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Ionic movements mediated by monensin in frog skeletal muscle

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Monensin-mediated ionic movements were studied in frog skeletal muscle. The ionophore, which forms electrically neutral complexes with monovalent cations, induced dose dependent fluxes of Na^+, K^+ and H^+ in and out of the fibers. Monensin concentrations ([MON]) ranged from 2 to 40 μM. In the presence of normal Ringer's solution the following maximum ionic exchanges were generated by monensin (in pmol cm^{-2} s^{-1}): (1) Na_i^+/Na_o^+ 112, (2) Na_i^+/H_o^+ 30.7, (3) K_i^+/Na_o^+ 14.2 (4) H_i^+/Na_o^+ 49. The maximum net fluxes produced by these exchanges (i.e. for [MON] = ∞) are (in pmol cm^{-2} s^{-1}): Na^+ (inward) 32.5, K^+ (outward) 14.2, H^+ (outward) 18.3. The last one appears to be largely offset by a passive (monensin-independent) H^+ influx down an inwardly directed electrochemical gradient promoted by pH reduction of the T-tubular lumen content as a consequence of the monensin-mediated net H^+ efflux. Maximum unidirectional cationic fluxes mediated by monensin amounted to 206 pmol cm^{-2} s^{-1} and had the following composition: influx: 85% Na^+ and 15% H^+; efflux: 69% Na^+, 7% K^+, 24% H^+.

Introduction

Monensin is a carboxylic ionophore with a marked selectivity for sodium over other monovalent cations. The monensin-cation complex is electrically neutral and its formation requires the deprotonation of the terminal carboxyl group of the ionophore [1,2].

The purpose of this work was to study how, and to which extent, monensin would affect the movements of Na^+ and K^+ across the sarcolemma of frog muscle fibers, a preparation where the fluxes of these two ions in the absence of the ionophore have been extensively investigated. It was found that addition of the ionophore to the normal external medium increases the unidirectional fluxes of these two ions leading to an exchange of external Na^+ for internal K^+ and H^+ in a saturable [MON]-dependent fashion.

As far as we know this is the first report on monensin-mediated ionic transport across the sarcolemma of skeletal muscle fibers.

Materials and Methods

Experiments were performed on isolated sartorius muscle from the Argentine frog *Leptodactylus ocellatus* at room temperature (20 to 22°C). Monensin and ouabain were purchased from Sigma Chemical Co., USA.

The stock solutions of monensin were made by disolving the antibiotic in ethanol (5 to 25 mM). Control experiments indicated that ethanol concentrations as high as 10 mM in the experimental solutions did not affect, per se, Na^+ and K^+ fluxes.

$^{24}Na^+$ was obtained from Comisión Nacional de Energía Atómica CNEA, Argentina).

Normal bathing medium had the following composition (in mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Tris maleate buffer, 2 mM (pH 7.15). In some experiments Na^+ was replaced either by $Tris^+$ or Mg^{2+}.

When Mg^{2+} was used to replace Na^+, 76.7 $MgCl_2$ was substituted for 115 mM NaCl. In some experiments (indicated in the text) Tris maleate buffer was replaced by phosphate buffer (2.15 mM Na_2HPO_4, 0.85 mM NaH_2PO_4).

Net fluxes of Na^+ and K^+ were determined by atomic absorption spectrophotometry (Shimadzu AA 630-12) by comparing Na^+ and K^+ content in paired muscles: one member of the pair in the presence of a given [MON] and the other (control) in its absence, at different times of exposure to those solutions.

H^+ efflux promoted by 40 μM was estimated by incubating four to eight sartorii (total weight ranging from 195 to 580 mg) for 2 h in 5 ml of Ringer (phosphate buffer) containing 40 μM monensin (experimental muscles) and in the same medium without

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monensin (paired control muscles). Monensin-mediated H^+ efflux was calculated, using the Henderson-Hasselbalch equation, from the difference between the amounts of proton released by muscles in the presence and in the absence of the ionophore.

Na^+ efflux was measured using $^{24}Na^+$ as previously described [3]. Briefly, muscles were first loaded with $^{24}Na^+$ by exposing them to normal Ringer's solution labelled with the isotope for 2.5 h. Subsequently, they were washed in a series of tubes containing 3 ml of unlabelled media at intervals varying between 10 and 15 min. At the end of the experiment, muscles and washout tubes were counted in a gamma counter. The rate of release of the isotope was calculated using a microcomputer and expressed in terms of rate of fractional loss (^{Na}k, unit min^{-1}).

Na^+ influx measurements were performed in paired sartorii, one of them in normal Ringer and the other in the same medium plus 2 μM monensin. Preparations were equilibrated for 1 h in their respective solutions and then exposed to the same media labelled with $^{24}Na^+$ for 17 min. Subsequently they were washed in a series of tubes containing unlabelled Na^+ free (Mg^{2+}) Ringer plus 30 μM ouabain. Influx was calculated from extrapolation to time = 0 of the slow monoexponential component of the washout. Extrapolated values were corrected for back-flow as previously described in detail [4].

Influx measurements are expressed in terms of unit area of superficial sarcolemma (T tubules excluded) using the relationship 430 cm^2 per g of muscle [5]. Under this conditions the value of the extracellular space (Na^+ space) is on the average 29% v/w [6] and intracellular solids amounted to 16.8 ± 1.5% w/w ($n = 76$) or 11% ± 1.0 v/w (assuming a density 1.5 g cm^{-3}). This leads to an intracellular water volume on the order of 60% v/w.

Non isotopic K^+ efflux into K^+ free media was determined by atomic absorption spectrophotometry following the same methodology used for $^{24}Na^+$ efflux measurements and also expressed in terms of rate of fractional loss (^{K}k).

If not otherwise stated all experimental solutions contained 30 μM ouabain to block active Na^+/K^+ transport. As a rule, exposure of muscles (control and experimental) to ouabain containing medium began 1 h before exposing the experimental ones to monensin.

Student's *t*-test was used to estimate statistical significance of differences. Values are expressed as means ± 1 S.E. A non-linear regression software was used for curve fitting.

Results

Fig. 1A shows the effect of 2 μM monensin on the rate of $^{24}Na^+$ fractional loss (^{Na}k) from a pair of sartorii in the presence of normal Ringer's solution and in the same medium, plus 30 μM ouabain. The marked increase of ^{Na}k produced by the ionophore is apparently unrelated to the activity of the Na^+ pump. The efflux increase is completely reversible upon withdrawal of the ionophore although the off is clearly slower than the on effect.

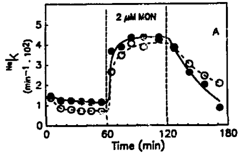

Fig. 1. Effect of 2 μM monensin on the rate of fractional loss of $^{24}Na^+$ (^{Na}k) from paired sartorii bathed in normal Ringer, one of them in the presence of 30 μM ouabain from time = 0 to the end of the experiment (○) and the other in ouabain-free medium (●).

Na^+ influx was also considerably increased by 2 μM monensin: in four muscles exposed to this concentration of the ionophore it was 9.8 ± 2.1 pmol cm^{-2} s^{-1} while in the paired controls it was 1.6 ± 0.5 pmol cm^{-2} s^{-1}.

The fact that both efflux and influx are markedly increased by monensin, suggests that in the sarcolemma, as it occurs in artificial membranes and red blood cells under physiological conditions [1,2,7], the ionophore mediates a Na^+/Na^+ exchange. If that were the case, then, the replacement of external Na^+ by some other cation with little or no affinity for the ionophore, should produce a sizable reduction of ^{Na}k. This notion was supported by experiments like the one depicted in Fig. 2 which shows the effect of 3.5 μM monensin on ^{Na}k first in the absence of external Na^+ (replaced by Mg^{2+}) and subsequently in its presence. It is clear that in Na^+ free medium the response to

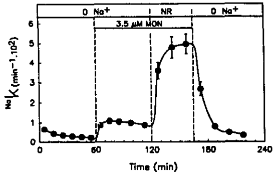

Fig. 2. Na^+ dependence on the monensin-mediated increase in $^{24}Na^+$ rate of fractional loss (^{Na}k). Effect of 3.5 μM monensin, first in the absence of Na^+ (replaced by Mg^{2+}) and subsequently in its presence. All solutions contained 30 μM ouabain. Symbols represent means ± 1 S.E. (errors bars absent when smaller than symbol) from four experiments.

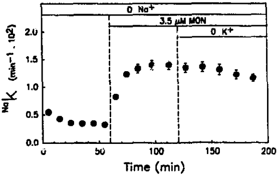

Fig. 3. Increase in the rate of $^{24}Na^+$ fractional loss (^{Na}k) in Na^+-free medium (Tris replacement) produced by 3.5 μM monensin is not significantly affected by withdrawal of K^+ from the external medium. Ouabain (30 μM) was present throughout. Means ± 1 S.E. (error bar absent when smaller than symbol) from four experiments.

monensin is considerably smaller than that in the presence of Na^+. This is in agreement with results obtained in artificial membranes and red blood cells showing a much greater preference of monensin for Na^+ than for any other cation which makes the ionophore to act mainly as an exchange diffusion carrier for Na^+. When $Tris^+$ instead of Mg^{2+} was used to replaced external Na^+, a similar result was obtained.

The possibility that a significant fraction of the increase in Na^+ efflux generated by monensin in Na^+ free media might represent an exchange of internal Na^+ for external K^+ was ruled out by experiments like the one shown in Fig. 3 where it can be seen that the suppression of K^+ from the external medium has no significant effect on the ^{Na}k increase mediated by the ionophore. This reflects the small probability of K^+ complexation at the external side of the membrane likely due to both, the low $[K^+]_o$ as compared with $[Na^+]_o$, and the higher affinity of Na^+ for monensin.

Another possible mechanism that could account for the remaining monensin-mediated efflux in Na^+ free media is a Na_i^+/H_o^+ exchange. The data in Fig. 4A suggest that, indeed, this might be the case. Thus, in Na^+ free medium an increase of the external pH from 7.2 to 9.2, produced a marked reduction of the monensin-mediated ^{Na}k increase. Such a reduction would even be larger were it not for the fact that the increase in pH induced, in the absence both of monensin and Na^+, an increase in ^{Na}k of about 65% (Fig. 4B) whose nature, we cannot account for at present. On the other hand, a reduction of external pH to 6.2 produced an increase of monensin-mediated ^{Na}k (Fig. 5A), an effect not seen in the absence of monensin (Fig. 5B).

As might be expected the stimulation of Na^+ efflux by monensin is dose-dependent. Fig. 6 illustrates the relationship between the increment of ^{Na}k and [MON] in the presence and in the absence of external Na^+. It can be seen that under those two conditions the magnitude of the effect tends to saturate as [MON] increases. A simple explanation for this behavior would be that there is a maximum number of monensin molecules that can be lodged in the sarcolemma.

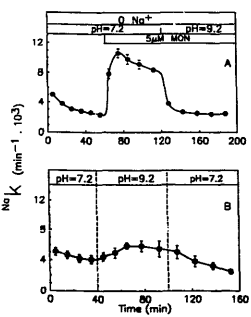

Fig. 4. (A) Effect of increasing pH from 7.2 to 9.2 on monensin (5 μM) mediated increase in $^{24}Na^+$ fractional loss in the absence of Na^+ (replaced by Mg^{2+}). Means from five experiments. (B) Effect of the same increase in pH under similar experimental conditions but in the absence of monensin. Means from four experiments. All solutions in A and B contained 30 μM ouabain. Error bars: ± 1 S.E. (absent when smaller than symbols).

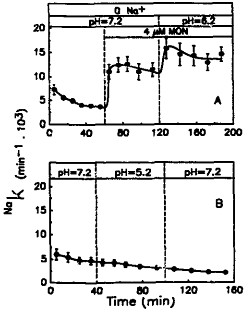

Fig. 5. (A) Effect of decreasing pH from 7.2 to 6.2 on monensin (4 μM) mediated increase of ^{Na}k in Na^+-free medium (Mg^{2+} replacement). Means from four experiments. (B) Lack of effect of a pH reduction to 5.2 on ^{Na}k in Na^+ free medium and in the absence of monensin. Means from four experiments. All solutions in A and B contained 30 μM ouabain. Error bars: ± 1 S.E. (absent when smaller than symbol).

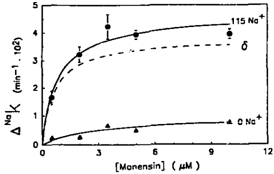

Fig. 6. Increase in fractional loss rate of $^{24}Na^+$ ($\Delta^{Na}k$) produced by monensin as function of inophore concentration from muscles in the presence (●) and, their paired companions, in the absence (▲) of Na^+. Lower curve: fit of the data obtained in Na^+-free medium (filled triangles) to eqn. 1 ($\Delta^{Na}k_m = 0.0097$ min^{-1}; $K_{0.5} = 2.87$ μM). It represents the Na_i^+/H_o^+ exchange component of $\Delta^{Na}k$. Middle curve (δ): fit of the differences between paired values (at $[Na^+]_o$ = 115 mM and $[Na^+]_o = 0$) to Eqn. 1 ($\Delta^{Na}k_m = 0.0379$ min^{-1}; $K_{0.5}$ = 0.66 μM). It represents the Na_i^+/Na_o^+ component of $\Delta^{Na}k$. Upper curve: the sum of lower and middle curves given by $\Delta^{Na}k = \{0.0379\,[MON]/(0.66+[MON])\} + \{(0.0097\,[MON]/(2.87+[MON])\}$. Each pair of symbols represent means from four to eight pairs of muscles and error bar (absent when smaller than symbol) ± 1 S.E. All solutions contained 30 μM ouabain.

If the ionophore induces Na_i^+/Na_o^+ and Na_i^+/H_o^+ exchanges, it seems reasonable to assume that under our experimental conditions, in the presence of normal Ringer's solution the monensin-mediated increase in ^{Na}k is given by the sum of those two components. Hence the data obtained in Na^+ free medium should represent the Na_i^+/H_o^+ while the difference between the ^{Na}k increase induced by monensin in the presence of Na^+ (115 mM) and that in its absence, would represent the Na_i^+/Na_o^+ exchange. Both these components were fitted to the following hyperbolic function,

$$\Delta^{Na}k = \Delta^{Na}k_m[MON]/(K_{0.5}+[MON]) \qquad (1)$$

where $\Delta^{Na}k$ is the increment in the rate of Na^+ fractional loss at a given [MON], $\Delta^{Na}k_m$ is the maximum $\Delta^{Na}k$ (i.e. $\Delta^{Na}k$ for [MON] = ∞) and $K_{0.5}$ the [MON] at which $\Delta^{Na}k = \Delta^{Na}k_m/2$. The values of the parameters yielded by fitting the Na_i^+/Na_o^+ component to equation 1 are: $\Delta^{Na}k_m = 0.0397$ min^{-1} and $K_{0.5} = 0.66$ μM and for the Na_i^+/H_o^+ component: $\Delta^{H}k_m = 0.0097$ min^{-1} and $K_{0.5} = 2.87$ μM.

The upper curve in Fig. 6 represents the sum of the curves fitted to Na_i^+/Na_i^+ (middle curve) and the Na_i^+/H_o^+ (lower curve) components. It is apparent that the upper curve is a reasonably good fit of the $\Delta^{Na}k$ values obtained in the presence of normal Ringer. The simplest conclusion to be drawn from this analysis is that monensin, as it does in artificial lipid membranes, in addition to sodium exchange, induces a sodium/proton exchange accross the sarcolemma. The maximum monensin-mediated increase in ^{Na}k is about four times greater (0.0379/0.0097 = 3.91) for Na_i^+/Na_o^+ than for Na_i^+/H_o^+ exchange.

The intracellular content of Na^+ was also altered by monensin. Fig. 7A shows the time-course of the change in Na^+ content of muscles in the presence of 5 μM monensin. Determinations were done at 30, 60 and 120 min exposure to the ionophore and in the presence of 30 μM ouabain to block the Na^+ pump. Paired control muscles were kept in the same medium but without monensin. The difference in Na^+ content under these two conditions was taken as the monensin-mediated increase in Na_i. It can be seen that the increase in Na_i^+ produced by the ionophore was linearly related to time. The slope of the line represents a monensin-mediated net Na^+ influx which in this experiment amounted to 0.183 μM g^{-1} min^{-1} of muscle or 7.10 pmol cm^{-2} s^{-1}. A similar linear relationship was found in the presence of monensin concentrations ranging from 1 to 40 μM.

Since monensin increases $[Na^+]_i$, the ionophore should mediate an exchange of Na_o^+ for an intracellular cation other than Na^+. Given the abundance

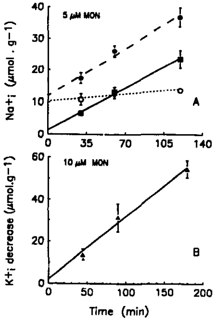

Fig. 7. (A) intracellular Na^+ content as a function of exposure time to 5 μM monensin plus 30 μM ouabain (●) and in the presence of 30 μM ouabain alone (○) in paired preparations. Differences between paired values are represented by filled squares. Linear regresion lines were fitted to each set of values. The slope of the solid line represents the net Na^+ influx mediated by monensin. Each pair of values (●, ○) were obtained from eight muscles (four pairs) Symbols indicate means ± 1 S.E. (error bar absent when smaller than symbol). (B) Reduction in intracellular K^+ mediated by 10 μM monensin as a function of exposure time to the ionophore. Values were obtained following the same procedure used in A to calculate the monensin-mediated increase in Na_i^+. The slope of the line (regresion fit) represents the net K^+ efflux induced by the ionophore whose value is 0.295 ± 0.033 μM g^{-1} min^{-1} or 11.4 ± 1.3 pmol cm^2 s^{-1}. Each filled triangle is the mean value obtained from four pairs of muscles (error bar = ± 1 S.E.).

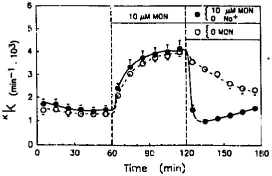

Fig. 8. Rate of fractional loss of K^+ (${}^{K}k$) from four pairs of muscle into K^+ free medium determined by AA spectrophotometry. At the 60-min mark all preparations were exposed to 10 μM monensin which produced a considerable efflux increase. From the 120-min mark to the end of the run four muscles (one member of each pair) were bathed in monensin-free medium (○), while their paired companions were exposed to Na^+-free medium (Mg^{2+} replacement) and the same [MON] (●). Notice that although both maneuvers abolish the effect of monensin, Na^+ withdrawal acts much faster than ionophore supression. All solutions contained 30 μM ouabain. Symbols represent means ± 1 S.E. (error bar absent when smaller than symbol).

of K^+ in the cytosol, a possibility that seemed likely was that Na^+ uptake could occur through a K_i^+/Na_o^+ exchange. The experiment shown in Fig. 8 indicates that, indeed, Na^+ uptake is linked, at least partly, to a monensin-mediated K^+ exit. It can be seen that 10 μM monensin produced a marked release of K_i^+ which was canceled either by withdrawal of the drug or by Na^+ free medium despite the presence of monensin. $[Na^+]_o = 0$ quickly abolished the monensin-mediated increase in the rate of K^+ fractional loss, while withdrawal of monensin from the external medium without changing $[Na^+]_o$ resulted in a much slower effect. This difference is not surprising since the release of the ionophore from the membrane matrix would almost certainly take a longer time than that required for the Na^+ clearance of the extracellular space.

It is likely that in the absence of external Na^+ and K^+ a fraction of the ionophore molecules reaching the external side of the membrane as monensin-K^+ complexes might return to the internal side in their protonated form. As shown in Fig. 6 and below, about 20% of monensin molecules moving outwards associated with Na^+ return to the inner side protonated (and the rest complexed with Na^+). Probably, a similar proportion of protonated and Na^+ complexed monensin molecules would move back to the inner side after releasing K^+ in the external medium. This, however, is not apparent in Fig. 8 where in Na^+ free medium one would expect ${}^{K}k$ to fall to a level somewhat higher than the basal one. This suggests that the K_i^+/H_o^+ exchange might be very small, although, its magnitude remains to be determined.

Fig. 7B illustrates the time course of the K^+ loss mediated by 10 μM monensin under conditions identical to those used to determine Na^+ uptake. As in the case of Na_i^+ increase, K^+ loss was linearly related to the exposure time to the ionophore. We found that monensin-mediated net Na^+ influx (${}^{Na}J$) was greater than net K^+ efflux (${}^{K}J$) regardless of [MON]. This is illustrated in Fig. 9. It is clear that throughout the whole range of [MON]'s (1 to 40 μM) ${}^{Na}J > {}^{K}J$. Data in Fig. 9A are fitted to a hyperbolic function (similar to Eqn. 1), that is, $J = J_m[\mathrm{MON}]/(K_{0.5} + [\mathrm{MON}])$. The parameters of the curves, i.e., maximum flux (J_m) and $K_{0.5}$ are: ${}^{Na}J_m = 32.5 \pm 5.4$ pmol cm^{-2} s^{-1}, ${}^{K}J_m = 14.2 \pm 2.8$ pmol cm^{-2} s^{-1}, ${}^{Na}K_{0.5} = 11.6 \pm 5.2$ μM, ${}^{K}K_{0.5} = 6.7 \pm 4.2$ μM. As can be seen in panel B, for physiological ionic gradients across the sarcolemma ${}^{Na}J/{}^{K}J$ ratio, is about 2 and apparently independent of [MON].

In cylindrical cells the relationship between Na^+ efflux (${}^{Na}J_o$) and ${}^{Na}k$ is given by: ${}^{Na}J_o = {}^{Na}k[Na^+]_i r/2$ [8], where r is the fiber radius. Fig. 10A shows $[Na^+]_i$ as a function of [MON] after 1 h exposure to the ionophore, a time at which the effects of monensin are well established. From these data and the values of ${}^{Na}k$ in the presence of monensin (also at 1 h exposure) and in its absence, the increase in J_o (ΔJ_o, i.e., the mon-

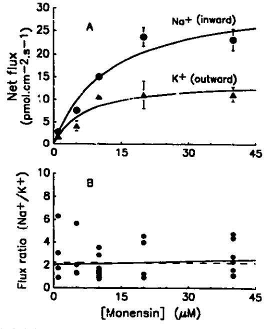

Fig. 9. (A) Net fluxes (J) of Na^+ (●) and K^+ (▲) mediated by monensin determined in the same preparation as a function of the ionophore concentration. Data were fitted to a hyperbolic function of the form $J = J_m[\mathrm{MON}]/(K_{0.5} + [\mathrm{MON}])$, where J_m represent the maximum flux and $K_{0.5}$ the [MON] at which $J = J_m/2$. The parameters' values are ${}^{Na}J_m = 32.5 \pm 5.6$ pmol cm^{-2} s^{-1}, ${}^{Na}K_{0.5} = 11.6 \pm 5.2$ μM, ${}^{K}J_m = 14.3 \pm 2.8$ pmol cm^{-2} s^{-1}, ${}^{K}K_{0.5} = 6.7 \pm 4.2$ μM. Each symbol represents the mean from 4 to 12 muscles. Data were obtained as shown in Fig. 7. (B) ${}^{Na}J/{}^{K}J$ from all preparations used [32]. The solid line represents the linear regresion fit to data and the dashed line denotes the mean of all values.

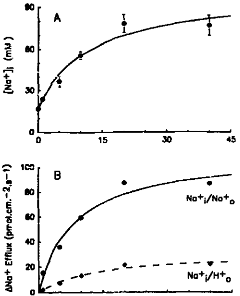

Fig. 10. (A) Intracellular Na$^+$ concentration as a function of [MON] for 1 h exposure to the ionophore and in the presence of 30 μM ouabain for 2 h. Values are fitted to equation: $[Na^+]_i = \{(83.9 [MON])/(11.6 + [MON])\} + 16.7$ mM. The value of $[Na^+]_i$ for [MON] = 0 (16.7 mM) represents the mean from 32 muscles, the rest of the filled circles are means from 4 to 12 muscles. In normal Ringer in the absence of monensin and ouabain $[Na^+]_i = 13.5 \pm 1.3$ ($n = 32$). Error bars: ±1 S.E. (absent when smaller than symbol. (B) Na_i^+/Na_o^+ (●) and Na_i^+/H_o^+ (◆) efflux components as a function of [MON] calculated according to Eqn. 2 (see text).

ensin mediated Na$^+$ efflux) can be estimated using the following equation:

$$\Delta^{Na}J_o = ({}^{Na}k'[Na^+]_i' - {}^{Na}k[Na^+]_i)r/2 \qquad (2)$$

where primed terms represent values in the presence of monensin. A mean fiber diameter of 62 μm has been estimated for this preparation [5] and therefore an r value of 31 μm was used. Fig. 10B shows Na_i^+/Na_o^+ and Na_i^+/H_i^+ components of Na$^+$ efflux mediated by monensin as a function of ionophore concentration. The fitting of hyperbolic dose-response curves to the data yielded a maximum Na$^+$ efflux of 112 ± 13 pmol cm^{-2} s^{-1} for Na_i^+/Na_o^+ exchange and 30.7 ± 4.3 pmol cm^{-2} s^{-1} for Na_i^+/H_o^+ exchange. The $K_{0.5}$ values were 8.5 ± 2.9 and 12.7 ± 4.4 μM for Na_i^+/Na_o^+ and Na_i^+/H_o^+ exchange, respectively. This means that in the presence of normal Ringer's solution, about 80% of monensin molecules moving out associated with Na$^+$, return to the inner face of the sarcolemma as monensin-Na$^+$, while 20% do so in the protonated form.

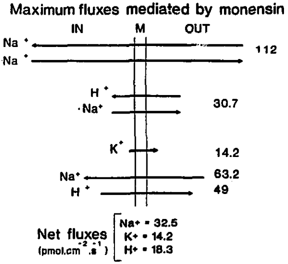

Fig. 11. Maximum unidirectional and net fluxes mediated by monensin across the sarcolemma (M). Numbers on the right denote values of fluxes in pmol cm^2 s^{-1}. The value of the sixth arrow from the top is given by 32.5 (net Na$^+$ influx) + 30.7(Na_i^+/H_o^+ exchange) = 63.2 pmol cm^{-1} s^{-1}. Total cationic influx is 205.9 pmol cm^2 s^{-1} and since the ionophore mediates electrically neutral ionic exchanges it should be equal to total cationic efflux. The value of the lower H$^+$ arrow is given by 205.9 − (112 + 30.7 + 14.2) = 49 pmol cm^{-2} s^{-1}.

Maximum unidirectional and net fluxes mediated by monensin are summarized in Fig. 11. Total Na$^+$ influx calculated as the sum of all Na$^+$ inward going arrows in Fig. 11 is 175.2 pmol cm^{-2} s^{-1}. In excellent agreement with this estimate the measurement of total Na$^+$ influx yielded a maximum value of 177 pmol cm^{-2} s^{-1} (Fig. 12). The difference between Na$^+$ gain and K$^+$ loss mediated by monensin would, in all probability, be accounted for by a H$^+$ efflux carried by the ionophore in its undissociated form, as it occurs in red blood cells (Painter and Pressman, 1982). Thus, the maximum mediated net H$^+$ flux (i.e. for [MON] = ∞) would be 32.5-14.2 = 18.3 pmol cm^{-2} s^{-1} and for [MON] = 40 μM, the highest concentration used here, it would be 13 pmol cm^{-2} s^{-1}. This means that, for example, 200

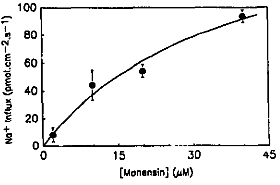

Fig. 12. Monensin-mediated Na$^+$ influx as a function of [MON]. Experiments were performed on paired muscles. ^{24}Na$^+$ influx was measured in both members of the pair one in the presence of normal Ringer containing 30 μM ouabain plus monensin and the other in the presence of the same medium but without monensin. The influx difference between the two muscles represents the monensin-mediated influx. Each circle is the mean from four muscle pairs and error bars represent ± 1 S.D. Curve corresponds to the fitting of the data to a hyperbolic Michaelian function whose parameters are: Maximum influx = 177+57 pmol cm^{-2} s^{-1} and [MON] for half-maximum influx = 37 μM.

TABLE I

Apparent H^+ efflux mediated by 40 μM MON (Δ^HJ)

Expt.	^HJ (pmol cm^{-2} s^{-1})		Δ^HJ
	control	MON	
a	1.051	1.101	0.050
b	1.368	1.635	0.267
c	1.018	1.300	0.282
d	0.489	0.561	0.072
e	0.446	0.574	0.128
Means ± 1 S.E.	0.874 ± 0.177	1.034 + 0.209	0.159 + 0.049 *

* $P < 0.05$.

mg of muscle in the presence of 40 μM monensin would release 4 μmol of H^+ ion in 1 h. Such a release of H^+ would represent an increase in $[H^+]_o$ of 0.8 mequiv. If the external medium were 5 ml of phosphate-buffered normal Ringer's solution ($[Na_2HPO_4]$ = 4.3 mequiv.; $[NaH_2PO_4]$ = 0.85 mequiv.), according to the Henderson-Hasselbalch equation, the reduction of pH_0 (ΔpH_o) would be: $\Delta pH_o = \log (4.3(0.85 + 0.8)/0.85\ (4.3-0.8)) = 0.38$. This possibility was tested in five experiments where it was measured the pH change produced in 5 ml of Ringer containing 40 μM monensin by the addition of 203 to 598 mg of muscle. Paired control muscles underwent a similar treatement except that the medium was monensin free (see Methods). Table 1 shows that, under these conditions, the estimated monensin-mediated H^+ efflux (Δ^HJ^m) is about two orders of magnitude lower than that calculated as the difference between Na^+ and K^+ net fluxes. Biological membranes are highly permeable to H^+, this discrepancy, therefore, might reflect the presence in the sarcolemma of a monensin independent H^+ back-flow. Such a possibility is strenghened by the fact that about 4/5 of monensin-mediated H^+ efflux should take place through T-tubular membrane. T-tubular volume represents about 0.3% of fiber volume [9] and the ratio between tubular surface area and volume (a/v) is on the order of $5 \cdot 10\ cm^{-1}$. It is not unreasonable, therefore, to assume that [H] in the tubular lumen ($[H^+]_T$) may raise considerably in the presence of monensin, producing a large increase in the inwardly directed H^+ electrochemical gradient. If the inward driving force on H^+ were large enough and considering that H^+ permeability in frog muscle fibers is about 10^{-3} cm s^{-1} [10], most H^+ ions released by monensin in the tubular lumen would flow back passively to the cytosol. Consequently, little or no change in pH_0 would occur under experimental conditions where, otherwise, a change on the order of 0.5 to 1 pH units would be expected. The model discussed in the Appendix suggests that this might well be the case.

Discussion

In this work we have investigated the effect of monensin on Na^+ and K^+ movements through the sarcolemma of frog skeletal muscle fibers, a preparation were the fluxes of these iones through a variety of pathways are reasonably well characterized.

Upon deprotonation, monensin forms electrically neutral complexes with alkali metal ions. The ionophore Na^+/H^+ selectivity ratio is 10 [1]. We found that the maximum monensin-mediated Na^+ efflux, 142.7 pmol cm^{-2} s^{-1} (= 112 + 30.7, Fig. 11), is 10-times greater than the maximum monensin-mediated K^+ efflux (14.2 pmol cm^{-2} s^{-1}), even though under our experimental conditions, $[Na^+]_i$, although significantly increased (84 mM, see Fig. 10A), was still lower than $[K^+]_i$ (about 134 mM) *. This is a measure of the preference of monensin for Na^+ over K^+ at the inner side of the membrane. The fact that in the external medium $[Na^+]_o$ is 46-times greater than $[K^+]_o$, explains why we were unable to detect a monensin-mediated K^+ influx: the probability of K^+ complexation at the external membrane interface is much lower than at the internal one.

At variance with channel-forming molecules (gramicidin, for instance), ionic movements mediated by ionophores like monensin are insensitive to membrane potential and rate limited by the ionophore's diffusion time across the membrane [12]. Therefore, the fact that mediated Na^+ fluxes are greater than mediated K^+ efflux does not mean that the monensin-Na^+ complex moves faster than the monensin-K^+ complex across the sarcolemma. It rather shows that within the membrane the number of monensin molecules complexed with Na^+ is greater than that associated with K^+.

As shown in Fig. 11, of the total maximum cationic influx mediated by monensin (205.9 pmol cm^{-2} s^{-1}), 85% (175.2) corresponds to Na^+ and 15% (30.7) to H^+. The composition of cationic efflux, on the other hand, is 69% (142.7) Na^+, 7% (14.2) K^+ and 24% (49) H^+. These values correspond to 1 h exposure to [MON] = ∞. Maximum $[Na^+]_i$ under these conditions was estimated to be 84 mM (see Fig. 10A).

When maximun monensin-mediated Na^+ influx and efflux are compared with $[Na^+]_i$ (see above and Fig. 10A) and $[Na^+]_o$, it is found that the monensin-mediated Na^+ influx/efflux ratio (175.2/142 = 1.23) is close to the $[Na^+]_o/[Na^+]_i$ ratio (115/84 = 1.37). This suggests that the degree of complexation at each side of

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* For a surface/muscle weight ratio of 430 cm^2/g and 60% of intracellular water (see Methods), a net outward flux of 14.2 pmol cm^{-2} s^{-1} represents a K^+ loss of about 6 mmol per 1 of intracellular water per hour. Therefore, assuming a normal $[K^+]_i$ of 140 mM [11], we have: 140 − 6 = 134 mM.

the membrane is proportional to the corresponding $[Na^+]$.

From the data in (Fig. 9) it can be estimated that 1 h exposure to $[MON] = 10\ \mu M$, a concentration that produces about half-maximum net fluxes, will increase $[Na^+]_i$ by 39 mM and reduce $[K^+]_i$ by 22 mM, respectively. The difference, 17 mM, represents the exit of H^+ due to the outward movement of the ionophore in its protonated form.

In addition to the cytosolic buffering power, which is about 30 mM H^+/pH unit [13–16], other mechanisms may contribute to attenuate the cytoplasmic alkalinization produced by the electroneutral exchange of Na_o^+ for K_i^+ and H_i^+. One of them would be the slower pace or stoppage of Na^+/H^+ exchange due to reduction of Na^+ and H^+ gradients as a consequence of the $[Na^+]_i$ increase and reduction in $[H^+]_i$. In this regard, it should be mentioned that cytosolic alkanization induced by monensin has been shown to occur in chick skeletal muscle fibers [17] bringing the Na^+/H^+ exchanger close to equilibrium where little or no exchange takes place.

Another system that could act indirectly as a buffer is the Na^+/Ca^{2+} exchanger which in frog muscle accounts for about one third of Ca^{2+} efflux [18]. Thus, the reduction of the Na^+ gradient promoted by monensin could produce an increase in $[Ca^{2+}]_i$ which in turn, might displace H^+ from binding sites in some proteins or by Ca^{2+}/H^+ exchange between organelles and cytosol as it occurs in heart Purkinje fibers [19].

The fact that under experimental conditions where the calculated proton efflux is about 13 pmol $cm^{-2}\ s^{-1}$ (i.e. in 40 μM MON) we could only detect a pH_o change corresponding to a H^+ efflux of only 0.16 pmol $cm^{-2}\ s^{-1}$, suggested the possibility that a sizable fraction of H^+ ions may passively flow back to the cytosol. This back-flow, provided that an adequate electrochemical proton gradient is formed, would be favored by the high membrane permeability to H^+ ions. T tubules with their large surface/volume ratio seem to be a suitable structure for the buildup of the necesary inward driving force on H^+. It is reasonable to assume that monensin distributes homogeneously throughout the sarcolemma and therefore, about 80% of the H^+ ions moving outward as the ionophore protonated form will be released in the tubular lumen which represents less than 1% of the fiber volume. The model in the appendix shows that, in the presence of monensin, H^+ concentration in the tubules ($[H^+]_T$) may be large enough to generate a back-flow similar in magnitude to the H^+ efflux mediated by monensin. Even in the surface membrane there might be a significant H^+ back flow. It has been shown that pH at the surface of rat skeletal muscle and sheep Purkinje fibers is lower than in the bulk solution [20], so that, conceivably, in the presence of monensin, $[H^+]$ in the unstirred layer might raise suficiently to generated a back-flow also at the surface sarcolemma. Proton back-flow may then represent an important contribution to the maintenance of cytosolic pH near its normal value in spite of the presence of monensin which, otherwise, would produce an appreciable intracellular alkalinization.

In summary, in frog muscle fibers monensin mediates rather large ionic exchanges, mainly Na^+/Na^+, but also Na^+/H^+ and Na^+/K^+, which result in a net gain of Na^+ and a net loss of K^+ and H^+. The calculated maximum net fluxes mediated by the ionophore in the presence of normal Ringer's solution are 32.5, 14.2 and 18.3 pmol $cm^{-2}\ s^{-1}$ for Na^+, K^+ and H^+, respectively, that is, 7 Na_o^+ for 3 K_i^+ and 4 H_i^+.

Appendix

The rate of change of tubular proton concentration ($[H^+]_T$) can be estimated using Equation A – 1 which is similar to that used by Bezanilla et al. [21] for tubular Na^+ depletion, with an additional term (constant field flux equation, [22]) to account for passive back-flow,

$$d[H^+]_T/dt = {}^{M}J^{H}(a/v) - k_H([H^+]_T - [H^+]_o) - \left\{(a/v)P_H\beta([H^+]_i e^{\beta} - [H^+]_T)(1-e^{\beta})^{-1}\right\} \quad \text{(A-1)}$$

where ${}^{M}J^{H}$ is the flux of H^+ mediated by monensin (mol $s^{-1}\ cm^{-2}$ of tubular membrane), k_H is the rate constant for H^+ diffusion from the tubules to the external medium whose value is assumed to be 1 s^{-1} and equal to that of other ions in the tubule to satisfy electroneutrality (Hodgkin and Horowicz, 1959; Bezanilla et al., 1972), P_H represents H^+ permeability of tubular membrane and $\beta = VF/RT$ (V: membrane potential = –90 mV; F, R and T have their usual meaning) and $a/v = 5 \cdot 10^5\ cm^{-1}$ (tubular area to volume ratio).

Under steady-state conditions ($d[H^+]_T/dt = 0$) and solving for $[H^+]_T$,

$$[H^+]_T = \left\{{}^{M}J^{H}(a/v) + k_H[H^+]_o - (a/v)P_H\beta[H^+]_i e^{\beta}(1-e^{\beta})^{-1}\right\} / \left\{k_H - (a/v)P_H\beta(1-e^{\beta})^{-1}\right\} \quad \text{(A-2)}$$

Let us use a numerical example to have an estimate of the sort of $[H^+]_T$ values that should be expected in the presence of monensin at steady state. Thus, for $[MON] = 40\ \mu M$ the calculated H^+ efflux mediated by the ionophore is about 13 pmol $s^{-1}\ cm^{-2}$ of surface membrane (see results) or, assuming 4 cm^2 of tubular membrane associated to each cm^2 of surface membrane, a ${}^{M}J^{H}$ of 2.6. pmol $cm^{-2}\ s^{-1}$. If in addition,

$[H^+]_o = 63.1$ nM ($pH_o = 7.2$). $[H^+]_i = 126$ nM ($pH_i = 6.9$) [13,24] and $P_H = 10^{-3}$ cm s^{-1} [10], Eqn. A-2 yields $[H^+]_T = 718$ nM, that is $pH_T = 6.14$. It should be noticed that in the numerator the second and the third terms are considerably smaller than the first one ($2 \cdot 10^4$ and 200 times, respectively) and therefore, both can be neglected. Similarly, the second term in the denominator is more than three orders of magnitude larger than k_H. Eqn. A-2, then reduces to,

$$[H^+]_T = {}^{M}J^{H}(1 - e^{\beta})/P_H\beta \qquad \text{(A-3)}$$

If back-flow component in Eqn. A-1 were ignored, then, at steady state,

$$[H^+]_T = ({}^{M}J^{H}(a/v)/k_H) + [H^+]_o \qquad \text{(A-4)}$$

The $[H^+]_T$ value for ${}^{M}J^{H} = 2.6$ pmol cm^{-2} s^{-1} will be 1300 μM, that is, 1800-times greater than that with back-flow component.

In steady-state conditions net passive H^+ flux (${}^{P}J^{H}$) is given by,

$${}^{P}J^{H} = P_H\beta([H^+]_i e^{\beta} - [H^+]_T)(1 - e^{\beta})^{-1} \qquad \text{(A-5)}$$

While under control (MON free) conditions ($pH_i = 6.9$; $pH_0 = 7.2$; $V = -90$ mV) $[H^+]_T$ is about 18-times greater than $[H^+]_i e^{\beta}$, in the above example, $[H^+]_T/[H^+]_i e^{\beta} = 206$. Therefore, Equation A-5 can be reduced to,

$${}^{P}J^{H} = -P_H\beta[H^+]_T(1 - e^{\beta})^{-1} \qquad \text{(A-6)}$$

and from Eqns. A-3 and A-6, the net H^+ efflux is,

$${}^{P}J^{H} + {}^{M}J^{H} = 0 \qquad \text{(A-7)}$$

This means that most if not all protons released by monensin in the tubular lumen would return to the cytosol by passive back-flow which would contribute to keep pH_i near its normal value.

In the presence of phosphate buffered Ringer ($[HPO_4^{2-}] = 4.30$ mequiv.; $[H_2PO^-] = 0.85$ mequiv.) it can be estimated that a reduction in tubular pH (ΔpH) will require (from Henderson-Hasselbalch equation) a release into the tubular lumen of $4.30 \cdot 0.85\,(10^{\Delta pH} - 1)/(4.30 + 0.85 \cdot 10^{\Delta pH}) = x$ mmol l^{-1} of H^+. For $\Delta pH = 1$, $x = 2.57$ mmol l^{-1}. From the tubular surface/volume ratio ($5 \cdot 10^5$ cm^{-1}) and the magnitude of ${}^{M}J^{H}$ (2.6 pmol cm^{-2} s^{-1}) it can be estimated that the time required for a pH reduction of 1 unit and thus, to reach steady state, in our example, would be on the order of 2 s.

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