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# ENHANCEMENT OF CALCIUM INFLUX IN HUMAN PLATELETS BY CGP 28392, A NOVEL DIHYDROPYRIDINE

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SUMMARY. CGP 28392, a novel compound structurally related to the dihydropyridine $Ca^{2+}$-entry blockers, causes a dose-dependent increase in intracellular free $Ca^{2+}$ in human platelets, as measured with the Quin-2 $Ca^{2+}$ indicator, with a semimaximal effective concentration of $2.2 \times 10^{-7}$ M. This effect occurs in a concentration range in which CGP 28392 competes for specific [$^3$H]nitrendipine binding in guinea pig heart membranes. It can be inhibited by nitrendipine. The data presented furnish direct evidence of the $Ca^{2+}$-entry- stimulating properties of CGP 28392 and indicate the presence of dihydropyridine-susceptible structures in human platelets.

Transmembranal influx of $Ca^{2+}$ through specific channels plays a key role in physiological regulations, e.g. of vascular smooth-muscle tone and the contractility of myocardial cells. Structurally different groups of specific $Ca^{2+}$-channel blockers, such as dihydropyridines (e.g. nifedipine), phenylalkylamines ( e.g. verapamil) and the benzothiazepine diltiazem, are known to reduce the influx of $Ca^{2+}$ through specific channels (1).

Recently, structural modification of dihydropyridine molecules led to the discovery of a novel class of compounds (2,3,4,) which act in the opposite way to $Ca^{2+}$-entry blockers. These agents (e.g. CGP 28392, see Fig.1) induce an increase in cardiac contractility and vasomotor tone , which can be competitively inhibited by $Ca^{2+}$- entry blockers. Thus, these putative $Ca^{2+}$-entry stimulators are believed to promote $Ca^{2+}$ influx via an action on specific

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Abbreviation: $[Ca^{2+}]_i$, intracellular free calcium.

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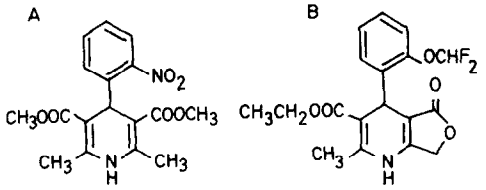


Fig.1 Chemical structure of nifedipine (A) and CGP 28392 (B)

sites linked to $Ca^{2+}$ channels. So far, direct evidence of an increase in $[Ca^{2+}]_i$ induced by these compounds is lacking.

Platelets afford a suitable model for in vitro investigations of hormone- and drug-induced changes in $[Ca^{2+}]_i$ using the Quin-2 fluorescent selective $Ca^{2+}$ chelator probe (5,6). We will show that CGP 28392 increases $[Ca^{2+}]_i$ by augmenting $Ca^{2+}$-influx in platelets, as measured by the Quin-2 method, and that this effect can be competitively inhibited by nitrendipine. It is also shown that this effect occurs at concentrations in which CGP 28392 competes for the binding of [$^3$H]nitrendipine at specific dihydropyridine-binding sites on cardiac membranes.

## MATERIAL AND METHODS

Materials Ketanserin was obtained from Janssen Pharmaceuticals, forskolin from Calbiochem and yohimbine from Böhringer Ingelheim. Quin-2 was kindly provided by Dr.T.Rink, Cambridge, UK. All other drugs were obtained fom the Chemistry Department of CIBA-GEIGY.

Measurement of free intracellular $Ca^{2+}$ in platelets. Citrated blood was obtained by venepuncture from healthy volunteers, who had fasted overnight and had taken no drugs during the previous six weeks. After centrifugation at 140 x g for 20 min at room temperature, platelets were separated from the platelet-rich plasma by gel filtration at room temperature on a sepharose 2B-CL column (Ø 44mm x 150 mm), eluted with 10 mM Hepes buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM $Na_2HPO_4$ and 6 mM glucose (pH 7.4 at 37°C). After gel filtration, the platelets ($2-3x10^8$ cells/ ml) were loaded with Quin-2 by incubation in the above buffer containing 5 µM Quin-2-tetra-acetoxymethylester for 30 min at 37°C. To remove extraneous dye, the platelets were again gel-filtered as described above and heated to 37°C. $CaCl_2$ (1mM) was added to restore extracellular calcium. This was monitored by recording the fluorescence at 340 nm excitation and 490 nm emission wavelength at 37°C in a spectrofluorometer. The platelets were lyzed with Triton X-100 (0.01%) to measure basal $[Ca^{2+}]_i$ (6). When equilibrium of $[Ca^{2+}]_i$ had been reached, drugs were added, and the change in fluorescence was recorded as above, until a new steady state was obtained. In order to relate the change in fluorescence induced by drugs to basal $[Ca^{2+}]_i$ levels, measurements in samples in the absence of drugs were interposed. For calculation of $[Ca^{2+}]_i$, the autofluorescence of drugs was subtracted. If drugs were used to check for possible interference with the rise in $[Ca^{2+}]_i$ induced by CGP 28392, they were preincubated for 10 min at steady state before CGP 28392 was added. Control experiments performed in the absence of platelets showed that none of the drugs changed fluorescence during the observation period.

Radioligand binding assay. Pirbright white, male guinea pigs (CIBA-GEIGY Animal Breeding Unit, Stein, Switzerland) were sacrified by cervical dislocation. Hearts were rapidly removed and cleaned of connective tissue, membranes were prepared as described (7) with the exception that $MgCl_2$, EDTA and phenylmethylsulfonylfluoride were omitted in the buffers used. The membranes were frozen in liquid nitrogen and stored at -80°C.

(±)[$^3$H]nitrendipine (New England Nuclear, 72-87 Ci/mmol) competition binding experiments were performed under sodium light. Membranes (protein 30-70 µg) were incubated for 30 min at 25°C with the radioligand and competitors in a final volume of 1 ml of 50 mM Tris, pH 7.4 . Kinetic experiments showed that under these conditions equilibrium was reached. After incubation, samples were rapidly diluted with 10 ml of ice-cold 50 mM Tris-HCl, pH 7.4, and filtered under vacuum through Whatman GF/C filters. The filters were washed twice with the same buffer and radioactivity was measured by liquid scintillation counting. Binding in the presence of $10^{-6}$M nitrendipine was defined as nonspecific. Protein was determined by a modified Lowry method (8), using bovine serum albumin as standard.

Data analysis. Values given are means ± standard deviation. Dose response curves were analyzed by nonlinear regression (9) and competiton binding experiments by a Mass Action Law Model for one and two binding sites (10).

## RESULTS AND DISCUSSION

The dihydropyridine derivative CGP 28392 ($10^{-6}$M) increased $[Ca^{2+}]_i$ in human platelets from basal levels of 119 ± 9 nM to 197 ± 20 nM (n=9). As shown in Fig.2A, this increase was dose-related, and the concentration needed to give a semimaximal increase in $[Ca^{2+}]_i$ was 220 ± 10 nM (n=4). The increase in free $Ca^{2+}$ induced by CGP 28392 reached a plateau in about 15 min regardless of the concentration used. In the presence of 6 x $10^{-9}$M nitrendipine

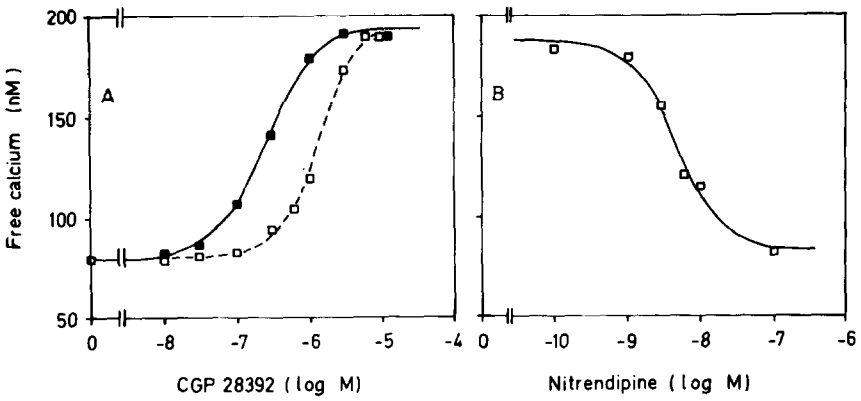


Fig.2 Increase in intracellular free calcium in human platelets induced by CGP 28392 and inhibition of this effect by nitrendipine. The data shown are representative of 2-4 similar experiments performed in duplicate each. A. Dose-dependent increase in free intracellular calcium induced by CGP 28392 alone (solid line) and in the presence of 6 x $10^{-9}$M nitrendipine (broken line) with mean semimaximal effective concentrations of 220 ± 10 nM (n=4) and 1480 nM (n=2). B. Inhibition of the CGP 28392 ($10^{-6}$M)-induced increase in intracellular free calcium by nitrendipine. The semi-maximal inhibitory concentration of nitrendipine was 6.3 ± 1.3 nM (n=3).

(Fig.2A) the dose-response curve of CGP 28392 was shifted to the right by a factor of 7. As shown in Fig.2B, nitrendipine-inhibition curves (n=3) with an $IC_{50}$ of 6.3 ± 0.7 nM and a slope factor close to unity (1.25 ± 0.21) indicate competitive interaction between these two compounds.

Ketanserin, yohimbine and forskolin in concentrations up to $10^{-5}$M did not inhibit the effect of $10^{-6}$M CGP 28392. This indicates that the rise of $[Ca^{2+}]_i$ is not due to an action of CGP 28392 on alpha-2 and serotonin receptors, nor is it related to a direct effect on adenylate cyclase. The latter assumption is in accordance with the observation that CGP 28392 does not change cAMP content in cultured myocardial cells (H. Porzig and H. Reuter, Bern, personal communication). Furthermore, it was found that CGP 28392 had no effect on beta and histaminergic receptors and did not inhibit $Na^+K^+$ ATPase (4).

Verapamil and diltiazem (up to $10^{-5}$M) did not alter the effect of CGP 28392. This could be due to different reasons: 1) It is assumed that they act distant from the dihydropyridine site (11), which may be lacking on human platelets. 2) The $Ca^{2+}$-influx-inhibiting potency of verapamil and diltiazem is dependent on depolarization of the membranes, the so-called "use dependence" (12), and thus the state of the platelet membrane may prevent access of these two drugs to their site of action. Up to $3x10^{-6}$M, the isomer of CGP 28392 with the $OCHF_2$ substituent in para- instead of ortho- position on the phenyl ring had no significant effect on $[Ca^{2+}]_i$. This is in accordance with the binding data (see Fig.3) and with the structure-activity relationship of dihydropyridine $Ca^{2+}$-entry blockers (13). It further indicates that the effect of CGP 28392 is not due to an unspecific action on the platelet membrane.

In the presence of 3mM EDTA, the CGP 28392-induced increase of $[Ca^{2+}]_i$ was reduced by 89 ± 3 % (n=7), showing that the increase of $[Ca^{2+}]_i$ is due mainly to $Ca^{2+}$ influx, rather than to redistribution of intracellular $Ca^{2+}$.

Although there have been no reports of successful direct identification of dihydropyridine-binding sites in human platelets by the radioligand

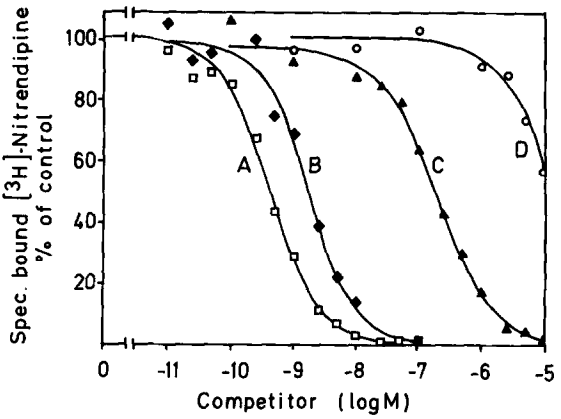


Fig.3 Inhibition of [$^3$H]nitrendipine binding to guinea pig heart membranes by nitrendipine (A), nifedipine (B), CGP 28392 (C) and the isomer of CGP 28392 with the $OCHF_2$-substituent in para- instead of ortho-position on the phenyl ring (D). Inhibition curves were determined in the presence of 150 pM [$^3$H]nitrendipine. The data given are representative of 3-4 experiments each assayed in duplicate. The following Kd values were obtained (mean ± SD): nitrendipine 0.204 ± 0.008 nM, nifedipine 0.799 ± 0.182 nM, CGP 28392 131 ± 16 nM.

binding technique (14), it has been found that therapeutic doses of nifedipine decrease platelet aggregation, providing evidence of the presence of dihydropyridine-sensitive structures in platelets (15). Assuming similar densities of $Ca^{2+}$-channels to those reported in cardiac cells (16), it can be calculated that a platelet may possess less than twenty $Ca^{2+}$channels per cell. Therefore, the failure to detect such putative structures by radioligand binding studies may be explained by the inadequate specific radioactivity of tritiated radioligands, which is insufficient for measuring such low numbers of binding sites. We consequently performed [$^3$H]nitrendipine binding studies in guinea pig heart membranes, yielding results similar to those found in other systems (17-19) in order to relate the affinity of CGP 28392 at specific dihydropyridine-binding sites to the $Ca^{2+}$-entry-stimulant properties of this compound. As shown in Fig.3, CGP 28392 inhibits [$^3$H]-nitrendipine binding in a dose-dependent manner, similarly to nifedipine or nitrendipine. From these experiments the dissociation constant of CGP 28392 has been calculated to be 131 ± 16 nM (n=4). The computerized data analysis was in accordance with a model implying one single class of binding sites. The concentration of CGP 28392 inhibiting the [$^3$H]nitrendipine binding in

guinea pig heart membrane is thus close to that found to raise $[Ca^{2+}]_i$ in human platelets. In summary, the present data show that CGP 28392 exerts $Ca^{2+}$-entry-stimulating effects in human platelets in concentrations similar to those at which it binds at specific [$^3$H]nitrendipine binding sites in cardiac membranes. The $Ca^{2+}$-stimulating effect can be antagonized by the $Ca^{2+}$-entry blocker nitrendipine. They also indicate the presence of dihydropyridine-susceptible structures in human platelets.

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