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Effects of dihydropyridines and inorganic calcium blockers on aggregation and on intracellular free calcium in platelets

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$[Ca^{2+}]_i$ increase is necessary in physiological platelet activity, particularly aggregation and release. The increase of $[Ca^{2+}]_i$ observed during platelet activation depends in part on Ca^{2+} influx from the extracellular medium. The participation of voltage-operated Ca^{2+} channels as a pathway for Ca^{2+} entry is controversial. In the present study we have attempted to reinvestigate this problem by measuring aggregation and $[Ca^{2+}]_i$ changes in platelets activated by ADP or thrombin and incubated with organic or inorganic blockers of calcium channels. The main findings of the present paper can be summarized as follows: (i) Ni^{2+}, Co^{2+} and Mn^{2+}, well known inorganic blockers of Ca^{2+} channels, inhibited platelet aggregation induced by ADP or thrombin in a dose-dependent manner, Ni^{2+} being the most effective agent. (ii) Thrombin induced a rise in free $[Ca^{2+}]_i$ in platelets incubated both in 1 mmol/l Ca^{2+}-containing medium and in nominally Ca^{2+}-free medium; the rise of free $[Ca^{2+}]_i$ was in the first case up to 370 ± 31 nmol/l and in the second case up to 242 ± 26 nmol/l, indicating that this observed difference was due to Ca^{2+} entry from the extracellular medium. Co^{2+} and Ni^{2+} abolished that difference by inhibiting Ca^{2+} influx. (iii) Nisoldipine, nitrendipine and nimodipine (10–50 nmol/l) inhibited in a dose-dependent manner platelet aggregation induced by either ADP or thrombin in platelets incubated in normal-Ca^{2+} normal-K^+ medium, also, aggregation was inhibited to a similar extent in platelets incubated in normal-Ca^{2+} high-K^+ medium. (iv) Nisoldipine – the most effective dihydropyridine to inhibit platelet aggregation – also inhibited Ca^{2+} influx in platelets incubated in normal-Ca^{2+} medium, either in normal-K^+ or high-K^+ media. Our data support the existence of voltage-operated, dihydropyridine-sensitive calcium channels (L-type) and a physiological role for them in platelet function.

Introduction

Ca^{2+} has a central role in platelet activation. Cytosolic free calcium ($[Ca^{2+}]_i$) increase is observed in physiological platelet activity, i.e., aggregation and release reaction. The intracellular increase of Ca^{2+} observed during platelet response results both from the internal calcium release and from calcium influx, in different proportions depending on the agonist [1]. The major pathway for calcium entry in a great variety of cells are receptor-mediated and voltage-gated Ca^{2+} channels [2–4] as well as Na^+/Ca^{2+} exchange [5]. The role of Na^+/Ca^{2+} exchange has been described in platelets [6] but its role in platelet Ca^{2+} transport is still unclear [7]. It is known that at least three classes of the receptor-mediated calcium entry are involved in platelet function [8], although the presence of voltage-operated Ca^{2+} channels in these cells is controversial [9–12].

Platelets suspended in physiological medium show a resting membrane potential (V_m) of about -60 mV [13–15], and changes in this V_m modify the platelet aggregating response [15]. It has recently been pointed out that voltage-gated K^+ channels appear to play a role in setting the resting potential [16,17]. On the other hand it is well established that ADP and thrombin induce platelet depolarization mediated by changes in Na^+ influx [18–20]. Consistent with these findings we have reported that low Na^+ media or the presence of tetrodotoxin inhibit not only platelet aggregation induced by the same agonists but also the increase in $[Ca^{2+}]_i$ [21]. As a whole, these data lend support to the

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notion that a voltage-depend mechanism (voltage-operated Ca^{2+} channels?) in involved in the entry of Ca^{2+} in platelets. It has been reported that high $[K^+]_o$, which induces platelet membrane depolarization, increases the thrombin-induced Ca^{2+} influx, but not resting $[Ca^{2+}]_i$ [22]. This finding would indicate, however, that depolarization may be necessary to activate the voltage-operated pathway for Ca^{2+} entry but that an additional signal, probably generated by the platelet agonists, is required. In another approach to this problem, several authors have used blockers of voltage-operated Ca^{2+} channels, with controversial results. Thus, while Rink [23] and Zschauer et al. [24] found no effect of dihydropyridines, which have specific affinity for L-type Ca^{2+} channels [25], on platelet activity or Ca^{2+} influx, other authors [26–28] found the opposite, i.e., an inhibition of platelet aggregation and Ca^{2+} influx in dihydropyridine-treated platelets.

In the present study we have attempted to shed light on this controversy. We have studied platelet aggregation and $[Ca^{2+}]_i$ changes induced by ADP or thrombin in normal-K^+ and high-K^+ depolarized platelets. These experiments were performed with the use of three different inorganic blockers of Ca^{2+} channels and three different dihydropyridines, and they allow us to obtain evidence in support of a role of a voltage-operated dihydropyridine-sensitive Ca^{2+} channel in platelets.

Materials and Methods

Preparation of platelets. Human blood was obtained from healthy volunteers of both sexes, ranging in age from 21 to 32 years. They had not taken antiplatelet drugs for at least 2 weeks. Blood was anticoagulated with 1/6 vol. acid citrate/dextrose (ACD). Blood was centrifuged at $700 \times g$ for 10 min to obtain platelet-rich plasma (PRP). Platelets were obtained from PRP as described elsewhere [15] and resuspended in a standard medium containing (in mmol/l): NaCl (140), KCl (5.4), $MgCl_2 \cdot 6H_2O$ (1.0, dextrose (10) and Hepes (10), maintained at 37°C, and pH 7.4. In K^+-induced depolarization experiments, platelet suspensions were performed substituting KCl for NaCl at the desired concentration. Isotonicity was maintained at 294–304 mosmol/kg H_2O and a pH value of 7.4. Platelets suspended in normal- or high-K^+ media were used after incubation with $MnCl_2$, $CoCl_2$, $NiCl_2$ at concentrations ranging from 10 to 100 μmol/l, and with nitrendipine, nisoldipine or nimodipine at concentrations ranging from 10 to 100 nmol/l, at room temperature for 15 min. Nitrendipine, nisoldipine and nimodipine were a generous gift from Prof. C. Gónzalez (Valladolid, Spain).

Aggregation experiments. Aggregation was assessed at 37°C with platelets suspended in the different media using Born's method [29] and constant stirring at 1100 rpm with a stirring bar (0.5 cm length). Aliquots of platelet suspensions (500 μl) were placed in siliconized glass cuvettes (diameter 0.8 cm) for use in a Chronolog 400 aggregometer equipped with a Yew recorder. The platelet count was adjusted to $2.5 \cdot 10^8$/ml, and 1.0 mmol/l $CaCl_2$ was added. In ADP-induced platelet aggregation 0.5 mg/ml fibrinogen (Kabi, Sweden) was added. Final concentrations of 5 μmol/l ADP (Stago, Asnières, France) and thrombin (Behring) 0.1 U/ml were used in saline in a volume of 10 μl. Platelet aggregation was evaluated by measuring, in each case, the maximal deflection obtained after 5 min of curve registration computed as a percentage of maximal aggregation.

Measurement of cytosolic free Ca^{2+}. The measurement of $[Ca^{2+}]_i$ was performed using the fluorescent calcium indicator fura-2 according to Pollock [30]. Platelets obtained as above were loaded with 1 μmol/l fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) and washed as described previously [21]. The cell count was adjusted to $2.5 \cdot 10^8$ platelet/ml. Fura-2 fluorescence was measured at 37°C in a Kontron SFM-50 spectrofluorimeter with 340 nm excitation and 500 nm emission in the presence and in the absence of 1 mmol/l Ca^{2+}. $[Ca^{2+}]_i$ values monitored by observed fluorescence were calculated as described elsewhere [21,30].

Measurement of platelet membrane potential (V_m). In the experiments carried out with high extracellular K^+, V_m was monitored using the fluorescent potentiometric dye diS-C_3(5) (Nippon, Kankoh-Shikiso, Kenkuysho, Japan) as described previously [15]. The V_m for 5.4, 20 and 40 mM extracellular K^+ correspond to -63.8 ± 5.6, -50.2 ± 5.8 and -40.6 ± 4.6 (mean $\pm$ S.D.; $n = 10$), respectively [15]. All the V_m values obtained in the present study were in this range.

Results

Effects of inorganic blockers of Ca^{2+} channels on platelet aggregation and on $[Ca^{2+}]_i$ level

Fig. 1 shows the effects of Co^{2+}, Ni^{2+} and Mn^{2+} on platelet aggregation in normal-K^+ (V_m, -63.8 mV and high-K^+ depolarized platelets (V_m, -40.6 mV). Control platelets activated by ADP 5 μmol/l or thrombin 0.1 U/ml showed a significantly inhibited aggregation when divalent cations were present in the medium at doses ranging from 10 to 100 μmol/l ($P < 0.001$ in all cases). Ni^{2+} was the cation with largest capacity to block this aggregating response. High-K^+ depolarized platelets (V_m, -40.6 mV) showed a significantly higher ($P < 0.001$) aggregating response to ADP or thrombin than platelets suspended in normal-K^+ medium. In these high-K^+ depolarized platelets, divalent cations also inhibited the aggregating response induced by ADP or thrombin but by a smaller percentage.

The basal level of $[Ca^{2+}]_i$ in resting platelets was

101 ± 15 nM (mean ± S.D., $n = 11$) both in Ca^{2+} containing and in nominally Ca^{2+}-free medium. In control Ca^{2+} containing saline, 0.1 U/ml thrombin induced a rise in $[Ca^{2+}]_i$ from the basal level to 370 ± 31 nmol/l ($P < 0.001$), but in platelet suspended in Ca^{2+}-free medium the $[Ca^{2+}]_i$ increase, due to a Ca^{2+} mobilization from intracellular stores, was only up to 242 ± 26 nmol/l ($P < 0.001$). Thus, the difference between the two levels should represent Ca^{2+} entry from the extracellular medium (Fig. 2A). In resting platelets suspended in control Ca^{2+} 1 mmol/l containing saline or in Ca^{2+}-free medium, Ni^{2+} or Co^{2+} at concentrations of 10, 50 and 100 μmol/l did not induce any significant change of fluorescence. On the contrary, in platelets activated by thrombin 0.1 U/ml in Ca^{2+}-containing medium, the same concentrations of Ni^{2+} and Co^{2+} reduced $[Ca^{2+}]_i$ to levels almost identical to those observed in Ca^{2+}-free medium indicating that they completely block the Ca^{2+} influx (Fig. 2B). The same protocol carried out in Ca^{2+}-free medium did not in-

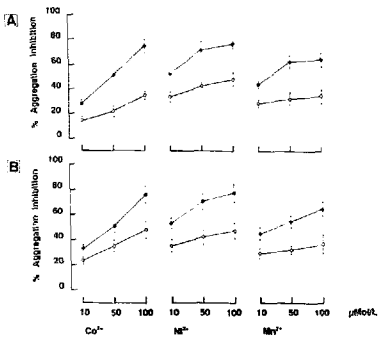

Fig. 1. Effects of divalent ions Co^{2+}, Ni^{2+} and Mn^{2+} on platelet aggregation induced by (A) ADP 5 μmol/l and (B) thrombin 0.1 U/ml in control platelets (●) and depolarized platelets (○). Ordinate represents inhibition of platelet aggregation in percentage; each point represents the mean of seven experiments ± S.D.

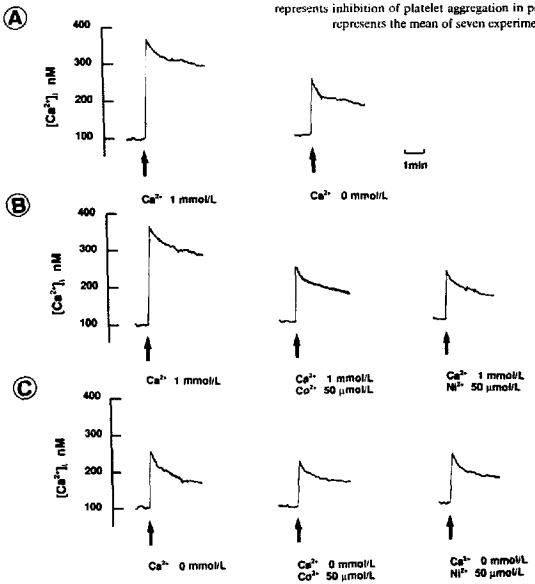

Fig. 2. Changes of $[Ca^{2+}]_i$ induced by thrombin (arrows) at final concentration 0.1 U/ml in (A): 1 mmol/l Ca^{2+}-containing medium and nominally Ca^{2+}-free medium, (B): 1 mmol/l Ca^{2+}-containing medium in the presence of Co^{2+} or Ni^{2+} 50 μmol/l, and (C): nominally Ca^{2+}-free medium in the presence of Co^{2+} or Ni^{2+} 50 μmol/l. The traces of each group shown are from the same batch of platelets and are replicates of single representative recordings.

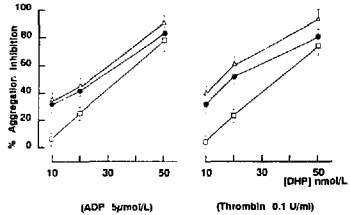

Fig. 3. Effects of dihydropyridines (DHP): nitrendipine (●), nisoldipine (△) and nimodipine (□) on platelet aggregation induced by ADP 5 μmol/l and thrombin 0.1 U/ml. Ordinate represents inhibition of platelet aggregation in percentage; each point represents the mean of seven experiments ± S.D.

duce significant changes in Ca^{2+} peak with respect to their controls in Ca^{2+}-free medium (Fig. 2C). For well known technical reasons [31], Mn^{2+} could not be used in these series of experiments.

Effects of dihydropyridines on platelet aggregation and on free $[Ca^{2+}]_i$ level

The effects of different concentrations of nimodipine, nitrendipine and nisoldipine on platelet aggregation induced by ADP and thrombin are shown in Fig. 3. Aggregation induced by 5 μmol/l ADP or 0.1 U/ml thrombin was inhibited by dihydropyridines in a dose-dependent manner. The IC_{50} of nisoldipine, nitrendipine and nimodipine were 21, 25 and 33 nmol/l, respectively, when ADP was used as inducer, and 15, 25, and 35 nmol/l, respectively, when the inducer was thrombin.

TABLE I

Effects of nisoldipine on platelet aggregation induced by ADP or thrombin in control and K^+-depolarized platelets

Results are expressed as % of maximal aggregation ± S.D. $n = 8$ in all cases. * $P < 0.01$; ** $P < 0.001$, with respect to the control value for each group.

Nisoldipine (nmol/l)	Aggregating inducer	Platelet aggregation: $[K^+]_o$, (V_m)	
		5.4 mmol/l (−63.8 mV)	40 mmol/l (−40.6 mV)
0	ADP (5 μmol/l)	21.03 ± 3.0	39.1 ± 3.5
10		15.1 ± 4.1 (28%) *	17.3 ± 3.6 (56%) *
20		11.3 ± 3.3 (45%) **	15.2 ± 2.1 (61%) **
50		2.1 ± 1.9 (90%) **	4.1 ± 1.2 (90%) **
0	Thrombin (0.1 U/ml)	58.5 ± 2.9	70.5 ± 3.8
10		37.5 ± 4.3 (36%) *	39.1 ± 4.2 (44%) *
20		23.7 ± 2.5 (61%) **	28.2 ± 3.1 (60%) **
50		4.2 ± 2.8 (93%) **	8.4 ± 3.2 (88%) **

Nisoldipine, the most effective dihydropyridine found in these experiments, was assayed in high-K^+ depolarized platelets. Table I shows the effects of 10, 20 and 50 nmol/l nisoldipine on platelet aggregation induced by 5 μmol/l ADP and 0.1 U/ml thrombin in normal and high-K^+ depolarized platelets. In control platelets nisoldipine significantly inhibited ($P < 0.001$) the platelet aggregation induced by ADP (28%) and by thrombin (36%). The same dose of nisoldipine significantly inhibited ($P < 0.001$) the enhanced platelet aggregation of control-K^+ depolarized platelets induced by either ADP (56%) or thrombin (44%). It is interesting to note that the absolute value of the inhibition of aggregation was similar for each inducer, ADP and thrombin, in both control and K^+-depolarized platelets.

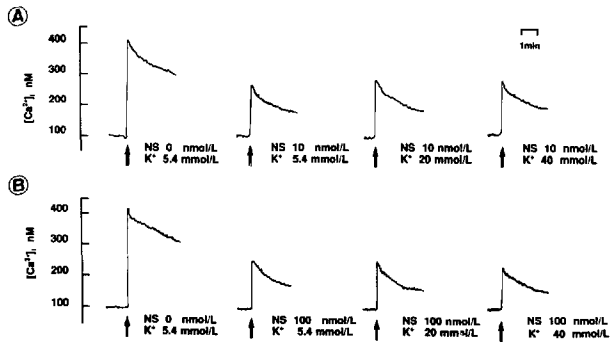

Fig. 4. Changes of $[Ca^{2+}]_i$ induced by thrombin (arrows) at final concentration 0.1 U/ml in platelets suspended in media containing different extracellular $[K^+]$ in (A): the presence of nisoldipine 10 nmol/l and (B) the presence of nisoldipine 100 nmol/l. The traces of each group shown are from the same batch of platelets and are replicates of single representative recordings.

Fig. 4A shows sample recordings of the effects of nisoldipine 10 nmol/l and Fig. 4B of nisoldipine 100 nmol/l on the free $[Ca^{2+}]_i$ level in thrombin-activated platelets. Nisoldipine produced a significant inhibition of the $[Ca^{2+}]_i$ rise induced by thrombin in platelets suspended in control normal-Ca^{2+} normal-K^+ medium. In two batches of thrombin-activated K^+-depolarized platelets (V_m −50.2 mV and −40.6 mV, respectively), the same doses of nisoldipine also induced a significant inhibition of the $[Ca^{2+}]_i$ rise. Note that at 10 nmol/l, nisoldipine produced a maximal effect, completely abolishing the influx of Ca^{2+} and rendering $[Ca^{2+}]_i$ at levels observed in Ca^{2+}-free medium or in Ca^{2+}-containing medium in the presence of inorganic blockers.

Discussion

The results presented in Fig. 1 of this study show that well-known inorganic Ca^{2+}-channel blockers Ni^{2+}, Co^{2+} and Mn^{2+} inhibit platelet aggregation induced by ADP and thrombin in a dose-dependent manner. Also, these blockers inhibit the $[Ca^{2+}]_i$ rise induced by thrombin, both in platelets incubated in 1 mmol/l Ca^{2+}-containing medium and in nominally Ca^{2+}-free medium (Fig. 2). Of the inorganic channel blockers tested, Ni^{2+} was the most effective in inhibiting platelet aggregation. The presence of inorganic channel blockers does not affect the basal $[Ca^{2+}]_i$ level observed in non-stimulated platelets suspended in 1 mmol/l Ca^{2+}-containing medium or that observed in platelets suspended in nominally Ca^{2+}-free medium. These data indicate the ability of Ni^{2+} and Co^{2+} to inhibit extracellular Ca^{2+} influx. These results agree with previous observations [24,26,32] and indicate once again the presence of functional calcium channels in human platelets. However, these data do not allow us to assume voltage-operated or receptor-operated characteristics, because divalent cations, Ni^{2+} and Co^{2+}, block both types of channels [25,33].

Nisoldipine, nitrendipine and nimodipine at the nanomolar range inhibit in a dose-dependent manner and to a similar extent platelet aggregation induced by either ADP or thrombin in normal-Ca^{2+} normal-K^+ incubated platelets or in normal-Ca^{2+} high-K^+ media. Nisoldipine, the most effective dihydropyridine to inhibit platelet aggregation, also inhibits Ca^{2+} influx in platelets incubated in normal-Ca^{2+} medium, with normal-K^+ or high-K^+ medium. High K^+ per se in the absence of either inducer or nisoldipine did not induce changes in $[Ca^{2+}]_i$. Also, at 10 nmol/l nisoldipine produces its maximum effect, completely abolishing the influx of Ca^{2+} and rendering $[Ca^{2+}]_i$ at levels observed in Ca^{2+}-free medium or in Ca^{2+}-containing medium in the presence of inorganic blockers.

It has been reported that dihydropyridines fail to block platelet activity [23,24]. Other authors observed that nitrendipine antagonizes the $[Ca^{2+}]_i$ increase induced by ADP, adrenaline, thrombin and CGP-28392, a dihydropyridine calcium ionophore in platelets [27], as well as in other cells [34]. The effects observed in our experiments indicate that dihydropyridines are able to act as effective Ca^{2+}-channel blockers in platelets. Perhaps the discrepancy between present data and those aforementioned could be explained by the different doses of inducers used.

Dihydropyridines inhibited platelet aggregation to a greater extent than did Ca^{2+} rise. This observation could be explained from other complex effects of dihydropyridines reported in platelets, such as the inhibition of cyclic AMP phosphodiesterase [35], inhibition of TXA_2 [32], α-adrenergic receptor antagonism [36] and particularly the inhibition of fibrinogen binding [37].

However, it should be noted that binding studies have shown that human platelets seemed to be devoid of specific receptors for dihydropyridines [38]. However, it has been reported for other tissues that the affinity of dihydropyridines for calcium channels is conditioned by their functional state [39,40]. In this sense, it has been shown in adrenomedullary tissue that [^{3}H]nitrendipine binding depends on V_m and is considerably enhanced by depolarization [39]. The dihydropyridine binding experiments carried out in human platelets were performed only on resting platelets. Furthermore, the fact that high K^+ itself does not increase $[Ca^{2+}]_i$ would indicate that in addition to V_m changes, another signal (the agonist (e.g., ADP or thrombin) or a intracellular signal generated by the agonist) is required. This additional signal might be necessary for the dihydropyridine interaction with the Ca^{2+} channel. Thus, the lack of evidence for specific dihydropyridine receptors in human platelets could be explained in this manner and, therefore, should be reinvestigated.

It is known that in different tissues dihydropyridines at nanomolar concentrations are able to block the voltage-operated Ca^{2+} channels [41,42]. Under our conditions, the inhibitory effect obtained with such concentrations suggests that dihydropyridines may be acting on this type of Ca^{2+} channels. It has been described that some dihydropyridines such as nicardipine at the micromolar range are able to block Ca^{2+} influx induced by several agonists [28], this effect being attributed to receptor-operated calcium channel inhibition. However, it is necessary to point out that an inhibition of calcium flow through the voltage-operated calcium channel also occurs in some cases at μM concentrations [43,44]. In addition, several authors have suggested the presence of a vestigial population of voltage-operated Ca^{2+} channels in human platelets or the presence of a receptor operated Ca^{2+} channel whose permeability could be increased by membrane depolarization, as occurs in other cells [22,45].

In conclusion, the data presented in this study dem-

onstrate that dihydropyridine-sensitive mechanisms participate in the Ca^{2+} entry pathway during platelet activation and suggest that some population of L-type Ca^{2+} channels may be present in platelets.

Acknowledgements

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