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Inhibition by xipamide of amiloride-induced acidification in cultured rat cardiocytes

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Abstract

The diuretic drug xipamide improves myocardial relaxation in hypertensive patients with left ventricular hypertrophy, but its mechanism of action is unknown. Here, xipamide was tested in cultured rat heart myogenic H9c2 cells and newborn cardiomyocytes for its effects on cell acidification (and Ca^{2+} mobilization). In H9c2 cells, blocking Na^+/H^+ exchange with amiloride (2 mM) provoked cell acidification with rate = 0.82 ± 0.17 pH units/h ($n = 6$). Xipamide 1 μM maximally inhibited 50 ± 7% ($n = 9$) of cell acidification. The action of xipamide required the presence of HCO_3^- and was antagonized by the HCO_3^--transport blocker DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid). Conversely, the carbonic anhydrase (EC 4.2.1.1) inhibitor acetazolamide failed to prevent xipamide action. Finally, xipamide was without significant effect on the Ca^{2+} signals induced by endothelin-1, vasopressin or the Ca^{2+} ionophore ionomycin. In newborn rat cardiomyocytes, xipamide reduced amiloride-induced cell acidification at similar concentrations as in H9c2 cardiocytes, but with a slightly higher extent of maximal inhibition (70–80%). In conclusion, xipamide reduced amiloride-dependent cell acidification in the rat heart myogenic H9c2 cell line and in newborn rat cultured cardiomyocytes. This action of xipamide seems to be related to a complex interaction with DIDS-sensitive HCO_3^- movements. Prevention of cell acidification by xipamide could be involved in the beneficial effects of this compound in myocardial relaxation and left ventricle filling in hypertensive patients with left ventricular hypertrophy. © 1997 Elsevier Science B.V.

Keywords: Diuretic; Xipamide; Amiloride; Cardiocyte; H9c2 heart cell line; (Rat, newborn); Cell membrane

1. Introduction

Xipamide is a diuretic drug used for the treatment of high blood pressure and edema of cardiac, hepatic or renal origin (for review see Prichard and Brodgen, 1985). We have previously shown that xipamide inhibits the Na^+-dependent Cl^-/HCO_3^- exchanger in human erythrocytes, and suggested that its salidiuretic properties may result from the inhibition of the same carrier in the early distal segment of the renal tubule (Nazaret et al., 1987).

Ollivier et al. (1989) reported that xipamide improves myocardial relaxation and left ventricular filling in hypertensive patients with left ventricular hypertrophy. According to these authors, since xipamide acts through a functional rather than structural effect, the explanation of this phenomenon should be searched for in the intracellular ionic environment (Ollivier et al., 1989).

These observations suggested to us that xipamide could perhaps interfere with HCO_3^- movements across cardiac cell membranes. Indeed, the Cl^-/HCO_3^- exchanger is present in cardiac cell membranes and regulates cell pH (Nakanishi et al., 1992; Wu et al., 1994; Vandenberg et al., 1994; Kusuoka et al., 1994; Xu and Spitzer, 1994). Moreover, in a preliminary study with the rat embryonic heart cell line H9c2, we found that xipamide significantly alkalinized the cell interior (Hannaert et al., 1990; for references on H9c2 cells see Hescheler et al., 1991; Sipido and Marban, 1991; Mejia-Alvarez et al., 1994).

A well established link between myocardial relaxation and cell pH arises from the numerous studies showing that preventing cell acidification (e.g., ischemia-induced) improves muscle relaxation (for review see Okada and Ochi, 1992). Therefore, cultured rat heart myogenic H9c2 cells

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were acidified by fully blocking Na^+/H^+ exchange activity with amiloride, and xipamide was tested alone or in the presence of inhibitors of HCO_3^- membrane movements or carbonic anhydrase activity. To discard direct actions on Ca^{2+}-dependent relaxant mechanisms, xipamide was further tested for its effects on cell Ca^{2+} mobilization induced by different agonists. Finally, the inhibition of cell acidification by xipamide in H9c2 cells was confirmed in cultured newborn rat cardiomyocytes.

2. Materials and methods

2.1. Cultured rat heart myogenic H9c2 cells

The myogenic H9c2 cell line, derived from embryonic rat heart tissue, although not contractile, exhibits many of the native cardiomyocyte membrane features (for instance, they express L-type Ca^{2+} channels and they are electrophysiologically responsive, see Hescheler et al., 1991; for other references on the properties of H9c2 cells, see also Sipido and Marban, 1991; Mejia-Alvarez et al., 1994). H9c2 cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were grown in 75 cm^2 flasks (Costar) under a 5% CO_2 atmosphere in air saturated with water vapor at 37°C. Culture medium was renewed every 2 days. To passage the cells, cultures were rinsed with a Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline solution and incubated in the same medium containing 0.05% trypsin + EGTA for 4 min at room temperature.

For pH_i or cytosolic Ca^{2+} measurements, 200 μl of H9c2 cell suspension (containing about $(10–20)\times10^3$ cells) were plated on 9×35 mm glass coverslips 1–3 days before the experiments.

2.2. Cultured newborn rat cardiomyocytes

Isolation and culture of newborn rat cardiomyocytes were performed according to a previously published method (Dassouli et al., 1993). For pH_i measurements, $(20–50)\times10^3$ cells were seeded on glass coverslips 2–5 days before the experiments.

2.3. Measurement of pH_i

The pH-sensitive dye 2′-7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to measure cytosolic pH (pH_i). Adherent cells were incubated for 15 min with Ringer medium containing the acetoxymethyl ester of BCECF (1 μM) at 37°C in the dark. The composition of the basal Ringer medium was (mM): NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 5, 3-[*N*-morpholino]propanesulfonic acid (MOPS)-Tris 10 (pH 7.4 at 37°C). After BCECF loading, the coverslips with adherent cells were gently washed three times by immersion in new Ringer solution and were kept for 15 min at 37°C in new Ringer medium to allow for full BCECF hydrolysis. Coverslips were then mounted on a Teflon holder and placed at a 30° angle to the incident light beam in cuvettes containing 2 ml of HCO_3^--Ringer where 25 mM NaCl were substituted for 25 mM $NaHCO_3$ (pH = 7.4, adjusted with MOPS). Fluorescence was recorded every 2 s on a Shimadzu RF 5000 spectrofluorimeter (Roucaire, Vélizy-Villacoublay, France) at 508 nm (excitation wavelength) and 525 nm (emission wavelength). Coverslip-adherent H9c2 cell autofluorescence was negligible (i.e., < 1%). pH calibration was performed in the presence of 10 μmol/l nigericin, in 135 mM KCl saline solutions of different pH.

2.4. Measurement of cytosolic Ca^{2+}

Cytosolic free Ca^{2+} concentrations were monitored by using the Ca^{2+}-sensitive dye fura-2. H9c2 cells adherent to coverslips were loaded with 2.5 μM acetoxymethyl ester of fura-2 in Ringer solution for 45 min at room temperature in the dark. Cells were washed three times with Ringer medium and transferred to dye-free Ringer for an additional 15 min at 37°C for complete de-esterification of the dye. Fura-2 fluorescence emission was monitored in a Shimadzu spectrofluorimeter at 505 nm from cells alternatively stimulated at 345 and 380 nm using slits at 5 nm. Ionomycin 20 μM (or digitonin 0.05% v/v) and EGTA 10 mM were used for calibration. Intracellular Ca^{2+} was calculated using the method of Grynkyewicz et al. (1985):

$$Ca^{2+}\ (\mathrm{nM}) = K_d \times (R - R_{min})/(R_{max} - R) \times F_0380/F_s380$$

with K_d = fura dissociation constant at 37°C (= 224 nm); R = 340/380 nm fluorescence ratio; R_{max} = fluorescence ratio in high cytosolic Ca^{2+}; R_{min} = fluorescence ratio in low cytosolic Ca^{2+}; F_0380/F_s380 = ratio of fluorescences at 380 nm in low Ca^{2+} to that of high Ca^{2+}.

Autofluorescence of non-loaded cells measured in the same experimental conditions were subtracted before computation of fluorescence ratios for determination of cytosolic free Ca^{2+}.

2.5. Measurement of internal sodium content

Internal sodium content of cultured cells was measured according to a previously published protocol (Hannaert et al., 1986). Briefly, after careful washing of the cells $((2–4)\times10^5$ cells/well) with ice-cold isotonic $MgCl_2$ (112 mM) and cell lysis in 0.02% Acationox, sodium concentrations in the lysates were measured by flame photometry (Eppendorf 700, Roucaire, Les Ulis, France).

2.6. Solutions and reagents

BCECF and fura-2 were purchased from Molecular Probes (Eugene, OR, USA). Xipamide was provided by

Laboratoires Solvay-Pharma (Suresnes, France). 4,4′-Di-isothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and other chemicals were purchased from Sigma-Coger (Paris, France). The fluorescent dyes, xipamide, amiloride, DIDS, endothelin-1 and vasopressin were added from freshly prepared concentrated stock solutions. Dimethyl sulfoxide (DMSO) was used for the dyes, xipamide and amiloride; DMSO was added in reference conditions; control experiments have shown that the final DMSO concentrations ($< 0.2\%$, v/v) had no effect per se.

2.7. Statistical analysis

Results were expressed as mean ± S.E.M. Statistical significance between mean values was evaluated by using the two-tailed Student's *t*-test.

3. Results

3.1. H9c2 cardiocytes

3.1.1. Amiloride-induced cellular acidification

H9c2 cells incubated in HCO_3^--containing Ringer's medium had a cytosolic basal pH of 7.22 ± 0.03 ($n = 9$), whereas internal pH was 7.09 ± 0.04 ($n = 7$) in the nominal absence of HCO_3^- (external pH = 7.40). Addition of 2 mM amiloride induced a rapid cell acidification (Fig. 1). The initial rate of this process was 0.82 ± 0.17 pH units/h ($n = 6$); it was reversible upon removal of amiloride (cf., Fig. 1), and in our conditions ($Na^+ = 145$ mM) the maximal rate of amiloride-induced acidification was already obtained with 1 mM amiloride (0.84 ± 0.20 pH units/h, $n = 2$).

3.1.2. Effect of xipamide on amiloride-induced cellular acidification

Xipamide added simultaneously with amiloride had little or no effect on amiloride-dependent cell acidification. Conversely, this acidification process was significantly reduced when cells were preincubated with xipamide: for instance, Fig. 2 shows the inhibitory effect (about 50%) of 100 μM xipamide after a 3 min preincubation. Since we observed that the same extent of inhibition (50%) was attained with 5–10 μM xipamide after a 10–15 min preincubation (cf., Fig. 3), we decided to use a 15 min pre-incubation period to carry out all the following experiments.

Xipamide was tested in concentration-response curves for its effects on amiloride-dependent cell acidification. Fig. 3 shows that xipamide 1 μM inhibited $50 \pm 7\%$ ($n = 9$) of cell acidification. No further inhibition was observed at higher xipamide concentrations.

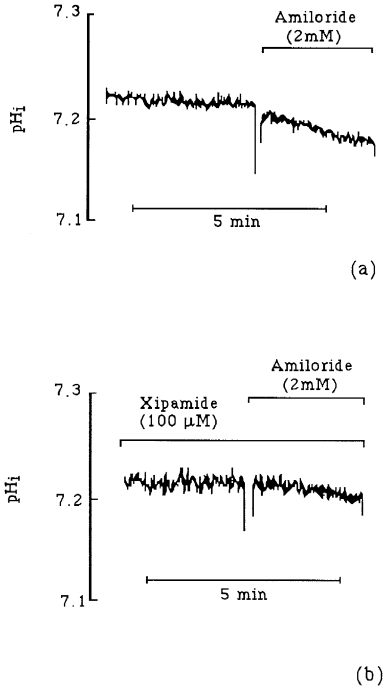

Fig. 2. Reduction by xipamide of amiloride-dependent acidification in H9c2 cells (representative traces of 4 distinct experiments). Top: control experiment. Bottom: 100 μM xipamide.

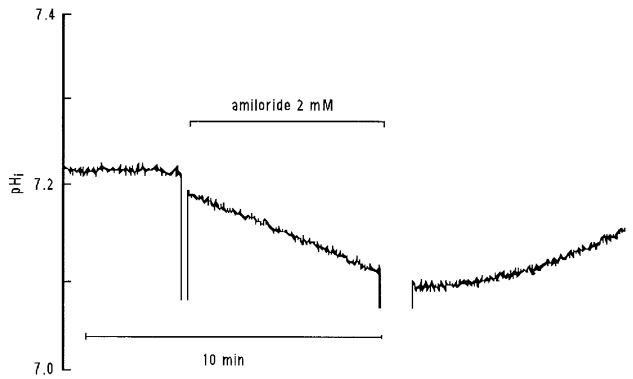

Fig. 1. Acidification by amiloride (2 mM) of rat heart myogenic H9c2 cells (initial rate = 0.82 ± 0.17 pH units/h, $n = 6$). This process was reversible upon removal of amiloride.

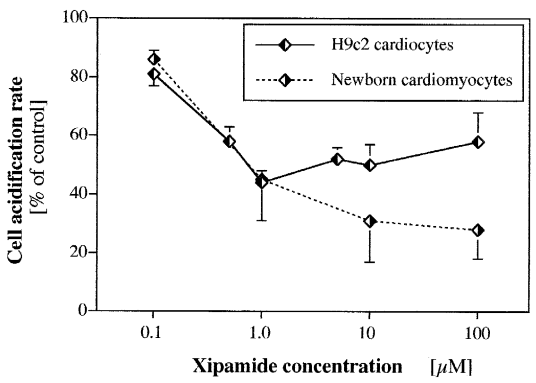

Fig. 3. Inhibition by xipamide of amiloride-dependent acidification in H9c2 cells and newborn rat cardiomyocytes. Cells were preincubated for 15 min with xipamide. Values are given as mean ± S.E.M. ($n = 4$–9).

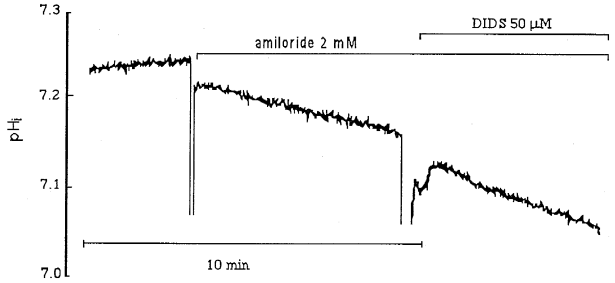

Fig. 4. Effect of DIDS on xipamide action in H9c2 cells. Amiloride-dependent acidification was not antagonized upon addition of 50 μM DIDS. Indeed, cell acidification rate was slightly enhanced by DIDS.

3.1.3. Action of xipamide on DIDS-sensitive HCO_3^- carriers

Amiloride-dependent acidification was not antagonized upon addition of 50 μM DIDS: indeed, the rate of cell acidification was slightly enhanced by DIDS (cf., Fig. 4). Moreover, DIDS prevented and even reverted xipamide antagonism on cell acidification: in the presence of both amiloride and DIDS (20 μM), xipamide increased the acidification process by 46 ± 17% ($n = 3$).

The possible involvement of a DIDS-sensitive HCO_3^--dependent membrane transport system was further explored by performing experiments in a nominally HCO_3^--free medium: in these conditions, xipamide (10 μM) inhibited amiloride-dependent cell acidification by only 25 ± 8% ($n = 3$) ($P < 0.05$ vs. (50% at 10 μM xipamide in the presence of HCO_3^-), see Fig. 3).

Finally, at constant external sodium (145 mM), bicarbonate (25 mM) induced an increase of 32 ± 5% ($n = 14$) of internal sodium content. This value was not significantly modified by 10 μM xipamide (29 ± 7%, $n = 3$), nor by 100 μM xipamide (27 ± 5%, $n = 3$).

3.1.4. Xipamide and furosemide-sensitive Cl^- carriers

Cells were preincubated for 15 min with furosemide (100 μM) and amiloride-dependent acidification was tested as before. Furosemide was not able to antagonize amiloride-dependent acidification (0.96 ± 0.24 pH units/h, $n = 3$, vs. control = 0.82 ± 0.17 pH units/h, n.s.). It is interesting to mention that hydrochlorothiazide (10 μM) was also unable to significantly modify amiloride-dependent acidification (0.81 ± 0.19 pH units/h, $n = 3$, n.s. versus control).

3.1.5. Action of xipamide on carbonic anhydrase

The carbonic anhydrase inhibitor acetazolamide was tested on amiloride-induced intracellular acidification. A significant reduction of the acidification rate was observed at concentrations higher than 2 μM, with a maximal inhibition at 10 μM acetazolamide (54 ± 11%, $n = 3$); this was similar to 10 μM xipamide alone, which inhibited 50 ± 7% of the acidification rate ($n = 9$; cf., Fig. 3). Moreover, the two drugs tested simultaneously exerted additive inhibitory effects (94 ± 5%, $n = 6$). Finally, acetazolamide (10 μM) failed to modify the shape and IC_{50} (approx. 0.2 μM) of the dose–response curve for xipamide (data not shown).

3.1.6. Effect of xipamide on agonist-induced Ca^{2+} mobilization

Basal cytosolic free Ca^{2+} content in H9c2 cells was 96 ± 10 nM ($n = 7$). Xipamide (10 μM) was unable to significantly modify basal cytosolic Ca^{2+} (Table 1). Moreover, as shown in Table 1, xipamide also failed to significantly affect the Ca^{2+} signals induced by endothelin-1 (100 nM), vasopressin (25 nM) or the Ca^{2+} ionophore ionomycin (1.4 μM).

3.2. Newborn rat cardiomyocytes

Newborn rat cardiomyocytes incubated in HCO_3^--containing Ringer's medium had a cytosolic basal pH of 7.18 ± 0.06 ($n = 7$; external pH 7.40). Addition of 2 mM amiloride induced a rapid and reversible cell acidification, with initial rate of 0.54 ± 0.07 pH units/h ($n = 18$).

As for H9c2 cells (cf., above), amiloride-dependent cell acidification was measured in cells preincubated for 15 min with different concentrations of xipamide. Fig. 3 shows that preincubation with xipamide reduced acidification rate in newborn cardiomyocytes at concentrations similar to those observed in H9c2 cardiocytes, but with a slightly higher extent of maximal inhibition (70–80% vs. 40–50%, Fig. 3).

4. Discussion

Xipamide partially reduced the acidification process initiated by blocking Na^+/H^+ exchange with amiloride in

Table 1
Xipamide and Ca^{2+} mobilization in H9c2 cardiocytes

Condition	Cytosolic free Ca^{2+} (nM)		Statistical significance
	Control	+ 10 μM xipamide	
Control (6)	96 ± 10	129 ± 16	n.s.
100 nM endothelin-1 (11)	201 ± 7	227 ± 7	n.s.
25 nM vasopressin (6)	165 ± 17	205 ± 16	n.s.
1.4 μM ionomycin (6)	222 ± 28	250 ± 31	n.s.

Values are given as mean ± S.E.M. The number of experiments is indicated in parentheses. Statistical significance was tested by using paired Student's *t*-test.

both cultured H9c2 cardiocytes (40–50%) and newborn rat cardiomyocytes (70–80%). These actions required xipamide concentrations (approx. 1 μM) much lower than therapeutical ones. In this respect, pharmacokinetic studies in human volunteers revealed that following oral administration of xipamide 20 mg, a maximum plasma concentration of about 10 μM occurs within 1 h (Knauf and Mutschler, 1984). On the other hand, preventing cell acidification is known to improve muscle relaxation (for review see Okada and Ochi, 1992). Therefore, our findings could be related to the mechanism underlying the observation by Ollivier et al. (1989) that xipamide improves myocardial relaxation and left ventricular filling in hypertensive patients with left ventricular hypertrophy.

A mechanism involving cell pH was further suggested by the observation that xipamide failed to modify basal cytosolic Ca^{2+} or to antagonize Ca^{2+} mobilization triggered by endothelin-1, vasopressin or ionomycin. Indeed, cytosolic free Ca^{2+} in cardiocytes is strongly dependent on pH_i, particularly during hypoxia/reoxygenation disturbances (for review, see Karmazyn and Moffat, 1993). Thus, cytosolic Ca^{2+} should participate as a secondary step in the mechanism of action of xipamide.

The mechanism of action of xipamide on cell pH is unclear. Two arguments suggested to us that HCO_3^- transport was perhaps involved in xipamide action: (i) in (nominally) HCO_3^--free media, xipamide action was strongly reduced, and (ii) DIDS, a non-selective blocker of Cl^-/HCO_3^- exchangers, antagonized the action of xipamide. Therefore, we suspected that xipamide was inhibiting the Na^+-dependent Cl^-/HCO_3^- exchanger, as previously shown in human erythrocytes (Nazaret et al., 1987). However, this mechanism can be discarded because: (i) in cardiocytes, the Na^+-dependent Cl^-/HCO_3^- exchanger is known to catalyze HCO_3^- influx and thus inhibition by xipamide of this HCO_3^- carrier would be expected to enhance cell acidification, (ii) HCO_3^- increased net cell Na content, but xipamide was unable to affect this phenomenon, and (iii) xipamide acts on cardiocyte pH at much lower concentrations than those required to inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger (25 μM, see Nazaret et al., 1987). Nonetheless, it seems likely that in our experiments higher concentrations of xipamide were indeed exerting an inhibition of the Na^+-dependent Cl^-/HCO_3^- exchanger, at least in H9c2 cardiocytes, since in this model the antagonistic potency of xipamide versus acidification was consistently reduced beyond 5–10 μM as compared to lower concentrations (cf., Fig. 3). The fact that this phenomenon was not observed in newborn rat cardiomyocytes might be due to a relative greater expression and/or activity of the Na^+-dependent Cl^-/HCO_3^- in H9c2 cells. This hypothesis deserves further investigations.

The Na^+-independent Cl^-/HCO_3^- exchanger mediates HCO_3^- efflux in cardiocytes (Xu and Spitzer, 1994) and can be therefore a candidate of xipamide action. A cell alkalinisation by xipamide via the inhibition of Na^+-independent Cl^-/HCO_3^- exchanger can explain the requirement of HCO_3^- and the inhibition by DIDS. This interesting hypothesis requires further investigation by using sophisticated techniques to measure Na^+-independent Cl^-/HCO_3^- fluxes and to dissociate the differential pharmacological inhibitions of DIDS (and perhaps xipamide) versus the Na^+-independent and the Na^+-dependent Cl^-/HCO_3^- exchangers (for example, see Madshus and Olsnes, 1987).

Besides DIDS-sensitive HCO_3^- carriers, other mechanisms regulate cell pH in cardiocytes and can be affected by xipamide. Among these, the Na^+-H^+ exchanger could not have contributed to xipamide action since it was fully blocked by amiloride. Likewise, the involvement of carbonic anhydrase can also be excluded, because xipamide was still able to act in the presence of the carbonic anhydrase inhibitor acetazolamide. Finally, the intervention of a putative Na^+-HCO_3^- cotransport in the action of xipamide on amiloride-induced acidification can also be excluded since, as the Na^+-dependent Cl^-/HCO_3^- exchanger does, it would catalyze HCO_3^- entry and thus would alkalinize the cytosol.

In conclusion, xipamide significantly reduced the acidification induced by amiloride in the rat heart myogenic H9c2 cell line and in cultured cardiomyocytes from newborn rats. This action of xipamide seems to be related, at least in part, to a complex interaction with DIDS-sensitive membrane HCO_3^- movements. Prevention of cardiomyocyte acidification by xipamide could explain the beneficial effects of this compound in myocardial relaxation and left ventricle filling in hypertensive patients with left ventricular hypertrophy.

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