Journal of Physiology (1992), **447**, *pp.* 275–292
With 8 figures
Printed in Great Britain

α_1-ADRENERGIC EFFECTS ON INTRACELLULAR pH AND CALCIUM AND ON MYOFILAMENTS IN SINGLE RAT CARDIAC CELLS

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(*Received 7 March 1991*)

SUMMARY

1. The cellular effects of α_1-adrenoceptor stimulation by phenylephrine were studied in the presence of propranolol in single cells isolated from the ventricles of rat hearts.

2. Phenylephrine (10–100 μM) induced a biphasic pattern of inotropism in these cells: a transient negative followed by a sustained positive inotropic effect as usually observed in cardiac tissues.

3. In Snarf-1-loaded cells, phenylephrine induced an alkalinization. This effect was reversible on wash-out and inhibited by prazosin, an α_1-adrenoceptor antagonist.

4. The α_1-adrenoceptor-mediated increase in intracellular pH (pH_i) was 0·1 pH unit in HEPES buffer containing 4·4 mM-$NaHCO_3$ and in Krebs buffer containing 25 mM-$NaHCO_3$.

5. The alkalinization was blocked by the Na^+–H^+ antiport blocker, ethyl-isopropylamiloride (EIPA).

6. The recovery from an acidosis induced by a NH_4Cl pre-pulse was accelerated by phenylephrine. The phenylephrine-induced alkalinization was attributed to activation of the Na^+–H^+ antiport.

7. Despite its ability to increase pH_i, phenylephrine did not alter Ca^{2+} current amplitude and kinetics.

8. Ca^{2+} transients recorded in Indo-1-loaded cells were not augmented by phenylephrine. Diastolic calcium level was decreased.

9. In single skinned cells, the Ca^{2+} sensitivity of the contractile proteins was increased by a pre-treatment with phenylephrine even when the α_1-adrenoceptor-mediated alkalinizing effect had been prevented by EIPA.

10. These results lead us to propose that the α_1-adrenergic-induced positive inotropic response of heart muscle could result from an increased sensitivity of the myofilaments to Ca^{2+} ions. This α_1-adrenoceptor-mediated Ca^{2+} sensitization could result both from an intracellular alkalinization and from a direct effect on contractile proteins.

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INTRODUCTION

For many years, β-adrenoceptors have been considered as the exclusive adrenergic receptor population through which catecholamines exert their actions on heart muscle. However, α_1-adrenoceptors were also found to co-exist with β-adrenoceptors in myocardial cells (Williams & Lefkowitz, 1978; Wagner & Brodde, 1978). Contrary to β-adrenoceptor stimulation, the selective stimulation of α_1-adrenoceptors produces an increase in twitch contractile force without an increase in cyclic AMP (Endoh, 1986).

Several mechanisms could lead to the positive inotropic effect of the α_1-adrenergic agonists. These neuromediators could increase the influx of calcium through the voltage-dependent Ca^{2+} channels primarily because they prolong the duration of the action potential (Ledda, Marchetti & Muggelli, 1975; Vogel & Terzic, 1989; Fedida, Shimoni & Giles, 1989). In addition, α_1-adrenergic agonists induce the hydrolysis of polyphosphoinositides (Brown & Jones, 1986; Poggioli, Sulpice & Vassort, 1986; Scholz, Schaefer, Schmitz, Scholz, Steinfath, Lohse, Schwabe & Puurunen, 1988). One product, inositol trisphosphate ($InsP_3$), has been proposed to facilitate the release of calcium from the sarcoplasmic reticulum, thus increasing the availability of Ca^{2+} ions to the myofilaments. However $InsP_3$-induced Ca^{2+} release in cardiac preparations is still controversial (Movsesian, Thomas, Selak & Williamson, 1984; Nosek, Williams, Zeigler & Godt, 1986; Vites & Pappano, 1990). More recently α_1-adrenergic agonists were suggested to increase the Ca^{2+} sensitivity of the contractile proteins (Endoh & Blinks, 1988). The exact mechanism of this sensitization is not known. It could have at least two origins. First, it could be due to an intracellular alkalinization. An increase of pH_i is well known to induce such an effect (Fabiato & Fabiato, 1978). In this regard, phenylephrine has already been shown to increase pH_i in Purkinje fibres (Breen & Pressler, 1988), in suspensions of isolated cardiac cells (Wallert & Fröhlich, 1989) and in atrial muscle (Terzic, Anagnostopoulos & Vogel, 1991). This alkalinization was abolished by amiloride and its derivatives, known to block the Na^+–H^+ antiport. Moreover, blockers of this antiport significantly reduced the positive inotropic effect of phenylephrine (Otani, Uriu, Hara, Inoue, Omori, Cragoe & Inagaki, 1990; Terzic & Vogel, 1990, 1991). Secondly, the increase in myofibrillar responsiveness to Ca^{2+} might be also consequential to an alteration of the contractile proteins by phosphorylation (Pucéat, Clément, Lechêne, Pélosin, Ventura-Clapier & Vassort, 1990).

Most of these results were obtained on multicellular preparations or suspensions of cardiac cells under various experimental conditions, and it remained undefined as to what are the cellular effects of α_1-adrenoceptor agonists that are responsible for the positive inotropic effect. Thus, in the present study, we investigated in single myocytes isolated from the same heart the effects of α_1-adrenergic agonists on several parameters of excitation–contraction coupling using and combining powerful techniques (whole-cell patch-clamp, monitoring of intracellular Ca^{2+} or pH by microfluorimetry, and force measurement on single chemically skinned cells). We demonstrated that neither Ca^{2+} current nor Ca^{2+} transient were significantly altered by the α_1-adrenergic agonist, phenylephrine; rather, the positive inotropy could be attributed to an increase in the Ca^{2+} sensitivity of the myofilaments following both an alkalinization as well as a change in their intrinsic properties.

METHODS

Isolation of cardiac cells

Ventricular myocytes were dissociated from hearts of urethane-anaesthetized (0·2 g/100 g) male Wistar rats (200–250 g) using the collagenase method as previously described in detail (Pucéat *et al.* 1990). The yield of rod-shaped viable cardiomyocytes is routinely 80%. Following the enzymatic dissociation the isolated cardiac cells appear to contain functional α_1-adrenoceptors (Ravens, Wang & Wettwer, 1989; Vogel & Terzic, 1989; Pucéat *et al.* 1990).

Solutions

The HEPES-buffered solution contained (mM): NaCl, 117; KCl, 5·7; $NaHCO_3$, 4·4; NaH_2PO_4, 1·2; $CaCl_2$, 1·8; $MgCl_2$, 1·7; HEPES, 20. The pH was adjusted to 7·4 at 32 °C with NaOH. In the Krebs bicarbonate-buffered solution, 20 mM-HEPES was replaced by 20 mM-$NaHCO_3$. The pH was maintained at 7·4 by bubbling with CO_2 5%/O_2 95%. Unless otherwise indicated the cells were bathed in HEPES buffer. To block possible β-adrenergic effects of the α-sympathomimetic, phenylephrine, all solutions contained the β-adrenoceptor antagonist, propranolol (3 μM).

The external solution used during electrophysiological experiments was the same HEPES solution except that 5·7 mM-KCl was replaced by 20 mM-CsCl. To record calcium current (I_{Ca}) and intracellular pH (pH_i) without buffering pH_i, the patch electrode (0·5–1 MΩ) contained (mM): CsCl, 120; $MgCl_2$, 6·6; HEPES, 1; Na_2ATP, 5; Na_2-phosphocreatine, 5; Na_2GTP, 0·4; bis (*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 0·1; Snarf-1, 0·03; pH was adjusted to 7·2 at 32 °C with CsOH. This internal solution allowed the cell to contract in response to a depolarization. Because of its low affinity for H^+ ions, BAPTA was used instead of EGTA to chelate the contaminating Ca^{2+} of the solution.

Snarf-1/AM was purchased from Molecular Probes. Indo-1/AM and ionomycin were from Calbiochem. Nigericin, phenylephrine, propranolol, ascorbic acid and all salts were obtained from Sigma. Prazosin was a generous gift of Pfizer, Inc.

Recording of cell contraction

Contractions of an isolated cardiomyocyte were induced by electrical stimulation (3 V, 2 ms, i.e. two times above threshold) using a 60 μm Teflon-coated tungsten wire, recorded by a photomultiplier and displayed on-line on a chart recorder. The degree of shortening and re-lengthening of the unloaded cardiomyocyte was estimated from the change in white light intensity induced by the movement of a selected end-edge of the cell, out of focus, in a narrow slit of a rectangular diaphragm. Drift in baseline recording was eliminated by a home-made track-and-hold amplifier. This simple approach of estimating the amplitude of contractions was validated by triggering well-known positive inotropic behaviours such as post-rest and post-stimulative potentiations (see Fig. 1).

Measurement of Ca_i^{2+} and pH_i

A detailed account of the techniques utilized in this laboratory to record intracellular Ca^{2+} (Ca_i^{2+}) and pH_i has been recently presented elsewhere (Pucéat, Clément, Scamps & Vassort, 1991). In brief, Ca_i^{2+} and pH_i were respectively measured using the Ca^{2+}-sensitive dye Indo-1 and the pH-sensitive probe Snarf-1. The cells were loaded at room temperature either for 15 min with 4 μM-Indo-1/AM or for 25 min with 5 μM-Snarf-1/AM. Both probes were previously dissolved in dimethyl sulphoxide (DMSO) and pluronic acid. Cells attached to laminin-coated cover-slips were then transferred to the stage of an inverted microscope. The cardiomyocytes were continuously superfused with solutions pre-warmed at 32 °C. Ca_i^{2+} and pH_i of a single cell were recorded by microfluorimetry using a dual-emission wavelength system. The excitation light was provided by xenon lamp and attenuated by two neutral density filters. Two sets of dichroic mirrors and narrow range filters allowed the cell to be illuminated at 360 nm (Indo-1) or 514 nm (Snarf-1), and the emitted light to be recorded by two photomultipliers at 405 and 480 nm (Indo-1) or at 580 and 640 nm (Snarf-1). A computer program calculated the emission fluorescence wavelength ratio (F_{405}/F_{480} for Indo-1 or F_{640}/F_{580} for Snarf-1) which was then displayed on-line on a chart recorder. In addition, the two emission wavelengths were simultaneously tape-recorded for off-line analysis. When Ca^{2+} transients were measured, cells were externally stimulated with the tungsten electrode at 0·2 Hz.

Ca_i^{2+} and pH_i were respectively calibrated with the ionomycin and nigericin methods according to the protocols already described (Pucéat *et al.* 1991).

Estimate of the intracellular buffering capacity and pH_i recovery from an acid load

The classical approach for studying pH_i-regulating mechanisms is to rapidly perturb pH_i by applying to the cells an acid or alkaline load and then monitor the recovery towards the initial pH_i. A commonly used technique is the ammonia pulse protocol. In the present study we utilized this approach to first estimate the intracellular buffer capacity (β) as described by Roos & Boron (1981). The apparent value of β was calculated according to the following equation:

$$\beta(\text{mM/pH unit}) = [NH_4^+]_i/\Delta pH_i,$$

where ΔpH_i equals the difference between pH_i prior to and following the removal of 20 mM-NH_4Cl. The concentration of internal NH_4^+, $[NH_4^+]_i$, was calculated from the concentration of external NH_4^+ and its pK_a using the Hendersson–Hasselbalch equation. It should be noted that in most experiments β was estimated in the absence of inhibitors of acid-extruding transporters and its value might be overestimated. Second, the ammonia pulse protocol was also used to estimate the ability of the cell to recover from the acidification (or alkalinization) induced by the removal (or application) or 20 mM-NH_4Cl (see legend to Fig. 5).

Electrophysiology

Voltage-clamp recordings were carried out with the whole-cell patch-clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). For monitoring I_{Ca}, ventricular cells were depolarized from −50 mV holding potential to 0 mV for 200 ms every 4 s with a patch-clamp amplifier (RK-300, Biologic, France). I_{Ca} was recorded on a Gould Brush recorder and stored on magnetic tape.

Measurement of force developed by myofilaments

After collagenase dissociation and before skinning 10^5 cells/ml were incubated with 10 μM-phenylephrine and 1 μM-propranolol for 5 min at 37 °C in HEPES solution containing 1 mM-calcium and 0·5% bovine serum albumin (BSA). For experiments performed in the presence of ethylisopropylamiloride (EIPA; 10 μM), the amiloride derivative was applied for 10 min before and maintained during the incubation with phenylephrine and propranolol.

Following the incubation period, cardiomyocytes were decanted and immediately chemically skinned by shaking for 6 min at 20 °C in a relaxing solution at pH 7·1 in the presence of 0·3% (v/v) Triton X-100. After they were rinsed twice with the relaxing solution, the skinned myocytes were kept at 4 °C for up to 8 h.

As previously described (Pucéat *et al.* 1990) the tension developed by the myofilaments of a single skinned cell was evaluated by using a transducer (AME, Horten, Norway). One extremity of the skinned cell was glued to a thin glass rod, attached to the transducer by the aid of an optical adhesive (Norland Products Inc., North Brunswick, USA) which polymerizes within 3 min when exposed to UV light. The other end of the cell was fixed by sucking into a glass pipette. The sarcomere length was adjusted to 2·1 μm. The isolated skinned myocyte was superfused with solutions at different pCas (-log[Ca]). Solutions contained (mM): phosphocreatine, 12; potassium acetate, 78·4; sodium acetate, 30·6; magnesium acetate, 3·5; imidazole, 30; EGTA, 10; dithiothreitol, 0·3; Na_2ATP, 3·5; and $CaCO_3$, 35 μM (pCa 9) or 10 mM (pCa 4·5). The pH was adjusted to 7·1 at room temperature with acetic acid. Various pCas were obtained by mixing activating (pCa 4·5) and relaxing (pCa 9) solutions as already described in Pucéat *et al.* (1990). This enabled us to establish pCa–tension curves which were then used to determine myofilament calcium sensitivity. Experiments were performed at room temperature (22 ± 2 °C).

RESULTS

Effect of phenylephrine on cell contraction

Figure 1 shows a typical recording of cell shortening measured in a cardiac cell stimulated at a basal frequency of 0·6 Hz. Increasing briefly the stimulation frequency to 6 Hz was followed by a potentiation of cell contraction upon returning to the basal frequency (post-stimulative potentiation). Then, within a few beats, the cell recovered its basal shortening amplitude. Potentiation of cell contraction was

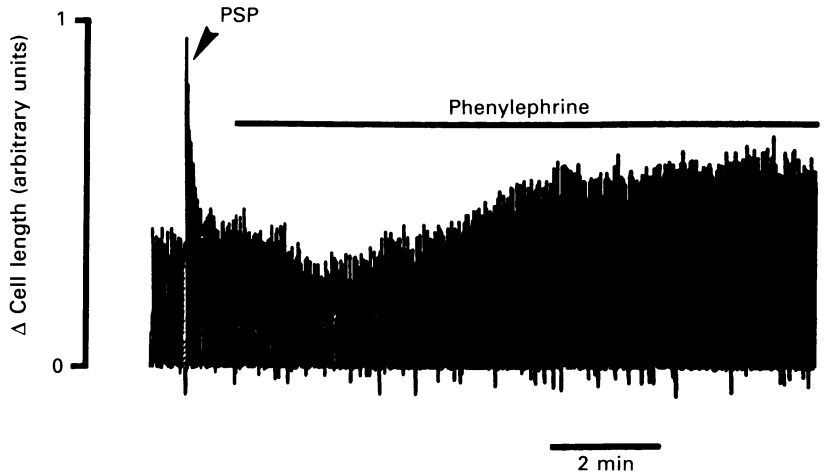

Fig. 1. Effect of phenylephrine on contraction in a rat cardiomyocyte. The shortening of an unloaded isolated ventricular cell was estimated from the variations in light intensity recorded by a photomultiplier (see Methods). The cell was stimulated at 0·6 Hz. A post-stimulative potentiation (PSP) was observed after ten stimulations at 6 Hz (arrow-head). After recovery, phenylephrine (100 μM) was applied as indicated. All solutions contained 3 μM-propranolol.

TABLE 1. Basal pH_i and phenylephrine (100 μM)-induced alkalinization in single rat cardiac cells bathed in bicarbonate-poor and -rich buffers

	Basal pH_i	Phenylephrine pH_i
HEPES	7·18±0·03 (n = 36)	+0·10±0·01 (n = 18)
Krebs	7·01±0·01 (n = 5)	+0·08±0·01 (n = 5)

pH_i of Snarf-1-loaded cardiomyocytes was monitored in HEPES-buffered solution (containing 4·4 mM-$NaHCO_3$) or in Krebs solution (containing 25 mM-$NaHCO_3$); n, number of cells investigated, means±S.E.M.

also observed when the basal stimulation was stopped for 10 s and then stimulation resumed (post-rest potentiation, not shown).

Application of the α_1-adrenoceptor agonist phenylephrine (10 or 100 μM, in the presence of 3 μM-propranolol) induced a biphasic inotropic effect. The initial negative inotropic effect, measured as a decrease in cell shortening, was followed after 1–2 min by a sustained positive inotropic effect. This time course pattern of phenylephrine's effect was consistently observed in five cells so tested. These results are in agreement with the generally reported effect of α_1-adrenergic agonists on force of contraction in multicellular preparations.

Effect of phenylephrine on pH_i

The basal intracellular pH (pH_i) of single cardiac cells, bathed in HEPES buffer containing $NaHCO_3$ (4·4 mM) and the β-adrenoceptor antagonist, propranolol (3 μM), was 7·18±0·03 (n = 36, Table 1). Intracellular pH remained stable without noticeable 'run-down' for a period of at least 20 min.

The application of phenylephrine (100 μM) produced an alkalinization (Fig. 2*A*). This agonist-induced increase in pH_i reached a maximal averaged value of $0{\cdot}10 \pm 0{\cdot}01$ pH unit ($n = 18$ cells, Table 1) above the baseline in 5 min. Following the removal of phenylephrine, the pH_i value reversed to the pre-drug value within about

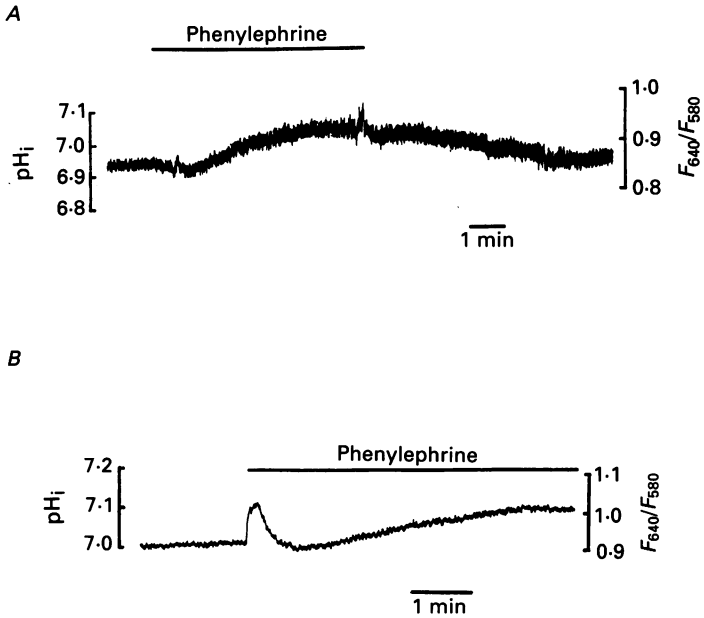

Fig. 2. α_1-Adrenergic stimulation increases pH_i in single rat cardiac cells. *A*, phenylephrine (100 μM) added to a HEPES-buffered solution containing 4·4 mM-$NaHCO_3$ reversibly increased pH_i in a Snarf-1-loaded cell. *B*, similar effects were observed when phenylephrine (100 μM) was transiently applied to a cell bathed for several hours in a Krebs solution. The transient alkalinization upon applying phenylephrine is an artifact related to the change between solutions. The right-hand scale indicates the ratio of Snarf-1 fluorescence at the two emitted wavelengths. The left-hand scale shows values of pH_i determined as described in the Methods section.

5–6 min (Fig. 2*A*). Phenylephrine at 10 μM produced a slightly smaller increase in pH_i ($0{\cdot}07 \pm 0{\cdot}01$ pH units, $n = 9$ cardiomyocytes). In some cells, the alkalinization was preceded by a transient small acidosis whose origin was not investigated.

Some experiments were performed in Krebs buffer containing 25 mM-$NaHCO_3$, a solution in which intracellular buffering would be expected to increase. Cells were kept for at least 3 h in Krebs buffer following the dissociation. Under these conditions, pH_i was significantly more acidic than in HEPES (see Table 1). However, phenylephrine (100 μM) still induced an alkalinization (Fig. 2*B*). The magnitude of the increase in pH_i averaged $0{\cdot}08 \pm 0{\cdot}01$ pH units ($n = 5$ cells, Table 1). Thus, both in $NaHCO_3$-poor and -rich buffers, phenylephrine produced a noticeable and reversible alkalinization.

To find out whether the effect of phenylephrine on pH_i was mediated via the α_1-adrenoceptors and did not reflect a non-specific effect, we tested if the selective α_1-adrenoceptor antagonist, prazosin, blocked the alkalinizing effect of phenylephrine.

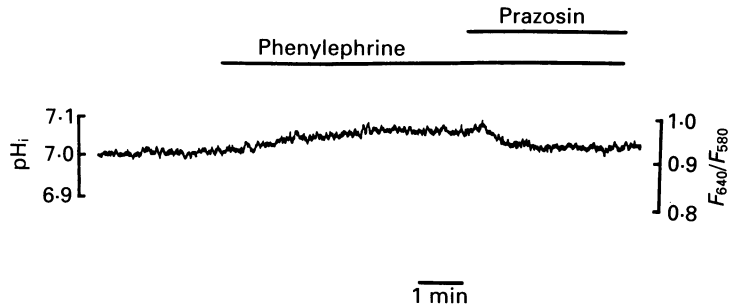

Fig. 3. Inhibition of the phenylephrine-induced increase in pH_i by prazosin. The effects of phenylephrine (100 μM) on a Snarf-1-loaded single cardiac cell were rapidly antagonized after switching to a solution containing 0·1 μM of the α_1-adrenoceptor antagonist. The right-hand scale indicates the ratio of Snarf-1 fluorescence at the two emitted wavelengths. The left-hand scale shows values of pH_i determined as described in the Methods section.

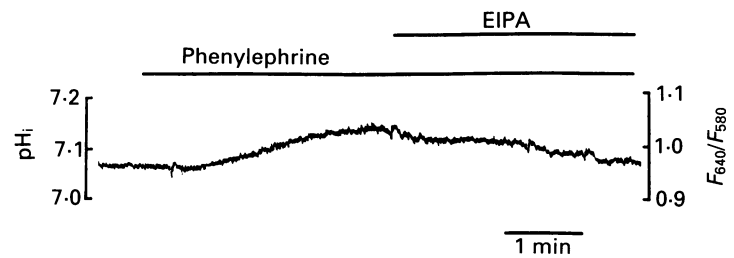

Fig. 4. Inhibition of the phenylephrine-induced increase in pH_i by EIPA. Alkalinization was first induced by exposing a Snarf-1-loaded cardiac cell to 100 μM-phenylephrine. The further addition of 10 μM-EIPA, an inhibitor of the Na^+–H^+ antiport, abolished this increase in pH_i. The right-hand scale indicates the ratio of Snarf-1 fluorescence at the two emitted wavelengths. The left-hand scale shows values of pH_i determined as described in the Methods section.

Prazosin (100 nM) *per se* had no effect on pH_i but it completely abolished within 1 min the increase in pH_i due to phenylephrine in all four cells so tested (Fig. 3). Thus, we conclude that the effects of phenylephrine are due to the activation of α_1-adrenoceptors.

Origin of the α_1-adrenoceptor-mediated alkalinization

We investigated whether the α_1-adrenergic agonist produced the observed alkalinization by stimulating the Na^+–H^+ antiport, one of a number of different mechanisms known to regulate pH_i. If this hypothesis is correct then EIPA, a potent and selective blocker of the Na^+–H^+ antiport, should inhibit the increase in pH_i induced by phenylephrine. In the experiment illustrated in Fig. 4, 10 μM-EIPA completely reversed the action of the agonist on pH_i. This result was repeated in eight single cardiomyocytes in which EIPA always inhibited the alkalinizing effect of phenylephrine within 1–3 min. In some experiments, EIPA was added prior to phenylephrine. By itself, EIPA did not markedly affect basal pH_i but it prevented phenylephrine producing an alkalinization (data not illustrated). Thus, the alkalinizing effect of α_1-adrenoceptor agonists is entirely EIPA sensitive.

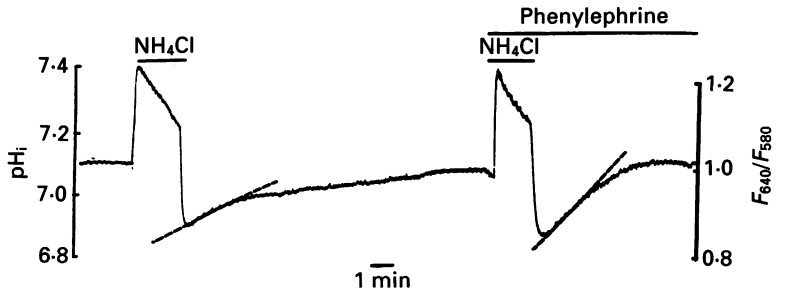

Fig. 5. Phenylephrine facilitates the recovery from an acid load. A Snarf-1-loaded cardiomyocyte was submitted to a pulse of NH_4Cl (20 mM for 1·5 min), first in the absence and then in the presence of phenylephrine. Upon removal of NH_4Cl, an acidosis occurred with a similar amplitude in both conditions; however, the recovery from this acid load was about twice as fast in the presence as in the absence of phenylephrine. The right-hand scale indicates the ratio of Snarf-1 fluorescence at the two emitted wavelengths. The left-hand scale shows values of pH_i determined as described in the Methods section.

TABLE 2. Phenylephrine (100 μM) facilitates the recovery from an acid load induced by the removal of NH_4Cl in isolated rat cardiac cells bathed with HEPES solution

	Control ($n = 7$)	Phenylephrine ($n = 7$)
Time of half-recovery (min)	3·13±0·26	1·32±0·12**
dpH_i/dt (pH units/min)	0·05±0·01	0·12±0·02**
β (mM/pH unit)	34·6±7·8	40·7±8·0
$\beta\ dpH_i/dt$ (mM/min)	1·73±0·33	4·82±1·20*

dpH_i/dt represents the rate of recovery from an acid load triggered by the removal of 20 mM-NH_4Cl, β is the estimated cell buffering capacity and $\beta\ dpH_i/dt$ the equivalent acid efflux rate; n, number of cells investigated, means±S.E.M.; $^{**}P \leqslant 0{\cdot}01$, $^{*}P \leqslant 0{\cdot}025$, Student's t test.

However, EIPA could have inhibited the effect of phenylephrine through a mechanism independent of the blockade of Na^+–H^+ exchange. To further confirm that phenylephrine stimulates the Na^+–H^+ antiport, the technique of the NH_4Cl pulse was used (Roos & Boron, 1981). Specifically, we took advantage of the fact that the removal of NH_4Cl, following its brief application to a cardiac cell, triggers an acidosis whose recovery towards its initial baseline pH_i mainly depends on the EIPA-sensitive Na^+–H^+ antiport. As expected for an activator of the Na^+–H^+ antiport, phenylephrine accelerated the return to the baseline pH_i value following the removal of 20 mM-NH_4Cl (Fig. 5; Table 2). The intracellular buffering capacity of the cell appeared not to be altered by the α_1-adrenergic agonist (Table 2) even when β was estimated in $NaHCO_3$-free HEPES buffer containing 10 μM-EIPA (data not shown).

Effect of phenylephrine on the calcium current

Previous experiments have shown that alkalinization induces an increase in amplitude of the L−type Ca^{2+} current (Kurachi, 1982). It is controversial whether α_1-adrenergic agonists can increase I_{Ca} amplitude (Brückner & Scholz, 1984;

Hescheler, Nawrath, Tang & Trautwein, 1988; Hartmann, Mazzoca, Kleiman & Houser, 1988). To find out if the alkalinization induced by phenylephrine could increase I_{Ca} in rat cardiac cells, whole-cell patch-clamp recording and pH_i monitoring by microfluorimetry were performed simultaneously. Under our experimental

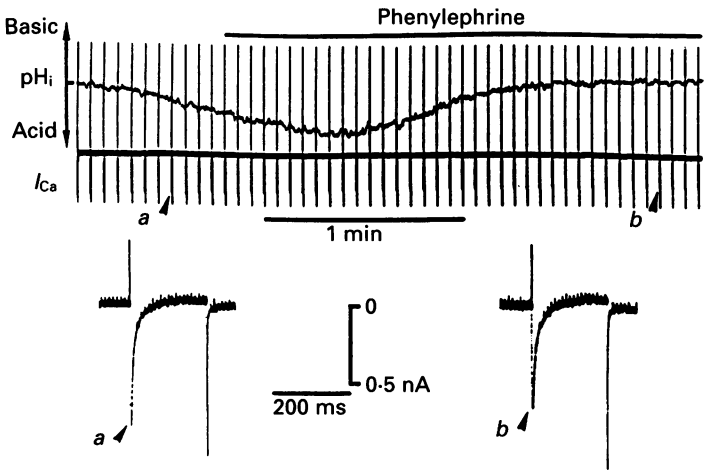

Fig. 6. Effects of phenylephrine during simultaneous recordings of Ca^{2+} current and pH_i. Continuous recording of membrane currents under whole-cell patch-clamp was performed while pH_i was simultaneously measured in a Snarf-1-loaded cardiac cell. Repetitive 200 ms depolarizing pulses to 0 mV, applied every 4 s from a −50 mV holding potential, were used to trigger L-type Ca^{2+} currents. The application of 100 μM-phenylephrine induced an alkalinization with no increase in Ca^{2+} current amplitude and no change in I_{Ca} run-down; *a*, calcium current trace in control; *b*, calcium current trace in the presence of phenylephrine.

conditions, while phenylephrine still induced an alkalinization, there was no concomitant increase of I_{Ca} amplitude nor obvious modifications in its kinetics (Fig. 6). Throughout the experiments, a 'run-down' of I_{Ca} was observed. It was favoured by the −50 mV holding potential and the low BAPTA concentration used to allow the cell to contract (Belles, Malécot, Hescheler & Trautwein, 1988; Schouten & Morad, 1989). As seen in Fig. 6, application of phenylephrine did not affect the 'run-down' of I_{Ca}. Moreover, when cardiac cells were electrically stimulated, pH_i did not remain stable but rather acidified (see also Presler, 1988). Similar results were obtained in the seven cells so investigated. Phenylephrine also did not alter I_{Ca} in rat cells under whole-cell patch-clamp conditions, with no concomitant microspectrofluorometry measurement. The α_1-adrenoceptor agonist also did not vary the holding current.

Effect of phenylephrine on the Ca^{2+} transient

Basal intracellular free Ca^{2+} content, Ca_i^{2+}, of single rat cardiac cells loaded with Indo-1 and stimulated at 0·2 Hz was 72±8 nM ($n = 6$). This value is similar to the one previously obtained in quiescent cells (69±5 nM; Pucéat *et al.* 1991). Figure 7

shows that the application of 100 μM-phenylephrine on a electrically driven single cell did not change the magnitude of the Ca^{2+} transient. It should be noted, however, that the diastolic Ca_i^{2+} was slightly decreased. The α_1-adrenergic agonist also did not significantly alter the kinetics of the transient (Fig. 7). Indeed, time to peak of the

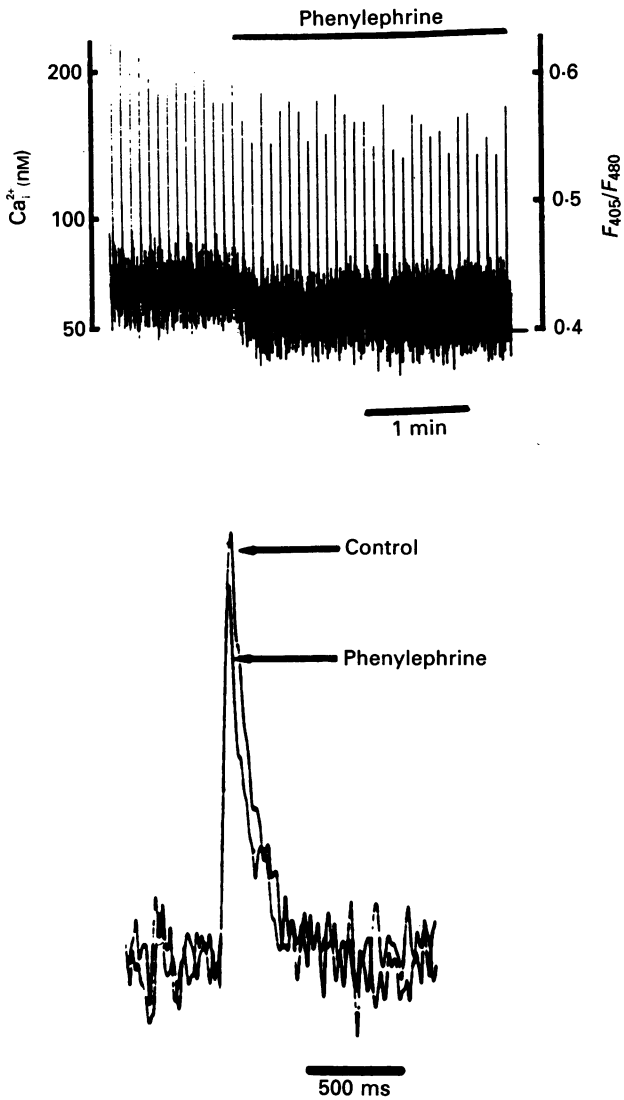

Fig. 7. Effects of phenylephrine on Ca^{2+} transients. A single Indo-1-loaded cardiac cell was electrically stimulated at a frequency of 0·2 Hz, and superfused with a HEPES solution containing 100 μM-phenylephrine. The lower panel depicts two superimposed Ca^{2+} transients in the absence and in the presence of the α_1-adrenoceptor agonist. The averages of ten successive Ca^{2+} transients in the absence and in the presence of phenylephrine revealed no significant change in the kinetics. The right-hand scale indicates the ratio of Indo-1 fluorescence at the two emitted wavelengths. The left-hand scale shows values of Ca_i^{2+} determined as described in the Methods section.

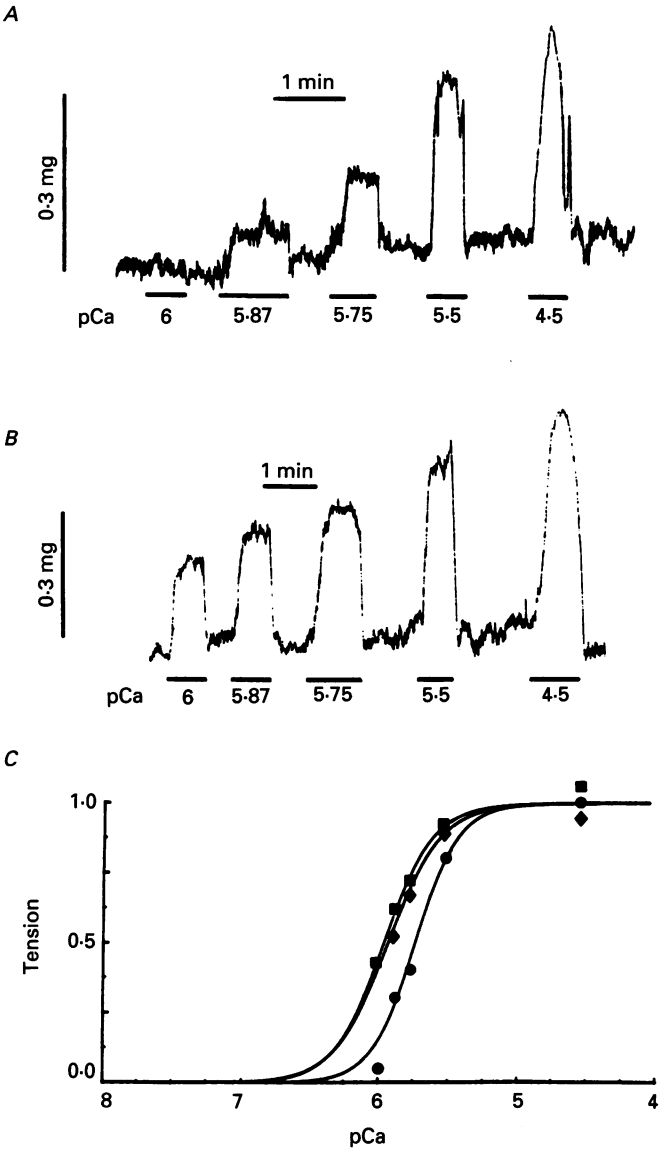

Fig. 8. Effects of phenylephrine in the absence and presence of EIPA on Ca^{2+} sensitivity of single skinned cardiac cells. Continuous recording of tension developed by single skinned cells superfused with EGTA-buffered solutions of known calcium concentration. *A*, cell exposed for 10 min to EIPA (0·1 μM). *B*, cell exposed for 5 min to EIPA plus phenylephrine (10 μM). *C*, tension–pCa relationships established in three single skinned cardiac cells isolated from the same heart. The cells were exposed to EIPA (0·1 μM) (●), to phenylephrine (10 μM) (■) or to phenylephrine plus EIPA (◆) to prevent the phenylephrine-induced alkalinization. Curves are drawn according to the Hill equation using a linear regression fit. pCa_{50} and n_H are respectively 5·72, 5·94, 5·91 and 2·70, 2·37, 2·21 in EIPA, phenylephrine and phenylephrine plus EIPA.

Ca^{2+} transient was 100 ± 6 ms in control and 114 ± 10 ms ($n = 5$) in the presence of phenylephrine; Ca_i^{2+} returns towards its baseline level within 237 ± 12 and 221 ± 13 ms ($n = 5$) in control and in the presence of the α_1-adrenoceptor agonist, respectively. Control experiments showed large increases in Ca^{2+} transients during the application of β-adrenergic agonists.

Effect of phenylephrine on calcium sensitivity of the myofilaments

To investigate whether phenylephrine-induced alkalinization could be involved in the increase in calcium sensitivity that is due to phosphorylation of cardiac contractile proteins, cardiomyocytes were incubated with phenylephrine alone or in the presence of the Na^+–H^+ antiport inhibitor, EIPA. Figure 8 shows the tension–pCa relationships established on three skinned cells from the same heart, after they have been incubated in the presence of EIPA, phenylephrine or phenylephrine and EIPA. The incubation, with EIPA alone, prior to skinning at 37 °C for 10 min, had no effect on Ca^{2+} sensitivity of contractile proteins. The half-activation, pCa_{50}, and the slope coefficient, n_H, were respectively $5{\cdot}78 \pm 0{\cdot}01$ and $2{\cdot}76 \pm 0{\cdot}12$ ($n = 14$) and were similar to previously reported control values obtained under similar conditions (Pucéat *et al.* 1990). Thus, EIPA on its own had no effect on the contractile properties of skinned cells. Phenylephrine, in the presence of propranolol induced a large leftward shift of the tension–pCa relationship as previously reported (Pucéat *et al.* 1990). Furthermore, phenylephrine produced similar effects in cells pre-treated with EIPA. In the cells investigated, pCa_{50} and n_H were $5{\cdot}95 \pm 0{\cdot}01$ and $2{\cdot}73 \pm 0{\cdot}10$ ($n = 8$) in phenylephrine and $5{\cdot}92 \pm 0{\cdot}01$ and $2{\cdot}30 \pm 0{\cdot}04$ ($n = 3$) in phenylephrine plus EIPA respectively. Thus, the increase in Ca^{2+} sensitivity of the myofilaments induced by α_1-adrenergic stimulation occurs with the same magnitude in the presence of the Na^+–H^+ antiport inhibitor, and appears not to be dependent on the alkalinizing ability of α_1-adrenoceptor agonists.

DISCUSSION

The present results obtained in rat isolated ventricular cells suggest that the positive inotropic effect induced by α_1-adrenergic agonists (present study; see also Endoh, 1986; Hartmann *et al.* 1988; Ravens *et al.* 1989) could not be attributed to significant changes in the Ca^{2+} current, nor in the Ca^{2+} transients as indicated by our measurements with the fluorescent dye, Indo-1. Instead, α_1-adrenergic stimulation increases the sensitivity of the myofilaments to Ca^{2+} ions in at least two independent ways: a direct effect on the contractile proteins and a secondary effect consequent to an intracellular alkalosis.

Several studies have shown that the myocardial response to α_1-adrenergic stimulation can differ between almost none to relatively large effects similar to those seen with β-adrenergic stimulation. We selected rat ventricular cells since it was shown that stimulation of both types of adrenoceptors might equally increase contractile activity in rat heart; however, while the β-adrenergic effect developed monophasically, the α_1-adrenergic response is characterized by a biphasic or triphasic pattern (Skomedal, Osnes & Oyes, 1982). The β-adrenergic stimulation, mediated by an increase in cyclic AMP, leads to an initial increase in the L-type Ca^{2+}

current, the only Ca^{2+} current reported in rat ventricular cells (Scamps, Legssyer, Mayoux & Vassort, 1990). The α_1-adrenergic effect occurs without an increase in cyclic AMP level (Schümann & Endoh, 1975; Brückner & Scholz, 1984), but with a simultaneous increase in inositol trisphosphate (Poggioli *et al.* 1986; Otani, Otani & Das, 1988; Scholz *et al.* 1988). The latter suggests that protein kinase C (PKC) could be activated by diacylglycerol, the second product of polyphosphoinositide breakdown (Berridge, 1984). In rat single cardiomyocytes, we did not observe an α_1-adrenergic effect on I_{Ca} under whole-cell-clamp conditions. At first sight, this result was surprising for at least two reasons. First, it has been reported that activation of PKC increases and then decreases ^{45}Ca influx and the probability of Ca^{2+} channels being open in ventricular myocytes (Lacerda, Rampes & Brown, 1988). Second, it is known that an alkalinization increases the Ca^{2+} current (Kurachi, 1982); however in this last study, the applied changes in pH were of much larger magnitude than the one we observed. A lack of augmentation in Ca^{2+} current has always been reported in other mammalian species (guinea-pig and rabbit, Hescheler *et al.* 1988; feline, Hartmann *et al.* 1988), while in frog heart we demonstrated some increase (Alvarez, Mongo & Vassort, 1987). During these previous studies, it was not known whether the intracellular alkalinization observed under α_1-adrenergic stimulation was taking place due to the internal dialysis of the myocytes through the patch pipette. Our present experiments which combine I_{Ca} and pH_i recordings clearly demonstrate that the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channel was not affected by α_1-adrenergic stimulation despite the concomitant alkalinization.

As already proposed by Brückner & Scholz (1984), who were the first to describe minute changes in peak Ca^{2+} current (but which were associated with prolonged inactivation in their double sucrose gap study), other mechanisms may account for the positive inotropic effect of α_1-adrenergic stimulation. One candidate would be an enhancement of Ca^{2+} influx. This could be consequential to the prolongation of the action potential duration (Ledda *et al.* 1975; Vogel & Terzic, 1989; Fedida *et al.* 1989). Another possibility was suggested by Jahnel, Nawrath, Carmeliet & Vereecke (1991) who showed that α_1-adrenergic stimulation induced membrane depolarization in rat atrial cells. This would activate the Na^+ window current and consequently increase Ca^{2+} influx by the Na^+–Ca^{2+} exchange. In another study, direct Ca_i^{2+} measurements on Fura-2-loaded hamster cardiac cells showed an increase of the time-averaged Ca_i^{2+} under α_1-adrenergic stimulation (Sen, Liang, Colucci & Smith, 1990). Such an enhancement of Ca^{2+} influx is only partially supported by the elegant demonstration that in rabbit papillary muscle under the influence of phenylephrine, the height of the aequorin signal increases much less than with comparable positive inotropic concentrations of β-adrenergic agonists (Endoh & Blinks, 1988). In the present study, isolated rat ventricular cells loaded with Indo-1 showed no change in peak Ca^{2+} transient nor in its kinetics after α_1-adrenergic stimulation (Fig. 7). Indirectly, these observations suggest that under our experimental conditions, $InsP_3$ does not significantly interfere with Ca^{2+} handling by the sarcoplasmic reticulum although the $InsP_3$ level is expected to be markedly increased (Poggioli *et al.* 1986). We cannot exclude the possibility that the putative $InsP_3$-induced Ca^{2+} release was overwhelmed by a mechanism which leads to the small decrease in diastolic Ca_i^{2+} that we report. Several mechanisms could conceivably account for the latter observation.

First, it has been reported that phenylephrine increased the Ca^{2+} uptake by mitochondria (Crompton, Kessar & Al Nasser, 1983). Secondly, the expected PKC activation by α_1-adrenergic agonists could stimulate the Ca-ATPase of the sarcoplasmic reticulum (Movsesian, Nishikawa & Adelstein, 1984) and/or accelerate extrusion of Ca^{2+} ions by Na^+–Ca^{2+} exchange (Philipson, Bersohn & Nishimito, 1982; Zaza, Kline & Rosen, 1990). Moreover, it has been shown that phorbol esters, direct PKC activators, induce a decrease in diastolic Ca_i^{2+} in rat ventricular myocytes (Capogrossi, Kaku, Filburn, Pelto, Hansford, Spurgeon & Lakatta, 1990). Consequently, it could be suggested that the decreased diastolic Ca_i^{2+} accounts for the initial negative inotropic effect induced by phenylephrine as reported in this study as well as in multicellular preparations. Finally, the possible major reason for the difference between the reported observations in Ca_i^{2+} variations could be related to tissue as well as to species differences as pointed out by Endoh, Hiramoto, Ishihata, Takanashi & Inui (1991).

The present study demonstrates, for the first time in single cardiac cells, that the α_1-adrenoceptor agonist phenylephrine (10 and 100 μM), in the presence of β-adrenoceptor blockade, produces an intracellular alkalinization. This effect can be ascribed to stimulation of the sarcolemmal α_1-adrenoceptors since it was abolished by the selective α_1-adrenoceptor antagonist, prazosin. Previously, a similar alkalinizing effect of α_1-adrenergic agonists has been described in multicellular preparations using ion-selective microelectrodes (Terzic *et al.* 1991) and in cell suspensions using single-excitation and emission wavelength fluorescence measurements (Wallert & Fröhlich, 1989; Iwakura, Hori, Watanabe, Kitabatake, Cragoe, Yoshida & Kamada, 1990). This is significant since intact cardiac muscle also contains non-muscle elements which could affect the direct postsynaptic action of an α_1-adrenoceptor agonist. Furthermore, the single-cell/dual-emission approach used here offers advantages over cell suspension studies. As already pointed out by Iwakura *et al.* (1990), the interpretation of their results should be cautious due to methodological limitations which included dye leakage or cardiac cell death during the recording periods. Two findings suggest that the α_1-adrenoceptor-mediated increase in pH_i was due to activation of the Na^+–H^+ antiport. First, the phenylephrine-induced alkalinization was completely abolished or prevented by EIPA, a selective and potent inhibitor of Na^+–H^+ exchange (Fig. 4). Second, phenylephrine is capable of enhancing the recovery from an acid load (Fig. 5). This recovery cannot be attributed to an increase buffering capacity of the cells, but is known to be mainly governed by the Na^+–H^+ exchange (Vaughan-Jones, 1988).

Though intracellular pH is a critical regulator of force (Vaughan-Jones, Eisner & Lederer, 1987; Orchard & Kentish, 1990) and alkalinizing transport systems are present in cardiac muscle (Frelin, Vigne, Ladoux & Lazdunski, 1988; Vaughan-Jones, 1988), pharmacological modulation of pH_i has not been considered as a potential means by which cardiac contractility could be regulated. However, such a possibility appears attractive since a modest intracellular alkalinization, e.g. 0·1 pH unit, can cause pronounced positive inotropic and tonotropic effects (Vaughan-Jones *et al.* 1987). Further, in many non-cardiac tissues, intracellular alkalinization via receptor-mediated activation of Na^+–H^+ antiport has been demonstrated. The receptors involved often stimulate the break-down of phosphatidylinositols and seem

to be coupled to the Na^+–H^+ antiport by activating protein kinase C (Vigne, Frelin & Lazdunski, 1985).

The alkalinization induced by α_1-adrenergic stimulation develops with a slow time course and can reach 0·1 pH unit. As shown in skinned cardiac preparations (Fabiato & Fabiato, 1978), this leads to a significant increase in myofilament sensitivity to Ca^{2+} ions. Such an effect of α_1-adrenergic agonists might account for part of the positive inotropy reported in the present study. However, another mechanism might be involved since preventing pH_i changes by the use of hexamethylamiloride, a potent Na^+–H^+ antiport inhibitor, suppresses only half of the inotropic effect (Terzic & Vogel, 1991), although others reported a 'significant correlation between variations in alkalinization and inotropy', both effects being antagonized by EIPA (Gambassi, Blank, Spurgeon, Chung, Lakatta & Capogrossi, 1990). We have previously shown that α_1-adrenergic stimulation of isolated rat cells increased Ca^{2+} sensitivity of their contractile proteins after fast chemical skinning in a solution buffered at pH 7·1 (Pucéat *et al.* 1990). These results are extended by demonstrating that such an effect of α_1-adrenergic agonists occurs even when pH_i changes are prevented by EIPA. Besides indirectly indicating that EIPA does not affect the α_1-adrenergic receptor, this observation excludes the hypothesis that myofilament alterations are dependent or modulated by the alkalinization. Rather, it is suggested that the increase in Ca^{2+} sensitivity of the contractile proteins is induced by the activation of both Ca^{2+}–calmodulin myosin light chain kinase and protein kinase C (Clément, Pucéat, Walsh & Vassort, 1990). The change in Ca^{2+} sensitivity induced by phenylephrine could reach 0·18 pCa unit, while it is estimated that an alkalinization of 0·1 pH unit could account for a leftward shift of 0·07 in pCa_{50} (according to Fig. 1 in Fabiato & Fabiato, 1978), with no change in the slope in both cases. Such rough approximation could further suggest that during maximal α_1-adrenergic stimulation, the increase in Ca^{2+} sensitivity of the myofilaments depends on two mechanisms: changes in pH_i could only partially account for the overall positive inotropic effect.

In conclusion, while it is well established that a majority of drugs that enhance myocardial contractility do so by affecting intracellular calcium (reviewed by Reiter, 1988), this appears not to be the case with α_1-adrenergic agonists. Changes in Ca^{2+} transients are not significant in rat cardiac cells following α_1-adrenergic stimulation. Instead, the increase in twitch tension should be mostly attributed to an enhancement of Ca^{2+} sensitivity of the myofilaments as first suggested by Endoh & Blinks (1988). This Ca^{2+} sensitization could have two origins: not only the α_1-adrenergic neuromodulation would increase the internal pH by stimulating the Na^+–H^+ antiport, but it would also alter the contractile proteins themselves. In this regard, α_1-adrenoceptor agonists represent a novel class of positive inotropic agents.

A.T. was supported in part by the Association for US/French Biomedical Cooperation and is a recipient of the Fellowship Award for Careers in Clinical Pharmacology from the Pharmaceutical Manufacturers Association Foundation.

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