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The Na^+-dependent regulation of the internal pH in chick skeletal muscle cells. The role of the Na^+/H^+ exchange system and its dependence on internal pH

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The internal pH (pH_i) of chick muscle cells is determined by the transmembrane Na^+ gradient. Li^+, but not K^+, Rb^+ or Cs^+, can substitute for Na^+ for regulating the internal pH of chick muscle cells. Pharmacological evidence using amiloride and amiloride analogs has shown that the Na^+/H^+ exchange system is the membrane mechanism that couples the pH_i to the transmembrane Na^+ gradient. The pH_i dependence of the amiloride-sensitive Na^+/H^+ exchange mechanism was defined. Internal H^+ interacts cooperatively with the Na^+/H^+ exchange system, in contrast with external H^+, thus indicating an asymmetrical behaviour of this exchanger. The half-maximum effect for the activation by the internal H^+ of the Na^+ transporting activity of the amiloride-sensitive Na^+/H^+ exchange was observed at pH 7.4. The Hill coefficient of the H^+ concentration dependence is higher than 3. Insulin was shown to have no effect on the pH_i of chick muscle cells.

Key words: intracellular pH/Na^+/H^+ exchange/chick muscle

Introduction

Significant changes in internal pH (pH_i) occur during the activation of a number of cell processes. Well studied examples are the reinitiation of mitotic activity (Schuldiner and Rozengurt, 1982; Moolenaar *et al.*, 1983), the fertilization of sea urchin eggs (Johnson and Epel, 1981), the induction of motility and respiration in sea urchin sperm (Christen *et al.*, 1982; Lee *et al.*, 1983) and the differentiation of the slime mold (Gross *et al.*, 1983). The pH_i appears to be regulated by several membrane mechanisms that exchange ions: the amiloride-sensitive Na^+/H^+ exchanger (Aickin and Thomas, 1977; Roos and Boron, 1981), and the different Cl^-/HCO_3^- exchange systems that are inhibited by stilbene derivatives (Roos and Boron, 1981; Boron *et al.*, 1981; Thomas, 1982). However it is not known how these different pH_i regulating mechanisms are distributed in different cell types and to what extent they contribute to the regulation of the intracellular pH in each of these cells.

The purpose of this paper is to show that the pH_i of chick muscle cells is determined by the transmembrane Na^+ gradient which under physiological conditions is maintained by the (Na^+,K^+) ATPase. Pharmacological evidence using new potent derivatives of amiloride shows that the coupling between the Na^+ and H^+ gradients is produced by the amiloride-sensitive Na^+/H^+ exchanger that has been previously characterized in these cells (Vigne *et al.*, 1982b).

Results

Table I presents the mean pH_i values of chick muscle cells that have been incubated under different experimental conditions. These values were obtained with two different types of pH_i measurements (Christen *et al.*, 1982): using the radiolabelled weak acid dimethyloxazolidinedione (DMO) and the fluorescent weak base 9-aminoacridine (9AA). The two methods give very similar results and for convenience fluorescence measurements of pH_i using 9AA have been used in all other experiments described here.

The Na^+-dependent regulation of the pH_i of chick muscle

pH_i values of chick muscle cells were determined under different conditions of transmembrane Na^+ gradient. The first set of conditions corresponds to experiments in which no Na^+ gradient was maintained across the membrane ($[Na]_i = [Na]_o$). This situation was obtained by substituting all external Na^+ with choline chloride so that the internal Na^+ concentration decreased to < 1 mM; the internal pH is then 7.10. In the second type of conditions, equal concentrations of Na^+ were maintained on both sides of the membrane by treating muscle cells with ouabain and with a neurotoxin mixture including sea anemone toxin and veratridine. These neurotoxins maintain voltage-dependent Na^+ channels in an open form (Frelin *et al.*, 1981) thus allowing the passive equilibration of intracellular and extracellular Na^+ concentrations when the (Na^+,K^+)ATPase has been blocked with ouabain. Figure 1 shows that when $[Na^+]_i = [Na^+]_o$, the internal pH was 7.10 and that this value was independent of the internal and external Na^+ concentrations. In a third set of experiments the internal pH of chick muscle cells was determined under different conditions of inward Na^+ gradient. Cells were loaded with different concentrations of Na^+ from 2 mM to 140 mM (see Materials and methods) and then shifted to an external medium containing 140 mM Na^+ to create an inward Na^+ gradient. Figure 1 shows that imposing an inward Na^+ gradient of increasing amplitude across the plasma membrane of chick muscle cells produced a progressive alkalinization of the cell from pH 7.10 to pH 7.25.

Finally, different conditions of outward Na^+ gradient were created by first loading muscle cells with different Na^+ concentrations from 2 mM to 140 mM (see Materials and

Table I. Intracellular pH values of chick muscle cells incubated under different experimental conditions

Experimental conditions	Internal pH	
	9AA	DMO
Na^+-free	7.10 ± 0.02	7.11 ± 0.03
140 mM Na^+	7.31 ± 0.08	7.37 ± 0.04
Na^+-free + 1 μg/ml nigericin	6.64 ± 0.05	6.67 ± 0.11
140 mM K^+ + 1 μg/ml nigericin	7.45 ± 0.01	7.34 ± 0.06

pH_i measurements were obtained either with 9AA or with DMO. Each value is the mean of 4 – 9 independent determinations performed on different sets of primary cultures. The standard errors of the means are indicated.

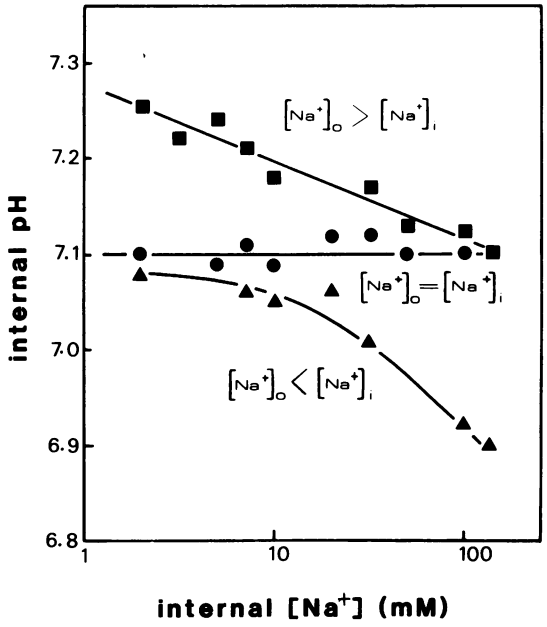

Fig. 1. The dependence of the pH_i of chick muscle cells on the transmembrane Na^+ gradient. Muscle cells were loaded with different concentrations of Na^+ as described in Materials and methods and then shifted to an incubation medium that contained the same concentration of Na^+ as the intracellular space (●), to a 140 mM Na^+ medium to create an inward Na^+ gradient (■) or to a Na^+-free medium to create an outward Na^+ gradient (▲). Intracellular pH values were determined from the equilibrium distribution ratio of free 9AA.

methods) and then shifting these cells to a Na^+-free medium. Figure 1 shows that under these conditions, an acidification of the cellular cytoplasm was observed. A pH_i value of 6.87 was obtained for the largest outward Na^+ gradient ($[Na]_o$ = 0 mM, $[Na]_i$ = 140 mM). All these experiments taken together clearly indicate that the internal pH of muscle cells is determined by the transmembrane Na^+ gradient.

It has been seen before that when Na^+-depleted muscle cells were incubated in a Na^+ free medium containing choline chloride, the internal pH was 7.10 (Table I). Substituting all the external choline chloride with NaCl produced a cell alkalinization to pH 7.35 (Table I). This result is expected since, in the absence of ouabain, raising the external Na^+ concentration progressively increased the magnitude of the Na^+ gradient. The dose-response curve for the alkalinizing effect of external Na^+ is presented in Figure 2. Substitution of external choline with Li^+ also produced a cellular alkalinization which was similar to that observed with Na^+ (Figure 2). Substitution of choline by K^+, Rb^+ or Cs^+ had no effect on the pH_i of muscle cells. These results indicate that the membrane system that couples the Na^+ and H^+ gradients will accept Li^+ instead of Na^+ and that ions like K^+, Rb^+ or Cs^+ are not acceptable as Na^+ substitutes.

Pharmacology of the Na^+-dependent mechanism of pH_i regulation

Amiloride is the classical inhibitor of the Na^+/H^+ exchange system. However, it was previously reported for a variety of cell types (Rindler *et al.*, 1979; Vigne *et al.*, 1982b) that the binding of amiloride to its receptor site on the Na^+/H^+ exchanger is competitively antagonized by Na^+ or Li^+ ions. Consequently high concentrations of amiloride unfortunately are required to block the activity of the Na^+/H^+ exchange system in the presence of high Na^+ or Li^+ concentrations.

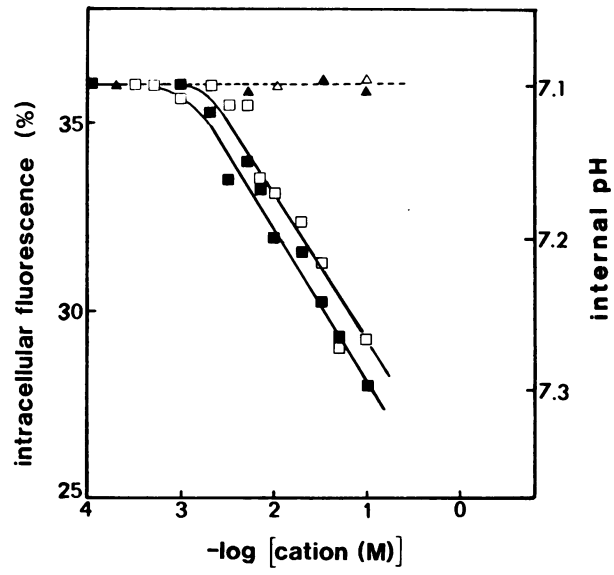

Fig. 2. The dependence on extracellular Na^+ or Li^+ concentrations of the pH_i of chick muscle cells. Muscle cells were incubated in the presence of various concentrations of Na^+ (■) or of Li^+ (□) and the pH_i was determined from the distribution ratio of free 9AA. In some experiments 10 μM EIPA was added to the incubation medium that contained Na^+ (▲) or Li^+ (△).

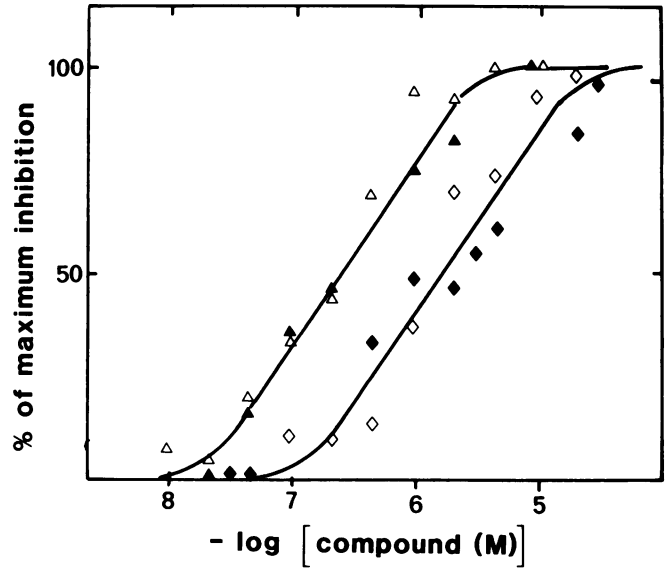

Fig. 3. Dose-response curves for DMA and EIPA action on the pH_i and on $^{22}Na^+$ uptake by chick muscle cells. For pH_i measurements, muscle cells were incubated in a 50 mM Na^+ medium in the presence of varying concentratons of DMA (◇) or of EIPA (△). The inhibitory actions of DMA (◆) and EIPA (▲) on the initial rate of $^{22}Na^+$ uptake by chick muscle cells were determined using a 50 mM Na^+ incubation medium.

$^{22}Na^+$ flux studies have recently shown (Vigne *et al.*, 1983, 1984a) that N-5 substituted derivatives of amiloride are much more potent than amiloride itself for inhibiting the Na^+/H^+ exchanger of chick muscle cells, 3T3 fibroblasts and cardiac cells so that some of them can be used even in the presence of physiological Na^+ concentrations. Two amiloride analogs: dimethylamiloride (DMA) and ethylisopropylamiloride (EIPA) are among the most active inhibitors found (Vigne *et al.*, 1983, 1984a). Figure 2 shows that 10 μM EIPA inhibits the Na^+- or Li^+-induced variations of pH_i.

The dose-response curves for DMA and EIPA inhibition

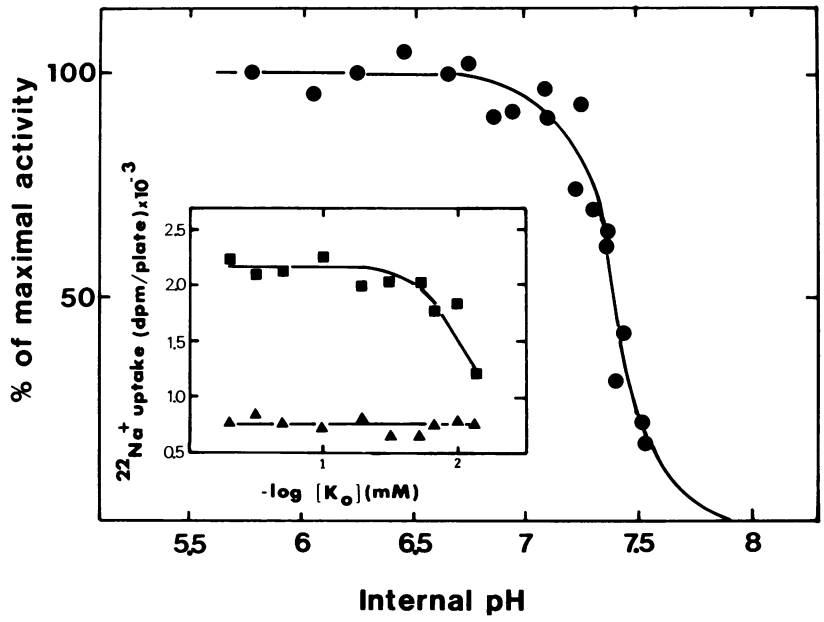

Fig. 4. The pH_i dependence of the activity of the amiloride-sensitive Na^+/H^+ exchange system of chick skeletal muscle cells. Cells were equilibrated for 30 min in Na^+-free media of different K^+ concentrations in the presence of 1 μg/ml of nigericin, and then shifted to a 3 mM Na^+ medium that contained 5 mM K^+ and no nigericin to determine the initial rate of $^{22}Na^+$ uptake in the presence (▲) or in the absence (■) of 0.1 mM amiloride. **Main panel:** the dependence on internal pH of the initial rate of amiloride-sensitive $^{22}Na^+$ uptake.

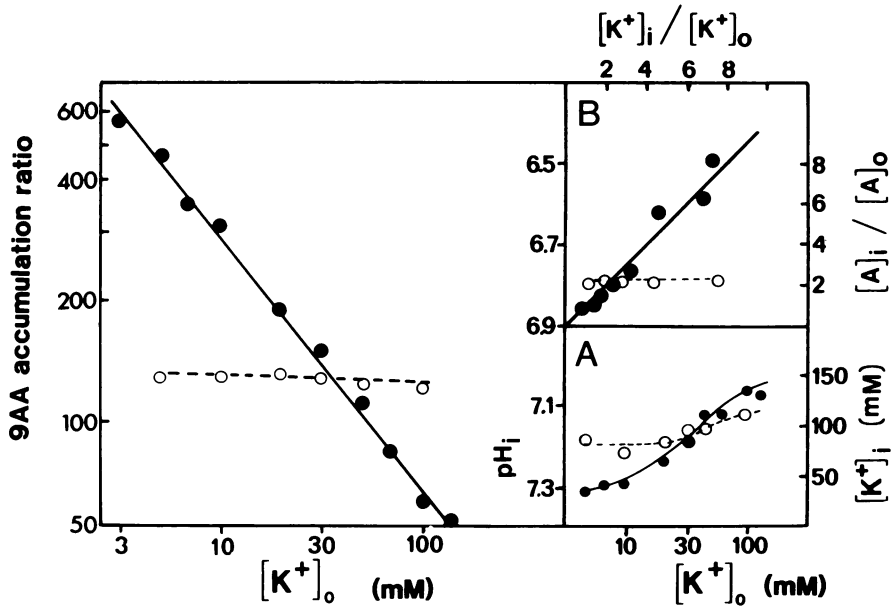

Fig. 5. Calibration of intracellular pH measurements with nigericin. **Main panel:** dependence on external K^+ concentration of the equilibrium distribution ratio of 9AA. Experiments were performed in the absence (○) or in the presence (●) of 1 μg/ml of nigericin. **(A)** The dependence on external K^+ concentration of the internal K^+ concentration after equilibration of muscle cells in a Na^+-free medium in the absence (○) or in the presence (●) of 1 μg/ml of nigericin. **(B)** The relationship between the equilibrium distribution ratio of free 9AA and the K^+ distribution ratio. The equilibrium distribution ratio of free 9AA was calculated by assuming that 1.8% of the total intracellular 9AA is in a free form (Vigne *et al.*, 1984b).

of the cellular alkalinization induced by 5 mM Na^+ are compared in Figure 3 with dose-response curves for DMA and EIPA inhibition of the activity of the Na^+/H^+ exchange system followed by $^{22}Na^+$ flux experiments. The close correlation of the inhibitory effects is a strong indication that the mechanism that links the pH_i to the Na^+ gradient in chick muscle cells is the amiloride-sensitive Na^+/H^+ exchange system. Both from pH_i measurements and from $^{22}Na^+$ flux studies, $K_{0.5}$ values determined for EIPA and DMA are at 0.2 and 1 μM, respectively.

The pH_i dependence of the Na^+/H^+ exchange system

The pH_i dependence of the Na^+/H^+ exchange system was studied using the following procedure: (i) cells were first incubated with nigericin an ionophore which promotes the transmembrane exchange of K^+ for H^+ in the presence of various concentrations of external K^+ to impose different values of pH_i (see Figure 5), (ii) the activity of the Na^+/H^+ exchanger was then measured using $^{22}Na^+$ uptake experiments in the presence of a low external Na^+ concentra-

tion (3 mM).

The inset of Figure 4 shows that the total rate of $^{22}Na^+$ uptake decreased as the external K^+ concentration was increased i.e., as the pH_i was raised. Conversely, the rate of the amiloride-insensitive $^{22}Na^+$ uptake component (which is a measure of the passive permeability of the membrane to Na^+) was independent of the external K^+ concentration and hence of the pH_i. The amiloride-sensitive component of $^{22}Na^+$ flux is a measure of the activity of the Na^+/H^+ exchanger. Its dependence on pH_i is presented in the main panel of Figure 4. The half-maximum effect for the activation by internal H^+ of the Na^+ transporting activity of the amiloride-sensitive Na^+/H^+ exchanger was observed at pH 7.4. The Hill coefficient characterizing this titration curve is higher than 3.

Is there an influence of insulin on the internal pH of muscle cells?

Moore (1981) reported that insulin produced an alkalinization of frog skeletal muscle cells. The observed alkalinization was dependent on external Na^+ and was prevented by amiloride. Therefore, he suggested that insulin exerts its action on pH_i in frog skeletal muscle cells by activating the Na^+/H^+ exchanger of the plasma membrane. We found that in chick muscle cell cultures, the addition of 1 μM insulin did not produce any change in the pH_i. We also found that insulin had no action on the rate of amiloride-sensitive $^{22}Na^+$ uptake. We extended this study to show that insulin had no effect on pH_i in rat skeletal muscle cells and in IM9 lymphoma cells grown in culture and no effect on the amiloride-sensitive $^{22}Na^+$ flux measured in 3T3 fibroblasts, chick or rat skeletal muscle cells (data not shown). Therefore, an activation of the Na^+/H^+ exchanger which would produce cellular alkalinization does not appear to be a general response of muscle cells to insulin action.

Discussion

When an inward Na^+ gradient is imposed on muscle cells, a cellular alkalinization occurs. Conversely, imposing an outward Na^+ gradient produces a cellular acidification (Figure 1). When no Na^+ gradient is imposed across the plasma membrane, the pH_i of chick muscle cells is independent of the external or internal Na^+ concentration (Figure 1). These observations indicate that the transmembrane Na^+ gradient controls the pH_i in chick muscle cells. In this function, Na^+ can be replaced by Li^+ but not by K^+, Rb^+ or Cs^+. Such an ionic specificity is the same as that of the amiloride-sensitive Na^+/H^+ exchange system that has been characterized biochemically in a variety of cell types (Rindler *et al.*, 1979; Moolenaar *et al.*, 1981; Vigne *et al.*, 1982b).

Further evidence in favor of the involvement of a Na^+/H^+ exchange system as a central mechanism linking the pH_i to the Na^+ gradient was obtained by pharmacological studies using amiloride analogs. Amiloride is a well known inhibitor of the Na^+/H^+ exchanger. Highly potent derivatives of amiloride have been found recently with affinities for the Na^+/H^+ exchange system which can be as much as 200 times that of amiloride itself (Vigne *et al.*, 1983, 1984a). The interest of these derivatives is that, because of their high affinity, they can be used in the presence of relatively high external Na^+ concentrations in spite of the known competition of Na^+ and amiloride (Rindler *et al.*, 1979; Vigne *et al.*, 1982b) for the same receptor site. The two amiloride analogs tested, EIPA and DMA, block the Na^+-dependent mechanism of pH_i regulation (Figure 2 and 3). Dose-response curves observed for the inhibition by these compounds of the Na^+-induced cell alkalinization are identical to dose-response curves for the inhibition of the amiloride-sensitive $^{22}Na^+$ uptake measured at identical external Na^+ concentrations (Figure 3).

In squid giant axons, snail neurons and barnacle muscle fibers, the pH_i appears also to be regulated by the transmembrane Na^+ gradient (Roos and Boron, 1981; Thomas, 1982; Boron *et al.*, 1981). However, in these cells, the Na^+-dependent mechanism of pH_i regulation also involves Cl^- and HCO_3^- in addition to Na^+; it is insensitive to amiloride inhibition but it can be blocked with stilbene derivatives. It therefore seems different from the amiloride-sensitive, Na^+-dependent mechanism of pH_i regulation that occurs in chick muscle cells.

The activity of the Na^+/H^+ exchange system in chick muscle cells varies with the external pH (Vigne *et al.*, 1982b). The maximal rate of Na^+ transport is higher at alkaline pH. An ionizable group with a pK of 7.6 is essential for the system to be functional. The Hill coefficient of the external pH dependence is near 1.0 which shows that no cooperativity in the function of the Na^+/H^+ exchanger is observed in response to variations of the external pH (Vigne *et al.*, 1982b). We also previously showed that the activity of the Na^+/H^+ exchange system is increased when the cytoplasm is more acidic, for instance during transient exposures to NH_4^+-containing solutions (Vigne *et al.*, 1982b; Frelin *et al.*, 1983). In this paper we analyse the pH_i dependence of the activity of the Na^+/H^+ exchange system of chick skeletal muscle cells. In contrast to the external pH dependence of the system, the internal pH dependence is very steep, being all or none in less than one pH unit, between pH 7.1 and pH 7.7, and with an apparent pK near 7.4 (Figure 4). This value is close to the internal pH of chick muscle cells incubated at a physiological Na^+ concentration (7.3–7.4, Table I). This implies non-identical properties of interaction of internal and external H^+ with the Na^+/H^+ exchange system. The steep dependence on pH_i suggests that the Na^+/H^+ exchange system can rapidly respond to a small cellular acidification by excluding H^+. This behaviour is exactly what is expected if the Na^+/H^+ exchange has an important role in the maintenance of the intracellular pH.

Finally, in spite of the fact that it is well known that chick muscle cells have insulin receptors on their plasma membrane (Sandra and Przybylski, 1979); we have been unable to observe any activation of the Na^+/H^+ exchange system by the polypeptide hormone and no insulin-dependent change in the pH_i. This conclusion differs from that reached by Moore (1981) for the action of insulin on frog skeletal muscle cells which seemed to respond to insulin by an alkalinization of their internal medium. Insulin was also found to be without effect by itself on the activity of the Na^+/H^+ exchange system and on the pH_i in other cellular types like 3T3 fibroblasts and human foreskin fibroblasts (Moolenaar *et al.*, 1983). Fibroblasts have a Na^+/H^+ exchange system that can be activated by serum and growth factors in relation to their mitogenic effect (Pouysségur *et al.*, 1982; Moolenaar *et al.*, 1981; Frelin *et al.*, 1983).

Materials and methods

Dulbecco-Vogt modification of Eagle's minimum essential medium, M 199 culture medium and fetal calf serum were purchased from Gibco. Nigericin, veratridine and ouabain were from the Sigma Chemical Co. Tetrodotoxin was from the Sankyo Chemical Co. 9AA and insulin were from Fluka. Amiloride, DMA and EIPA were obtained from Dr. E.J.Cragoe,Jr. (Merck Sharp and Dohme). The sea anemone (*Anemonia sulcata*) toxin II was purified in this laboratory as previously described (Schweitz *et al.*, 1981). $^{22}NaCl$ was from the Commissariat à l'Energie Atomique (Saclay, France). [^{14}C]DMO (50 mCi/mmol) was from New England Nuclear.

Cell cultures

Myoblasts from 9- to 12-day old chick embryo pectoralis muscle were prepared and grown as previously described (Vigne *et al.*, 1982b).

The Earle solution used for incubating cells had the following composition: 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and it was buffered with 25 mM Hepes-Tris at pH 7.4. When concentrations of Na^+ and K^+ ions had to be varied, choline chloride was used as a substitute to these two cations to maintain osmolarity. All solutions used to incubate the cells were freshly prepared and were nominally bicarbonate free.

Intracellular K^+ and Na^+ measurements

Muscle cell cultures were rapidly washed with an ice-cold 0.1 M $MgCl_2$ solution and treated with 2 ml of 5% trichloroacetic acid. The supernatant was harvested and the K^+ or Na^+ content of the cell was determined by flame spectrometry. Intracellular K^+ or Na^+ concentrations were then calculated by using an intracellular water space of 5 μl/mg of protein (Vigne *et al.*, 1982a). Cell proteins were determined according to Hartree (1972) after dissolution of the cells into 4 ml of 0.1 N NaOH.

pH_i measurements

Weak acids and weak bases equilibrate rapidly across cellular membranes in their unchanged form and can be used as probes for measuring the intracellular pH (Roos and Boron, 1981). Two types of molecules have been used in this work: the weak acid DMO and the weak base 9AA. Both types of molecules have been previously used to measure pH_i values in a variety of subcellular and cellular systems (Roos and Boron, 1981; Christen *et al.*, 1982; Thomas *et al.*, 1979). The distribution ratio of [^{14}C]DMO across the muscle cell membrane was measured as previously described (L'allemain *et al.*, 1984). The cellular uptake of 9AA is conveniently measured using fluorescence techniques (Christen *et al.*, 1982; Lee *et al.*, 1983). The quenching of 9AA fluorescence due to the penetration of the probe in muscle cells was measured with a Perkin Elmer MPF_3 fluorescence spectrometer (excitation 382 nm, emission 454 nm). The equilibrium of 9AA across the plasma membrane is reached in 15–20 min. A 30 min incubation of muscle cells in the presence of 1 μM 9AA was routinely used to determine the distribution ratio of 9AA. The calibration of the fluorescence method using 9AA for pH_i determinations was made by the null point technique (Babcock, 1983) using nigericin. Nigericin is an ionophore which promotes the transmembrane exchange of K^+ for H^+ (Reed, 1979). Figure 5A shows the relationship between the external ($[K^+]_o$) and internal ($[K^+]_i$) concentrations of K^+ in the absence and in the presence of nigericin. At 40 mM external K^+, nigericin does not change the transmembrane distribution of K^+. This result means that, in these particular conditions, and in the absence of nigericin, the transmembrane proton gradient is equal to the transmembrane K^+ gradient:

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[K^+]_i}{[K^+]_o}$$

Knowing $[H^+]_o$, $[K^+]_i$ and $[K^+]_o$, one can easily calculate that the internal pH value is 7.06 for an external pH of 7.42. In order for 9AA to be a useful probe for measuring the pH_i, the two representations relating the distribution ratio of 9AA to $[K^+]_o$ in the absence and in the presence of nigericin should also intersect at $[K^+]_o$ = 40 mM. This result is indeed the one observed as shown in the main panel of Figure 5. At $[K^+]_o$ = 40 mM nigericin does not change the distribution of 9AA across the membrane indicating again that it does not produce internal pH changes in these particular conditions of external pH. The value of pH_i = 7.06 which is found in these conditions will be called the reference pH ($pH_{i,ref}$). Changes in conditions of the external medium (and of course the internal medium) will produce changes in pH_i. New values of pH_i under any new experimental conditions can be calculated as:

$$pH_i = pH_{i,ref} - \log \frac{[A]_i/[A]_o}{[A]_{i_{ref}}/[A]_{o_{ref}}}$$

where $[A]_i/[A]_o$ is the distribution ratio of 9AA. Another demonstration of the quantity of 9AA to measure internal pH is presented in the inset B of Figure 5 where it is shown that, as expected, in the presence of the K^+/H^+ exchanger nigericin:

$$\frac{[K^+]_i}{[K^+]_o} = \frac{[H^+]_i}{[H^+]_o} \text{ at all values of } \frac{[K^+]_i}{[K^+]_o}$$

The concentration of 9AA routinely used in this work is 1 μM; however the distribution ratio of 9AA across the membrane under a given set of conditions is independent of 9AA concentration between 0.1 μM and 10 μM.

Imposing Na^+ gradients of varying amplitude across the plasma membrane of chick muscle cells

Transmembrane Na^+ gradients of varying amplitude were imposed across the plasma membrane of chick skeletal muscle cells by controlling the membrane permeability to Na^+ ions with ouabain which blocks the (Na^+,K^+)ATPase and with neurotoxins like the sea anemone toxin, veratridine and tetrodotoxin which interact with the voltage-dependent Na^+ channels. The properties of interaction of ouabain, sea anemone toxin, veratridine and tetrodotoxin with the (Na^+,K^+)ATPase and the Na^+ channels of chick muscle cells have been previously described (Frelin *et al.*, 1981; Vigne *et al.*, 1982a).

Loading muscle cells with varying concentrations of Na^+ was achieved by equilibrating muscle cells with 0.2 mM ouabain, 10 μM sea anemone toxin II and 0.1 mM veratridine in a medium that contained different concentrations of Na^+ from 2 mM to 140 mM. These conditions allowed the passive equilibration of internal and external Na^+ concentrations (Frelin *et al.*, 1981). To create an inward Na^+ gradient of known amplitude, cells loaded with varying concentrations of Na^+ were shifted to a 140 mM Na^+ medium that contained 0.2 mM ouabain to maintain the (Na^+,K^+)ATPase in an inhibited state (to prevent Na^+ efflux through the pump) and with 0.1 μM tetrodotoxin to close Na^+ channels which had been opened by the sea anemone toxin and veratridine (to prevent Na^+ influx through Na^+ channels). An outward Na^+ gradient of varying amplitude was obtained by shifting cells that had been loaded with known concentrations of Na^+ to a Na^+-free medium supplemented with 0.2 mM ouabain and 0.1 μM tetrodotoxin.

The internal pH dependence of the Na^+/H^+ exchanger

The ability of nigericin to couple the H^+ gradient to the K^+ gradient was used to impose different pH_i values in chick muscle cells. Cells were equilibrated for 30 min in a Na^+-free medium at pH 7.4 that contains 1 μg/ml of nigericin and varying concentrations of K^+ from 2 mM to 140 mM. Figure 5B shows that under these conditions the H^+ and K^+ gradients are equalized so that the pH_i can be calculated from the K^+ distribution ratio. Cells were then shifted to a 3 mM Na^+, 2 mM K^+ medium at pH 7.4 which was supplemented with 0.1 mM bumetamide to inhibit the Na/K/Cl co-transport (Rindler *et al.*, 1982) with 1 μCi/ml of $^{22}Na^+$, 0.2 mM ouabain and in the presence or in the absence of 0.1 mM amiloride. It was checked that, as we had previously reported (Frelin *et al.*, 1983), the addition of a concentration of Na^+ as low as 3 mM did not alter the pH gradient that has been created by nigericin in the presence of K^+ gradient. Initial rates of $^{22}Na^+$ uptake were then determined as previously described (Vigne *et al.*, 1982b).

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