Intracellular pH measurements using the fluorescence of 9-aminoacridine

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9-Aminoacridine is a fluorescent weak base which distributes across the plasma membrane according to the pH gradient, and can be used to monitor changes in intracellular pH in eukaryotic cells. A calibration technique using the ionophore nigericin is described which allows us to measure intracellular pH values of chick skeletal muscle cells.

Intracellular pH *9-Aminoacridine* *Nigericin* *Monensin* *Skeletal muscle*

1. INTRODUCTION

Cytoplasmic pH variations produce drastic changes in the behaviour of eukaryotic cells. Changes in pH_i are important for mediating the action of serum and growth factors in quiescent fibroblasts [1,3]; they are also involved in the action of insulin on frog skeletal muscle [4]. Variations of the cytoplasmic pH have been reported to occur, for example, during the fertilization of sea urchin oocytes [5], during the induction of respiration and motility of sea urchin sperm [6,7], and during differentiation of a slime mold [8].

Different techniques have been used to measure variations of pH_i [9,10]. The main ones involve the use of specific pH microelectrodes, NMR methods, radioactive or fluorescent probes which distribute across the plasma membrane according to the pH gradient, and in situ generated fluorescent probes which respond to a change in pH by a change in their fluorescence spectrum. Fluorescence is of course one of the most convenient ways to measure cytoplasmic pH variations and fluorescent probes have been used with success in recent years [2,6,7,9,10]. The weak base 9-AA is one example. The purpose of this paper is to show, using skeletal muscle cells in culture, how 9-AA can be used in a quantitative way to measure pH_i and variations of pH_i using a calibration technique involving the ionophore nigericin.

Abbreviations: 9-AA, 9-aminoacridine; DMO, dimethyloxazolidinedione; pH_i, internal pH

2. MATERIALS AND METHODS

9-AA was from Fluka; nigericin and monensin were from Sigma. [^{14}C]DMO (50 mCi/mmol) was from New England Nuclear. Skeletal muscle cell cultures were prepared from 9–12-day-old chick embryos as in [11]. Cultures used in this work consist of differentiated myotubes.

For pH_i measurements, muscle cells were incubated in a medium consisting of 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose buffered at pH 7.42 with 25 mM Hepes–Tris and supplemented with 1 μM 9-AA. When the Na^+ or K^+ concentrations of this medium were changed, choline chloride was substituted to maintain osmolarity. The 9-AA concentration of incubation media was determined by measuring its fluorescence using a Perkin Elmer MPF3 fluorescence spectrometer with excitation at 382 nm and emission at 454 nm. The uptake of 9-AA by muscle

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cells was determined from the disappearance of 9-AA from the incubation media. Intracellular pH measurements using [^{14}C]DMO were performed as in [3].

3. RESULTS AND DISCUSSION

9-AA is a fluorescent weak base which partitions across the plasma membrane according to the transmembrane pH gradient [6,7]. Fig.1 shows the time course of 9-AA uptake by chick skeletal muscle cells. At an external pH of 7.4, 20–30 min incubation is sufficient to reach full equilibration of the probe across the plasma membrane. Depolarizing membrane conditions obtained by incubating cells in 145 mM K^+ medium had no influence on the time course and equilibrium level of 9-AA accumulation (fig.1). This indicates that the membrane permeability of the charged form of 9-AA is negligible as compared to the permeability of the unchanged form.

Nigericin, an electroneutral ionophore that exchanges K^+ for H^+ [13], was used to show that the accumulation of 9-AA responds primarily to the transmembrane pH gradient. When muscle cells were incubated in Na^+-free, 5 mM K^+ medium in the presence of 1 μg/ml nigericin, the internal K^+ content decreased from 90 to 36 mM and higher amounts of 9-AA were accumulated (fig.1). Conversely, when muscle cells were incubated in 145 mM K^+ medium in the presence of 1 μg/ml nigericin, the internal K^+ content increased from 90 to 130 mM and less 9-AA was accumulated (fig.1).

Nigericin has been used here to determine pH_i values by a null point technique [14] and to calibrate the evaluation of pH_i values obtained with 9-AA. At a given external K^+ concentration, the addition of nigericin normally changes the internal K^+ concentration as discussed above. However, there is a particular value of the external K^+ concentration at which the addition of nigericin to muscle cells produces no change in the internal K^+ content. At this point, and even in the absence of nigericin, the transmembrane K^+ and H^+ gradients are equal:

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

Fig.2 shows that such an equilibrium is reached for an external K^+ concentration of 40 mM. Under

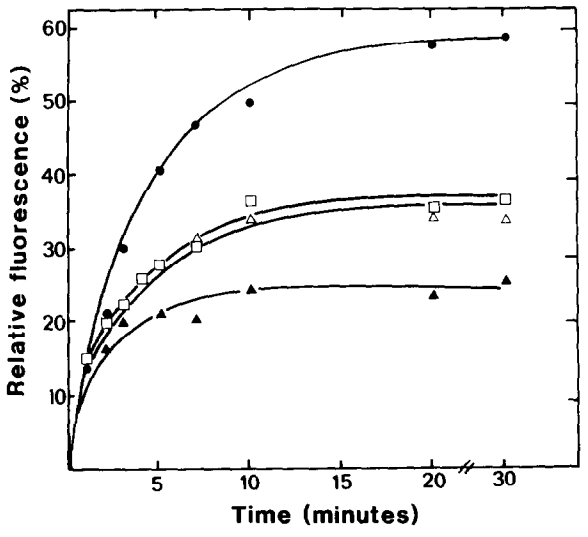

Fig.1. Time course of 9-AA accumulation by chick skeletal muscle cells in culture: Cells were incubated for the times indicated in Na^+-free, 5 mM K^+ medium (●,□), or Na^+-free, 145 mM K^+ medium (△▲) in the presence (●,▲) or absence (□,△) of 1 μg/ml nigericin. Uptake is expressed as % of the extracellular 9-AA that has been accumulated by muscle cells.

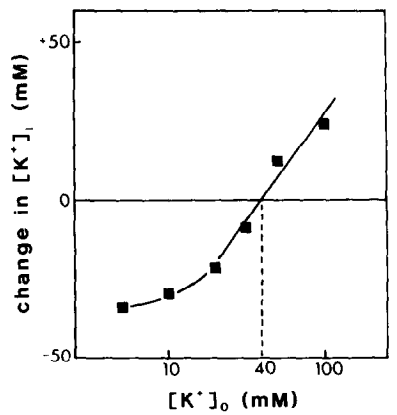

Fig.2. Determination of the pH_i of muscle cells by a null point method using nigericin. Muscle cells were incubated for 30 min in Na^+-free medium of different $[K^+]$ in the presence or absence of 1 μg/ml nigericin, and internal K^+ was determined by flame photometry for both sets of cultures. The ordinate indicates the change in $[K^+]_i$ that is induced by the presence of nigericin.

these conditions, the internal K^+ concentration is 92 mM. One can then easily calculate that, for an external pH of 7.42, the pH_i is 7.06. This value will be called the reference pH, $pH_{i\,ref}$. This value of the pH_i of chick muscle cells incubated in Na^+-free, 40 mM K^+ medium was independently determined from the distribution of the weak acid [^{14}C]DMO. This value was 7.11 ± 0.03 which agrees well with the pH_i value determined above.

For 9-AA to be a useful probe to measure pH_i, one should of course observe that the two representations relating the distribution ratio of 9-AA to the external K^+ concentration in the absence and presence of nigericin should also intersect at $[K^+]_o$ = 40 mM. When nigericin does not produce variations of internal K^+, it should not produce variations of pH_i. This is exactly what is shown in the main panel of fig.3.

At an external K^+ concentration of 40 mM, the distribution ratio of K^+ is 2.3, while the distribution ratio of 9-AA is 127. The difference between these values when K^+ and H^+ are at equilibrium across the membrane is due to intracellular binding of the acridine dye [6], leading to an overestimation of the distribution ratio of free 9-AA. This binding event is useful to give an intense fluorescence signal [6]. A comparison of the values of the K^+ and 9-AA distribution ratio indicates that only

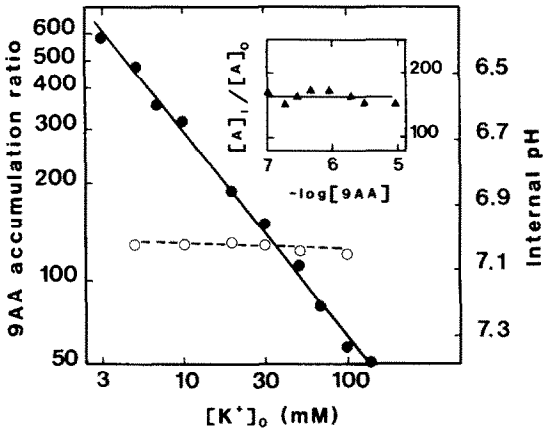

Fig.3. The dependence on $[K^+]_o$ of the equilibrium distribution ratio of 9-AA. Chick muscle cells were incubated in Na^+-free media of different $[K^+]$ in the absence (○) or presence (●) of 1 μg/ml of nigericin. Inset: dependence on external 9-AA concentration of the distribution ratio of 9-AA. Muscle cells were incubated in Na^+-free, 5 mM K^+ medium.

1.8% of the total intracellular 9-AA accumulated by chick muscle cells is in the free form. Similar experiments carried out using chick cardiac cells have shown that in these cells, 2.1% of the 9-AA that is accumulated is in the free form. This value was reported to be 1% in sea urchin sperm [6].

The inset of fig.3 shows that the distribution ratio of 9-AA is independent of the external concentration of 9-AA used between 0.1 and 10 μM. This result means that there must be a large excess of intracellular binding sites for 9-AA and that the ratio of free to bound 9-AA is constant. Calculations of pH_i from the distribution of 9-AA were done using the following equation [6]:

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

where $[A]_i/[A]_o$ is the distribution ratio of 9-AA for cells of unknown pH_i, $pH_{i\,ref}$ and $[A]_{i\,ref}/[A]_{o\,ref}$ are the pH_i and 9-AA distribution ratio for cells incubated under reference conditions (40 mM external K^+, 1 μg/ml nigericin). This relationship has been found useful for determining pH_i values of

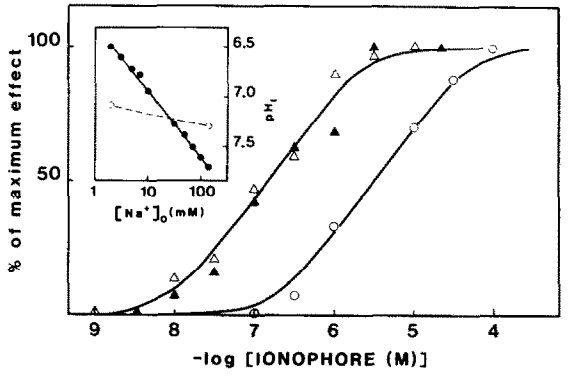

Fig.4. Dose-response curves for the action of nigericin and monensin on the pH_i of chick muscle cells. Incubation of muscle cells with nigericin in Na^+-free, 5 mM K^+ medium (△) produced a cell acidification from pH 7.05 to 6.45. Incubation with nigericin in Na^+-free, 145 mM K^+ medium (▲) produced a cell alkalinisation from pH 7.07 to 7.35. Incubation of muscle cells with monensin in 140 mM Na^+, 5 mM K^+ medium produced a cell alkalinisation from pH 7.30 to 7.65 (○). Inset: relationship between the equilibrium distribution ratio of 9-AA and external Na^+ concentration for chick muscle cells incubated in the presence (●) or absence (○) of 0.1 mM monensin.

chick muscle cells incubation in a variety of experimental conditions [15].

Fig.4 shows an illustration of the use of 9-AA to measure variations in pH_i induced by nigericin and monensin. Monensin is another electroneutral ionophore that catalyses exchange of Na^+ for H^+ [13]. Increasing incorporation of the two ionophores in the muscle membrane produced graded variations in the internal pH. When chick muscle cells are incubated in Na^+-free, 5 mM K^+ medium, pH_i decreases from 7.05 in the absence of nigericin to 6.45 at a concentration of nigericin giving maximal effect. Conversely, when chick muscle cells are incubated in Na^+-free, 145 mM K^+ medium, the pH_i increases from 7.07 in the absence of nigericin to 7.35 in the presence of a maximally active concentration of nigericin. In both experiments the concentration of nigericin which produced half-maximal change in pH_i is observed at 0.15 μM (fig.4). The inset of fig.4 shows that monensin is able to couple the internal pH to the Na^+ gradient, just as nigericin was shown to couple the internal pH to the transmembrane K^+ gradient. The main panel of fig.4 shows that the pH_i increases from a value of 7.30 when muscle cells are incubated in 140 mM Na^+ medium in the absence of monensin to a value of 7.65 at a maximal concentration of monensin. The concentration of monensin which produces half-maximal change in pH_i is observed at 3 M.

In conclusion, 9-AA appears to be a sensitive and convenient tool to analyze in further studies the role of the different membrane systems that are involved in pH_i regulation, such as the Na^+/H^+ exchange system.

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