulation increased MAP from 146 ± 10 to 159 ± 10 mmHg, and the 5-Hz stimulation increased MAP from 141 ± 8 to 157 ± 8 mmHg (P < 0.05 for both stimulation paradigms). The maximal HR response was from 181 ± 13 to 186 ± 7 beats/min with 3-Hz stimulation, and it was from 187 ± 7 to 189 ± 15 beats/min with 5-Hz stimulation (P > 0.05 for both paradigms). The changes in tension and MAP during 10-min stimulation are shown in Fig. 2.

Fig. 3. Dialysate ATP concentrations in resting and contracting muscle. Control, stimulation, and recovery: 10 min before, during, and after muscle contraction, respectively. Open bars, resting muscle; solid bars, contracting muscle. Values are means ± SE. Electrical stimulations induced at 3 (A) and 5 Hz (B) significantly elevated ATP dialysate concentrations in contracting muscle but not in the opposite control muscle. *Control vs. stimulation, P < 0.05.
3 and 5 Hz significantly increased the dialysate ATP concentration in contracting muscle (n = 8). ATP increased from 0.317 ± 0.11 μM at rest to 0.793 ± 0.15 μM (150% increase; P < 0.05) during 3-Hz stimulation. At 5 Hz, ATP increased from 0.325 ± 0.08 to 0.985 ± 0.13 μM (200%; P < 0.05). ATP concentrations at 3- and 5-Hz frequency of stimulation are not different. Changes in ADP, AMP, and adenosine during twitch muscle contractions evoked by 3- and 5-Hz stimulations are shown in Table 1.

To examine whether tension developed during the two paradigms were linked to the dialysate ATP concentration, a linear regression analysis was performed and a significant relationship was demonstrated (r = 0.909, P < 0.001; Fig. 4).

Stimulation of the ventral roots did not significantly increase the dialysate ATP concentration in the triceps surae of the opposite noncontracting hindlimb (3 Hz: rest 0.347 ± 0.08; stimulation: 0.330 ± 0.08 μM; 5 Hz: rest 0.328 ± 0.08; stimulation: 0.329 ± 0.09 μM).

Fig. 4. Relationship between developed muscle tensions and elevated dialysate ATP. A regression analysis was used for this relationship. Muscle tensions were induced by electrical stimulation of the L7 and S1 ventral roots at 3 and 5 Hz (n = 8 cats).

5-Hz Stimulation After Section of the Dorsal Roots

Section of the L7 and S1 dorsal roots eliminated the pressor response to ventral roots stimulation at 5 Hz (Fig. 2). Ventral roots stimulation of 5 Hz evoked a peak tension response of 4.4 ± 0.5 kg (P < 0.05), and the dialysate ATP rose (0.311 ± 0.09 to 0.919 ± 0.13 μM, 195%; P < 0.05; Fig. 5). ATP concentrations with and without dorsal roots sectioned were not different.

Table 1. Interstitial ADP, AMP, and adenosine during muscle contraction evoked by electrical stimulation of the L7 and S1 ventral roots

<table>
<thead>
<tr>
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<th>ADP, μM</th>
<th>AMP, μM</th>
<th>Adenosine, μM</th>
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<tr>
<td></td>
<td>Control</td>
<td>Stimulation</td>
<td>Recovery</td>
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<tr>
<td>3 Hz</td>
<td></td>
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<tr>
<td>Resting muscle</td>
<td>0.28 ± 0.08</td>
<td>0.29 ± 0.08</td>
<td>0.25 ± 0.05</td>
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<tr>
<td>Contracting muscle</td>
<td>0.29 ± 0.09</td>
<td>0.54 ± 0.11 *</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>5 Hz</td>
<td></td>
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<tr>
<td>Resting muscle</td>
<td>0.24 ± 0.06</td>
<td>0.25 ± 0.08</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>Contracting muscle</td>
<td>0.22 ± 0.05</td>
<td>0.65 ± 0.12 *</td>
<td>0.30 ± 0.10</td>
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<tr>
<td>5 Hz (DR section)</td>
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<tr>
<td>Resting muscle</td>
<td>0.27 ± 0.7</td>
<td>0.29 ± 0.08</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>Contracting muscle</td>
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<td>0.69 ± 0.10 *</td>
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<td>5 Hz (Paralysis)</td>
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</tr>
<tr>
<td>Contracting muscle</td>
<td>0.31 ± 0.11</td>
<td>0.33 ± 0.10</td>
<td>0.30 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE. Control, stimulation, and recovery: 10 min before, during, and after muscle contraction, respectively; resting, resting muscle; contracting, contracting muscle; DR section, cutting L7 and S1 dorsal roots. * Significant difference from control, P < 0.05.

Fig. 5. Dialysate ATP concentrations in contracting muscle after section of the dorsal roots (A) and muscle paralysis (B). Control, stimulation, and recovery: 10 min before, during, and after muscle contraction, respectively. Open bars, resting muscle; solid bars, contracting muscle. Values are means ± SE. A: electrical stimulation of 5 Hz induced after section of the dorsal roots significantly elevated dialysate ATP (* control vs. stimulation, P < 0.05). B: electrical stimulation of 5 Hz induced after muscle paralysis did not increase ATP.
The changes in ADP, AMP, and adenosine during this paradigm are shown in Table 1.

**5-Hz Stimulation After Paralysis of Muscle**

After muscle paralysis, electrical stimulation of the L7 and S1 ventral roots evoked no change in muscle tension or in the cardiovascular responses (Fig. 2). Additionally, the increase in dialysate ATP evoked by the electrical stimulation was eliminated (0.394 ± 0.04 to 0.393 ± 0.04 μM, n = 4; Fig. 5). ADP, AMP, and adenosine values are shown in Table 1.

**DISCUSSION**

**Study Findings**

In the present study, we have shown that contraction of skeletal muscle induced by electrical stimulation of the ventral roots caused significant increases in blood pressure (Fig. 2) and dialysate ATP concentrations (Fig. 3). The increases in ATP were 150 and 200%, respectively, for 3- and 5-Hz stimulations. Moreover, the increase in dialysate ATP was linked to muscle tension (Fig. 4). The rise in ATP was not eliminated by sectioning the dorsal roots, and no change was seen in ATP in the control limb (Figs. 3 and 5). Finally, the ATP response was eliminated by paralysis (Fig. 5).

Thus muscle cell contraction is the necessary and sufficient stimulus for the rise in ATP concentrations. If the ATP response were part of the muscle reflex, we would have expected an effect of dorsal root sectioning. If interstitial ATP were released from sympathetic nerves, then we should have seen an increase in interstitial ATP in the opposite limb. Finally, the fact that tension was linked to interstitial ATP and the rise in interstitial ATP was eliminated during paralysis supports that the ATP came from skeletal muscle cells and not from motor nerves.

ATP released from the muscle cells during contraction can act as a stimulus for transduction of the muscle pressor reflex. The free nerve endings of groups III and IV muscle afferents residing in the muscle interstitium respond to chemical and/or mechanical stimulation to evoke a muscle pressor reflex. ATP-sensitive P2X receptors have been identified from a subpopulation of small-diameter afferent sensory neurons in dorsal root ganglia and are expressed on their central and peripheral nerves (7, 27, 51, 52). Activation of ATP-sensitive P2X receptors by injection of α,β-methylene ATP into the blood supply of the hindlimb evokes a skeletal afferent-mediated pressor response (16, 25). ATP also sensitizes the muscle pressor response to muscle stretch via P2X receptors (25). The pressor response to static hindlimb contraction in cats is attenuated when P2X-receptor blockers are administered (17). These observations viewed together with the present report suggest that contracting muscle cells release ATP into the interstitial space where the free nerve endings of group III and IV muscle afferents reside. Elevated ATP then stimulates these afferent nerves to mediate the exercise pressor reflex via P2X receptor.

In skeletal muscle, adenosine and adenine nucleotides (ATP, ADP, and AMP) have been reported to be present in the interstitial space (8, 10, 28, 45). Previous studies have shown that interstitial adenosine concentration was increased in the extracellular medium of contracting primary rat skeletal muscle cells (18, 28). It has been suggested that the breakdown of ATP to adenosine is the main source of extracellular adenosine because adenosine concentrations fall if ectonucleotidases are inhibited (38, 46). This suggests that adenosine levels may be reflective of interstitial ATP. Interstitial adenosine and adenosine nucleotide have also been shown to rise with knee extension in humans and during obturator nerve stimulation-induced muscle contraction in the dog (19, 36). The source of interstitial nucleotidase is unclear.

In the present study, we employed electrical stimulation of the L7 and S1 ventral roots to induce muscle contraction in decerebrate cats, and we showed that contracting muscle cells are the major source of elevated interstitial ATP. We believe that the failure of ATP to rise in the "control" limb was due to enhanced nucleotidase activity (42) that can be seen with sympahtoexcitation. If this were the case, we would have expected a rise in the concentrations of one or more of the ATP breakdown products.

Elevated adenosine and ATP have been shown to be released from nerves (10, 45). In these reports, adenosine and ATP samples were collected from the bath in which the isolated skeletal muscle preparations were electrically stimulated (10, 45). The concentration of adenosine and ATP were much lower than those reported in this paper. Thus it is possible that in this report a relatively small amount of ATP was released from sympathetic and/or motor nerves.

The membrane permeability to ATP is very low, in agreement with the molecular size of ATP, and its extracellular concentration remains low because of ectonucleotidase activity (15). However, arterial occlusion and exercise may increase extracellular ATP by ~50-fold from basal levels in human venous plasma (14). The potential mechanisms by which ATP can be released from skeletal muscle cells are not entirely clear. ATP can be released by exocytosis from platelets, epithelium and nerves like other neurotransmitters (12, 15). The cystic fibrosis transmembrane conductance regulator (CFTR) has recently been suggested to act as an ATP channel and enable intracellular ATP to cross the cell membrane (1–3, 53). Other studies have shown that mechanical stimulation of the epithelial cell surface, rather than CFTR activation, is sufficient to release ATP (54). It is known that mechanical stimulation of epithelial and neuronal cells is a sufficient stimulus for ATP release (37, 53). Evidence also suggests that gadolinium, an inhibitor of stretch-activated channels, suppresses ATP efflux from eukaryotic cells (6). Moreover, the rise is seen with increased extracellular ATP rat hepatocytes and epithelial cells volume is inhibited by gadolinium (39). The precise mechanism by which ATP exits muscle cells cannot be determined.
from data in the present report, and further studies are needed to evaluate this issue.

Conclusions

The results of this report demonstrate that contracting muscle elevates interstitial ATP concentrations. Muscle afferent nerve endings reside in the muscle interstitium. When these findings are viewed in conjunction with prior work showing that arterial injections of ATP evokes and sensitizes the muscle pressor response, they suggest that ATP is released from contracting muscle cells and stimulate P2X receptors on muscle afferent nerves as an endogenous muscle afferent stimulant. This in turn leads to stimulation and sensitization of the exercise pressor reflex.

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