N\textsuperscript{G}-Nitro-L-arginine Methyl Ester Potentiates the Effect of Aminophylline on the Isolated Rat Hemidiaphragm

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Abstract. The effects of different concentrations of \textsuperscript{N}\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) (0.3, 1, 3, and 10 mM), a non-selective inhibitor of NOS, on the effect of aminophylline on the isometric contraction of the isolated rat hemidiaphragm were investigated. The muscle contractions were induced by direct subtetanic electrical stimulation. Aminophylline (0.36 – 3.60 mM) produced a typical concentration-dependent increase in both parameters of the isometric contraction: tension developed (Td) and the maximum rate of rise of tension (dT/dt max). The second series of additions of aminophylline produced a more pronounced effect. L-NAME (0.3, 1, 3, and 10 mM, 30 min of incubation without stimulation) itself did not change Td and dT/dt max. However, L-NAME (1, 3, and 10 mM) produced a statistically significant potentiation of the effect of aminophylline on Td and dT/dt max.

Keywords: aminophylline, L-NAME, rat hemidiaphragm

Many sympathomimetic substances and aminophylline (AMPh) are known to modulate cAMP metabolism, and at the same time, to influence the basal characteristics of the contraction of skeletal muscle. It is known that aminophylline modulates cAMP level (via inhibition of phosphodiesterase (PDE)) and produces an increase in the isometric contraction of the skeletal muscle. Several types of PDE exist, each with different properties and tissue distributions. In the skeletal muscle, aminophylline inhibits several isoforms of the enzyme: PDE1, PDE2, PDE4, and PDE5 (1).

It was shown that aminophylline, given in a cumulative manner (0.36 – 3.60 mM), produced a concentration-dependent increase in the parameters of the isometric contraction of the isolated rat hemidiaphragm during direct single pulse and subtetanic stimulation (2). The repeated series of additions of aminophylline into the bathing medium during subtetanic stimulation produced a more pronounced potentiation of the parameters of the isometric contraction (Td and dT/dt max) (3). Such in vitro findings were in agreement with the clinical observations on the beneficial effects of aminophylline on the fatigue diaphragm (4, 5). This effect of aminophylline is still of a great therapeutic importance in the treatment of asthma and chronic obstructive pulmonary diseases.

In skeletal muscle, all isoforms of nitric oxide synthase (NOS) have recently been identified (6). Neuronal NOS (nNOS) is predominantly localized postsynaptically at the neuromuscular junction (7) and is most evident in fast muscular fibers. It was postulated that nitric oxide originated from nNOS has a major role in the skeletal muscle contractility (8).

The activity of both constitutive isoforms (nNOS and endothelial NOS (eNOS)) is regulated by calcium ions (9). Calcium-dependent calmodulin binding is a principal regulator of nitric oxide synthesis for both constitutive NOS isoforms (6).

Balon and Nadler (10) have reported that in the skeletal muscle, NO is synthesized continually at low rates by NOS. Under their experimental conditions, the synthesis of NO increased during repetitive isometric contractions up to 200%. Other authors have postulated that NO can modulate skeletal muscle contractility via at least two mechanisms: first, through the cGMP-NO pathway (11) and second, NO may also act directly by interacting with calcium release channels of the sarcoplasmic reticulum (6, 11 – 13).
Enzyme NOS is activated during muscle contraction and the synthesized nitric oxide activates soluble guanylyl cyclase (sGC), which, in turn, increases the level of cGMP. Furthermore, cGMP can activate or inhibit cGMP-dependent phosphodiesterases leading to decreased or increased cAMP levels.

The aim of the present study was to determine the possible interaction between $N^\text{G}$-nitro-L-arginine methyl ester (L-NAME) (a potent inhibitor of nitric oxide synthase) and aminophylline on the contractility of the isolated rat diaphragm.

The experiments were performed on the isolated rat hemidiaphragm. Wistar rats were bred and kept under ordinary laboratory conditions. The investigation conforms to the Guide for the Care and Use of the Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

The isolated hemidiaphragm from male and female rats (200 – 250 g) was suspended in an isolated organ bath of 15 ml capacity. The muscle was immersed in Tyrode solution with a double amount of glucose and bubbled with a mixture of 97% O$_2$ and 3% CO$_2$. The composition of Tyrode solution was as follows: 136 mM NaCl, 2.81 mM KCl, 0.105 mM MgCl$_2$, and 1.08 mM CaCl$_2$, 0.417 mM NaH$_2$PO$_4$, 11.9 mM NaHCO$_3$, and 11.1 mM dextrose. The temperature of this solution was about 36°C. Two paladore wires (palladium 30% and silver 70%) were used. The diaphragm was secured to one of these wires at several points along the rib line. The other electrode was placed around the upper part of the diaphragm, but was not in contact with the muscle. The initial tension after the 30-min equilibration period was about 5 g.

The preparation was stimulated directly by subtetanic electrical stimulation. The frequency of stimulation was 14 Hz for 2 s, a train of pulses being applied every 12 s (5 times/min). The isometric contractions were recorded with a microdisplacement myograph transducer (F 50; Narco-Bio-System, Inc., Houston, TX, USA) and displayed on paper (Physiograph IV polygraph). Both tension developed (Td) and the maximum rate of rise of tension (dT$_{\text{max}}$) were obtained simultaneously (for details, see Ref. 3).

The experimental designs (1 – 5) are shown in Fig. 1. The effects of the drugs used were expressed as percent differences between means was assessed for significance (ANOVA followed by post hoc 2-sided Dunnett’s test), when appropriate. Values of $P<0.05$ were taken as statistically significant (SPSS 8.0 for Windows).

Aminophylline given in a single concentration (EC$_{50}$ = 1.08 mM) produced an increase in both Td and dT$_{\text{max}}$ (up to 24% and 10%, respectively) (Experimental design 1, experiments without L-NAME). The second addition of a single concentration of aminophylline did not significantly potentiate muscle contractility in comparison with the first addition (not shown in the figure).

Aminophylline given in a cumulative manner (0.36 – 3.60 mM) produced a concentration-dependent increase in both Td (Fig. 2A-1) and dT$_{\text{max}}$ (not shown) of the isolated rat hemidiaphragm during direct subtetanic electrical stimulation. The effect of each concentration of aminophylline developed slowly, reaching its maximum 4 – 6 min after the addition of the drug into the bathing medium. The 2nd series of additions of aminophylline (0.36 – 3.60 mM) on the same preparation produced more pronounced potentiation of Td and dT$_{\text{max}}$ (Experimental design 2).

In a separate series of experiments, the isometric contractions of the stimulated hemidiaphragm under basal conditions, i.e., without any drug, were recorded (Experimental design 3, Fig. 2A-2). These experiments represent the time-related controls for the experiments with the repeated additions of aminophylline (0.36 – 3.60 mM) (Experimental design 2, Fig. 2A-1). No significant fluctuations of Td above and below the baseline were recorded (Fig. 2A-2).

In addition, 30 min of incubation without electrical stimulation did not change Td in comparison with the corresponding control; i.e., C-30 was only negligibly different from C (Fig. 2A-1, 2A-2).

The next step was to investigate the effects of different concentrations of L-NAME (0.3, 1, 3, and 10 mM) on the parameters of the isometric muscle contraction (Td and dT$_{\text{max}}$) of the previously untreated muscle (Experimental design 4). After recording the control, L-NAME was added into the bathing medium and left for 30 min, without electrical stimulation of the hemidiaphragm. It was found that L-NAME did not significantly change either Td or dT$_{\text{max}}$ in comparison with the corresponding controls (L-NAME-30 vs C and L-NAME-30 vs C-30, $P>0.05$). The results are not shown in the figure.

Finally, the effect of different concentrations of L-NAME (0.3, 1, 3, and 10 mM) on the muscle pretreated with single (Experimental design 1) or cumulative concentrations of aminophylline (Experimental designs 2 and 5) was investigated in a separate series of experiments. It was found that L-NAME (3 and 10 mM only)
produced a further increase in Td (Fig. 2B) of the muscle pretreated with cumulative concentrations of aminophylline. The same parameter (Td) of the muscle pretreated with a single concentration of aminophylline (1.08 mM) was not affected by L-NAME (not shown).

The interaction between aminophylline and L-NAME was again investigated in a separate series of experiments (Experimental design 1, 2, and 5). L-NAME (0.3, 1, 3, and 10 mM) in the muscle already pretreated with a single concentration of aminophylline (1.08 mM) did not change the effect of the second addition of the same concentration of aminophylline on Td (Experimental design 1) (the results are not shown). However, L-NAME (1, 3, and 10 mM) in the muscle already pretreated with cumulative concentrations of aminophylline (0.36 – 3.60 mM) significantly potentiated the effect of

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**Fig. 1.** Experimental designs (1 – 5). Experimental design 3 represents the time-related control for the experiments shown as Experimental design 2. C: the value of Td or dT/dt max of the electrically stimulated muscle under basal conditions; C-30: the value of Td or dT/dt max of the electrically stimulated muscle recorded after 30 min of incubation without L-NAME, as well as without electrical stimulation; L-NAME-30: the value of Td or dT/dt max of the electrically stimulated muscle recorded after 30 min of incubation with L-NAME (0.3, 1, 3, or 10 mM), as well as without electrical stimulation; AMPh (1.08 mM): a single concentration of aminophylline; AMPh (0.36 – 3.60 mM): cumulative concentrations of aminophylline.
the second series of addition of the same cumulative concentrations of aminophylline on Td (Experimental designs 2 and 5). The results are shown in Table 1.

Such effects of L-NAME were not concentration-related. Specifically, L-NAME (1 mM) produced statistically significant potentiation of the effect of all concentrations of aminophylline (0.36, 1.08, 2.16, and 3.60 mM), during direct submaximal electrical stimulation, after 30 min of incubation without or with L-NAME (0.3, 1, 3, or 10 mM) (Experimental design 2 and 5, respectively). ΔTd (%): the mean percentage change of Td from the corresponding control (C and C-30 for the first and second series of additions of aminophylline, respectively). Each vertical bar represents the mean ± S.E.M. of 6 experiments. P<0.01, significantly different from the effect of aminophylline during the first series of additions (paired Student’s t-test). B: Change of Td of the isolated hemidiaphragm of the rat pretreated with cumulative concentrations of aminophylline (AMP: 0.36, 1.08, 2.16, and 3.60 mM), during direct submaximal electrical stimulation, after 30 min of incubation without or with L-NAME (0.3, 1, 3, or 10 mM) (Experimental design 2 and 5, respectively). ΔTd (%): the mean percentage change of Td from the corresponding control (C). C-30: the value of ΔTd of the electrically stimulated muscle recorded after 30 min of incubation without L-NAME, as well as without electrical stimulation (Experimental design 2). L-NAME(0.3–10)-30: the value of ΔTd of the electrically stimulated muscle recorded after 30 min of incubation with L-NAME (0.3, 1, 3, or 10 mM), as well as without electrical stimulation (Experimental design 5). Each vertical bar represents the mean ± S.E.M. of 6 separate experiments. P<0.05 and P<0.01, significantly different from C-30 (ANOVA with post hoc 2-sided Dunnett’s test).

Fig. 2. Aminophylline and L-NAME on the isolated hemidiaphragm of the rat. A: The effects of the repeated series of additions of aminophylline (AMP-cumulative concentrations of 0.36, 1.08, 2.16, and 3.60 mM) on the isometric contraction of the isolated rat hemidiaphragm during direct submaximal stimulation (A-1, Experimental design 2) and the corresponding time-related controls (without any treatment) (A-2, Experimental design 3). ΔTd (%): the mean percentage change of Td from the corresponding control (C and C-30 for the first and second series of additions of aminophylline, respectively). Each vertical bar represents the mean ± S.E.M. of 6 experiments. P<0.01, significantly different from the effect of aminophylline during the first series of additions (paired Student’s t-test).
Table 1. The effect of a second series of addition of aminophylline (AMPh) (0.36 – 3.60 mM) without and with l-NAME (0.3, 1, 3, and 10 mM) on Td of the isolated hemidiaphragm of the rat (Experimental designs 2 and 5, respectively)

<table>
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<tr>
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<th>AMPh without l-NAME</th>
<th>AMPh without l-NAME</th>
<th>AMPh without l-NAME</th>
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<td></td>
<td>vs l-NAME_{&lt;1} + AMPh</td>
<td>[\Delta Td (%) ± S.E.M. (n)]</td>
<td>vs l-NAME_{&lt;1} + AMPh</td>
<td>[\Delta Td (%) ± S.E.M. (n)]</td>
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<tr>
<td>AMPh_{0.36}</td>
<td>24.25 ± 5.59 (4)</td>
<td>12.83 ± 2.23 (6)</td>
<td>14.83 ± 3.0 (6)</td>
<td>15.62 ± 1.85 (8)</td>
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<td>P = 0.4607</td>
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<tr>
<td>AMPh_{1.16}</td>
<td>70.25 ± 8.11 (4)</td>
<td>77.60 ± 8.15 (5)</td>
<td>44.25 ± 3.97 (4)</td>
<td>74.4 ± 5.02 (5)</td>
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<td>P = 0.0213*</td>
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<tr>
<td>AMPh_{2.16}</td>
<td>95.00 ± 0.95 (4)</td>
<td>135.60 ± 10.40 (5)</td>
<td>81.60 ± 5.89 (5)</td>
<td>123.40 ± 8.29 (5)</td>
</tr>
<tr>
<td>P = 0.0024*</td>
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<tr>
<td>AMPh_{3.16}</td>
<td>102.75 ± 10.62 (4)</td>
<td>153.40 ± 19.25 (5)</td>
<td>110.40 ± 9.82 (5)</td>
<td>161.80 ± 7.83 (5)</td>
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<td>P = 0.0347*</td>
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\(\Delta Td\%): the mean percentage change of Td from C-30 or l-NAME_{<1} – 102.75 (before the addition of the second series of AMPh, i.e., after 30 min of incubation without stimulation); n: number of experiments; AMPh without l-NAME (Experimental design 2) vs the corresponding AMPh with l-NAME (Experimental design 5), unpaired Student’s t-test, *P<0.05.

and pattern of electrical stimulation (14 vs 20 Hz) and/or periods of incubation. Therefore, we have postulated that under our experimental conditions (Experimental design 4), the production of nitric oxide in the untreated muscle is insufficient. Thus, inhibition of NO synthesis by l-NAME (0.3 – 10 mM, 30 min of incubation) could not produce any significant change in the muscle contractility.

In our experiments, the NOS inhibitor did not influence diaphragm contractility under basal conditions (i.e., previously untreated muscle, Experimental design 4), but increased Td of the pretreated hemidiaphragm with cumulative AMPh (Experimental design 5, Fig. 2B). The possible explanation takes into consideration AMPh-induced modulation of calcium current and cAMP. In stimulated striated muscle, aminophylline activates sarcoslemal L-voltage calcium current via cAMP and cAMP-dependent protein kinases. It is already known that nitric oxide does not affect calcium current under basal conditions, but modulates sympathomimetic-activated L-calcium channels in cardiomyocytes (16). It is quite possible to presume that such a mechanism works in the skeletal muscle also.

It is known that nNOS is a membrane bound and calcium-dependent constitutive isoform (7). It binds calmodulin only when local calcium levels are increased (8). Production of NO by nNOS can be upregulated during periods of repetitive isometric contraction (10). Therefore, the observed l-NAME-increased muscle contractility after pretreatment of the muscle with cumulative, millimolar concentrations of AMPh (Fig. 2B) is most likely due to blockade of NOS (i.e., the loss of the inhibitory effect of nitric oxide on muscle contraction).

There are at least two possible explanations why pretreatment with single AMPh (1.08 mM) influenced neither the effects of l-NAME nor the effects of the second addition of the same, single concentration of AMPh (Experimental design 1). First, the concentration of AMPh used in the pretreatment (1.08 mM) could be insufficient for the upregulation of nNOS. Second, the time of exposure of hemidiaphragm to the single aminophylline pretreatment was probably not long enough for the activation of nNOS. This problem remains to be elucidated in further experiments, some of which are in progress, using different concentrations and times of exposure of hemidiaphragm to a single aminophylline pretreatment, as well as using the experimental designs with combined exposure of the muscle to single and cumulative AMPh.

The effects of aminophylline were potentiated by l-NAME (Table 1). As NO production was blocked by l-NAME, the amount of nitric oxide was not enough to activate sGC. Decreased cGMP-induced stimulation of PDE2 leads to decreased degradation of cAMP (1). A raised cytosolic concentration of cAMP facilitates phosphorylation of calcium-ion-conducting L-type channels and increases the likelihood of the channel being activated in response to stimulation. This mechanism could probably explain the potentiation of the effect of aminophylline in the presence of l-NAME.

The apparent difference between the interaction of
L-NAME (1 mM) and L-NAME (3 and 10 mM) with the higher concentrations of AMPH (2.16 and 3.60 mM) (Table 1) could be explained by the fact that L-NAME itself only in higher concentrations (3 and 10 mM) produced a significant, probably near maximal increase of Td (ΔTd: 49% and 46%, respectively) (Fig. 2B). Because of that, AMPH (2.16, and 3.60 mM) given in the second series in the presence of higher concentrations of L-NAME could not cause further increase of the diaphragm contractility.

In conclusion, this study provides the first evidence about the interaction between the NO system inhibitor (L-NAME) and a substance that modulates both the cAMP system and calcium metabolism in the skeletal muscle (aminophylline). This interaction between drugs that have been known to influence the skeletal muscle contractility via nitric oxide and cAMP/calcium system may be of a great clinical importance for the treatment of certain respiratory and neurological diseases.

References

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