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# CYCLIC AMP-PHOSPHODIESTERASE IIIA1 INHIBITORS DECREASE CYTOSOLIC $Ca^{2+}$ CONCENTRATION AND INCREASE THE $Ca^{2+}$ CONTENT OF INTRACELLULAR STORAGE SITES IN HUMAN PLATELETS

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**Abstract**—The effect of cyclic AMP-phosphodiesterase (cAMP-PDE) inhibitors on $Ca^{2+}$ homeostasis in human platelets was studied using both quin-2 (2-(-bis-(acetylamino)-5-methyl-phenoxy)methyl-6-methoxy-8-bis-(acetylamino)quinoline) and chlorotetracycline (CTC) to measure changes in cytosolic $Ca^{2+}$ as well as changes in the amount of $Ca^{2+}$ accumulated in intracellular storage sites. At therapeutic concentrations (1 $\mu$M) milrinone and R 80 122 but not enoximone decreased the cytosolic $Ca^{2+}$ concentration in the resting platelet while the $Ca^{2+}$ content in intracellular stores was increased. These observations are in accord with the proposed mechanism of action of cAMP-PDE inhibitors on cardiomyocites and highlight the particular role of cAMP in regulation of $Ca^{2+}$ homeostasis.

In platelets, as well as in many other cells, the cellular response to extracellular signals is regulated by two antagonizing signal transduction systems. Excitatory pathways, for all stimuli, except for adrenaline, lead to an increase in cytosolic $Ca^{2+}$, whereas inhibitory platelet agonists provoke an elevation of cAMP that inhibits platelet activation (for a review see Ref. 1). One of the molecular links that regulates the interplay between both signal transducing systems, is the control of the cytosolic $Ca^{2+}$ level by cAMP. In that, cAMP appears to stimulate resequestration of $Ca^{2+}$ after excitatory stimulation [2–4].

Many $Ca^{2+}$ transport mechanisms that have been demonstrated in platelets ($Na^+/Ca^{2+}$ exchange, ATPase-dependent $Ca^{2+}$ pumps and mitochondrial $Ca^{2+}$ transport), also operate in cardiac tissue and seem to be regulated in a similar way. In comparison to the cardiac sarcolemma and cardiac sarcoplasmic reticulum, both calmodulin and cAMP can regulate the ATP-dependent $Ca^{2+}$ transport mechanisms in platelets [5–7]. Platelet cAMP-dependent protein kinase appears to play a major stimulatory role in $Ca^{2+}$ sequestration in intracellular stores [8–10], by phosphorylation of a 22 kDa protein that resembles cardiac phospholamban [5, 9]. In cardiac tissue, increased accumulation of $Ca^{2+}$ in a store that is involved in excitation–contraction coupling has been repeatedly suggested as a possible molecular basis for positive inotropy. Recently we were able to demonstrate that the mechanism of action of ouabain in the platelet was similar to that proposed for cardiac muscle [11]. Using 2-(-bis-(acetylamino)-5-methyl-phenoxy)methyl-6-methoxy-8-bis-(acetylamino)quinoline (quin-2†) and chlorotetracycline (CTC) as intracellular probes for measuring, respectively, cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) and $Ca^{2+}$ in intracellular stores, we found the glycoside to increase $Ca^{2+}$ in both pools. In accordance with earlier findings [12], $[Ca^{2+}]_{cyt}$ was positively related to the $Ca^{2+}$ level in intracellular stores. In comparison with the ouabain-induced positive inotropic effect on cardiac muscle, the ouabain-induced increase in $Ca^{2+}$ accumulated in intracellular stores enhances the platelet $Ca^{2+}$ dependent functional responses [11, 13, 14].

In view of the striking similarities between $Ca^{2+}$ homeostasis in platelets and cardiomyocytes, we investigated whether the same experimental approach could give clues on the mechanism of action of cyclic AMP-phosphodiesterase (cAMP-PDE) inhibitors.

Inhibitors of the cAMP-PDE IIIA1 (cGMP-inhibited family according to Beavo and Reifsnyder [15]) are known for many years to increase the contractile force of the heart [16, 17]. By analogy to ouabain, this increase in contractile force has been attributed to an increased amount of $Ca^{2+}$ retained in the intracellular stores: the latter being a consequence of a cAMP-induced increase in $Ca^{2+}$ sensitivity of the sarcoplasmic reticular $Ca^{2+}$ pump [18] and by an accelerated turnover of this ion pump [19]. Furthermore, this increased $Ca^{2+}$ pump activity is believed to contribute to an important extent to the observed increased rate of relaxation (lusitropic effect) of the heart [17] by enhanced removal of activator $Ca^{2+}$ from the cytosol.

R 80 122, a newly synthesized 1,2,3,5-tetrahydro-2-oxoimidazo[2,1-*b*]quinazoline derivative, is a potent and selective cAMP-PDE IIIA1 inhibitor [20]. Milrinone and enoximone are two well-documented cardiotonic drugs [21, 22].

In this work we present data obtained in intact cells that reinforce the proposed mechanism of action of these cAMP-PDE inhibitors on $Ca^{2+}$ homeostasis.

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† Abbreviations: cAMP-PDE, cyclic AMP-phosphodiesterase; CTC, chlorotetracycline; quin-2, 2-(-bis-(acetylamino)-5-methyl-phenoxy)methyl-6-methoxy-8-bis-(acetylamino)quinoline; $[Ca^{2+}]_{cyt}$, cytosolic free $Ca^{2+}$ concentration.

## MATERIALS AND METHODS

Chlorotetracycline, was obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.), quin-2 acetoxy methylester came from Calbiochem (La Jolla, CA, U.S.A.), ouabain from Janssen Chimica (Beerse, Belgium) R 80 122 ((*E*)-*N*-cyclohexyl-*N*-methyl-2-[[[phenyl(1,2,3,5 - tetrahydro - 2 - oxoimidazole[2,1 - *b*] - quinazolin - 7 - yl)methylene]amino]oxy]acetamide) was synthesized in the Department of Chemical Research (Janssen Research Foundation, Beerse, Belgium). Milrinone and enoximone were purchased from the companies of their origin. Chlorotetracycline and ouabain were dissolved in 0.9% NaCl; R 80 122, milrinone and enoximone in dimethyl sulphoxide (DMSO). The cyclic AMP radioassay kit was obtained from Amersham International (Amersham, Bucks, U.K.).

*Platelet isolation and handling.* Blood collection from healthy human volunteers and preparation of platelet-rich plasma (PRP) were carried out as described recently [11]. Except for quin-2 experiments, platelets were isolated by repeated centrifugation, and suspended in buffer A (25 mM Hepes, 125 mM NaCl, 2.7 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 0.1 mM EGTA, 0.5 mM $Na_2HPO_4$, 0.1% bovine serum albumin at pH 7.4). In CTC experiments $MgSO_4$ and bovine serum albumin were omitted. In all experiments, platelets were preincubated for 15 min in buffer with 1 mM $CaCl_2$ to allow them to recover from the isolation procedure and to restore normal $Ca^{2+}$ homeostasis, before any compound was added. The final solvent concentration in the platelet suspension never exceeded 0.1%. All incubations were at 37°.

*Fluorescence measurements using quin-2 as a probe.* Platelets were loaded with quin-2 and isolated by gel filtration exactly as described before [11]. Fluorescence measurements on drug-treated platelets and control platelets were performed alternatively, exactly as described previously [11], and were corrected for a slight fluorescence of milrinone at $10^{-6}$ M, when this compound was tested.

*Fluorescence measurements using CTC as a probe.* We used the same procedure as described before [11]: 10 $\mu$M CTC was added to washed platelet suspension (2 mL, $2 \times 10^8$ platelet/mL) in the cuvette at 37°, in the presence of 1 mM external $Ca^{2+}$ and fluorescence was measured (390 nm excitation; 530 nm emission). The addition of CTC gives rise to an instantaneous increase of fluorescence (fast phase), followed by a slow increase of fluorescence which we monitored for 15 min (slow phase). The rapid change ($A_{fast}$) is due to fluorescence of the Ca–CTC complex in the aqueous phase and binding of the Ca–CTC complex on the outer surface of the plasma membrane whereas the slow fluorescence increase ($A_{slow}$) arises primarily from the binding of the Ca–CTC complex to the inner surfaces of $Ca^{2+}$ sequestering organelles and is proportional to the free $Ca^{2+}$ concentration in these organelles [12].

We calculated the CTC ratio as the amplitude of the slow phase over the amplitude of the fast phase ($A_{slow}/A_{fast}$) taken 15 min after CTC addition. It represents a quantitative index of net $Ca^{2+}$ accumulated into intracellular $Ca^{2+}$ sequestering pools/unit surface area of platelets [12].

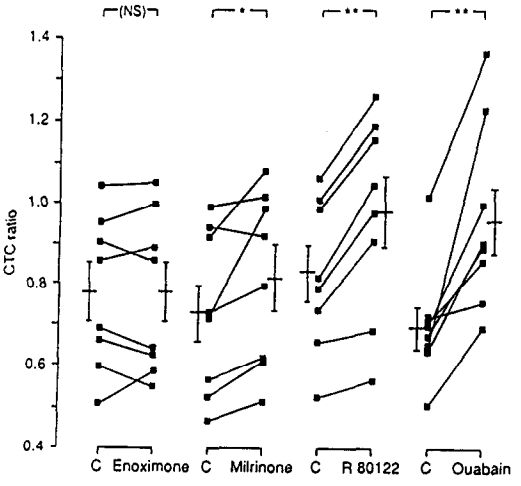


Fig. 1. The effect of different compounds on the platelet CTC ratio. Washed platelets were incubated with the indicated drugs at $10^{-6}$ M in the presence of 1 mM extracellular $Ca^{2+}$. After 35 min CTC was added, and CTC ratio determined as described in Materials and Methods. Results represent the individual measurements which are each means of duplicate determinations as well as the mean and SE (N = 8 NS, not significant; *P < 0.05; **P < 0.01.

$A_{fast}$ in drug-treated platelets never differed significantly from control platelets (Student's *t*-test for paired data).

*Determination of cAMP.* cAMP was determined by means of a radioimmunoassay kit. $HClO_4$ was added to the platelets to a final concentration of 0.4 N on ice. After 5 min, pH was neutralized by the addition of 1.2 vol. of 0.4 M $KHCO_3$. Precipitate of protein and $KClO_4$ was discarded after centrifugation (900 *g*, 10 min) at room temperature. Further steps were performed exactly as described by the manufacturer.

*Data presentation.* In each test, drug-treated platelets were compared with control platelets. Data are presented as the mean and SE. The asterisks in the figures denote the statistical evaluation of data performed by Wilcoxon's signed rank test. Values of $P < 0.05$ were considered significant.

For quin-2 experiments each measurement on drug-treated platelets was compared with the preceding control measurement.

## RESULTS

Platelets were incubated in the presence of R 80 122, milrinone or enoximone and $Ca^{2+}$ in intracellular stores or $[Ca^{2+}]_{cyt}$ was measured using, respectively, CTC and quin-2 as intracellular probes. The drugs were tested at 1 $\mu$M, a concentration close to therapeutic plasma levels [21, 22] and compared with the same concentration of ouabain. The results shown in Fig. 1 demonstrate that milrinone, R 80 122 and ouabain increased the amount of $Ca^{2+}$ in

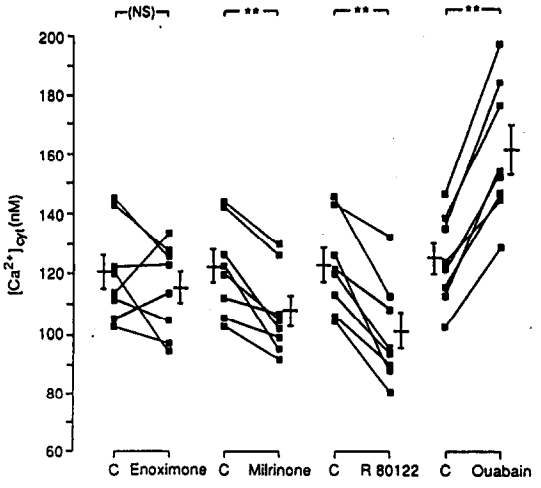


Fig. 2. The effect of different compounds on the $[Ca^{2+}]_{cyt}$ in resting platelets. Quin-2 loaded platelets were incubated with the indicated drugs at $10^{-6}$ M or their solvent in the presence of 1 mM extracellular $Ca^{2+}$ and basal $[Ca^{2+}]_{cyt}$ was determined after 50 min. Results represent the individual measurements which are means of duplicate determinations as well as the mean and SE (N = 8). NS, not significant; $^{**}P < 0.01$.

Table 1. Changes in cyclic AMP on addition of different drugs to isolated human platelets

| | Cyclic AMP (pmol/$10^8$ platelets) | |
|---|---|---|
| Control | 932 ± 76 | |
| Enoximone | 1241 ± 107 | $P < 0.01$ |
| Milrinone | 1912 ± 225 | $P < 0.01$ |
| R 80 122 | 1988 ± 319 | $P < 0.01$ |
| Ouabain | 814 ± 36 | NS |

Washed platelets were incubated for 50 min in the presence of the indicated agents at 1 μM. Results represent means ± SE from eight experiments with single samples. P-values are obtained by Wilcoxon's signed rank test.

intracellular stores that can be detected by CTC. Enoximone had no effect on CTC ratio.

In accordance with our earlier findings [11], the ouabain-induced increase in these intracellular pools is accompanied by an increase of resting $[Ca^{2+}]_{cyt}$, measured using quin-2 as a probe (Fig. 2). In contrast, both milrinone and R 80 122 decreased the resting $[Ca^{2+}]_{cyt}$ while enoximone again had no effect. Table 1 illustrates that under these experimental conditions both milrinone and R 80 122 increased platelet cAMP to a similar extent while enoximone was far less potent.

## DISCUSSION

To our knowledge, this is the first time that drugs, at therapeutic concentrations, have been found to be able to decrease the resting $[Ca^{2+}]_{cyt}$. Even more striking is our observation that cAMP-PDE inhibitors affected $[Ca^{2+}]_{cyt}$ and $Ca^{2+}$ content in intracellular stores in an opposite manner. This observation strongly contrasts with the effect of ouabain (Figs 1 and 2; [11]) and the earlier observations [12] that an increase in $[Ca^{2+}]_{cyt}$ leads to a higher $Ca^{2+}$ content in intracellular stores. The observed decrease in $[Ca^{2+}]_{cyt}$ accompanied by an increase of $Ca^{2+}$ in intracellular stores on treatment with cAMP-PDE inhibitors, implies that the apparent equilibrium between $[Ca^{2+}]_{cyt}$ and $Ca^{2+}$ in intracellular stores can be disrupted by cAMP-PDE inhibitors. These data therefore clearly evidence that although $[Ca^{2+}]_{cyt}$ decreased, the uptake in intracellular stores, mediated by the $Ca^{2+}$ pump activity, was increased and confirm the observations from experiments on cAMP-stimulated $Ca^{2+}$ transport activity on purified sarco(endo)plasmic reticulum [10, 18, 19].

The fact that increased uptake of $Ca^{2+}$ into intracellular stores co-exists with a decreased level of cytosolic $Ca^{2+}$ further implies, at least, that the latter store is not replenished up to normal homeostatic levels by uptake of extracellular $Ca^{2+}$. Whether additional extrusion of cytosolic $Ca^{2+}$ through the plasma membrane by means of $Ca^{2+}$ ATPases or $Na^+/Ca^{2+}$ exchange activity is required to maintain the decreased $[Ca^{2+}]_{cyt}$, cannot be decided from these experiments.

The putative involvement of cAMP in mediating the changes in $Ca^{2+}$ homeostasis is illustrated by the elevation of platelet-cAMP on treatment with ouabain and milrinone (Table 1). The lack of effect of enoximone on platelet $Ca^{2+}$ homeostasis, fits with the minor changes in platelet cAMP content.

In conclusion, the combination of the CTC and quin-2 method to monitor $Ca^{2+}$ in intracellular stores and in the cytosol, respectively enabled us to identify profound effects of cAMP-PDE inhibitors on $Ca^{2+}$ homeostasis of intact resting platelets. On the one hand, the increased accumulation of $Ca^{2+}$ in intracellular stores of the platelet, reflects the hypothesized mechanism of positive inotropy in cardiac tissue, much like ouabain. On the other hand, and in contrast to ouabain, the particular feature of cAMP-PDE inhibitors, to reduce $[Ca^{2+}]_{cyt}$ presumably by enhanced removal of activator $Ca^{2+}$ from the cytosol, most likely explains the increased rate of relaxation (lusitropic effect) of the heart on treatment with these drugs.

Furthermore, the striking similarities between the effects of drugs measured on $Ca^{2+}$ homeostasis of intact platelets and their proposed effects in the cardiac cells points to the involvement of the same biochemical entities in regulation of $Ca^{2+}$ homeostasis in both cell types.

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