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POLYENE MACROLIDE ANTIBIOTICS: INDIRECT STIMULATION OF THE Na^+/H^+ EXCHANGER OF BALB/c B LYMPHOID CELL LINE, A20

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Abstract—The fluorescent pH probe, 2′-7′-bis (carboxyethyl) 5-carboxyfluorescein, was used to follow changes in internal pH (pH_i) induced by aromatic polyene antibiotics in the BALB/c lymphoid cell line A20. The antibiotics studied were vacidin, which contains a free carboxylic group in the position C18 of the macrolide ring, and vacidin glycyl methyl ester and perimycin, which are without free carboxylic groups. Although all of them induced transmembrane Na^+ and K^+ movements, only vacidin had protonophoric activity, as previously demonstrated for red blood cells [Cybulska B *et al.*, *Biochem Pharmacol* **38**: 1755–1762, 1989]. However, with all three antibiotics, pH_i changes were observed in A20 cells. It was demonstrated that the transmembrane H^+ movements resulted to different degrees, principally in the case of perimycin and vacidin glycyl methyl ester, or partially in the case of vacidin, from the stimulation of the Na^+/H^+ exchanger by the induced Na^+ permeability. The non-aromatic polyene antibiotic amphotericin B had a low ability to increase proton permeability.

It has been proven that antibiotics, classified as large macrolide ring polyenes, form channels through sterol-containing membranes, for example of fungi (ergosterol containing) or mammalian cells (cholesterol containing). These channels mainly increase the permeability to ions and non-electrolytes of small size (for a review see Ref. 1). A large difference in biological activity between aromatic and non-aromatic heptaenes, two subgroups of the large macrolide polyenes, has been observed. The aromatic heptaenes, vacidin A, gedamycin and perimycin, are characterized by a biological activity on eucaryotic microorganisms two orders of magnitude higher than the representatives of non-aromatic heptaenes, like amphotericin B (AmB‡) or nystatin, commonly used in therapy. In spite of that, aromatic heptaenes have had restricted applications due to their high toxicity for animal cells [2]. For human red blood cells, the origin of the higher toxicity (haemolytic effect) of aromatic polyene antibiotics possessing a free carboxyl group in the position C18 of the macrolide ring, as compared with that of AmB, has been shown to come from their high protonophoric activity. The aromatic polyene antibiotics devoid of this free carboxyl group are much less haemolytic and have a much lower protonophoric activity, although they induce K^+ leakage to the same extent [3–5].

The present work was done to determine whether polyene macrolides induce intracellular pH changes in animal cells other than red blood cells, which could be responsible for their toxicity. Extrapolation of the results obtained with red blood cells to other animal cells is not straightforward because the regulation of transmembrane ion fluxes is strongly dependent on the cell type considered. We chose to study a lymphoma B cell line because we have already analysed the influence of polyene antibiotics upon its intracellular ion concentrations [6] and have a good knowledge of its internal pH [7].

MATERIALS AND METHODS

Polyene macrolide antibiotics. Polyene antibiotics were purified and their semisynthetic derivatives obtained according to Czerwinski *et al.* [8]. The following antibiotics have been used: aromatic heptaenes: vacidin A, vacidin A methoxycarbonylmethylamide (VGlyMe), perimycin; non-aromatic heptaenes: AmB. Samples of antibiotics were dissolved in dimethylsulfoxide at a concentration of 5 mg/mL (approximately 5×10^{-3} M). These stock solutions were kept at 0° and used within 1 month of their dissolution. No changes in their spectral properties and their activities were observed during this period. Exact concentrations were determined by absorption between 430 and 330 nm after 500-fold dilution of the stock solutions in methanol. They were calculated based on $\varepsilon_{382} = 120{,}000$ for aromatic heptaenes and $\varepsilon_{409} = 150{,}000$ for AmB. Proper dilution of the stock solution was carried out before each set of experiments.

Cell culture. BALB/c B lymphoid line A20 was a gift from P. Sarthou (Département d'Immunologie, Institut Pasteur, France) and was cultivated in RPMI 1640, supplemented with 10% foetal calf serum (RPMI-S), 50 μM β-mercaptoethanol, 1 mM sodium

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‡ Abbreviations: BCECF, 2′-7′-bis (carboxyethyl) 5-carboxyfluorescein; EIPA, ethylisopropylamiloride; AmB, amphotericin B; VGlyMe, vacidin A methoxycarbonylmethylamide; VMe, vacidin methyl ester; RPMI-S, RPMI 1640 supplemented with 10% foetal calf serum.

pyruvate, 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin (Flow Laboratories, les Ulis, France.

The medium was inoculated with 3–4 × 10^5 cell/mL. Cells were grown in suspension, at 37°, in humidified air containing 5% CO_2. Usually, 24-hr cultures were used. Viability, assayed by Trypan blue exclusion, was around 90% during experiments.

Media and reagents. Intracellular pH (pH_i) measurements were carried out in Na^+ medium or choline medium. Na^+ medium was composed of 130 mM NaCl, 4 mM KCl, 1 mM (NaH_2PO_4), 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 25 mM HEPES, pH 6.8 or 7.4. Choline medium was composed of 135 mM choline Cl^- (Sigma), 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 25 mM HEPES, pH 7.4 adjusted with KOH. Usually Na^+ medium and choline medium were mixed with 330 mM sucrose in proportion 9:1 (v:v). In a medium devoid of sucrose, partial lysis of cells was observed. Other reagents were acetoxymethylester of 2'-7'-bis (carboxyethyl) 5-carboxyfluorescein (BCECF) (Molecular Probes, Eugene, OR, U.S.A.), ethyl-isopropylamiloride (EIPA) (gift from Dr E. J. Cragoe), digitonin (Sigma) and nigericin (Sigma).

Intracellular pH measurement with trapped fluorescent indicator BCECF. Determination of pH_i was performed by a modification of Thomas' method [9]. A20 cells in the growing phase were centrifuged and resuspended in growing medium at a concentration of 10^7 cells/mL. Cells were loaded by incubation with 1 μM acetoxymethylester of BCECF for 20 min at 37°, 5% CO_2. The cell suspension was diluted 10 times with RPMI-S and incubation was continued for 20 min at 37°, 5% CO_2. Cells were collected by centrifugation (1200 rpm, 10 min without cooling), and washed once with RPMI-S. Washed cells were resuspended in RPMI at a concentration of 5 × 10^7 cells/mL (stock cell suspension). The stock cell suspension was kept on ice. This procedure was used because it was observed that A20 lymphocytes, at room temperature, have a tendency to lyse when kept in Na^+ medium for a long time, even in the presence of sucrose. In RPMI-S at 0°, lysis and loss of viability during 4 hr was negligible. When required, 0.2 mL of this stock suspension was briefly centrifuged (10 sec in Eppendorf minicentrifuge) and resuspended in 2 mL of Na^+ or choline medium, prewarmed to 37°, in a fluorescence quartz cuvette; final cell concentration was 5 × 10^5 cells/mL. The fluorescence signal was recorded with a Jobin-Yvon JY 3D spectrofluorimeter, in a cell holder thermostated at 37° and equipped with a magnetic stirrer. Excitation and emission wavelengths were set at 508 and 535 nm, respectively. It was checked that the antibiotics do not quench BCECF fluorescence in solution.

The conversion of fluorescence intensity into pH units was carried out with a calibration curve obtained by disrupting the cells with digitonin and recording the fluorescence signals of media of known pH [10].

RESULTS

Principle of the method

To investigate the effect of various polyene macrolide antibiotics on intracellular pH (pH_i) we examined the changes in fluorescence of logarithmically growing A20 lymphocytes loaded with the pH-sensitive indicator BCECF.

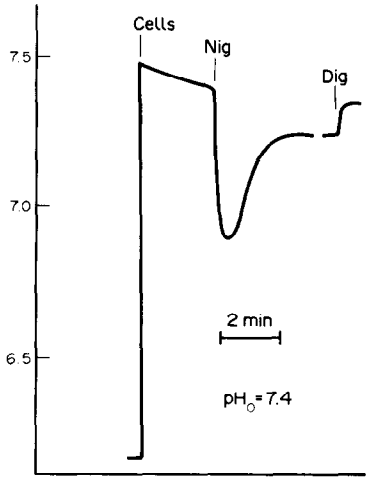

Fig. 1. Changes in fluorescence of BCECF-loaded A20 cells, induced by the K^+/H^+ antiporter nigericin (2 × 10^{-6} M) (Nig) and corresponding internal pH variations. Cells were suspended in Na^+ medium. pH 7.4 at 37°, at a concentration of 5 × 10^5 cells/mL. Digitonin (Dig) (final concentration 10^{-4} M) was used to lyse cells and to make pH calibrations at the end of experiments.

Changes in fluorescence were observed after the addition of nigericin, a known K^+/H^+ antiporter, indicated that the fluorescence of BCECF is responsive to pH_i changes. The example shown in Fig. 1 was observed with cells suspended in Na^+ medium, pH 7.4. The calibration of fluorescence as a function of pH was carried out in the presence of digitonin at the end of each experiment. Lymphocytes A20 from logarithmically growing cultures had an extracellular pH of 7.4–7.5. The addition of nigericin (2 × 10^{-6} M) to the cell suspension caused a drastic reduction in fluorescence intensity followed by a slower increase until fluorescence attained an equilibrium value. The extent of the decrease and the equilibrium values were dependent on the pH of the medium. The biphasic response of pH_i was consistent with the transport selectivity $K^+ > Na^+ > Li^+$ observed for nigericin in other systems [11]. Indeed, in a medium of pH 7.4 with 130 mM Na^+ and a low K^+ concentration, a decrease in fluorescence reflected an intracellular acidification due to the obligatory K^+/H^+ exchange driven mainly by the K^+ gradient, directed from the cells to the medium. A subsequent increase in fluorescence reflected the outflux of protons resulting from the nigericin-induced Na^+/H^+ exchange driven by the gradient of Na^+ directed into the cells. Fluorescence stabilized when equilibrium in the distribution of Na^+, K^+ and H^+ was reached. Addition of a membrane disrupting agent, digitonin, selectively released cytosolic contents but left intracellular organelles intact [10]. The second phase, pH recovery, could be significantly slowed by decreasing

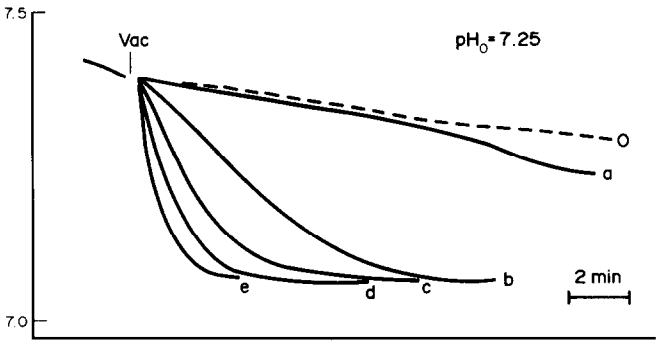

Fig. 2. Internal pH (fluorescence-related) changes in BCECF-loaded A20 cells induced by vacidin A (Vac) at various concentrations: 10^{-7} M (a); 5×10^{-7} M (b); 10^{-6} M (c); 2.5×10^{-6} M (d); 5×10^{-6} M (e). Cells were suspended in Na^+ medium, pH 7.25 at 37°, at a concentration of 5×10.5 cells/mL.

nigericin concentration and was not observed in a medium in which Na^+ was replaced by K^+. Biphasic pH change induced by nigericin was also observed when an inhibitor of Na^+/H^+ exchanger (EIPA) was added before the ionophore.

Effect of vacidin A: concentration dependence

A20 lymphocytes suspended in Na^+ medium pH 7.2 had a relatively stable intracellular pH of ~7.4. The 2% decrease in fluorescence observed within 10 min was due only to leakage of the probe from an intracellular medium with pH 7.4 to a more acidic extracellular solution. Under these conditions, vacidin appeared to be very efficient in inducing proton permeability in A20 lymphocytes. The effect of various vacidin concentrations on these cells is shown in Fig. 2. The time course of pH_i changes was different from that observed with nigericin and reflected low intercationic selectivity (Na^+/K^+) of the pathway formed by vacidin [4]. The time of pH_i equilibration was dependent on the antibiotic concentration. For 5×10^{-7} M, equilibrium pH_i was reached within 5 min. For higher concentrations it was faster.

However, it was difficult to compare the initial rates or half-times because at a vacidin concentration of 10^{-7} M permeability developed slowly (lag time). It is known that for polyene macrolides, a threshold concentration is needed to induce permeability. This can be explained by the fact that in aqueous media, polyene macrolide antibiotics undergo self-association; it was recently proven for AmB that the permeabilizing efficiency of monomers and self-associated forms was different in cholesterol-containing membranes [12].

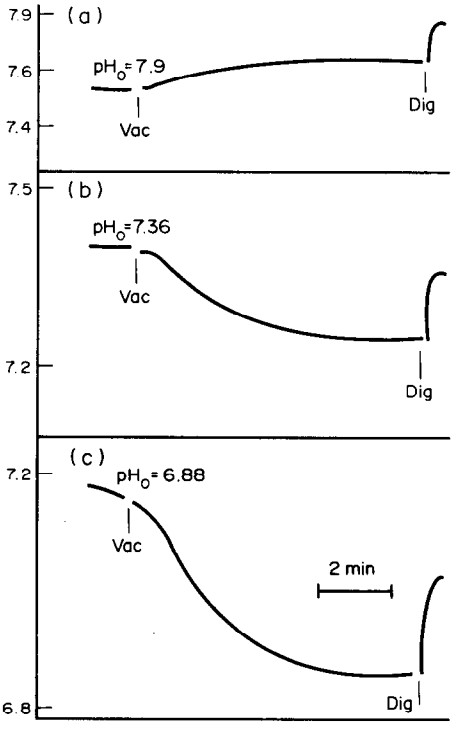

Fig. 3. Influence of pH_o on the pH_i variations of A20 cells, induced by the addition of vacidin A (vac). pH_o: 7.9 (a); 7.36 (b); 6.88 (c). Vacidin concentration: 5×10^{-7} M. Cells were suspended in Na^+ medium, at 37°, at a concentration of 5×10^5 cells/mL.

Vacidin action: extracellular pH effect

The extent and direction of pH changes were dependent on the extracellular pH. When cells were suspended in a medium of pH 7.4, the pH_i was 7.4. Upon addition of 5×10^{-7} M vacidin, the pH_i decreased by 0.1 U (Fig. 3b). When A20 cells maintained at pH 7.4 were resuspended at an external pH = 6.9, the pH_i decreased spontaneously from 7.4 to 7.2 and stabilized at this level. Addition of vacidin initiated a rapid influx of protons into the cells: after 6 min, a new equlibrium at pH_i = 6.8 had been established (Fig. 3c). The pH_i of the equilibrium always stabilized at a lower level than the pH_o (extracellular pH); addition of digitonin always induced an abrupt increase in pH.

Upon the creation of a pH gradient from cells into the medium (pH_o 7.9) a spontaneous increase in pH_i

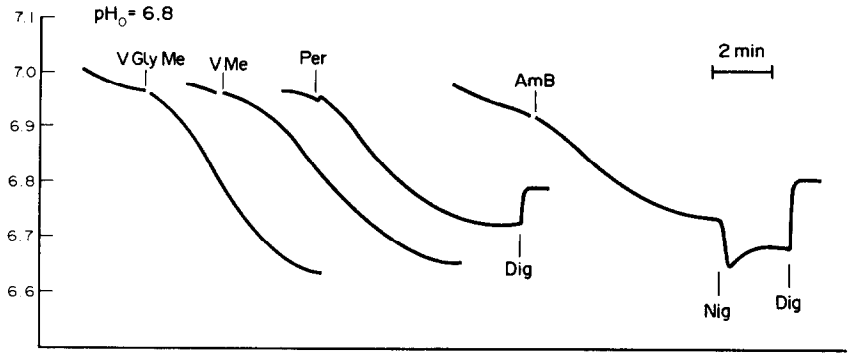

Fig. 4. Internal pH changes induced by various polyene antibiotics, in A20 cells. Cells were suspended in Na^+ medium, $pH_i = 6.8$ at 37°, at a concentration of 5×10^5 cells/mL. VGlyMe, 5×10^{-7} M; VMe, 5×10^{-7} M; perimycin (Per), 5×10^{-7} M; AmB, 5×10^{-6} M. Final addition of nigericin (Nig) and digitonin (Dig) is indicated .

was observed. Subsequent addition of vacidin induced alkalinization of the intracellular compartment (Fig. 3a). However at pH levels higher than 7.5, the relationship between the fluorescence intensity of BCECF and pH was no longer linear, due to the pK_a value of the probe. The results are only qualitative, indicating the directions of pH changes.

Other antibiotics

In previous experiments performed on erythrocytes [3, 5], it has been found that the induction of proton permeability is dependent on the presence of a free carboxyl group in the molecule of an aromatic heptaene. Results shown in Fig. 4 indicate that the derivatives of vacidin without free carboxyl groups, vacicin methylester (VMe) and VGlyMe, as well as perimycin, induce a decrease in pH_i in a manner similar to that of vacidin. In contrast, the non-aromatic heptaene AmB, which possesses a free carboxyl group, was remarkably less efficient: a 10 times higher concentration of AmB was necessary to obtain a similar change in pH_i as that observed with the aromatic heptaenes. The results obtained with the aromatic polyene antibiotics suggest that intracellular pH changes induced in A20 lymphocytes might not depend on the protonophoric properties of the antibiotic studied.

Effect of EIPA

One of the first effects of the aromatic heptaenes on cells, independent of the presence or absence of a free carboxyl group in the molecule, is an increased permeability of the cell membrane to monovalent cations. In Na^+-containing medium, antibiotics induced a cation flux, according to their concentration gradients, and intracellular K^+ was quickly replaced by Na^+. If external pH is lower than pH_i, stimulation of the constitutive system of Na^+/H^+ exchange might then be expected. Under these conditions, pH_i changes should be sensitive to Na^+/H^+ exchanger inhibitors. In Fig. 5 is shown the effect of aromatic heptaenes in the presence of EIPA, a relatively specific inhibitor of the Na^+/H^+ exchanger in animal cells [13]. When EIPA (final concentration 10^{-5} M) was added to A20 lymphocyte suspensions prior to polyene, the decrease in intracellular pH caused by VGlyMe (Fig. 5a) and perimycin (Fig. 5b) was almost completely inhibited. On the other hand, vacidin A induced a decrease in pH_i in the presence of the inhibitor but at a slower rate than observed in the absence of EIPA (Fig. 5c). Similar inhibitory effects were observed with amiloride (final concentration 10^{-4} M). AmB caused only small pH_i changes, not sensitive to EIPA (Fig. 5d).

Na^+/H^+ exchange may be reversed by imposing an appropriate in > out Na^+ gradient; this was done by suspending the cells in choline medium. In this Na^+-free medium at pH 7.4, spontaneous acidification was negligible. Addition of antibiotics induced a rapid decrease in pH_i (Fig. 6a, b). When Na^+ was added, pH_i recovery was observed. EIPA completely inhibited the initial acidification as well as pH recovery for VGlyMe and perimycin. In the case of vacidin, only partial inhibition of recovery was observed.

It should be noted that the extensively used method of cell acidification with NH_4Cl could not be used here; indeed both polyenes, vacidin A and perimycin, increased permeability to NH_4^+ (data not shown).

DISCUSSION

By a fluorimetric technique allowing the continuous monitoring of intracellular pH, we have shown that the several aromatic heptaenes induced intracellular pH changes in the A20 cell line, independent of their protonophoric activity. The protonophoric properties of polyene macrolide antibiotics appeared to be very important for their hemolytic activity. Only those antibiotics (vacidin, gedamycin) which increased permeability to both monovalent cations (Na^+, K^+) and protons were strongly hemolytic. It was proposed that hemolysis is a consequence of stimulation of Cl^-_{in}/HCO_{3-out} exchange, due to the increase in intracellular pH resulting from the exchange of extracellular cation for intracellular

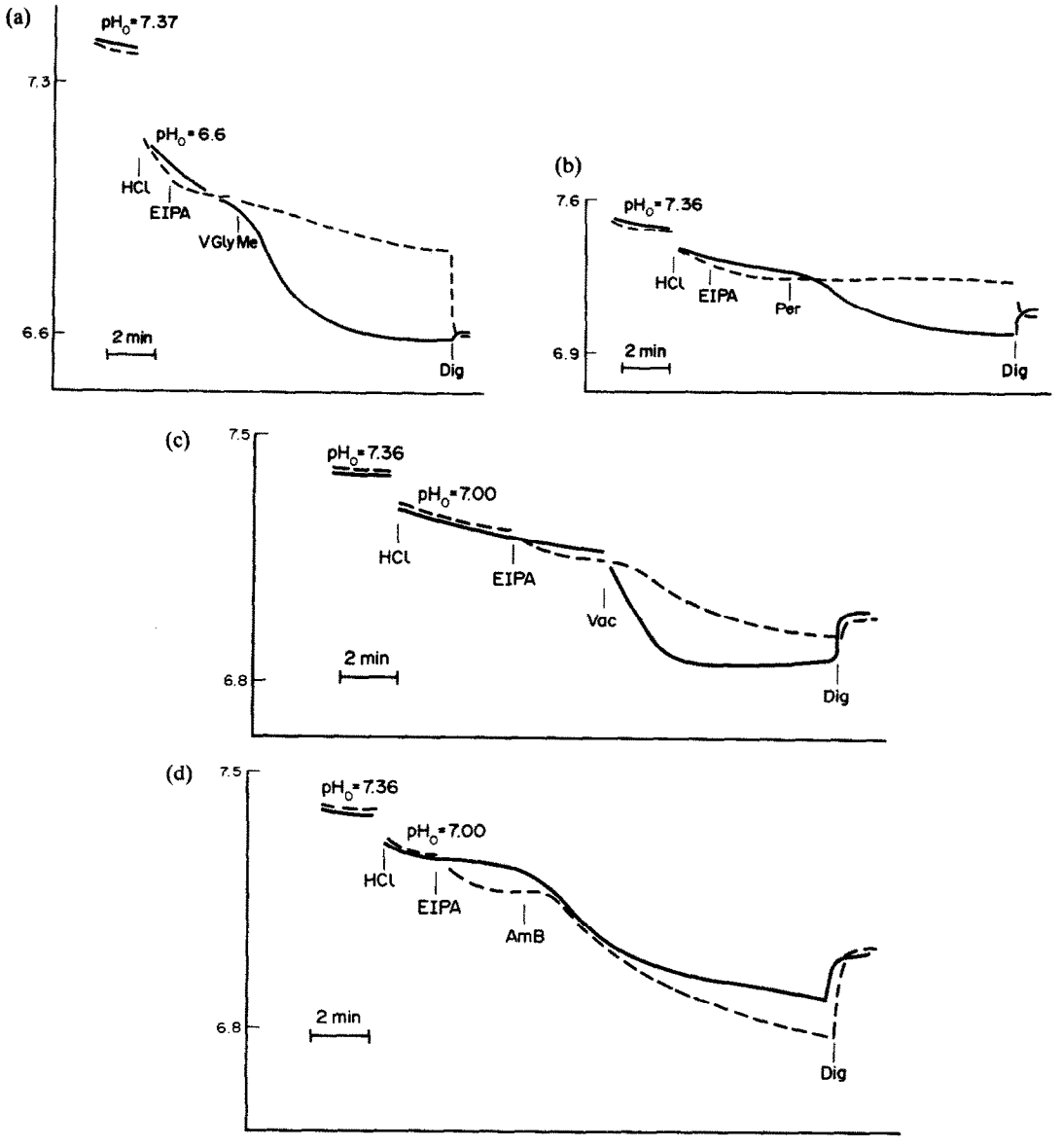

Fig. 5. Influence of EIPA on the polyene-induced internal pH changes in A20 cells. Continuous lines: recordings in the absence of EIPA. Dotted lines: recordings in the presence of EIPA (10^{-5} M). VGlyMe, 5×10^{-7} M, $pH_o = 6.6$ (a); perimycin (Per), 5×10^{-7} M, $pH_o = 7$ (b); vacidin (Vac), 5×10^{-7} M, $pH_o = 7$ (c); AmB, 5×10^{-6} M, $pH_o = 7$ (d).

proton [3]. For the antibiotics (perimycin) that did not induce permeability to protons, hemolysis is dependent on conductive influx of Cl^-, which is much slower than electroneutral HCO_3^-/Cl^- exchange. Protonophoric properties of the compound were related to the presence of a free carboxyl group in the molecule. It has to be stressed that this is not a general rule for all polyene macrolide antibiotics. AmB, which has a free carboxyl group, induced only small and transient pH changes under the same experimental conditions. The decrease in hemolytic activity was not observed in a series of AmB derivatives substituted at their carboxyl group [14]. The relationship between substitution of carboxyl group and proton permeability induction in lymphoma cells A20 was different from that in red blood cells. In fact, antibiotics without a free carboxyl group (VMe, VGlyMe, perimycin) were as effective in increasing permeability to protons as vacidin A. AmB was 10 times less effective than aromatic heptaenes (Figs 1, 4). The extent and direction of pH_i change were dependent on ionic (Na^+, K^+) and pH gradients (Fig. 3). The A20 cells at the logarithmic phase of growth had a pH_i of around 7.4. In Na^+ medium pH 7.4, a small degree of acidification of the cytoplasm occurred, due to a net ionic Na^+

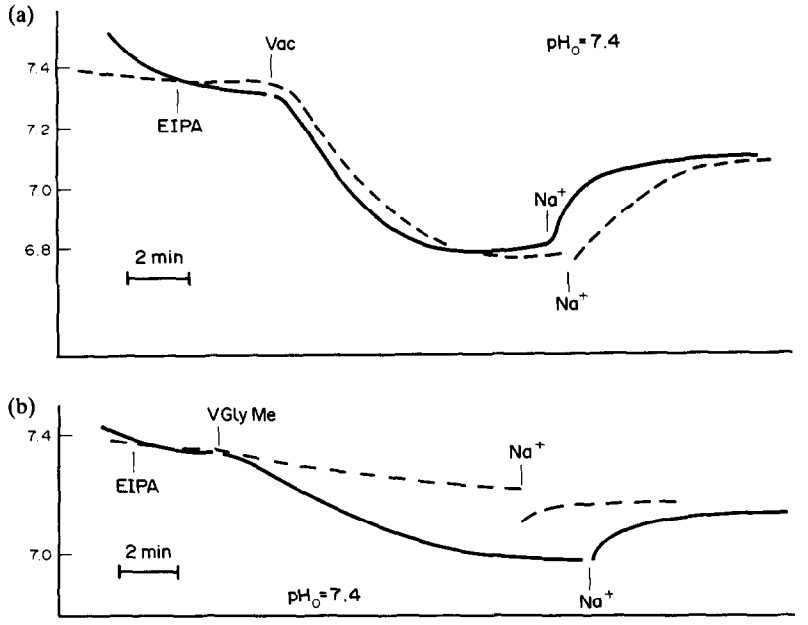

Fig. 6. Influence of EIPA on vacidin- and VGlyMe-induced pH_i changes in A20 cells suspended in Na^+-free medium. Continuous line: recordings in the absence of EIPA. Dotted line: recordings in the presence of EIPA (10^{-5} M). Vacidin (Vac) 5×10^{-7} M (a); VGlyMe 5×10^{-7} M (b). Cells were suspended in Na^+-free medium, $pH_o = 7.4$ at 37°. Addition of EIPA, antibiotics and Na^+ is indicated.

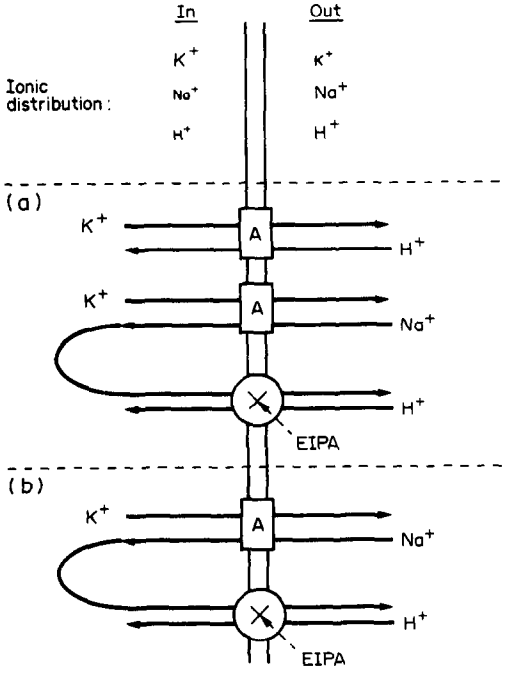

Fig. 7. Schemes for mechanism of proton flux induced by polyenes antibiotics (A): (a) vacidin; (b) perimycin or VGlyMe.

gradient directed into the cells. At external pH 6.9, acidification was more important, because both the Na^+ and H^+ gradients were directed into the cells and both ions could be exchanged for intracellular K^+. At pH 7.9, a slight alkalinization of the cytoplasm was observed (Fig. 3). These results suggested that pH_i change might be secondary to the changes in the cationic composition of the cytoplasm.

Aromatic heptaenes are efficient channel-forming ionophores, initially inducing in Na^+ medium a flux of Na^+ and H^+ according to their concentration gradients. Therefore, the intracellular concentration of sodium drastically increases and might induce an exchange of Na_i for H_o via the constitutive Na^+/H^+ exchanger localized in the cell membrane, by a reverse mechanism of that which takes place under normal physiological conditions.

This hypothesis has been proven by the effect of a specific inhibitor of the Na^+/H^+ exchanging system on polyene-induced pH_i changes. In Na^+ medium of slightly acidic pH, the pH_i decrease caused by perimycin or VGlyMe was inhibited by the amiloride derivative EIPA. At the same time, the pH_i decrease caused by vacidin was only partially inhibited. These results proved that H^+ flux occurring upon action of aromatic heptaenes without protonophoric activity is mediated by a Na^+/H^+ exchanger, the natural pH_i regulating system of animal cells. For vacidin, which has protonophoric properties, the proton flux is mediated by the Na^+/H^+ exchanger as well as by the antibiotic itself.

The confirmation of such a mechanism of proton permeability in cells treated with aromatic heptaenes

has been obtained in experiments performed in choline chloride medium. Vacidin A-induced acidification of the cell interior was due mainly to K^+/H^+ exchange. Recovery of pH_i caused by addition of NaCl to the external medium was only partially sensitive to amiloride derivative. This proved that the proton flux was mainly due to this antibiotic effect. In analogous conditions with perimycin and VGlyMe, which in sodium-free medium induce electrogenic movement of K^+, pH_i changes were completely inhibited by EIPA, showing that a decrease in pH_i and recovery are controlled by the pH_i regulating system, which mediates a reverse or normal Na^+/H^+ exchange, depending on the Na^+ gradient (Fig. 7). It is interesting to note that EIPA is without effect on the vacidin-induced acidification rate of cells suspended in Na^+-free medium (Fig. 6a) while it slows down that of cells suspended in Na^+-containing medium (Fig. 5c). Indeed, in both cases the driving force is the K^+ gradient. In the first case, Na^+ is not present in the external medium and the driving force (i.e. K^+ gradient) can only be abolished by H^+ influx in order to have no net flux of charges. Therefore, H^+ influx is dominated by the K^+/H^+ exchange (due to vacidin) while the Na^+/H^+ exchange (due to the Na^+/H^+ exchanger) is secondary. Inhibition of the Na^+/H^+ exchanger by EIPA has minor consequences.

In contrast, in the presence of external Na^+, the major exchange due to vacidin is K^+ over Na^+ and not K^+ over H^+. As a consequence, without EIPA H^+ influx is due almost exclusively to the action of the Na^+/H^+ exchanger activated by the very important gradient of Na^+ resulting from vacidin-induced Na^+/K^+ exchange. With EIPA, vacidin-induced exchanges of Na^+/K^+ and H^+/K^+ are in direct competition and acidification is slower than that due to the Na^+/H^+ exchanger.

AmB, as seen with red blood cells, has a very low ability to increase proton permeability, an observation which is in agreement with former results obtained with HL 60 leukemia cells [15]. The pH_i changes induced, if any, were not sensitive to EIPA.

In conclusion, concerning the biological properties of polyene macrolides, it can be seen that selectivity of the pathway formed by these antibiotics is important in their action on animal cells. However it is not the only determinant of the action because the antibiotic may indirectly stimulate other ion movements in a manner depending on the native constitution of the membrane and therefore the cell type.

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