Sarcolemmal Membrane Changes Related to Enzyme Release in the Imipramine/Serotonin Experimental Animal Model

Lawrence M. Silverman1 and Hans-Dieter Gruemer2

We report specific findings in the imipramine-serotonin animal model that are consistent with sarcolemmal membrane alterations. Among these findings are cytoplasmic enzyme release, diminished uptake of α-aminoisobutyrate (an amino acid analog), decreased oxygen consumption in isolated rat diaphragm, and ribosuria. Furthermore, we describe for the first time the release of the MB isoenzyme of creatine kinase from a source other than cardiac tissue; that is, isolated diaphragms from imipramine-serotonin-treated animals released increased amounts of MB isoenzyme as compared to diaphragms from control animals. We believe the similarities between this animal model and the human disease (Duchenne muscular dystrophy) support a genetically determined generalized membrane abnormality in the pathogenesis of this form of muscular dystrophy.

Additional Keyphrases: creatine kinase • isoenzyme MB, release from diaphragmatic tissue • Duchenne muscular dystrophy • α-aminoisobutyrate • platelets • animal model of human disease • rats • membrane permeability

Although Duchenne muscular dystrophy (DMD) is the most frequent form of muscular dystrophy, the pathogenesis of this hereditary disease is unknown. Increased release of cytoplasmic enzymes such as creatine kinase (CK; EC 2.7.3.2), lactate dehydrogenase (EC 1.1.1.27), aldolase (EC 4.1.2.13), pyruvate kinase (EC 2.7.1.40), and of aspartate- and alanine-amino-transferases (EC 2.6.1.1 and EC 2.6.1.2) into the sera of Duchenne dystrophy patients has led several investigators to postulate changes in the membrane permeability of skeletal muscle as the underlying pathogenesis in DMD (1–3). CK has been considered the most sensitive and specific indicator of muscle damage (2) because it is predominantly found in cardiac and skeletal muscle. An increase in the activities of both the MM and MB isoenzymes of CK in serum has been reported in DMD (4–8). Increased MB activity in serum has recently been suggested to be specifically related to myocardial damage (9, 10); however, serum MB activity in DMD patients does not correlate well with the clinical cardiomyopathy (11).

Other tissues have been shown to have altered membrane function in DMD. Matheson and Howland (12) have reported increased morphological changes in erythrocytes from DMD patients, detected by scanning electron microscopy, and Bosmann et al. (13) have shown a difference in electrophoretic mobility of erythrocytes in DMD. Platelets have also been shown to have a decreased uptake of serotonin (5-hydroxytryptamine) in patients with DMD (14). Because imipramine has been shown to simulate this biochemical defect (15), an animal model of the defect was developed by use of imipramine (16). In combination with serotonin, imipramine produces skeletal muscle lesions that are histologically similar to those seen in the skeletal muscles of DMD patients.

We have studied this animal model, to examine the manifestations of skeletal muscle permeability changes and the relationship of these membrane changes to specific cellular functions known to be altered in DMD.

Previous reports on the imipramine-serotonin experimental model described increased activity of CK in plasma, specifically the MM and MB isoenzymes of CK (17), and in the activities of lactate dehydrogenase and aspartate aminotransferase (18). We now report an increased release of total CK and of its MB isoenzyme from diaphragms of imipramine-serotonin-treated animals and the presence of ribose in their urine. In addition, studies on the isolated rat diaphragm showed that imipramine and serotonin diminished oxygen consumption and that imipramine alone altered the in vitro uptake of an amino acid analog, α-aminoisobutyric acid. Results of in vivo studies with imipramine and serotonin were similar. These findings are consistent with those described in the skeletal muscle sarcolemma of the Bar Harbour mouse (19) and in Syrian hamster heart (20), two genetically determined animal models of muscular dystrophy, which suggests that imipramine induces changes in the sarcolemma of muscle that may be related to the defect described in the platelets of DMD patients. Furthermore, these same studies suggest that a genetically determined generalized membrane defect may be present in DMD patients that affects skeletal muscle, platelets, and perhaps erythrocyte membranes.

The usefulness of this animal model in the study of muscular dystrophy is even more apparent when one considers enzyme release. Increased membrane permeability may be associated with increased release of
cytoplasmic enzymes; moreover, any additional alterations of membrane function found in this animal model lend support to the supposition that there is permeability dysfunction in the human disease it simulates. Thus, the model may be used to explain to some extent the enhanced release of intracellular enzyme into the sera of Duchenne dystrophy patients. It is hoped that an understanding of enzyme release in DMD may lead to a better understanding of cell leakage in other pathological conditions.

**Methods and Materials**

To produce the experimental myopathy, we injected male Wistar rats (weight, 120–180 g) intraperitoneally with imipramine (Tofranil, 12.5 mg/ml solution; Geigy Pharmaceuticals, Ardsley, N. Y.) for three days, a dosage of 10 mg/kg body weight each day. Six hours after the third imipramine injection, serotonin/creatinine sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio 44128) was injected intraperitoneally, in a dose of 100 mg/kg body weight. This injection schedule reportedly (18) produces the skeletal muscle lesions and chemical changes characteristic of this experimental myopathy. Urinary ribose was determined in samples collected overnight, with the rats in metabolism cages, after the serotonin injection. Ribose was analyzed by a modification of Bial's test for pentoses (23). The colored product was scanned with an SP 1800 spectrophotometer (Fye Unicam Limited, Cambridge, England) and found to have a characteristic spectrum, which we used to discriminate ribose from other pentoses. Additional verification was obtained by two-dimensional thin-layer chromatography on Silica Gel G plates (Brinkman Instruments, Inc., Westbury, N. Y. 11590) with butanol/acetic acid/water (60/30/10 by vol) in one direction and phenol (880 g/liter solution) in the other direction.

To study enzyme release, we removed diaphragms from imipramine-serotonin-treated animals (15 min after the serotonin injection) and from control animals that were injected intraperitoneally with NaCl (9 g/liter) instead of imipramine and serotonin. The diaphragms were washed in NaCl (9 g/liter), weighed on a torsion balance, and then placed in a glass scintillation vial containing 1 ml of Krebs–Ringer–bicarbonate buffer (24). These vials were incubated in a water bath under continuous shaking at 37 °C in a constant O2/CO2 (95/5 by vol) atmosphere for 30 min. Diaphragms were then blotted and weighed again on a torsion balance, to assess water uptake. Total CK and CK-MB isoenzyme activities in the bath were measured by a modified Rosalki procedure (Test Kit 45-UV; Sigma Chemical Co., St. Louis, Mo. 63178). CK isoenzymes were fractionated and measured by the column-chromatographic procedure of Mercer (21), modified (17) to increase resolution. Serum was used for studies of patients but plasma was used for animal studies, because clotting has been reported to increase CK release from platelets in rats (22).

In the study of in vitro uptake of [14C]-α-aminoisobutyric acid (20 mCi/liter; New England Nuclear, Boston, Mass. 02118), diaphragm hemispheres from control rats were excised, blotted, weighed, and then added to scintillation vials containing 1 ml of the Krebs–Ringer–bicarbonate solution. Imipramine and serotonin were added so that the final concentration of each was 4 mmol/liter. Control vials contained only Krebs–Ringer–bicarbonate solution in an additional volume equal to the volume of imipramine and serotonin added to the test vials. The diaphragms were incubated in the vials for 5 min in the water bath. [14C]-α-Aminoisobutyric acid was then added to each vial (0.5 μCi/vial) and incubation was continued under identical conditions for 30 min. The diaphragms were removed, blotted, and weighed as described and solubilized overnight in “Soluene-100” (Packard Instrument Company, Inc., Downers Grove, Ill. 60515), 1 ml/100 mg wet weight. Solubilized tissue, 0.1 ml, was placed in 10 ml of scintillation cocktail “3a40” (Research Products International Corp., Elk Grove, Ill. 60007) as was 0.10 ml of bath solution before counting in a Packard TriCarb Model 524 scintillation counter, with use of the channels ratio method for quench correction.

Rate studies were performed by varying the amount of substrate, [14C]-α-aminoisobutyric acid, in various incubation mixtures (see Figure 1 for details). In this sense, the active uptake of α-aminoisobutyrate was compared to enzyme/substrate kinetics (20), and standard double-reciprocal plots were prepared.

For in vivo study of α-aminoisobutyrate uptake, [14C]-α-aminoisobutyric acid was injected intraperitoneally into 15 imipramine-serotonin-treated rats and 15 saline-treated controls. This injection schedule was repeated once a week for three weeks to ensure that all rats were affected. Thirty minutes after the last serotonin dose, we injected 1 μCi of [14C]-α-aminoisobutyrate into the animals, which were then bled via cardiac puncture at the times indicated below in Figure 3. Blood was obtained in heparinized syringes and the whole blood was centrifuged at 2000 rpm for 10 min; 0.10 ml of the plasma was added to 10 ml of 3a40 counting mixture and counted as described.

A temperature-controlled oxygen electrode system (Model M53; Yellow Springs Instrument Co., Yellow Springs, Ohio 45387) was used to measure oxygen consumption. Whole diaphragms were placed in 4 ml of oxygenated Krebs–Ringer–bicarbonate buffer in the 37 °C chamber equipped with a magnetic stirrer. Baseline oxygen consumption rates were established over a 15-min period. Freshly excised diaphragms were then used to measure alterations of the baseline rate when imipramine, serotonin, ouabain, or combinations of these were added to the chamber. Final concentrations of imipramine and serotonin were 4 mmol/liter and of ouabain, 2 mmol/liter.

Purified Na,K-ATPase (EC 3.6.1.3; Sigma Chemical Co.) from porcine brain (0.3 U/mg protein) was used for in vitro studies of inhibition. ATP (Sigma) was assayed by the method of Bucher (25) by use of Sigma Test Kit No. 366-UV. Imipramine and serotonin were added so that the final concentrations of each were 4 mmol/liter.
In this way, we studied the effects of imipramine and serotonin on Na,K-ATPase hydrolytic activity.

Results

In vitro experiments: Uptake of labeled α-aminoisobutyrate into isolated diaphragms was inhibited by 80–90% when imipramine and serotonin were added to the bath solution (Table 1). In control experiments, without imipramine or serotonin, the radioactivity of the intracellular water (26) was 17-fold that of the external bath solution after the 30-min incubation; addition of imipramine and serotonin (each, 4 mmol/liter) decreased this ratio to 1.7. Additional experiments with only imipramine or serotonin showed that the inhibition was entirely attributable to imipramine.

Double-reciprocal plots of labeled α-aminoisobutyrate distribution (intracellular radioactivity minus extracellular radioactivity) vs. labeled α-aminoisobutyrate concentration in the bath solution demonstrated an increase in the apparent K_m for α-aminoisobutyrate (from 0.3 to 0.7 mmol/liter) when this amino acid was added to the incubation fluid at various concentrations (Figure 1).

Addition of imipramine (to a final concentration of 4 mmol/liter), followed by equimolar serotonin, resulted in cessation of oxygen consumption for 30–45 s (Figure 2). Repetitive introductions of serotonin after an initial addition of imipramine resulted in recurrent interruptions in oxygen consumption. When serotonin was added before imipramine, oxygen consumption was not affected; pretreatment of diaphragms with ouabain, an inhibitor of (Na^+ + K^+)-stimulated, Mg-dependent ATPase (EC 3.6.1.3), eliminated the effects of imipramine and serotonin.

When diaphragms from imipramine-serotonin-treated rats were incubated in the bath solution, release of total CK and its MB isoenzyme was increased. Control rats, given only saline (NaCl, 9 g/liter) released an average of 280 U of total CK and 12 U of CK-MB activity per liter during the 30-min incubation; in contrast, diaphragms from the imipramine-serotonin-treated animals released an average of 390 U of total CK and 41 U of CK-MB activity per liter during the same incubation time (Table 2). The total increase in wet weight (from water uptake) was less than 5% in all diaphragms.

Table 3 shows that addition of imipramine (4 mmol/liter, final concentration) to the Na,K-ATPase solution decreased the hydrolytic activity of this enzyme by 76%, comparable to the results of Nag and Ghosh (27) in synaptosomes. Serotonin had no effect on (Na^+ + K^+)-stimulated ATPase activity, and neither imipramine nor serotonin affected the coupling enzymes in the assay in a blank determination.

In vivo experiments: Figure 3 shows the in vivo disappearance of labeled α-aminoisobutyrate from the plasma of imipramine-serotonin-treated animals as compared to saline-treated controls. The distribution volume of α-aminoisobutyrate (28) was calculated to be 10.6-fold the total body-water space (taken to be 60% of the total body weight) in the saline controls, while the calculated distribution volume of α-aminoisobutyrate is only 6.3-fold the total body water space in the imipramine-serotonin-treated animals, a decrease of about 40%. Urine samples from test and control rats had similar radioactivities, indicating that renal elimination mechanisms were not affected by the injected com-
Table 3. (Na\(^+\) + K\(^+\))-ATPase Assay, Effect of Imipramine

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Absorbance change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>4</td>
<td>0.222</td>
</tr>
<tr>
<td>ATP + (Na(^+) + K(^+))-ATPase</td>
<td>4</td>
<td>0.047</td>
</tr>
<tr>
<td>ATP + imipramine + (Na(^+) + K(^+))-ATPase</td>
<td>4</td>
<td>0.179</td>
</tr>
<tr>
<td>(Na(^+) + K(^+))-ATPase acty</td>
<td></td>
<td>[0.222 - 0.047] = 0.79</td>
</tr>
<tr>
<td>(Na(^+) + K(^+))-ATPase acty</td>
<td></td>
<td>[0.222 - 0.179] = 0.19</td>
</tr>
<tr>
<td>with imipramine</td>
<td></td>
<td>[0.79 - 0.19] \times 100% = 76%</td>
</tr>
<tr>
<td>% Inhibition</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Our results demonstrate that imipramine produces changes in the sarcolemma of isolated rat diaphragm preparation, and that these changes affect the transport of radiolabeled \(\alpha\)-aminoisobutyrate into diaphragm. Two observations suggest that this may be related to sarcolemmal (Na\(^+\) + K\(^+\))-stimulated ATPase. As previously shown by Nag and Ghosh (27) for synaptosomes, addition of imipramine to a (Na\(^+\) + K\(^+\))-stimulated ATPase preparation markedly decreases enzymatic hydrolysis of ATP. In addition, we observed cessation of oxygen consumption in the isolated rat diaphragm when it was incubated with imipramine followed by serotonin addition, but not with the reverse situation. Furthermore, we saw no effect on oxygen consumption under identical conditions with ouabain preincubation. These findings suggest that this effect was mediated via (Na\(^+\) + K\(^+\))-stimulated ATPase, although changes produced by ouabain may have prevented membrane interaction with imipramine. Analogous in vivo studies, of animals injected with imipramine and serotonin in the manner described to produce skeletal muscle lesions similar to DMD, also showed a defect in the uptake of labeled \(\alpha\)-aminoisobutyrate from plasma. Because serotonin alone had no effect on in vitro \(\alpha\)-aminoisobutyrate uptake, these in vivo changes were also presumed to be related to membrane alterations induced by imipramine.

Plasma from animals with the experimental myopathy has increased CK and CK-MB isoenzyme activities, as well as increased activities of certain other enzymes that are present in muscle (18). The likelihood that these increases are related to increased membrane permeability in skeletal muscle is supported by our observations that diaphragms from imipramine/serotonin-treated rats release increased amounts of total CK and its MB isoenzyme into an isotonic bath solution. An analogous study has recently been reported by Hallak and Wilkinson (30), in which enzyme release from intact lymphocytes was demonstrated by using chlorpromazine, a phenothiazine structurally similar to imipramine. Furthermore, our study showed an increase in the ratio of MB to MM release from the diaphragms incubated in imipramine, as compared to controls.

One interesting aspect of this increased CK release

boulds. The limitations of this approach for determining distribution volume of \(\alpha\)-aminoisobutyric acid have been discussed elsewhere (28).

Absorption spectra of the colored derivative from Bial's test showed maxima at 410, 540, and 600 nm for ribose standards and for six urine specimens from imipramine/serotonin-treated rats. Three normal human controls and three normal rat controls showed no significant absorption other than a broad peak at 410 nm. Urine specimens from the six imipramine/serotonin-treated rats gave a spot on two-dimensional thin-layer chromatograms that was indistinguishable from that seen for a ribose standard.

Discussion

A genetically determined, generalized membrane defect affecting skeletal muscles as well as other membranes (such as the erythrocyte membrane) has been postulated (7). The imipramine/serotonin animal model has been shown to have several parallels to DMD: the histologically demonstrable lesions are similar and show a predilection for proximal muscles, and activities of total CK and of its MB isoenzyme, as well as of lactate dehydrogenase and of aspartate \(\alpha\)-aminotransferase are increased so that they are within the ranges observed in DMD patients' sera. We have shown in this study that ribosuria is also present in the imipramine/serotonin animal model, as it is in patients with progressive muscular dystrophy (29).
from skeletal muscle relates to the increased CK and MB isoenzyme activities found in DMD patients' sera. Although some investigators have suggested a cardiac origin for the MB isoenzyme in Duchenne dystrophy patients (9), we have raised the possibility that increased MB activity in the serum originates at least partly from skeletal muscle. A previous study (17) showed that the increase in MB activity in DMD patients' sera does not correlate well with the cardiomyopathy seen in DMD patients. The present study demonstrates direct release of the MB isoenzyme from rat diaphragms and supports the concept that increased MB isoenzyme release results from increased sarcolummal membrane permeability of skeletal muscle.

In conclusion, we report several in vivo and in vitro findings that are consistent with a sarcolummal membrane defect in the imipramine-serotonin-treated rat. Concomitant with enzyme release are alterations in amino acid uptake, oxygen consumption, and in skeletal muscle morphology typical of human DMD. These changes are uniquely related to compromised muscle function with minimal necrosis in the experimental animal; thus, our findings are related to tissue damage of a reversible nature, and the observed membrane abnormalities are likewise reversible. These findings also demonstrate that an increase in serum enzyme activity can be based on functional membrane changes without necrosis as a prerequisite.

We are obliged to Dr. J. R. Mendell for his advice and stimulating discussions. This work was supported by NIH Training Grant No. GM01865 and by the National Muscular Dystrophy Association of America. Excerpts have been taken from a dissertation submitted by L. M. Silverman to the Ohio State University Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

References