Comparative Aspects of Trypsin-Induced Changes in Blood Platelets Membrane Potential

Ľ. VAREČKA and J. POGÁDY

Laboratory of Psychiatric Research, Institute of Medical Bionics, Psychiatric Hospital, 902 18 Pezinok, Czechoslovakia

Abstract. Changes in the fluorescence of the potential-monitoring dye, 3, 3′-dipropylthiodicarbocyanine iodide, were measured in suspensions of thrombin or trypsin-activated blood platelets of several mammalian species. Fluorescence changes were shown to reflect changes in the membrane permeability for monovalent ions. The results suggest that in all species studied (man, rabbit, guinea-pig, and sheep) the platelet membrane becomes permeable for K^+ ions following activation. In all but the sheep platelets the increase in Na^+ permeability followed that of K^+ permeability. In human platelets it was demonstrated that the membrane becomes permeable for Na^+ ions when platelets were exposed to treatment which is known to increase the cytoplasmic Ca^{2+} concentration. The increase in Na^+ permeability could be inhibited by extracellular Ca^{2+} ions, and enhanced by their removal. The present results extend those obtained previously in experiments with pig platelets (Varečka et al. 1978) and suggest that the changes in the membrane potential and monovalent ion permeability are not causative factors of the platelet secretion. They accompany the secretion, being mediated by the rise in the cytoplasmic Ca^{2+} concentration.

Key words: Platelet activation — Membrane potential — Ca^{2+} involvement — Interspecies differences

Introduction

Experiments with various secretory cells showed that the secretion was accompanied by changes in the membrane permeability for monovalent ions (for a review see Rubin 1974; Putney 1978). Rubin (1974) pointed out that the latter are not in a causative relationship with the initiation of the secretion. Of all membrane permeability changes only those for Ca^{2+} ions seem to be involved in initiating secretion. The function and significance of changes in the membrane permeability for monovalent ions have not yet been defined.

Activation of blood platelets resulting in the initiation of the secretion, and expression of other platelet functions, is also accompanied by changes in the membrane permeability for monovalent ions. This was documented indirectly by

the measurement of the fluorescence of a potential-monitoring dye (Horne and Simons 1978a; Varečka et al. 1978; Horne et al. 1981), or by direct measurements of K^+-efflux (Lages et al. 1977), and Na^+-influx (Feinberg et al. 1977). However, the problem concerning the expression of the increase of the membrane permeability for Na^+ and K^+ ions under various experimental situations, and in platelets from various mammalian species has remained unclear. In particular the response of the potential-monitoring dye, diS-C_3-(5), to platelet stimulation was found to be different in human (Horne and Simons 1978a) and pig platelets (Varečka et al. 1978). The analysis of the response in pig platelets showed that the fluorescence changes reflected the increase in K^+-permeability, and that they may have represent the opening of the Ca^{2+}-activated K^+-channel. In contrast, the response in human platelets could be inhibited by amiloride suggesting that the Na^+-channel may have been operative (Horne and Simons 1978).

The aim of the present study was to analyze the trypsin-induced response of the diS-C_3-(5) fluorescence in platelet suspensions in order to investigate the link between the fluorescence changes and membrane permeability for monovalent ions, and to compare fluorescence responses between the platelet suspensions of several mammalian species.

Material and Methods

Blood platelet suspensions were prepared from blood collected into EDTA (final concentration 5 mmol/l). Healthy volunteers, mostly staff members of our laboratory, served as donors of human blood for our experiments. Blood of experimental animals was drawn by cardial puncture (rabbit, guinea-pig), or by puncture of the jugular vein (sheep). The blood was centrifuged for 25 min ($120 \times g_{av}$, at 4°C) and platelets were spun down from the platelet-rich plasma by another centrifugation (25 min, $700 \times g_{av}$, at 4°C), and were suspended and washed (3 times) in the medium containing: (in mmol/l) 30 Tris-Cl, pH 7.5; 125 NaCl; 5 KCl; 5 glucose and 0.5 EGTA. The resulting suspension was stored on ice until used. Platelets prepared according to this procedure were tested for the presence of the membrane potential using the method of Sims et al. (1974). The values of the membrane potential of about minus 70 mV were found assuming the intracellular K^+ concentration of 100 mmol/l (Varečka 1979).

The extent of the secretory reaction and changes in the fluorescence of diS-C_3-(5) (referred to as "dye") used to monitor changes in the membrane potential (Sims et al. 1974) were measured as described previously (Varečka et al. 1978).

A 23187, valinomycin, and soybean trypsin inhibitor were purchased from Calbiochem, Luzern, Switzerland; gramicidin from Serva, Heidelberg, F.R.G.; thrombin (topical) from Imuna, Šarišské Michaľany, Czechoslovakia; heparin from Spofa, Prague, Czechoslovakia; and trypsin (crystalline), from Lachema, Brno, Czechoslovakia. All other reagents of analytical grade were commercial products of Lachema, Brno. The dye (3, 3′-dipropylthiodicarbocyanine iodide) was kindly donated by Dr. Alan Waggoner, Department of Chemistry, Amherst College, Amherst, Ma, U.S.A.

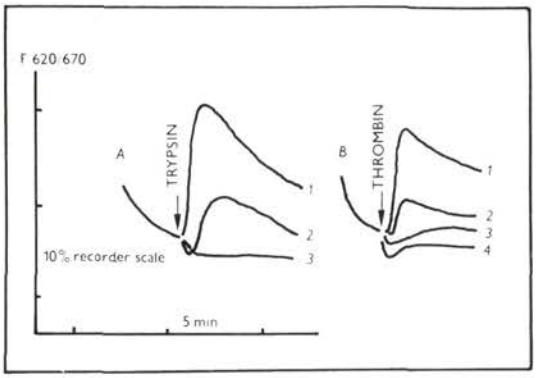

Fig. 1. Changes in the dye fluorescence induced by various concentrations of trypsin and thrombin in suspensions of washed human platelets. Platelets were suspended in a medium containing (in mmol/l): 10 Tris-Cl, pH 7.5; 145 NaCl; 0.2 KCl; 5 glucose; to the final concentration of protein (biuret) 0.15 mg/ml. To this suspension, ethanolic solution of the dye was added (final concentration 0.7 μmol/l, 0.17% (v/v) ethanol), and the fluorescence was recorded at the excitation wavelength 620 nm, and emission wavelength 670 nm. During the measurements the suspension was continuously stirred. The fluorescence, was left to equilibrate, and the following concentrations of trypsin or thrombin were added: *A*-trypsin: 1 — 7.4 μg/ml, 2 — 1.8 μg/ml, 3 — 0.37 μg/ml *B*-thrombin: 1 — 11.6 U/ml, 2 — 4.0 U/ml, 3 — 2.3 U/ml, 4 — 1.1 U/ml. This figure shows tracings of actural recording obtained in a single experiment that have been normalized to the same steady level of fluorescence prior to the addition of trypsin (A), or trombin (B). The measurements were performed at room temperature. No changes in fluorescence were seen if trypsin or thrombin were added to the medium from which the cells were omitted.

Results

Unlike the pig blood platelets which responded to the secretion stimulus by monophasic uptake of the dye (Varečka et al. 1978), human platelets exhibited a complex change in the fluorescence after the addition of thrombin or trypsin. Similarly as Horne and Simons (1978a), we have also observed an early decrease in the fluorescence followed by an increase which was also transient (Fig. 1*A*, trace 2, Fig. 1*B*, trace 2, 3). When higher concentrations of the proteolytic enzymes were used, the early decrease in the fluorescence diminished, and finally disappeared. A transient response in the direction of the depolarization was only observed. All these responses were completely inhibited by the soybean trypsin inhibitor, or heparin if trypsin, or thrombin were used as the secretion inducers (not shown). This suggests that the proteolytic action of the secretion inducers was substantial for the observed fluorescence changes to occur. In experiments illustrated in Fig. 2*A*, and 2*B* the dependence of both phases of the fluorescence change on the composition of the medium in which the concentrations of monovalent cations

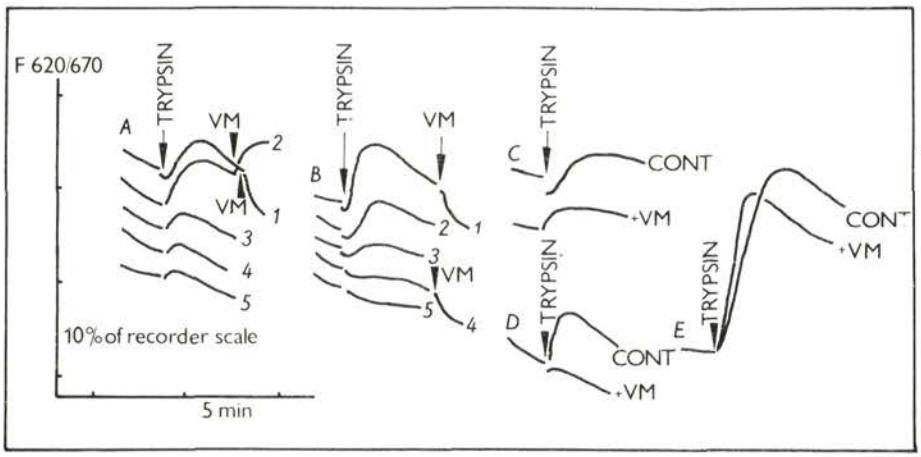

Fig. 2. The effect of monovalent ion and sucrose concentrations on trypsin-induced changes in the dye fluorescence. The effect of valinomycin. Human platelets were suspended in a medium containing (in mmol/l): 10 Tris-Cl, pH 7.5; 5 glucose; 145 mixture of NaCl and KCl (*A*, *C*, *D*, *E*); or iso-osmotic mixture of NaCl, KCl, and sucrose (*B*). Other experimental conditions as in Fig. 1. *A*. Concentrations: K^+ (mmol/l): 1 — 0.2, 2 — 11.5, 3 — 34.5, 4 — 69, 5 — 138. Trypsin — 37 μg/ml, valinomycin 1 μg/ml. *B*. Concentrations (mmol/l): K^+ — 0.2; Na^+: 1 — 145, 2 — 108.8, 3 — 72.5, 4 — 36.2, 5 — 5.2. Trypsin 37 μg/ml valinomycin 1 μg/ml *C*. Concentrations: K^+ — 0.2 mml/l; Na^+ — 145 mmol/l; trypsin — 2.5 μg/ml; valinomycin — 1 μg/ml. *D*. Concentrations: KCl — 138 mmol/l; NaCl — 7 mmol/l; trypsin — 37 μg/ml; valinomycin — 1 μg/ml. *E*. Concentrations as in *C*; the concentration of trypsin was 37 μg/ml. This figure shows tracings of actual recordings obtained in three independent experiments (A and B, C, D and E). In A, B, C the tracings were ordered vertically, and their position does not reflect the actual fluorescence value prior to the addition of trypsin. In D, E the tracings have been normalized to the same level of fluorescence prior to the addition of trypsin.

were varied keeping the osmolarity constant, was tested. It is clear that the latter phase of the fluorescence (increase) was significantly, but not absolutely, dependent on the presence of Na^+ ions in the medium (Fig. 2*A*), and there was an absolute dependence of this phase on the presence of Na^+ ions if NaCl was substituted with an equal volume of iso-osmotic sucrose (Fig. 2*B*). In other experiments (not presented here) (Varečka 1979) it was shown that trypsin did not cause any change in fluorescence if blood platelets suspended in Na^+-containing medium were preincubated with gramicidin, a monovalent cation ionophore, which only poorly discriminates between Na^+, and K^+ ions (Pressman 1976). These results suggest that the later phase of the fluorescence change corresponds to the depolarization of the membrane due to the opening of the Na^+ channel, in agreement with the oservations that there is an influx of Na^+ but not of Cl^- during the activation of human platelets (Feinberg et al. 1977), and that the fluorescence of the same dye was inhibited by amiloride, a blocker of the passive Na^+ movement across cell membranes (Horne and Simons 1978b).

The different response of the dye fluorescence to the substitution of KCl, or sucrose for NaCl suggested that these two substituents of Na^+ ions were not equivalent. Although the different fluorescence response in these substituents could be ascribed to several factors (e.g. changes in the contribution of the surface potential, or anion concentration), the experimental observations showed below suggest that it was due to the fact that the membrane becomes permeable also to K^+ ions during the trypsin-induced platelet activation.

The time-course of the early phase of the fluorescence change observed in the experiment (Fig. 2A) was critically dependent on the external K^+ concentration. At significantly low concentrations (e.g. 0.2 mmol/l), the early phase exhibited a short lag and the depolarization phase started slowly. Under these conditions the subsequent addition of valinomycin (a K^+-specific ionophore) resulted in hyperpolarization of the membrane (Fig. 2*A*, trace 1). When the external K^+ concentration was increased, the early lag was absent, and the depolarization started steeply, and immediately after the addition of trypsin. In this case, the subsequent addition of valinomycin induced depolarization of the membrane (Fig. 2*A*, trace 2). These results indicate that the fluorescence change induced by trypsin or thrombin in the suspension of human platelets has two components: one — associated with the increase in membrane permeability for K^+ ions, which, upon increasing the external K^+ concentration in the milimolar range, turns from hyperpolarization to depolarization; and another — associated with the increase in membrane permeability for Na^+ and diminishing monophasically with the decrease in Na^+ concentration in the range of $10^{-1} - 10^{-2}$ mol/l.

The association of the early phase of the fluorescence change with the increase in membrane permeability for K^+ was tested independently using valinomycin (Fig. 2*C*, *D*, *E*). Valinomycin pretreatment of the suspension preincubated with the dye abolished completely both the early phase of the fluorescence and the lag, whereas the depolarization remained almost unchanged (Fig. 2*C*). If a high trypsin concentration was used to activate blood platelets, resulting in disappearance of the early phase of the fluorescence, pretreatment of blood platelets with valinomycin markedly accelerated the depolarization phase (Fig. 2*E*). The trypsin-induced increase in the fluorescence in K^+-rich, and Na^+-free medium was also completely inhibited by the pretreatment of platelets with valinomycin (Fig. 2*D*). This suggests that in this medium only the K^+-associated increase in membrane permeability occurs. The suggestion that the membrane of human platelets becomes also permeable for K^+ ions following platelet activation was thus fully supported by the experiments with valinomycin.

It should be mentioned that the depolarization phase of the platelet response to the addition of trypsin was not always homogeneous. In approximately 1/3 of experiments two phases could be distinguished in the depolarization. In a single experiment two resolved peaks in the direction of the depolarization instead of one

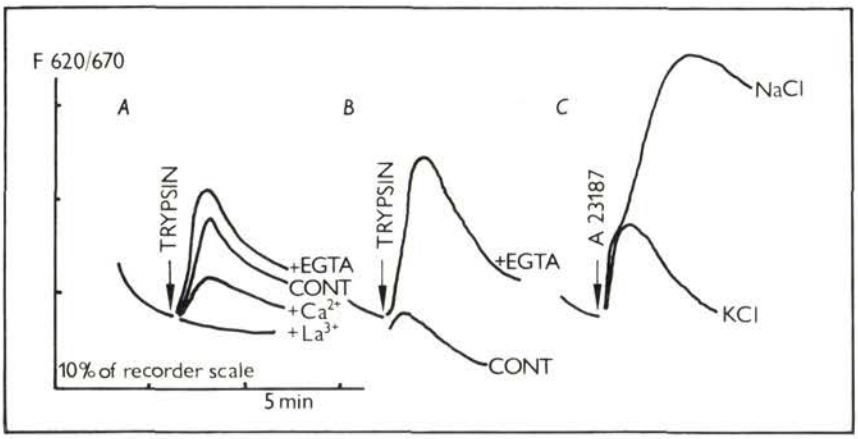

Fig. 3. The effect of Ca^{2+}, La^{3+}, and EGTA on the trypsin-induced changes in dye fluorescence in suspensions of human platelets (*A*, *B*). A 23187 — induced changes (C). *A*, *B*: The experiments were performed as described in Fig. 1, except that the concentration of trypsin was 37 μg/ml, and the medium was supplemented with 1.4 mmol/l EGTA (*A*, *B*); 1.6 mmol/l Ca^{2+} (*A*); or 1.1 mmol/l La^{3+} (*A*) after the addition of the dye, and 5 min prior to the addition of trypsin. *C*: Upper trace — the composition of the medium was the same as in Fig. 2*C*. Other conditions as in Fig. 1, except that 1 μg/ml of A 23187 was used to induce secretion. Lower trace: the composition of the medium was the same as in Fig. 2*D*. Other conditions as in Fig. 1, except that 1 μg/ml of A 23187 was used to induce the secretion. This figure shows tracings of actual recordings obtained in three independent experiments (A, B, C). The tracings have been normalized to the same steady level of fluorescence prior to the addition of trypsin.

were even observed. In this case, the depolarization phases were also preceded by the usual hyperpolarization and lag. This suggests that the membrane permeability changes induced by the platelet-activating agents might represent a more complex process. So far, we have failed in creating conditions enabling to observe these fine changes in a controlled manner.

The increase in K^+ permeability in pig platelets during the secretion was shown to be inhibited by EGTA, and Ca^{2+} antagonists, respectively (Varečka et al. 1978); this was in agreement with the known fact that the pig platelet secretion could also be inhibited by EGTA (Grette 1962; Kinlough-Rathbone et al. 1974). Unlike in pig platelets, human platelet secretion is not dependent on the presence of extracelular Ca^{2+}, and cannot be inhibited by EGTA (Mürer 1968; Feinstein et al. 1976). This enabled us to study the effect of EGTA, Ca^{2+}, and Ca^{2+} antagonists on the trypsin-induced fluorescence changes described above.

As shown in Fig. 3*A*, EGTA enhanced the trypsin-induced depolarization. As a matter of fact, in some platelet preparations which responded to trypsin by a very small fluorescence change, the latter could be restored by previous addition of EGTA (Fig. 3*B*). The presence of external Ca^{2+} depressed the depolarization (Fig. 3*A*). The potency of Ca^{2+} in this respect varied significantly in various

preparations of blood platelets. In a extreme case, 1.1 mmol/l Ca^{2+} inhibited the depolarization completely. The same concentration range of La^{3+} ions which inhibited the human platelet secretion (unpublished data) caused a complete inhibition of both K^+- and Na^+-associated change in the dye fluorescence (Fig. 3*A*).

If the divalent ionophore A 23187 was used as the secretion inducer, a response similar to that induced by trypsin was observed as to the dependence on the Na^+ ion concentration (Fig. 3*C*), and to the effect of EGTA (not shown). These experiments suggested that the platelet activation-associated permeability changes are not dependent on the way the platelets become activated, and that they are triggered by the increase in intracellular Ca^{2+}.

In order to study the occurrence of trypsin-induced permeability changes in platelets of other mammalian species experiments were conducted in rabbit, guinea-pig, and sheep platelets under identical conditions as used in the experiments described above. Rabbit and guinea-pig platelets were found to exhibit similar responses as did human platelets, whereas sheep platelets responded by hyperpolarization (not shown). These results suggest that the opening of K^+ channel which occurs following the increase in cytoplasmic Ca^{2+} concentration might be a general phenomenon accompanying the platelet activation in contrast to the increase in Na^+ permeability which is absent in platelets of certain mammalian species.

Discussion

The results presented here in demonstrate that secretion inducers increase the permeability of the human platelet membrane for K^+-ions. This was also the case in experiments in which high concentrations of thrombin or trypsin were used, and no hyperpolarization-like changes were observed due to the stimulation with these agents.

It has been found that the occurrence of hyperpolarization-like changes in the fluorescence, i.e. visualization of the increase in the membrane permeability for K^+ ions, is dependent on the concentration of the secretion stimulus in the suspension (Fig. 1). A "pure" depolarization-like change in the fluorescence was only observed after sufficiently high concentrations of thrombin or trypsin. However, early hyperpolarization-like fluorescence changes were even seen in the presence of high concentrations of the secretion stimulus in several few human blood platelet preparations (see Fig. 2*A*, *B*). Horne et al. (1981) reported the ability of thrombin to induce depolarization- or hyperpolarization-like changes to be dependent on the purity of the thrombin reparation used. Although topical thrombin was only used in our experiments, the possibility that some factors other than the actual enzyme

concentration might play any role in triggering the fluorescence changes observed could be ruled out, since crystalline trypsin showed similar effects as did topical thrombin (Fig. 1).

The time course of fluorescence changes showed that the increase in the membrane permeability for K^+ ions proceded that for Na^+ ions. Since it has been shown that the increase in the K^+ membrane permeability occurs as a phenomenon accompanying Ca^{2+} entry into the pig platelet cytoplasm (Varečka et al. 1978; Varečka 1979), the same could be expected to occur as for the Na^+-associated changes. This could be well documented by the following experimental evidence:

1. The secretion responses of rabbit (Sneddon and Williams 1973) and human platelets (unpublished results) were found to be independent on the presence of Na^+ ions in the medium.

2. The opening of Na^+ channel could be inhibited by amiloride, while the secretion was blocked by this drug at low concentrations of thrombin only (Horne and Simons 1978b).

3. In pig and sheep platelets, the secretion was not accompanied by the opening of Na^+ channel.

In pig platelets the increase in the K^+ permeability during the secretion was ascribed to the opening of the Ca^{2+}-activated K^+-channel (Varečka et al. 1978; Varečka 1979); the latter becomes activated after the intracellular Ca^{2+} concentration has increased (for a review see Lew and Ferreira 1978). Our experimental results suggested that the increase in Na^+ permeability in trypsin-treated human platelets was also due to an increase in the intracellular Ca^{2+} concentration (Fig. 3*A*, *C*). On the other hand, in milimolar concentrations extracellular Ca^{2+} ions inhibited the activation of Na^+-channel (Fig. 2*A*), while a decrease in extracellular Ca^{2+} to submicromolar concentrations stimulated it significantly. Such a side-specific effect of Ca^{2+} ions on the Na^+ translocation across the cytoplasmic membrane has recently been observed in cultured kidney cells (Taub and Saier 1979) and fibroblasts (Villereal 1981). These characteristics, together with the sensitivity to amiloride, suggest that the transport systems which mediate the electrogenic influx of Na^+ ions might be the same in these cells.

Our experiments were performed in Ca^{2+}-free medium using EGTA-treated platelets. The experiments of Horne and Simons (1978a) were performed in the presence of EGTA. In both cases, no aggregation of platelets was seen throughout the experiments. At the same time, these conditions resulted in the expression of Na^+ permeability-associated membrane depolarization. These experiments contrast with those of McIntyre and Rink (1982) who found only very small, or no effect of platelet-aggregating agents on the platelet membrane potential as measured by the same technique as used in our experiments under conditions favourable for the initiation of platelet aggregation. These authors measured fluorescence changes in the absence of extracellular Ca^{2+} in platelets prepared

without EGTA or EDTA treatment. This indicates that the expression of Na^+-associated depolarization may be dependent on the membrane-bound Ca^{2+} rather than on the concentration of ionized Ca^{2+} in the medium.

The existence of permeability changes for monovalent ions during the activation of blood platelets of various species suggests that they may represent a general phenomenon which accompanies the secretion, similarly as observed in other secretory cells (Rubin 1974). These permeability changes result from the increase in the cytoplasmic Ca^{2+} concentration, in contrast to nerve cells in which changes of the permeability for monovalent ions result in inward movement of Ca^{2+} ions (Baker et al. 1971). This difference has also been confirmed by the fact that in one pilot experiment both changes in the membrane potential and secretion induced by trypsin were not inhibited by 10^{-5} mol/l tetrodotoxin, a specific inhibitor of the Na^+ channel in excitable tissues (unpublished results). Therefore, the suggestion by several authors (Sneddon 1973; Bennett et al. 1979) that blood platelets could serve as a simple model of the nerve endings cannot be extended on the mechanisms by which the platelet secretion is triggered.

The absence of Na^+-associated potential changes in pig and sheep platelets also confirms that the increase in the permeability for Na^+ ions is not operative in the secretion triggering mechanism. This difference could be basically due to the absence of Na^+ channels in these species, or to their functional incompetence. So far, we have not been able to distinguish between these two possibilities. Future comparative studies may improve our understanding of the function of the Na^+ channel in blood platelets. Not least, these pluripotent cells could in this respect serve as a useful model of other non-excitable cells.

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