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Na+–Ca2+ exchange function underlying contraction frequency inotropy in the cat myocardium

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In most mammalian species, an increase in stimulation frequency (ISF) produces an increase in contractility (treppe phenomenon), which results from larger Ca^{2+} transients at higher frequencies, due to an increase in sarcoplasmic reticulum Ca^{2+} load and release. The present study attempts to elucidate the contribution of the Na^+–Ca^{2+} exchanger (NCX) to this phenomenon. Isolated cat ventricular myocytes, loaded with $[Ca^{2+}]_i$- and $[Na^+]_i$-sensitive probes, were used to determine whether the contribution of the NCX to the positive inotropic effect of ISF is due to an increase in Ca^{2+} influx (reverse mode) and/or a decrease in Ca^{2+} efflux (forward mode) via the NCX, due to frequency-induced $[Na^+]_i$ elevation, or whether it was due to the reduced time for the NCX to extrude Ca^{2+}. The results showed that the positive intropic effect produced by ISF was temporally dissociated from the increase in $[Na^+]_i$ and was not modified by KB-R7943 (1 or 5 μM), a specific blocker of the reverse mode of the NCX. Whereas the ISF from 10 to 30 beats min^{-1} (bpm) did not affect the forward mode of the NCX (assessed by the time to half-relaxation of the caffeine-induced Ca^{2+} transient), the ISF to 50 bpm produced a significant reduction of the activity of the forward mode of the NCX, which occurred in association with an increase in $[Na^+]_i$ (from 4.33 ± 0.40 to 7.25 ± 0.50 mM). However, both changes became significant well after the maximal positive inotropic effect had been reached. In contrast, the positive inotropic effect produced by ISF from 10 to 50 bpm was associated with an increase in diastolic $[Ca^{2+}]_i$, which occurred in spite of a significant increase in the relaxation rate and at a time at which no increases in $[Na^+]_i$ were detected. The contribution of the NCX to stimulus frequency inotropy would therefore depend on a decrease in NCX-mediated Ca^{2+} efflux due to the reduced diastolic interval between beats and not on $[Na^+]_i$-dependent mechanisms.

The Na^+–Ca^{2+} exchanger (NCX) is a sarcolemmal Ca^{2+} transport system present in the plasma membrane of almost every cell type. The exchanger is an electrogenic counter ion transporter in which three Na^+ are exchanged for each Ca^{2+}. Thus, the function of this exchanger is controlled by the membrane potential as well as by the gradients of Na^+ and Ca^{2+} across the cell membrane. In cardiac myocytes, the NCX usually operates in the forward mode, in which Na^+ enters the cell and Ca^{2+} is extruded, playing a central role in the control of muscle relaxation and diastolic calcium (Bers *et al.* 1989; Bassani *et al.* 1994). By these actions, the NCX indirectly regulates the Ca^{2+} stored in the sarcoplasmic reticulum (SR). During the action potential, the NCX can also operate in the reverse mode (Blaustein & Lederer, 1999; Bers, 2001; Pieske *et al.* 2002), in which Ca^{2+} enters the cell and Na^+ is extruded. This could increase the SR Ca^{2+} content and also modulate SR Ca^{2+} release, working in synergism with L-type Ca^{2+} channels (Litwin *et al.* 1998). Under conditions in which $[Na^+]_i$ is increased, the activity of the forward mode of the

NCX would be limited and that of the reverse mode would be favoured. Thus, an increase in $[Na^+]_i$ may increase $[Ca^{2+}]_i$ because either Ca^{2+} efflux via the forward mode of the NCX is reduced (failing to match Ca^{2+} influx), or Ca^{2+} influx via the reverse mode of the exchanger is increased.

A typical example of how the NCX can influence myocardial contractility is provided by cardiac glycosides. Experimental evidence indicates that the positive inotropy produced by these agents is due to Na^+–K^+ pump blockade, increases in $[Na^+]_i$ and increases in $[Ca^{2+}]_i$. Recent experiments in rat myocytes have shown that the positive inotropic effect of strophanthidin is due to inhibition of Ca^{2+} efflux via the forward mode of the exchanger. The Ca^{2+} influx mode of the NCX seems to be only responsible for the toxic effects of these drugs and not for the inotropic effects (Satoh *et al.* 2000).

The NCX has been also associated with the mechanisms involved in the increase in contractility produced by increasing stimulation frequency (treppe phenomenon;

Langer *et al.* 1971; Cohen *et al.* 1982; Wang *et al.* 1988; Bers, 2001). The positive staircase results from an increase in the intracellular Ca^{2+} transient (Ca_iT), mainly due to an increased SR Ca^{2+} content at higher stimulation frequencies. The increase in SR Ca^{2+} content would mainly result from an increase in Ca^{2+} influx per unit time and a reduced Ca^{2+} efflux between beats (reviewed in Bers, 2001). Moreover, additional mechanisms, like an increase in the activity of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) produced by higher average $[Ca^{2+}]_i$ (Bassani *et al.* 1995; Li *et al.* 1997) or the frequency-dependent facilitation of L-type Ca^{2+}-current described at higher frequencies (Zygmunt & Maylie, 1990) would further enhance SR Ca^{2+} uptake and release. In this scenario, one of the mechanisms by which the NCX could contribute to the positive staircase is through the reduced time available for the exchanger to extrude Ca^{2+}, which would favour the increase in SR Ca^{2+} content. But in addition, and as in the case of cardiac glycosides, the increase in contraction frequency is also associated with an increase in $[Na^+]_i$ produced by the larger number of depolarizations per unit time (Cohen *et al.* 1982; Wang *et al.* 1988; Despa *et al.* 2002). The increase in $[Na^+]_i$ may either reduce the activity of the forward (Ca^{2+}-efflux) mode of the NCX limiting Ca^{2+} extrusion, which would increase SR Ca^{2+} content, or favour the reverse (Ca^{2+}-influx) mode, which would increase SR Ca^{2+} content and enhance SR Ca^{2+} release (Litwin *et al.* 1998; Bers 2001). Indeed, previous studies have suggested that an increase in $[Na^+]_i$ is a significant mediator of the positive force–frequency staircase (Cohen *et al.* 1981; Boyett *et al.* 1987; Lee *et al.* 1987). Whether the NCX contributes to stimulus frequency inotropy by either increasing Ca^{2+} influx or decreasing Ca^{2+} efflux due to the frequency-induced elevation in $[Na^+]_i$, or whether the NCX-dependent increase in $[Ca^{2+}]_i$ is simply due to the reduced time between beats to extrude Ca^{2+} via the forward mode of the exchanger, has never been explored. The present experiments were undertaken with the aim of answering these questions.

METHODS

Myocyte isolation

All experiments were performed in accordance with the guidelines for Animal Care of the Scientific Committee of the University of La Plata School of Medicine. Cats were anaesthetized by intra-abdominal injection of sodium pentobarbitone (35 mg (kg body weight)$^{-1}$). Cat myocytes were isolated according to the technique previously described (Vila Petroff *et al.* 2000) with some modifications. Briefly, the hearts were attached via the aorta to a cannula, excised and mounted in a Langendorff apparatus. They were then retrogradly perfused at 37°C at a constant perfusion pressure of 70–80 mmHg with Krebs-Henseleit solution (K-H) of the following composition (mM): 146.2 NaCl, 4.7 KCl, 1.35 CaCl$_2$, 10.0 Hepes, 0.35 NaH$_2$PO$_4$, 1.05 MgSO$_4$, 10.0 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100 % O$_2$. After a stabilization period of 4 min, the perfusion was switched to a nominally Ca^{2+}-free K-H for 6 min.

Hearts were then recirculated with collagenase (118 u ml^{-1}), 0.1 mg ml^{-1} pronase and 1 % bovine serum albumin (BSA), in K-H containing 50 μM CaCl$_2$. Perfusion continued until hearts became flaccid (15–25 min). Hearts were then removed from the perfusion apparatus by cutting at the atrio-ventricular junction. The isolated myocytes were separated from the undigested tissue and rinsed several times with a K-H solution containing 1 % BSA and 500 μM CaCl$_2$. After each wash, myocytes were left for sedimentation for 10 min. Myocytes were kept in K-H solution at room temperature (20–22° C) until use. Rod-shaped myocytes with clear and distinct striations and obvious marked shortening and relaxation on stimulation were used. Cells that were unstable at low frequencies, that did not show an obvious positive inotropic effect in response to an increase in stimulation frequency, or that showed early signs of deterioration, such as large decreases in resting cell length at low stimulation frequencies, were discarded. Experiments were performed at room temperature.

Indo-1 fluorescence and cell-shortening measurements

The isolated myocytes were loaded at room temperature with the cell-permeant acetomethyl ester form of indo-1 (17 μM for 9 min) according to the bulk method described by Spurgeon *et al.* (1990) and left for de-esterification for 45 min. Cells were then placed on the stage of an inverted microscope (Nikon Diaphot 200) adapted for epifluorescence. Myocytes were continuously superfused with K-H (pH 7.4) at a constant flow of 1 ml min^{-1} and field-stimulated via two platinum electrodes on either side of the bath (square waves, 2 ms duration and 20 % above threshold) at the frequencies indicated in the text and figures. The excitation light was centred at 350 nm through the × 40 objective and the fluorescence emitted by the cell passed a barrier filter of 400 nm, a 450 nm dichroic mirror, and was recorded at 410 and 490 nm. Fluorescence signals were sampled at rate of 10^3 Hz and averaged. Background fluorescence was subtracted from each signal before obtaining the 410 nm/490 nm fluorescence ratio. The diastolic fluorescence ratio was measured as the mean value over a 100 ms period after the twitch was completed. The systolic fluorescence ratio was determined directly from the peak of the recorded ratio. The ratio of the indo-1 emission at the two wavelengths was taken as an index of the $[Ca^{2+}]_i$. The indo-1 fluorescence was used to measure systolic, diastolic and amplitude of the Ca_iT. The rate of Ca_iT decline was assessed by the time to half-relaxation, $t_{1/2}$, and by the time constant of Ca_iT decline, τ.

The stage of the microscope was illuminated with red light (640–750 nm) through its normal bright-field illumination optics to allow simultaneous measurement of fluorescence and shortening. Resting cell length and cell shortening were measured by a video-based motion detector (Crescent electronics, UT, USA) and stored by software (PowerLab/400 ADInstruments) for off-line analysis. Relaxation was assessed by time to half-relaxation, $t_{1/2}$. Cell shortening was continuously monitored on-line and stored by software (PowerLab/400 ADInstruments).

SBFI fluorescence measurements

For $[Na^+]_i$ measurements, the isolated myocytes were loaded with the cell-permeant acetomethyl ester form of sodium-binding benzofuran isophthalate (SBFI AM). Myocytes were incubated for 120 min at 37°C under regular gentle shaking with 10 μM 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

Table 1. Effect of increasing stimulation frequency on contraction and Ca^{2+} transient parameters of single cat myocytes

Contractile parameters					
Frequency (bpm)	10	20	30	40	50
L_0 (μm)	138.16 ± 7.78	137.95 ± 7.72	137.69 ± 7.77	137.27 ± 7.72	136.67 ± 7.70*
TA (% of L_0)	1.35 ± 0.23	2.21 ± 0.38*	3.25 ± 0.50*	4.14 ± 0.47*	5.05 ± 0.59*
$t_{\frac{1}{2}}$ contraction (ms)	177.94 ± 20.39	155.19 ± 23.60*	105.53 ± 13.43*	82.95 ± 10.2*	79.85 ± 9.45*
Indo-1 fluorescence ratio (410 nm/490 nm)					
Diastolic	0.71 ± 0.04	0.71 ± 0.04	0.72 ± 0.04	0.76 ± 0.03	0.87 ± 0.05*
Systolic	0.81 ± 0.04	0.83 ± 0.03*	0.86 ± 0.04*	0.91 ± 0.04*	1.04 ± 0.09*
Amplitude	0.09 ± 0.01	0.12 ± 0.02*	0.14 ± 0.02*	0.15 ± 0.03*	0.17 ± 0.04*
τ	495.45 ± 41.43	383.41 ± 25.71*	271.58 ± 26.76*	213.19 ± 14.81*	198.80 ± 16.39*
$t_{\frac{1}{2}}$ transient (ms)	319.44 ± 40.32	275.68 ± 17.64*	229.55 ± 14.54*	196.26 ± 15.20*	195.49 ± 12.32*
n	8	8	8	8	8

bpm, beats per minute; L_0, resting cell length; TA, twitch amplitude; $t_{\frac{1}{2}}$ contraction, half-relaxation time of contraction; τ, time constant of Ca^{2+} transient decline; $t_{\frac{1}{2}}$ transient, half-relaxation time of indo-1 fluorescence transient. Values are means ± S.E.M. *Significant *vs.* the corresponding value at 10 bpm value ($P < 0.05$).

340AF15) 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 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 nm/590 nm fluorescence ratio.

Fluorescence signals of SBFI-loaded myocytes were calibrated at the end of each experimental day by superfusion with 2.0 μM gramicidin, 5 μM monensin and 0.05 mM ouabain, according to Harootunian *et al.* (1989). Calibration media containing various Na$^+$ concentrations were made from appropriate mixtures of 150 mM Na$^+$ and 0 mM Na$^+$ solutions. The relationship between the SBFI fluorescence ratio and Na$^+$ activity was linear in the physiological range (4–16 mM). The values of [Na$^+$]$_i$ were estimated for each cell from this *in vivo* calibration.

Papillary muscles

Papillary muscles were dissected from the right ventricle and mounted vertically in a chamber to contract isometrically. Mean cross-sectional area of the muscles were calculated from the muscles' length and weight, assuming a uniform cross-section and a density of 1.0. The mural end of the muscle was firmly fixed to the bottom of the chamber by a small clamp and the tendinous end to a force transducer (Statham G1-4-250 or Hewlett-Packard FTA-10-1) via a stainless steel wire. Developed tension was monitored on-line and stored by software (PowerLab/400 ADInstruments). The muscles were paced to contract at the frequencies indicated in the text and figures, at constant temperature (30 °C), and maintained with the same solution used for isolated myocytes experiments. Contractility was assessed by the developed tension (DT) and relaxation was assessed by time to half-relaxation ($t_{1/2}$).

After the papillary muscles were mounted, they were stretched until they reached the length at which maximal DT occurred and then allowed to stabilize for 1 h.

Materials

Collagenase type B was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA); pronase from Boerhinger Mannheim Corp. (GmbH, Mannheim, Germany); BSA essentially fatty acid free, thapsigargin, ryanodine, caffeine and ouabain from Sigma

Chemical Co. (St Louis, MO, USA); indo-1 AM and SBFI AM from Molecular Probes Inc. (Eugene, OR, USA); and KB-R7943 2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea methane-sulphonate from Tocris Cookson Inc. (Ballwin, MO, USA). All other chemicals were of the purest reagent grade available.

Statistics

All data are presented as means ± S.E.M. Comparisons within groups were assessed by Student's *t* test, either paired or unpaired as appropriate. A value of $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Effect of increasing stimulation frequency on myocyte contraction and intracellular calcium transients

Using indo-1-loaded cardiac myocytes we examined the effects of increasing stimulation frequency from 10 beats min^{-1} (bpm) to 50 bpm, in steps of 10 bpm, on contraction and the associated Ca$_i$T. Figure 1*A* and *B* shows a representative example of this type of experiment. The increase in frequency produced a rate-dependent increase in the amplitude of contraction and Ca$_i$T (positive inotropic effect) that was associated with a parallel increase in the rate of Ca$_i$T decline and of twitch relaxation (relaxant effect). Increasing stimulation frequency also resulted in a modest but gradual increase in diastolic [Ca^{2+}], as well as a reduction in diastolic cell length. Figure 1*C* shows the overall results of these experiments ($n = 8$), indicating the parallel increase in contraction and Ca$_i$T amplitude produced upon an increase in stimulation frequency. Additionally, the average results of increasing frequency on contraction, Ca$_i$T and their kinetics are provided in Table 1. The increase in contraction frequency produced a significant positive inotropic and relaxant effect from 20 to 50 bpm. The increase in diastolic [Ca^{2+}] and the decrease in resting cell length, although present in
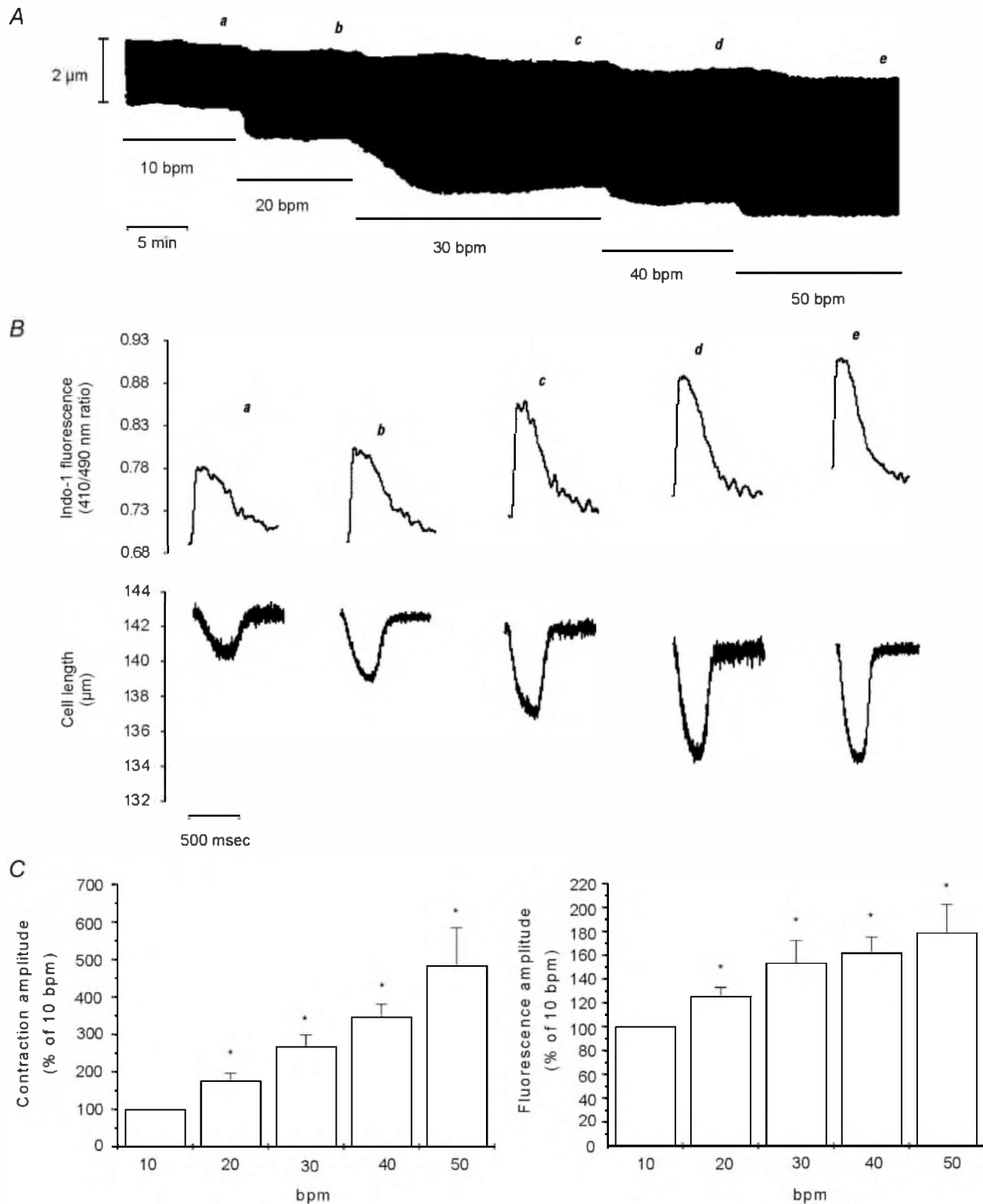
Figure 1. Effect of increasing stimulation frequency on myocyte contraction and Ca_iT

A, a typical continuous recording of cell length at the stimulation frequencies indicated in the figure (bpm, beats min^{-1}). *B*, traces of the individual twitch contractions and the associated indo-1 fluorescence transients at the moment indicated by the letters *a–e* on *A*. The traces show, from *a* to *e*, a gradual increase in diastolic $[Ca^{2+}]_i$, rate of Ca_iT decline and rate of twitch relaxation, and reduction in diastolic cell length. *C*, bar graphs depicting the overall results of the effect of increasing stimulation frequency on myocyte contraction amplitude and indo-1 transient amplitude, expressed as a percentage of the value at 10 bpm. Data are means ± S.E.M. of 8 cells, * $P < 0.05$ with respect to 10 bpm. Increasing stimulation frequency from 10 to 50 bpm resulted in parallel increases in contraction and Ca_iT amplitude.

most of the experiments from 30 bpm and beyond, only attained significant levels at 50 bpm. In a parallel group of experiments performed in the presence of 1 μM ryanodine (Ry) plus 1 μM thapsigargin (Tg) to functionally abolish SR function (Vila Petroff *et al.* 2000), the positive inotropic effect of increasing stimulation frequency was still observed. However, the increase in diastolic $[Ca^{2+}]$ and the decrease in resting cell length attained significant levels at lower frequencies of stimulation than in the absence of these drugs. In the absence of a functional SR, a significant
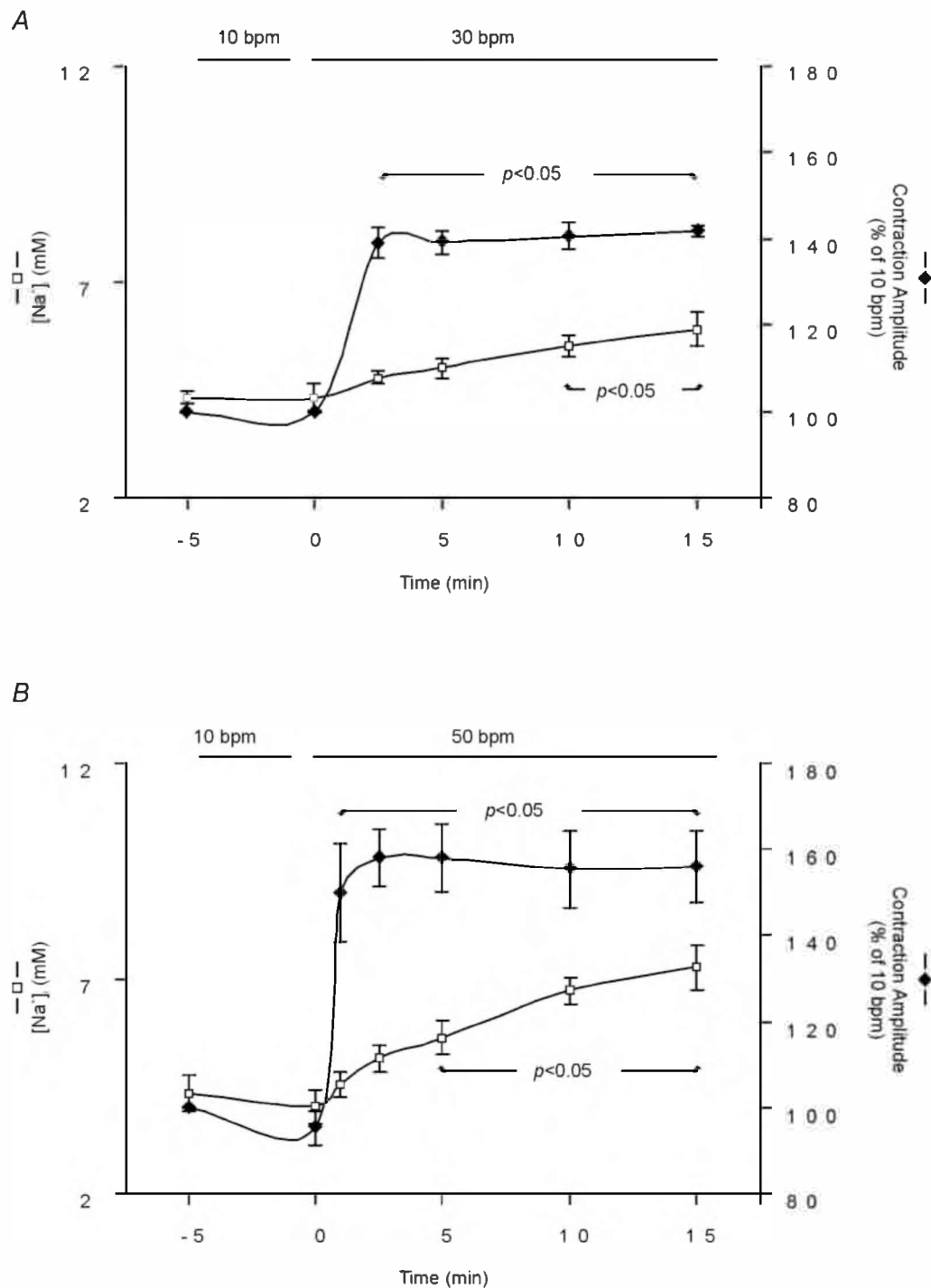

Figure 2. Effect of increasing stimulation frequency on contraction and $[Na^+]_i$

The graphs show the time course of simultaneous $[Na^+]_i$ and contraction amplitude measurements, during an increase in stimulation frequency from 10 to 30 bpm (A, $n = 6$) and from 10 to 50 bpm (B, $n = 5$). Data are means ± s.e.m. Increasing stimulation frequency either from 10 to 30 bpm or from 10 to 50 bpm resulted in a temporal dissociation between the frequency-dependent positive inotropic effect and the increment in $[Na^+]_i$.

increase in diastolic $[Ca^{2+}]$ ($8.9 \pm 2.3\%$) and a significant decrease in resting cell length ($6.2 \pm 1.4\%$) were already observed at 30 bpm.

Effect of increasing stimulation frequency on myocyte contraction and cytosolic $[Na^+]$

In SBFI-loaded cells, we examined the time course of the changes in cytosolic $[Na^+]$ and contraction during an increase in stimulation frequency. Figure 2*A* shows that increasing stimulation frequency from 10 to 30 bpm resulted in a rapid increase in contraction amplitude that reached its maximum after approximately 2.5 min, whereas the $[Na^+]_i$ increase developed slowly, reaching statistical significance only after 10 min of the change in frequency. Similarly, an increase in frequency from 10 to 50 bmp resulted in a faster increase in contraction amplitude than in $[Na^+]_i$ (Fig. 2*B*). Figure 3*A* shows a representative example of the effect of increasing frequency in one step from 10 to 50 bpm on myocyte contraction. The increase in frequency induced an initial increase in contraction amplitude that reached its maximum after 1 min and was followed by a slow decay until a new steady state was reached. The initial increase in contraction was followed by a decrease in diastolic cell length, which then continued to decrease while contraction amplitude declined. The gaps in the continuous chart recording correspond to the intervals during which $[Na^+]_i$ was measured. The values obtained are represented below the recording. Note that 1 min after the increase in stimulation frequency, the positive inotropic effect reached its maximum, whereas $[Na^+]_i$ was not affected (vertical dotted line). Figure 3*B* shows the overall results of these experiments, indicating the time course of the effect of increasing frequency from 10 to 50 bpm on resting cell length and $[Na^+]_i$. The increase in frequency produced a significant decrease in resting cell length within 1 min, whereas the increase in $[Na^+]_i$ became significant only after 5 min of the change in frequency. Furthermore, similar experiments in which $[Ca^{2+}]_i$ instead of $[Na^+]_i$ was measured during the increase in stimulation frequency from 10 to 50 bpm, showed that both diastolic and Ca_iT amplitude were significantly increased within 1 min of the change in stimulation frequency by 7 ± 0.9 and $46 \pm 19\%$, respectively ($n = 4$). The increase in diastolic $[Ca^{2+}]$ and the decrease in resting cell length occurred in association with the typical relaxant effect of the increase in stimulation frequency (decrease in the time to half-relaxation, $t_{1/2}$, and in the time constant of Ca_iT decline, τ). The striking dissociation between the changes at the level of contraction (resting cell length and contraction amplitude as well as diastolic and Ca_iT amplitude) and the increase in $[Na^+]_i$ produced by increasing stimulation frequency, may be taken as evidence that the NCX does not participate through a Na^+-dependent mechanism in the positive inotropic effect of increasing stimulation frequency. However, subsarcolemmal $[Na^+]_i$ gradients,

not detectable by the method of SBFI fluorescence used herein, could significantly modulate the function of the NCX, either by slowing the forward mode or favouring the Ca^{2+} influx mode of the exchanger (Leblanc & Hume, 1990; Lipp & Niggli, 1994; Litwin *et al.* 1998; Blaustein & Lederer, 1999). In either case, there would be an increase in $[Ca^{2+}]_i$, which would contribute to the increase in myocardial contractility produced by increasing contraction frequency. In an attempt to clarify this possibility, three different strategies were followed: (1) we studied the effects of increasing stimulation frequency after specific inhibition of the reverse mode of the NCX; (2) we studied the rate of Ca_iT decline mediated by the forward mode of the exchanger on caffeine-induced contractures, after an increase in contraction frequency; (3) we compared the time course of $[Na^+]_i$ and contractility changes produced by ouabain, a typical positive inotropic intervention mediated by the NCX (Satoh *et al.* 2000), with that of increasing stimulation frequency.

Lack of contribution of the reverse mode NCX to the frequency-induced positive inotropic effect

To examine the possible contribution of the reverse mode of the NCX, we performed experiments in the presence and absence of a specific blocker of the reverse mode NCX, KB-R7943 (KBR) (Iwamoto *et al.* 1996). Figure 4*A* shows a representative trace from a group of control experiments where the ability of 1 μM KBR to inhibit the reverse mode of the NCX was examined. This concentration of KBR did not affect basal contractility levels (Table 2). For these experiments, SR function was completely blocked by pretreatment of cells for 30 min with 1 μM Ry + 1 μM Tg. Caffeine-induced Ca_iTs in the presence and absence of Ry + Tg were used to assess the ability of these drugs to effectively inhibit SR function. The top traces in Fig. 4*A* show that application of 25 mM caffeine for 10 s induced a Ca_iT that was not reproducible when caffeine was applied after incubation with Ry + Tg. These results indicate the failure of the SR to accumulate and release Ca^{2+} in the presence of Ry + Tg. Similar results have previously been published (Vila Petroff *et al.* 2000). Figure 4*A* shows that, when extracellular Na^+ was abruptly removed (replaced with choline chloride), which changes the thermodynamic driving force of the NCX to favour Ca^{2+} influx, the indo-1 fluorescence ratio rose, as expected for Ca^{2+} influx via the NCX. A similar method of challenging the cell with a Na^+ gradient to force the NCX to work in the reverse mode was previously used by us (Vila Petroff *et al.* 2000), and others (Ladilov *et al.* 1999, Satoh *et al.* 2000). The reproducibility of the Ca^{2+} increase produced by two cycles of Na^+ removal was established in additional experiments (results not shown). KBR at 0.15 and 1 μM partially and completely prevented, respectively, the increase in the fluorescence ratio produced by extracellular Na^+ removal, which would indicate that these concentrations of KBR were able to inhibit the reverse mode of the NCX. Similar results were
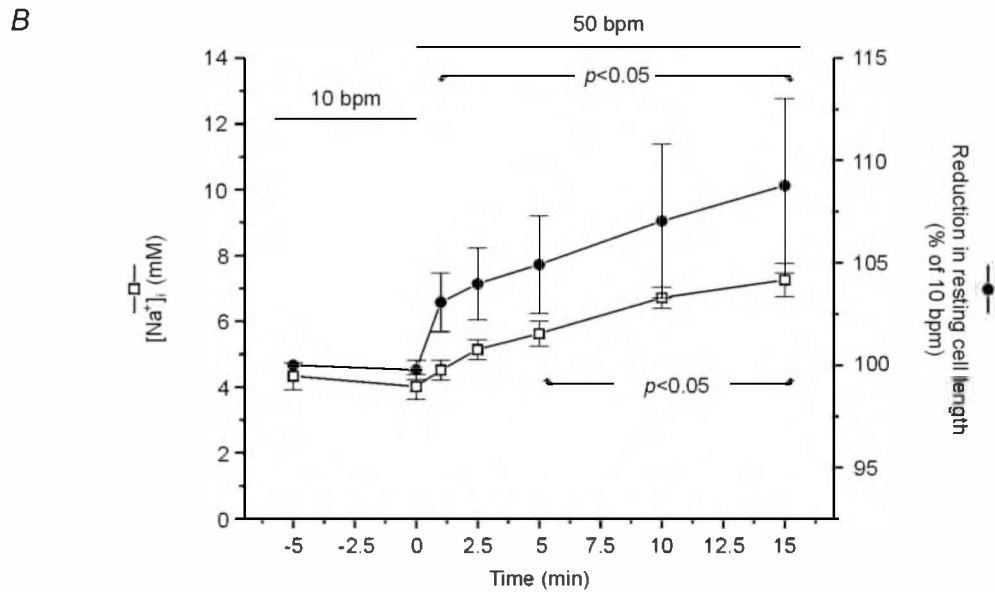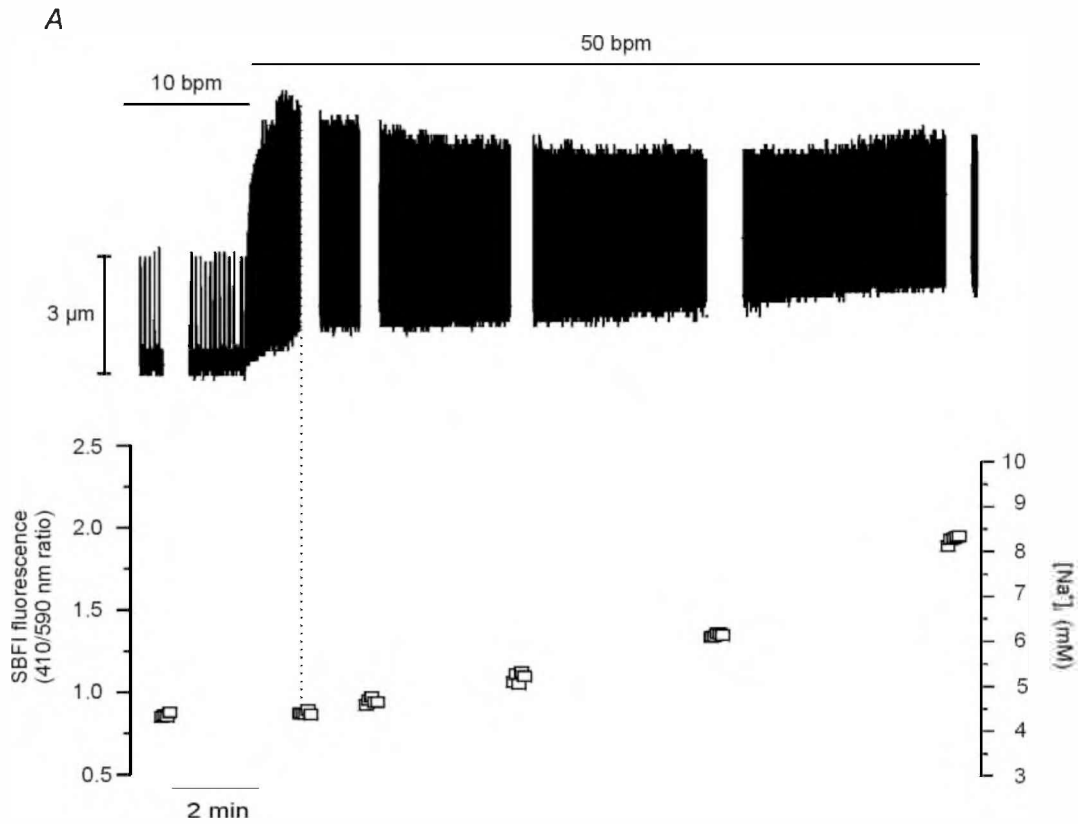
Figure 3. Effect of increasing stimulation frequency on contraction amplitude, resting cell length and [Na⁺]ᵢ

A, continuous recording of cell length when frequency was increased from 10 to 50 bpm. The gaps in the continuous chart recording correspond to the measurements of $[Na^+]_i$, plotted below. The increase in stimulation frequency induced a slow increase in $[Na^+]_i$ that was temporally dissociated from both the positive inotropic effect and the increase in resting length evoked by the increase in contraction frequency. *B*, overall results indicating the temporal dissociation between the reduction in resting cell length and the increase in $[Na^+]_i$. Data are means ± s.e.m. of 5 cells. Two of the five cells studied had a rather high increase in resting cell length after the first 5 min of the increase in stimulation frequency and are responsible for the large error bars observed. Repeated mean $[Na^+]_i$ values from Fig. 2 are placed here for visual comparison.
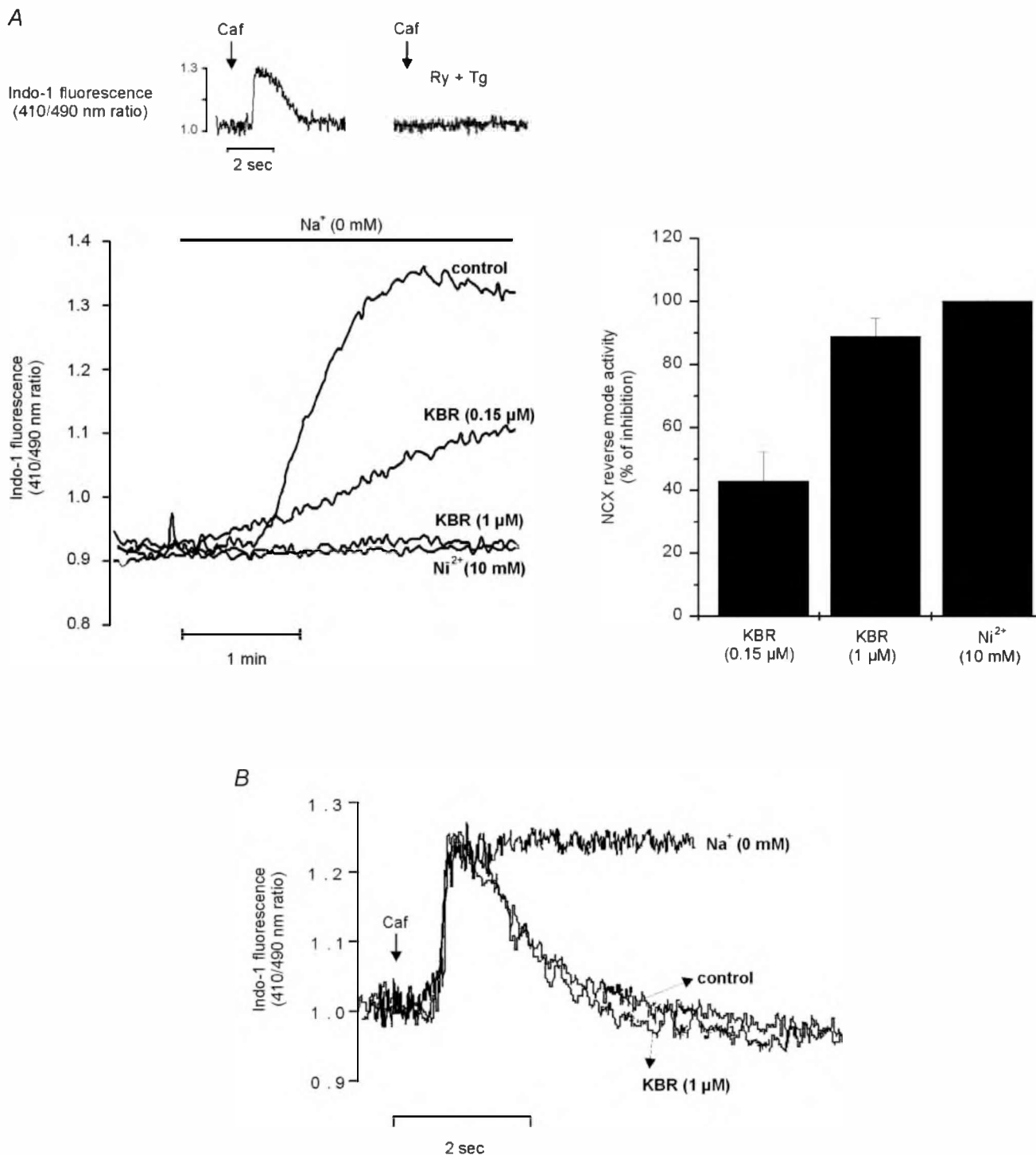
Figure 4. Effect of KBR on the reverse and forward mode NCX

A, the top traces show the ability of 1 μM Ry + 1 μM Tg to completely prevent the caffeine (caf)-induced Ca$_i$T. The traces below depict the Ca^{2+} measurements in the absence and presence of KBR. In the absence of a functional SR, 1 μM KBR and 10 mM Ni^{2+} were able to completely prevent the increase in Ca^{2+} entry produced by the activation of the reverse mode of the NCX due to external Na$^+$ removal (control). Application of 0.15 μM KBR, in contrast, only partially prevented this increase. The bar graph on the right depicts the overall results of these experiments expressed as percentage inhibition of NCX reverse mode activity. *B*, the caffeine-induced Ca$_i$T does not relax in zero [Na$^+$] solution. The absence of the declining phase shows that (1) caffeine is capable of completely inhibiting SR Ca^{2+} uptake and (2) the declining phase of the Ca$_i$T of the caffeine contracture is mostly due to Ca^{2+} extrusion via the NCX. Application of 1 μM KBR did not alter the [Ca^{2+}]$_i$ decline of the caffeine-induced Ca$_i$T, indicating the lack of effect of the drug on the forward mode of the NCX.

Table 2. Effect KB-R7943 on contraction and Ca^{2+} transient parameters of single cat myocytes at 10 bpm

	L_0 (μm)	TA (% of L_0)	$t_{1/2}$ contraction (ms)	Indo-1 ratio (410 nm/490 nm)		n
				Amplitude	$t_{1/2}$ transient (ms)	
Control	137 ± 6	1.02 ± 0.20	211 ± 20	0.16 ± 0.05	340 ± 21	6
KB-R7943 (1 μM)	137 ± 6	1.14 ± 0.25	212 ± 24	0.15 ± 0.04	320 ± 13	6

L_0, resting cell length; TA, twitch amplitude; $t_{1/2}$ contraction, half-relaxation time of contraction; $t_{1/2}$ transient, half-relaxation of indo-1 fluorescence transient. Values are means $\pm$ s.e.m.

obtained in four other cells. Additionally, in a further attempt to assess whether the reverse mode NCX was fully inhibited by 1 μM KBR, we performed experiments where the exchanger was forced to work in the reverse mode in the presence of 10 mM Ni^{2+}, a concentration capable of fully blocking the NCX (Kimura et al. 1987). The results depicted in Fig. 4A indicate that, similar to 1 μM KBR, in the presence of Ni^{2+} there was no detectable increase in indo-1 fluorescence. The bar graph on the right of Fig. 4A shows the overall results of these experiments. The results indicate that the inhibition produced by 1 μM KBR was not significantly different from that produced by 10 mM Ni^{2+}.

To assess the effect of KBR on Ca^{2+} efflux via the NCX, the rate of Ca^{2+} decline from a caffeine-induced Ca$_i$T, produced by a fast (10 s) application of 25 mM caffeine,

was used. During the caffeine-induced contracture, the declining phase of the Ca$_i$T in the cat is almost entirely due to Ca^{2+} extrusion from the cell via the NCX (Puglisi et al. 1996). Consistent with the findings of Puglisi et al. (1996), Fig. 4B shows that the caffeine-induced Ca$_i$T does not relax when caffeine is applied in a zero [Na$^+$] solution, to fully inhibit NCX function. The absence of this declining phase indicates that, in the presence of caffeine, SR uptake is completely inhibited. Figure 4B also shows that KBR failed to affect the rate of Ca^{2+} decline of the caffeine-induced Ca$_i$T, indicating that, at the concentration used, the blocker does not interfere with forward mode activity of the exchanger.

Figure 5 depicts a representative example of the positive inotropic response to increments in contraction frequency
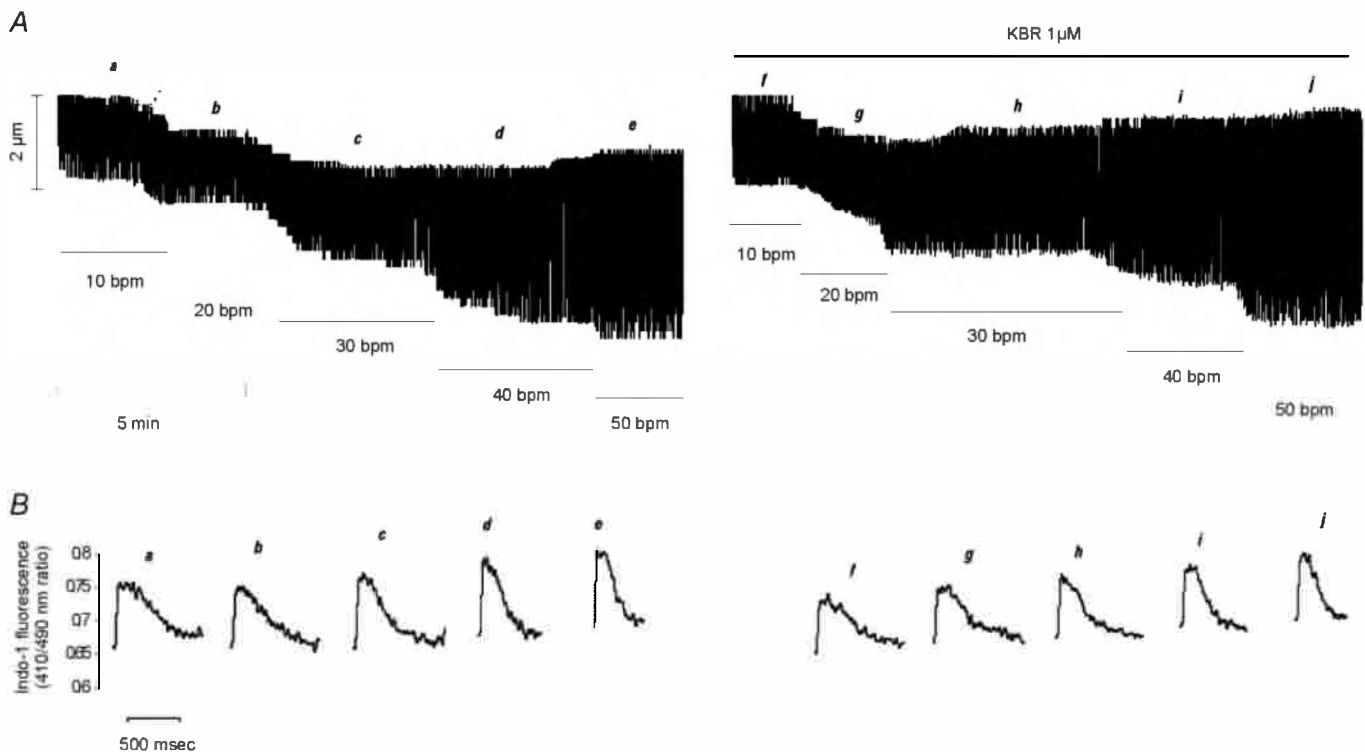

Figure 5. Failure of KBR to prevent the frequency-induced positive inotropic effect

A, typical continuous recording of cell length showing a similar increase in cell shortening induced by increasing stimulation frequency either in the absence or in the continued presence of the specific blocker of the reverse mode of the NCX. B, traces of the individual Ca$_i$T at the steady state of each frequency indicated by letters a–j in A.

in the absence and presence of 1 μM KBR. Figure 5*A* shows a continuous chart recording of cell length and Fig. 5*B*, on an expanded time scale, shows the Ca$_i$T acquired at the steady state of each stimulation frequency. The continued presence of 1 μM KBR did not affect the increase in contraction and Ca$_i$T amplitude associated with the increase in stimulation frequency. The overall results of these experiments, expressed as a percentage of the value at 10 bpm, are depicted in Fig. 6*A*. Although in the presence

of KBR the increase in contractility produced by increasing stimulation frequency to 50 bpm tended to be lower than in the absence of the NCX inhibitor, this difference did not attain significant levels. This tendency results from the fact that in five out of eight experiments, the presence of KBR produced a modest decrease in the enhancement of Ca$_i$T amplitude and shortening evoked by increasing stimulation frequency to 50 bpm.
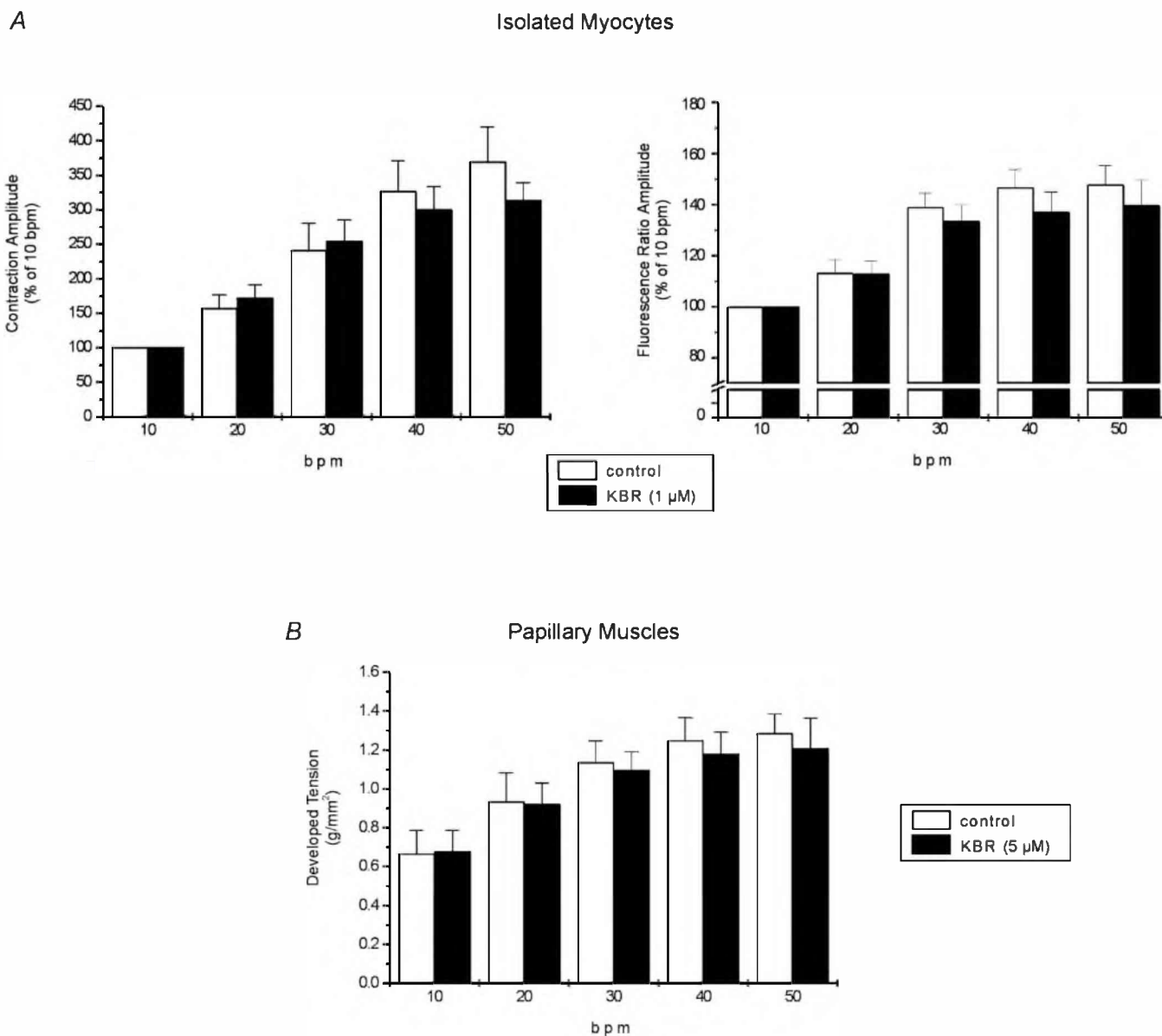

Figure 6. Lack of contribution of the reverse mode of the NCX to the stimulation frequency inotropy

A, overall results of increasing stimulation frequency on contraction and indo-1 fluorescence amplitude in isolated myocytes under control conditions and after administration of 1 μM KBR. Data are means ± s.e.m. of 10 cells expressed as a percentage of the value at 10 bpm. The increase in contraction and Ca$_i$T amplitude had a similar magnitude in the absence and in the presence of drug. *B*, overall results of force–frequency relationship on papillary muscles with ($n = 9$) and without ($n = 5$) 5 μM KBR. Data are means ± s.e.m. The increase in developed tension was not statistically different between treated and non-treated muscles. These results exclude a contribution of the reverse mode of the NCX in the frequency-induced positive inotropic effect.

Because higher concentrations of KBR seriously depress basal cardiac myocyte contraction and Ca_iT, we performed a parallel group of experiments in papillary muscles contracting isometrically, incubated for 1 h with a fivefold higher concentration of the blocker. In this preparation, 5 μM KBR did not significantly reduce the basal developed tension (5 ± 3 %). Figure 6B shows the overall results of these experiments. These results are in agreement with those obtained in the isolated myocytes, indicating that KBR fails to significantly affect the typical force–frequency relationship. Taken together, the results failed to demonstrate a significant contribution of the reverse mode of the NCX to the increment in contractility induced by increasing stimulation frequency, in cat ventricle, in the range of frequencies explored.

Role of forward mode NCX in the frequency dependence of contraction

Figure 7A shows representative traces of the caffeine-induced Ca_iT recorded after the rapid application of caffeine (25 mM) at the steady state of 10 and 30 bpm (upper panel) and at the maximum positive inotropic effect (1 min) and the steady state of 50 bpm (lower panel). The amplitude of the caffeine-induced Ca_iT was larger when caffeine was applied after the train of stimuli at either 30 or 50 bpm than after that at 10 bpm. In contrast, the amplitudes of the caffeine-induced Ca_iTs recorded at stimulation frequencies of 30 and 50 bpm were of similar magnitude. These results are consistent with an increase in SR Ca^{2+} load at the higher frequencies (Frampton *et al.* 1991) and also indicate that at a stimulation frequency of 30 bpm the SR is already maximally loaded. The rate of Ca_iT decline of the caffeine-induced Ca_iT was not significantly affected when stimulation frequency was increased from 10 to 30 bpm, whereas it was significantly prolonged at 50 bpm at the time at which a significant increase in $[Na^+]_i$ was observed (beyond 5 min of increasing stimulation frequency, trace *d*). When the caffeine pulse was applied only 1 min after the stimulation frequency was changed to 50 bpm (peak of the positive inotropic effect), the rate of Ca_iT decline was not affected, while the amplitude of the caffeine-induced Ca_iT was already increased (trace *c*). These findings on the kinetics of the caffeine-induced Ca_iT, can be better observed in the superimposed traces of the individual caffeine-induced Ca_iT at the frequencies studied, normalized to a similar peak of the Ca_iT. Because the rate of twitch Ca_iT decline depends on the peak $[Ca^{2+}]_i$ (Bers & Berlin 1995), an increase in the rate of Ca_iT decline produced by this mechanism could mask a plausible decrease in NCX forward mode activity at the stimulation frequency of 30 bpm or after stimulating at 50 bpm for 1 min. To rule out this possibility, the rate of the caffeine-induced Ca_iT decline at a given $[Ca^{2+}]_i$ was investigated according to the method described by Hulme & Orchard (1998). The exponential curve fitted to the declining phase of the

caffeine-induced Ca_iT was differentiated to give the rate of change of $[Ca^{2+}]_i$, ($d[Ca^{2+}]_i/dt$), and plotted against the undifferentiated $[Ca^{2+}]_i$ (Fig. 7B). This method allows analysis of the instantaneous rate of $[Ca^{2+}]_i$ decline as a function of $[Ca^{2+}]_i$, independently of peak $[Ca^{2+}]_i$. The slopes were similar for the linear regressions obtained from the caffeine-induced Ca_iTs recorded at 10 or 30 bpm or after 1 min of the increase in frequency to 50 bmp. These results indicate that for any given change in $[Ca^{2+}]_i$, the rate of Ca_iT decline at 30 bpm and after 1 min of 50 bpm were not different from that obtained at 10 bpm. In contrast, when the frequency of stimulation was maintained at 50 bpm, the rate of Ca_iT decline was decreased for any given change in $[Ca^{2+}]_i$.

The bar graphs in Fig. 7C represent the overall data from these experiments. The left panel shows the similar increase in the caffeine-induced Ca_iT amplitude produced by increasing frequency from 10 to 30 or 50 bpm after either 1 min of stimulation or at the steady state. The right panel shows the failure of the increase in contraction frequency to 30 or to 50 bpm for 1 min to affect the time course of Ca_iT decline (assessed by the time to half-relaxation of the caffeine-induced Ca_iT). This finding is in contrast to the significant prolongation observed in the rate Ca_iT decline when it was measured after the increase in $[Na^+]_i$ (produced by increasing contraction frequency to 50 bpm) had reached significant levels (see Fig. 2). The results obtained indicate that forward mode NCX activity is diminished at the highest stimulation frequency used (50 bpm). However, the reduced activity observed became evident after the maximal positive inotropic effect occurred and was not associated with any further increase of the inotropic effect of increasing stimulation frequency.

Effect of ouabain on myocyte contraction and cytosolic $[Na^+]$

The time course of the changes in cytosolic $[Na^+]$ and contraction after the administration of 10 μM ouabain was examined in SBFI-loaded myocytes. The overall results of these experiments, shown in Fig. 8A, demonstrate that, in contrast to the results obtained with the increase in contraction frequency (Fig. 2), the positive inotropic effect of cardiac glycosides is tightly associated to the increase in $[Na^+]_i$. In an additional group of experiments, the effect of ouabain at 10 bpm and of increasing contraction frequency from 10 to 50 bpm was studied in an attempt to make a more accurate comparison of both inotropic interventions. In Fig. 8B, the $[Na^+]_i$ level measured at the peak of the positive inotropic effect was compared with that associated with the same positive inotropy evoked by ouabain. Whereas the positive inotropic effect of ouabain was associated with a large and significant increase in $[Na^+]_i$ from 4.2 ± 0.2 to 12.39 ± 1.5 mM, there was no detectable increase in $[Na^+]_i$ associated with the maximal positive inotropic effect at the stimulation frequency of
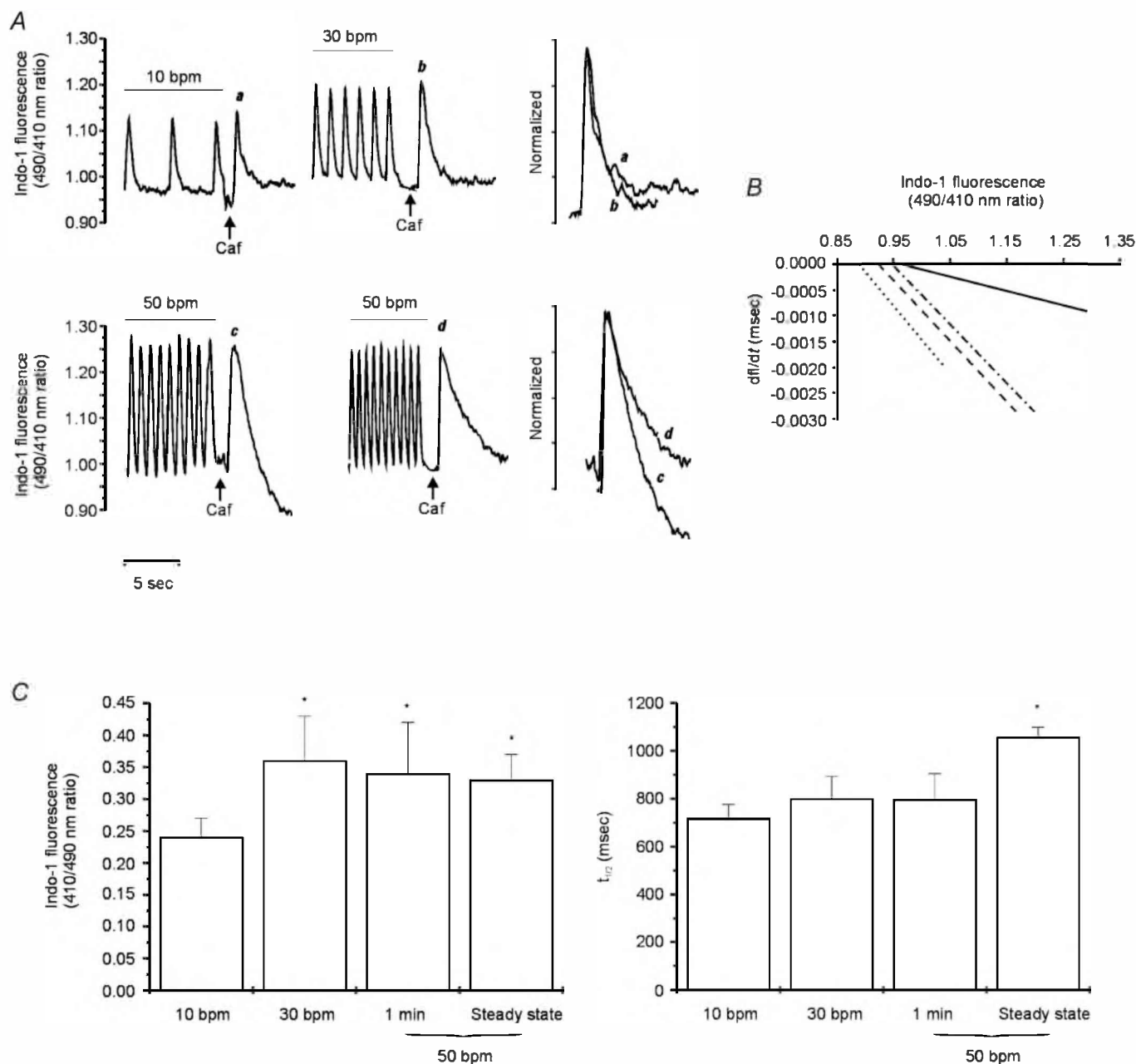
Figure 7. Contribution of the forward mode of the NCX to the contraction frequency inotropy

A, caffeine-induced Ca_iT after a train of stimuli at 10 (*a*), 30 (*b*) and 50 bpm, either after 1 min of the change in stimulation frequency (*c*) or after having reached the steady state (*d*). Increasing stimulation frequency from 10 to 30 or 50 bpm resulted in similar increases in the amplitude of the caffeine-induced Ca_iT. The superimposed and normalized traces show the caffeine-induced Ca_iTs under the conditions mentioned (*a* and *b* above and *c* and *d* below) for a better comparison of the rate of Ca_iT decline. The declining phases of the caffeine-induced Ca_iTs were not different at 10 or 30 bpm or after 1 min at 50 bpm. However, a prolongation of the rate of Ca_iT decline was observed at the steady state of 50 bpm. *B*, plots of the rates of the caffeine-induced Ca_iT decline (dfl/d*t*) against $[Ca^{2+}]_i$ (indo-1 fluorescence) at stimulation frequencies of 10 (dotted line), 30 (dashed line) and 50 bpm for 1 min (dotted–dashed line) and 50 bpm at the stage where $[Na^+]_i$ was significantly increased (continuous line). *C*, the bar graphs show the overall results of the caffeine-induced Ca_iT at 10 (*n* = 17), 30 (*n* = 13) and 50 bpm either after 1 min of the change in stimulation frequency (*n* = 9) or at the steady state (*n* = 11), on indo-1 fluorescence amplitude and the time to half-relaxation ($t_{1/2}$). Data are means ± S.E.M. These results indicate that the activity of forward mode of the NCX was reduced well after the maximal positive inotropic effect of increasing frequency at 50 bpm was reached.
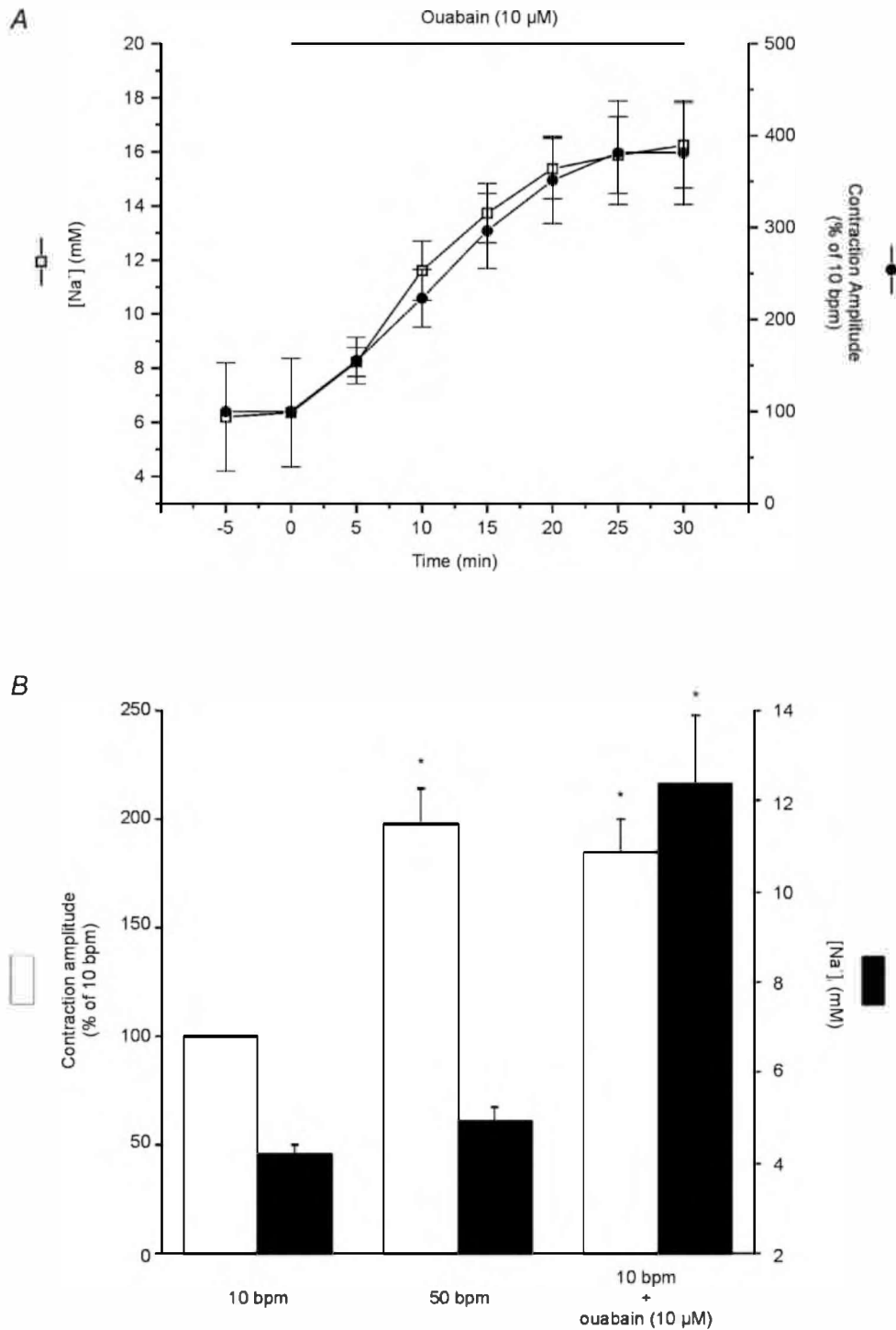
Figure 8. Effect of ouabain vs. frequency on contraction and [Na$^+$]$_i$

A, time course of ouabain-induced positive inotropic effect and the increase in [Na$^+$]$_i$. The increase in contraction and [Na$^+$]$_i$ induced by ouabain are tightly associated. B, overall results of the effect of 10 μM ouabain at 10 bpm and of increasing stimulation frequency from 10 to 50 bpm on contraction amplitude (expressed as percentage of 10 bpm control) and [Na$^+$]$_i$ (expressed in mM). Data are means $\pm$ s.e.m. of 8 cells, * $P < 0.05$. The contractility was matched by ouabain to the maximal increase in contractility evoked by increasing stimulation frequency. For a similar increase in contraction, the positive inotropic effect of ouabain was associated with a large and significant increase in [Na$^+$]$_i$, whereas that of contraction frequency occurred in the absence of any detectable increase in [Na$^+$]$_i$.

50 bpm, in agreement with the results shown in Fig. 2*B*. It is important to point out that, whereas the increase in contractility produced by the change in stimulation frequency, as shown in other experiments (see Fig. 3), was associated with a significant reduction in resting cell length ($5 \pm 1.2\%$), the administration of ouabain, for a similar inotropic effect, did not significantly affect resting cell length.

DISCUSSION

Frequency potentiation of contractility represents a potent inotropic mechanism in the myocardium of most species, including human non-failing hearts (Cohen *et al.* 1982; Wang *et al.* 1988; Schillinger *et al.* 1998; Alpert *et al.* 1998). The increase in contractility is the consequence of an increase in intracellular Ca^{2+} transients, produced by an enhanced SR Ca^{2+} load and release at the higher frequencies (reviewed in Bers, 2001). The NCX has been largely associated with the subcellular mechanisms that converge to produce this enhancement. Although direct experimental evidence is still lacking, the participation of the NCX in the positive staircase has mainly been related to the increase in $[Na^+]_i$ that occurs with the increase in the frequency of stimulation (Langer 1971; Cohen *et al.* 1982; Lee *et al.* 1987; Boyett *et al.* 1987; Wang *et al.* 1988), and/or to the reduced time between contractions for Ca^{2+} extrusion via the forward mode of the exchanger (Cohen *et al.* 1982; Bers 2001; Antoons *et al.* 2002). In this study, a dissection of the possible mechanisms by which the NCX may be involved in the stimulation frequency-induced positive inotropic effect was performed.

The results showed that in the cat ventricle (1) the positive inotropic effect produced by increasing contraction frequency was temporally dissociated from the increase in $[Na^+]_i$; (2) the positive inotropic effect of increasing stimulation frequency was not affected by inhibiting the reverse mode of the NCX; (3) the increase in stimulation frequency from 10 to 30 bpm did not produce any significant inhibition of the forward mode of the NCX; (4) the increase in stimulation frequency from 10 to 50 bpm produced a significant reduction in the activity of the forward mode of the NCX, which occurred in association with a significant increase in $[Na^+]_i$. However, these changes became significant well after the maximal positive inotropic effect was reached and did not contribute further to the positive inotropic effect of increasing contraction frequency; (5) the increase in stimulation frequency from 10 to 50 bpm produced an increase in diastolic $[Ca^{2+}]$ associated with a decrease in resting cell length, which occurred in parallel with the increase in contractility and before the detection of any significant increase in $[Na^+]_i$. Taken together, the results indicate that neither the reverse nor the forward mode of the NCX contributes significantly to the increase in

contractility produced by increasing frequency via a Na^+-dependent mechanism, in the frequency range explored. The contribution of the NCX to the positive inotropism of increasing contraction frequency would be indirect and dependent on the reduced time between beats. The reduced diastolic interval would preclude the NCX from compensating for the increase in Ca^{2+} influx. This failure of the NCX to match Ca^{2+} influx became evident by the significant increase in diastolic $[Ca^{2+}]$ that occurred either when the SR was disabled in the presence of Ry and Tg or at the higher contraction frequency explored, i.e. 50 bpm, a condition in which the SR is fully saturated.

Ca^{2+} influx via the NCX and stimulation frequency-induced increase in contractility

The role of the reverse mode of the NCX in cardiac excitation–contraction coupling is still a matter of debate. Previous studies suggest that Na^+ entry via I_{Na} could cause a rise in local submembrane $[Na^+]_i$ and local elevation of $[Ca^{2+}]_i$ via the NCX and consequent triggering of SR Ca^{2+} release (Leblanc & Hume, 1990; Lipp & Niggli, 1994; Litwin *et al.* 1998). Although there is experimental evidence indicating that the Ca^{2+}-influx mode of the NCX cannot trigger Ca^{2+} sparks directly (Lopez-Lopez *et al.* 1995), recent experiments have suggested that the reverse mode of the NCX may locally regulate resting Ca^{2+} concentration in the restricted space of the diadic cleft, and modulate the threshold for triggering Ca^{2+} sparks (Litwin *et al.* 1998; Goldhaber *et al.* 1999). Litwin *et al.* (1998) further proposed that the net Ca^{2+} influx by the I_{Ca} and the reverse NCX may not sum linearly, but rather act in synergism in the modulation of Ca^{2+} release. In line with this concept, the results of Viatchenko-Karpinski & Györke (2001), indicated that the reverse mode of the NCX may play a role in the β-adrenergic enhancement of Ca^{2+}-induced Ca^{2+} release by effectively contributing as a Ca^{2+} trigger. A similar role for the reverse mode of the NCX might be expected in the positive staircase phenomenon. In the present experiments, KBR tended to decrease the enhancement of both Ca_iT amplitude and shortening produced by increasing stimulation frequency, mainly at 50 bpm. However, this effect was modest and did not reach significant levels. Moreover, stimulation frequencies higher than 50 bpm failed to further increase contractility and Ca_iT in cat myocytes (results not shown). Thus, the present results do not support the view of a significant contribution of the reverse mode of the NCX in the positive inotropic effect of increasing contraction frequency in the cat ventricle.

A limitation of the above conclusion is that it relies on the ability of KBR to preferentially block the Ca^{2+} influx (reverse) mode of the NCX rather than the extrusion (forward) mode (Iwamoto *et al.* 1996). This reported property of KBR has recently been used to examine the possible contribution of the reverse mode of the exchanger

to the influx of Ca^{2+}, under different physiological or pathological conditions (Ladilov *et al.* 1999; Satoh *et al.* 2000; Perez *et al.* 2001; Vittone *et al.* 2002). However, several reports claim that KBR is not specific in blocking the reverse mode of the exchanger, and that several other currents may be blocked as well (Satoh *et al.* 2000; Lu *et al.* 2002; Reuter *et al.* 2002). The results shown in Fig. 4*A* indicate that the concentration of KBR used in the present experiments in cat isolated myocytes (1 μM) was able to selectively block the Ca^{2+} influx mode of the NCX, without affecting the Ca^{2+} efflux mode (Fig. 4*B*). Figure 4*A* shows, in addition, that 1 μM KBR is a concentration sufficiently high to produce a virtually complete inhibition of the reverse mode of the NCX, when compared with the inhibition produced by 10 mM Ni^{2+} (Fig. 4*A*). Moreover, this concentration of KBR failed to significantly affect basal contractility and relaxation parameters (Table 2). This latter finding would further indicate that the contribution of the Ca^{2+} influx mode of the NCX to the excitation–contraction coupling in the cat ventricle is minimal and barely significant, under basal conditions, in agreement with what has been found in other species (Satoh *et al.* 2000). More important for the conclusion of this study, the lack of effect of KBR on basal contractility would also suggest that the L-type Ca^{2+} current is not affected by this concentration of the drug. Supporting our own data, previous experiments, also performed in isolated cat myocytes under the same conditions as in the present experiments, have shown that concentrations of KBR up to 5 μM failed to affect the inward NCX current (Aiello *et al.* 2002) and that 1 μM of the inhibitor does not affect the L-type Ca^{2+} current (E. A. Aiello & H. E. Cingolani, personal communication). Similar results were obtained by Watano *et al.* (1996), in guinea-pig ventricular myocytes.

Thus, although KBR may be an imperfect agent, our results seem to indicate that in our experimental conditions, KBR behaved as a specific blocker of the reverse mode NCX, and support the conclusion that this mode of the NCX does not significantly participate in the positive staircase in the cat ventricle.

Ca^{2+} efflux via NCX and stimulation frequency-induced increase in contractility

The NCX plays an important role in the decrease in cytosolic Ca^{2+} that induces relaxation in several species. In isolated cat myocytes, it has been shown that the NCX is responsible for approximately 30 and 50 % of cyto-solic Ca^{2+} decline, at 25 and 35 °C, respectively, and a stimulation frequency of 0.5 Hz (Puglisi *et al.* 1996). A decrease in Ca^{2+} extrusion by this mechanism would therefore favour SR Ca^{2+} loading due to an increase in diastolic $[Ca^{2+}]$. As a consequence, myocardial contractility would increase. The present results are not consistent with a reduction in the activity of the Ca^{2+} efflux mode of the NCX, when stimulation frequency was increased from 10 to 30 bpm. Although the increase in contraction frequency did produce an increase in SR Ca^{2+} content, the rate of Ca^{2+} decline of the caffeine-induced contracture was similar at the two different frequencies. This was so, even after correction for the higher amplitude of the caffeine-induced Ca_iT at 30 bpm, which, by increasing by itself the rate of Ca^{2+} decline, could have masked a possible reduction of Ca^{2+} efflux via the NCX (Bers & Berlin, 1995). At the higher contraction frequency explored (50 bpm), there was a reduction in the activity of the forward mode of the NCX (Fig. 7), which could contribute to the increase in diastolic $[Ca^{2+}]_i$ and to the decrease in diastolic cell length produced by increasing stimulation frequency. However, there was a temporal dissociation between the increase in $[Na^+]_i$ that mediates the reduction in NCX forward mode activity, and the increase in contractility produced by increasing stimulation frequency. Whereas the increase in contractility and SR Ca^{2+} load fully developed within 1 min, the increase in $[Na^+]_i$ and the reduction in the activity of the forward mode of the NCX reached significant levels only several minutes after stimulation frequency was increased to 50 bpm and the maximal positive inotropic effect was reached. At 50 bpm, the development of the maximal positive inotropic effect occurred in parallel with an initial increase in diastolic $[Ca^{2+}]$ and with a decrease in resting cell length. In the absence of any significant decrease in the Ca^{2+} efflux mode of the NCX, this increase in diastolic $[Ca^{2+}]$ and decrease in resting cell length would reflect either a decrease in the rate of Ca^{2+} removal by the SR or an insufficient time between beats for Ca^{2+} extrusion via the NCX. Since the increase in diastolic $[Ca^{2+}]$ occurred in association with the typical relaxant effect of increasing frequency, which seems to be dependent on the SR (Bassani *et al.* 1995; DeSantiago *et al.* 2002), the rate of SR Ca^{2+} removal must be increased rather than decreased at higher stimulation frequencies, which would exclude the first of the two possibilities considered above. Thus our results argue in favour of an indirect, Na^+-independent contribution of the NCX to the stimulation frequency-induced positive inotropic effect. This action might also be present at lower stimulation frequencies, i.e. 30 bpm, but would be undetectable in the presence of a functional and unsaturated SR. However, it became evident at 50 bpm, due to the fact that the SR was maximally loaded (Fig. 7), or when the SR was disabled in the presence of Ry and Tg. Furthermore, the increase in $[Na^+]_i$, and the associated reduced activity of the NCX, that occurred later than the maximal positive inotropic effect was reached, did not further modify the positive staircase. The results therefore exclude the participation of a Na^+-dependent reduction in the activity of the forward mode of the NCX in the positive inotropic effect induced by the increase in stimulation frequency. The reduced activity of the NCX is more likely to be responsible for the slow increase in diastolic $[Ca^{2+}]$ and decrease in resting cell

length associated with the increase in $[Na^+]_i$. A lack of contribution of a $[Na^+]_i$-dependent reduction in NCX forward mode activity to contraction frequency inotropy can be also drawn from the experiments performed using cardiac glycosides, the typical NCX-dependent inotropic intervention (Satoh *et al.* 2000). In contrast to what was observed with the increase in stimulation frequency, ouabain produced an increase in contractility that occurred in close association with the increase in $[Na^+]_i$ (Fig. 8*A*). Moreover, for an increase in contractility similar to the maximal effect evoked by increasing contraction frequency, ouabain produced a large and significant increase in $[Na^+]_i$ whereas the maximal positive inotropic effect of the increase in contraction frequency occurred in the absence of any detectable increase in $[Na^+]_i$ (Fig. 8*B*).

Although the positive inotropic effect of increasing contraction frequency has been largely associated with an increase in $[Na^+]_i$ (Cohen *et al.* 1982; Wang *et al.* 1988; Despa *et al.* 2002), there is also evidence indicating that this association is not so straightforward and should be supported by parallel increases in contractility and $[Na^+]_i$. In this scenario, our results indicate that frequency-dependent increases in $[Na^+]_i$ are not necessary for a positive force–frequency relationship. Similarly, Antoons *et al.* (2002) observed, in mouse myocytes, a stimulation frequency-induced increase in $[Na^+]_i$ that was rather slow to explain the increase in contraction. Furthermore, careful examination of the experiments presented by Cohen *et al.* (1982) reveals that, when stimulation frequency is increased from 0.1 to 3 Hz, there is an immediate, large increase in contraction followed by a small progressive secondary increase. The increase in $[Na^+]_i$ observed in these experiments was closely associated with the secondary increase in contraction. A dissociation between simulation-frequency inotropy and the increase in $[Na^+]_i$ has also been observed in the rat and the failing human heart, where a negative force–frequency relation has been shown to occur in parallel with a frequency-dependent increase in $[Na^+]_i$ (Maier *et al.* 1997; Pieske *et al.* 2002). It has been shown that the relationship between $[Na^+]_i$ and force is highly non-linear (Eisner *et al.* 1984). A similar non-linear relationship must be expected between $[Na^+]_i$ and shortening of unloaded cells. Thus, it might be argued that small elevations in $[Na^+]_i$ could lead to a significant increase in shortening, which then saturates due to cell stiffness, while $[Na^+]_i$ continues to rise. This possibility seems unlikely, however, because (1) as described above, neither the activity of the forward mode nor that of the reverse mode of the NCX, was affected at the moment of the initial maximal inotropic effect produced by increasing stimulation frequency; (2) the lack of further increase in unloaded shortening observed when the elevation in $[Na^+]_i$ reached significant levels was associated with a lack of increase in the amplitude of Ca_iT, which cannot be attributed to limitations due to cell stiffness. This lack of contractile and Ca_iT amplitude response may be more likely

to be associated with a saturated SR at 50 bpm, as shown in the caffeine-induced contractures (Fig. 7). Furthermore, slower Ca_iT decline and elevated diastolic $[Ca^{2+}]$ may also slow the recovery of Ca^{2+} channels between beats, and this could further limit the extent of enhancement of systolic function at higher heart rates (Sipido *et al.* 1998).

The results presented herein would also indicate that sub-sarcolemmal $[Na^+]_i$ gradients, that might have occurred during the increase in stimulation frequency, are not sufficient to modulate NCX activity. If this were the case, a dissociation between the increase in $[Na^+]_i$ and the activity of the NCX should have been detected. However, this was not the case in the present experiments.

In summary, the present results indicate that the NCX does not participate directly in the positive inotropic effect of increasing stimulation frequency in the cat ventricle. The reduced diastolic interval produced by the increase in frequency, by precluding the exchanger from extruding the Ca^{2+} that entered the cell in each beat, would constitute the only mechanism by which the NCX contributes to the increase in SR Ca^{2+} load and positive inotropism of the increase in stimulation frequency. Since the NCX is the main mechanism of Ca^{2+} extrusion from the cell in the cat myocardium, the increase in diastolic $[Ca^{2+}]_i$ that occurred immediately after the stimulation frequency was increased, in the absence of significant changes in $[Na^+]_i$, and tightly associated with an increase in contractility, gives strong support to this conclusion.

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