EFFECTS OF ADENYLATE CYCLASE ACTIVATORS ON PORCINE SKELETAL MUSCLE IN MALIGNANT HYPERPYREXIA

A. T. R. SIM, M. D. WHITE AND M. A. DENBOROUGH

Malignant hyperpyrexia (MH) is an often fatal complication of general anaesthesia, which is attributed to a dysfunction of skeletal muscle (Denborough, 1980). While the precise site of the abnormality associated with MH remains unknown (Gronert, 1980), it is thought that an increase in myoplasmic Ca\(^{2+}\) concentration can account for most of the features of MH (Denborough, 1980). The concentration of Ca\(^{2+}\) in the myoplasm is regulated by the sarcoplasmic reticulum (SR), and the enzyme adenylate cyclase has been found to influence SR Ca\(^{2+}\)-transport in cardiac muscle (Kirchberger and Tada, 1976) and slow skeletal muscle (Schwartz et al., 1976). Furthermore, halothane, which precipitates an MH episode (Gronert, 1980) and induces MH-susceptible (MHS) skeletal muscle to contract in vitro (Ellis et al., 1971), activates the adenylate cyclase system (Sprague, Yang and Ngai, 1974). Consequently, this link between adenylate cyclase and MH has been investigated. Although increased concentrations of cyclic AMP (cAMP), and increases in adenylate cyclase activity, have been reported in MHS human muscle (Willner, Cerri and Wood, 1981; Ellis et al., 1984), no difference has been found between control and MHS porcine skeletal muscle, in relation to adenylate cyclase activity (Ono, Topel and Althen, 1976, 1977). Since MHS skeletal muscle characteristically contracts in response to various reagents in vitro (Moulds and Denborough, 1974a), we have investigated the effect of adenylate cyclase activation on the contractility of control, and MHS porcine, skeletal muscle in vitro.

SUMMARY

The effect of adenylate cyclase activation on the in vitro contractures of control and malignant hyperpyrexia susceptible (MHS) porcine muscle was investigated. While fluoride and molybdate ions potentiated drug-induced contractures in control muscle, other activators of adenylate cyclase (forskolin and noradrenaline) did not. Furthermore, fluoride and molybdate had no effect on MHS skeletal muscle contractility. Cyclic AMP content, basal adenylate cyclase activity and molybdate-stimulated adenylate cyclase activity of MHS skeletal muscle was not significantly different from that of control muscle. It is concluded that increased activity of adenylate cyclase does not represent the primary skeletal muscle defect which predisposes to porcine MH.

MATERIALS AND METHODS

Pigs susceptible to MH were bred from crosses of MHS Landrace and Large White breeds. Animals used as controls were either commercially purchased, unrelated animals or MH-negative siblings of MHS pigs.

Susceptibility to MH was assessed by the in vitro contracture testing of skeletal muscle, according to Okumura, Crocker and Denborough (1979).

Anaesthesia and surgical procedures were carried out using the method of Okumura, Crocker and Denborough (1979), except that the neurolept agent Stresnil (4-fluor-4-(4-(2-pyridyl)-1-piperazinyl)-butyrophenone) was used for premedication.

For pharmacological studies, the method described by Moulds and Denborough (1974b) was used. Muscle preparations which did not produce
TABLE I. The effect of adenylate cyclase activators on drug-induced contractures in porcine control skeletal muscle. Control muscle fibres were pretreated for 5 min with the appropriate adenylate cyclase activator and then exposed to the appropriate diagnostic reagent. Results are expressed as the mean contractures (g) ± SEM. For comparisons between pre-treatment and no pre-treatment: *P < 0.05 **P < 0.01; ***P < 0.001 (Student's paired t test)

<table>
<thead>
<tr>
<th>Diagnostic reagent</th>
<th>Pretreatment</th>
<th>Sodium molybdate</th>
<th>Sodium fluoride</th>
<th>Forskolin</th>
<th>Noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>20 mmol litre⁻¹</td>
<td>20 mmol litre⁻¹</td>
<td>100 μmol litre⁻¹</td>
<td>100 μmol litre⁻¹</td>
</tr>
<tr>
<td>3% Halothane</td>
<td></td>
<td>0.37 ± 0.07</td>
<td>0.29 ± 0.13</td>
<td>0.01 ± 0.01</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>(n = 21)</td>
<td>(n = 19)***</td>
<td>(n = 17)***</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Caffeine</td>
<td></td>
<td>0.28 ± 0.06</td>
<td>0.13 ± 0.04</td>
<td>0.0</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>2 mmol litre⁻¹</td>
<td>(n = 16)</td>
<td>(n = 16)***</td>
<td>(n = 17)***</td>
<td>(n = 12)*</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Suxamethonium</td>
<td></td>
<td>0.09 ± 0.02</td>
<td>0.13 ± 0.05</td>
<td>—</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>1 mmol litre⁻¹</td>
<td>(n = 19)</td>
<td>(n = 19)***</td>
<td>(n = 12)*</td>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>Potassium chloride</td>
<td></td>
<td>0.24 ± 0.12</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>80 mmol litre⁻¹</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a twitch response equivalent to a tension of 0.75 g or more, were rejected.

Drugs and chemicals were added directly into the organ bath at the final concentrations indicated in the text. Forskolin (Calbiochem) and dantrolene sodium (Norwich Eaton) were dissolved in dimethyl sulphoxide, final concentrations of which in the organ bath did not exceed 0.4% (v/v) and had no effect on muscle integrity. When used, thymol-free halothane was administered by passing carbogen through a Dragewick halothane vaporizer.

Adenylate cyclase activity was determined by measuring the change in cAMP content of skeletal muscle homogenates in the presence of ATP and a phosphodiesterase inhibitor. Samples of muscle were obtained at biopsy, frozen immediately in liquid nitrogen and stored at −70°C until required. No sample was stored for more than 4 weeks, and control and MHS samples of equivalent storage age were assayed at the same time, in each study. Using a mortar and pestle, cooled to −70°C, approximately 0.5 g of muscle sample was ground to a fine powder and homogenized in 20 volumes of homogenization buffer using a Polytron at maximum setting for 2×10⁻³ s intervals. The homogenization buffer contained EDTA 4 mmol litre⁻¹, potassium chloride 150 mmol litre⁻¹, Tris-HCl 10 mmol litre⁻¹, pH 7.4. Aliquots (50 μl) of muscle homogenate were added to 50 μl of assay buffer at 0°C. The assay buffer contained ATP 1 mmol litre⁻¹, creatine phosphate 20 mmol litre⁻¹ and creatine phosphokinase 100 u ml⁻¹, theophylline 20 mmol litre⁻¹, magnesium chloride 50 mmol litre⁻¹, Tris-HCl 50 mmol litre⁻¹, pH 7.4. The reaction was started by transferring the samples to a 37°C water bath. After 10 min, the reaction was stopped by the addition of 5% TCA 150 μl (w/v) and the protein precipitated by centrifugation at 2000 g for 15 min. The supernatant was collected, extracted with water-saturated ether and assayed for cAMP content using a competitive-binding assay kit (Amersham Radiochemicals). Experiments were performed in duplicate. Dose–response curves for different chemicals were carried out on muscle from one animal.

All adenylate cyclase activators used did not interfere with the cAMP assay. Adenylate cyclase activity was expressed as the amount of cAMP formed per mg of protein per min.

Protein estimations were carried out using the method of Peterson (1977).

RESULTS

Pharmacological action of adenylate cyclase activators on skeletal muscle

The results obtained when control porcine skeletal muscle fibres were pretreated with adenylate cyclase activators and then exposed to 3% halothane, caffeine 2 mmol litre⁻¹, suxamethonium 1 mmol (Anectine, Burroughs Wellcome) or potassium chloride 80 mmol litre⁻¹ are shown in table I. These activators, alone, did not stimulate contractures in control or MHS skeletal muscle. Pre-treatment with 20 mmol litre⁻¹ of fluoride or molybdate significantly...
potentiated contractures in response to halothane, caffeine and suxamethonium (table I). Molybdate also increased the magnitude of the potassium chloride-induced contractures, but this was not statistically significant. Pretreatment with equivalent concentrations of sodium chloride in place of NaF + Na₂MoO₄ had no effect.

Lower concentrations of molybdate also significantly potentiated drug-induced contractures in control muscle. Using molybdate 10 mmol litre⁻¹, mean contractures of 0.26 ± 0.08 g (n = 7) were obtained in response to 3% halothane, while molybdate 2.5 mmol litre⁻¹ produced a mean contracture of 0.125 ± 0.05 g (n = 3).

While fluoride and molybdate potentiated drug-induced contractures in control skeletal muscle, forskolin and noradrenaline (100 µmol litre⁻¹) did not (table I). Furthermore, noradrenaline up to 2 mmol litre⁻¹ failed to potentiate drug-induced contractures (results not shown).

When these experiments were carried out with MHS porcine muscle, the results were different from those obtained with control muscle (table II). Fluoride and molybdate 20 mmol litre⁻¹ produced no significant changes in drug-induced contractures. Forskolin and noradrenaline also failed to have any effect on contractures induced by the same drugs in MHS tissue (table II).

The effect of dantrolene on fluoride and molybdate-potentiated contractures was also examined (table III). Dantrolene was added to the organ bath after fluoride and molybdate and before the diagnostic reagent. Treatment of muscle fibres with dantrolene 6 µmol litre⁻¹ prevented fluoride- and molybdate-potentiation of contractures in response to 3% halothane and caffeine 2 mmol litre⁻¹ (table III). Dantrolene was equally effective in reversing fluoride- and molybdate-potentiated drug-induced contractures (not shown).
TABLE IV. Adenylate cyclase activity of control and MHS porcine skeletal muscle. Where appropriate, sodium molybdate 20 mmol litre\(^{-1}\) with or without dantrolene 6 \(\mu\)mol litre\(^{-1}\) was included in the assay buffer. Results are expressed as the mean adenylate cyclase activity ± SEM. There was no significant difference between control and MHS samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>None</th>
<th>Molybdate 20 mmol litre(^{-1})</th>
<th>Molybdate 20 mmol litre(^{-1}) + dantrolene 6 (\mu)mol litre(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.79 ±0.97</td>
<td>30.17 ±6.55</td>
<td>24.08 ±5.25</td>
</tr>
<tr>
<td>((n = 8))</td>
<td>((n = 8))</td>
<td>((n = 5))</td>
<td></td>
</tr>
<tr>
<td>MHS</td>
<td>8.06 ±1.34</td>
<td>28.12 ±4.31</td>
<td>25.23 ±3.1</td>
</tr>
<tr>
<td>((n = 8))</td>
<td>((n = 7))</td>
<td>((n = 5))</td>
<td></td>
</tr>
</tbody>
</table>

Adenylate cyclase activity of control and MHS skeletal muscle

Cyclic-AMP content of control samples varied between 8.46 and 30 pmol/mg protein and between 12.8 and 41.3 pmol/mg protein for MHS samples. The mean cAMP content of MHS skeletal muscle homogenates (22.39 ±3.7 pmol/mg protein) was not significantly different from that of control muscle (18.19 ±2.49 pmol/mg protein). Furthermore, basal adenylate cyclase activity in MHS skeletal muscle homogenates was not significantly different from the basal adenylate cyclase activity of control samples (table IV).

The effect of adenylate cyclase activators on skeletal muscle adenylate cyclase activity was also studied. Dose-response curves showed that the inclusion of fluoride, molybdate, forskolin and noradrenaline in the assay buffer produced a concentration-dependent increase in adenylate cyclase activity in control samples (fig. 1). These agents were thus shown to activate adenylate cyclase in porcine skeletal muscle as they do in other tissues. However, there was no significant difference between control and MHS samples in relation to molybdate-stimulated adenylate cyclase activity (table IV). Furthermore, dantrolene had no significant effect on molybdate-stimulated adenylate cyclase activity in control or MHS samples (table IV).

DISCUSSION

Adenylate cyclase activity has been reported to be increased in MHS humans (Willner, Cerri and Wood, 1981; Ellis et al., 1984), but not in MHS pigs (Ono, Topel and Althen, 1976, 1977). While the results of these different investigations may reflect a real difference and species variability, it may be noted that the use of an animal model allows more stringent attention to the choice of controls, muscle type and experimental conditions. In the investigation of human MH, controls are obtained from various sources and may involve different muscle types (Willner, Cerri and Wood, 1981) and anaesthetic conditions (Gronert and Van Dyke, 1984). However, variations in the concentrations of circulating catecholamines (Halper, Pflug and Porter, 1977) and adenylate cyclase activity in different muscle types (Festoff, Oliver and Reddy, 1977) may lead to spurious differences between subjects. The animals used in the present study were subject to the same experimental conditions and results showed that there was no difference between control and MHS porcine muscle in relation to cAMP content and basal adenylate cyclase activity.

While fluoride and molybdate increased the sensitivity of control muscle to the diagnostic reagents, other activators of adenylate cyclase (forskolin and noradrenaline) did not. Fluoride has been shown to activate adenylate cyclase only in broken cell preparations (Perkins and Moore, 1971) while forskolin is a general activator of adenylate cyclase in all systems, including whole cell preparations (Seamon and Daly, 1981). Since the muscle preparations used in this study were essentially intact, it is unlikely that fluoride increased the sensitivity of control muscle through a direct action on adenylate cyclase. Furthermore, it is not known whether molybdate stimulates adenylate cyclase in whole cell preparations, but it is generally believed to have a mechanism of action similar to that of fluoride (Richards and Swislocki, 1979). Although fluoride and molybdate were both found to stimulate adenylate cyclase activity in skeletal muscle homogenates,
ADENYLATE CYCLASE AND MALIGNANT HYPERPYREXIA

related to activation of adenylate cyclase activity. It is also concluded that the primary defect associated with MH does not reside in the adenylate cyclase system.

The finding that fluoride and molybdate did not increase the contractile response of MHS muscle suggests that MHS muscle has a decreased sensitivity to fluoride and molybdate. Both fluoride and molybdate are potent inhibitors of protein phosphatases (Hollander, 1971) and, as such, might interfere with the phosphorylation state of certain proteins involved in muscle contractility. Experiments to explore this hypothesis have been started.

REFERENCES


