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Effects of the antithrombitic agent PCA 4230 on agonist-induced Ca^{2+} entry and Ca^{2+} release in human platelets

Maria Teresa Alonso, Carlos Villalobos and Ana Sanchez

Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, Valladolid (Spain)

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We have studied the effects of the antithrombitic agent PCA 4230 on the entry of Mn^{2+}, used here as a Ca^{2+} surrogate for Ca^{2+} channels, and on the release of Ca^{2+} from the intracellular stores in stimulated human platelets loaded with fura-2. PCA 4230 prevented receptor-operated calcium entry activated by thrombin, ADP and collagen with no modification of the Ca^{2+} release from the intracellular stores. PCA 4230 also inhibited cytochrome *P*-450-mediated *O*-dealkylase activity with the same concentration-dependence as the thrombin-induced Mn^{2+} entry. These results suggest that the inhibitory effects of PCA 4230 on Ca^{2+} influx may be due to its interaction with cytochrome *P*-450, which has been proposed recently to be involved in the activation of receptor-operated Ca^{2+} channels. In addition, PCA 4230 inhibited both PAF-induced Ca^{2+} entry and Ca^{2+} release, behaving as a PAF-antagonist. All these effects contribute to explain the antithrombitic action of PCA 4230.

Introduction

The increase of the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) plays a central role in platelet activation [1]. Platelet agonists induce an increase of $[Ca^{2+}]_i$ which is due to both mobilization of Ca^{2+} from the intracellular stores to the cytoplasm and Ca^{2+} entry through the plasma membrane [2]. Mobilization of Ca^{2+} is mediated by inositol 1,4,5-trisphosphate, produced on activation of phospholipase C [3]. Calcium entry takes place through receptor-operated Ca^{2+} channels (ROCC) whose characteristics are poorly known at present [4].

PCA 4230 is a new dihydropyridine derivative with potent in vivo and in vitro antithrombitic activity and weak cardiovascular effects [5]. Here we have studied the effects of PCA 4230 on Ca^{2+} movements induced by several agonists in fura-2-loaded platelets. A new procedure was used which allows simultaneous and independent monitoring of calcium release from the intracellular stores and activation of ROCC [6,7].

Experimental

Platelets were prepared from fresh blood, loaded with fura-2 (Molecular Probes) as described previously [3] and suspended at $2 \cdot 10^8$ cells/ml in a solution containing (in mM): NaCl, 145; KCl, 5; $MgSO_4$, 1; glucose, 10; Na-Hepes 10, pH 7.4. Mn^{2+}, used here as a Ca^{2+} surrogate for plasma membrane Ca^{2+} channels [6–8], was added to the cell suspension 1 min before agonists. Fura-2 fluorescence was measured at three excitation wavelengths, 340, 360 and 380 nm, with the emission set at 530 nm [9]. This allowed independent calculation of $[Ca^{2+}]_i$ (from the ratio of the fluorescences excited at 340 and at 380 nm, Ref. 9) and the entry of Mn^{2+} (from the decay of the fluorescence excited at 360 nm, which is insensitive to Ca^{2+} but quenched by Mn^{2+}, Ref. 8). Alternatively, Ca^{2+} movements were followed using the dual emission dye indo-1 [9], which was loaded into the cells in the same way as fura-2. Measurements were performed at 360 nm excitation and 410 and 485 nm emission and $[Ca^{2+}]_i$ was estimated from the ratio of both fluorescence emissions. The uptake of Mn^{2+} was calculated from the quenching of both fluorescences (F_{tot}). For these purposes F_{tot} was estimated as $F_{410} + k \cdot F_{485}$ where k is a

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; EGTA, [ethylenbis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAF, platelet-activating factor, (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine); ROCC, receptor-operated calcium channel.

Correspondence: A. Sanchez, Departamento de Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005-Valladolid, Spain.

proportionality factor that cancels out the sensitivity of F_{ion} to $[Ca^{2+}]$ [7].

PCA 4230 (ALTER Laboratories, Spain) was stored in the dark at room temperature and fresh solutions were prepared every day in dimethylsulfoxyde. Thrombin and PAF were purchased from Calbiochem-Behring, collagen from Horm (Germany), and ADP from Boehringer (Germany).

Results and discussion

Fig. 1 shows the effects of thrombin on the entry of Mn^{2+} and on $[Ca^{2+}]_i$ in platelets loaded with either fura-2 (left) or indo-1 (right). The incubation medium was nominally Ca^{2+}-free and contained 0.2 mM Mn^{2+}. This allowed simultaneous monitoring of the activity of ROCC (quenching of fluorescence by Mn^{2+} entry, upper panels) and mobilization of Ca^{2+} from the cellular stores ($[Ca^{2+}]_i$ increase, lower panels). Thrombin produced both, acceleration of Mn^{2+} uptake and release of Ca^{2+} from the intracellular stores. PCA 4230 inhibited the acceleration of Mn^{2+} uptake (Fig. 1A) without significant modification of the release from the stores (Fig. 1B).

PCA 4230 absorbs light with a maximum near 340 nm. This distorts the excitation spectrum of fura-2. These artifacts were corrected by calibrating the fluorescence signal within a cell suspension containing the same PCA 4230 concentration as that used in the experiments. In order to check that these corrections were adequate, the experiments were repeated in indo-1-loaded cells. Even though PCA 4230 also quenched measurably indo-1 fluorescence, the shape of the emission spectrum was little modified. Since the calculation of $[Ca^{2+}]_i$ is done, in this case, from the ratio of two emission readings, quenching had little effect on the final outcome. Fig. 1C shows that PCA 4230 prevented the thrombin-induced quenching of fluorescence by entering Mn^{2+} in indo-1-loaded platelets (Fig. 1C) with little effect on the increase of $[Ca^{2+}]_i$ (Fig. 1D). These results confirm the validity of the measurements performed in fura-2-loaded cells and indicate that PCA 4230 selectively inhibits the activation by thrombin of the plasma membrane Ca^{2+} (Mn^{2+}) pathway without interfering with the release of Ca^{2+} from the intracellular stores.

In order to investigate whether the inhibitory effect of PCA 4230 required time to develop, the cells were incubated with the inhibitor for different periods of time before thrombin addition. With 50 μM PCA 4230 no inhibitory effect was found with the shortest preincubation period (7 s) whereas inhibition was maximum after 5 min preincubation. With 5 μM PCA 4230 maximum inhibitory effect required even longer times to develop (10–15 min, results not shown). The release of Ca^{2+} from the intracellular stores was not modified by PCA 4230 in any of the conditions tested. Fig. 2 shows the inhibition of thrombin-induced Mn^{2+} influx by different concentrations of PCA. In all the cases the cells were preincubated with the drug for 5 min before

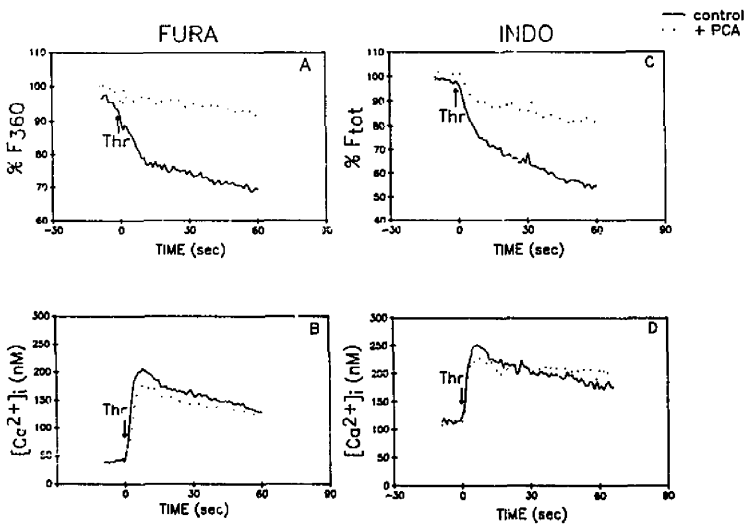

Fig. 1. Effects of PCA 4230 on the uptake of Mn^{2+} (A, C) and on the release of Ca^{2+} from the intracellular stores (B, D) in fura-2-loaded (left) or indo-1-loaded human platelets (right) stimulated with thrombin. The incubation medium, nominally Ca^{2+}-free, contained 0.2 mM $MnCl_2$. Platelets were incubated with 50 μM PCA 4230 (dotted lines) for 5 min prior to addition of thrombin, 1 U/ml. Fluorescence decays (F_{360} and F_{ion}) were normalized to 100% at the time of thrombin addition.

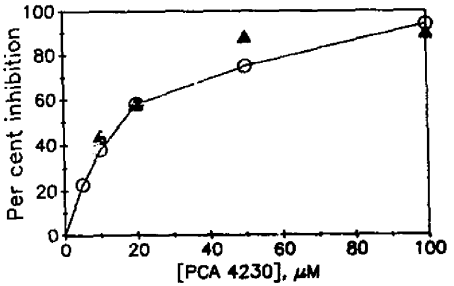

Fig. 2. Inhibition by PCA 4230 of thrombin-induced Mn^{2+} entry in human platelets (circles) and of ethoxyresorufin *O*-dealkylase activity of liver microsomes (triangles). Mn^{2+} uptake was measured as in Fig. 1A. The enzyme activity was assayed as described elsewhere [10,11]. Cells were preincubated with PCA 4230 for 5 min before addition of thrombin or ethoxyresorufin.

thrombin addition. The inhibition was concentration-dependent, half-maximal effect being reached at about 16 μM.

The effects of 50 μM PCA 4230, preincubated with the cells for 5 min before agonist addition, were also tested in platelets stimulated with either platelet activating factor (PAF), ADP or collagen (Fig. 3). As reported before [6], all these three agonists produced both, increase of Mn^{2+} uptake (upper panels) and mobilization of Ca^{2+} from the intracellular stores (lower panels). As shown above for thrombin, PCA 4230 (dotted lines) prevented the acceleration of Mn^{2+} uptake induced by all the agonists tested (Fig. 3A). Ca^{2+} mobilization from the stores was not affected, except when PAF was the stimulus (Fig. 3B). This finding may reflect additional activity of PCA 4230 as a PAF antagonist, which is consistent with previous reports [12].

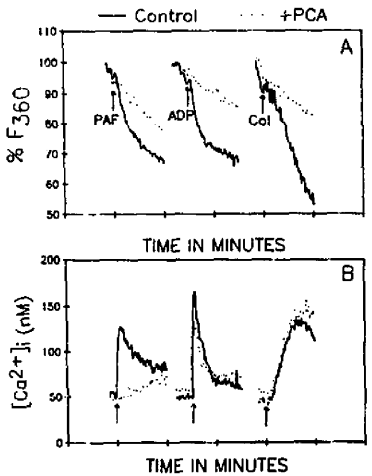

Fig. 3. Effect of PCA 4230 on the uptake of Mn^{2+} (A) and on the release of Ca^{2+} from the intracellular stores (B) induced by PAF, ADP and collagen in fura-2-loaded human platelets. Concentrations used were 20 ng/ml PAF, 10 μM ADP and 10 μg/ml collagen. Other details as in Fig. 1.

As a whole, the above results indicate selective antagonization of ROCC by PCA 4230, with no interference with the mechanisms responsible for Ca^{2+} release. This selectivity is unusual since most platelet inhibitors simultaneously antagonize both mechanisms [1,6,8]. Cytochrome *P*-450 inhibitors are a conspicuous exception since they selectively block plasma membrane Ca^{2+} influx [13]. It has been proposed recently that activation of plasma membrane Ca^{2+} entry by agonists may be indirect, secondary to the emptying of the intracellular Ca^{2+} stores. Under this view emptying of the stores by agonists (or by other means) would activate a microsomal cytochrome *P*-450, which would, in turn, promote the opening of plasma membrane Ca^{2+} channels [14]. These mechanisms have been documented to be responsible for agonist-induced Ca^{2+} entry in human neutrophils [15] and platelets [13].

The above findings are consistent with the effect of PCA 4230 on Ca^{2+} influx being due to an action on cytochrome *P*-450. In order to investigate this view, we tested the effects of PCA 4230 on cytochrome *P*-450-dependent 7-ethoxyresorufin dealkylation by liver microsomes. PCA 4230 inhibited dealkylation at concentrations close those found to inhibit thrombin-induced Mn^{2+} influx. These results have been shown without comment in Fig. 2, where they are compared with the inhibition of the thrombin-induced Mn^{2+} entry. Inhibition of agonist-induced Ca^{2+} entry by PCA 4230 may contribute to explain the antithrombitic effects of this drug in vivo [16].

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