Mechanism of adenosine-induced vasodilation in rat diaphragm microcirculation

CHANG-WEN CHEN, HAN-YU CHANG, AND TZUEN-REN HSIEU
Department of Internal Medicine, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan, Republic of China

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ADENOSINE HAS BEEN SHOWN to be a vasodilator in most vascular beds except the renal or placenta tissues (27). The effector mechanisms underlying the biological action of adenosine are believed to be mediated by the specific receptors known as P1 purinoceptors (10, 14). At least four adenosine receptor subtypes are currently known: A1, A2A, A2B, and A3 receptors (12). All of these receptors are members of the G protein-coupled receptor family, which is tightly linked to adenylate cyclase (AC) (11). A1 and A3 receptors mediate the inhibition of AC activity, and A2 receptors enhance AC activity (11). Both A1 and A2 receptors have been reported to contribute to the vasodilation effect of adenosine (14).

The ATP-dependent potassium (KATP) channel has been shown to participate in adenosine-induced vasodilation (AIV) at least in some vascular beds (4, 14, 20, 23, 25, 26). Evidence suggests that adenosine may act on A1 receptors to produce vasodilation via activation of the KATP channel (4, 23). Stimulation of A1 receptors leads to reduced AC activity, and reduced cAMP is unlikely to mediate vasodilation; thus an important consequence is that activation of the KATP channel may be coupled to A1 receptors by a pertussis toxin-sensitive G protein but is independent of the AC-cAMP pathway (27). On the other hand, some reports indicate that AIV is mediated by A2 receptors (13, 23–25). As stimulation of the A2 receptors leads to enhanced AC activity and increased cAMP levels, the KATP channel may be opened through cAMP-dependent protein kinase (19). In resting or contracting skeletal muscles, adenosine produces vasodilation in a dose-dependent manner and is partly responsible for the development of functional hyperemia (6, 18). The A2 receptor is thought to be responsible for AIV in skeletal muscle (27).

The mechanism by which adenosine modulates blood flow in the diaphragmatic vascular bed is a topic of considerable interest, because the diaphragm is the principle respiratory muscle. Because the microcirculation supplies nutrients to the tissue, understanding blood flow control in the diaphragm microvascular bed is of great importance. Danialou et al. (7) first described the mechanisms of AIV on the rat diaphragm microcirculation under resting conditions using an intravital microscope. They concluded that activation of the A1 receptor contributes to the vasodilation response of adenosine, but the A2 receptor plays almost no role in this response. The KATP channel and nitric oxide are also involved in the vasodilation process. The conclusions of Danialou et al. (7) contradict the conventional view that AIV in skeletal muscle is mediated via the A2 receptor. The A1 receptor predominantly contributes to vasodilation in skeletal muscle only under...
conditions of systemic hypoxia (3). Actually, Danialou’s study is the only demonstration of the predominant role of A1 receptors in AIV in the resting skeletal muscles. Laser Doppler flowmetry (LDF) has also been widely used to monitor microcirculatory blood flow (31). In the present study, we used LDF in our previously developed rat diaphragm microcirculation model (5) to clarify the role of the KATP channel and different adenosine receptors on AIV in rat diaphragm microcirculation. We first assessed the role of the KATP channel-blocker glibenclamide on adenosine and adenosine agonist-induced vasodilation. Different adenosine antagonists were used to further elucidate the roles of each adenosine subtype on AIV. Finally, the role of AC in AIV was evaluated.

METHODS

Animal preparation. Male Sprague-Dawley rats (age 8–10 wk, weight 300–400 g) were housed at the Laboratory Animal Center of the College of Medicine at National Cheng Kung University. All animals were acclimatized to a 12:12-h light-dark cycle and were maintained on Purina Rat chow and tap water ad libitum. The animals were fasted overnight but allowed free access to water the day before the experiment.

The animals were initially anesthetized with pentobarbital sodium (30 mg/kg ip) followed by 50% wt/vol urethan (1.2–1.5 g/kg iv) and placed in a supine position on a rodent operating table (Harvard Apparatus, South Natick, MA). After a tracheostomy with polyethylene (PE)-240 tubing, a tracheostomy with polyethylene ( PE)-240 tubing, muscle relaxant (gallamine triethiodide, 60 mg/kg) was administered and the rats were artificially ventilated at tidal volumes of 6–7 ml/kg and a rate of 70–80 breaths/min (model 683, Harvard Apparatus). The adequacy of ventilation was monitored with the aid of a micro CO2 analyzer (model JS-02282, Polaris, Jerusalem, Israel) through a T-shaped outlet (model 8110–20, Cole-Palmer Instruments, Chicago, IL). A side port connected with a syringe pump (model 55–3000, Harvard Apparatus) was used for the administration of drugs. The infusion rate was set at 1% of the superfusing fluid rate to standardize the final concentration of test agents.

Laser Doppler flowmetry. A commercially available laser Doppler flowmetry monitor (Laserflo BPM+, Vasamedics, St. Paul, MN) equipped with a small-caliber probe (model P443–3) was used to measure microvascular flow rates. The signal from the LDF was output as a direct current (Q_{LD}, updated eight times per second). The time constant was set at 1 s.

LFD signals were recorded continuously in all experiments. The probe, held in an MM-3 micromanipulator (Narishgi Instruments, Tokyo, Japan), was placed perpendicular to the surface of the diaphragm with the probe tip just touching the water film of the superfusate on the surface of the diaphragm. A site on the left costal diaphragm without visible large vessels was chosen and confirmed by visualization with a long-working-distance stereoscopic zoom microscope (SMZ-1, Nikon, Tokyo, Japan). After stable readings were obtained, the probe was kept in the same fixed position for the duration of the experiments. An average reading time of 30 s was required to provide a stable signal that was independent of vasomotion. At the end of the experiments, the animals were killed with an intravenous injection of saturated potassium chloride. The postmortem signal was considered as biological zero and subtracted from the LDF values.

Drugs. Urethan, gallamine triethiodide, adenosine, glibenclamide, and forskolin were obtained from Sigma Chemical (St. Louis, MO). N-[(2S)-2-phenyl-cyclopentyl] azacyclotridecan-2-imine-hydrochloride (MDL-12330A), 2-(2-carboxyethyl)-phenyl-amino-5'-N-ethylcarboxamidoadenosine (CGS-21680), 5'-N-ethylcarboxamido-adenosine (NECA), R-N'-phenoxyproppyladenosine (R-PIA), 8-cyclopteny-1,3-dipropoxypentane (DPCPX), and 8-(p-sulfophenyl)-theophylline (SP-7-P) were purchased from Tocris Cookson (Bristol, UK). Glibenclamide and DPCPX were dissolved in DMSO (final concentration 0.1%) and NaOH (final concentration 0.0001 N). 8-P-SPT was dissolved in NaOH (final concentration 0.0001 N). Forskolin and MDL-12330A were dissolved in ethanol. CGS-21680, NECA, R-PIA, and ZM-241385 were stored in DMSO (final concentration <0.1%). Urethan was prepared in saline at a 50% wt/vol concentration.

Experimental protocols. Experiments were initiated after a 30- to 45-min stabilization period. Arterial blood gas and
hematocrit were determined. The animals used in this study met the following criteria during the stabilization period: 1) PSYS > 80 mmHg; 2) pH 7.35–7.50 and P O2 > 100 mmHg; 3) Hct SYS > 40%; 4) Q LDF showed a greater than twofold increase compared with baseline values after topical application of adenosine at 10-4 M; and 5) no obvious hemorrhage in the muscle tissue under investigation. Ten series of experiments were performed. Each series of experiments was performed in six rats.

Series 1–4 assessed the role of KATP channel blocker glibenclamide on adenosine and adenosine agonist-induced vasodilation response in rat diaphragm microcirculation. The vasodilation response of rat diaphragm microcirculation to adenosine (final concentrations 10-5 M, 3.2 × 10-5 M, and 10-4 M), nonselective adenosine agonist NECA (final concentrations 10-5 M, 3.2 × 10-5 M, and 10-7 M), specific A2A receptor agonist CGS-21680 (final concentrations 10-8 M, 3.2 × 10-8 M, and 10-9 M), and specific A1 receptor agonist R-PIA (final concentrations 10-7 M, 3.2 × 10-7 M, and 10-8 M) were assessed before and after continuous suffusion of the KATP blocker glibenclamide (3.2 × 10-8 M) for 30 min. Noncumulative concentration responses to one test agent only were obtained in each animal. Each concentration was administered until a stable response was obtained, and a diaphragm recovery period of 5 min was allowed after application of each concentration. Series 5–7 assessed the role of adenosine antagonists on the AIV response in rat diaphragm microcirculation. In series 5, after baseline values were recorded, adenosine was applied to the diaphragm in sequentially increasing concentration of 10-5 M, 3.2 × 10-5 M, 10-4 M, and 10-3 M. After QLDF returned to baseline, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a specific A1 receptor antagonist was applied to the preparation at a concentration of 5 × 10-8 M for 15 min by continuous suffusion and the effects of adenosine at these concentrations were recorded again. DPCPX was continuously suffused during the tests. In the experiments of series 6, 8-p-SPT, a nonselective adenosine receptor antagonist was used at a concentration of 3 × 10-5 M. In series 7, ZM-241385, a selective A2A receptor antagonist, was used at a concentration of 10-6 M. In series 8 and 9, either no agent or vehicle (DMSO at a final concentration of 0.1% + 0.0001% NaOH) were used as suffusing agents (time and vehicle control). In series 10, the role of AC on the AIV response in rat diaphragm microcirculation was assessed. The AC inhibitor MDL-12330A and the AC activator forskolin were used in this series. After a basal recording was completed, adenosine at 10-5 M, 3.2 × 10-5 M, and 10-4 M, and forskolin at 2 × 10-6 M were sequentially suffused to the preparation. After QLDF had returned to baseline levels, MDL-12330A at 10-6 M was applied topically for 15 min and a second run of adenosine and forskolin at the same concentrations was done after MDL-12330A was stopped.

Data acquisition and statistical analysis. PSYS and QLDF inputs were fed into the chart recorder (Gould RS 3200 polygraph) for continuous recording. The output of pressure signals, heart rate, and the analog output from the LDF were directed into a multichannel analog interface unit, where the data were sampled at 10 Hz with a 12-bit analog-to-digital converter (AT codas, Dataga Instrument, Akron, OH) and stored in a personal computer. Recording periods complicated by artifacts were excluded before data analysis. The average LDF signal during a recording time of 30 s was defined as one measurement.

Results are expressed as means ± SE. Responses to adenosine, forskolin, R-PIA, CGS-21680, and NECA were measured as a stable increase in QLDF and expressed as a percentage of baseline values. Baseline QLDF immediately before the start of suffusion with adenosine, forskolin, R-PIA, CGS-21680, and NECA represented 100% values for calculation of the percentage of the maximal change in QLDF. Differences in mean values between groups were analyzed for statistical significance using two-way repeated-measures ANOVA. Differences in mean values among groups were analyzed for statistical significance using one-way repeated-measure ANOVA followed by Student’s t-test with Bonferroni correction if necessary. When appropriate, Student’s t-test for paired data was also used. A probability value of P < 0.05 was considered statistically significant. The number of observations (measurements) was denoted by n.

RESULTS

Systemic and microcirculatory variables. The results are based on experiments carried out on 60 rats that met the inclusion criteria for P SYS, arterial blood gases, and Hct SYS. P SYS was 99.6 ± 9.3 mmHg. The heart rate was 406 ± 3.7 beats/min. Arterial pH was 7.41 ± 0.01, arterial P O2 was 133.9 ± 3.1 mmHg, and arterial P CO2 was 35.6 ± 0.4 mmHg. Hct SYS was 45.6 ± 3.7%. The resting rate for QLDF was 297.0 ± 8.2 mV. No significant differences were found in baseline P SYS, heart rate, and QLDF among animals in the 10 experimental groups as shown in Table 1 (P = 0.152, 0.192, and 0.358, respectively). Eight typical tracings from each series of experiments are shown in Fig. 1.

Effect of KATP channel on adenosine and adenosine agonist-induced vasodilation response. Figure 2 illustrates that adenosine and several adenosine agonists elicited significant vasodilation responses in a dose-dependent manner. All of these drugs elicited a similar degree of incremental microcirculatory flow (P > 0.05) under the different ranges of concentration used in this study. As shown in Fig. 2, CGS-21680 and NECA elicited similar degrees of vasodilation using concentra-

Table 1. Baseline mean systemic arterial blood pressure, heart rate, and diaphragmatic microvascular blood flow recorded by laser Doppler flowmetry among rats of 10 experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>P SYS, mmHg</th>
<th>HR, beats/min</th>
<th>Q LDF, mV</th>
</tr>
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<tbody>
<tr>
<td>Ado + GLB</td>
<td>104.3 ± 5.3</td>
<td>415 ± 15</td>
<td>332 ± 33</td>
</tr>
<tr>
<td>NECA + GLB</td>
<td>99.5 ± 3.4</td>
<td>421 ± 9</td>
<td>272 ± 15</td>
</tr>
<tr>
<td>CGS-21680 + GLB</td>
<td>89.3 ± 3.0</td>
<td>390 ± 9</td>
<td>274 ± 22</td>
</tr>
<tr>
<td>R-PIA + GLB</td>
<td>99.3 ± 3.5</td>
<td>407 ± 6</td>
<td>302 ± 25</td>
</tr>
<tr>
<td>Ado + DPCPX</td>
<td>100.7 ± 5.4</td>
<td>408 ± 13</td>
<td>323 ± 19</td>
</tr>
<tr>
<td>Ado + 8-p-SPT</td>
<td>105.3 ± 3.2</td>
<td>388 ± 16</td>
<td>306 ± 45</td>
</tr>
<tr>
<td>Ado + ZM-214385</td>
<td>99.5 ± 2.4</td>
<td>388 ± 9</td>
<td>272 ± 22</td>
</tr>
<tr>
<td>Ado + vehicle</td>
<td>100.5 ± 2.5</td>
<td>422 ± 11</td>
<td>256 ± 26</td>
</tr>
<tr>
<td>Ado + Forskolin+MDL-12330A</td>
<td>102.2 ± 2.7</td>
<td>408 ± 10</td>
<td>303 ± 21</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ado, adenosine; GLB, glibenclamide; R-PIA, R-N6-phenylisopropyladenosine; CGS-21680, 2-p-(2-carboxyethyl)phenyl-amino-5-N-ethylcarboxamidoadenosine; NECA, 5-N-ethylcarboxamido-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; 8-p-SPT, 8-(p-sulfophenyl)-thephophine; FORSK, forskolin; MDL-12330A, N-(cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride. P SYS, mean systemic arterial blood pressure; HR, heart rate; Q LDF, laser Doppler flowmetry.
trations 10 times lower than R-PIA. The vasodilation response induced by all of these drugs could be significantly inhibited by pretreatment with the K<sub>ATP</sub> channel blocker glibenclamide (3.2 × 10<sup>-6</sup> M).

**Selectivity of adenosine receptor antagonist.** Figure 3 shows that the vasodilation response elicited by incremental concentrations of adenosine was not blocked by DPCPX, yet 8-p-SPT and ZM-241385 significantly attenuated the AIV response. In the time and vehicle control groups, the vasodilation response induced by incremental concentrations of adenosine remained the same throughout the experiments (P > 0.05, data not shown).

**Effects of AC inhibitor MDL-12330A on adenosine- and forskolin-induced vasodilation responses.** Figure 4 shows that the vasodilation response elicited by incremental concentrations of adenosine or with a single concentration of forskolin could be significantly attenuated by pretreatment of MDL-12330A for 15 min.

**DISCUSSION**

Although adenosine is known to be an important skeletal muscle vasodilator, only one study has provided information regarding its mechanism of action in diaphragm microcirculation (7). The results of the
present study suggest that AIV in diaphragm microcirculation is mediated via binding of the A2A receptor, which is coupled to AC stimulation and opening of the KATP channel. However, our finding of adenosine subtype involvement in AIV in this vascular bed is in disagreement with the finding of Danialou et al. (7).

Classification of purinoceptors has been based on a combination of structural and pharmacological information (17). At least four adenosine purinoceptors are known and have been cloned, i.e., A1, A2A, A2B, and A3, and all of them are coupled to guanine nucleotide binding proteins (11). The availability of adenosine agonists has enabled differentiation among individual subtypes (14, 17). A number of adenosine agonists exist with different individual subtype potencies (10, 14). NECA is a nonselective agonist with high potency at A1 ($K_i = 6.3 \text{ nM}$), A2A ($K_i = 10.3 \text{ nM}$), and A2B ($K_i = 2 \text{ \mu M}$). In this study, nanomolar ranges (10–100 nM) of NECA elicited an increase in diaphragmatic microcirculatory flow in a dose-dependent manner. Because a high concentration of NECA (micromolar range) is required for stimulation of the A2B receptor (8), it seems unlikely that the A2B receptor is the adenosine subtype responsible for NECA-induced vasodilation. CGS-21680 elicited a similar degree of increase in diaphragmatic microcirculatory flow in a dose-dependent manner using the same concentrations as in the experiments with NECA. These findings suggest that the density of the A2A receptor is abundant in this vascular bed.

R-PIA is an agonist with high potency at the A1 receptor ($K_i = 1.2 \text{ nM}$) and intermediate potency at the A2 receptor ($K_i = 124 \text{ nM}$) (14). In this study, R-PIA also elicited similar degrees of incremental diaphragmatic microcirculatory flow using a concentration 10 times higher (100 nM to 1 \text{ \mu M}) than NECA or CGS-21680. In human pulmonary arteries or guinea pig coronary vasculature where the A2 receptor was known to mediate AIV, R-PIA was found to elicit a dose-relaxation curve similar to NECA with an agonist concentration 10 times higher (22, 32). Therefore, the concentration of R-PIA used could also increase microcirculatory flow via the A2 receptor. These findings suggest that AIV in this vascular bed is most likely mediated by the A2A receptor. This result is also compatible with recent studies on rat or cat hindlimb vasculature, where the A2A receptor was shown to mediate AIV or contribute to functional hyperemia (3, 28).

To further clarify the issue of adenosine subtype involvement in AIV, several adenosine antagonists with different potencies at individual subtypes were

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**Fig. 2. Dose-response curves of diaphragmatic microcirculatory blood flow measured by laser Doppler flowmetry (QLDF) in response to increasing doses of topical application of ADO (A), NECA (B), CGS-21680 (C), and R-PIA (D) before (●) and after (●) suffusion with $3.2 \times 10^{-6} \text{ M}$ glibenclamide; $n = 6$ for each. Responses are shown as a percentage increase of baseline QLDF and are given as means ± SE. **$P < 0.01$ vs. postglibenclamide.
used (10, 29). DPCPX is a selective A1-receptor antagonist exhibiting 700-fold selectivity for the A1 over the A2 receptor (2). At a concentration of ∼50 nM, DPCPX has been shown to be highly selective for A1-receptor antagonism and without effect on the A2 receptor (2, 23, 32). However, in this study, DPCPX (50 nM) failed to attenuate AIV, which suggests that AIV in this vascular bed is unlikely to be mediated via the A1 receptor.

Fig. 3. Dose-response curves of diaphragmatic microcirculatory QLDF before (●) and after (●) topical application of ADO antagonists; n = 6 for each. A: DPCPX, 5 × 10⁻⁸ M. B: 8-p-SPT, 3 × 10⁻⁶ M. C: ZM-241385, 10⁻⁸ M. Responses are shown as a percentage increase of baseline QLDF and are given as means ± SE; *P < 0.05, **P < 0.01 vs. postadenosine antagonists.

Fig. 4. A: dose-response curves of diaphragmatic microcirculatory QLDF to increasing doses of ADO before (●) and after (●) suffusion with MDL-12330A (10⁻⁵ M) for 15 min. B: forskolin (2 × 10⁻⁶ M)-induced vasodilation response in diaphragm microcirculation before (−MDL) and after (+MDL) suffusion with MDL-12330A (10⁻⁵ M) for 15 min. Responses are shown as a percentage increase of baseline QLDF and are given as means ± SE; n = 6 for each. *P < 0.05, **P < 0.01 vs. post-MDL-12330A.
8-p-SPT is a nonselective antagonist that blocks both the A1 and A2 receptors. Although 8-p-SPT is regarded as a low-potency A2-receptor antagonist (10), it acts as an effective antagonist to the A2 receptor at a concentration of 30 μM (32). In this study, AIV in diaphragm microcirculation was significantly attenuated by 8-p-SPT, suggesting that the A2 receptor is involved in AIV. Moreover, ZM-241385, a nonxanthine adenosine-receptor antagonist, exhibiting at least 100-fold higher affinity at the A2A receptor than at the A2B receptor, 1,000-fold lower affinity at the A1 receptor, and 500,000-fold lower affinity at the A3 receptor (28, 29), was used for further discrimination. It has been reported that an intravenous dose of ZM-241385 up to 2 mg/kg produced only inhibition of the A2A receptor in dog hindlimb vasculature (28). Therefore, ZM-241385 at a concentration of 1 μM, which is much less than 2 mg/kg, should inhibit only the A2A receptor in the rat diaphragmatic microvasculature. In this study, AIV in diaphragm microcirculation was also significantly blunted by ZM-241385, suggesting that the A2A receptor is involved in AIV. As the A3 receptor has been characterized as being resistant to blockade by 8-p-SPT or ZM-241385 and as having an equal agonist potency profile for R-PIA and NECA (16, 29), it is unlikely to be involved in AIV of this vascular bed. Therefore, antagonist studies have also favored a predominant role of the A2A receptor in mediating AIV response in rat diaphragm microvasculature.

The AC system is the most extensively studied effector system coupled to the adenosine receptor (14). In rabbit mesenteric arterial myocytes, activation of AC results in increased intracellular cAMP levels, which can regulate phosphorylation of myosin light chain kinase by cAMP-dependent protein kinase with subsequent vasodilation (19). MDL-12330A, an AC inhibitor (30), was used in this study to test the hypothesis of AC involvement in AIV in the diaphragm. After administration of 10 μM MDL-12330A, AIV was significantly attenuated, suggesting decreased AIV is mediated via inhibition of AC. This result was further supported by the inhibitory effect of MDL-12330A on forskolin, an AC activator, which induced vasodilation in diaphragm vascular bed. These findings indicate that activation of AC is involved in the AIV.

The involvement of the KATP channel in AIV varies in different vascular preparations. The KATP channel appears to be involved in AIV of rat hindlimb vasculature (4) or guinea pig coronary artery (25) but not in rat pulmonary circulation (16) or porcine epicardial vessels (21). In this study, adenosine and vasodilation induced by the adenosine agonists NECA, CGS-21680, and R-PIA could be significantly blunted by pretreatment with the KATP channel blocker glibenclamide, suggesting that the KATP channel is at least partly responsible for AIV in the rat diaphragm vascular bed.

The results obtained from the present study are in contrast with a recent study of AIV in rat diaphragm microcirculation. Using an intravital microscope, Danialou et al. (7) concluded that AIV in rat diaphragm microcirculation was mediated predominantly via the A1 receptor. This discrepancy could have several explanations. First, the measuring instrument and anesthetics used were different in these two studies. In this study, we mainly used urethan for general anesthesia; however, in Danialou’s study only pentobarbital sodium was used. Second, differences in the spatial variation of the diaphragm vascular bed may have involved different mechanisms of AIV in different sized arterioles (9, 15). Only diameter changes of the superficial arterioles were reported in Danialou’s study due to limitations of the intravital microscope (1), but the measurement by LDF consists mostly of capillary flow. Therefore, discrepancies in the results of our study may have been due to different sites of measurement.

A third possible explanation for the discrepancy may be related to the inclusion criteria used to select rats for study. In Danialou’s study, no inclusion criteria were mentioned; however, in our study strict inclusion criteria were used to ensure appropriate hemodynamic and oxygenation status of the rats. It is known that activation of the A1 receptor contributes predominantly to AIV in rat limb muscles during systemic hypoxia (3).

In conclusion, we have provided functional evidence that the predominant mechanism of AIV in resting rat diaphragm microcirculation is mediated via stimulation of the A2A receptor. The binding of adenosine to the A2A receptor is coupled to the activation of AC and the opening of the KATP Channel. This information may be valuable in respiratory muscle pathophysiology because the diaphragm is the principal respiratory muscle.

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