



Contribution of phosphodiesterase isozymes to the regulation of the L-type calcium current in human cardiac myocytes

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1 To determine the contribution of the various phosphodiesterase (PDE) isozymes to the regulation of the L-type calcium current ($I_{Ca(L)}$) in the human myocardium, we investigated the effect of selective and non-selective PDE inhibitors on $I_{Ca(L)}$ in single human atrial cells by use of the whole-cell patch-clamp method. We repeated some experiments in rabbit atrial myocytes, to make a species comparison.

2 In human atrial cells, 100 μM pimobendan increased $I_{Ca(L)}$ (evoked by depolarization to +10 mV from a holding potential of -40 mV) by $250.4 \pm 45.0\%$ ($n=15$), with the concentration for half-maximal stimulation (EC_{50}) being 1.13 μM . $I_{Ca(L)}$ was increased by 100 μM UD-CG 212 by $174.5 \pm 30.2\%$ ($n=10$) with an EC_{50} value of 1.78 μM in human atrial cells. These two agents inhibit PDE III selectively.

3 A selective PDE IV inhibitor, rolipram (1–100 μM), did not itself affect $I_{Ca(L)}$ in human atrial cells. However, 100 μM rolipram significantly enhanced the effect of 100 μM UD-CG 212 on $I_{Ca(L)}$ (increase with UD-CG 212 alone, 167.9 ± 33.9 , $n=5$; increase with the two agents together, $270.0 \pm 52.2\%$; $n=5$, $P<0.05$). Rolipram also enhanced isoprenaline (5 nM)-stimulated $I_{Ca(L)}$ by $52.9 \pm 9.3\%$ ($n=5$) in human atrial cells.

4 In rabbit atrial cells, $I_{Ca(L)}$ at +10 mV was increased by $22.1 \pm 9.0\%$ by UD-CG 212 ($n=10$) and by $67.4 \pm 12.0\%$ ($n=10$) by pimobendan (each at 100 μM). These values were significantly lower than those obtained in human atrial cells ($P<0.0001$). Rolipram (1–100 μM) did not itself affect $I_{Ca(L)}$ in rabbit atrial cells. However, $I_{Ca(L)}$ was increased by $215.7 \pm 65.2\%$ ($n=10$) by the combination of 100 μM UD-CG 212 and 100 μM rolipram. This value was almost 10 times larger than that obtained for the effect of 100 μM UD-CG 212 alone.

5 These results imply a species difference: in the human atrium, the PDE III isoform seems dominant, whereas PDE IV may be more important in the rabbit atrium for regulating $I_{Ca(L)}$. However, PDE IV might contribute significantly to the regulation of intracellular cyclic AMP in human myocardium when PDE III is already inhibited or when the myocardium is under β -adrenoceptor-mediated stimulation.

Keywords: Phosphodiesterase inhibitor; phosphodiesterase isozymes; human atrial cells; L type Ca^{2+} current; whole-cell voltage-clamp method

Introduction

Ca^{2+} influx via the L-type calcium current ($I_{Ca(L)}$) is the major factor in the regulation of cardiac excitation-contraction coupling. $I_{Ca(L)}$ is known to be regulated by adenosine 3':5'-cyclic monophosphate (cyclic-AMP)-dependent phosphorylation in cardiac tissues. Thus, β -adrenoceptor-mediated stimulation activates adenylate cyclase to produce cyclic AMP, which finally phosphorylates $I_{Ca(L)}$ by activating cyclic AMP-dependent protein kinase (Kameyama *et al.*, 1986). The intracellular cyclic AMP concentration is regulated not only by the rate of cyclic AMP production by adenylate cyclase, but also by the rate of its hydrolysis by phosphodiesterase (PDE). On the basis of their particular primary protein sequence, PDE isozymes are classified into four families (PDEs I to IV) in the heart (Beavo & Reifsnnyder, 1990). These PDE isozymes have been shown to be of major importance as targets for pharmacological interventions in the heart (Beavo & Reifsnnyder, 1990). Moreover, in recent years, several types of PDE inhibitors have been shown to be clinically useful in the failing heart (DiBanco *et al.*, 1989; Kubo *et al.*, 1992).

In the human myocardium, the effect of β -adrenoceptor agonists on $I_{Ca(L)}$ has been well established (LeGrand *et al.*, 1991; Ouadid *et al.*, 1991; Piot *et al.*, 1996). However, a relatively small number of studies has been made of the effects of PDE inhibitors on $I_{Ca(L)}$ (Li *et al.*, 1994). Further, the regulation of cyclic AMP hydrolysis has not been well documented and it remains unclear whether and to what extent the different types of PDE isozymes contribute to the regulation of $I_{Ca(L)}$ in

human cardiac myocytes. For this reason, we thought it important to study thoroughly the effects of PDE inhibitors on $I_{Ca(L)}$ in human cardiac myocytes. Since the activity of cardiac $I_{Ca(L)}$ is strongly regulated by cyclic AMP-dependent phosphorylation, the measurement of $I_{Ca(L)}$ in isolated cardiac myocytes may be used as a probe for studying cyclic AMP metabolism by PDE isozymes.

To evaluate the contribution of the various PDE isozymes to the regulation of $I_{Ca(L)}$ in the human myocardium, we designed an investigation of the effect of various selective and non-selective PDE inhibitors on $I_{Ca(L)}$ in single human atrial cells by use of the whole-cell voltage-clamp method. We repeated some experiments in rabbit atrial myocytes, to make a species comparison. Pimobendan and its primary metabolite, UD-CG 212, are known to inhibit selectively the PDE III isozyme, the IC_{50} being 0.57–1.7 μM for pimobendan and 0.05–0.22 μM for UD-CG 212 (Brunkhorst *et al.*, 1989; Bethke *et al.*, 1991; 1992; Cheffoy *et al.*, 1992). On the other hand, rolipram is the most potent inhibitor known of PDE IV, with an IC_{50} value of 1.4–3.0 μM in guinea-pig (Bethke *et al.*, 1992) and human myocardium (Reeves *et al.*, 1987). We therefore chose to investigate the effects of these PDE inhibitors on $I_{Ca(L)}$ in the present study. Our results show that $I_{Ca(L)}$ is significantly enhanced by the selective PDE III inhibitor pimobendan and by UD-CG 212 in human atrial cells to an extent greater than that seen in other species. Experiments examining the combined effect on $I_{Ca(L)}$ of a PDE III inhibitor and a PDE IV inhibitor or of isoprenaline and a PDE IV inhibitor demonstrated that PDE IV, as well as PDE III, can contribute significantly to the regulation of $I_{Ca(L)}$ in human atrial cells.

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Methods

Preparation of single cells

The cell isolation procedure for human atrial myocytes was derived from a technique described previously (Escande *et al.*, 1987; Sakai *et al.*, 1995). Small segments of myocardium samples from right atrial appendages were obtained at the time of open-heart surgery, in accordance with the institutional guidelines for research on human subjects (Sakai *et al.*, 1995). Each patient had a normal right atrial pressure and a normal sinus rhythm and was between 1 and 45 years of age. The clinical diagnosis was congenital heart disease in 31 patients and valvular disease in 2 patients.

None of the patients were on medications such as Ca^{2+} -channel blockers, β -adrenoreceptor receptor blockers or PDE inhibitors. In brief, each segment of human myocardium was cut into pieces and incubated in warm Tyrode solution for 10 min. The strips were then cut into even smaller pieces and left in nominally Ca^{2+} -free Tyrode solution for 17 min. They were then transferred to a nominally Ca^{2+} -free Tyrode solution containing 0.8 mg ml^{-1} collagenase (Yakult, Tokyo, Japan) with 1 mg ml^{-1} protease (Type XX VII, Sigma Chemical Co, St. Louis, MO, U.S.A.) for 70–80 min at 37°C . The collagenase was then washed out by rinsing with high- K^+ , low Cl^- solution (Isenberg & Klöckner, 1982) and the digested tissue stored in the same solution. For the rabbit atrium, the method of cell isolation by use of enzymes was the same as already described elsewhere (Hagiwara *et al.*, 1992a,b; Sakai *et al.*, 1996; Matsuda *et al.*, 1996). All procedures used complied with institutional guidelines governing animal experimentation.

Electrical recordings

The whole-cell voltage-clamp method used was the same as that described previously (Hamil *et al.*, 1981; Hagiwara *et al.*, 1992a,b; Matsuda *et al.*, 1996; Sakai *et al.*, 1996). Patch pipettes were pulled from 1.25 mm borosilicate capillaries (D941-8.5-85 Clinitubes: Radiometer A/S, Copenhagen, Denmark). The resistance of each electrode, when filled with the pipette solution, was in the range 2–3 M Ω . The amplifier (TM-1000, ACT ME Laboratory, Tokyo, Japan) employed a 100 M Ω feedback resistor, and series resistance was partially compensated. The current-voltage (I - V) signals were stored on a video recorder (S-6000, Victor, Tokyo, Japan), with a PCM converter system (RP-880, NF Electronic Instruments, Tokyo, Japan) being used for computer analysis (PC 9801 RA, NEC, Tokyo, Japan). The current signals were fed from the video recorder to the computer via a 2.5 kHz, 8 pole Bessel-type low-pass filter. The liquid junction potential (-7.5 mV) between the pipette and the bathing solutions was corrected. For recording $I_{Ca(L)}$, the cells were usually depolarized every 10 s from a holding potential of -40 mV to a test potential of $+10 \text{ mV}$ for 300 ms. This test pulse is based on the voltage corresponding to the peak current in the I - V relationships obtained in both the control situation and after application of various PDE inhibitors. No leakage correction was applied. To avoid the effects of current run-down, the measurement of $I_{Ca(L)}$ during the cumulative application of PDE inhibitors was performed within a 20–30 min experimental period. Under our experimental conditions, $I_{Ca(L)}$ was stable over experimental periods of this length. $I_{Ca(L)}$ was measured as the peak inward current. Experiments were performed at $37 \pm 0.5^\circ\text{C}$.

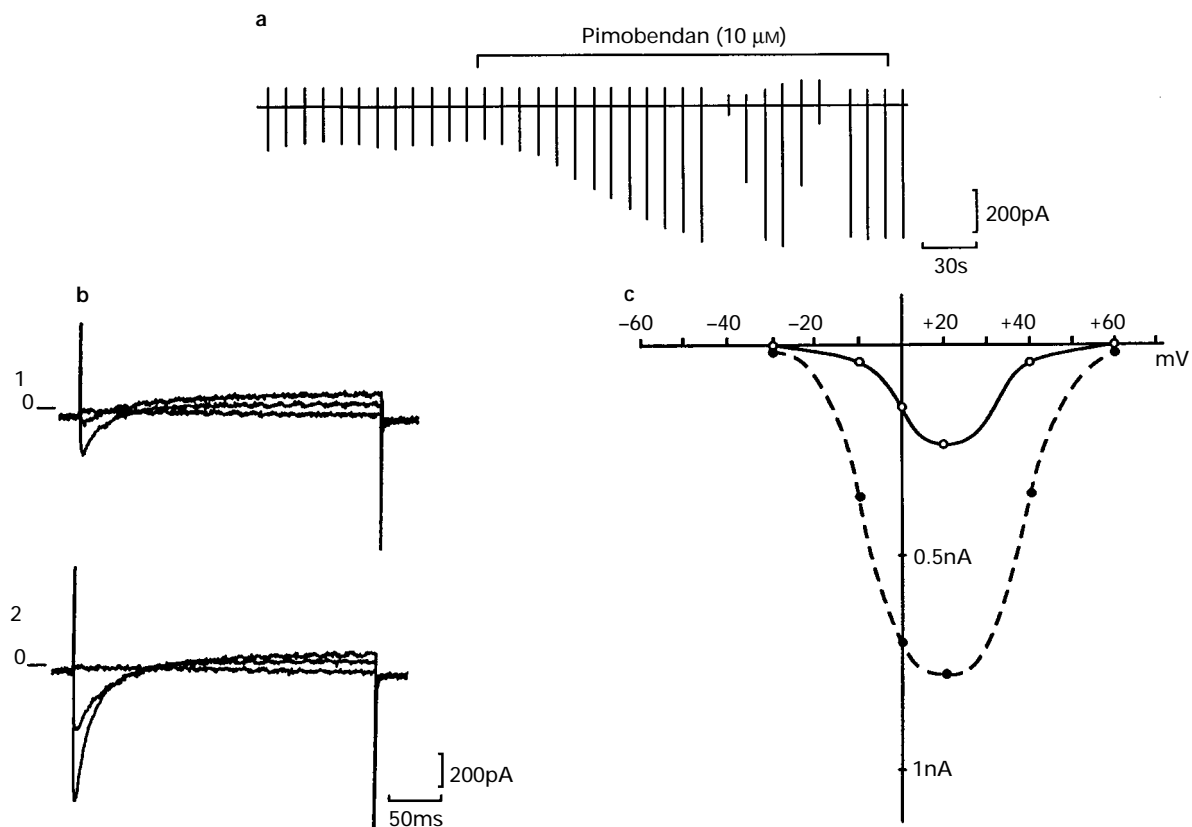


Figure 1 Effect of pimobendan on $I_{Ca(L)}$ in a human atrial cell. (a) Chart recording of $I_{Ca(L)}$ before and after application of $10 \mu\text{M}$ pimobendan. (b) $I_{Ca(L)}$ elicited by depolarizing pulses from a holding potential of -40 mV to -30 , $+10$ and $+30 \text{ mV}$ for 300 ms in control (b-1) and in the presence of $10 \mu\text{M}$ pimobendan (b-2). This dose of pimobendan increased $I_{Ca(L)}$ from 220 pA (b-1) to 750 pA (b-2) on depolarization to $+10 \text{ mV}$. (c) I - V relationships for $I_{Ca(L)}$ before (open circles) and during administration of $10 \mu\text{M}$ pimobendan (solid circles). The I - V relationships showed that $I_{Ca(L)}$ was increased by $10 \mu\text{M}$ pimobendan at every membrane potential examined. The data shown were obtained from a single cell.

Solutions

The normal Tyrode solution contained (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 0.33, glucose 5 and HEPES 5 (pH adjusted to 7.4 with NaOH). The standard pipette solution contained (in mM): CsOH 120, CsCl 20, aspartic acid 100, EGTA 10, $MgCl_2$ 2, Mg-ATP 5, K_2 -creatine phosphate 5, Na-GTP 0.1 and HEPES 5 (pH = 7.4 with CsOH). The standard external solution contained (in mM): NaCl 150, $CaCl_2$ 1.8, $MgCl_2$ 0.5 and HEPES 5 (pH = 7.4 with NaOH, Matsuda *et al.*, 1996).

Drugs

Pimobendan and UD-CG 212 cl (UD-CG212) were kindly provided by Boehringer Ingelheim Ltd. (Hyogo, Japan) and dissolved in dimethyl sulphoxide (DMSO) as a 100 mM stock solution. Rolipram was kindly provided by Schering Ltd. (Tokyo, Japan) and also dissolved in DMSO as a 100 mM stock solution. 3-Isobutyl-1-methylxanthine (IBMX, Sigma) was dissolved in DMSO and prepared as a 100 mM stock solution. Isoprenaline (Sigma) was made freshly as a stock solution of 1 mM in distilled water.

Statistical analysis

Paired and unpaired Student's *t* tests were used to evaluate the statistical significance of differences between means. Values of $P < 0.05$ were considered to indicate statistical significance. All statistical data are given as mean \pm s.d.

Results

Effects of selective PDE III inhibitors on $I_{Ca(L)}$ in human atrial cells

To evaluate the inhibitory effects on PDE III of pimobendan and UD-CG 212, we first examined their effects on the L-type calcium current ($I_{Ca(L)}$) in human atrial myocytes.

Figure 1 illustrates the effects of pimobendan on $I_{Ca(L)}$ at various membrane potentials (from a holding potential of -40 mV) in human atrial cells. Superfusing the particular cell illustrated in Figure 1a and b with $10 \mu M$ pimobendan increased $I_{Ca(L)}$ from 220 pA (Figure 1b-1) to 750 pA at $+10$ mV (Figure 1b-2) within 2 min. In 12 human atrial cells, $10 \mu M$ pimobendan increased $I_{Ca(L)}$ by $221.5 \pm 29.5\%$. Current-vol-

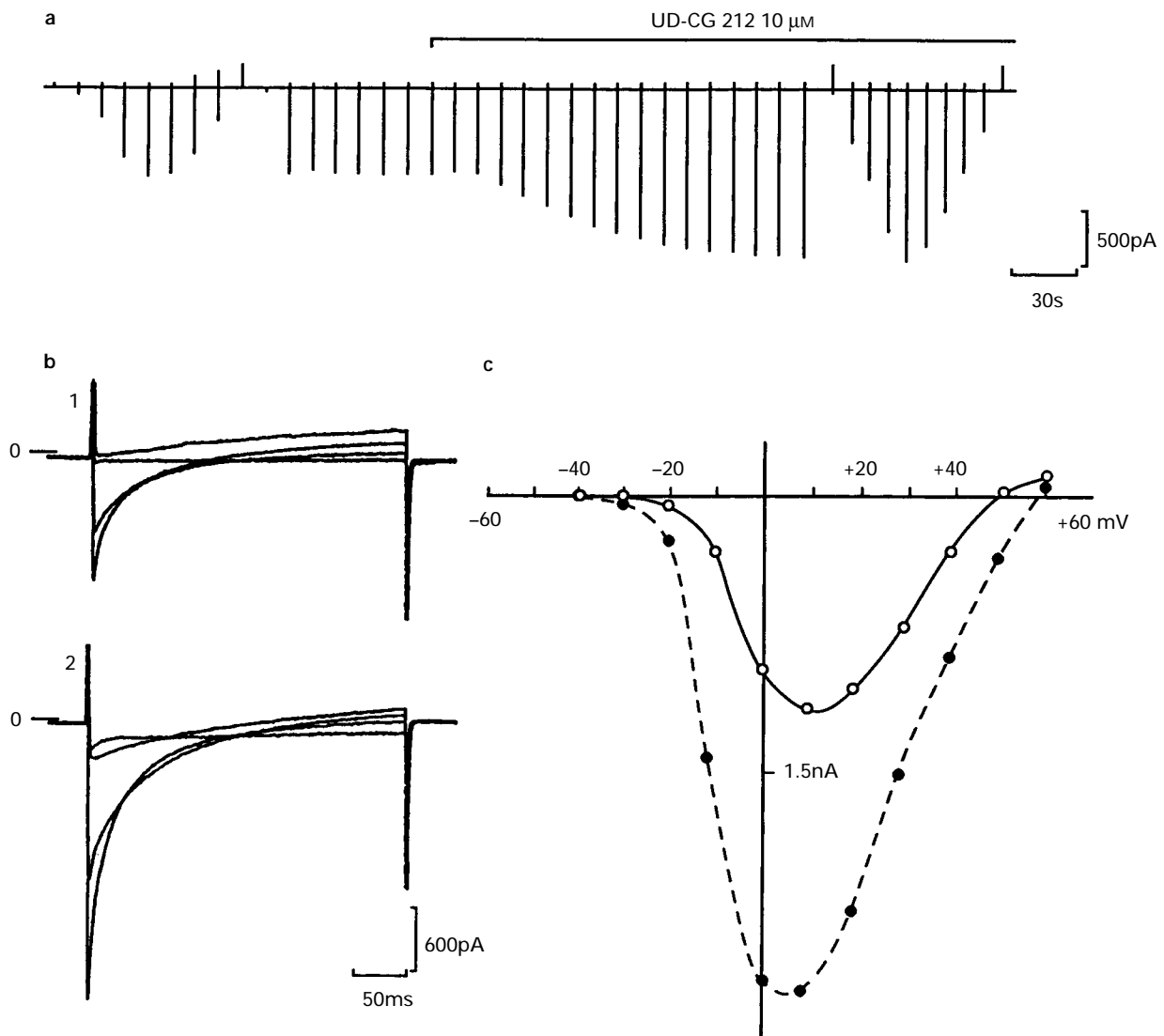


Figure 2 Effect of UD-CG 212 on $I_{Ca(L)}$ in a human atrial cell. (a) Chart recording showing peak $I_{Ca(L)}$ before and after treatment with $10 \mu M$ UD-CG 212. (b) $I_{Ca(L)}$ recorded at -20 , $+10$, $+30$ and $+50$ mV (from a holding potential of -40 mV), in control (b-1) and in the presence of $10 \mu M$ UD-CG 212 (b-2). This dose of UD-CG 212 increased $I_{Ca(L)}$ from 1150 pA (b-1) to 2450 pA (b-2) at $+10$ mV. (c) *I-V* relationships for $I_{Ca(L)}$ before (open circles) and during (solid circles) superfusion with $10 \mu M$ UD-CG 212. The data shown were obtained from a single cell.

tage (I - V) relationships (Figure 1c) showed that $I_{Ca(L)}$ was increased at every membrane potential.

Similar results were obtained on application of UD-CG 212, which is the primary metabolite of pimobendan, to human atrial cells. Figure 2 shows the effect of $10 \mu\text{M}$ UD-CG 212 on $I_{Ca(L)}$ at $+10 \text{ mV}$ (from a holding potential of -40 mV) in a human atrial cell. In this cell, UD-CG 212 increased $I_{Ca(L)}$ from 1150 pA (Figure 2b-1) to 2450 pA (Figure 2b-2). In 10 such cells, $10 \mu\text{M}$ and $100 \mu\text{M}$ UD-CG 212 increased $I_{Ca(L)}$ by $145.2 \pm 38.3\%$ and $174.5 \pm 30.2\%$, respectively.

The dose-response relationship for the increase in $I_{Ca(L)}$ produced by pimobendan is illustrated in Figure 3. On average, 1 , 10 and $100 \mu\text{M}$ pimobendan increased $I_{Ca(L)}$ by $129.3 \pm 20.3\%$ ($n = 11$), $221.5 \pm 29.5\%$ ($n = 12$) and $250.4 \pm 45.0\%$ ($n = 15$), respectively, in human atrial cells. The curve (Figure 3B) was derived by making a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca(L)}$ to the Michaelis equation. The values obtained by this method for the maximal effect

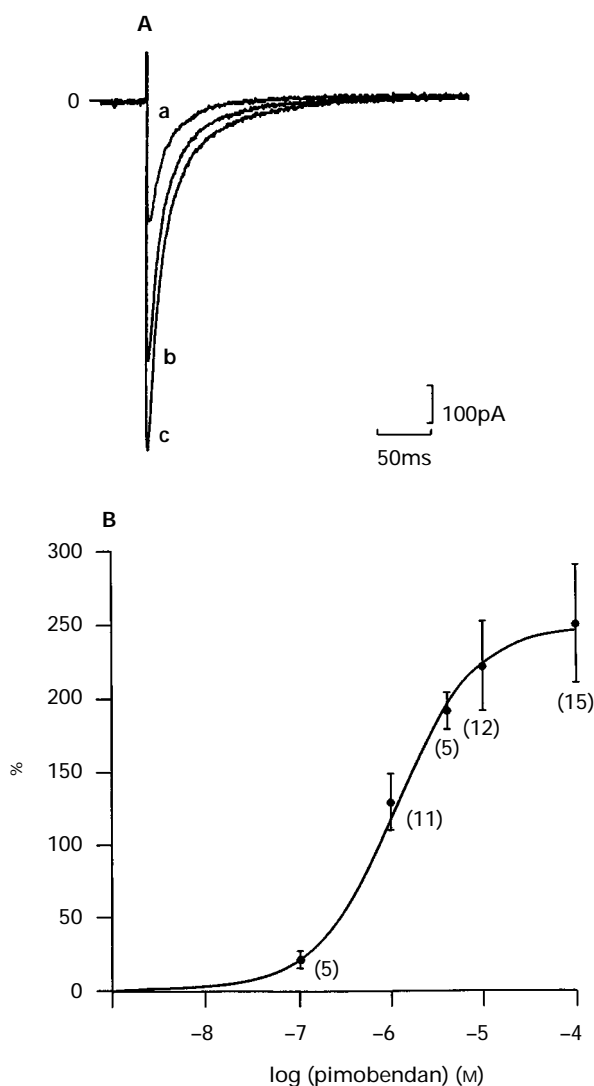


Figure 3 Dose-response relationships for the effects of pimobendan on $I_{Ca(L)}$ in human atrial cells. (A) In this cell, $I_{Ca(L)}$ was increased from 320 pA (a) to 700 pA (b) by $1 \mu\text{M}$ pimobendan and to 950 pA (c) by $10 \mu\text{M}$ pimobendan (in each case, depolarization to $+10 \text{ mV}$ from a holding potential of -40 mV). (B) Dose-response relationship derived by a nonlinear least-squares fit of the values for mean percentage increase in $I_{Ca(L)}$ to the Michaelis equation: the percentage increase in $I_{Ca(L)} = E_{\text{max}} (\text{pimobendan}) / [(\text{pimobendan}) + EC_{50}]$. Ordinate scale represents percentage increase in $I_{Ca(L)}$ over and above the size of the control response. Numbers in parentheses indicate the number of experiments. The values of E_{max} and EC_{50} obtained from this curve were 249.4% and $1.13 \mu\text{M}$, respectively, for pimobendan. Data points show the mean values and vertical lines the s.d.

(E_{max}) of pimobendan on $I_{Ca(L)}$ and for the concentration for half-maximal stimulation (EC_{50}) were 249.4% and $1.13 \mu\text{M}$, respectively, in human myocytes. The corresponding values for UD-CG 212 were 192.0% and $1.78 \mu\text{M}$, respectively. The increases in $I_{Ca(L)}$ induced by pimobendan and UD-CG 212 did not depend on the patient's disease or age.

Effect of a non-specific PDE inhibitor on $I_{Ca(L)}$ in human atrial cells

IBMX is non-selective and inhibits all four PDE isozymes found in the mammalian myocardium (Bethke *et al.*, 1992; Cheffoy *et al.*, 1992). We therefore investigated the effects of IBMX on $I_{Ca(L)}$ in human atrial cells. In 10 human atrial cells, $I_{Ca(L)}$ was increased by $270.3 \pm 71.5\%$ by $100 \mu\text{M}$ IBMX (data not illustrated). This value was significantly higher than that obtained with $100 \mu\text{M}$ UD-CG 212 ($174.5 \pm 30.2\%$, $n = 10$; $P < 0.005$). These results suggest that in human atrial cells a PDE isozyme other than PDE III may also make an important contribution to the regulation of the intracellular cyclic AMP concentration.

Enhanced effect on $I_{Ca(L)}$ produced by a combination of PDE III and PDE IV inhibitors in human atrial cells

Recently, it has been shown that a significant positive inotropic effect can be induced by a combined application of selective PDE III and PDE IV inhibitors in both rabbit and rat ventricular cells (Shahid & Nicholson, 1990). Rolipram is the most potent inhibitor known of PDE IV in guinea-pig (Bethke *et al.*, 1992) and human myocardium (Reeves *et al.*, 1987). However, there are no experimental data concerning the effect of a PDE IV inhibitor on $I_{Ca(L)}$ in single human cardiac myocytes. We therefore evaluated the effects of rolipram alone, and of a combination of rolipram and the PDE III selective inhibitor UD-CG 212 on $I_{Ca(L)}$ in human atrial cells.

Application of 1 – $100 \mu\text{M}$ rolipram alone did not affect the control amplitude of $I_{Ca(L)}$ in human atrial cells ($n = 5$, data not illustrated). However, rolipram did enhance the effect of UD-CG 212 on $I_{Ca(L)}$. In the human atrial cell illustrated in Figure 4, $I_{Ca(L)}$ was significantly increased (by 141% , Figure 4B-b) by $100 \mu\text{M}$ UD-CG 212 (mean increase by $167.9 \pm 33.9\%$, $n = 5$). When $100 \mu\text{M}$ rolipram was also applied, the total increase in $I_{Ca(L)}$ was by 251% (Figure 4B-c) in that particular cell (mean increase by $270.0 \pm 52.2\%$, $n = 5$). The increase in $I_{Ca(L)}$ induced by the combination of UD-CG 212 and rolipram ($100 \mu\text{M}$ of each) was not significantly different from that observed with $100 \mu\text{M}$ of the non-selective inhibitor IBMX ($270.3 \pm 71.5\%$, $n = 10$).

Similar results were obtained on application of pimobendan and rolipram in human atrial cells. In the presence of pimobendan at $1 \mu\text{M}$ (a concentration close to the EC_{50} value for this drug, viz. $1.13 \mu\text{M}$; Figure 3B), application of 0.1 – $100 \mu\text{M}$ rolipram increased $I_{Ca(L)}$ in a dose-dependent manner with an EC_{50} value of $0.3 \mu\text{M}$ (data not illustrated).

Rolipram also increased the isoprenaline-stimulated $I_{Ca(L)}$ in human atrial cells (Figure 4C). In the human atrial cell illustrated in Figure 4C, $I_{Ca(L)}$ was increased by 210% by 5 nM isoprenaline (Figure 4C-b). An application of $10 \mu\text{M}$ rolipram enhanced the effect of isoprenaline by 60% in this particular cell (Figure 4C-c). In 5 such experiments, $10 \mu\text{M}$ rolipram enhanced the effect of 5 nM isoprenaline on $I_{Ca(L)}$ by $52.9 \pm 9.3\%$. These results suggest that, in human atrial cells, inhibition of PDE IV, when the intracellular cyclic AMP concentration is already increased by inhibiting PDE III or by previous administration of a β -adrenoceptor agonist, might have a significant effect on $I_{Ca(L)}$, by further increasing the intracellular cyclic AMP concentration.

Effects of selective PDE III inhibitors on $I_{Ca(L)}$ in rabbit atrial myocytes

To compare the relative contribution of PDE III to the regulation of intracellular cyclic AMP in man with the situation in

other species, we next examined the effects of pimobendan and UD-CG 212 on $I_{Ca(L)}$ in rabbit atrial myocytes.

Figure 5 illustrates the effect of UD-CG 212 on $I_{Ca(L)}$ in a single rabbit atrial cell. UD-CG 212 increased $I_{Ca(L)}$ by 29% in this cell when given at 100 μM (Figure 5B-b). On the other hand, the non-selective PDE inhibitor IBMX (100 μM) increased it by 197% (Figure 5B-c). In 10 rabbit atrial cells, 10 and 100 μM UD-CG 212 increased $I_{Ca(L)}$ by $26.1 \pm 16.4\%$ and $22.1 \pm 9.0\%$, respectively, whereas 100 μM IBMX increased it by $254.3 \pm 61.1\%$. In rabbit atrial cells, $I_{Ca(L)}$ was increased by $34.2 \pm 20.5\%$ and $67.4 \pm 12.0\%$ by 10 and 100 μM pimobendan, respectively ($n=10$, data not illustrated).

Thus, in rabbit atrial cells the non-selective PDE inhibitor, IBMX, induced a marked increase in $I_{Ca(L)}$ and the increase in $I_{Ca(L)}$ was not significantly different from that seen in human atrial cells. However, the increases in $I_{Ca(L)}$ induced by selective PDE III inhibitors were significantly smaller ($P < 0.0001$) than those obtained in cells from the human atrium (Figures 1 and

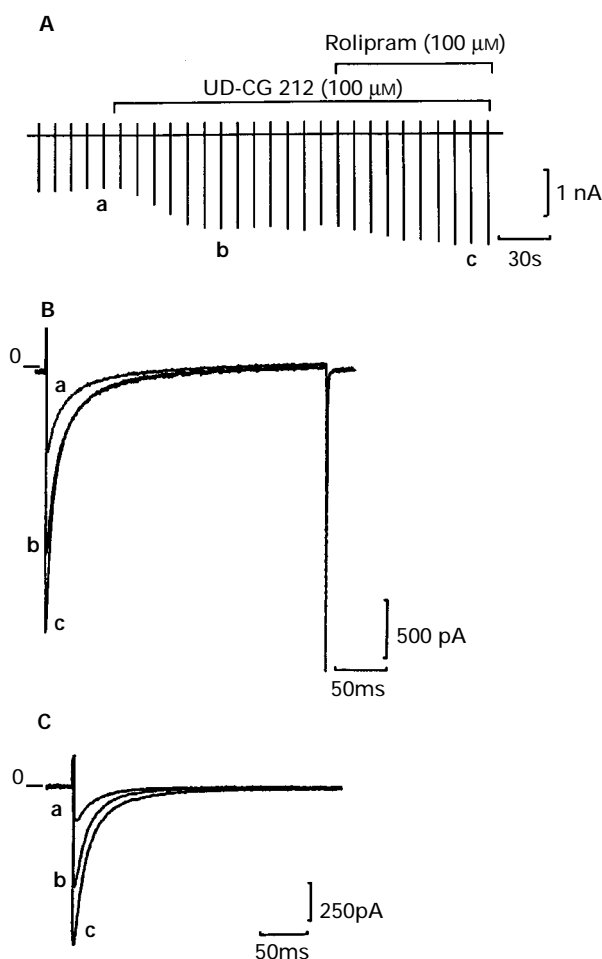


Figure 4 Enhanced effect on $I_{Ca(L)}$ caused by the combined application of inhibitors of PDE III and PDE IV in a human atrial cell. (A) Chart recording of $I_{Ca(L)}$ before (a) and after treatment with 100 μM UD-CG 212 (b) and 100 μM UD-CG 212 plus 100 μM rolipram (c). (B) After administration of 100 μM UD-CG 212, $I_{Ca(L)}$ at +10 mV was increased from 680 pA (a) to 1640 pA (b) in this cell. During the administration of 100 μM UD-CG 212 plus 100 μM rolipram, $I_{Ca(L)}$ was further increased to 2390 pA (c). The amplitude of $I_{Ca(L)}$ in (c) was 45.7% greater than that obtained with 100 μM UD-CG 212 alone (b). (C) Enhancement by rolipram of isoprenaline-stimulated $I_{Ca(L)}$. $I_{Ca(L)}$ recorded at +10 mV (from a holding potential of -40 mV) in control (a), after superfusion with 5 nM isoprenaline (b) and with 5 nM isoprenaline plus 10 μM rolipram (c). Isoprenaline 5 nM increased $I_{Ca(L)}$ from 200 pA (a) to 625 pA (b). Rolipram 10 μM enhanced the 5 nM isoprenaline-stimulated $I_{Ca(L)}$ by 60% in this cell (c). This experiment was carried out in a different cell from that used for (A) and (B).

2). Our results clearly indicate that the effects of PDE III inhibitors in increasing $I_{Ca(L)}$ was significantly greater in human atrial cells than in rabbit atrial cells. Thus, there seems to be a considerable species difference in the distribution of the PDE III isozyme.

Enhanced effect on $I_{Ca(L)}$ produced by a combination of PDE III and PDE IV inhibitors in rabbit atrial cells

We also examined the enhanced effect on $I_{Ca(L)}$ induced by a combination of PDE III and PDE IV inhibitors in rabbit atrial cells. Figure 6 illustrates just such an effect. $I_{Ca(L)}$ was elicited by constant depolarizing pulses from a holding potential of -40 mV to +10 mV. UD-CG 212 100 μM in-

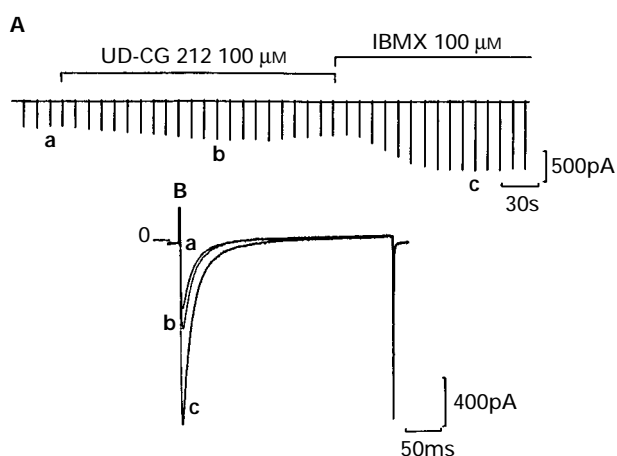


Figure 5 Effect of UD-CG 212 and IBMX on $I_{Ca(L)}$ in a rabbit atrial cell. (A) Chart recording of $I_{Ca(L)}$ before (a) and after addition of 100 μM UD-CG 212 (b) followed by 100 μM IBMX (c). The peak current was elicited every 10 s by a depolarizing voltage step from a holding potential of -40 mV to a test potential of +10 mV for 300 ms. (B) After administration of 100 μM UD-CG 212, the peak $I_{Ca(L)}$ was increased from 525 pA (a) to 675 pA (b) in this cell. After administration of 100 μM IBMX, $I_{Ca(L)}$ was increased to 1560 pA (c).

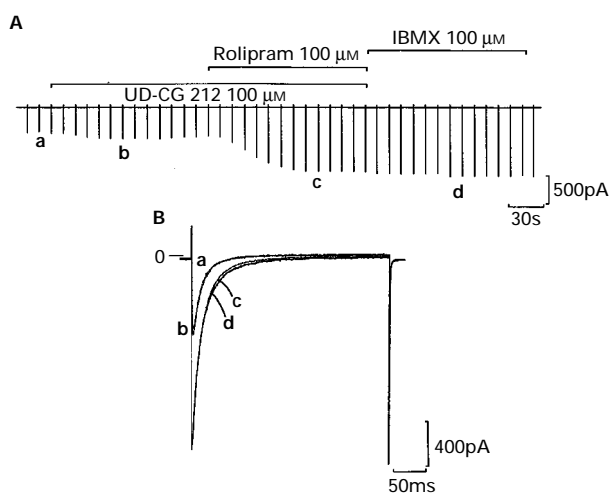


Figure 6 Enhanced effect on $I_{Ca(L)}$ caused by the combined application of inhibitors of PDE III and PDE IV in a rabbit atrial cell. (A) Chart recording of $I_{Ca(L)}$ before (a) and after treatment with 100 μM UD-CG 212 (b), 100 μM UD-CG 212 plus 100 μM rolipram (c) and, sequentially, 100 μM IBMX (d). Basic experimental protocol was as in Figure 5. (B) After administration of 100 μM UD-CG 212, $I_{Ca(L)}$ (at +10 mV from a holding potential of -40 mV) was only slightly increased, from 600 pA (a) to 690 pA (b) in this particular cell. During the administration of 100 μM UD-CG 212 plus 100 μM rolipram, $I_{Ca(L)}$ was further increased, to 1700 pA (c). The subsequent application of 100 μM IBMX (d) had no additional effect on $I_{Ca(L)}$ over and above that seen in the presence of 100 μM UD-CG 212 plus 100 μM rolipram.

creased $I_{Ca(L)}$ by only 15% in the cell illustrated (Figure 6B-b). However, application of 100 μ M rolipram while the UD-CG 212 was still present raised the overall increase to 183% (Figure 6B-c). No further increase was observed on the subsequent application of 100 μ M IBMX (Figure 6B-d). In 7 such experiments, $I_{Ca(L)}$ was increased by $215.7 \pm 65.2\%$ by the combination of 100 μ M UD-CG 212 and 100 μ M rolipram. This value was almost 10 times larger than that obtained in the same cells for the effect of 100 μ M UD-CG 212 alone ($22.8 \pm 3.6\%$, $n=7$, $P < 0.0001$) and it was not significantly different from that observed in the same cells with 100 μ M IBMX ($233.4 \pm 29.0\%$, $n=7$). In rabbit atrial cells, as in human atrial cells, application of 1–100 μ M rolipram alone did not itself affect $I_{Ca(L)}$ ($n=5$). However, 10 μ M rolipram increased the stimulatory effect of 5 nM isoprenaline on $I_{Ca(L)}$ by $47.3 \pm 6.1\%$ ($n=5$) in rabbit atrial cells. These results indicate that the potentiating effect of the PDE IV inhibitor, rolipram, in the rabbit atrium was essentially the same as that observed in the human atrium. However, the potentiating effect was significantly greater in rabbit atrial cells than in human atrial cells.

Comparison of the effects of different PDE inhibitors on $I_{Ca(L)}$ in human and rabbit atrial cells

Figure 7 summarizes the effects of different PDE inhibitors on $I_{Ca(L)}$ in rabbit (a) and human atrial cells (b). In rabbit cells, 100 μ M rolipram was ineffective. The mean increases in $I_{Ca(L)}$ induced separately by 100 μ M UD-CG 212 and pimobendan were $22.1 \pm 9.0\%$ ($n=10$) and $67.4 \pm 12.0\%$ ($n=10$), respectively. This finding suggested that the selective PDE III inhibitors, UD-CG 212 and pimobendan, were not very potent at increasing $I_{Ca(L)}$ in rabbit atrial cells. The increase in $I_{Ca(L)}$ induced by the combination of 100 μ M of each of UD-CG 212 and rolipram ($215.7 \pm 65.2\%$, $n=7$) was about 10 times that obtained with 100 μ M UD-CG 212 alone; this value was not significantly different from that observed with 100 μ M of the non-selective inhibitor IBMX.

In human atrial cells, 100 μ M rolipram was ineffective, just as in rabbit atrial cells. The mean increases in $I_{Ca(L)}$ induced separately by UD-CG 212, pimobendan and IBMX (each at 100 μ M) were $174.5 \pm 30.2\%$ ($n=10$), $250.4 \pm 45.0\%$ ($n=15$) and $270.3 \pm 71.5\%$ ($n=10$), respectively. The increase in $I_{Ca(L)}$ induced by 100 μ M UD-CG 212 was significantly smaller than that obtained with 100 μ M IBMX ($P < 0.005$). However, the effect of 100 μ M pimobendan on $I_{Ca(L)}$ was not significantly different from that seen with 100 μ M IBMX. The lack of a significant difference between the effects of $I_{Ca(L)}$ induced by 100 μ M pimobendan and 100 μ M IBMX may be explained if pimobendan does not have an entirely selective effect on PDE III (see Discussion). The increase in $I_{Ca(L)}$ induced by the combined presence of 100 μ M of each of UD-CG 212 and rolipram ($270.0 \pm 52.2\%$, $n=5$) was only 1.5 times that obtained with 100 μ M UD-CG 212 alone ($P < 0.05$) in human atrial cells. These results clearly indicate that the effect of PDE III inhibitors in increasing $I_{Ca(L)}$ was significantly greater in human than in rabbit atrial cells, whereas the potentiating effect of the PDE IV inhibitor, rolipram, was significantly greater in rabbit atrial cells than in human atrial cells.

Discussion

Contribution of PDE III to the regulation of $I_{Ca(L)}$ in human atrial cells

According to recent investigations on the role of PDE in cardiac tissues from mammalian species, cyclic AMP is hydrolyzed to 5'-AMP by at least four classes of PDE isozymes (PDEs I–IV, Reeves *et al.*, 1987; Weishaar *et al.*, 1987a,b; Beavo & Reifsynder, 1990; Fischmeister & Hartzell, 1991). A Ca^{2+} -calmodulin-dependent PDE (PDE I) hydrolyzes both

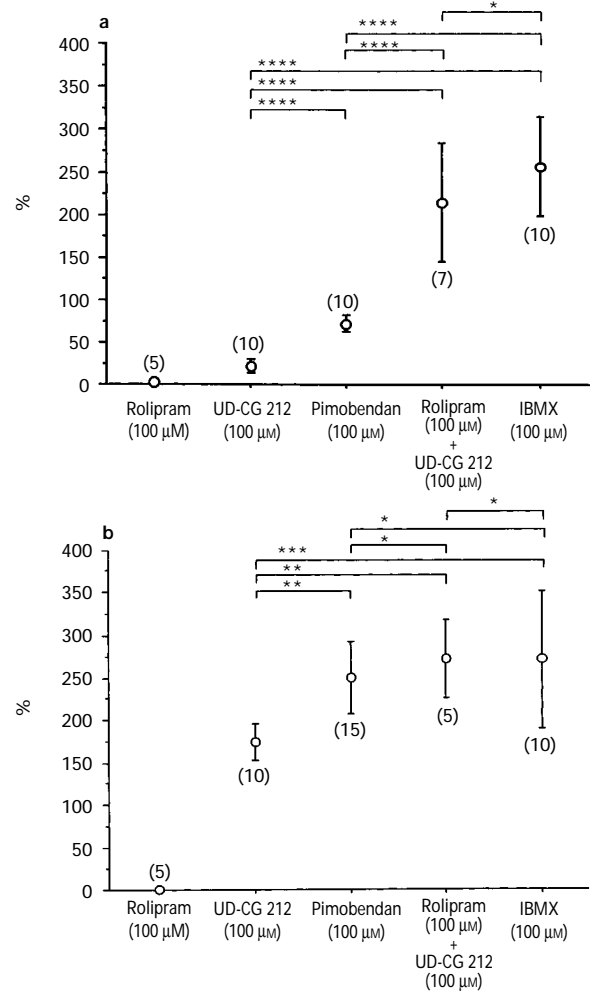


Figure 7 Comparison of the effects of various PDE inhibitors on $I_{Ca(L)}$ in human and rabbit atrial cells. Each panel shows the average percentage increase in $I_{Ca(L)}$ induced by treatment with 100 μ M of each of the following: rolipram, UD-CG 212, pimobendan and with 100 μ M rolipram plus 100 μ M UD-CG 212, and with 100 μ M IBMX. Numbers in parentheses indicate the number of experiments. Data points show the mean values and vertical lines the s.d. (a) In rabbit atrial cells, 100 μ M rolipram had no effect on $I_{Ca(L)}$. At 100 μ M, UD-CG 212 and pimobendan increased $I_{Ca(L)}$ by $22.1 \pm 9.0\%$ and $67.4 \pm 12.0\%$, respectively. Rolipram plus UD-CG 212 (each at 100 μ M) increased $I_{Ca(L)}$ by $215.7 \pm 65.2\%$, an increase which was not significantly different from that obtained with 100 μ M IBMX ($233.4 \pm 29.0\%$). (b) In human atrial cells, 100 μ M rolipram had no effect on $I_{Ca(L)}$. At 100 μ M, UD-CG 212 and pimobendan increased $I_{Ca(L)}$ by $174.5 \pm 30.2\%$ and $250.4 \pm 45.0\%$, respectively. Rolipram plus UD-CG 212 (each at 100 μ M) increased $I_{Ca(L)}$ by $270.0 \pm 52.2\%$, an increase which was not significantly different from that obtained with 100 μ M IBMX ($270.3 \pm 71.5\%$). * Not significant, ** $P < 0.05$, *** $P < 0.005$, **** $P < 0.0001$.

cyclic AMP and cyclic GMP. A cyclic GMP-stimulated PDE (PDE II) hydrolyzes both cyclic AMP and cyclic GMP with a low affinity. A cyclic GMP-inhibited PDE (PDE III) hydrolyzes cyclic AMP with a high affinity. A cyclic AMP-specific PDE (PDE IV), which hydrolyzes only cyclic AMP with a high affinity, is also referred to as 'cyclic GMP-insensitive PDE'. There is no experimental evidence of PDEs I and II mediating an increase in the force of contraction in mammalian cardiac tissues (DiBanco *et al.*, 1989; Beavo & Reifsynder, 1990; Ouadid *et al.*, 1991; Kubo *et al.*, 1992; Muller *et al.*, 1992). In contrast, inhibition of the low K_m , cyclic AMP-specific cardiac isozymes (PDE III and PDE IV) is considered to contribute significantly to positive inotropic effects (Beavo & Reifsynder, 1990; Shahid & Nicholson, 1990; von der Leyen *et al.*, 1991; Muller *et al.*, 1992). We therefore chose to investigate the ef-

fects of PDE III and IV inhibitors on $I_{Ca(L)}$ in human atrial cells in the present study.

Two points should be made about species differences. First, that the effect of selective PDE III inhibitors on $I_{Ca(L)}$ in human atrial cells was much greater than in rabbit atrial cells in our study, and much greater than that obtained in guinea-pig ventricular cells (Westfall *et al.*, 1992). In that study, $I_{Ca(L)}$ was increased by $21 \pm 5\%$ by $100 \mu\text{M}$ UD-CG 212, an effect similar in magnitude to that obtained in our rabbit atrial cells ($22.1 \pm 9.0\%$, $n = 10$; Figure 5). For this reason, we suggest that the distribution of PDE III might differ between man and other species. Second, while PDE III seems to be the dominant isozyme regulating intracellular cyclic AMP in the heart of many species (Rapundalo *et al.*, 1989; Beavo & Reifsynder, 1990; Böhm *et al.*, 1991), the positive inotropic efficacy of PDE III inhibitors appears to depend on the intracellular location of the enzyme (Masuoka *et al.*, 1990). Thus, species like dog and monkey, in which PDE III is associated with the membranous fraction of the ventricular homogenate, are far more sensitive to the positive inotropic action of PDE III inhibitors *in vivo* than are the rat and guinea-pig, whose PDE III activity is predominantly cytosolic in location (Weishaar *et al.*, 1987a,b). Consequently, Weishaar *et al.* (1987a) postulated that the membrane-bound form of PDE III exerts a greater effect on ventricular contractility than the cytosolic form. Taking these conclusions together with the relatively powerful effect of PDE III inhibitors on $I_{Ca(L)}$ in human cells, we think it likely that, in human atrial cells, membrane-bound PDE III, rather than cytosolic PDE III, contributes significantly to the regulation of $I_{Ca(L)}$ (by modulating cyclic AMP concentration).

Contribution of PDE IV to the regulation of $I_{Ca(L)}$ in human atrial cells

In rat and rabbit ventricular myocardium, PDE III, together with the cyclic GMP-insensitive PDE IV, is known to represent the main regulator of intracellular cyclic AMP (Shahid & Nicholson, 1990; Akita *et al.*, 1994). However, the contribution of PDE IV to the regulation of $I_{Ca(L)}$ in the human myocardium has not been properly evaluated. According to biochemical measurements (Shahid & Nicholson, 1990; Bethke *et al.*, 1991; 1992; Cheffoy *et al.*, 1992), the selectivity ratio (IC_{50} for PDE IV/ IC_{50} for PDE III) for PDE inhibitors is nearly 1 for IBMX, 172 for pimobendan, 400–800 for UD-CG 212, but less than 0.03 for rolipram in rabbit, guinea-pig and human myocardium (Shahid & Nicholson, 1990; Bethke *et al.*, 1991; 1992; Cheffoy *et al.*, 1992; Westfall *et al.*, 1992).

In our human atrial cells, the PDE IV inhibitor, rolipram, despite inducing no change in $I_{Ca(L)}$ itself, potentiated the effect of the PDE III inhibitor, UD-CG 212, to such an extent that their combined effect was as great as that of the non-selective inhibitor IBMX (Figure 4B). Rolipram also enhanced the stimulating effect of isoprenaline on $I_{Ca(L)}$ in human atrial cells (Figure 4C). These data suggest that inhibition of PDE IV can exert a considerable influence over the regulation of cyclic AMP concentration in human atrial cells, but only when associated with inhibition of PDE III or after the prior administration of a β -adrenoceptor agonist.

Results consistent with this idea have been obtained by others, in non-human species. Thus, Shahid & Nicholson (1990) found that the PDE IV selective inhibitor, rolipram, was inactive in both rabbit and rat ventricles, whereas the non-selective PDE inhibitor, IBMX, and the dual PDE III/IV inhibitor, Org 30029, both increased cardiac contractile force in these two species. Similar results have been obtained in the guinea-pig atrium, i.e. rolipram was able to exert a positive inotropic effect in the presence of either the cyclic AMP-dependent positive inotropic agent, forskolin, or a PDE III inhibitor (Muller *et al.*, 1990). In the present study, we obtained a similarly enhanced effect on $I_{Ca(L)}$ in rabbit atrial cells by using a combination of rolipram and UD-CG 212 (Figure 6) or rolipram and isoprenaline. However, the enhancement was much greater in rabbit than in human atrium.

In the present experiment, the effect of $100 \mu\text{M}$ IBMX on $I_{Ca(L)}$ was significantly larger than that obtained with $100 \mu\text{M}$ UD-CG 212 ($P < 0.005$). However, the effect of $100 \mu\text{M}$ pimobendan was not significantly different from that seen with $100 \mu\text{M}$ IBMX in human atrial cells (Figure 7b). It has been shown that the selectivity ratio (IC_{50} for PDE IV/ IC_{50} for PDE III) is 400–800 for UD-CG 212 and 172 for pimobendan (Cheffoy *et al.*, 1992) and that the IC_{50} is $151 \mu\text{M}$ for the effect of pimobendan on PDE IV. These observations suggest that higher concentrations of pimobendan, e.g. $100 \mu\text{M}$, may inhibit PDE IV to a small extent, as well as inhibiting PDE III. Therefore, we suggest that this may be why $100 \mu\text{M}$ pimobendan increased $I_{Ca(L)}$ to an extent similar to that seen with the non-selective inhibitor IBMX in the present study.

Reeves *et al.* (1987) and Muller *et al.* (1992) showed that, for cyclic AMP, PDE III has a K_m value of $0.14\text{--}0.33 \mu\text{M}$ whereas PDE IV has a K_m value of $2\text{--}3.3 \mu\text{M}$ (in human and guinea-pig ventricle). These observations indicate that PDE III and PDE IV differ by a factor of about 10 in their apparent K_m values for cyclic AMP. On this basis, Muller *et al.* (1992) hypothesized that PDE IV might play a significant role in regulating both the cyclic AMP level and cardiac contraction only when the intracellular cyclic AMP concentration reached a level high enough to be compatible with the K_m value of this enzyme, whereas PDE III might play a functional role when cyclic AMP concentrations were lower. Possibly, similar mechanisms might underlie the regulation of $I_{Ca(L)}$ by the combination of a PDE III inhibitor with a PDE IV inhibitor or of isoprenaline with a PDE IV inhibitor in the present experiments. Thus, taking these results together with ours, it seems likely that PDE IV would make a significant contribution to the regulation of intracellular cyclic AMP in the human myocardium only when PDE III is already inhibited, or when the myocardium is under β -adrenoceptor-mediated stimulation.

Functional significance

In general, the cardiotoxic effect of PDE III inhibitors ultimately results from either an increase in the concentration of intracellular free Ca^{2+} that is available to interact with the contractile proteins, an increased sensitivity of the myofilaments to Ca^{2+} , or both (Sholtz & Meyer, 1987). Böhm *et al.* (1991) suggested that, although both PDE III inhibition and the sensitization of troponin-C to Ca^{2+} are related to the positive inotropic effects of pimobendan or UD-CG 212, the combination of these actions could have functional advantages over mechanisms involving only one of them. Furthermore, an increase in the binding of Ca^{2+} to troponin-C depends on an increase in the intracellular concentration of free Ca^{2+} and is achieved either via a cyclic AMP-dependent increase in the Ca^{2+} current or by cyclic AMP-dependent phospholamban phosphorylation (Lee *et al.*, 1989; Böhm *et al.*, 1991). We used atrial specimens in the present experiments. However, the effects of a given PDE III inhibitor on $I_{Ca(L)}$ are almost identical in human ventricular and atrial tissues (Li *et al.*, 1994). Furthermore, the properties of the PDE isozymes and the inhibitory effects of the PDE inhibitors on these isozymes do not differ between failing and non-failing human myocardial tissues (von der Leyen *et al.*, 1991). Therefore, it seems likely that the positive inotropic effect of PDE III inhibitors depends on an increase in $I_{Ca(L)}$ modulated by cyclic AMP-dependent phosphorylation. Moreover, it is noteworthy that an inhibition of PDE IV, if associated with the application of catecholamines or inhibition of PDE III, could increase $I_{Ca(L)}$ in human atrial cells. Thus, both PDE III and PDE IV isozymes might contribute to the regulation of $I_{Ca(L)}$ in the human myocardium.

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