Characterisation of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca^{2+} current in rat ventricular myocytes

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Running title: *PDE subtypes and Ca current in rat heart*

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ABSTRACT

1 The effects of several phosphodiesterase (PDE) inhibitors on the L-type Ca current (I_{Ca}) and intracellular cAMP concentration ($[cAMP]_i$) were examined in isolated rat ventricular myocytes. The presence of mRNA transcripts encoding for the different cardiac PDE subtypes was confirmed by RT-PCR.

2 IBMX (100 μM), a broad-spectrum PDE inhibitor, increased basal I_{Ca} by 120% and $[cAMP]_i$ by 70%, similarly to a saturating concentration of the β-adrenoceptor agonist isoprenaline (1 μM). However, MIMX (1 μM), a PDE1 inhibitor, EHNA (10 μM), a PDE2 inhibitor, cilostamide (0.1 μM), a PDE3 inhibitor, or Ro 20-1724 (0.1 μM), a PDE4 inhibitor, had no effect on basal I_{Ca} and little stimulatory effects on $[cAMP]_i$ (20-30%).

3 Each selective PDE inhibitor was then tested in the presence of another inhibitor to examine whether a concomitant inhibition of two PDE subtypes had any effect on I_{Ca} or $[cAMP]_i$. While all combinations tested significantly increased $[cAMP]_i$ (40-50%), only cilostamide (0.1 μM) + Ro 20-1724 (0.1 μM) produced a significant stimulation of I_{Ca} (50%). Addition of EHNA (10 μM) to this mix increased I_{Ca} to 110% and $[cAMP]_i$ to 70% above basal, i.e. to similar levels as obtained with IBMX (100 μM) or isoprenaline (1 μM).

4 When tested on top of a sub-maximal concentration of isoprenaline (1 nM), which increased I_{Ca} by ≈40% and had negligible effect on $[cAMP]_i$, each selective PDE inhibitor induced a clear stimulation of $[cAMP]_i$ and an additional increase in I_{Ca}. Maximal effects on I_{Ca} were ≈8% for MIMX (3 μM), ≈20% for EHNA (1-3 μM), ≈30% for cilostamide (0.3-1 μM) and ≈50% for Ro 20-1724 (0.1 μM).

5 Our results demonstrate that PDE1-4 subtypes regulate I_{Ca} in rat ventricular myocytes. While PDE3 and PDE4 are the dominant PDE subtypes involved in the regulation of basal I_{Ca}, all four PDE subtypes determine the response of I_{Ca} to a stimulus activating cAMP production, with the rank order of potency PDE4>PDE3>PDE2>PDE1.

Keywords: rat heart; L-type Ca^{2+} current; intracellular cAMP; phosphodiesterase subtypes; phosphodiesterase inhibitors; MIMX (PDE1 inhibitor); EHNA (PDE2 inhibitor); cilostamide (PDE3 inhibitor); Ro 20-1724 (PDE4 inhibitor); β-adrenoceptor agonist.

Abbreviations:

EHNA, *Erythro*-9-[2-Hydroxy-3-nonyl]adenine

IBMX, 3-isobutyl-1-metylxanthine

I_{Ca}, L-type calcium current

MIMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine

PDE, phosphodiesterase

PDE1, Ca^{2+}/calmodulin-activated PDE

PDE2, cGMP-stimulated PDE

PDE3, cGMP-inhibited PDE

PDE4, low K_m cGMP-independent PDE

PKA, cAMP-dependent protein kinase

Introduction

Phosphorylation of cardiac L-type Ca^{2+} channels by cAMP-dependent protein kinase (PKA) plays a determinant role in the hormonal regulation of myocardial contraction. PKA increases the mean open probability of individual Ca^{2+} channels which results in an increase in the macroscopic L-type calcium current (I_{Ca}) (McDonald *et al.*, 1994). Activation of PKA usually results from an increased production of cAMP by activation of membrane receptors positively coupled to adenylyl cyclase via stimulatory G proteins (G_s). The best documented of such a regulation is the positive inotropic effect of sympathomimetic amines, such as isoprenaline (Hartzell *et al.*, 1991; Hove-Madsen *et al.*, 1996). However, cardiac myocytes, as most other cell types, also possess a negative feedback mechanism to adenylyl cyclase activation which is constituted of the cyclic nucleotide phosphodiesterases (PDEs), a family of enzymes that break down cAMP into 5'-AMP (Beavo, 1995). Cyclic nucleotide PDE activity, at any given location within the cell, will counterbalance the synthesis of cAMP and determine the extent of PKA activation and, hence, of protein phosphorylation. In particular, at the sarcolemmal membrane, this balance between adenylyl cyclase and PDE activities will control the degree of I_{Ca} stimulation upon hormonal activation (Fischmeister & Hartzell, 1991; Hove-

Madsen *et al.*, 1996). Other factors are involved, such as cAMP compartmentation (Jurevicius & Fischmeister, 1996), PKA tethering to the membrane (Gao *et al.*, 1997), or phosphatase activity (Wiechen *et al.*, 1995).

Cyclic nucleotide PDEs exist in multiple molecular forms (Stoclet *et al.*, 1995, Loughney & Ferguson, 1996) and at least four different subtypes have been shown to coexist in the heart muscle (Shahid & Nicholson, 1990; Bode *et al.*, 1991; Dubois *et al.*, 1993; Taira et al., 1993, Engels et al., 1994; Kostic et al. 1997): 1) a Ca^{2+}/calmodulin-activated PDE (PDE1) which hydrolyses cAMP and cGMP; 2) a cGMP-stimulated PDE (PDE2) which hydrolyses cAMP and cGMP; 3) a cGMP-inhibited PDE (PDE3) which hydrolyses cAMP with high affinity (low K_m); 4) and PDE4, a cGMP-independent PDE which hydrolyses selectively cAMP with high affinity. All these PDEs can be inhibited by xanthine derivatives, such as 3-isobutyl-1-metylxanthine (IBMX) or caffeine, which have been frequently used to evaluate the role of PDEs in the metabolism of cAMP in cardiac preparations (see e.g. Katano & Endoh, 1993).

The use of selective or non selective PDE inhibitors has provided some information about the functional importance of PDEs in I_{Ca} regulation (Fischmeister &

Hartzell, 1991). IBMX stimulates basal I_{Ca} in rat (Méry *et al.*, 1991) and guinea pig ventricular myocytes (Ono & Trautwein, 1991; Levi *et al.*, 1989; Shiriyama & Pappano, 1996) and enhances the stimulatory effect of cAMP in frog ventricular myocytes (Fischmeister & Hartzell, 1990; Méry *et al.*, 1995). *Erythro*-9-[2-Hydroxy-3-nonyl]adenine (EHNA), a selective PDE2 inhibitor (Méry *et al.*, 1995; Podzuweit *et al.*, 1995), stimulates basal I_{Ca} in human atrial cells (Rivet-Bastide *et al.*, 1997), and antagonizes the inhibitory effect of intracellular cGMP or NO-donors on isoprenaline- or cAMP-stimulated I_{Ca} in frog ventricular myocytes (Méry *et al.*, 1995). Inhibition of PDE3 with milrinone results in an increase in basal I_{Ca} in human atrial myocytes (Kirstein *et al.*, 1995). Similarly, application of intracellular cGMP in guinea pig ventricular (Shiriyama & Pappano, 1996; Ono & Trautwein, 1991) or human atrial myocytes (Rivet-Bastide *et al.*, 1997) or activation of guanylyl cyclase by NO donors in human atrial myocytes (Kirstein *et al.*, 1995) leads to an increase in I_{Ca} mediated by cGMP inhibition of PDE3. Ro 20-1724 or rolipram, two selective PDE4 inhibitors, enhance the response of I_{Ca} to cAMP, isoprenaline or forskolin in frog cardiomyocytes (Fischmeister & Hartzell, 1990; Méry *et al.*, 1995).

Despite these previous studies, to our knowledge the respective contribution of any given PDE subtype to the regulation of I_{Ca} or to the control of intracellular cAMP

concentration has not been examined at once in the same mammalian cardiac preparation. Thus, the aim of the present study was to get some insight into the respective roles of the four different PDE subtypes in the regulation of cardiac I_{Ca} and intracellular cAMP levels in freshly isolated rat ventricular myocytes. After verifying the expression of PDE1-4 subtypes in rat heart by RT-PCR, we have examined how selective inhibition of one PDE subtype or simultaneous inhibition of two PDE subtypes modulate basal or isoprenaline-stimulated I_{Ca} and intracellular cAMP. I_{Ca} was recorded using the whole-cell patch-clamp technique and intracellular cAMP was measured using radioimmunoassay. Selective PDE inhibition was achieved using 8-methoxymethyl-3-isobutyl-1-methylxanthine (MIMX) for PDE1, EHNA for PDE2, cilostamide for PDE3, and Ro 20-1724 for PDE4 (for reviews, see Beavo, 1995; Stoclet *et al.*, 1995).

A preliminary account of this work was presented at the XVIIIth European Section Meeting of the International Society for Heart Research, Versailles, France, July 1997 (Verde *et al.*, 1997).

Methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* n°L358, 18 December 1986) and the French decree n°87/748 of October 19, 1987 (*J Off République Française*, 20 October 1987, pp. 12245-12248). Authorisations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (n°04226, April 12, 1991).

Preparation of rat ventricular myocytes

Rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (200-250 g) as described (Méry *et al.*, 1991), with slight modifications. Briefly, the rats were subjected to anesthesia by intraperitoneal injection of penthotal and hearts were excised rapidly. The ionic composition of the Ca^{2+}-free Ringer solution was as follows: 117 mM NaCl, 57 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 $MgCl_2$, 11.7 mM D-glucose, 10 mM sodium phosphocreatine, 20 mM taurine, and 21 mM HEPES, adjusted to pH 7.1 with NaOH at room temperature. For enzymatic dissociation, 1 mg/ml collagenase A (Boehringer Mannhein, Mannhein, Germany) and 300 µM EGTA were

added to the Ca^{2+}-free Ringer solution, so that the free Ca^{2+} concentration was adjusted to 20 μM. The hearts were perfused retrogradedly at a constant flow of 6 ml/min and at 37ºC by Ca^{2+}-free solution during 5 min followed by 1 h of perfusion at 4 ml/min with the same solution containing collagenase. The ventricles were then separated from atria, chopped finely and agitated gently to dissociate individual cells. The resulting cell suspension was filtered on a gauze and the cells were allowed to settle down. The supernatant was discarded and cells resuspended four more times in Ca^{2+}-free solution containing a progressively increasing calcium concentration. The cells were maintained at 37ºC until use.

Measurement of rat PDE mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from rat isolated ventricular myocytes and rat ventricular tissue using the Trizol RNA purification system (Gibco BRL, Cergy-Pontoise, France). All RNAs were checked in 1% formaldehyde agarose gel. cDNA was prepared from mRNA with random hexanucleotide primers (20 pmol/μg RNA) using MMLV reverse transcriptase

and the conditions recommended by the manufacturer (Clontech, Palo Alto, CA, USA). Fifty ng of cDNA were amplified in 50 µl PCR reaction mixture (200 µM dNTPs final concentrations) containing 2.5 U of Taq polymerase in the buffer supplied by the manufacturer (Bioprobe Sytems, Montreuil, France), and 1 µM primers. Oligonucleotide primers designed against the C-terminal region of each PDE sub-type were as follows:

PDE1C (Genbank accession number L41045): Forward primer 5′-TTTTCTCCTCTGTGTGACCG-3′, reverse primer 5′-GTGTTCCGTTGACTTGACCT-3′, fragment size 505 bp;

PDE2A2 (Genbank accession number U21101): Forward primer 5′-GAAGGACTATCAGCGAATGC-3′, , reverse primer 5′-GGATGGTGAACTTGTGGGAC-3′, fragment size 461 bp;

PDE3B1 (Genbank accession number Z22867): Forward primer 5′-CACCCAGGAAGAACAAATGC-3′, reverse primer 5′-AAGCCAGCAGCATCATAGGA-3′, fragment size 551 bp;

PDE4A5 (Genbank accession number L27057): Forward primer 5′-TACAGTGGTGGAAGTGGCAG-3′, reverse primer 5′-GAGCAGAGATGATGGCAGAA-3′, fragment size 278 bp;

PDE4 B2 (Genbank accession number L27058): Forward primer 5′-GTTCTCCTCTCTACGCCAGCA-3′, reverse primer 5′-ACTTGGTAGGGTTGCTCAGGTC-3′, fragment size 454 bp;

PDE4 D3 (Genbank accession number U09457): Forward primer 5'-GCGTCCTCCTCCTTGATAACTATT-3', reverse primer 5'-CTGACTCGCCATCTTCCTCTAA-3', fragment size 447 bp.

The PCR products were separated on 1.7% agarose gel containing 0.01% ethidium bromide and photographed under U.V. irradiation at 320 nm. To assess relative quantities of cDNA from each sample, a second PCR amplification was conducted with primers directed to the rat housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously described (Grimaldi *et al.*, 1998). All PCR procedures were performed as follows: 40 cycles and 35 cycles for isolated myocytes and ventricular tissue respectively (45 sec at 94°C, 50 sec at 50°C and 1 min at 72°C) and a final elongation (7 min at 72°C).

Cyclic AMP radioimmunoassay

Freshly isolated ventricular myocytes were prepared as described above from hearts of adult male Wistar rats weighting 200-250 g. Rod-shaped myocytes were counted using a Mallassez cell and resuspended in an adequate volume of the Ca^{2+}-free Ringer solution supplemented with 1 mM Ca^{2+}, in order to obtain a density of 10^5 cell/ml. Each

incubation tube contained 500 µl of this cell suspension to which was added successively 5 µl of a 200x stock solution of the appropriate drug and 500 µl of Ringer solution. Each condition including control (i.e. in absence of any drug) was tested in triplicate. Incubation was carried out at room temperature (20-24°C) and lasted 15 min, allowing the cells to settle down. After this period, the supernatant was discarded and replaced by 500 µl of cold (4°C) ethanol to stop the reaction. Ethanol was evaporated (1 hour at 37°C and low pressure) and the pellet was homogenised in 200 µl of buffer for cAMP assay (cAMP radioimmunoassay kit, Immunotech, Marseille, France). After centrifugation at 4500 rpm for 5 min, the supernatants were diluted 10 times in the same buffer and cyclic AMP was assayed according to the instructions provided by the manufacturer.

Electrophysiological experiments

The whole cell configuration of the patch-clamp technique was used to record the high-threshold L-type calcium current (I_{Ca}) on Ca^{2+}-tolerant rat ventricular myocytes. The cell was routinely depolarized every 8 s from -50 to 0 mV for 400 ms. The use of a holding potential of -50 mV allowed the elimination of fast sodium currents. Potassium currents

were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. For the determination of current-voltage relationships for I_{Ca} (see Fig. 4A) and I_{Ca} inactivation curve (see Fig. 4B), a double pulse voltage clamp protocol was used (Kirstein *et al.*, 1995). Briefly, every 4 s, the membrane potential of the cell, which was normally maintained at its holding value of -50 mV, experienced the following sequence of events: -50 mV for 10 ms, different potentials values ranging from -100 to +100 mV for 200 ms, -50 mV for 3 ms, and 0 mV for 200 ms (see inset in Fig. 4B). Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage-clamped using a patch-clamp amplifier (model RK-400; Bio-Logic, Claix, France). Currents were analog-filtered at 3 KHz and digitally sampled at a frequency of 10 kHz using a 12-bit analog-to-digital converter (DT2827; Data translation, Marlboro, MA, USA) connected to a PC compatible (386/33 Systempro; Compaq Computer Corp., Houston, TX, USA). All experiments were done at room temperature (21-25ºC).

Solutions and Drugs

Control external Cs^+ Ringer solution contained: 107.1 mM NaCl, 20 mM CsCl, 4 mM $NaHCO_3$, 0.8 mM NaH_2PO_4, 1.8 $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of a 250 µm inner diameter capillary tubing from which the external solution was flowing at a rate of 10 µl/min (Méry *et al.*, 1991). Patch electrodes (0.5-1 mΩ) were filled with control internal solution containing: 119.8 mM CsCl, 5 mM EGTA, 4 mM $MgCl_2$, 5 mM sodium phosphocreatine, 3.1 mM Na_2ATP, 0.42 mM Na_2GTP, 0.062 mM $CaCl_2$ (pCa 8.5) and 10 mM HEPES, adjusted to pH 7.3 with CsOH.

Isoprenaline, IBMX, MIMX and EHNA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cilostamide was purchased from Calbiochem (Meudon, France), and Ro 20-1724 was gently provided by Hoffman-La-Roche (Switzerland).

Data Analysis

The maximal amplitude of I_{Ca} was measured as the difference between the peak inward current and the end-pulse current (I_{200} or I_{400}), which was the current amplitude at

the end of the 200 or 400 ms duration pulse (Kirstein *et al.*, 1995). Currents were not compensated for capacitive and leak currents. Cell membrane capacitance and series resistance were measured by exponential analysis of current responses to 1 mV step changes in membrane potential. Membrane capacitance was 130.2 ± 5.0 pF and series resistance 4.0 ± 0.2 MΩ (n=137 different cells). The on-line analysis was made possible by programming a PC compatible computer in Pascal language to determine for each depolarization, peak, and steady state current value. The results are expressed as mean ± S.E.M. In each experimental condition the effects of the drugs tested on I_{Ca} or cAMP levels are expressed as percent change with respect to the values of these parameters under basal conditions, i.e., in the absence of any hormonal stimulation. The variations in I_{Ca} or cAMP induced by the PDE inhibitors were tested for statistical significance by Student's *t* test.

Results

Expression of PDE (1-4) in rat heart

Semi-quantitative RT-PCR analysis with primers specific for rat PDE genes was performed on rat ventricular tissue as well as on isolated rat ventricular myocytes in order to check for the expression of PDE1C, PDE2A, PDE3B and PDE4 (A, B and D) subtypes (Fig.1). Earlier studies have provided direct or indirect evidence for the presence of these PDE gene products in rat cardiac tissues (Bode *et al.*, 1991; Taira *et al.*, 1993; Engels *et al.*, 1994; Kostic *et al.*, 1997). Fig. 1 shows that rat ventricles (Fig. 1A) as well as isolated rat ventricular myocytes (Fig. 1B) express significant amounts of transcripts for each PDE subtype, suggesting the presence of these enzymes in this tissue. In addition, we found that PDE2 and PDE4 transcripts were more abundant than those coding for PDE1 and PDE3.

(Figure 1 near here)

Effect of PDE inhibitors on basal I_{Ca}

I_{Ca} was measured in isolated rat ventricular myocytes using the whole-cell patch-clamp technique (Hamill *et al.*, 1981). The effects of PDE inhibitors were first examined on basal I_{Ca}, i.e. in the absence of a stimulated cAMP production. Basal I_{Ca} amplitude was on average 827.3 ± 36.8 pA at 0 mV membrane potential, and mean I_{Ca} density, which represents the ratio of I_{Ca} amplitude to membrane capacitance, was 6.9 ± 0.3 pA/pF (n=137). Fig. 2 shows a typical experiment in which the effect of an extracellular application of Ro 20-1724 (0.3 μM), a PDE4 inhibitor, was compared with the effect of 100 μM IBMX, a broad-spectrum PDE inhibitor. As shown, application of Ro 20-1724 produced no effect on basal I_{Ca}. Table 1 shows that similar results were obtained with MIMX (1 μM), a PDE1 inhibitor, EHNA (10 μM), a PDE2 inhibitor, and cilostamide (0.1 μM), a PDE3 inhibitor, when the compounds were applied under basal conditions.

(Figure 2 near here)

The lack of effect of selective inhibitors of PDE1-4 on I_{Ca} may indicate that none of the four PDEs is active under basal conditions. Alternatively, it may indicate that inhibition of a single subtype of PDE does not lead to a sufficient increase in cAMP to stimulate basal I_{Ca}. To discriminate between these two hypotheses, we examined the effect of IBMX, a broad-spectrum PDE inhibitor, on basal I_{Ca}. The individual experiment of Fig. 2 and the summary data of Table 1 show that application of 100 μM IBMX, a concentration which inhibits all PDE subtypes, produced a >2-fold and reversible stimulation of I_{Ca}. This result indicates that, while inhibition of any single PDE subtype is insufficient to enhance basal I_{Ca}, complete inhibition of all PDEs produces a sufficient accumulation of cAMP to activate basal I_{Ca}.

(Table 1 near here)

We next wanted to examine which PDE subtype had a dominant activity under basal condition. To do this, we tested different combinations of selective PDE inhibitors for their effect on basal I_{Ca}. In the experiment shown in Fig. 3, the myocyte was initially exposed to 0.1 μM cilostamide which had no effect on basal I_{Ca} (see also Table 1).

However, when Ro 20-1724 (0.1 μM) was applied to the cell in combination with cilostamide, I_{Ca} increased by ≈40%. Addition of EHNA (10 μM) on top of the two other PDE inhibitors induced a further increase in I_{Ca}. All the stimulatory effects were completely reversible upon washout of the drugs. Fig. 4 shows the current-voltage (Fig. 4A) and the inactivation relationships (Fig. 4B) of I_{Ca} obtained in the experiment of Fig. 3, under control condition, in the presence of cilostamide + Ro 20-1724 or cilostamide + Ro 20-1724 + EHNA. As shown, the combination of cilostamide and Ro 20-1724 and the addition of EHNA increased I_{Ca} essentially by the same amount at every membrane potential (Fig. 4A) which indicates that the stimulatory effects of the drugs were not dependent on membrane potential. The inactivation curve of I_{Ca} (Fig. 4B) was also unchanged by the presence of the drugs which indicates that neither the voltage-dependent nor the Ca-dependent inactivation process (McDonald *et al.*, 1994) was modified.

(Figures 3 and 4 near here)

Each of the selective PDE inhibitors was tested in the presence of another inhibitor. This led to six different experimental conditions, and the results of these experiments are summarized in Table 1. Of all the combinations tested, only Ro 20-1724 and cilostamide had a significant stimulatory effect on basal I_{Ca}. The dual inhibition of PDE3 and PDE4 resulted in a ≈50% increase in basal I_{Ca}, an effect which corresponded to ≈40% of the stimulatory effect observed in the presence of 100 μM IBMX (Table 1). At this concentration, IBMX increased maximally I_{Ca}, since its effect was not statistically different from the stimulation induced by a saturating concentration (1 μM) of isoprenaline (Table 1). PDE1 inhibition (with 1 μM MIMX) had no effect on basal I_{Ca} under any condition tested (data not shown). PDE2 inhibition (with 10 μM EHNA) had no effect unless PDE3 and PDE4 were simultaneously blocked (data not shown). As shown in Table 1, the concomitant inhibition of PDE2, PDE3 and PDE4 with a combination of EHNA, cilostamide and Ro 20-1724 led to a similar increase in I_{Ca} as that produced by IBMX or isoprenaline.

Effect of PDE inhibitors on isoprenaline-stimulated I_{Ca}

We next examined the respective contribution of each PDE subtype in the regulation of phosphorylated Ca^{2+} channels. To do this, we examined the effects of each selective PDE inhibitor on I_{Ca} which had been stimulated by isoprenaline. However, it was important to use a non-saturating concentration of the β-adrenoceptor agonist in order to allow I_{Ca} to be further increased by the drugs. A concentration of 1 nM isoprenaline was chosen, which increased I_{Ca} by 38.1 ± 4.1% (n=82, Fig. 5), i.e. which produced ≈30% of the maximal stimulatory effect observed with 1 μM isoprenaline or 100 μM IBMX (Table 1).

(Figure 5 near here)

Fig. 5 shows a typical experiment in which the effects of PDE2 and PDE3 inhibition with EHNA and cilostamide, respectively, were successively tested on a rat ventricular myocyte exposed to 1 nM isoprenaline. While, as shown above, 100 nM cilostamide or 10 μM EHNA had no effect on basal I_{Ca}, as low as 30 nM cilostamide or 3 μM EHNA

induced a net and reversible stimulation of I_{Ca} on top of that obtained with isoprenaline alone. A similar finding was obtained with PDE4 inhibition by Ro 20-1724. Indeed, Fig. 6 shows that application of 30 nM Ro 20-1724 induced a net further increase in I_{Ca} in the presence of 1 nM isoprenaline, while this drug had no effect on basal I_{Ca} when used at a 3-fold higher concentration (Table 1).

(Figure 6 near here)

Fig. 7 summarizes the results of several similar experiments in which various concentrations of EHNA (Fig. 7A), cilostamide (Fig. 7B) and Ro 20-1724 (Fig. 7C) were tested for their effect on I_{Ca} pre-stimulated by 1 nM isoprenaline. While all these drugs induced an additional increase in I_{Ca}, the maximal stimulatory effect was the highest with Ro 20-1724 (≈50% at 30-100 nM), intermediate with cilostamide (≈30% at 0.3-1 μM) and the lowest with EHNA (≈20% at 1-10 μM). Under the same conditions (with 1 nM isoprenaline), PDE1 inhibition with 3 μM MIMX produced only a weak but significant ($p<0.05$) additional stimulation of I_{Ca} (8.1 ± 1.2%, n=5).

(Figure 7 near here)

Effect of PDE inhibitors on intracellular cAMP concentration

To get further insights into the mechanism of action of the different PDE inhibitors tested on I_{Ca}, we examined their effects on intracellular cAMP concentration ($[cAMP]_i$) in isolated rat ventricular myocytes. Fig. 8 shows the results of cAMP assays performed in ventricular myocytes isolated from three different rat hearts (symbols) together with the mean values (bars). Although one preparation responded by a large increase in $[cAMP]_i$ with any of the four PDE inhibitors tested (Fig. 8A, filled circles), on average the effects of MIMX (1 μM), EHNA (10 μM), cilostamide (0.1 μM) and Ro 20-1724 (0.1 μM) on basal $[cAMP]_i$ were small (20-30%) and not statistically significant (Fig. 8A). However, when the inhibitors were used in combination to achieve a concomitant inhibition of two PDE subtypes (tested only on PDE2-4), $[cAMP]_i$ increased by 40-50% and the effect became statistically significant ($p<0.05$, Fig. 8A). $[cAMP]_i$ increased further (to ≈80%) when all three PDE subtypes were inhibited, an effect which was similar to that obtained with a maximal concentration of IBMX (100 μM, Fig. 8A). Fig. 8B shows the effects of the selective PDE inhibitors on $[cAMP]_i$ when applied in the presence of a sub-maximal concentration of isoprenaline (1 nM). Although the preparation that responded to any

single PDE inhibitor also responded by a large increase in $[cAMP]_i$ with 1 nM isoprenaline (Fig. 8B, filled circles), the average stimulatory effect of 1 nM isoprenaline was small (20%) and not statistically significant (Fig. 8B). However, in the presence of isoprenaline, any single application of PDE inhibitor (tested only on PDE2-4) induced a large (60-70%) increase in $[cAMP]_i$ (Fig. 8B), which was similar to the effect obtained with 1 μM isoprenaline (Fig. 8B) or 100 μM IBMX (Fig. 8A).

(Figure 8 near here)

Discussion

In the recent years, it has become well established that cardiac I_{Ca} as well as myocardial contractility is modulated by cAMP-dependent phosphorylation. An increase in cAMP concentration activates cAMP-dependent protein kinase (PKA), which phosphorylates various proteins, including the L-type Ca^{2+} channels, leading to an increase in the mean open probability of individual channels (McDonald *et al.*, 1994; Hartzell *et al.*, 1991; Hove-Madsen *et al.*, 1996). The cAMP signal, which is generated at the level of the sarcolemmal membrane by adenylyl cyclase, is terminated by cyclic nucleotide phosphodiesterases, a class of enzymes that hydrolyse cAMP into 5'-AMP (Beavo, 1995).

At present, four different subtypes of PDE (PDE1-4) have been characterised in mammalian hearts (Taira *et al.*, 1993; Engels *et al.*, 1994; Kostic *et al.*, 1997). Their intracellular localisation and/or respective activities may vary depending on the animal species and/or the cardiac tissue (for reviews, see Beavo, 1995; Stoclet *et al.*, 1995). In our study, we have confirmed by RT-PCR the presence of mRNA transcripts encoding for all four PDE subtypes in isolated rat ventricular myocytes.

Investigation of their functional role has been made possible by the development of pharmacological agents that inhibit selectively their activity. A number of compounds, such as the bypiridine derivatives amrinone and milrinone, have long been used as selective PDE3 inhibitors with therapeutic applications. Among these, cilostamide is by far the most potent inhibitor of PDE3 (Reeves & England, 1990). The antidepressants Ro -20-1724 or rolipram potentiate the positive inotropic effect of forskolin on the heart by acting as selective inhibitors of PDE4 (Muller *et al.*, 1990). The adenosine deaminase inhibitor EHNA was recently characterised as a potent PDE2 inhibitor, which is also selective of this PDE subtype as far as other PDEs are concerned (Méry *et al.*, 1995; Podzuveit *et al.*, 1995). Potent and selective inhibitors of PDE1 are still to come, although the 8-methoxymethyl-derivative of IBMX, MIMX, has been shown to possess some selectivity at the PDE1 subtype (Wells & Miller, 1988).

In the present study, we used the broad-spectrum PDE inhibitor IBMX as well as the selective inhibitors MIMX, EHNA, cilostamide and Ro 20-1724 to assess the respective roles of PDE1-4 in the regulation of basal and stimulated I_{Ca} and $[cAMP]_i$ in rat ventricular myocytes. Some of these PDE inhibitors have been shown earlier to increase I_{Ca} by elevating $[cAMP]_i$. For instance, application of IBMX leads to a large

stimulation of basal I_{Ca} in guinea-pig (Mubagwa *et al.*, 1997), rabbit (Akita *et al.*, 1994; Kajimoto *et al.*, 1997) and human cardiomyocytes (Kajimoto *et al.*, 1997). Similarly, we found that IBMX also increased basal I_{Ca} in rat ventricular myocytes. This indicates that, in all these preparations, i) there is a substantial basal activity of adenylyl cyclase which determines a basal cAMP synthesis, and ii) there is a significant basal PDE activity which limits the degree of cAMP-dependent phosphorylation of L-type Ca^{2+}-channels. The situation in mammals differs from what was found in frog ventricular myocytes, where IBMX had no effect on I_{Ca} unless the current had been pre-stimulated by isoprenaline, forskolin or cAMP (Fischmeister & Hartzell, 1990; 1991). Thus, in frog cardiomyocytes, unlike in most mammalian species, basal adenylyl cyclase activity is insufficient to generate enough cAMP to lead to a stimulation of I_{Ca} even when all PDE activity is blocked.

For this reason, selective PDE inhibitors, such as EHNA or milrinone, have no effect on basal I_{Ca} in frog myocytes (Fischmeister & Hartzell, 1990; Méry *et al.*, 1995) while they do stimulate basal I_{Ca} in human atrial myocytes (Kirstein *et al.*, 1995; Kajimoto *et al.*, 1997; Rivet-Bastide *et al.*, 1997). Similarly, PDE3 inhibitors stimulate basal I_{Ca} in

rabbit cardiomyocytes (Kajimoto *et al.*, 1997) and exert a positive inotropic effect in guinea-pig left atria (Muller *et al.*, 1990). However, in this study, we found that none of the selective PDE inhibitors used alone had any significant effect on basal I_{Ca} or $[cAMP]_i$ in rat ventricular myocytes. Thus, inhibition of a single PDE subtype is insufficient to raise cAMP concentration near Ca^{2+} channels to the threshold concentration necessary for I_{Ca} stimulation. The likely explanation for this is that other PDE subtypes accounted for cAMP hydrolysis under such condition. To test this hypothesis, we tried to block two PDE subtypes at the same time using combinations of PDE inhibitors. The only 'duo' of inhibitors that was found to enhance basal I_{Ca} was cilostamide and Ro 20-1724, indicating that PDE3 and PDE4 were the dominant PDE subtypes regulating basal cAMP concentration and I_{Ca} in rat ventricular myocytes. These results are in agreement with a number of biochemical studies showing a much higher specific activity of PDE3 and PDE4 compared with PDE1 and PDE2 in rat heart (Shahid & Nicholson, 1990; Bode *et al.*, 1991; Dubois *et al.*, 1993; Picq *et al.*, 1996). Also, functional experiments have shown that inhibition of PDE3 or PDE4 alone had no effect on the contractile activity of rat heart while a dual PDE3 and PDE4 inhibition produced a strong positive inotropic effect (Shahid & Nicholson, 1990).

Surprisingly, however, we found that not only cilostamide and Ro 20-1724 but also any other 'duo' combination of PDE2-4 inhibitors induced a substantial elevation in $[cAMP]_i$ in rat ventricular myocytes. This difference between I_{Ca} and $[cAMP]_i$ measurements may indicate the presence of different pools of cAMP inside cardiac myocytes, which are not all associated with L-type Ca^{2+} channels. Such a hypothesis is supported by recent findings of a strong cAMP compartmentation near L-type Ca^{2+} channels in frog ventricular myocytes exposed to isoprenaline (Jurevicius & Fischmeister, 1996). It is also supported by a large body of evidence for distinct intracellular localisation of the different PDE subtypes. For instance, a substantial amount of PDE3 and PDE4 activity was found in a membrane-bound fraction of rat cardiomyocytes (Weishaar *et al.*, 1987; Shahid *et al.*, 1990; Kaasic & Ohisalo, 1996; Okruhlicova *et al.*, 1996) which may accentuate the role of these PDE subtypes in the control of sarcolemmal processes, such as cAMP-dependent phosphorylation of L-type Ca^{2+} channels. PDE3 and PDE4 have also a 5 to 30-fold lower K_m for cAMP than PDE2 (Bode *et al.*, 1991; Beavo, 1995), which should attenuate the role of PDE2 in I_{Ca} regulation when $[cAMP]_i$ near the membrane is low. This may explain why EHNA did not affect I_{Ca} unless PDE3

and PDE4 were blocked or when their activity was saturated by an enhanced cAMP synthesis in the presence of isoprenaline.

Regulation of I_{Ca} by PDE1 in heart remains unclear. In this study, we found that the PDE1 inhibitor MIMX (Wells & Miller, 1988) had negligible effects on basal I_{Ca} and increased only by 8% isoprenaline stimulated I_{Ca}. However, a major drawback of our experiments is that the solution used in the patch-pipette was adjusted to a free Ca^{2+} concentration of pCa 8.5 using 5 mM EGTA. Although the Ca^{2+} buffering capacity of the pipette solution should only weakly affect the Ca^{2+} concentration near the membrane, as evidenced by the presence of a large Ca^{2+}-mediated inactivation of I_{Ca} under these conditions, it might abolish a Ca^{2+}-calmodulin dependent activation of PDE1 in a remote part of the cell. Nystatin-perforated patch-clamp experiments would be necessary to elucidate the role of PDE1 in the control of I_{Ca}. However, it should be mentioned that biochemical studies have shown that rat heart possesses a much lower PDE1 activity as compared to other PDE subtypes (Dubois *et al.*, 1993, Shahid & Nicholson, 1990, Bode *et al.*, 1991).

To the exception of MIMX, all other PDE inhibitors tested exerted a clear stimulation of I_{Ca} after application of 1 nM isoprenaline. This low concentration of the β-

adrenoceptor agonist moderately increased the activity of adenylyl cyclase localised in the membrane, bringing the cAMP concentration above threshold for cAMP-dependent phosphorylation of L-type Ca^{2+} channels. In these conditions, EHNA, cilostamide or Ro 20-1724 were found to further enhance I_{Ca} as well as $[cAMP]_i$. Thus, unlike what was observed under basal conditions, inhibition of any single PDE2-4 subtype stimulates Ca^{2+} channel activity when the intracellular cAMP level is above threshold. At their maximal effects, the rank order of potency of the different PDE inhibitors on isoprenaline stimulated I_{Ca} was Ro 20-1724>cilostamide>EHNA>MIMX. However, in the case of Ro 20-1724, increasing the concentration above the maximal effective concentration (100 nM) produced lower stimulatory effects on I_{Ca} (Fig. 7C). While, we have no clear explanation for this effect, a similar phenomenon was observed in contractile experiments on guinea-pig heart by Muller *et al.* (1990) with another PDE4 inhibitor, rolipram. An interesting possibility is that the increase in cAMP and PKA activity that results from PDE4 inhibition leads to phosphorylation and activation of PDE4 as has been shown recently in thyroid cells (Sette & Conti, 1996).

In summary, our results demonstrate that PDE2, PDE3 and PDE4 regulate I_{Ca} in rat ventricular myocytes. PDE3 and PDE4 are the dominant PDE subtypes involved in the regulation of basal I_{Ca}. Each of these two PDEs possesses a sufficient enzymatic activity to fully hydrolyse alone the cAMP produced by the basal activity of adenylyl cyclase. This double hydrolytic system may prevent intracellular cAMP from rising immoderately in the absence of stimulatory hormones or neuromediators. When the cell is challenged by a stimulus activating cAMP production, such as a β-adrenoceptor agonist, all PDE subtypes become determinant in regulating the intracellular cAMP concentration. Hence, the response of I_{Ca} to a sympathetic stimulation will be determined by the activity of each PDE subtype, with the rank order of potency PDE4>PDE3>PDE2>PDE1.

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Table 1. Effect of PDE inhibitors and isoprenaline on basal I_{Ca}.

Drugs & concentrations	%variation over basal I_{Ca}	n=
MIMX 1 μM	2.6 ± 3.3	9
EHNA 10 μM	0.1 ± 1.4	10
Cilostamide 0.1 μM	-3.1 ± 2.0	12
Ro 20-1724 0.1 μM	-1.3 ± 2.9	12
MIMX 1 μM + EHNA 10 μM	-1.8 ± 1.7	4
MIMX 1 μM + cilostamide 0.1 μM	-0.9 ± 1.9	3
MIMX 1 μM + Ro 20-1724 0.1 μM	0.6 ± 1.1	3
EHNA 10 μM + cilostamide 0.1 μM	3.9 ± 2.8	6
EHNA 10 μM + Ro 20-1724 0.1 μM	0.7 ± 1.7	4
Cilostamide 0.1 μM + Ro 20-1724 0.1 μM	47.7 ± 7.8*	16
Cilostamide 0.1 μM + Ro 20-1724 0.1 μM + EHNA 10 μM	107.2 ± 22.3*	4
IBMX 100 μM	120.3 ± 10.0*	8
Isoprenaline 1 μM	143.4 ± 27.1*	7

The results are the means ± S.E.M. of the number of experiments indicated in the last column. They are expressed in % variation over basal I_{Ca} amplitude. The asterisks indicate when the effects were statistically significant at the $p<0.01$ level (*).

Figure 1. Expression analysis of PDE1, PDE2, PDE3 and PDE4 transcripts in rat ventricle

RT-PCR analysis was performed on mRNA extracted from whole ventricular tissue (*A*) and isolated ventricular myocytes (*B*) and in the presence (+RT) or in the absence (-RT) (as a negative control) of the reverse transcriptase. The PCR products were analysed on a 1.7% agarose gel and photographs of the ethidium bromide stained gels are shown. The PCR primers used for this analysis and expected length of the PCR products are described in Methods. Positive controls were performed in a second PCR using rat glyceraldehyde 3-phosphate dehydrogenase primers (GAPDH). Positions of two molecular weight markers are indicated in bp. Note that the PCR procedure was 40 cycles and 35 cycles for isolated myocytes and ventricular tissue respectively. This figure is representative of three separate determinations of the PDE mRNA expressions obtained by RT-PCR.

Figure 2. Effects of Ro 20-1724 and IBMX on I_{Ca} in a rat ventricular myocyte

Each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. The cell was first superfused with control Cs^+ Ringer solution and then exposed to the drugs during the periods indicated by the solid lines. 0.3 μM Ro 20-1724 had no effect on I_{Ca}, while application of 100 μM IBMX produced a strong and reversible stimulation of I_{Ca}. The individual current traces shown on the *upper part* were obtained at the times indicated by the corresponding letters in the *bottom graph*. The *dotted line* indicates the zero current level.

Figure 3. Effects of cilostamide, Ro 20-1724 and EHNA on I_{Ca} in a rat ventricular myocyte

The cell was superfused for several minutes with a control solution and then challenged with different drugs during the periods indicated by the solid lines. While application of 0.1 μM cilostamide (Cil) had no effect on basal I_{Ca}, application 0.1 μM Ro 20-1724 (Ro) on top of cilostamide or of EHNA (10 μM) on top of cilostamide and Ro 20-1724 induced a net increase in I_{Ca} Test pulses were 0 mV except during the recording of current-voltage (I-V) relationships. The individual current traces shown on the *upper part* were obtained at the times indicated by the corresponding letters in the *bottom graph*. The *dotted line* indicates the zero current level.

Figure 4. Effects of cilostamide, Ro 20-1724 and EHNA on the current-voltage and inactivation relationships of I_{Ca} in a rat ventricular myocyte

A, current-voltage relationships and *B*, inactivation curves for I_{Ca} obtained in the experiment shown in Fig. 3 in control (*filled squares*), in the presence of 0.1 μM cilostamide and 0.1 μM Ro 20-1724 (Cil + Ro, *open circles*), and in the presence of 0.1 μM cilostamide, 0.1 μM Ro 20-1724 and 10 μM EHNA (Cil + Ro + EHNA, *open triangles*). Inactivation curves were obtained using a double-pulse protocol indicated in the inset and described in Methods.

Figure 5. Effects of cilostamide and EHNA on I_{Ca} pre-stimulated by isoprenaline

The cell was superfused for few minutes with a control solution and then challenged with different drugs during the periods indicated by the solid lines. Application of 1nM isoprenaline (Iso) slightly increased I_{Ca}. Addition of 30 nM cilostamide (Cil) or of 3 or 10 μM EHNA in the continuing presence of isoprenaline induced an additional and reversible increase in I_{Ca}. The individual current traces shown on the *upper part* were obtained at the times indicated by the corresponding letters in the *bottom graph*. The *dotted line* indicates the zero current level.

Figure 6. Effects of RO 20-1724 on I_{Ca} pre-stimulated by isoprenaline

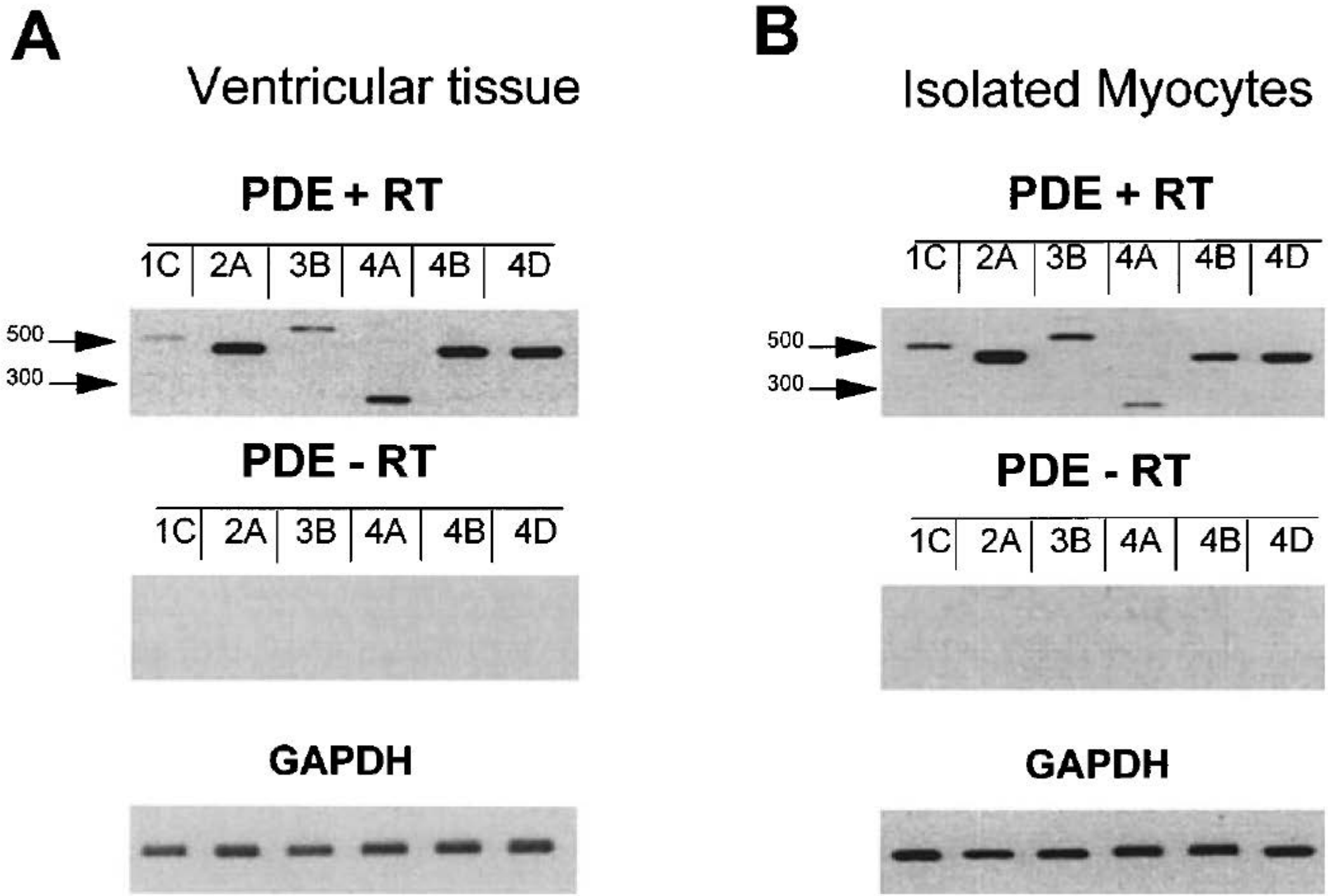
The cell was superfused for few minutes with a control solution and then challenged with different drugs during the periods indicated by the solid lines. Application of 1nM isoprenaline (Iso) increased I_{Ca}. Addition of 30 nM Ro 20-1724 (Ro) in the continuing presence of isoprenaline induced an additional and reversible increase in I_{Ca}. The individual current traces shown on the *upper part* were obtained at the times indicated by the corresponding letters in the *bottom graph*. The *dotted line* indicates the zero current level.

Figure 7. Effects of EHNA, cilostamide and RO 20-1724 on I_{Ca} pre-stimulated by isoprenaline

Summary of the effects of different concentrations of EHNA (*A*), cilostamide (*B*) and Ro 20-1724 (*C*) on I_{Ca} pre-stimulated by 1 nM isoprenaline in rat ventricular myocytes. The bars indicate the means and the lines the S.E.M. of the numbers of experiments indicated near the bars. The effects are expressed in % variation over the amplitude of I_{Ca} in the presence of isoprenaline alone. Statistical significant differences from I_{Ca} amplitude in isoprenaline alone are indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

Figure 8. Effects of PDE inhibitors and isoprenaline on $[cAMP]_i$ in isolated rat ventricular myocytes

The selective PDE inhibitors MIMX (1 μM), EHNA (10 μM), cilostamide (0.1 μM) and Ro 20-1724 (0.1 μM) were tested as indicated in three different preparations of rat ventricular myocytes (corresponding to the different symbols used in *A* and *B*) in the absence (*A*) or presence (*B*) of 1 nM isoprenaline. The effects are expressed in % increase over basal cAMP concentration which was, respectively, 59 (○), 52 (■) and 22 (●) pmoles per mg protein, assuming that each myocyte contained 15 ng protein. Each individual measurement was done in triplicate. The bars indicate the average response in each condition. Separate experiments were performed with IBMX (100 μM, *A*) and isoprenaline (1 μM, *B*), and the corresponding bars indicate the mean and the s.e.m. of the number of preparations indicated near the bars. Statistical significant differences from basal cAMP concentration are indicated as: *, $p<0.05$.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PDE + RT
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PDE - RT
1C
2A
3B
4A
4B
4D
GAPDH
B
Isolated Myocytes
PDE + RT
1C
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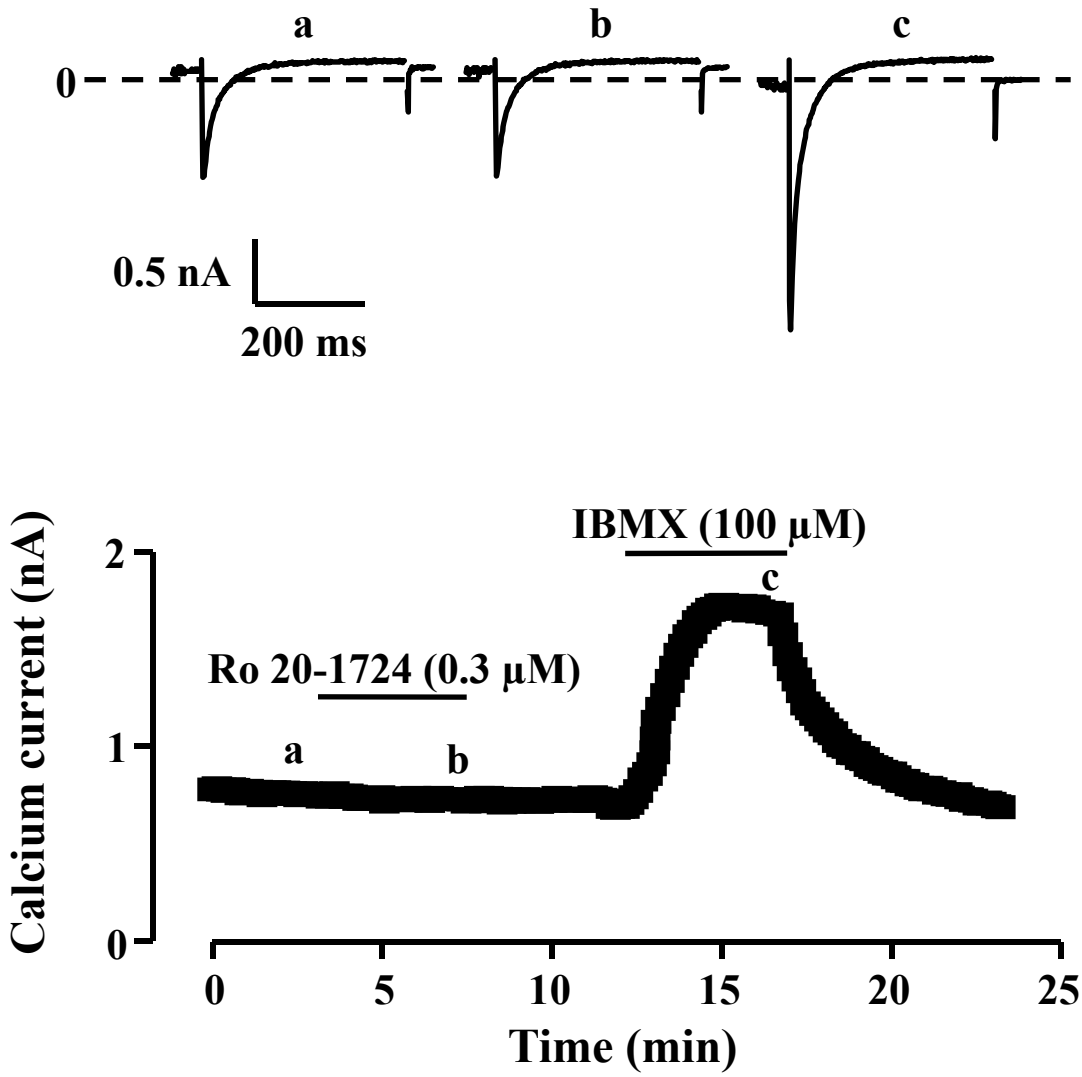

Figure 2
Verde et al.

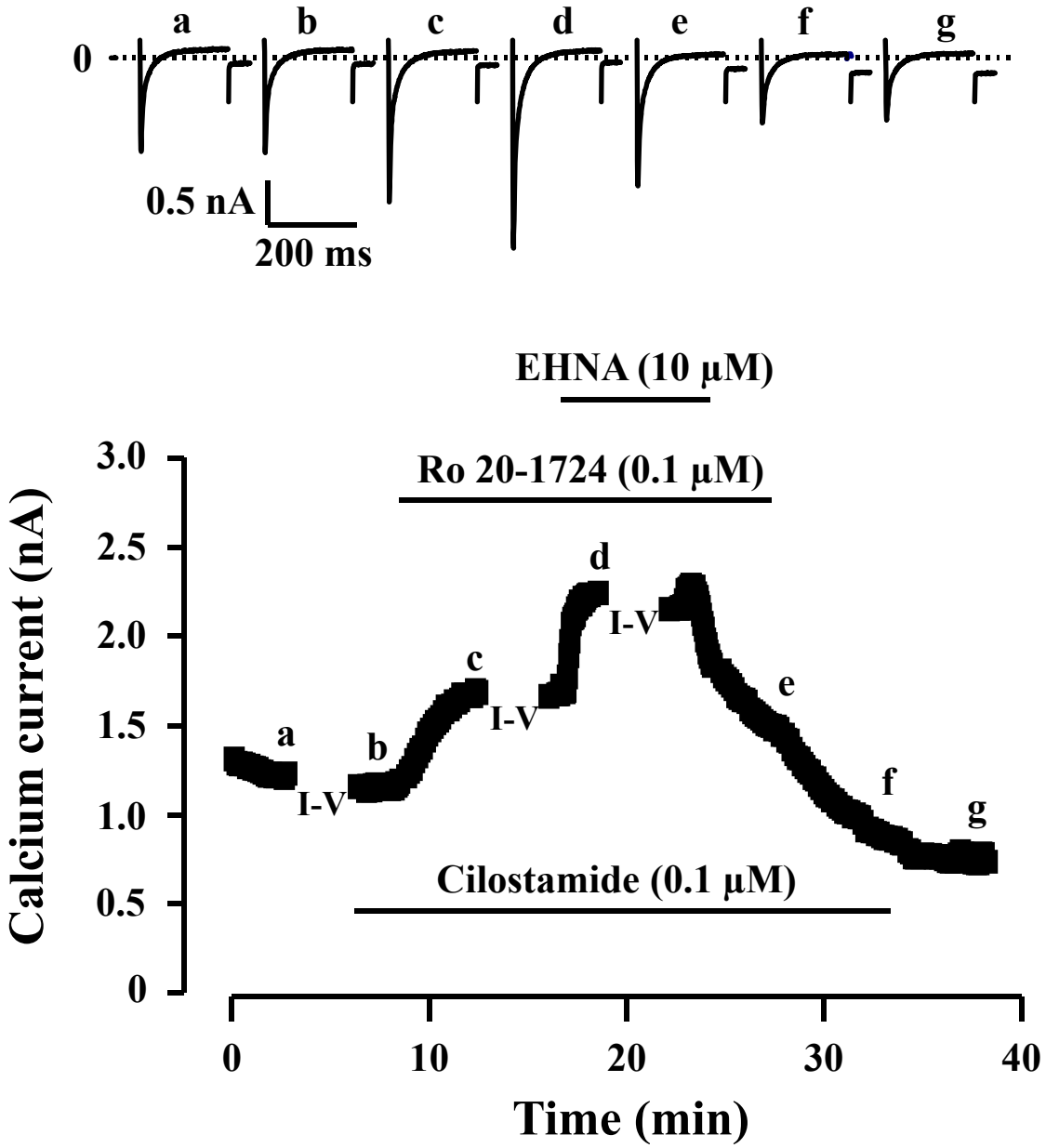

Figure 3
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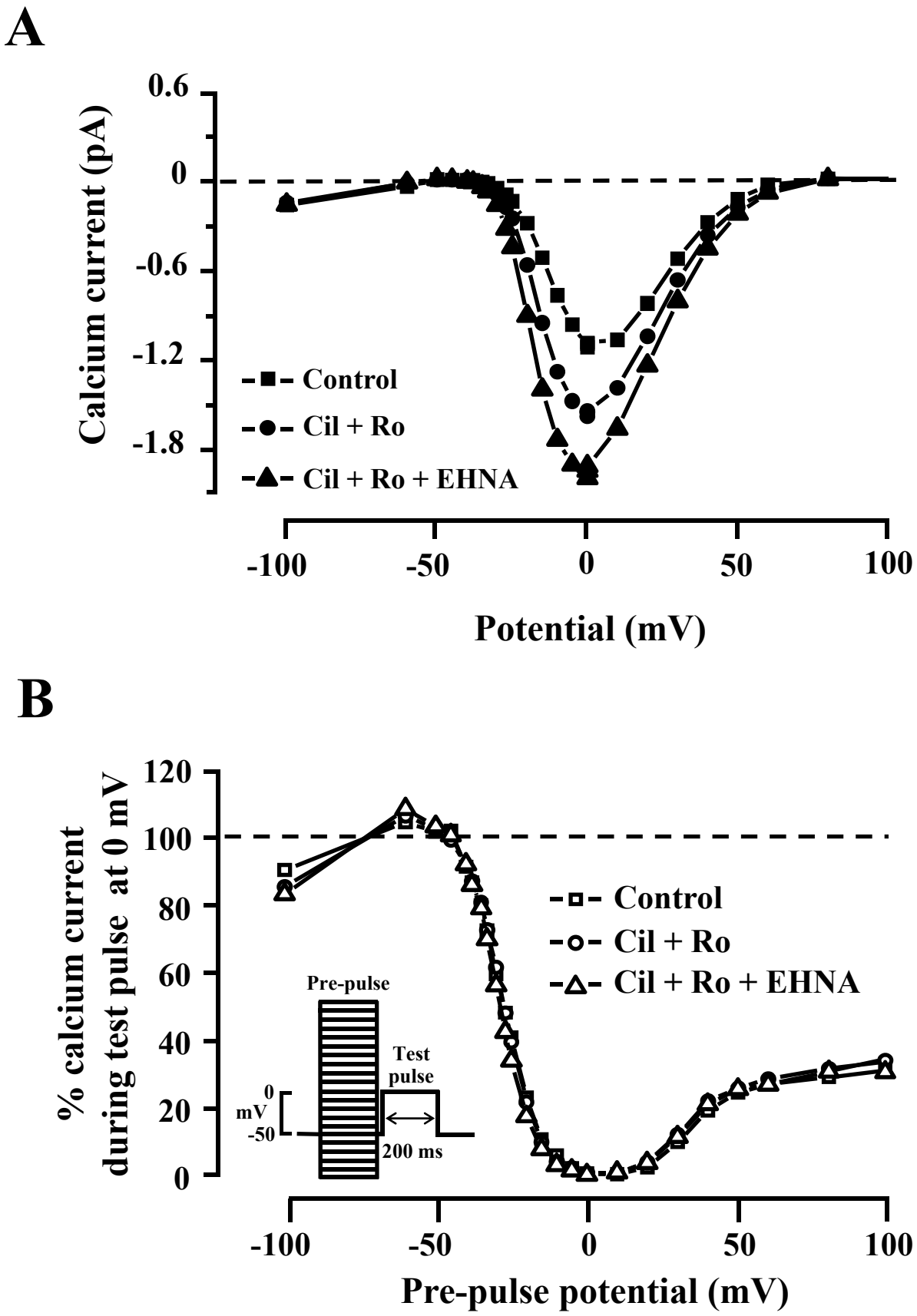

Figure 4
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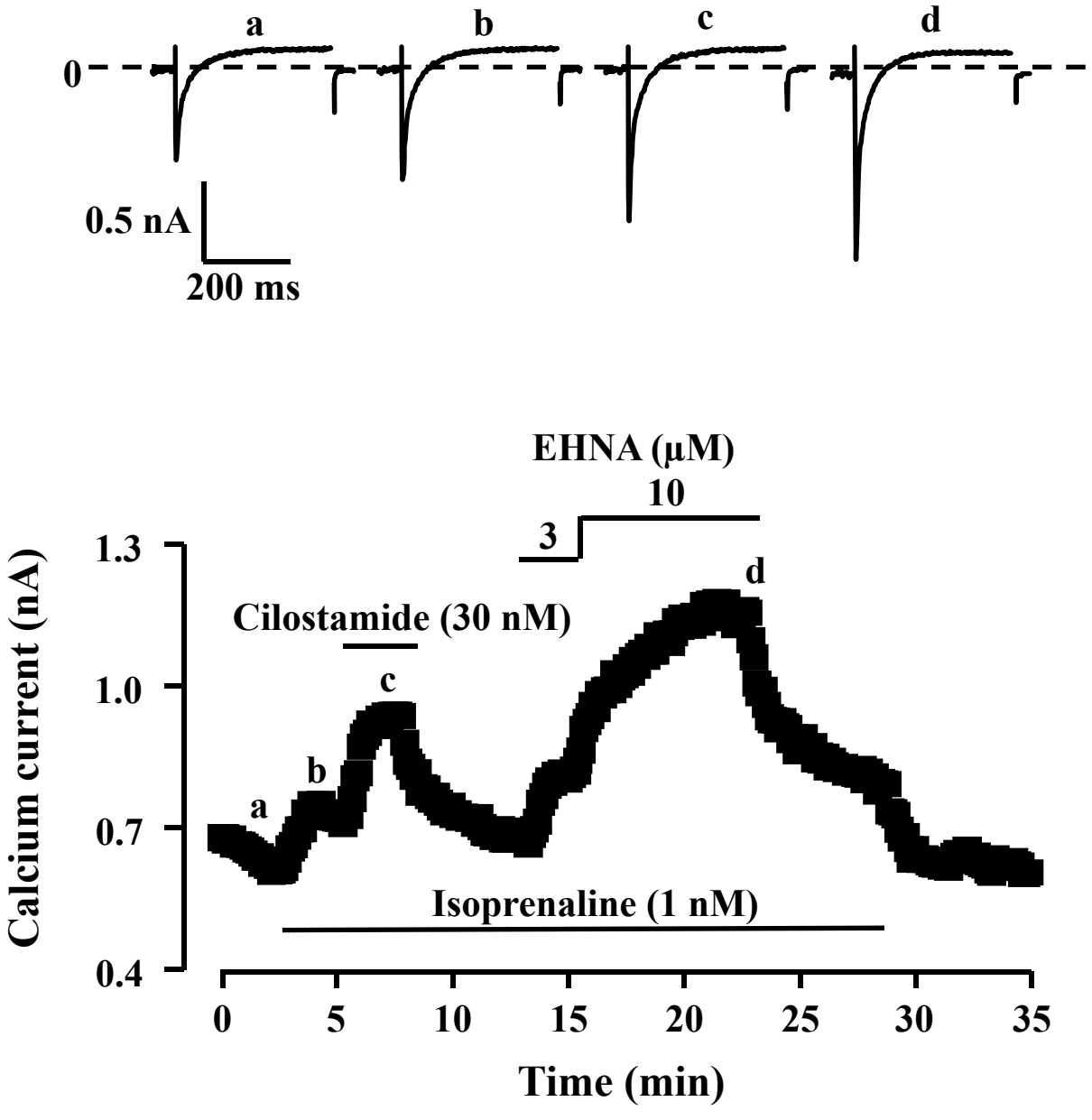

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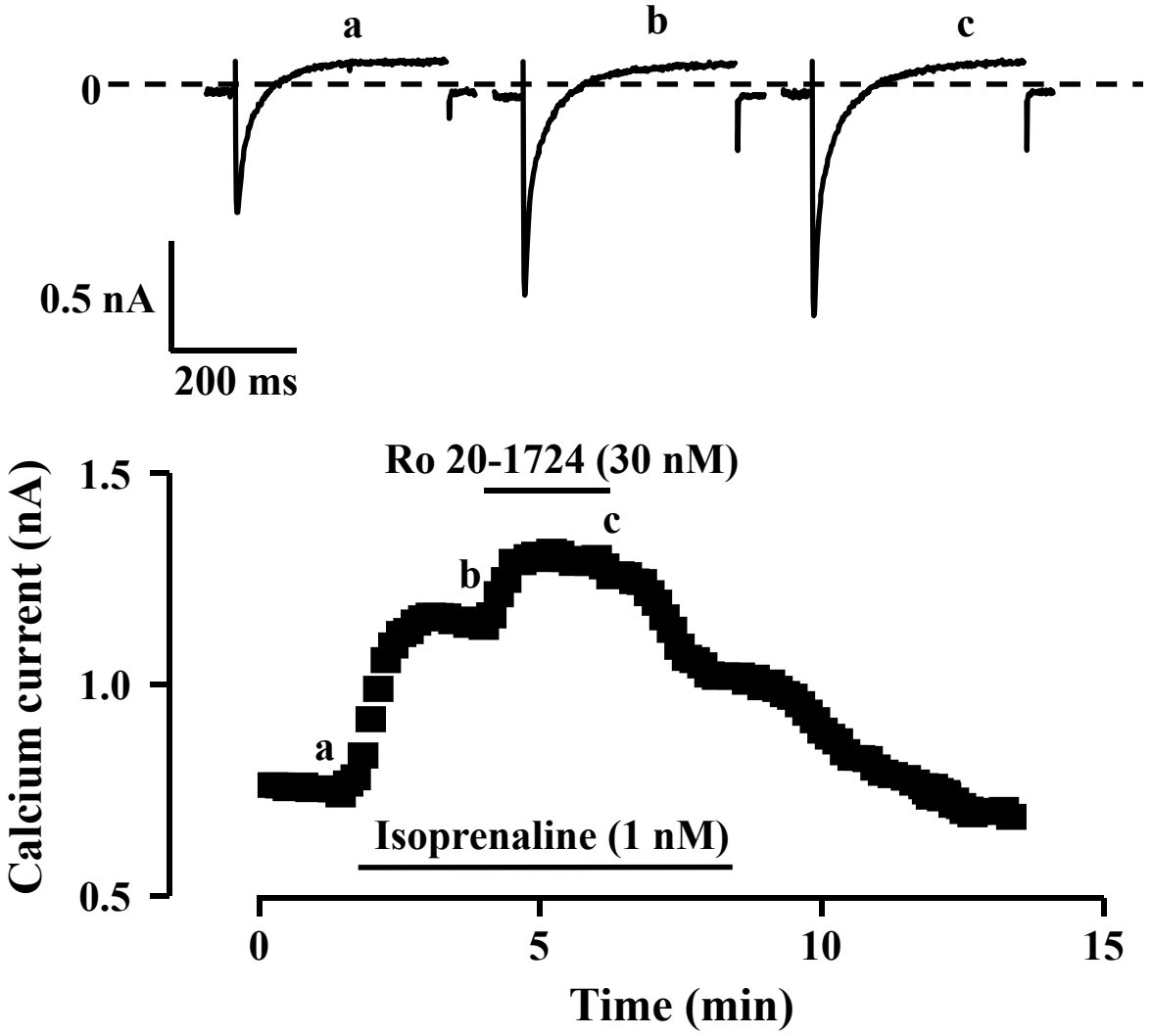

Figure 6
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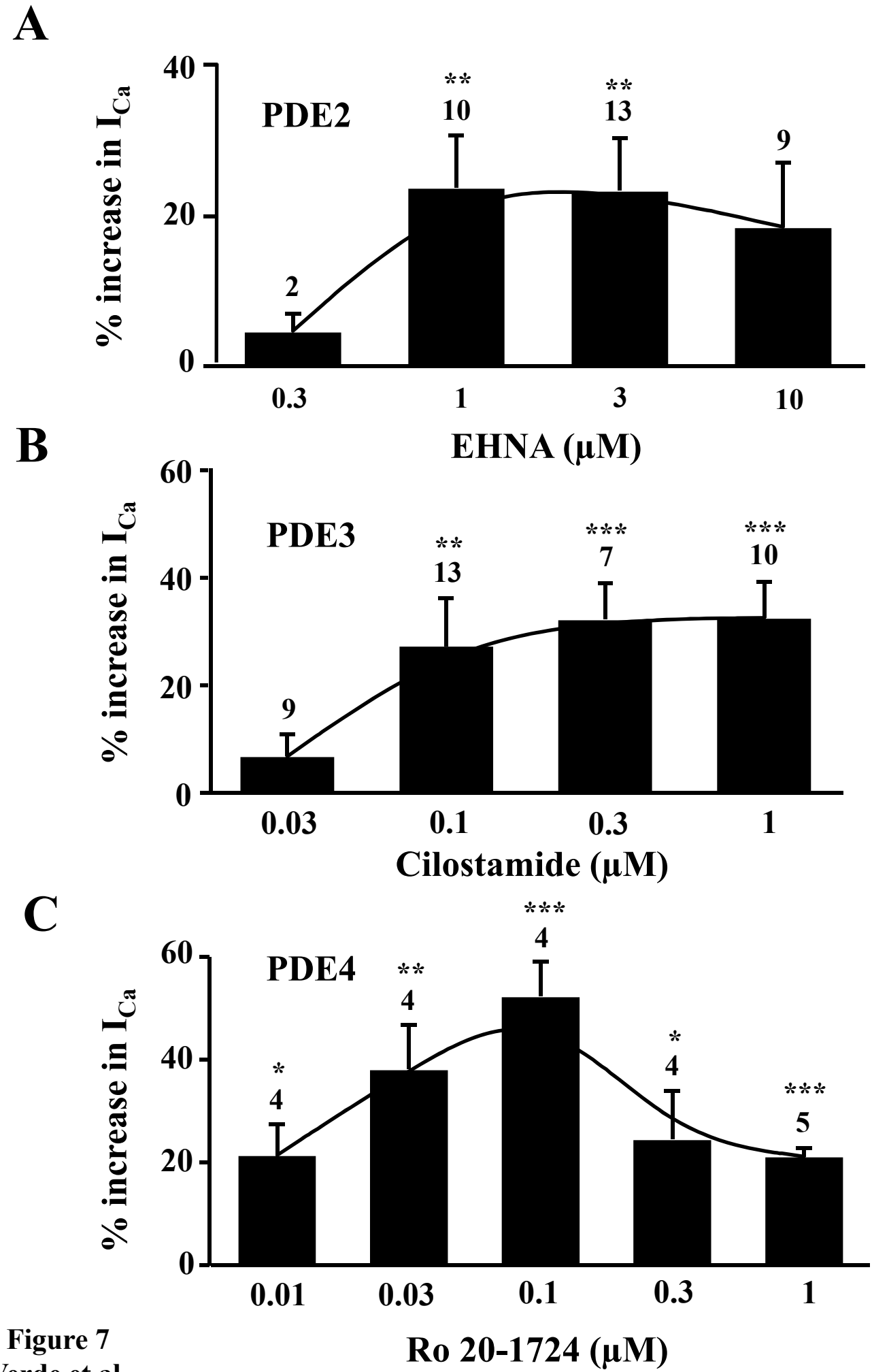

Figure 7
Verde et al.