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MONITORING OF THE ACTIVATION OF RECEPTOR-OPERATED CALCIUM CHANNELS IN HUMAN PLATELETS

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Summary: Activation of receptor-operated calcium channels has been monitored by measurements of the quenching of the fluorescence of intracellularly trapped fura-2 by Mn entering from the extracellular medium. Release of calcium from intracellular stores was followed simultaneously by measurements of the ratio of the fluorescence excited at 340 and 380 nm. Thrombin, ADP, platelet-activating-factor (PAF) and collagen, all produced both release of calcium from the intracellular stores and uptake of Mn from the extracellular medium. The uptake of Mn, but not the increase of $(Ca^{2+})_i$, was blocked by nickel. These results suggest the existence of plasma membrane calcium channels which can be activated by the different agonists tested here. The activation of calcium channels was very fast and transient with ADP and PAF, fast and maintained with thrombin, and delayed with collagen.

Several agonists have been proposed to activate platelets by stimulating calcium entry on the basis either of measurements of ^{45}Ca uptake or of differences in the intracellular Ca^{2+} increase observed when the agonists were added in Ca^{2+}-containing or Ca^{2+}-free media (1-4). Calculations of initial rates of uptake from these data are difficult, however, either because of poor temporal resolution or because of simultaneous Ca^{2+} movements from other compartments (5). Incorporation of Ca^{2+} channels from membrane vesicles obtained from thrombin-stimulated platelets into planar lipid bilayers has been achieved recently (6). This approach demonstrates the existence of receptor-operated channels in a more conclusive way, but does not allow a study of the activation process in the intact cell.

It has been shown previously that thrombin and ADP induce manganese uptake in human platelets. This was evidenced by the quenching of the fluorescence of intracellularly loaded quin2 or fura-2 (7,8), excited at 340 nm. However simultaneous and reverse changes of fluorescence produced by modifications of $(Ca^{2+})_i$ prevented precise measurements. It has also been shown that Mn uptake can be followed in human endothelial cells by the quenching of the fura-2 fluorescence excited at 360 nm (9). We have taken

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advantage of the spectral characteristics of fura-2 to estimate independently the entry of Mn and the changes in $(Ca^{2+})_i$. Fluorescence from the cell suspension was excited simultaneously at three different wavelengths, 360, 340 and 380 nm. The first excitation wavelength is sensitive to Mn and not affected by changes of $(Ca^{2+})_i$ whereas the reverse applies to the ratio of the fluorescences excited at 340 and 380 nm. Alternatively, Mn uptake can also be estimated from the decrease of the corrected addition of fluorescences excited at 340 and 380 nm. Using this procedure the effects of several platelet agonists have been studied.

METHODS

Platelets were prepared from fresh blood anticoagulated with acid-citrate-dextrose as described previously(10). Apyrase (20 ug/ml) was added to prevent activation by traces of ADP during manipulation. Platelets were suspended at 10^8 cells/ml in Hepes-buffered saline solution containing no calcium (11) and loaded with fura-2 by incubation with 2 uM fura-2/AM (Molecular Probes, Eugene, OR, U.S.A.) for 45 min at 37ºC. The cells were then washed once to remove extracellular dye, resuspended at about 10^8 cells/ml in the same medium and used within 60 min. The experiments were performed at 37 ºC with magnetic stirring. Fluorescence was measured using a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Kent). The system can alternate up to six different excitation filters within 3-30 mseconds and separates the emission readings for each excitation wavelength. Time resolution was fixed at 1 second. The emission monochromator was a 530 (10 nm HWW) filter. This wavelength, somewhat longer than usual, was chosen to allow simultaneous measurements of intracellular pH using another dye. Excitation filters were set at 340, 360 and 380 nm (HWW, 10 nm).

RESULTS AND DISCUSSION

Fig. 1 shows the fluorescence records obtained at three excitation wavelengths in platelets stimulated by thrombin under two different conditions. In EGTA-containing medium (upper part) there was an increase of $(Ca^{2+})_i$ caused by release of calcium from the intracellular stores. This was shown by an increase of the fluorescence excited at 340 nm (F_{340}) and a symmetrical decrease of the fluorescence excited at 380 nm (F_{380}). The fluorescence excited at 360 nm (F_{360}) was not changed. The calibration at the end of the record shows again that F_{360} is not affected by (Ca^{2+}). The right side of the figure shows the $(Ca^{2+})_i$ peak, calculated from F_{340}/F_{380} (12). The lower part of the figure refers to platelets suspended in nominally Ca^{2+}-free medium (without EGTA). When Mn was added there was a rapid decrease in fluorescence at all the three wavelengths which reflects quenching of extracellular fura-2 by Mn followed by a slow decrease due to the entry of Mn to the cells under basal conditions. Addition of thrombin produced a fast increase of F_{340}, reflecting the increase of $(Ca^{2+})_i$ by release from intracellular stores, followed by a rapid decrease below the

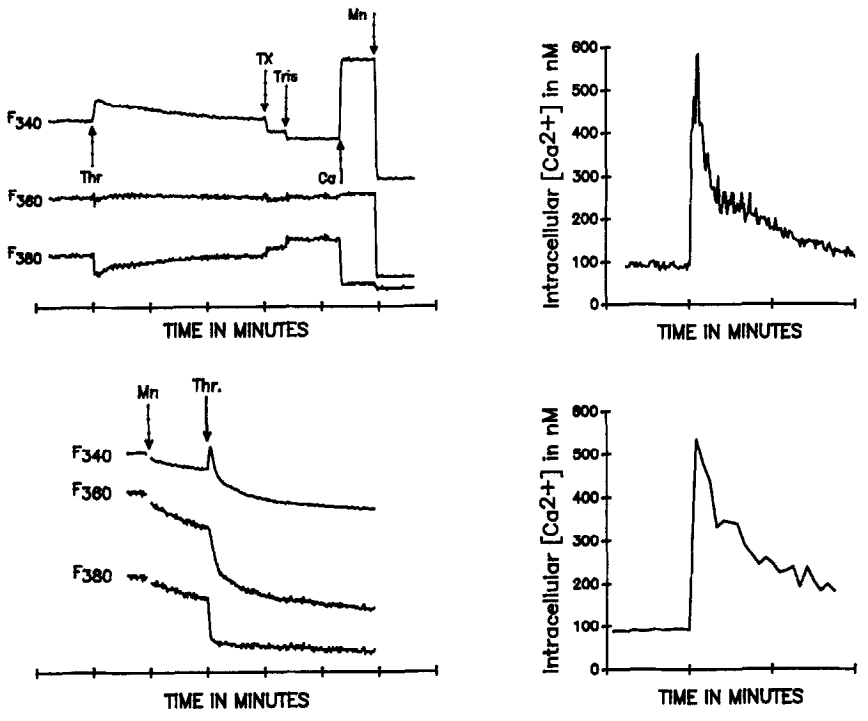

Fig. 1. Changes of fluorescence (left) and estimated $(Ca^{2+})_i$ (right) of fura-2-loaded platelets stimulated with thrombin. Fluorescence excited at 340, 360 and 380 nm (F_{340}, F_{360} and F_{380}, respectively) was measured simultaneously. In the upper part 1 mM EGTA was added about 2 min before thrombin. Other additions: thrombin, 1 U/ml (Thr), triton X-100, 5 % (TX), tris-base, 20 mM (Tris), $CaCl_2$, 2 mM (Ca), $MnCl_2$, 5 mM (Mn). In the lower part platelets were suspended in nominally Ca^{2+}-free medium. The experiment was started with the addition of 0.2 mM $MnCl_2$ followed 1 min later by thrombin. $(Ca^{2+})_i$ was calculated from the ratio F_{340}/F_{380} (12). Integration time for the signals was 1 second except for $(Ca^{2+})_i$ in the last experiment where 5 seconds integration time was used to reduce noise.

resting level, reflecting the quenching by entering Mn. F_{380} decreased very rapidly because at this wavelength both the increase of $(Ca^{2+})_i$ and the uptake of Mn have the same effect on fluorescence. Finally, F_{360} fell more slowly reflecting the real time course of Mn uptake. The value of $(Ca^{2+})_i$ calculated from the ratio F_{340}/F_{380} is shown on the right. It can be seen that the ratio continues to be a good measurement of $(Ca^{2+})_i$ even when Mn is quenching a part of the fluorescence. This is to be expected of the mechanism for fluorescence emission of these dyes and the relative values of K_d for Ca and Mn (12). Mn uptake could be equally well measured from the quenching of the "total fluorescence" calculated as $F_{tot} = (F_{340} + F_{380} \times K)$, where K stands for the ratio of the values of $(F_{max} - F_{min})$ obtained at 340 and 380 nm, respectively. This was checked in several experiments and, in general, F_{tot} was preferred over F_{360} to estimate Mn uptake.

Fig. 2 shows the effects of thrombin, ADP, PAF and collagen on $(Ca^{2+})_i$ and Mn uptake in human platelets. Three different conditions were

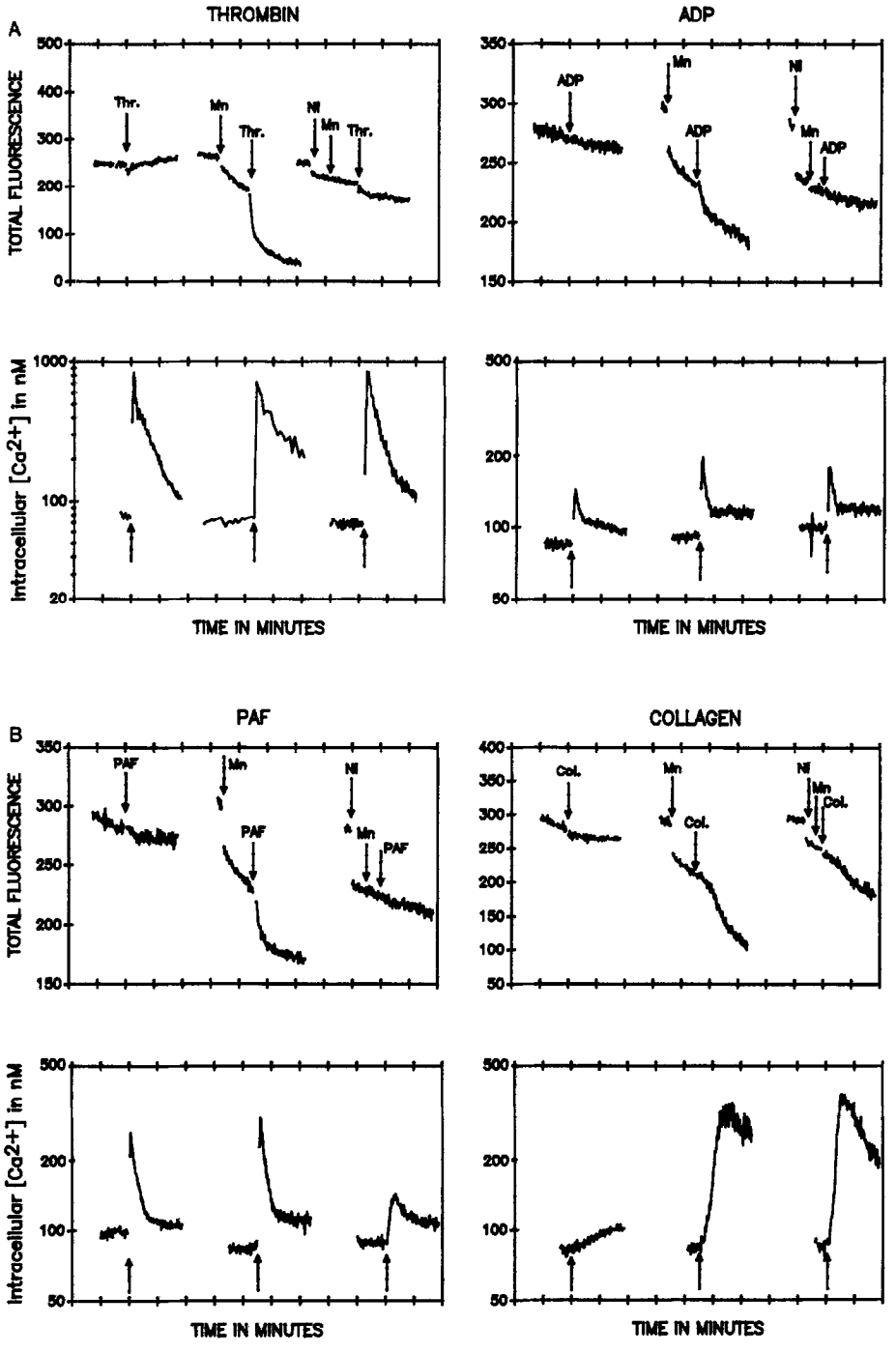

Fig. 2. Effects of thrombin (1 U/ml), ADP (20 uM), PAF (20 ng/ml) and collagen (10 ug/ml) on the quenching of fura-2 fluorescence by Mn and on $(Ca^{2+})_i$. Total fluorescence was estimated as $F_{340} + K \cdot F_{380}$ (see methods). The first record in each series, which contains no Mn, shows how changes of $(Ca^{2+})_i$ do not modify total fluorescence. Intracellular (Ca^{2+}) was estimated from F_{340}/F_{380}. Three pairs of records are presented for each agonist which correspond from left to right to measurements performed either in EGTA-containing medium, in nominally Ca^{2+}-free medium to which the first addition was Mn (0.2 mM), or in Ca^{2+}-free medium to which the first addition was Ni (5 mM).

tested (from left to right): Ca^{2+}-free (EGTA) medium, Mn-containing medium and (Ni+Mn)-containing medium. Ni has been reported to block calcium channels in platelets and other preparations (6-9,13). No calcium was added in any case. Thrombin and ADP produced similar $(Ca^{2+})_i$ peaks in all three conditions suggesting that release of calcium from the intracellular stores is not affected either by the uptake of Mn or by extracellular Ni. In the case of PAF the presence of Ni decreased the $(Ca^{2+})_i$ peak. Collagen did not produce a change of $(Ca^{2+})_i$ when added in EGTA medium, in agreement with previous reports (4,14). The inhibition by Ni of the effect of PAF could be fully overcome by increasing the concentration of the agonist. This suggests that Ni interferes either with the binding of PAF to the receptor or with an early step in the transducing mechanism. On the contrary, the results with collagen suggest that a divalent cation (Ca, Mn or Ni) is required for binding or transduction. This shows that removal of extracellular Ca^{2+} could have effects other than the obvious prevention of calcium entry and that the results of experiments performed in Ca^{2+}-free media should be interpreted with caution.

The uptake of Mn was accelerated by all four agonists, although the time courses differed. For ADP and PAF the activation was very fast and transient. The maximal rate of Mn uptake was usually reached within 1-2 seconds and returned rapidly to the basal value. With thrombin the maximum rate was reached within 1-4 seconds and its value was always smaller than that obtained with ADP or PAF in the same cell preparation. It was maintained near maximal values for at least 10 seconds and then returned slowly towards basal rates. With collagen there was a time lag of 15-20 seconds before any acceleration of Mn uptake could be detected and then the rate increased quickly towards maximal values and remained high for at least 20 seconds. Measurements after this period became unreliable because of microaggregation. In all the cases the effects on Mn entry were blocked by Ni, except, perhaps, for collagen where blockage may be incomplete. The acceleration of Mn uptake produced by PAF at concentrations high enough to produce maximal release of calcium from the intracellular stores was also completely blocked by Ni. The effects of all four agonists were also studied in medium containing 1 mM Ca^{2+}. All of them produced under this condition larger $(Ca^{2+})_i$ peaks than in EGTA medium, as reported previously (4). In all cases the $(Ca^{2+})_i$ changes obtained in (Ca+Ni) medium were the same as in media containing only Ni or (Ni+Mn).

The above results suggest that thrombin, ADP, PAF and collagen are all able to activate plasma membrane calcium channels in the human platelet. They do not give information on whether or not the channel population involved is the same for all the agonists tested. Kinetics of

activation/inactivation differed for each agonists, which suggests that, even if the channel population was the same, the activation mechanism could be different for each agonist. Our observations on kinetics are in agreement with the early changes in $(Ca^{2+})_i$ reported using stop-flow procedures (13). Finally, the present work shows that measurement of fura-2 fluorescence at two excitation wavelengths can be used to monitor simultaneously the release of calcium from intracellular stores and the entry of Mn, a calcium surrogate, from the extracellular medium.

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Note added in proof

After preparation of this manuscript a paper was published introducing much the same methodology and reporting similar results in human platelets stimulated by thrombin and ADP (Sage, S.O., Merrit, J.E., Hallam, T.J. and Rink, T.J. (1989) Biochem. J., 258, 923-926).

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