

## Authors' Reply to Commentary

## Do subcellular fractionation studies of skeletal muscle yield useful information regarding sarcolemmal components?

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Received 7 March 1996

Hansen and Clausen have suggested that poor sarcolemmal recovery, based upon measurements of Na,K-ATPase activity, may lead to misconceptions concerning the constituents of muscle membranes and possible changes with experimental manipulation. In their argument they cite a recent paper in which we investigated whether the GLUT4 glucose transporter and the  $\alpha_2$ -Na,K-ATPase subunit could be colocalised within membranes of intracellular origin. The major finding produced was that GLUT4 and the  $\alpha_2$ -Na,K-ATPase subunit were not in fact colocalised, according to the observation that membranes containing these proteins had different sedimentation properties; furthermore, GLUT4-containing vesicles, isolated by immunoprecipitation from an intracellular muscle fraction, were devoid of both the  $\alpha_2$ - and  $\beta_1$ -subunits of the Na,K-ATPase [1]. The work did not study translocation of Na,K-ATPase and GLUT4 between subcellular membrane fractions nor did it involve the fraction termed the plasma membrane, despite the impression given in the letter by Hansen and Clausen. Nevertheless, the issue of sarcolemmal recovery is an important one and the comments these correspondents made have important implications for those with an interest in muscle physiology and biochemistry; they therefore deserve discussion and further clarification.

A few points need to be emphasized regarding the data concerning isolated membrane fractions and its comparison with that obtained from intact tissues and also about the method used to assess recoveries of the sarcolemma. First, the membrane pellet KpNPPase activities shown in Table 1 of our paper [1] are those obtained from membranes pelleted at the bottom of the sucrose gradient and not in the crude homogenate. The enrichments shown were calculated with respect to the enzymatic activity in crude membranes (for which the data was not shown). If the sarcolemmal recovery is calculated based on the activities normally obtained in the homogenate, then it is about 1.6%. Secondly, the fractionation procedure we used leads to recovery of membranes enriched with sarcolemma but without any significant amounts of transverse-tubular (TT) membranes (based on the lack of reactivity with antibodies to TT membrane markers, e.g. the dihydropyridine receptor) [2]. The ouabain-binding data from intact muscle will reflect association of the glycoside to  $\alpha$ -subunits on both the sarcolemma and the TT. Since the TT membranes constitute nearly 60–70% of the total muscle surface area [3] and also house a sizable pool of immunoreactive Na,K-ATPase  $\alpha$ -subunits [2] their absence from our sarcolemmal fraction would imply that comparison of the amount of ATPase (based on data from this fraction alone) with that of intact tissue is not appropriate. Thirdly, we are in total agreement with Hansen and Clausen that the recovery of muscle

Na,K-ATPase activity, in what is termed the plasma membrane fraction, from a number of studies is small, ranging between 0.2 and 8.9% (reviewed in [4]). The precise reason for this remains poorly understood but in biochemistry it is generally accepted that whenever one attempts to purify membranes to a high degree the final yield is always compromised and this is ever more patent for muscle which is full of cytoplasmic proteins. Furthermore, the suggestion that K<sup>+</sup>-dependent phosphatase activity is susceptible to chemical reagents and physical treatments [5] may imply that measurement of its activity in isolated membrane fractions for calculation of recovery is not entirely satisfactory. This proposition is supported by a recent study [6] in which enzymatic recoveries were compared with those of immunological markers of the sarcolemma. Dombrowski et al. found that their sarcolemmal recoveries of 5'-nucleotidase and K<sup>+</sup>-stimulated phosphatase activity, relative to the starting muscle homogenate, were 5 and 8% respectively [6]. These recoveries are within the range of reported values [4]. However, if the recoveries were calculated based upon immunological markers of the sarcolemma, such as the  $\alpha_1$ -subunit of the Na,K-ATPase or the GLUT1 glucose transporter [7], then it was significantly higher (~15%) [6]; we have carried out similar analyses on our sarcolemmal fraction and obtain recoveries of a similar order of magnitude. This approach may be useful since it overcomes difficulties related to structural integrity, membrane orientation or loss, through inactivation, of enzymatic activity.

Accepting the limitations with regard to the use of enzymatic activities for calculation of sarcolemmal recoveries the central question remains: how representative is this enriched membrane fraction of the total sarcolemma and can studies with it yield meaningful data about sarcolemmal components? In our opinion there are several lines of evidence supporting the view that, despite the relatively low yield of this fraction, it does provide useful information about membrane proteins. Some of this evidence, relating to work concerning glucose transporters and the Na,K-ATPase, is discussed below.

First, immunological analyses of muscle membrane fractions with antibodies against GLUT1 and the GLUT5 hexose transporters reveals that both proteins are predominantly expressed in the fraction termed the plasma membrane [7,8] This finding is consistent with morphological studies carried out at both the light and electron microscopic level which show that both transporters are exclusively confined to the sarcolemma [7–9]. Moreover, it is noteworthy that the human GLUT5 transporter is not expressed in neural, vascular or connective tissue and thus its observed immunoreactivity in the human sarcolemmal fraction (observed using two distinct fractiona-

tion protocols, [8]) is unlikely to have arisen from sources other than the sarcolemma.

Secondly, data from a number of different laboratories, using different fractionation methods which all suffer from problems of low sarcolemmal yield, are qualitatively very similar with regard to the insulin-induced recruitment of GLUT4 from intracellular membrane stores to the sarcolemma [2,10–14]. The increased GLUT4 recovery in the sarcolemmal fraction is not a generalised response since within the same fraction there are no detectable changes in the abundance of proteins such as GLUT1 [7], the  $\alpha_1$ -subunit of the Na,K-ATPase [2,15] or  $\beta_1$ -integrin [13]. The redistribution of GLUT4 to the sarcolemma in response to insulin is consistent with that observed in adipocytes, which afford much higher recoveries of the plasma membrane [16]. Moreover, in our opinion, the increased GLUT4 abundance in the sarcolemmal fraction is likely to be representative of changes in the sarcolemma as a whole, based on observations showing that insulin treatment results in a marked increase in the number of glucose transporters at the cell surface of intact soleus muscles as judged using the exo-facial ATB-[2-<sup>3</sup>H]BMPA photolabel [17].

Thirdly, insulin also stimulates the subcellular redistribution of the  $\alpha_2$ - and  $\beta_1$ -subunits of the Na,K-ATPase in skeletal muscle [15]. This effect is specific for these two isoforms as the hormone does not affect the sarcolemmal abundance of the  $\alpha_1$ - and  $\beta_2$ -Na,K-ATPase subunits [15]. This observation is not an artefact introduced by contaminating non-muscle cell types since electronmicroscopy, using monoclonal and polyclonal antibodies to the  $\alpha_2$ -subunit, shows (a) significant intramuscular labelling of the  $\alpha_2$ -subunit and (b) that insulin induces a significant increase in the number of immunogold particles (i.e.  $\alpha_2$ -subunits) at the muscle surface [18].

One final piece of evidence which we wish to draw attention to concerns the finding that sarcolemmal fractions prepared from red or white muscle display distinct immunoreactivity patterns for the  $\beta_1$  and  $\beta_2$ -subunit isoforms. The  $\beta_1$ , but not the  $\beta_2$ , subunit is only detectable in fractions prepared from red (slow-oxidative, Type I) muscles, whereas, only  $\beta_2$  is detected in fractions prepared from white (fast-glycolytic, Type IIb) muscles [19]. It is difficult to attribute such findings to contamination of the sarcolemma by membranes of non-muscle origin which are unlikely to be separated in a muscle type-specific manner. Our protein data were also mirrored by distribution of RNA transcripts for these two  $\beta$ -isoforms in the different muscle types [19]. Such information not only underscores the muscle type-specific expression of  $\beta$ -subunit isoforms but strengthens the suggestion that information gleaned from studies using the sarcolemmal fraction is meaningful. Indeed, the biochemical observations made in isolated membranes have now been confirmed by immuno electron micro-

scopy, demonstrating that  $\beta$ -subunits are differentially expressed in muscles of different fibre type, and that insulin induces  $\beta_1$  redistribution [20].

The problem of poor sarcolemmal recovery from skeletal muscle is widely recognised and most investigators accept that the limitations of the isolation procedure make it far from perfect. Nevertheless a substantial amount of evidence has now accumulated clearly indicating that analyses of the sarcolemmal fraction have made major contributions to important new discoveries regarding sarcolemmal composition [8,19] and the dynamics of physiological membrane processes [2,11,15].

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