The Journal of Experimental Biology 209, 916-926
Published by The Company of Biologists 2006
doi:10.1242/jeb.02087

Contractile recovery from acidosis in toad ventricle is independent of intracellular pH and relies upon Ca^{2+} influx

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Accepted 10 January 2006

Summary

Hypercapnic acidosis produces a negative inotropic effect on myocardial contractility followed by a partial recovery that occurs in spite of the persistent extracellular acidosis. The underlying mechanisms of this recovery are far from understood, especially in those species in which excitation–contraction coupling differs from that of the mammalian heart. The main goal of the present experiments was to obtain a better understanding of these mechanisms in the toad heart. Hypercapnic acidosis, induced by switching from a bicarbonate-buffered solution equilibrated with 5% CO_2 to the same solution equilibrated with 12% CO_2, evoked a decrease in contractility followed by a recovery that reached values higher than controls after 30 min of continued acidosis. This contractile pattern was associated with an initial decrease in intracellular pH (pH_i) that recovered to control values in spite of the persistent extracellular acidosis. Blockade of the Na^+/H^+ exchanger (NHE) with cariporide (5 μmol l^{-1}) produced a complete inhibition of pH_i restitution, without affecting the mechanical recovery. Hypercapnic acidosis also produced a gradual increase of diastolic and peak Ca^{2+}_i transient values, which occurred immediately after the acidosis was settled and persisted during the mechanical recovery phase. Inhibition of Ca^{2+} influx through the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) by KB-R (1 μmol l^{-1} for myocytes and 20 μmol l^{-1} for ventricular strips), or of L-type Ca^{2+} channels by nifedipine (0.5 μmol l^{-1}), completely abolished the mechanical recovery. Acidosis also produced an increase in the action potential duration. This prolongation persisted throughout the acidosis period. Our results show that in toad ventricular myocardium, acidosis produces a decrease in contractility, due to a decrease in Ca^{2+} myofilament responsiveness, followed by a contractile recovery, which is independent of pH_i recovery and relies on an increase in the influx of Ca^{2+}. The results further indicate that both the reverse mode NCX and the L-type Ca^{2+} channels, appear to be involved in the increase in intracellular Ca^{2+} concentration that mediates the contractile recovery from acidosis.

Key words: toad, *Buffo arenarum*, ventricle, acidosis, muscle contraction.

Introduction

It has been recognized for many years that hypercapnic acidosis produces a negative inotropic effect which, at least in the mammalian heart, is caused by a decrease in myofilament Ca^{2+} responsiveness (Fabiato and Fabiato, 1978; Mattiazzi et al., 1979; Blanchard and Solaro, 1984; Marban and Kusuoka, 1987; Orchard et al., 1991). The initial impairment of contractility is followed by a partial recovery that occurs in spite of the persistent extracellular acidosis (Mattiazzi and Cingolani, 1977b; Fry and Poole-Wilson, 1981; Cingolani et al., 1990; Pérez et al., 1993). Considerable research has been done in mammalian heart, in order to elucidate the causes of this recovery (e.g. Orchard and Kentish, 1990). Among the possible mechanisms proposed are either a recovery of intracellular pH (pH_i), which would restore myofilament responsiveness to Ca^{2+} (Boron and De Weer, 1976; Kim and Smith, 1988; Cingolani et al., 1990) and/or an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Solaro et al., 1988; Nomura et al., 2002). The general consensus is that mechanical recovery in mammalian heart is completely or partially linked to the recovery of pH_i mediated by the Na^+/H^+ exchanger (NHE). The recovery of pH_i may not only restore myofilament Ca^{2+} responsiveness but could also, by increasing intracellular Na^+ concentration ($[Na^+]_i$), produce an increase in $[Ca^{2+}]_i$ by either reducing the forward (Ca^{2+} efflux) mode of the Na^+/Ca^{2+} exchanger (NCX) or by enhancing the reverse (Ca^{2+} influx) mode (Bountra and Vaughan-Jones, 1989; Cingolani et al., 1990; Terraciano and MacLeod, 1994). This increased $[Ca^{2+}]_i$ could contribute to load the sarcoplasmic reticulum (SR) which, by releasing Ca^{2+}, would

participate in the contractile recovery (Harrison et al., 1992). In addition, $[Ca^{2+}]_i$ may also increase by a displacement from intracellular buffering sites, because of a competition with H^+ ions (Lagerstrand and Poupa, 1980; Gambassi and Capogrossi, 1992). In ectoderms, the recovery from acidosis is largely variable among species, ranging from no recovery at all to a recovery that can exceed contractile values before acidosis (Gesser and Jorgensen, 1982; Driedzic and Gesser, 1994). The contractile response to acidosis in the amphibian heart, was earlier characterised in our laboratory (Mattiazzi and Cingolani, 1977a). Although qualitatively the mechanical pattern is similar to that observed in the mammalian ventricle, in the amphibian heart the negative inotropic effect of acidosis is less pronounced and the recovery of contractility is greater, reaching levels similar to or even higher than the control values before acidosis. The underlying mechanisms of this recovery are far from understood and studies from mammalian species might be misleading because of the particular excitation–contraction coupling (ECC) of amphibians, which differs from mammalian myocardium in several aspects. In contrast to the situation of mammalian heart, the SR of the amphibian heart is poorly developed (Page and Niedergerke, 1972), does not bind ryanodine, and the ryanodine receptors are not detectable (Tijskens et al., 2003). In accordance with these findings the Ca^{2+} induced-Ca^{2+} release mechanism is also absent (Fabiato, 1982). Since the SR does not contribute significantly to the Ca^{2+} transient, the L-type Ca^{2+} channels are the major sources of activating Ca^{2+} (Klitzner and Morad, 1983; Morad et al., 1988). Moreover, myocardial relaxation takes place mainly by Ca^{2+} efflux through the NCX (Chapman and Rodrigo, 1985; Shuba et al., 1998) instead of by the Ca^{2+} ATPase of the SR as in mammalian heart (Bers et al., 1993; Negretti et al., 1993). Of interest is the fact that the contractile mechanism of the neonatal mammalian ventricle shares some common features with the amphibian heart. In neonatal mammalian heart, the SR is poorly developed (Olivetti et al., 1980; Tanaka et al., 1989) and contractility depends mainly on sarcolemmal Ca^{2+} influx (Mahony, 1996). Moreover, the NCX also plays a major role in Ca^{2+} extrusion in the neonatal myocardium (Artman, 1992; Vetter et al., 1995). Thus, knowledge of the mechanisms responsible for the recovery from acidosis in toad ventricle might also contribute to a better understanding of the behaviour of neonatal mammalian heart during acidosis.

The present study was designed to elucidate the mechanisms involved in the contractile recovery from acidosis in the toad heart.

Materials and methods

The animals used in this study were maintained in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NHI Pub. No. 85-23, Revised 1996). Toads (*Buffo arenarum* Hensel) were killed by decapitation, the spinal cord was destroyed with a steel rod and the heart was then excised.

Toad ventricular strips

Ventricular strips were dissected from rings cut perpendicularly to the longitudinal axis of the toad ventricular wall. The methods used for mounting and stimulation were essentially similar to those previously described (Mattiazzi and Cingolani, 1977a). Briefly, ventricular strips were mounted vertically in a chamber to contract isometrically. One of the ends of the muscle was firmly fixed to the bottom of the chamber by a small clamp and the other to a force transducer (Harvard Apparatus, South Natick, MA, USA), *via* a stainless steel wire. The muscles were paced to contract at a constant frequency (10 beats min^{-1}), kept at a constant temperature (30°C) and superfused with a solution of the following composition (mmol l^{-1}): 120.37 NaCl, 2.5 KCl, 1.35 $CaCl_2$, 25 $NaHCO_3$, 0.35 NaH_2PO_4, 1.05 $MgSO_4$, 10.7 glucose (bicarbonate-buffered Ringer solution, BRS). This solution was equilibrated with a gas mixture of 5% CO_2 and 95% O_2 (pH 7.45±0.02).

Mechanical studies

Once the ventricular strips were mounted, they were stretched until they reached the length at which maximal developed tension (DT) occurred and then allowed to stabilize for 1 h. Hypercapnic acidosis was induced by switching the gas bubbling of the BRS from 5% CO_2 (pH_o 7.45±0.02) to 12% CO_2 (pH_o 6.83±0.02). Contractility was assessed by DT, and maximal rate of rise of tension ($+dT/dt$).

Action potential recording

Strips of ventricular wall were mounted with the epicardial side facing up, in a Plexyglass chamber and superfused with control solution at room temperature. After recording the control action potentials (AP), the perfusion solution was switched to BRS equilibrated with 12% CO_2, and the AP recorded for 20 min. In order to arrest contraction, 1 mmol l^{-1} 2,3-butanedione monoxime (BDM; Sigma, St Louis, MO, USA) was added to the BRS (Mulieri et al., 1989). Previous experiments have shown that this concentration of BDM has no significant effect on action potential duration (APD) (Gwathmey et al., 1991). Membrane potentials were measured by means of conventional electrophysiological techniques using glass microelectrodes filled with 3 mol l^{-1} KCl. The microelectrodes had resistances ranging from 10 to 20 MΩ and were coupled to a high input impedance electrometer (W.P. Instruments, New Haven, CT, USA), whose output was recorded on line by a data acquisition system (Power Lab/410, ADinstruments, Sydney, Australia), connected to a personal computer. Action potentials were elicited by supramaximal square pulses of 2 ms duration, generated by a stimulator (S 48 Grass, Quincy, MA, USA) and delivered by means of two thin tungsten external electrodes placed close to the preparation. The negative capacitance of the electrometer was adjusted before the action potential recording.

Toad ventricular myocytes

Toad myocytes were isolated according to the technique previously described (Vila-Petroff et al., 2000) with some

modifications (Fischmeister and Hartzell, 1986). Briefly, the hearts were attached via the aorta to a cannula, excised and mounted in a Langendorff apparatus. They were then retrogradly superfused at 30°C, at a constant perfusion flow of 2–4 ml min^{-1} with Hepes-buffered solution (HBS) of the following composition (mmol l^{-1}): 146.2 NaCl, 4.7 KCl, 1 $CaCl_2$, 10.0 Hepes, 0.35 NaH_2PO_4, 1.05 $MgSO_4$, 10.7 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100% O_2. After a stabilization period of 10 min, the perfusion was switched to a nominally Ca^{2+}-free HBS for 4 min. Hearts were then recirculated with 0.75 mg ml^{-1} collagenase (Worthington, Lakewood, NJ, USA), 0.075 mg ml^{-1} protease and 1.25% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA), in HBS containing 50 μmol l^{-1} $CaCl_2$. Perfusion continued for 14 min. Hearts were then removed from the perfusion apparatus by cutting at the atrial–ventricular junction. The tissue was minced and shaken in a Petri dish containing 20 ml of the same HBS used for digestion, with the addition of 0.7 mg ml^{-1} collagenase. After 10–15 min, the dissociated myocytes were separated from the undigested tissue and rinsed several times with HBS containing 1% BSA and increasing $CaCl_2$ concentrations from 50 μmol l^{-1} to 1 mmol l^{-1}. After each wash, myocytes were left for sedimentation for 10–15 min and finally kept in HBS at room temperature (20–22°C), until use. Quiescent myocytes with clear striations and an obvious marked shortening and relaxation on stimulation were used. At the beginning of the experiments, the cells were transferred to BRS equilibrated with 95% O_2/5% CO_2 and left to stabilize for 20 min. The protocols for hypercapnic acidosis were performed at room temperature. Intracellular acidosis was produced by switching the perfusion solution from BRS equilibrated with 5% CO_2 to BRS equilibrated with 12% CO_2.

pH_i measurements

After enzymatic isolation, myocytes were loaded with the membrane-permeant acetoxymethyl ester form of the fluorescent H^+-sensitive indicator SNARF-1/AM (Molecular Probes, Eugene, OR, USA). Cell suspensions (2 ml) were exposed to a final concentration of 4 μmol l^{-1} SNARF-1/AM. After 10 min, the myocytes were gently centrifuged for 2 min, diluted in Hepes buffer and stored at room temperature until use. pH_i and cell length were monitored on the stage of a modified inverted microscope (Nikon Diaphot 200, Tokyo, Japan), as previously described (Vila-Petroff et al., 2000). After excitation at 530±5 nm, the ratio of SNARF-1/AM emission at 590±5 nm to that of 640±5 nm, was used as a measure of pH_i, according to an *in vivo* calibration. This calibration was obtained from SNARF-1/AM-loaded myocytes exposed to solutions of varying pH values, containing 140 mmol l^{-1} KCl, 20 μmol l^{-1} nigericin, 1 μmol l^{-1} valinomycin and 1 μmol l^{-1} carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone, at room temperature.

$[Na^+]_i$ measurements

For $[Na^+]_i$ measurements the isolated myocytes were loaded with the cell permeant acetoxymethyl ester form of the sodium-binding benzofuran isophthalate (SBFI AM; Molecular Probes, Eugene, OR, USA). Myocytes were incubated for 120 min at 37°C under regular gentle shaking with 10 μmol l^{-1} SBFI AM and 0.01% (w/v) pluronic acid. Myocytes were washed and resuspended in 5 ml Hepes solution and kept for 15 min to ensure complete de-esterification of all residual intracellular SBFI AM. SBFI-loaded myocytes were used in the emission ratio mode, according to the technique previously described (Baartscheer et al., 1997). Briefly, fluorescence was excited (Omega optical XF1093 340AF15, Brattleboro, VT, USA) at 340 nm through the 40× objective. Emitted light passed a barrier filter of 400 nm, a 450 nm dichroic mirror and two narrow band interference filters of 410 nm and 590 nm. Fluorescence signals were sampled at a rate of 10^3 Hz and averaged. Background fluorescence was subtracted from each signal before obtaining the 410:590 fluorescence ratio. The ratio of the SBFI emission at the two wavelengths was taken as an estimation of the $[Na^+]_i$.

Myocyte shortening

Myocytes were placed in a flow chamber on the stage of an inverted microscope, superfused with BRS solution equilibrated with 5% CO_2, and electrically stimulated with square pulses (0.5 Hz, 50% above threshold). Resting cell length and cell shortening were recorded online using a photodiode array system and data acquisition software (Ion Optix, Milton, MA, USA).

Simultaneous measurement of myocyte $Ca^{2+}{}_i$ transients and shortening

In order to correlate the myocyte cell shortening with the simultaneous changes in $[Ca^{2+}]_i$, isolated myocytes were loaded with the cell permeant acetoxymethyl ester (AM) form of the fluorescent $Ca^{2+}{}_i$ indicator Fura-2 (Molecular Probes, Eugene, OR, USA). The dye stock was made in DMSO and pluronic acid. The cells were incubated with HBS containing 4 μmol l^{-1} Fura-2 for 20 min, then washed and left for de-esterification for 30 min. Fura-2-loaded cells were placed in a flow chamber on the stage of an inverted microscope adapted for epifluorescence. Myocytes were superfused with the BRS solution equilibrated with 5% CO_2, at a constant flow of 2 ml min^{-1}. Cell fluorescence at 510 nm was monitored with a photomultiplier tube during alternate excitation with light of 360 and 380 nm wavelengths. The ratio of the fluorescence at 360 nm excitation to that at 380 nm excitation, was taken as an estimation of $[Ca^{2+}]_i$.

Statistics

All data are presented as means ± s.e.m. Comparisons within groups were assessed by paired Student's *t*-tests. Analysis of variance (ANOVA) was used when required as indicated in the text. A value of $P<0.05$ was taken to indicate statistical significance.

Results

Effect of hypercapnic acidosis on ventricular strip contraction

Fig. 1A shows a continuous recording of the contractile response to acidosis induced by elevating the CO_2 of the gas mixture that equilibrates the medium from 5% to 12%, in muscle strips contracting at 10 beats min^{-1} and 30°C. An initial fall of developed tension was followed by a spontaneous recovery, which attained levels higher than the previous control. Fig. 1B shows the overall results of the time course of the effect of hypercapnic acidosis on developed tension. The maximal depression of contractility, occurring after 4 min of acidosis (82.30±1.31% of control) was followed by a recovery that reached values significantly higher than control (113.70±3.03%), after 30 min of persistent low pH_o. Similar results were obtained in an additional experimental series performed at 30 beats min^{-1} and 24°C (data not shown).

Changes in pH_i during hypercapnic acidosis

To measure the variation of pH_i during hypercapnic acidosis, experiments were performed in isolated toad myocytes, subjected to the same protocol used in the ventricular strips. Fig. 2A shows a typical experiment in which a single myocyte was subjected to two cycles of hypercapnic acidosis, first in the absence and then in the presence of 5 μmol l^{-1} of the NHE inhibitor, cariporide. In the absence of cariporide, pH_i fell from a basal value of 7.41 to 7.01, and returned to control values after 20 min. In the presence of cariporide, pH_i decreased from 7.37 to 6.95, and persisted at these low values throughout the 20 min of recording. Control experiments indicated that two successive cycles of acidosis in the same myocyte, do not affect pH_i recovery. The overall results of five experiments of the same type are shown in Fig. 2B. These results indicate that during a sustained hypercapnic acidosis, the NHE is the mechanism responsible for the restitution of pH_i to control values.

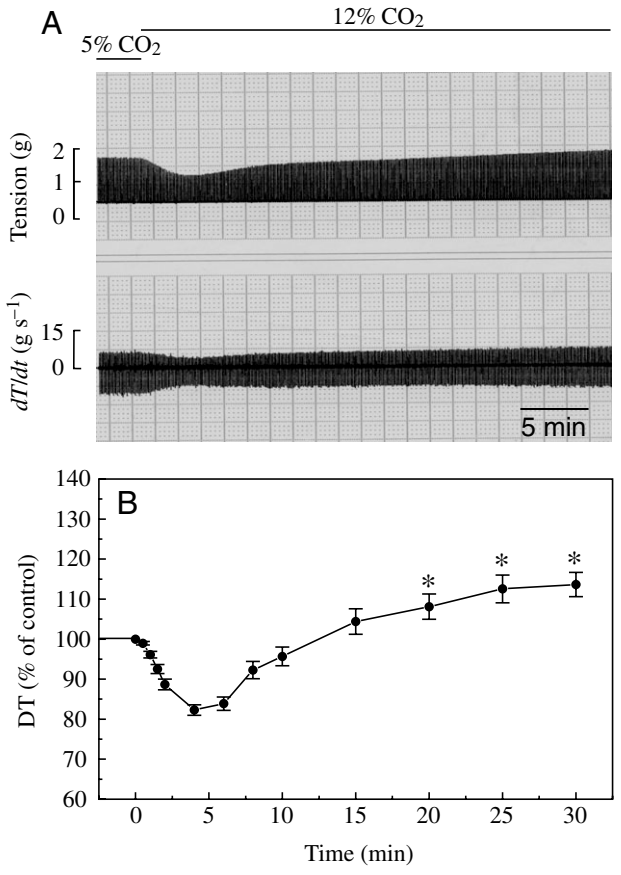

Fig. 1. Biphasic inotropic effect of hypercapnic acidosis on toad ventricle. (A) Continuous recording of developed tension (DT) and maximal rate of rise of tension (+*dT*/*dt*) during isometric contraction. Hypercapnia produced an abrupt decrease in contractility followed by recovery that exceeded control values. (B) Overall results of the effect of acidosis on DT. Data are means ± s.e.m. (*N*=49). **P*<0.05 with respect to values before hypercapnia.

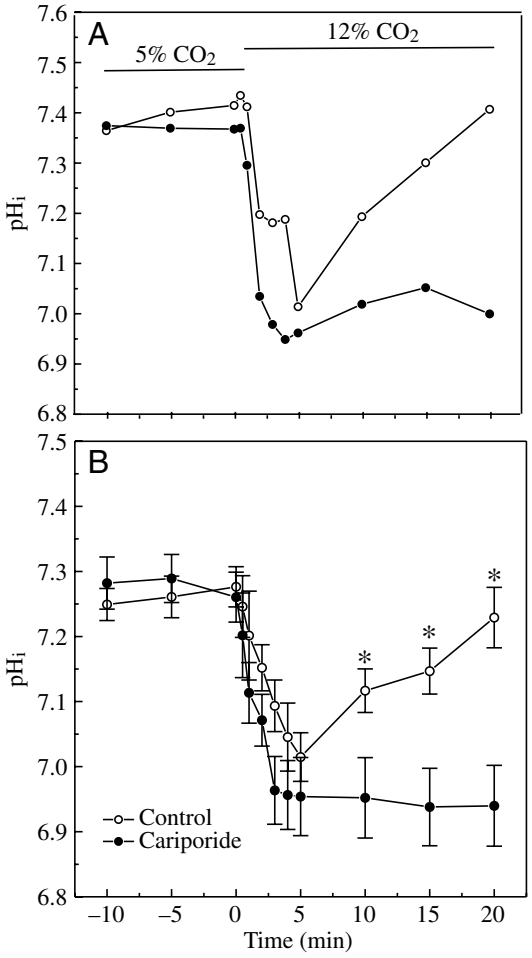

Fig. 2. Changes in pH_i in isolated toad myocytes subjected to hypercapnic acidosis in the absence or presence of Na^+/H^+ exchanger (NHE) inhibition. (A) In the the absence of cariporide, the initial fall in pH_i was followed by restitution towards the 5% CO_2 values, in spite of the persistent extracellular acidosis. No pH_i recovery was observed in the presence of cariporide (5 μmol l^{-1}). (B) Overall results of five experiments of this type. **P*<0.05 with respect to values with cariporide.

Independence of contractile recovery from pH_i restitution

In order to determine whether recovery of the pH_i through activation of the NHE is responsible for the mechanical recovery, myocyte shortening was recorded during hypercapnic acidosis in the presence and in the absence of 5 μmol l^{-1} cariporide. Fig. 3A,B shows overall results of the effect of the NHE inhibitor on the mechanical recovery during hypercapnic acidosis in isolated myocytes and ventricular strips. The presence of the NHE inhibitor failed to inhibit the contractile recovery, in spite of having abolished pH_i restitution, as shown in the experiments with isolated myocytes (Fig. 2A,B). Similar results were observed with 10 and 30 μmol l^{-1} of the drug, when used in ventricular strips (data not shown). All the doses tested did not affect the basal contractile and relaxation parameters (Table 1).

Taken together, the results indicate that the recovery of contractility in the toad ventricle is independent of pH_i recovery.

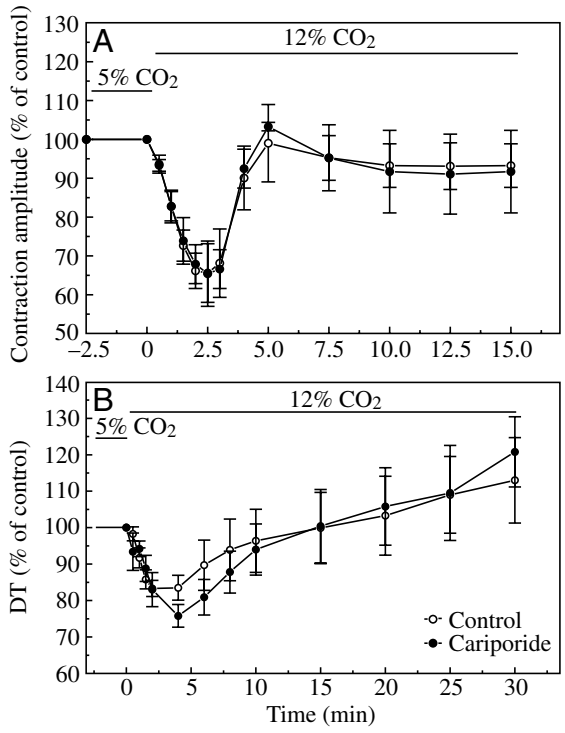

Fig. 3. Effect of persistent hypercapnic acidosis on contractile recovery in the presence and absence of the Na^+/H^+ exchanger (NHE) inhibition. (A) Isolated myocytes. During hypercapnic acidosis, contractility followed a pattern similar to that of ventricular strips, reaching control values after an initial fall. In spite of abolishing pH_i restitution with the NHE inhibitor cariporide (5 μmol l^{-1}), the inotropic recovery was not cancelled. (B) Ventricular strips. The presence of cariporide (5 μmol l^{-1}) did not modify the inotropic response of the toad ventricle to acidosis. There were no statistically significant differences between control and cariporide-treated preparations. Data are means ± s.e.m. of five experiments.

$[Ca^{2+}]_i$ and acidosis

Since contractile recovery from an acid load was not dependent on pH_i restitution, we explored the possibility that the contractile recovery was due to an increase in $Ca^{2+}{}_i$ levels.

Using electrically stimulated Fura-2-loaded toad cardiac myocytes we investigated the effect of hypercapnic acidosis on the unloaded contraction and intracellular Ca^{2+} transient (Ca_iT). Fig. 4A shows a representative example of the effect of a hypercapnic solution on myocyte contraction and the associated Ca_iT. As described earlier, hypercapnic acidosis produced a rapid decrease in cell shortening, followed by a recovery to control levels. The initial impairment in contractility occurred without a parallel decrease in the Ca_iT amplitude, but was associated with a large prolongation of the Ca_iT duration. The overall results of these experiments indicate that the decrease in contractility produced by hypercapnic acidosis is associated with a significant decrease in half relaxation time of cell shortening ($t_{1/2}$), a significant increase in diastolic and systolic $[Ca^{2+}]_i$ and a significant prolongation of the $t_{1/2}$ of Ca_iT decay (Fig. 4B). These results indicate that in the toad ventricle, the decrease in contractility evoked by hypercapnic acidosis is due to a decrease in myofilament responsiveness to Ca^{2+}, similar to mammalian heart. Contractility recovery occurs associated with a significant increase in both diastolic and systolic $[Ca^{2+}]_i$. Moreover, although the decrease in $t_{1/2}$ of cell shortening showed a

Table 1. *Effect of the different treatments on basal contractile parameters*

A Ventricular strips	*N*	DT (g)	$t_{1/2}$ (ms)
Control	5	1.07±0.19	101±5.57
Cariporide 5 μmol l^{-1}	5	1.08±0.24	101±6.25
Control	4	1.23±0.11	146±4.41
Cariporide 10 μmol l^{-1}	4	1.21±0.12	140±5.63
Control	6	0.95±0.10	111±6.40
Cariporide 30 μmol l^{-1}	6	1.05±0.22	115±4.47
Control	5	1.31±0.09	125±5.92
KB-R 20 μmol l^{-1}	5	1.27±0.09	128±6.44
Control	5	1.24±0.44	139±9.80
Nifedipine 0.5 μmol l^{-1}	5	0.71±0.30	130±7.07
B Isolated myocytes	*N*	Shortening (% of L_o)	$t_{1/2}$ (ms)
Control	5	4.13±0.60	471±25.80
Cariporide 5 μmol l^{-1}	5	4.51±0.42	466±30.60
Control	4	5.66±1.30	551±93.64
KB-R 1 μmol l^{-1}	4	6.03±1.27	563±75.07

DT, developed tension; $t_{1/2}$, half relaxation time of contraction; L_o, resting cell length.

Values are mean ± s.e.m.

tendency to recover, it did not reach control values and $t_{1/2}$ of Ca_iT decay remained significantly prolonged, suggesting that the reduced myofilament responsiveness to Ca^{2+} persists during the mechanical recovery from acidosis. Thus, the contractile recovery observed during hypercapnic acidosis is mainly due to a gradual increase in the $[Ca^{2+}]_i$. The cause for the sustained decrease in myofilament responsiveness to Ca^{2+} is not apparent to us but may suggest acidosis-induced alterations/modifications in the contractile proteins, which recover with a slower time course than the pH_i recovery. Indeed it is widely recognized that acidosis affects the different steps of Ca^{2+} signalling with different time courses (Endoh, 2001).

Taken together, these results indicate that the main mechanism underlying the contractile recovery during hypercapnic acidosis in the toad heart is an increase in $[Ca^{2+}]_i$ and occurs in spite of the lack of recovery of myofilament responsiveness to Ca^{2+}. Furthermore, in a species with a poorly developed SR such as the toad, the results showing that the increase in $[Ca^{2+}]_i$ during the recovery from acidosis is mostly due to the elevation in diastolic $[Ca^{2+}]_i$ would be consistent with an enhanced Ca^{2+} entry to the cell and failure to extrude this excess of Ca^{2+} during diastole. This could be explained by the NCX operating in the reverse mode, introducing Ca^{2+} into the cell. This extra Ca^{2+} may exceed the capacity of the NCX, during the diastolic period, to extrude all the Ca^{2+} that entered the cell.

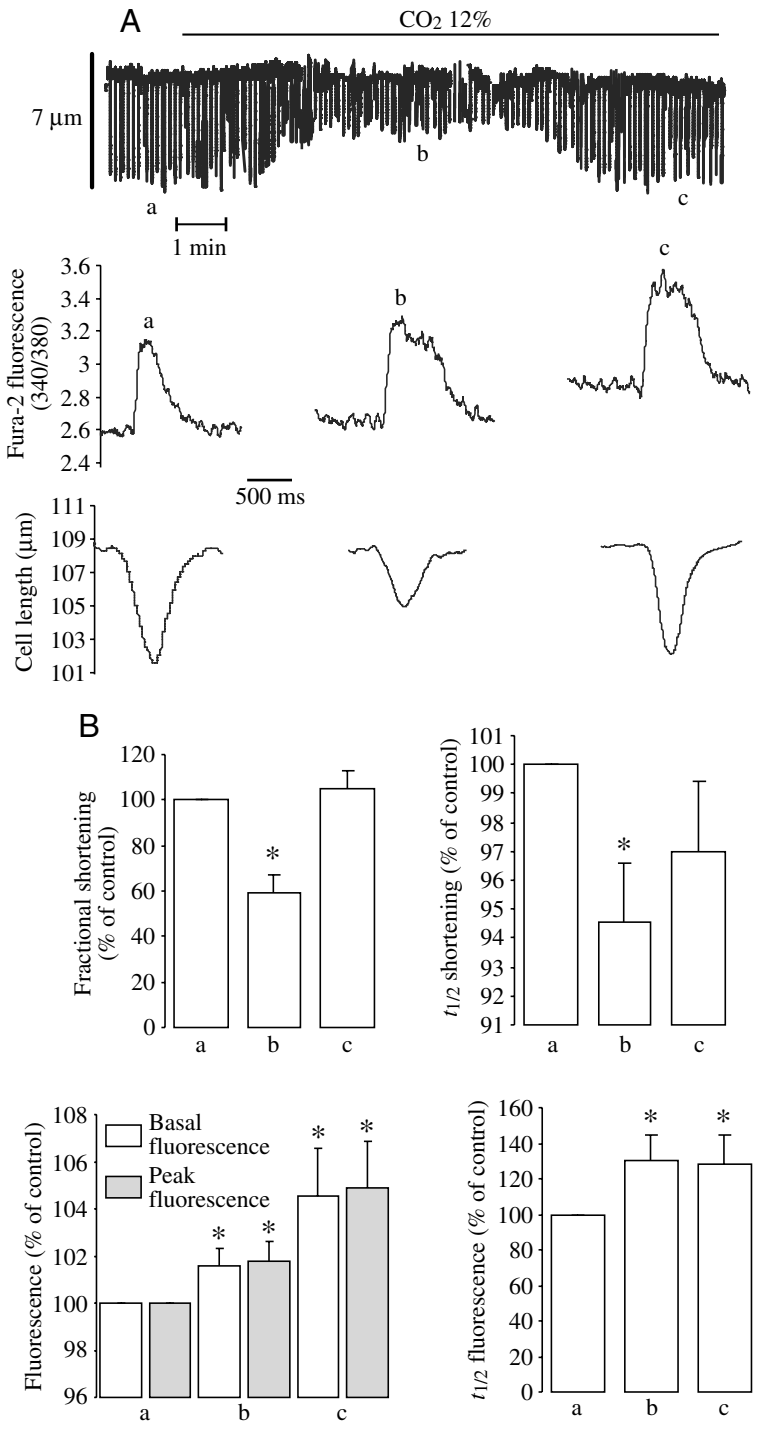

Fig. 4. Effect of hypercapnic acidosis on calcium transient (Ca_iT). (A) Top, a typical continuous recording of myocyte cell length during hypercapnic acidosis. Below, actual tracings of the individual twitch contractions and the Fura-2 fluorescence transients at the times indicated by letters a–c on the continuous chart. (B) Overall results of the effect of acidosis on Ca_iT, shortening and relaxation time of both parameters. The results indicate that during the recovery there is an increase in diastolic and peak systolic $[Ca^{2+}]_i$ with a prolongation of the Ca_iT (results from five different myocytes). *P<0.05 *vs* values before hypercapnia.

Mechanisms of Ca^{2+} increase during acidosis

The sarcoplasmic reticulum

Control experiments indicated that incubation of toad ventricular strips with ryanodine (Ry; 1 µmol l^{-1}) and thapsigargin (Ts; 1 µmol l^{-1} Sigma), administered together, did not affect either basal contractility (Ry–Ts: 98±3.27% of control) or the mechanical recovery during acidosis (114.8±7.14% *versus* 111.2±6.68% of preacidic values for control and Ry–Ts-treated muscles, respectively). In agreement with previous findings (Fabiato, 1982; Klitzner and Morad, 1983; Morad et al., 1988) these experiments indicate that the SR does not play a significant role in the ECC of this species or in the mechanical recovery from acidosis.

Influx of Ca^{2+} during acidosis

Among the mechanisms able to increase $[Ca^{2+}]_i$ in toad ventricle, Ca^{2+} influx through calcium channels and/or through the NCX working in the reverse mode, are plausible candidates. To investigate these possibilities we performed the following experiments in toad ventricular strips and isolated myocytes.

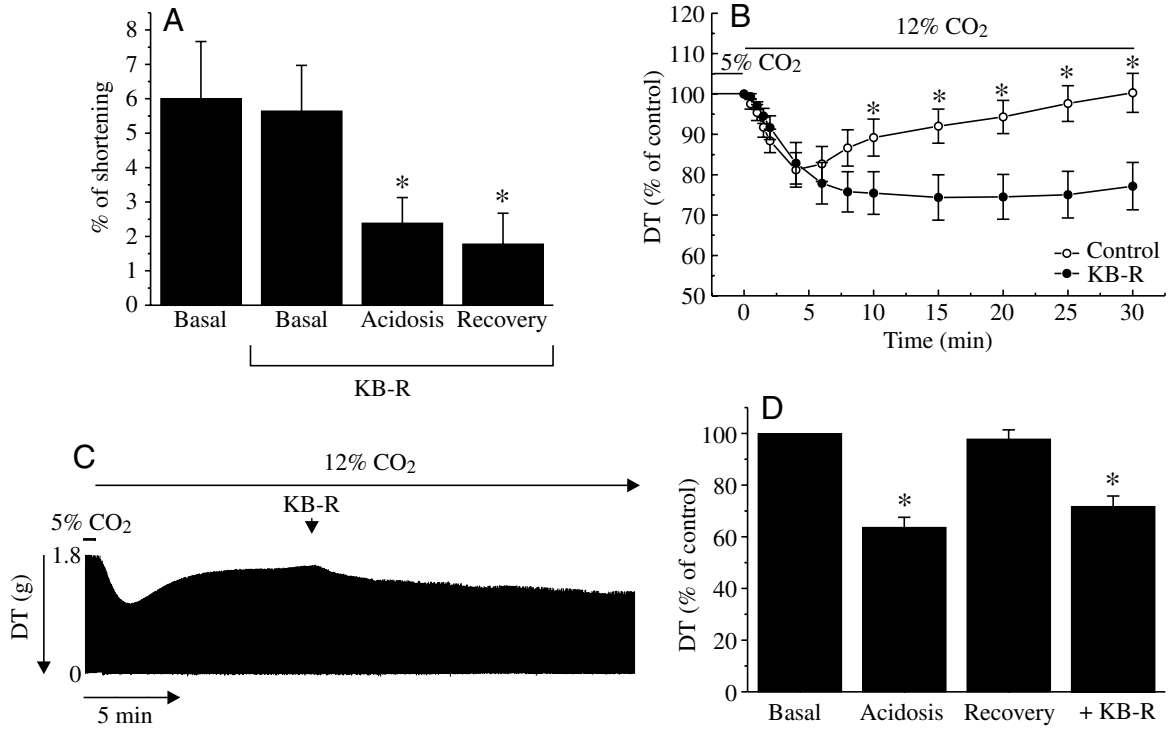

Fig. 5. Effect of Na^+/Ca^+ exchanger (NCX) inhibition on toad ventricular contractility during hypercapnic acidosis. (A) Preincubation of isolated myocytes with 1 μmol l^{-1} KB-R did not modify basal contractility but completely abolished the contractile recovery from acidosis (results from four different myocytes). (B) Similar results were obtained when ventricular strips were preincubated with 20 μmol l^{-1} (2-[2-[4-(4-nitro-benzyloxy)phenyl]ethyl] isothiourea methanesulphonate (KB-R) (N=5). (C) A continuous record of tension showing that KB-R reverses the contractile recovery: the addition of the Na^+/Ca^+ exchanger (NCX) inhibitor (20 μmol l^{-1}) to ventricular strips, once the recovery reached stabilization produced a fall of developed tension (DT) to the values before the recovery. (D) Overall results of these experiments (N=4). *P<0.05 *vs* values before hypercapnia.

Influx of Ca^{2+} through NCX

Hypercapnic acidosis was induced in the presence of KB-R, (2-[2-[4-(4-nitro-benzyloxy)phenyl] ethyl] isothiourea methanesulphonate; Tocris, Ellisville, MO, USA), an inhibitor of the reverse mode of the NCX (Iwamoto et al., 1996). Fig. 5A shows that preincubation of isolated myocytes with 1 μmol l^{-1} KB-R, a dose that did not affect basal shortening in this preparation (Table 1), abolished the contractile recovery from acidosis. Myocyte shortening decreased from 6.00±1.60% to 2.39±0.74% of resting cell length (L_o), after 2 min of hypercapnic acidosis and remained at these low values at the end of the experimental period (1.78±0.80% of L_o). Similar results were obtained after incubating ventricular strips with 20 μmol l^{-1} of KB-R. (Fig. 5B). To further confirm the participation of the NCX in contractile restitution, an additional set of experiments was designed in which the NCX inhibitor was added once the negative inotropic effect produced by the acidosis had recovered. In these conditions the addition of KB-R (20 μmol l^{-1}) to the ventricular strips completely suppressed the increase in developed tension, returning contractility to the values observed before the beginning of recovery (Fig. 5C). Fig. 5D shows the overall results of these experiments.

$[Na^+]_i$ measurements

The activity of the reverse mode of the NCX may be favored by an increase in $[Na^+]_i$. Since the mechanical recovery is independent of the activity of the NHE (Figs 2 and 3), a possible increase in $[Na^+]_i$ if responsible for the activation of this mode of the NCX, should occur by mechanisms different from those of the NHE. We therefore assessed $[Na^+]_i$ during hypercapnic acidosis, in the presence of NHE inhibition. Fig. 6A shows a continuous recording of $[Na^+]_i$ in the presence of 5 μmol l^{-1} cariporide, before and during hypercapnic acidosis, and after the addition of 10 μmol l^{-1} ouabain (Sigma), to inhibit the Na^+-K^+-ATPase pump. Whereas acidosis in the presence of cariporide failed to affect $[Na^+]_i$, the addition of ouabain evoked a significant increase. Fig. 6B depicts the overall results of these experiments.

Influx of Ca^{2+} through Ca^{2+} channels

The following experiments were conducted to elucidate the role of Ca^{2+} influx through L-type Ca^{2+} channels in the contractile recovery from acidosis. Ventricular strips were pretreated with 0.5 μmol l^{-1} nifedipine (Sigma) for 20 min. This nifedipine concentration decreased basal contractility to 53±4.25% of control (Table 1) and 10 μmol l^{-1} of nifedipine was sufficient to completely abolish contractility. When

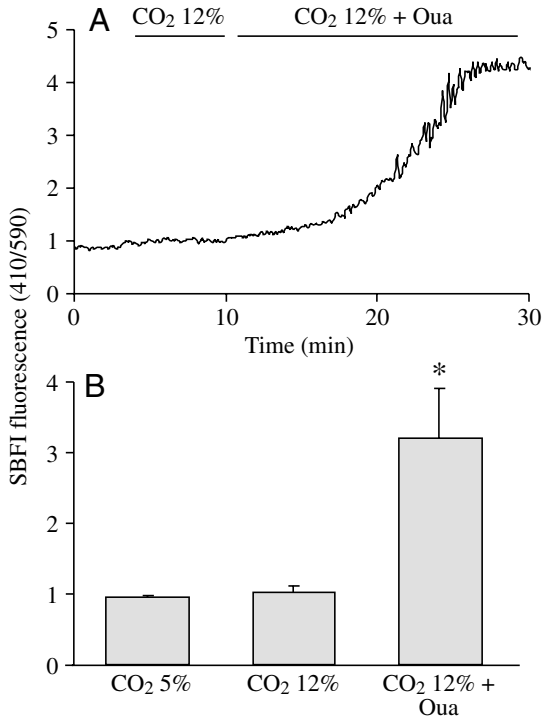

Fig. 6. Effect of hypercapnic acidosis and Na^+/K^+ ATPase-pump inhibition on intracellular Na^+ levels. (A) Typical recording of $[Na^+]_i$ in the continued presence of 5 μmol l^{-1} cariporide. Switching from a 5% to a 12% CO_2 buffered solution had no effect on Na^+_i levels, whereas addition of the Na^+/K^+ ATPase pump inhibitor, ouabain at 10 μmol l^{-1} during hypercapnic acidosis, substantially increased $[Na^+]_i$. (B) Overall results showing mean sodium-binding benzofuran isophthalate (SBFI) fluorescence values, indicating the lack of effect of hypercapnic acidosis on Na^+_i levels and the significant increase produced by ouabain. Values are means ± s.e.m., N=5 cells. *P<0.05 with respect to values before hypercapnia.

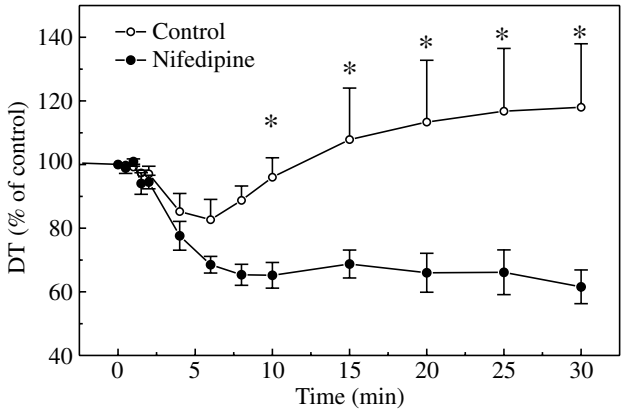

Fig. 7. Effect of blocking Ca^{2+} channels on contractile recovery. The treatment of ventricular strips with 0.5 μmol l^{-1} nifedipine abolished the contractile recovery from acidosis (N=5). *P<0.05 *vs* values with nifedipine. DT, developed tension.

hypercapnic acidosis was induced in the presence of 0.5 μmol l^{-1} nifedipine, the ventricular strips showed a complete inhibition of contractile recovery as shown in Fig. 7.

Taken together, these experiments indicate that the blockade of either of the two main pathways of Ca^{2+} influx in the amphibian ventricle is able to completely abolish the mechanical recovery.

Effect of acidosis on action potential duration

In order to elucidate whether hypercapnic acidosis alters action potential duration (APD) of toad ventricular myocardium, APs were monitored in ventricular strips superfused with BRS equilibrated with 5% CO_2 and then switched to BRS equilibrated with 12% CO_2. Fig. 8A, depicts a representative tracing of two APs recorded at control or at acid pH. Acidosis induced a prolongation of the repolarization phase. A significant lengthening of the time to 20% (APD_{20}), 50% (APD_{50}) and 90% (APD_{90}) of repolarization occurred after 3.40±0.02 min of acidosis and persisted during the 30 min period of recording. Overall results of these experiments are shown in Fig. 8B. Control experiments in which the strips were perfused with BRS (5% CO_2) for 30 min, failed to show detectable changes in APD.

Discussion

Acidosis-induced decrease in myocardial contractility is mediated by a decrease in Ca^{2+} myofilament responsiveness

In the mammalian species, there is agreement in the fact that acidosis induces an initial fall in myocardial contractility, due to a decrease in myofilament responsiveness to Ca^{2+} (Fabiato and Fabiato, 1978; Mattiazzi et al., 1979; Blanchard and Solaro, 1984; Marban and Kusuoka, 1987; Orchard et al., 1991). The present experiments extend this conclusion to the amphibian heart. We showed for the first time in the amphibian heart, that the decrease in contractility induced by acidosis occurred with no significant changes in Ca^{2+} transient amplitude, supporting the contention that this negative inotropic effect is mediated, also in this species, by a decrease in Ca^{2+} myofilament responsiveness.

Recovery of contractility during acidosis: independence of pH_i and dependence on $[Ca^{2+}]_i$

Depending on the species and the experimental conditions, the negative inotropic effect of acidosis is followed by a complete (Mattiazzi and Cingolani, 1977a; Gesser and Jorgensen, 1982), partial (Gesser and Jorgensen, 1982; Mattiazzi and Cingolani, 1977b; Cingolani et al., 1990; Pérez et al., 1993) or no recovery (Gesser and Jorgensen, 1982; Hoglund and Gesser, 1987) of contractility. The evidence to explain the increment in the contractile response has not been straightforward. Some investigators have found that intracellular acidosis was followed by restitution of pH_i and, since the contractile recovery was completely abolished by NHE inhibition, they suggested that pH_i restitution and the contractile recovery were entirely dependent on the activity of

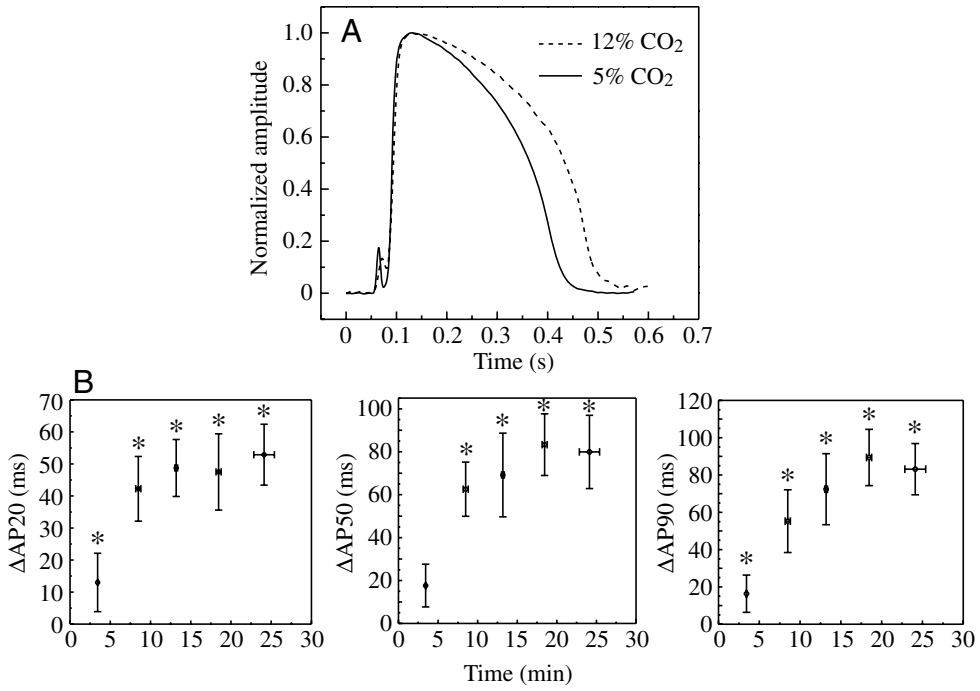

Fig. 8. Action potential duration of ventricular strips at control pH and during acidosis. (A) A representative tracing of AP under both conditions indicates a lengthening of repolarization. (B) Overall results showing hypercapnic acidosis-induced prolongation of the action potential (AP) duration at the time of the 20%, 50% and 90% of repolarization (N=6). *P<0.05 *vs* value before acidosis.

this ion exchanger (Pérez et al., 1993). Experiments performed in isolated ferret hearts showed that although NHE inhibition abolished the recovery of pH_i during respiratory acidosis, ventricular-developed pressure recovered partially (Cingolani et al., 1990). Of interest, Snow et al. (Snow et al., 1982) reported a recuperation of the contractile activity in amphibian hearts during hypercapnic acidosis, but they failed to detect a recovery of pH_i. Our results, in isolated myocytes, clearly showed an NHE-mediated pH_i restitution after hypercapnic acidosis in toad ventricle. However, the inhibition of the NHE with cariporide failed to abolish the positive inotropic response in both preparations, ventricular strips and myocytes. These results indicate that contractile recovery from acidosis, in the toad heart, is independent of pH_i recovery and appears to be the consequence of an increase in $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ during acidosis has already been reported in other species (Solaro et al., 1988; Nomura et al., 2002) and our results using Fura-2-loaded myocytes are consistent with this observation (Fig. 4).

Dependence of contractile recovery on Ca^{2+} influx: NCX vs L-type Ca^{2+}channels

In species with a poorly developed SR, Ca^{2+} influx from the extracellular space is the main source of activating contractile Ca^{2+} (Klitzner and Morad, 1983; Hoglund and Gesser, 1987; Vornanen, 1999; Tijskens et al., 2003). In agreement with these findings, our results indicate that blockade of SR function does not affect either basal contractility or the mechanical recovery from acidosis. Earlier experiments suggested that the major influx of Ca^{2+} in the amphibian heart occurs through the L-type Ca^{2+} channel (Tijskens et al., 2003). In line with these findings, the present results showed that inhibition of the NCX failed to affect basal contractility and 10 μmol l^{-1} nifedipine was able to completely block contraction. However, the results obtained with the inhibitor of the reverse mode of the NCX support the contention that the exchanger does play an important role in the recovery from acidosis. It could be argued that the concentration of KB-R used in ventricular strips is above that commonly used in mammalian heart. However, the concentration of KB-R necessary to block the reverse mode of the NCX seems to depend on the species or the stage of development [i.e. those with poorly developed SR require higher concentration (Woo and Morad, 2001; Huang et al., 2005)], and the experimental conditions [acid conditions require higher concentrations than normal pH (Ladilov et al., 1999; Schäfer et al., 2001)]. Nevertheless, similar results were obtained in isolated toad myocytes, using a much lower concentration of KB-R that also had no effect on basal contractility.

There are at least two, not mutually exclusive, mechanisms that may account for the increase in $[Ca^{2+}]_i$ through the reverse mode of the NCX during acidosis. First, a decrease in the transmembrane Na^+ gradient as a result of an increase in $[Na^+]_i$, which would favour the reverse mode of the NCX. The present results indicate that this possibility is unlikely. An increase in $[Na^+]_i$ could be expected during acidosis, from either the activation of the NHE and/or from the Na^+/CO_3H^- cotransporter or from an acidosis-induced inhibition of Na^+-K^+-ATPase activity (Speralakis and Lee, 1971; Balasubramanian et al., 1973). However, these explanations could be discarded based on the following findings. (1) The complete inhibition of pH_i recovery with cariporide would exclude the Na^+/CO_3H^- cotransporter as a significant mechanism in the regulation of pH_i; (2) The independence of contractile recovery from pH_i recovery, would exclude the participation of the NHE; (3) The lack of detection of any

significant increase in $[Na^+]_i$ during acidosis in the presence of NHE blockade, would indicate that the inhibition of the Na^+-K^+-ATPase pump is not significantly involved. A second possible mechanism that would favour the reverse NCX mode, is a prolongation of the time at which membrane potential is above the equilibrium potential for the NCX. During this time, the NCX would work in the Ca^{2+} influx mode. This possibility is supported by the present experiments. Although the effects of acidosis on APD in the mammalian heart are controversial (Chesnais et al., 1975; Fry and Poole-Wilson, 1981; Sato et al., 1985; Komukai et al., 2002), we clearly showed a prolongation of the AP at different repolarization times throughout the acidosis period in the toad ventricle. The mechanism of the AP prolongation was not explored in the present work. However, we showed that nifedipine was able to block mechanical recovery. Thus, it is tempting to speculate that acidosis, by increasing Ca^{2+} entry through L-type Ca^{2+} channels, might account for the prolongation of the AP, which in turn would favour the influx of Ca^{2+} through the reverse mode of the NCX. Although this is an attractive hypothesis, at least two different observations argue against its veracity. First, Ca^{2+} entry through L-type Ca^{2+} channels has been shown to be either decreased (Orchard and Kentish, 1990) or not changed by acidosis (Komukai et al., 2002); second, different experiments in mammalian ventricle have linked the prolongation of AP duration, during acidosis, to an inhibition of repolarizing K^+ currents rather than to activation of the Ca^{2+} inward current (Harvey and Ten Eick, 1989; Komukai et al., 2002). If this holds true for the amphibian heart, a possible explanation for our results might be that the decrease in myofilament responsiveness to Ca^{2+} (or any other intracellular effect of acidosis), triggers the activation of the two main pathways of Ca^{2+} influx to the cell, the L-type Ca^{2+} channels and the reverse mode of the NCX, both of which would be favoured, in addition, by the prolongation of the AP. However, this possibility cannot explain our finding that the separate inhibition of any of them precludes the recovery. A second possible explanation of the somewhat unexpected finding that nifedipine and KB-R are both able to block the mechanical recovery, would rely on the property of nifedipine, to decrease the AP duration (Go et al., 2005). This effect would negate the prolongation of the AP produced by acidosis and therefore the entry of Ca^{2+} through the NCX. If this were the case, nifedipine would indirectly preclude the activity of the NCX in the reverse mode. Clearly, further work is needed to clarify this issue.

Our results indicate that in toad ventricular myocardium a decrease in myofilament responsiveness to Ca^{2+} mediates the initial fall in contractility observed during hypercapnic acidosis, similar to what has been discussed in mammalian heart. The subsequent recovery of contractility is due to an increase in Ca^{2+} influx. While NCX appears to play a central role in the increase of the $[Ca^{2+}]_i$, participation of the L-type Ca^{2+} channels cannot be ruled out. In addition, the results provide clear evidence supporting the view that the mechanical recovery from acidosis is not a pH_i-dependent mechanism in the amphibian heart.

This study was supported by a grant PIP 02256 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and PICT 8592 from Agencia Nacional de Promoción Científica y Tecnológica to A.M. The technical assistance of Mrs Mónica Rando and of the student Luis Gonano is gratefully acknowledged. We also thank Dr Alejandro Aiello for helpful discussions of an early version of the manuscript. M.A.S. is an Established Investigator of the University of La Plata, Argentina. A.M., V.R.A. and M.G.V.P. are Established Investigators of CONICET, Argentina.

List of abbreviations

BRS	bicarbonate-buffered Ringer solution
DT	developed tension
pH_i	intracellular pH
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Na^+]_i$	intracellular sodium concentration
Ca_iT	calcium transient
KB-R	2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulphonate, inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NCX	Na^+/Ca^{2+} exchanger
APD	action potential duration

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