Skeletal Muscle Regulatory Volume Response by Monocarboxylate Transporters to Increased Extracellular Lactate

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Abstract

SKELETAL MUSCLE REGULATORY VOLUME RESPONSE BY MONOCARBOXYLATE TRANSPORTERS TO INCREASED EXTRACELLULAR LACTATE

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The purpose of this thesis was to investigate the role of lactate in the regulatory volume response of mammalian skeletal muscle to hypertonic challenge-induced cell shrinkage. It was demonstrated that adult mice skeletal muscle single fibres responded to increased extracellular osmolarity in a dose-dependent manner when exposed to NaCl or sucrose challenge. This regulatory response to sucrose and NaCl however was abolished when cells were pre-treated with bumetanide, a specific sodium-potassium-chloride cotransport (NKCC) inhibitor, demonstrating that the NKCC is primarily responsible for eliciting a regulatory volume increase (RVI). When cells were exposed to NaLac treatment, bumetanide incubation did not significantly diminish the ability of the cells to recover volume. Furthermore, these cells lost less volume compared to NaCl or sucrose control. Inhibiting the single muscle fibres with either monocarboxylate transport (MCT) inhibitor phloretin or pCMBS resulted in significantly greater volume loss and impaired volume recovery. Combined MCT inhibition of phloretin or pCMBS with NKCC inhibition (bumetanide) led to unexpected findings, whereby the cells lost very little volume. These data suggest that while skeletal muscle fibres may utilize the NKCC to regulate volume, the ability for these cells to employ the most efficient means of volume regulation involves the inclusion of lactate as well via MCT uptake.

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Tables of Contents

List of Figures

List of Tables

Literature Review

1.1 Introduction to cell volume regulation

The cell membranes of various cell types are permeable to the passage of water, accomplished through aquaporin water channels and osmotic gradients (Takata et al. 2004). At steady state, the osmolarity between the extracellular and intracellular environments are equal. When there is an increase or decrease in extracellular osmolarity an osmotic gradient is formed across the cell membrane. The difference in osmolarity between extracellular and intracellular environments can result in the influx or efflux of water. A counter movement of osmolytes would then occur across the gradient if there is an active mechanism for restoring cell volume. The solute movement sets up a new osmotic gradient that drives osmotically obliged movement of water into or out of the cell across the plasma membrane (Lang et al. 2009).

Fluctuations in volume can affect cellular signaling and overall structure and integrity of the cell (Lang, 2007), and hence is reasonable to consider that it be a highly regulated process. The regulation of cell volume is likely of similar importance in skeletal muscle, as in other tissues such as cardiac muscle, brain and the kidney that have been shown to have transport mechanisms to facilitate cell volume maintenance enabling sustained cell function. Because exercise causes a range of cellular state alterations that impact cell function, it is reasonable that governing cell volume be tightly regulated within the body. In addition, changes in extracellular osmolarity can occur as a consequence of several pathological situations such as diabetic hyperglycemia, hypoglycemia and hyponatremia. The importance of cell volume regulation is

demonstrated in a wide range of cell types, and maintaining a tolerable state of volume is an essential component for cell survival. It is this reason that the cell should possess adequate means to actively volume regulate (Haussinger et al. 1991; Haussinger et al. 1993; Stein, 2002; Friedrich et al. 2006).

The ability of cells to undergo volume changes lies in the semi-permeable nature of the plasma membrane. This selective membrane bilayer allows for only certain ions and molecules to pass through, by either diffusion or in more advanced instances a facilitated diffusion (Kraft, 2000). This selective property of the transporters within the membrane allows for specific uptake of desired solutes into the cell, and ultimately allows for the cell to arrive at and maintain set osmolarity status.

In a situation of intense exercise, solute flux occurs amongst compartments in the body, and consequently osmotic gradients are established. The ensuing event that occurs is resultant water flux, whereby water will follow the osmotic gradient to the side of the membrane where solute concentration (osmolality) is highest. An increase in osmolarity outside non-contracting cells causes the cells to lose volume, and since this volume loss is not a desired occurrence the cells therefore try to regain volume back after it is lost. This is attained by various solute transport processes, some involving amino acids and others including ions. The capability of cells to regulate ion movement has been shown in a range of cell types, from muscle to tumor cells, utilizing various processes including the $Na^{+}K^{+}2Cl^{-}$ cotransporter, in addition to other Ca2+ and Cl- transporters and channels (Petrunkina et al. 2004; Takeuchi et al. 2007).

While cells exhibit a response to initial exposure of either an increase or decrease in osmolarity through passive volume change in a manner similar to an ideal osmometer,

it has been demonstrated that they then elicit an active response shortly after the time of osmotic challenge to remedy the disturbance and in an attempt to return to a steady state volume. This is opposed to merely exhibiting a perfect osmometer response, whereby cells remain in a static volume state dependent on extracellular osmolarity. When exposed to an environment of increased extracellular osmolarity, cells will initially lose water only (i.e. no solutes), and thus cell shrinkage occurs. The active mechanism which then takes place allows for the uptake of solute by these transporters, resulting in a recovery back to the original isosmotic volume – a process known as a regulatory volume increase (RVI). This dynamic phenomenon of cell volume regulation occurs in many cell types, from protozoa to cardiac myocyte (Macknight et al. 1974, Larson et al. 1984; Macknight et al. 1989; Drenowska & Baumgarten, 1991; Rasmusson et al. 1993), as well as mammalian skeletal muscle (Wong et al. 1999; Lindinger et al. 2002; Gosmanov et al, 2002; Kristensen, 2006).

In contrast, exposure to hypotonic extracellular medium results in a situation where the extracellular osmolarity is less than inside the cell, resulting in ensuing water entry into the cell and consequent cell volume increase. The compensatory mechanism for this event, known as a regulatory volume decrease (RVD), involves the efflux of ions leading to water drawn out and causing the return of cell volume back down to its original isotonic volume (Lang et al. 2000).

As indicated above, upon exposure to anisosmotic fluid cells respond by exhibiting shrinkage or swelling. In the case of exposure to an increased osmolarity environment, cells will lose volume and be in a shrunken cell state. While cells are able to withstand short periods of volume perturbation, chronic exposure and prolonged

durations of reduced cell volume can be detrimental to overall cell survival (Waldegger et al. 1998). Cells that have the capacity to volume regulate to this situation will display the ability to upregulate volume, and effect a RVI. In the process of this RVI occurrence, certain transport pathways are activated, which results in the increase in content of intracellular osmolytes. The transport of osmolytes in some cells is achieved through a number of transporters including Na^+Cl^- cotransport and Na^+H^+ exchange, however the predominant route is considered to be through $Na^+K^+2Cl^-$ cotransport in skeletal muscle (Lang et al. 2000).

Skeletal muscle makes up 30-40% of the total body water in humans (Jaffrin et al. 1997) and, consequently, skeletal muscle water transport and its regulation has a significant impact on whole body water and ion balance. During moderate to high intensity exercise, contractile activity results in the production and accumulation of a large amount of osmolytes within working muscles. Consequently, water of the insterstitial fluid osmotically flows into the working muscles balance the elevated osmolarity, resulting in a significant water loss from the interstitial and consequently vascular compartments (Lundval, 1972; Lindinger et al. 1994). In order to maintain or restore blood volume, there is an osmotic movement of fluid from non-contracting muscles and other tissues (Lindinger et al. 1990), and this ultimately results in volume loss in the non-working muscle (Gosmanov et al. 2003). This sequence of events thus illustrates that it may be desirous and indeed advantageous to be able to produce an increase in NKCC driven solute transport within non-contracting muscle. It would act to counter the loss of water from these cells, to ultimately prevent or minimize the physiological disturbance caused by cellular dehydration.

The importance of cellular volume maintenance has also been demonstrated from studies of muscle contractility, for maximal isometric force production is dramatically reduced when cell volume is decreased as a result of elevated extracellular tonicity (Gulati and Babu, 1982). It is evident that the regulation of cell volume is important to all living cells, and should thus be a highly regulated process.

1.2 Transport mechanisms and inhibition studies

The ability of mammalian skeletal muscle to regulate cell volume lies in the function of specific transport mechanisms, and these transporters are involved in the selective uptake of solutes into the cell. The primary transport mechanism in mammalian skeletal muscle appears to be the sodium-potassium-chloride cotransporter (NKCC) for RVI (Lindinger et al. 2011). Following that, it is believed that the sodium-potassium pump (Na^+K^+ATPase) plays a key secondary role by providing the inward driving force for Na^+ and Cl^- to facilitate the maintained activity of the NKCC. The solute taken up by the aforementioned transporters then causes osmotically obliged uptake of water into the cell via the aquaporin (AQP) transporters.

1.2. a) The primary involvement of the NKCC in volume regulation

The cell membranes of most animal cells are permeable to water and therefore water must be at equilibrium across the membrane. With the exception of renal tubular epithelial cells, the osmolarity of the intracellular environment therefore is regulated in an attempt to be equal to that of the extracellular environment. Consequently, cell volume maintenance should then be determined by the regulation of intracellular osmolyte

content. In the 1970s, work on duck erythrocytes demonstrated the existence of ion transport mechanisms in the course of regulatory volume responses (Kregenow et al. 1973). It was soon after discovered that the RVI process was carried out by the electro-neutral cotransport of Na^+, K^+, and Cl^- ions in a 1:1:2 ratio (Geck et al. 1980).

Geck and colleagues first observed that the electroneutral cotransport of Na^+, K^+ and Cl^- ions in a 1:1:2 ratio was present in a variety of epithelial and nonepithelial cells, and later on linked to volume regulation in several tissue types. It is now known that the NKCC is present in a wide range of animal species, having an important role in fluid secretion. Two isoforms, NKCC1 and NKCC2, have been identified. The NKCC1 isoform is widely expressed in many tissues (Russell, 2000) and cell types including stomach, lung, trachea, pancreas and skeletal muscle. The NKCC2 isoform, on the other hand, is highly localized and is expressed exclusively in the thick ascending limb of the loop of Henle of the kidney.

Bumetanide is a pharmacological agent known to be a potent inhibitor of the NKCC. It belongs to a class of loop diuretics because it acts on the loop of Henle of the kidney, inhibiting the reabsorption of sodium and fluid, and is implicated in establishing the present day knowledge of the NKCC. This inhibitor reversibly binds to the NKCC through a concentration-dependent mechanism in a variety of preparations, with a half-inhibitory constant of ~1 x 10 -7 M (Russell, 2000). While bumetanide has a strong affinity towards inhibiting the NKCC, it also has the ability to block other transport processes such as Cl^-/HCO3- exchange (Jennings, 1985), Cl^- channels (Evans et al. 1986), and the potassium chloride cotransporter (KCC) (Lauf et al. 2001), when administered in higher concentrations. However, in keeping with a low concentration (below 10uM),

bumetanide's inhibitory effect is believed to be relatively specific for the NKCC (Russell, 2000).

Binding of bumetanide to the NKCC has been postulated to occur on the external face of the cotransporter on the plasmalemma, directly on the NKCC protein itself (Haas, 1989; Russell, 2000). As such, the adequate binding of the inhibitor to the NKCC involves three main properties: 1) the displacement of bound molecules by other members of the loop diuretic family with apparent affinities that match their potency as inhibitors of cotransport fluxes; 2) Na^+, K^+, and Cl^- all present in the binding medium to allow for specific binding to occur; 3) raising $[Cl^-]$ in the binding medium above the optimal level for binding results in a decrease in specific binding (Russell 2000).

The effect of bumetanide inhibition on the NKCC has been illustrated by the consequent impairment of RVI responses in cells made to shrink by inducing increased extracellular osmolarity. The effect is a decreased influx of Na^+, K^+ and Cl^-, and due to the resultant absence of an inwardly directed concentration gradient for Na+ and Cl^- the impairment in the osmotic influx of water causes cells to remain in a shrunken state following hypertonic challenge (Sitdikov et al. 1989; Urazaev, 1998). Lindinger et al. (2002) demonstrated complete abolishment of a RVI response in rat hindlimb when bumetanide preceded exposure to hypertonic perfusate over a 20 minute period, and similar responses have been shown in isolated intact and single mouse muscles (Lindinger et al. 2011). Similarly, Zhao et al. (2004) also displayed an inhibited RVI with bumetanide after a 29% extracellular tonicity increase in L6 myotubes. The Na-K-Cl cotransporters have the ability to elicit a RVI in many different cell types in a wide range of animal species**.** Evidence for the presence and function of NKCC activity in rat

skeletal muscle was established early on by Sitdikov and colleagues (1989), with Wong et al. (1999) being the first to show presence using histochemical staining. Soon after, independent experiments carried out on rat hindlimb skeletal muscle were also in support (Lindinger et al, 2002; Gosmanov et al, 2002), as well as human skeletal muscle (Kristensen, 2006). The literature on NKCC involvement in mammalian skeletal muscle volume regulation is rather novel. Prior to recent work (Lindinger et al. 2011) a Russian group of researchers had previously investigated the effects of increased osmolarity on volume changes in skeletal muscle and found it to actively regulate in the presence of increased extracellular osmolarity (Sitdikov et al. 1989; Sitdikov et al. 1991). However, no precise transport mechanisms were elucidated with the investigations. Since then, limited additional knowledge has been gained. Based on recent evidence (Lindinger et al. 2011), the involvement of the NKCC in eliciting this regulatory volume mechanism has been established, and this cotransporter is now regarded as the main contributor in effecting volume regulation in skeletal muscle cells, as previously shown in cardiac (Michea et al. 2001), brain (Jayakumar et al. 2008) and epithelial (Hamann et al. 2005) tissues.

1.2 b) Role of the Na^+K^+ATPase in volume regulation

A number of lines of evidence indicate that the RVI observed in skeletal muscle is not accomplished by the NKCC alone, but rather other functional transporters may also be involved in this process. Namely the Na^+/K^+ATPase, is believed to facilitate and maintain an effective gradient for sustaining increased NKCC activity, along with

aquaporin water channels that allow the passage of osmotically obliged water into the cell – whereby water molecules are transported as a result of solute transport. The Na^+/K^+ATPase is a transmembrane protein located in the plasma membrane of eukaryotic cells. This transporter is involved in maintaining the concentration gradients for Na^+ and K^+ across the plasma membrane, with bound ATP linked to the coupled extrusion of Na^+ ions and the uptake of K^+ ions. Inhibiting the Na^+K^+ pump with ouabain has lent support to the notion that the pump provides the ionic driving force for the NKCC (Lindinger & Grudzien, 2003). The inclusion of the Na^+/K^+ATPase in supporting the function of the NKCC is somewhat uncertain based on work by others. Data obtained from cultured L6 skeletal muscle myocytes (Sen et al. 1995) revealed that although the NKCC-mediated K^+ influx is dependent on extracellular [Na^+] and [Cl^-], it was regarded as being ouabain insensitive during short-term incubations. This finding suggests that the ouabain-sensitive Na^+K^+ATPase activity may in fact not be necessary, and also implies that a ouabain-insensitive Na^+K^+ pump activity may be present in these cells (Dostanic-Larson et al. 2006).

However, one must take into account the altered phenotype of cultured cells. In this cell line, the NKCC accounted for ~65% of K+ influx, compared to ~15% in muscle in vivo. It should also be noted that different pump alpha isoforms may be expressed in culture cells compared to in vivo cells (Zahler et al. 1996). As demonstrated by O'Neill (1999), a Cl^- gradient is believed to be the principle driving force, where a decrease in intracellular Cl^- would elevate the volume set point and thus magnify the degree of perceived cell shrinkage. In addition, others have suggested that membrane depolarization may occur in skeletal muscle after exposure to hyperosmotic conditions,

and that it is believed to be directly involved in activation of the NKCC (van Mil et al. 1997; Geukes Foppen, 2004). However, it is generally supported that the NKCC receives its driving force from the Na^+ gradient provided by the Na^+K^+ATPase.

1.2 c) Involvement of AQP4 in volume regulation

The aquaporins (AQPs) belong to a family of 10 regulated proteins which are expressed at the plasma membrane of many cells involved in fluid transport in various tissues in plants and animals (Verkman et al 1996; Agre et al. 2002). The mercurial insensitive water channel aquaporin-4 (AQP4) is the major water channel expressed in skeletal muscle fibres (Frigeri et al. 1998), and is considered to be responsible for the transport of water that follows the osmotic gradient produced by the NKCC (Nicchia et al. 2007). The AQP4 channel is present in greater quantity and thus may function to a greater extent in fast twitch EDL compared to slow twitch soleus muscle (Frigeri et al. 1998). While the AQP4 is believed to be the predominant player in facilitating water transport across the sarcolemma, subsequent work has proposed that other aquaporin isoforms may be involved as well. Exposing C2C12 cells, a mouse myoblastic cell line, to hypertonic solutions resulted in AQP5 expression (Hwang et al. 2002; Wakayama et al. 2002).

1.3 Mechanisms of cell volume regulation during increased extracellular osmolarity

Upon exposure to hyperosmotic solutions, cells shrink in response to the increase in extracellular osmolarity. Following the initial response to the increased extracellular osmolarity, it has been reported that some cells do not simply respond as perfect osmometers and remain volume depleted, as would be evident by the simple passive flux of water in accordance with the direction of osmolarity change (Lang et al. 1998). Rather, after the initial shrinkage a RVI is present, resulting in gradual recovery of volume back towards baseline status. This regulatory response has been demonstrated in various cell types, and is suggested to act in a dose-dependent manner (Bush et al. 2010; Lindinger et al. 2011). However, there are other groups that have not yielded similar results. Such has been the case with amphibian skeletal muscle (Fraser et al. 2005) as well as mouse skeletal muscle single fibres (Pickering et al. 2009), which under the experimental conditions, simply responded osmometrically in the face of increased osmolarity.

There is little evidence in support of such a regulatory response in mammalian skeletal muscle. The earliest report of the ability for skeletal muscle to regulate volume appears to come from Russian researchers some 20 -25 years ago (Sitdikov et al. 1989). This group assessed fibre volume in rats utilizing furosemide, a loop diuretic similar to bumetanide, in the presence of extracellular hyperosmolarity. Furosemide is also a potent Cl^- channel blocker and a recognized inhibitor of outwardly directed KCl cotransport (Hiki et al. 1999), as well as a blocker of calcium-dependent Cl^- channels (Hui and Chen, 1994). Nearly a decade later, further work surfaced to corroborate the presence of RVI in mammalian skeletal muscle (Lindinger et al. 2002), while others have also examined the relation between NKCC function and skeletal muscle, some with (Lindinger et al. 2011)

and albeit some without the inclusion of RVI (Zhao et al. 2004; Kristensen et al. 2006; Antolic et al. 2007). With the scope of investigations that have now been undertaken, it should be readily acknowledged that skeletal muscle cells do possess the ability to actively regulate volume in the presence of hyperosmotic challenge.

1.4 Muscle fibre type differences in volume regulation

Skeletal muscle is generally differentiated into two main fibre types by their unique structural, biochemical and functional characteristics. Type I fibres, also known as slow oxidative fibres, are red, and have slow contraction velocity although they are very resistant to fatigue as they are able to generate energy by oxidative processes. Type IIb fibres are referred to as fast twitch glycolytic, are white, and have the ability to generate energy by anaerobic processes with great force, but fatigue easily (Scott et al. 2001).

The muscle fibre type distinction of regulatory volume properties has been investigated in previous studies. The use of mice and rat skeletal muscle has been utilized commonly due to the distinct fibre type properties of unique muscle groups. The soleus muscle functions in postural control and is composed of 85% slow twitch oxidative fibres, while the EDL is mainly used for locomotion and comprises predominantly of fast-twitch muscle fibres (Scott et al. 2001). Two isoforms of the monocarboxylate transporter (MCT) are responsible for lactate transport in skeletal muscle. The distribution of the MCT1 is quite variable between different muscles and fibre type differences in MCT1 content are small. In contrast, the MCT4 is slightly more fibre type specific, with greater abundance in type II fibres as opposed to type I fibres (Pilegaard et al. 1999; Hashimoto et al. 2005; Metz et al. 2008).

Antolic et al. (2007) noted muscle fibre type dissimilarities in regulatory volume response to hyperosmotic exposure, where intact muscle cross sectional area was no different after inducing an increase in osmolarity in soleus fibres. In contrast, EDL increased cross sectional area following exposure to hyperosmotic conditions. More recent work on the other hand points to the view that both muscle fibre types express AQP4 in rats (Nicchia et al. 2007). Also, work by Stephenson and Lindinger (2007, unpublished) using intact mouse soleus and EDL support the notion that slow twitch fibres may in fact exhibit greater RVI effect in comparison to fast twitch EDL fibres which lost more volume and took longer to recover volume than the SOL. This observation of a lesser disturbance in slow twitch muscle may suggest a greater importance of volume maintenance in these muscle types, which are utilized more regularly as opposed to their fast-twitch counterpart. It has been shown that there is a lower expression of NKCC in fast twitch compared to slow twitch muscle (Wong et al. 1999; Fu et al. 1999). However, AQP4 expression is believed to be only present in fast twitch muscle (Frigeri et al. 1998; Nicchia et al. 2007). This would suggest that the regulatory mechanisms are more important in slow twitch muscle required for constant postural recruitment, as opposed to fast twitch muscle.

1.5 Lactate: production, accumulation, secretion and extraction

During high-intensity exercise, lactate formation results from a series of biochemical processes, through the utilization of glucose, and subsequent conversion to pyruvate in the cytosol, by way of the enzyme lactate dehydrogenase. Due to the presence of elevated lactate in fatigued muscles, it was previously assumed that the formation of

lactate directly caused muscle fatigue and was a consequence of lack of oxygen (Hill & Kupalov, 1929). However, this is not the case. Lactate can accumulate within the muscle fibres, but may also leave the muscles, passing down a concentration gradient through monocarboxylate transporters (MCTs) and into the extracellular fluid (Gladden, 2004). As lactate circulates in the blood it may diffuse down the concentration gradient, enter interstitial fluids of lower lactate concentration and cells through inwardly-directed MCTs, and thus be utilized by other tissues such as the liver, heart, adipose tissue and nonworking skeletal muscle. It is increasingly recognized that lactate is a metabolically useful carbohydrate and plays important physiological roles in resting, contracting and recovering skeletal muscle. For this reason, the potential involvement of lactate in facilitating the ever-important process of volume regulation is conceivable.

1.6 Lactate transporters (MCTs)

The transport of lactate across the sarcolemma is mediated by the SLC16A family of proton-linked membrane transport proteins, known as monocarboxylate transporters (MCTs). MCTs were first recognized in the mid 1990s, and there have been 14 separate isoform members identified (Halestrap, 2004). Of these, seven isoforms have been functionally characterized, and are responsible for governing the transport of a wide variety of endogenous and exogenous compounds including lactate, pyruvate, gamma-hydroxybutyrate, bumetanide, simvastatin acid and potentially other drugs (Halestrap et al. 2004; Bhattacharya & Boje, 2004; Murakami et al. 2005; Wang et al. 2007). The MCT1 and MCT4 isoforms are co-expressed in skeletal muscle. MCT1 is ubiquitously expressed in a variety of human, rat tissues, including skeletal muscle and the heart

(Bonen, 2000) and its expression is correlated with the oxidative capacity of various muscles (McCullagh et al. 1996; Baker et al, 1998). The MCT4 isoform is the predominant isoform in fast-twitch glycolytic muscle fibres, (Wilson et al, 1998) while the MCT1 isoform is more abundant in oxidative than glycolytic fibres. The fibre type distribution suggests that each MCT isoform may have distinct transport functions, with MCT1 acting more to take up lactate supplied to its interstitial fluids from the circulation or neighboring muscle cells, while MCT4 has a greater part in facilitating lactate efflux from the cells in which lactate production exceeds pyruvate oxidation. In accordance with this hypothesis is the fact that MCT4 is present in considerable quantity in intracellular pools, while no such presence was observed in the MCT1 isoform (Bonen, 2000).

A number of MCT inhibitors have been classified in accordance to their inhibition efficacy of the various MCT isoforms. Recent work investigating the potency of such inhibitors has found that phloretin and pCMBS have high affinity for blocking lactate transport. These are known to be effective agents against a wide range of MCT isoforms but especially in targeting MCT1 (mainly phloretin) and MCT4 (mainly pCMBS). pCMBS inhibits the MCT4 isoform with greater affinity compared to MCT1. Conversely, the greater binding affinity of phloretin to MCT1 influences the greater ability for restricting lactate influx through this specific isoform (Manning Fox et al. 2000). Similarly found by Dimmer and colleagues (2000), the MCT4 isoform was most effectively blocked by phloretin, while pCMBS was also found to display very effective inhibition resulting in a decrease in lactate uptake by more than 90%. The efficacy of these two inhibitors on the MCT isoforms of interest thus warrants the use of these two drugs in the specific targeting of the main routes of lactate transport into and out of the

muscle cells. The sarcolemma contains a high population of MCTs, and it is this abundance of the transporter that portrays its function within skeletal muscle. The uptake of lactate into muscle is involved in an important cell-to-cell lactate shuttle during exercise and recovery, as established through experiments by Brooks (1984). In addition to the sarcolemma, it has also been observed that MCT isoforms are detected in the transverse-tubule system across fibre types (Bonen, 2001; Hashimoto et al. 2005). There is ample evidence to show various MCT function within skeletal muscle, and with the potential abundance of lactate in circulation the presence of so many isoforms of lactate transporters would seem fitting to play a role in taking up lactate to be used within the muscle.

1.7 Lactate and its hypothesized role in cell volume regulation

Lactate is produced in large amounts during intensive exercise, the intracellular accumulation of which contributes to cell swelling within the working muscle. It is an osmotically active particle, with an osmotic coefficient close to 1 (Redetzki et al. 1972). During muscle contraction, lactate produced by type II fast twitch fibres may be taken up by non-contracting type I muscle fibres. This occurs through the lactate shuttle mechanism, whereby MCT1 predominantly plays a role in the uptake of lactate from the plasma and interstitium through MCT1 (Bergman et al. 1999; Brooks, 2002). There is valid reason to believe that this commonly found substrate may thus play a role in an important cellular process. With the ability of muscle to take up lactate, it then begs the question as to whether there is the possibility that lactate taken up by non-working muscle may help maintain and minimize volume losses by these cells during high

intensity exercise. Antolic et al. (2007) alluded briefly to the involvement of lactate in muscle cells recovering from anisosmotic conditions. A 4-fold and 2-fold increase in tissue lactate was found in EDL and soleus, respectively when exposed to hyperosmotic medium. Interestingly, the hypertonic challenge was induced with mannitol and in the absence of any extracellular lactate. Their findings are in accordance with the working hypothesis here, in that increased intracellular lactate would facilitate the influx of osmotically obliged water to assist in the recovery of cell volume. However, what is uncertain is the exact mechanism responsible for the resultant increase in intracellular lactate. Because lactate is such a biologically and biochemically active molecule, it is very plausible that the body does in fact make use of this substrate to allow the it to maintain working at an elevated rate during high intensity exercise and to aid in the recovery of a hindered state of bodily function.

1.8 Summary

Cells, including mammalian skeletal muscle, have the capacity to regulate volume during osmotic challenge through the activity of the NKCC. Since its earliest discovery in the 19th century, the role of lactate has been a common area of research focus in the broad field of exercise physiology. It is considered that the NKCC may in fact obtain its driving force created through other transport mechanisms such as the Na+K+ATPase. The importance of regulating cell volume is widely regarded as being critical to sustaining cell viability among all living things. With the aid of the AQP water channels, the possibility of MCTs functioning to facilitate this survival effort may therefore in fact be

rather likely. The present knowledge in the area is sparse, but the involvement of lactate in the regulatory volume mechanisms of muscle cells deserves further exploration.

Cell volume within skeletal muscle is faced with osmolarity challenges during various physiological conditions. Non-contracting muscle shrinks as a result of exercise-induced alterations in the plasma, causing elevated extracellular osmolarity. The accumulation of metabolic byproducts within the muscle leads to increased intracellular osmolarity, and interstitial water moves into the working tissue creating a state of cell swelling during the duration of exercise. At the same time, osmolyte release from the working muscle to the interstitium. To alleviate this state, water is donated from the non-working muscle to the arterial circulation, and the end result is a state of shrunken non-contracting muscle cells. The resultant effect of this cascade of osmolyte and fluid shift then is the utilization of the active mechanism of a regulatory volume increase, whereby the non-contracting muscles swell to regain the lost volume. The recovery process is mediated by the NKCC, which transports a sodium, potassium and two chloride ions in an electroneutral manner with osmotically obliged water following into the cell.

During exercise, contracting skeletal muscle produces an abundance of lactate which can be oxidized, or taken up by neighbouring cells. The presence of MCTs in muscle cells allow for the ability to take up and release of lactate. The present study investigated the potential role of MCTs in taking up lactate to be used in the muscle to facilitate the uptake of water and aid in the process of RVI. Using a single muscle fibre isolation technique, morphometric analysis of the cells were performed to obtain accurate measurements of volume change and regulation properties of the muscles.

Hypotheses

The primary purpose of the present experiments was to further investigate the regulatory volume mechanisms that take place in mammalian skeletal muscle in response to increased extracellular osmolarity. Building from this knowledge, an investigation on the involvement of lactate was further examined to specifically determine its involvement in effecting a regulatory volume response.

The purpose of the first set of experiments was to demonstrate the effect of increasing extracellular osmolarity through either NaCl or sucrose. Subsequent to showing a volume reduction and resultant cell shrinkage in response to the increased extracellular osmolarity, the working hypothesis was that a RVI would occur following exposure to the osmotic disturbance and return cell volume back to original baseline status.

The purpose of the second set of experiments was to reveal the involvement of the NKCC in performing a RVI, by utilizing the inhibitory drug bumetanide on the cotransporter in the presence of NaCl treatment. It was hypothesized that due to the inhibitory nature of bumetanide on the NKCC, adequate solute uptake to elicit a successful RVI would be impaired significantly, and therefore demonstrate the reliance of skeletal muscle on the NKCC to regulate volume.

The third set of experiments was carried out to investigate the impact of increased extracellular NaLac on volume response. Based on the premise that lactate is a metabolically active particle, and found in great abundance in muscle during exercise conditions, it was hypothesized that lactate is taken up via monocarboxylate transporters

to elicit an osmotic drive for the inward movement of water in maintaining cell volume in the face of increased extracellular osmolarity. In comparison to NaCl and sucrose treatment, it was further hypothesized that in the absence of any transport inhibitors the cell would show a greater capacity to maintain and regulate volume in the face of an extracellular osmolarity increase to lactate.

The purpose of the fourth set of experiments was to isolate the involvement of specific transporters by individually blocking with either bumetanide (NKCC) pCMBS (MCT) or phloretin (MCT), all in the presence of NaLac treatment. While previous data supports the strong involvement of the NKCC in causing a RVI, it was further hypothesized that skeletal muscle cells may employ the NKCC transporter in addition to MCTs to implement a complex system of volume regulation. It was hypothesized that although bumetanide will successfully block the function of the NKCC, the cells will alternatively utilize the MCTs inherent to the muscle cells and be able to take up lactate to still successfully elicit a RVI.

The final set of experiments was conducted to test the effects of combining the inhibition of both the MCT and NKCC. Based on results obtained from the previous experiment set, the data suggested that while significant transport inhibition was obtained and volume regulation was impaired, cells were still able to regulate cell volume to an extent. Therefore, the hypothesis of the present experiment was that blocking both the NKCC and MCT would lead to significantly greater volume regulation impairment.

Methods

2.1 Animal care

The animal care and use procedures used were approved by the University of Guelph Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. Adult (>2 months old) male and female C57BL/6 mice (Charles River, St. Hyacinthe, QC) were housed at the University of Guelph in light and temperature controlled quarters. Food (standard diet chow) and water were provided ad libitum.

2.2 Solutions

The type I collagenase (Sigma-Aldrich, Oakville, ON) solution that was used to dissociate individual fibres from the intact muscle was made 0.2% w/v in high-glucose (25mM) DMEM (Invitrogen, Burlington, ON). 10% Matrigel (BD Biosciences, Mississauga, ON) solution was prepared diluting in low-glucose (5.5mM) DMEM. Plating media consisted of 10% horse serum (Sigma-Aldrich) and 0.5% chick embryo extract (MP Biomedicals, Solon, OH) made in 85% low-glucose DMEM. Concentrated 1M stock of NaCl and NaLac solutions were both made in ddH_2O, and added to wells in a 6-well plate VWR International (Mississauga, ON) to increase osmolarity by ~30mM (Table 1). Each of the transport inhibitors (bumetanide and phloretin from Sigma-Adrich, Oakville, ON) and pCMBS (Toronto Research Chemicals, North York, ON) were dissolved and prepared in dimethyl sulfoxide (Sigma-Aldrich) to make up stock solutions of 100mM, When used to inhibit a transporter, an appropriate volume was added to plated fibres to reach a final desired concentration of 1mM.

2.3 Muscle preparation

EDL and peroneus muscles were removed from mice killed by cervical disarticulation following 15 seconds of 100% CO_2 anesthesia. Muscles were immediately transferred into collagenase solution and shaken gently in an incubator to commence muscle digestion. After allowing them to digest for 120 to 180 minutes, individual muscles were taken out one at a time to assess for optimal single fibre separation. The peroneus muscles were taken out of the collagenase solution to assess for digestion status. Upon determining the optimal degree of digestion of the EDL, the muscles were triturated in small petri plates to allow for single fibres to disperse from the muscle bundle. The EDL muscles were then transferred through consecutive plates in the same manner until an adequate number of fibres were visually attained. Mice EDL skeletal muscle is predominantly type IIB fast twitch fibres. Although there is a very small percentage of type I slow twitch fibres present in the EDL, adequate replication of the experiments was sufficient in concluding the responses collected are uniformly representative of fast twitch muscle type. The average muscle fibre diameter of mouse EDL muscle is 20 μm in width.

The wells of a 6-well plate were treated with 10% Matrigel made up in low-glucose DMEM in order to provide muscle fibres with an environment conducive to ensuing greatest likelihood of viability. The Matrigel was necessary to facilitate partial adhesion of the muscle fibres to the bottom of the plate wells to enable precise imaging. The Matrigel solution was removed after 1 minute, and the well filled with 1.5ml of low-glucose plating media. Individual fibres were then selected and picked to transfer into the media-contained wells. Upon obtaining sufficient number of fibres, entire plates were

then placed in the incubator (37^{o}C, 5% CO_2) for 60 to 90 minutes to allow for fibres to adhere onto the surface of the plate wells.

2.4 Experimental procedure

All experiments were conducted at room temperature (~21^{o}C). After removal of plated fibres from the incubator, plates were viewed under the dissecting microscope to ensure that fibres were viable and sufficiently adhered to the plate bottom. Plated fibres were then taken to the microscopy suite where they were kept at room temperature for the duration of the experiment. The plate containing fibres were mounted onto the stage of a Nikon Eclipse TE2000U inverted microscope and individual fibre positions (x-y-z coordinates) were then programmed into the SimplePCI software, allowing for sequential image capture between landmarks at fixed time intervals.

Transport inhibition experiments were conducted with NKCC inhibition (bumetanide) or combined NKCC and MCT inhibition (phloretin or pCMBS) during hypertonic stress. 30 µL of 50 mM stock bumetanide was added to the individual wells containing 1.5 ml plating medium to achieve a final concentration of 1.0 mM. In the phloretin trial, 15µL of 100mM stock was added to the well to induce a final concentration of 1mM. In experiments with pCMBS inhibition 15 µL was added from a 100 mM stock, and in each case inhibitors were allowed to incubate for approximately 30 minutes during the image landmarking period preceding the start of the hypertonic challenge. Baseline images of each landmarked fibre were taken three times prior to beginning the treatment protocol. Following the inhibitor incubation period, hypertonic challenge was administered by adding 45 µL of 1 M NaLac or NaCl stock solution to

achieve a 30% increase in osmolarity. Immediately following the application of hypertonic challenge, the image sequence was initiated and following a delay in duration of approximately 20 seconds for application of desired increased in extracellular osmolarity, sequential imaging of each landmarked fibre was then run for 30 minutes.

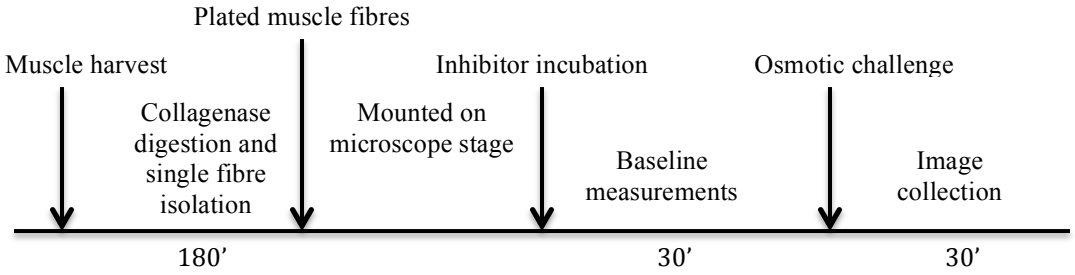


Figure 1 – Experimental timeline. Peroneus and EDL muscles were harvested from mice and allowed to digest in collagenase solution for up to 180 minutes. Muscles were removed from collagenase and single muscle fibres isolated. Viable fibres were transferred and plated onto Matrigel coated 6-well plates. Following successful plating of fibres, 6-well plates were placed in incubator for minimum 60 minutes to allow for fibre adhesion. Plates ready for experimental protocol are mounted onto microscope stage for landmarking, and inhibition drugs are administered for the applicable protocols. Following 30 minute quiescent period, baseline images were taken for individual landmarked sites. Immediately after application of osmotic challenge, image collection sequence was initiated and data collected for up to 30 minutes.

2.5 Data analysis and calculations

Image sequences extracted were quantified using Adobe Photoshop software. Each fibre was imaged at two distinctly separate positions at opposite ends, and individual fields were then measured at two different locations to obtain width data. The percent volume change for each time point measured over the 30 minute data collection protocol was calculated from individual muscle fibre width measurements in relation to baseline width measurements with the formula shown below. This yielded resultant muscle response curves as illustrated in Figure 2.

*Percent volume change at time T = (width at time T – baseline width) / baseline width * 100*

2.6 Statistics

One-way ANOVAs were used to identify significant differences between means with respect to time or treatment. When a significant F ratio was found, a post hoc test was performed to distinguish between different means. Statistical significance was accepted at $p<0.05$.

Results

3.1 Increased extracellular NaCl and sucrose cause cell volume loss and RVI

Preliminary experiments utilizing NaCl and sucrose treatment conditions yielded response curves which depict the initial cell volume loss and subsequent RVI as expected (Fig. 2), and previously shown in skeletal muscle cells (Lindinger et al. 2011). Upon initial exposure to increased extracellular osmolarity muscle fibres responded by rapidly losing cell volume, and within 200 sec reached a peak volume loss. This was followed immediately by a more gradual volume increase phase of recovery over the subsequent 15 min, establishing a steady state volume back near its original baseline volume in up to 30 min.

Under the imposed conditions, the decrease in cell volume was less than expected if there was no volume regulation. For example, imposing a 30% increase in extracellular osmolarity should result in a loss of approximately one-third of the original cell volume

(Fig. 3; dashed line). In the presence of a regulatory volume response though, the magnitude of peak volume loss (15%) was reduced to half of that theoretical amount. The 50 mM increase in NaCl gave a similar increase in extracellular osmolarity as a 100 mM increase in sucrose. When comparing increases in extracellular osmolarity induced by increased NaCl or sucrose, for the same increase in osmolarity sucrose caused a greater volume loss than NaCl. The volume loss in response to increased extracellular osmolarity occurred in a dose-dependent manner. The 50 mM NaCl treatment resulted in a greater magnitude of peak volume loss compared to 30 mM NaCl (Fig. 3). Similarly, exposing the cells to 100mM sucrose yielded a larger peak volume decrease compared to 60 mM sucrose. The results indicate that while skeletal muscle cells do lose volume in response to an increase in extracellular osmolarity, the volume loss also depends on the permeability of the sarcolemma to the solute.

3.2 Involvement of the NKCC in exerting a RVI effect in skeletal muscle

At low concentrations (below 10μM), bumetanide is a specific inhibitor of the NKCC (Russell, 2000), thus separate trials with the NKCC inhibitor bumetanide were performed to characterize the involvement of the NKCC in the volume loss and recovery responses. When the NKCC was blocked and cells were exposed to 30 mM NaCl, peak cell volume loss was not different to when the NKCC was fully functional (Fig. 4). The slope of the initial volume loss was also not significantly different between the two conditions. Upon reaching the lowest point of the volume loss curve at 200 s, volume recovery was observed within seconds of the peak loss. In control conditions cells

underwent a rapid recovery phase over the subsequent 200 s, followed by a more gradual recovery through the remainder of the 30 min collection time. On the other hand, in the bumetanide trial volume recovery was greatly attenuated in both rate and magnitude of peak volume recovery, but still showed recovery of ~1/3 of the total volume loss by 1500s (Fig. 4). Taken together, these data demonstrate the natural propensity of skeletal muscle cells to exhibit a RVI when faced with increased extracellular osmolarity. It is concluded that under NaCl treatment conditions, which represent an osmolarity increase similar to that of high intensity exercise, the RVI mechanism is highly dependent on a functional NKCC.

3.3 Volume recovery is also dependent on the uptake of lactate

The effects of an increase in extracellular osmolarity by raising extracellular NaLac was then investigated by evoking similar osmolarity increases as had been performed previously using NaCl, sucrose and NaLac. Compared to NaCl and sucrose, the peak volume decrease was significantly attenuated, resulting in only a 6.5±0.76 % volume loss, in the presence of NaLac (Fig. 5). In contrast, the volume loss response observed under NaCl and sucrose treatments was nearly 3-fold greater (14.9 ±0.91 % and 15.8 ±1.27 %, respectively). The rate of volume loss with the NaLac treatment during the initial cell shrinkage was also less compared to the rate observed in the NaCl and sucrose conditions, although the time elapsed to reach peak volume decrease was not different between the three treatments (Fig. 5). However, the rate of the recovery was much less in the presence of NaLac compared to in the NaCl and sucrose conditions. The ensuing 450

sec after peak volume loss in both the NaCl and sucrose conditions showed a much higher rate of volume recovery. Following this, a more gradual recovery phase is then observed for the remainder of the 30 min period, where the cells recovered at a rate similar to the NaLac condition. However, both the NaCl and sucrose conditions recovered slightly less volume compared to the NaLac trial. Neither of the treatment conditions though, resulted in a complete recovery back to their original baseline status prior to treatment. These results implicate lactate in volume recovery when lactate is present in the extracellular solution.

3.4 The lactate influx occurs through MCTs

Bumetanide inhibition of the NKCC in the NaLac treatment condition did not have an effect, in that it resulted in a similar response to the NaLac treatment in the absence of any inhibitors. There was no difference in the rate of volume loss between the two conditions, and the time to reach peak volume loss, the point at which volume began to recover, the rate of recovery, or the degree of peak volume recovery after 30 min were all similar between these two treatments (Fig. 6). These data demonstrate that although the NKCC was blocked with bumetanide, a RVI still occurred which suggests that an alternate means of regulating volume was utilized by the cells.

It is worth noting that while baseline measurements were only done immediately following inhibitor incubation and just prior to the osmotic challenge, the baseline values were very similar to the values obtained from the start of data collection in the presence

of the osmotic challenge, suggesting muscle cell volume was normal and not directly affected by inhibitor under isomotic conditions.

Investigation of the role of lactate in the RVI response focused on the MCTs by using the MCT inhibitors phloretin and pCMBS. After cells were exposed to MCT inhibitors, the decrease in cell volume induced by raising NaLac was greater with phloretin than with pCMBS (Fig. 6). The rate of volume loss with complete MCT1 inhibition (phloretin) was also greater compared to complete MCT4 inhibition (pCMBS), and the time to reach peak volume loss occurred sooner compared to pCMBS inhibition alone. Time to peak volume loss in the control (173 s), was less than with phloretin (196 s) and pCMBS (238 s) conditions, while bumetanide resulted in greatest time to reach maximum volume loss (299 s) prior to observing a recovery. The initial recovery stage with phloretin was delayed, with a temporary plateau of ~150 sec observed upon peak volume decrease prior to initiating a regain of volume. On the other hand, the decreased rate of volume loss with pCMBS compared to phloretin indicated an earlier activation of recovery in the pCMBS condition. In addition, an observable volume increase occurred much sooner following peak volume loss at the 250 sec mark in the pCMBS condition compared with phloretin (Fig. 6).

Coinciding with the dissimilarity between the two inhibitor responses, a distinct difference was observed during the remainder of the recovery phases. Peak volume recovery was similar between phloretin and control, regaining back to near baseline volume. However, the rate of volume recovery in the pCMBS condition maintained steady beyond the 30 min recovery period, resulting in a volume overshoot to 6% above the initial baseline volume (Fig. 6). pCMBS inhibition resulted in a time to peak volume

recovery of 1200 sec, while phloretin treatment required the full 1800 sec to reach maximum volume recovery back to 100%. This is in contrast to the responses observed with NaCl and sucrose (Fig. 5), where the fibres reached a peak volume recovery of 95% of its original cell volume during a similar 30 min period. While volume recovery is evident based on the data presented, there are clear dissimilarities between the responses of the various treatment conditions.

3.5 Combined inhibition of the NKCC and MCT did not result in greater cell volume disturbance

The results of the MCT inhibition experiments suggested a pronounced activation of the NKCC when cells were exposed to increased NaLac. Therefore it was hypothesized that blocking both the NKCC and MCT together will cause a much greater magnitude of volume loss and nearly complete inhibition of the rate and magnitude of volume recovery. In contrast to the hypothesis, combined inhibition of these transport proteins resulted in much smaller and slower volume decreases (Fig. 7) compared to blocking with either bumetanide, pCMBS or phloretin alone (Fig. 6) and compared to the NaLac control (Fig. 7). The rate of volume loss and the time to peak volume decrease were similar in both MCT inhibition conditions. The slopes of initial volume loss with combined inhibition of either phloretin or pCMBS with bumetanide were similar. The volume recovery in response to combined transporter inhibition conditions were biphasic. In the first phase, both conditions demonstrated recovery of volume at the same rate over the initial 200 sec following peak volume decrease. In the second segment of the

recovery phase, inhibition with pCMBS and bumetanide resulted in slower recovery rate from 400 sec onward, albeit steadily up to a full recovery to baseline volume. However, in the second phase of recovery in the phloretin and bumetanide condition, a divergent response was observed compared to that of the pCMBS and bumetanide condition (Fig. 7). From the point at 400 sec onward in the combined phloretin and bumetanide condition, the cell volume then stopped recovering and instead began to fall again. The volume decrease occurs at a steady rate through the remainder of the 30 min data collection period, showing no signs of forthcoming volume recovery. This is in contrast to the continuing recovery observed in the combined pCMBS and bumetanide trial. The volume loss in the combined phloretin condition fell at a rate reciprocal to the rate of ongoing recovery observed under combined pCMBS inhibition.

In summary, pCMBS-bumetanide and phloretin-bumetanide resulted in attenuated cell volume loss compared to all other conditions of increased extracellular osmolarity. Further, phloretin-bumetanide completely inhibited volume recovery, whereas with pCMBS-bumetanide volume recovery was complete. These data suggest that the ability of skeletal muscle to volume regulate is more multifaceted than initially expected.

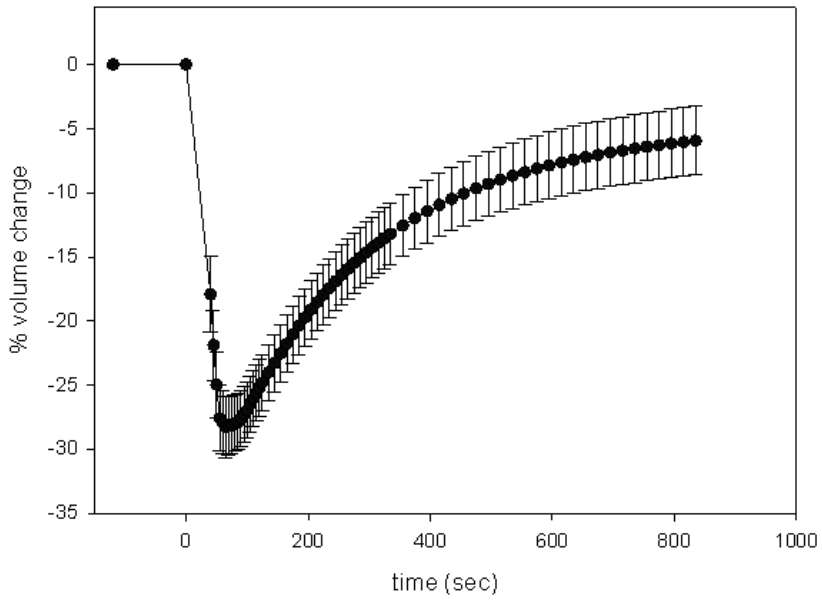


Figure 2 – Response curve of characteristic volume loss and recovery to increased osmolarity (+45mM NaCl) in single skeletal muscle fibres, n = 8 sections from 4 fibres from 2 mice.

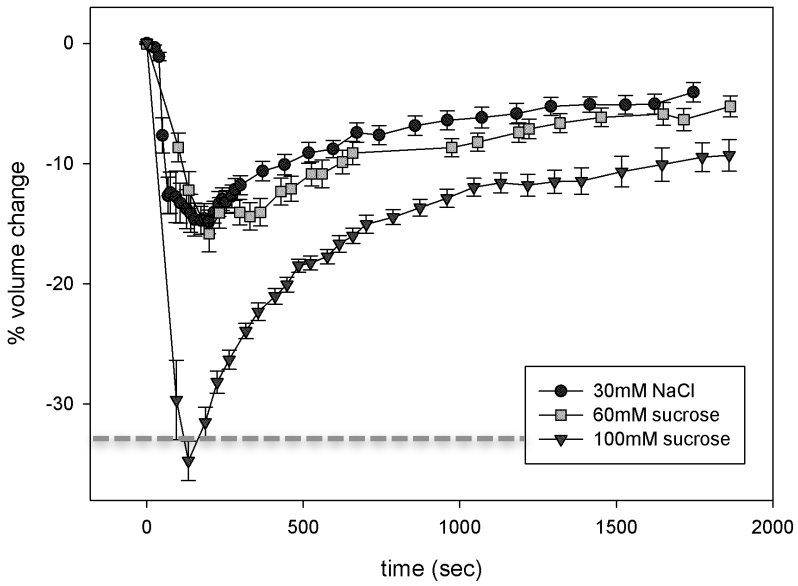


Figure 3 – Dose-dependent RVI responses to increased extracellular osmolarity induced by raising NaCl by 30mM (n = 6) and sucrose by 60 (n = 8) or 100 mM (n = 11) concentrations. Dashed line indicates expected theoretical volume loss to a 30mM extracellular osmolarity increase in the absence of regulatory volume mechanisms.

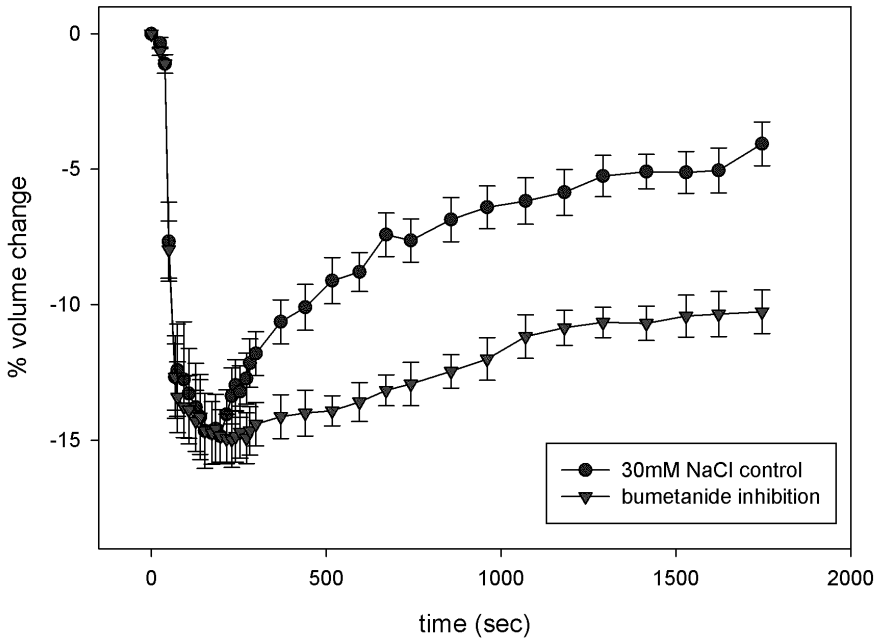


Figure 4 – Effect of inhibited NKCC with bumetanide (n = 6) on RVI response with extracellular osmolarity increase using NaCl (n = 6).

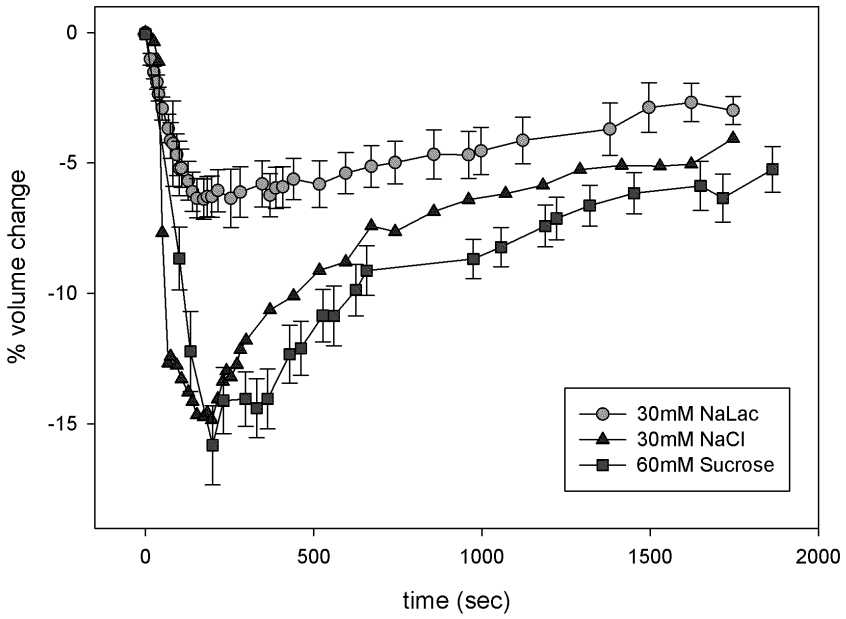


Figure 5 – The effect on increased extracellular osmolarity induced by raising NaLac (n = 30) on the regulatory volume response, compared to NaCl (n = 6) and sucrose (n = 11).

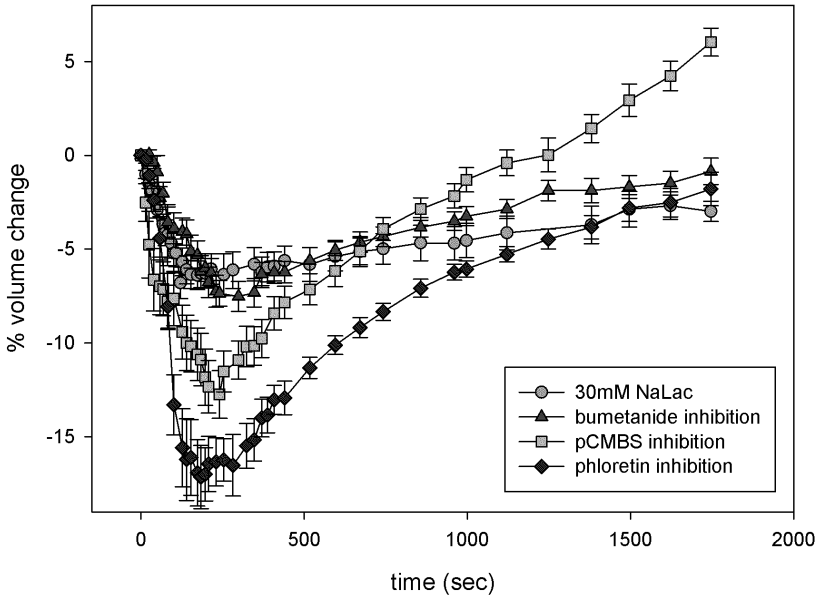


Figure 6 - Effect of blocking MCT on regulatory volume response with NaLac treatment in the presence of either bumetanide (n=39), pCMBS (n=11), phloretin (n=7) or control (n = 30).

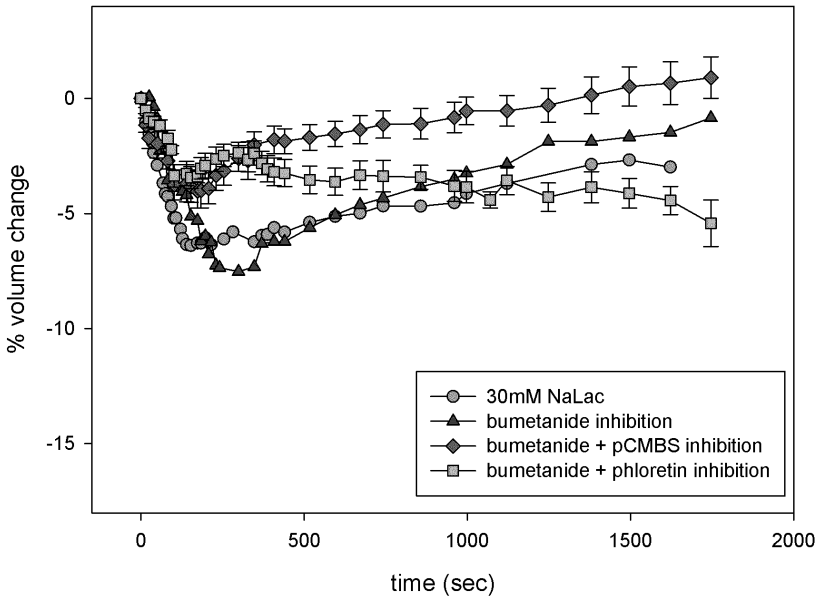


Figure 7 – Muscle volume response of NaLac treatment to combined inhibition of bumetanide with pCMBS (n = 13) or phloretin (n=19), bumetanide inhibition alone (n = 39) and control (n = 30).

Table 1 – Osmolarity measurements of incubating media following application of inhibitor drugs and 30mM NaLac treatment

	NaLac	Bumetanide	pCMBS	Phloretin	pCMBS + Bumetanide	Phloretin + Bumetanide
Osmolarity (mosmol/L)	381	687	479	547	615	841

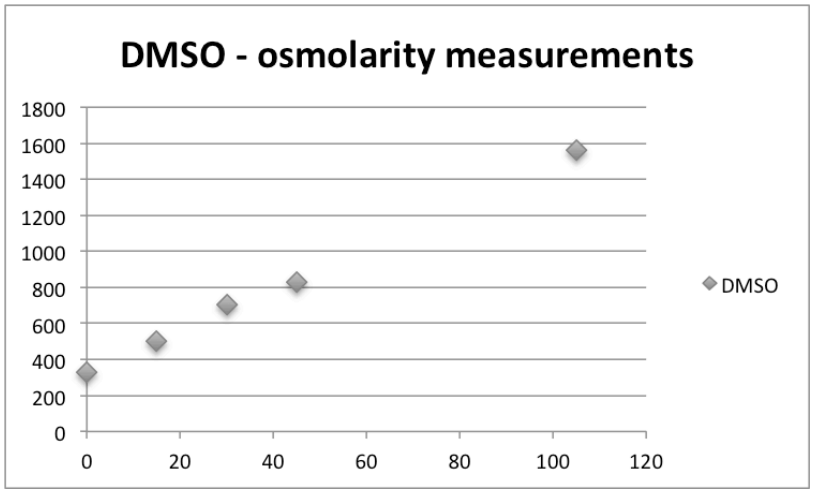
Table 2 – Osmolarity measurements (mosmol/L) of varying amounts of DMSO (µL) applied to 1.5mM wells of DMEM demonstrating effect of DMSO on increase in osmolarity.

Control (DMEM)	326 mosmol/L
15 µL DMSO	504
30 µL DMSO	706
45 µL DMSO	828
105 µL (0.7%) DMSO	1561

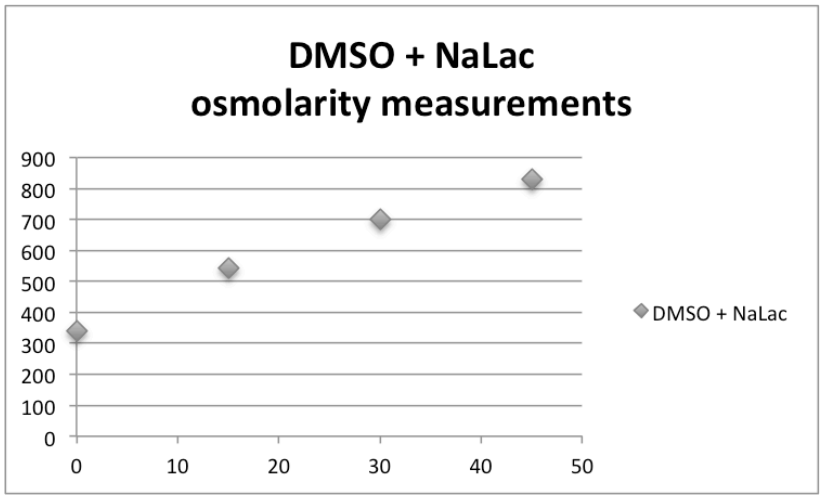
Table 3 – Osmolarity measurements (mosmol/L) of DMSO application (µL) to DMEM-containing wells following 30mM NaLac application demonstrating effect of DMSO on increase in final osmolarity of extracellular solution.

Control (plating media)	341 (mosmol/L)
15 µL DMSO + NaLac	544
30 µL DMSO + NaLac	701
45 µL DMSO + NaLac	830

**Figures shown below illustrate values presented in tables 2 and 3 (above)*



Dose-dependent increase in osmolarity (y-axis) related to increasing DMSO volume application (x-axis). DMSO was added to low-glucose DMEM.



Dose-dependent increase in osmolarity (y-axis) related to increasing DMSO volume application (x-axis). DMSO was added to low-glucose DMEM in the presence of 30mM NaLac treatment.

Discussion

The primary objective of the study was to investigate the involvement of MCTs in the volume responses of skeletal muscle to imposed increases in extracellular osmolarity induced by raising extracellular NaLac. The NaLac trials were preceded with experiments using NaCl or sucrose to raise extracellular osmolarity, to establish the presence of a regulatory volume response within single skeletal muscle fibres. Following the initial cell volume loss, an active RVI response occurred which returned cell volume back towards original baseline status. The RVI is bumetanide sensitive, which is in line with the notion of involvement of the NKCC. As shown previously using intact mouse soleus and EDL muscles, single mouse peroneus and EDL skeletal muscle fibres were observed to regulate volume in response to increases in extracellular osmolarity (Lindinger et al. 2011). In the present study, it was further demonstrated that skeletal muscle regulates volume in response to increased extracellular lactate, even in the presence of the NKCC inhibitor bumetanide. The results indicate that the MCT contributed to the RVI responses. The lactate-facilitated RVI response was sensitive to the MCT inhibitors phloretin and pCMBS, consistent with a primary contribution of MCT1. Lactate is taken up by the cells through MCT1 (Km = 4.5 mM) and, based on the premise that extracellular lactate may be in abundance in muscle during exercise, then it appears that the influx of lactate may aid in the restoration of cell volume in non-contracting muscle in vivo. The experiments, however, lead to unexpected results when cells were treated with both NKCC and MCT inhibitors combined – specifically the volume loss was greatly attenuated. It remains to be determined if this can be explained by high DMSO concentrations on muscle membrane permeability or if it is a bona fide effect of the use of both inhibitors. The

current observations suggest that skeletal muscle cells may elicit alternate means to volume regulate when faced with conditions that restrict its usual processes of RVI, i.e. activation of the NKCC. Taken together, the data suggests that the uptake of lactate through MCT1 plays a supporting role in skeletal muscle's ability to regulate volume in the face of osmolarity / ionic disturbances during high intensity exercise.

4.1 Experimental considerations: selecting methodological approach

The NKCC had been demonstrated (Sitdikov et al. 1989; Urazaev, 1998; Lindinger et al. 2002, 2011) to play a role in exerting a volume recovery response after cells were made to lose volume by raising extracellular osmolarity. Thus, the inhibitor bumetanide was selected to isolate the involvement of the specified cotransporter. Upon successfully completing this objective in displaying a regulatory response and demonstrating the function of the NKCC in contributing to a RVI using these single mouse fibres, it was then justified to extend the investigation towards lactate in exploring the potential involvement of this particularly abundant substrate in this regulatory process. Through these investigations, a greater understanding of the interplay between the NKCC and MCT were thus elucidated in the RVI response in skeletal muscle.

This regulatory volume increase response was previously demonstrated using intact whole soleus and EDL muscles removed from mice (Lindinger et al. 2011). In the present set of experiments, trials were conducted using single fibres isolated from whole EDL and peroneus muscle. This single fibre technique was also utilized by a previous

group, who appear to be the first researchers to have demonstrated the ability of muscle fibres to elicit a RVI in the face of hyperosmotic medium (Sitdikov et al. 1989).

The previous work involving intact muscles (Lindinger et al. 2011) was, however, performed with a different technique for assessing volume responses, whereby cell volume was indirectly measured using fluorescence of intracellular calcein. This surrogate indicator of volume change was assessed through shifts in intensity of calcein fluorescence. As a result, the kinetics of the cellular responses between the two protocols are slightly different (multiple cells versus single cells) but qualitatively very similar.

The intact muscle technique required the acquisition of light emitted from surface fibres of the intact muscle. Therefore the depth of light penetration within the muscle is a variable affecting the time course of responses and thus contributes to variation in the results. Similarly, diffusion of gases and nutrients from the bathing solution towards the inner fibres of the muscle may also occur gradually. The intact muscle technique is based on the premise that intensity of calcein fluorescence emitted by the muscle fibres is directly proportional to the volume change within the muscle. An increase in cell volume would result in a greater dispersion of intracellular calcein, thus resulting in a lower intensity of the fluorescence. Conversely, volume reduction would increase the density of the calcein and therefore produce a much brighter fluorescence emission. However, a true representation of calcein fluorescence might not be obtained for a certain amount appears to be sequestered within the muscle itself (Crowe et al. 1995) and thus insensitive to volume changes (Crowe et al. 1995; Lindinger et al. 2011).

The single fibre technique utilized in the present study, however, should be deemed to represent more closely the response present in vivo because bath perfusion of a single fibre is a better representation of blood perfusion of intact muscles. Single muscle fibres are equally exposed on all extracellular surfaces to the induced extracellular osmolarity increase, and any probable issues pertaining to diffusion kinetics is alleviated. This supports the application of this technique towards these experiments to investigate the properties of lactate in the regulatory volume response.

4.2 Experimental considerations: muscle viability

The success and usefulness of these experiments is dependent on the viability of the single muscle fibres throughout the experimental protocol. As previously demonstrated (Hawke et al. 2003, Shortreed et al. 2009, Pickering et al. 2009), the single muscle fibre preparation is a practical technique for investigating skeletal muscle responses to a variety of imposed perturbations. However, proper care must be practiced to ensure maintained viability throughout the experimental protocol.

The procedures for isolating single muscle fibres from the intact muscle may have induced alterations to the structural integrity of the fibres due to disruption of cytoskeletal extracellular matrix proteins involved in intercellular adhesion (Clark et al. 2002). These proteins are integrally connected to many sarcolemmal ion transport proteins, thus disruption could potentially affect the natural response of the muscle to such treatment conditions, resulting in confounded muscle observations. The process of using collagenase solution to incubate the intact muscles was performed under time- and

temperature-specific conditions, determined to be optimal for the long-term viability of the muscle cells. The ensuing trituration stage in the muscle fibre isolation process would also be susceptible to potential tissue damage. However, cautious selection of the individual fibres when collected minimizes the potential for including any sub-par fibres in the final plates of muscle fibres.

The matter of long term viability of the isolated mouse muscle may be questioned. Based on previous experiments intact muscles were kept sustainable for upwards of 10 hours at room temperature. It has been demonstrated (Hawke, TJ unpublished observations) that plated muscle fibres can be kept alive incubated in plating media at 37^{o} degrees for several days. While the tissues used in this experiment were isolated single fibres, which could cause for lesser capacity of maintaining its integrity, they were utilized in the protocol within 3 hours of being exposed to room temperature environment. Conversely, all individual fibres were visually verified under the microscope to be alive, have normal Z-disc patterning prior to running the experimental protocol. To further validate the viability of the single muscle fibres in the present experiments, a similar comparison in volume response can be made with the previously conducted studies which utilized the single fibre and intact muscle preparations (Lindinger et al. 2001). In addition, the inclusion of calcein as a fluorescing agent also points to cell membrane integrity and sustained viability (Crowe et al. 1995; Tanioka et al. 2007).

4.3 Experimental considerations: muscle volume measurement

The ability to quantify changes in cell volume also needs to be considered. It is not possible to directly measure the volume of skeletal muscle fibres due to the variable length and width of these cells. Also, the microscopic scale of the tissues do not allow for the manipulation required to impose the necessary handling of the fibres, and the environmental requirements of the fibres in a solution medium also prevent any possible real time assessment of fluid passage into and out of the cells. Therefore, other approaches must be used to indirectly evaluate volume properties. For these studies, single muscle fibres were individually quantified by cross-sectional measurements as an indirect measure of actual cell volume. The principle of this technique was similar to that performed on single frog muscle fibres accomplished previously (Usher-Smith et al. 2006). One of the assumptions this technique is based on is that the muscles are equally cylindrical in shape throughout the entire length of the fibre. Since it was not possible to view the fibres on a horizontal plane to verify this, it was presumed to be the case and a non-confounding factor. However, in light of the concern it should be noted that the method of volume measurement does allow for one to ensure that each muscle is fully viable and thus responsive in providing accurate data measurements.

4.4 Experimental considerations: DMSO effect on osmolarity and membrane permeability

A consideration that came to light was the effect of DMSO on increasing the osmolarity of the extracellular solution, as well as its potential impact on muscle

membrane permeability and muscle viability. DMSO was used as the dissolving agent to make up the stock solutions of the inhibitors (bumetanide, phloretin and pCMBS) due to the very low solubility these compounds have in aqueous solutions. It must be acknowledged that sizeable concentrations of DMSO will increase osmolarity of its surrounding solution; a solution with 0.7% DMSO has an osmolarity increased by 98.6 mOsm/L by addition of DMSO alone. This is cause for concern as it increases the induced osmotic challenge the muscle fibres are exposed to in solution.

A secondary concern is the impact DMSO has on cell membrane permeability, in which it may facilitate the uptake of substrate through the membrane. Investigation on the efficacy of a process whereby 10% DMSO pretreatment of cells is used to promote drug delivery by way of cell penetrating peptides suggests it as being a potentially novel drug delivery enhancement technique (Wang et al. 2010). If the membrane permeability properties of this delivery system holds promise, it may result in consequences for volume regulation for the expedited rate of solute uptake would lead to augmented volume recovery in the cell.

Attention has been drawn recently to the physiological effects of administered DMSO on skeletal muscle response. It is considered that the application and removal of DMSO causes biphasic volume changes (Pfaff et al. 1998). Fraser et al. have commented in an editorial (2011), that Randa temporaria cutaneous pectoris muscle fibres treated to Ringer's solution containing 100mM (0.7%) DMSO resulted in cell shrinkage. If this observation is accurate, it would be a worthy consideration as to how much of an increased osmotic response the application of DMSO has in addition to treatment through hypertonic solution. It would also imply that DMSO presence counters the volume

regulatory response that accompanies cell shrinkage and thus volume regulation should be impaired. However, it was noted that the possible influence of DMSO addition had not yet been studied in mammalian muscle. Data obtained from the present study illustrates the osmotic effect DMSO application has on plain low-glucose DMEM (Table 2) and plating media in the presence of NaLac treatment (Table 3). The present data involving mouse skeletal muscle demonstrates that RVI is observed even in the face of DMSO application. This finding then opposes the notion put forth by Fraser's group (2011), for volume was found to be upregulated even in the presence of DMSO. To address this concern, future experiments should be conducted by using as high a concentration of stock osmotic challenge solution as possible, to ensure the least amount of DMSO is added to the extracellular medium within the plate wells. Based on the solubility properties of the transport inhibitors involved in the present study, this would seem feasible and a worthy avenue to explore for subsequent experiments.

4.5 Experimental considerations: treatment application into wells

The method of inducing hypertonic challenge to the muscle fibres involved the application of a small volume of concentrated (1 M) stock solution of sucrose, NaCl or NaLac into each 1.5mL volume well containing 4 to 6 single fibres. A concern is that while data collection was initiated immediately following the application of treatment, each fibre in the well may not be exposed to the intended final osmolarity increase prior to even distribution within the medium. Upon mounting the plate onto the microscope stage, it was important to not induce unnecessary movements that could disrupt the

adhesion of the fibres to the bottom of the plates. While gentle swirling was performed, the certainty of equal exposure at the time of commencing data collection is unverified. Consequently, the time course of certain individual muscle fibre responses to the induced extracellular osmolarity increase may be delayed by as much as 30 seconds, which could produce some minor variation within the data time course. To account for this issue, the data were corrected using an average delay time of 20 seconds, and an adequate sample size was collected within each treatment condition per protocol to obtain statistically meaningful data. Finally, ensuring ample replication of each protocol over multiple experimental days was sufficient to justifiably dismiss it as a significant concern.

4.6 Cell volume regulation in response to increased extracellular osmolarity

An extracellular hypertonic environment is encountered physiologically during exercise when arterial osmolarity increases as a consequence of the accumulation of metabolic products such as lactate, produced from contracting muscle and from net flux of solute-poor fluid into contracting muscles (Lundvall, 1972; Lindinger et al. 1994; Gosmanov et al. 2003). Within contracting muscle, osmotically active metabolic products (creatine, inorganic phosphate, lactate) accumulate within the tissue, and as a result water flows osmotically into these cells; as a consequence volume is reduced within the plasma (vascular compartment and extracellular fluid) due to net water movement into the working muscle. It is important to regulate blood volume in order to preserve blood pressure and cardiac output, and to alleviate the subsequent state of hypovolemia fluid replacement is removed from non-contracting muscle (Lundvall, 1972). Following the

initial shift of fluid from the vascular compartment into the working muscle, there is a subsequent increase in the plasma osmolarity. In turn, the shift of fluid from the non-working muscles into the increased osmolarity environment of the vascular compartment protects plasma volume. The end result of this cascade of fluid movement within the various compartments is a state of cell shrinkage within the non-contracting muscle.

Based on our current understanding, it is presumed that when volume loss occurs from non-contracting muscles and other tissues, the regulatory volume response soon follows to assist in recovering volume back into the shrunken cells. An inability to do so has been linked to cellular apoptosis (Lang et al. 2009). The occurrence of the volume restoration process is referred to as the regulatory volume increase (RVI), which is considered to be primarily effected by the NKCC which is responsible for increasing intracellular concentration of osmolytes. The net solute influx ultimately draws water back into the cell to return volume towards baseline. Various transporters present on the cell surface allow for greater membrane permeability to Cl^-, Na^+ and K^+ as opposed to sucrose. Since the NKCC is capable of taking up NaCl to a greater propensity compared to sucrose, it is reasonable to expect the RVI response as seen (Fig. 3) whereby volume recovery is more optimal in the NaCl treatment condition in contrast to sucrose. Considering the difference in dosage of extracellular osmolarity increase, it is also logical that in the face of a greater magnitude of osmolarity increase that a greater duration of time is necessary to regain fluid volume back to baseline status.

Therefore, it is imperative in these cells that upon volume loss a subsequent RVI occurs to restore volume and proper cell function (Lang et al. 1998; Gosmanov et al. 2003). It is considered that the action of water flux back into these non-working muscles

is achieved through facilitated diffusion, by way of water channels, and it is the AQP4 water channel in which it has specifically been identified as the major aquaporin in mammalian skeletal muscle (Crosbie et al. 2002; Frigeri et al. 2004; Nicchia et al. 2007).

4.6.a Cell volume regulation in response to NaCl, sucrose, and NaLactate

Previous experiments have contrasted the response of skeletal muscle to induced extracellular osmolarity increase in the presence of NaCl and sucrose (Lindinger et al. 2011). While the effect of the two treatments yielded comparable responses, the osmolarity change stimulated by NaCl is considered to be more physiologically relevant compared to sucrose because exercise and sweating induced increases in extracellular osmolarity are more akin to the environmental changes imposed through NaCl application. In comparison to NaCl, of which Cl^- has a high conductance across the membrane, sucrose is not permeable across the sarcolemma. In addition, sucrose is not normally ingested in sufficient quantities to elicit, in vivo, the comparable levels achieved through the treatment of elevated extracellular sucrose in these experiments. Thus NaCl treatment conditions were used as a control for subsequent treatment protocols.

At a concentration of 60mM sucrose, the resultant effect of the increased extracellular osmolarity did not lead to a great distinction with the response of 30mM NaCl. While both these treatments increased osmolarity by 60mosmol/L, it was expected that the NaCl condition would have allowed for much more capable regulation of cell volume as opposed to sucrose treatment. This is because the presence of added Na^+ and Cl^- ions in the extracellular medium is expected to be taken up at a greater capacity as

opposed to sucrose being impermeable. While the two conditions show similar magnitudes of peak volume decrease, the NaCl condition did yield a response curve which depicted a slightly more rapid recovery following the volume loss (Fig. 5). Recovery began at 175 sec for NaCl while the sucrose condition did not begin to recover at an appreciative rate until nearly 350 sec. It is illustrated that the rapid recovery phase as well as the subsequent gradual recovery phase is steeper with NaCl compared to sucrose. In addition, there is marginally greater peak volume recovery under NaCl-induced osmolarity increase as opposed to sucrose.

Experiments examining the effect of the NaCl and sucrose treatments to increased extracellular osmolarity resulted in dose-dependent volume losses in muscle cells. Increases of 20 mM, 50 mM and 100 mM NaCl and 60 mM and 100 mM sucrose result in graded volume losses proportional to the degree of osmolarity change. This result demonstrates that the skeletal muscle's regulatory response may activate with a threshold property, and a similar minimum point may also need to be reached before activation of the transporter. It was demonstrated recently that at a 5% increase in extracellular osmolarity there was no discernable effect on either the soleus or EDL muscle volumes. It was also demonstrated that the magnitude of cell volume loss increased in accordance with the degree of extracellular osmolarity increase (Lindinger et al. 2011). In contrasting the effects of NaCl and sucrose on cell volume, it was observed that sucrose caused a greater peak volume loss for an equal osmolarity increase in NaCl. This is due to the fact that sucrose is not taken up by the cells, in contrast to NaCl treatment of which Cl^- passes through the sarcolemma through numerous Cl- channels (Bertran & Kotsias, 2000). The aforementioned experiments thus demonstrate the natural capacity of skeletal muscle

cells to exhibit a volume response, and also illustrates the characteristics of a typical regulatory volume increase when subjected to a step increase in osmolarity (Dulhunty et al. 1984).

It was hypothesized that in the presence of lactate, skeletal muscle has the ability to take up the substrate to assist in the process of RVI. Data comparing the two control conditions of NaCl and sucrose with the NaLac condition demonstrates the distinct differences in volume responses. Inducing an increased osmolarity in the absence of any transport inhibitors, it is evident that the NaLac treatment yielded a much reduced volume loss. Although the final peak volume recovery was similar between the three conditions, the response over the 30 min collection period is indicative of the presence of an augmented solute uptake mechanism in the presence of elevated extracellular lactate.

The cells in the NaCl and sucrose conditions, as previously mentioned, were able to exert an RVI based on the uptake of solutes via the NKCC (Lindinger et al. 2011). However, in the NaLac condition there are multiple indications of a much earlier onset of RVI. The rate of initial volume loss was less when comparing the slopes of the NaLac response to that of sucrose or NaCl. In addition, the resultant enhanced solute uptake in the NaLac condition and earlier onset of RVI resulted in a noticeably attenuated peak volume decrease. Compared to the NaLac condition, the rate of volume recovery was significantly greater in the other two conditions. While this may suggest greater RVI activity, it is reasonable to expect that the recovery rate in the NaLac condition be less when considering the greater than 2-fold difference in the initial volume loss between the treatment conditions.

Of notable interest is the consideration of what it takes to activate, and maximally activate, the NKCC. It was observed in all three treatment conditions that the peak activation occurred at about the same time after inducing cell shrinkage (~200 s). It may be possible that the cells recover volume in proportion to the magnitude of volume loss, as evident by the dose-response effects and the comparison between the NaLac condition vs. that of NaCl and sucrose. In essence, this would imply that the NKCC is being activated to a greater extent in these two conditions compared to NaLac, and that the maximum activation capability may extend well beyond the rates of osmolarity increase imposed in the present experiments. All things equal, this suggests that muscle cells may be capable of eliciting a RVI with a rate that is in proportion to the severity of the volume loss; consistent with the dose response data of Lindinger et al. (2011). Since the maintenance of cell volume is of great importance, this could explain such a response observed in these cells.

4.6.b Cell volume regulation in response to transport inhibition

Experiments have focused on the involvement of the NKCC in effecting a RVI with the use of the specific inhibitor bumetanide. At low concentrations (below 10mM), bumetanide effectively inhibits NKCC activity (Russell, 2000). In the presence of NaCl treatment, this would be exhibited by impairment of volume recovery in the muscle cells.

Based on the data obtained, it is convincing that blocking the NKCC had a negative impact on volume recovery. As a result, the point at which volume began to recover was delayed. The effect can also be seen in the rate of recovery, as the slope was

much less compared to the control condition. Furthermore, the diminished peak volume recovery can also be attributed to the inability of these muscle cells to adequately regulate volume in the face of NKCC inhibition. In contrast to previously conducted experiments with intact muscle where the inhibitor was rinsed out of the bathing solution following inhibitor incubation (Lindinger et al. 2011), the single fibres were pre-incubated in bumetanide and the inhibitor was not rinsed out but instead left in the extracellular medium during the osmotic challenge. This may account for subtle differences in RVI observed in the bumetanide treatment of intact muscle. Muscle in the presence of bumetanide would have had a raised osmolarity compared to controls due to the addition of DMSO being used to apply the inhibitory drug. Subsequent addition of NaCl, sucrose or NaLac would have imposed an increase in osmolarity above that.

While bumetanide is specific for the NKCC, it is possible that it may not completely block all NKCC transport proteins at the concentration used (1mM). Any functional NKCC still remaining would be able to continue solute uptake into the cell with ensuing osmotic water influx (Lang et al. 1998). This could explain the modest magnitude of volume that was in fact recovered in this experiment. This may also explain why a distinction in the magnitude of peak volume decrease was not discernable between the treatment and control condition under NaCl treatment. If the entire NKCC population were not inhibited, then that could account for the similar degree of volume loss within the two trials. It is possible that any residual uninhibited NKCC was able to prevent the amount of volume loss from reaching an even greater magnitude, although was evidently not enough to effect a successful volume recovery.

It was observed that the slope of the initial volume loss was not significantly different between NaCl control and NaCl treatment with bumetanide inhibition, which suggests a potential threshold for full activation of the cotransporter. It is likely that under a greater osmotic change than what was induced here, the NKCC would have activated sooner to elicit an effect prior to the cells reaching the point of maximum volume loss. This is supported by dose response data of Lindinger et al. (2011). The aforementioned characteristics thus demonstrate the involvement of the NKCC in the process of RVI, and the consequence of impeding the ability of the cell to take up solute in an attempt to regulate volume in an increased osmolarity environment.

The use of bumetanide in the presence of NaCl support the involvement of the NKCC in eliciting RVI, the same cannot be said for the incorporation of bumetanide when used with increases in extracellular NaLac. Even though the NKCCs were blocked in the bumetanide trial, a similar response curve was obtained to that of the NaLac control. This implies that although the NKCC may not be involved in volume regulation under these conditions there is still another mechanism of solute uptake and regulation of volume, pointing to the contribution of lactate in exerting the observed RVI response. It may be the case that when only NaCl was used as treatment, the absence of lactate precluded any involvement in the MCTs in exerting a regulatory volume response. However, when the treatment condition included lactate, the presence of the substrate allowed for the MCTs to utilize it in the regulatory response. Taken together, these data support the notion that skeletal muscle cells may be dependent on MCT uptake for optimal volume regulation when the conditions are favourable in the presence of lactate.

While the results thus far indicate the involvement of lactate in the course of RVI, subsequent experiments further support its contribution in this regulatory process. Inhibition of the MCTs employed the inhibitors phloretin and pCMBS, and as hypothesized blocking the MCT impeded the volume regulatory response. While both inhibitors have a high affinity for each of the involved MCT isoforms (MCT1 and MCT4), pCMBS at the concentration used in these experiments should have completely blocked MCT4 while phloretin at the concentration used in the present experiments should have completely inhibited MCT1 (Dimmer et al. 2000; Manning Fox et al. 2000). When either phloretin or pCMBS were used, a significantly greater volume loss was encountered compared to the control NaLac condition. This can be explained by the fact that upon inhibiting the MCTs, the uptake of lactate was impaired. However, in both the MCT inhibition conditions, full volume recovery was reached within the 30 min response period indicating potential recruitment and involvement of the NKCC in regaining volume. The fact that cells are capable of a near-full recovery following a greater volume loss compared to control may suggest that the cells are recruiting other means of solute uptake by way of different transporters.

While both phloretin and pCMBS involvement revealed similar negative impact on RVI, there were still dissimilarities between the two responses. This may be a result of differing MCT isoform specificity, explaining the greater inhibitory effect of phloretin compared to pCMBS. The MCT1 isoform is involved more in lactate influx, a role consistent with its low K_m of 4.5 mM (Broer et al. 1997), while MCT4 has a larger role in lactate efflux, consistent with its high K_m of 25–35 mM (Bonen, 2000, Dimmer et al. 2000). Since phloretin has a greater specificity towards MCT1 than MCT4, using 1 mM

phloretin completely blocks MCT1. Its effect on MCT4 however is approximately a 90% inhibition. Similarly, although pCMBS almost entirely blocks MCT4, its inhibitory effect on MCT1 is also approximately 90% (Dimmer et al. 2000; Manning Fox et al. 2000). The remainder of the uninhibited MCT1s would then still allow for a substantial amount of lactate flux due to its low K_m, although not so in the case of MCT4 because of its high K_m. The known transport kinetics of the MCT isoforms suggests that MCT1 has a much lower K_m compared to that of MCT4 (Bonen et al. 2006). Therefore, MCT1 would have a greater affinity for lactate compared to MCT4, and it would require less substrate (lactate) to permit the reaction to progress forward. It may be conceivable that these uninhibited lactate transporter populations are allowed to work in a state of overdrive because they are still fully functional and thus recruiting further mechanisms of solute uptake. It is also possible that the present experiments may not have been conducted over a long enough time period to allow for the observation of a plateau in the pCMBS condition to elucidate a more tangible explanation in the disparity between the two inhibition conditions. While 30 minutes was the standard protocol duration implemented in the experiments, near complete recovery of rat single muscle fibres exposed to hypertonic solutions were found to demonstrate complete recovery in up to 40 minutes (Sitdikov et al. 1989).

An interesting finding is the fact that when cells were treated with pCMBS they exhibit a volume overshoot, whereas an overshoot was absent in the phloretin condition. It is likely that there is activation of the NKCC in this condition, in which it is activated to assist in the overall regulation of the cell since the MCTs are partially inhibited. It may be that there is earlier and more rapid activation of the NKCC as well going on, in

addition to lactate influx through MCT1 – the primarily inward directed lactate transporter. The rate of volume recovery between the two MCT inhibitors is similar. Taking into account the fact that phloretin has greater affinity towards the MCT1, it may be possible that the pCMBS response curve is not indicative of a complete block of the MCTs. In other words, the phloretin response is suggestive of the characteristics involved in a response where lactate uptake is near completely blocked, while the pCMBS response is in fact allowing a small percentage of functional MCTs that are functioning to cause the regulatory response.

In support of this explanation is the difference in effect that the two inhibitors have on the recovery responses. pCMBS inhibition resulted in a much quicker time to recover full volume following peak volume loss. While pCMBS inhibits lactate efflux through MCT4, lactate will still go into the cells through MCT1 (90% inhibited) however the lactate will not be able to get back out. Therefore, the consequential effect is that solute is retained within the cell, allowing for a more rapid volume recovery than when MCT1 is fully blocked in the phloretin inhibition condition. This series of events may also help explain the volume overshoot under the pCMBS condition, in that there is retention of lactate in addition to Na^+, K^+ and Cl^- uptake. The likelihood of the presence and function of other MCT isoforms (Bonen 2000) or other transport proteins (Russel, 2000) within skeletal muscle may explain the some of the observed findings. While the MCT1 and MCT4 isoforms have been discussed and predominantly recognized as the main types within muscle, there are other lesser abundant isoforms that could also contribute to the transport of lactate within this tissue (Bonen, 2000).

The volume response to bumetanide inhibition alone highlights the difference between the effect of separately blocking either the NKCC or the MCT. Although the function of the NKCC was abolished, the response curve observed for bumetanide inhibition shows only a modest peak volume reduction compared to the larger peak volume decrease that is effected when the MCT is abolished via MCT inhibition alone. In comparison to the 7% peak volume loss under bumetanide inhibition, the peak volume loss under pCMBS was 13% while phloretin inhibition caused for a 17% loss (Fig. 6). Compared to both the bumetanide condition and NaLac control condition, this equates to a 50% greater magnitude of volume loss in the pCMBS trial, and a greater than 100% difference in peak volume loss during phoretin inhibition. When lactate is present in the solution, the regulatory volume response does not depend solely on the function of the NKCC. Therefore, the volume response in the face of NaLac treatment is not appreciably impacted whether in the presence or absence of a functional NKCC, which points to skeletal muscle having alternate means of eliciting volume increase independent of the NKCC.

In contrast to increasing the uptake of solute in an attempt to draw in water, the cell may possibly be restricting the rate of solute release from the muscle in an unidentified way as a means of obtaining a similar result of retaining solute. To maintain intracellular solute, the removal of lactate from within the muscle could be reduced while the cell is in this shrunken state. Detecting the decrease in intracellular solute, the cells may possess an unrevealed mechanism of limiting the magnitude at which solutes are extruded from the cell, which under normal conditions would be occurring at a higher rate. In addition, the MCT inhibitors which blocked the supposed uptake of lactate may in

fact be having a secondary inhibitory effect, by blocking the release of the intracellular lactate as well. Taken together, this would provide a possible rationale as to why solute is still elevated within the cell in the presence of an increase in extracellular osmolarity.

4.6.c Cell volume regulation in response to combined NKCC and MCT inhibition

While inhibiting either the NKCC or MCTs had a negative effect on volume regulation, blocking both the NKCC and MCT simultaneously resulted in unanticipated responses. The first was the reduction in peak volume decrease in comparison to the NaLac control condition. This proved to be contrary to what was expected, in that inhibiting both types of transporters was hypothesized to cause a greater and more rapid peak volume loss than the controls. Possible reasoning for such an occurrence may be that the cells elicited alternate mechanisms of retaining solute in a compensatory effort to maintain volume when it was detected that the usual means of solute transport (NKCC and MCTs) are blocked (Lang et al. 1998).

Since cell volume of the muscle fibres were only measured in each protocol following the application of hypertonic treatment, there is no confirmation that the cells did not encounter cell volume or cell structural changes during the period between application of the inhibitory drugs and the point at which the data collection commenced upon hypertonic challenge. Baseline measurements of each muscle fibre were taken prior to the increase in osmolarity exposure, but only after the inhibitors were applied. A limitation in the present protocol was that muscle fibre width was not measured both before and after application of the inhibitors. Since baseline measurements were taken

only following the application of inhibitors, it cannot be ruled out that there were cellular changes to the muscle fibres following the incubation of dual-inhibitors which may have lead to the aforementioned volume response. However, based on independent findings the result may be explained on the basis of greatly increased membrane permeability to solute as a result of 30 mM DMSO in the combined inhibitor trials.

While the cells were capable of volume recovery in the face of combined NKCC / MCT inhibition, it was done so in a biphasic manner. This is observed at the 320 s mark (Fig. 7). Both response curves of bumetanide-pCMBS and bumetanide-phloretin display a steeper recovery response phase beginning at 160 sec. The two treatment conditions then exhibit divergent responses. The data here is however in line with the previously mentioned set of data describing the response to inhibition with phloretin or pCMBS alone. The condition with bumetanide and pCMBS inhibition resulted in a more gradual recovery of volume back to complete recovery over the 30 min response period. On the other hand, the response to the condition of bumetanide combined with phloretin yielded a gradual volume loss which continued through the full 30 min period. The data here resembles that of the solo MCT inhibition conditions, where it was observed that the pCMBS condition ended up with a dramatic overshoot. On the other hand, the phloretin inhibition condition, while still recovered fully, did not have as strong of a RVI.

The greater inhibitory effect of phloretin inhibition compared to pCMBS (Fig. 6) is in line with the response viewed with phloretin and bumetanide inhibition together (Fig. 7), suggesting that phloretin may have a much greater inhibiting effect on MCT volume regulation. On the other hand, the surprisingly responsive RVI observed with pCMBS alone (Fig. 6) which resulted in an overshoot also parallels the findings seen when

pCMBS was combined with bumetanide inhibition (Fig. 7), where muscle fibres here exhibited a full volume recovery near 100% of original volume following an induced extracellular osmolarity increase.

It may be plausible that with the inhibition of the MCTs, an amplified volume regulatory response is recruited within the muscle cells. In the case of inhibiting with only the MCT inhibitors, the amplification of an over-compensatory volume regulation mechanism resembled that of the overshoot in the pCMBS condition. Due to the greater affinity of phloretin to MCT1, it may explain why the recovery response within the phloretin condition is diminished. On the other hand, when both types of transporters are inhibited, the amplified response is dampened due to the inability for the NKCC to exert its usual recovery effect. This then is accurately depicted in the dual-inhibition response curves, whereby dual inhibition with pCMBS displays a 'normal' recovery response but the dual inhibition with phloretin is presented by an inability for recovery.

It has been demonstrated that inhibition of NKCC activity using bumetanide has a depolarization effect on the membrane (van Mil et al. 1997; Geukes Foppen et al. 2002), so it would be noteworthy to see whether the two MCT inhibitors also have a similar effect on the muscle. If the presence of MCT inhibitors also contributes to polarization of the membrane potential, then it would increase the affinity and allow for greater capacity of solute uptake into the cell through other Na^+ transporters. As membrane potential increases sodium channels open, allowing sodium ions into the cell. This would consequently have an effect similar to that of lactate uptake, where volume would be regained in a much more rapid manner, or in this case depicted by a diminished volume loss. While this is a probable explanation for the effect of a reduced volume loss in the

face of dual inhibition, the present experiments did not account for such an occurrence and it would be an ideal area for future research.

The data presented here proposes that skeletal muscle cells are capable of eliciting an active regulatory volume increase in the face of increased osmotic disturbance, even when function of the NKCC is impaired. Once believed to be the primary player involved in controlling volume recovery following cell shrinkage to increase extracellular osmolarity, it would be welcome in considering other significant means of solute uptake and resultant water transport into the cell. Taken together, the rationalization of the aforementioned responses would then support the notion that perhaps while MCT uptake of lactate may be the primary means and the NKCC uptake of solutes the secondary means of volume regulation, under circumstances where the cell is exposed to extreme conditions the deployment of both transporters collectively may be imperative for maintaining adequate regulatory volume response.

4.7 Conclusions

The present study demonstrated that skeletal muscle is capable of regulating volume in the presence and absence of a functional NKCC. During lactate-induced hypertonic challenge, cells demonstrated an RVI of equal magnitude and rapidity as occurred in the presence of NKCC inhibition. Also, it was shown that MCT inhibition alone impaired RVI to a greater extent compared to just NKCC inhibition alone, for blocking the MCTs resulted in greater volume loss and reduced volume recovery. This suggests that skeletal muscle cells are not only dependent on the NKCC for carrying out a

successful RVI, but that they also have the capability to utilize lactate uptake through MCTs to effect RVI. The impact of combined NKCC and MCT inhibition may not have been adequately assessed in the present experiments, however the work does lend to possible means of explanation as to the reasons for the observed responses. Subsequent research is warranted to further explore down this avenue in better understanding the precise mechanisms of how these processes fully function.

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