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TRYPSIN AND CALCIUM IONS ELICIT CHANGES OF THE MEMBRANE POTENTIAL IN PIG BLOOD PLATELETS

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Summary: The addition of trypsin or thrombin or of Ca^{2+} ions to pig blood platelets was followed by a K^+- dependent change of the membrane potential similar to that produced by the ionophore valinomycin. The effect of trypsin and of Ca^{2+}, but not of valinomycin, was prevented by La^{3+} and by EGTA. It is proposed that upon the modification of the platelet surface by trypsin, (and by thrombin under physiological conditions) membrane Ca^{2+} move from the external to the internal side of the platelet surface membrane and open the gates of K^+ - specific channels.

Exocytosis appears to be an universal process of cellular secretion in eucaryots (1). Blood platelets may be expedient structures for the study of the mechanism of this process. They have even been considered as a suitable model of serotoninergic neurons (2). However, in contrast to nerve cells, in which exocytosis of transmitters is triggered by a change of the membrane potential, release of serotonin and of adenine nucleotides from blood platelets is induced by a number of chemical and mechanical stimuli (3) and the role of membrane potential, if any, in the platelet release reaction has not yet been assessed.

This paper shows that changes of the membrane potential accompany the exocytotic process induced by trypsin and thrombin in pig blood platelets. These changes reflect an increase in permeability of the platelet surface membrane for K^+ ions which may be mediated by dislocation of membrane Ca^{2+}.

Abbreviations: diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide; EGTA, bis-(aminoethyl)-glycolether-N,N,N′,N′-tetraacetic acid.

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Methods and Materials

Platelets were isolated from fresh pig blood collected into EDTA anticoagulant (final concentration of EDTA 5 mM) by first separating erythrocytes by centrifugation at 250xg for 15 min. and then the platelets as 800xg for 15 min. at room temperature. The platelets were washed twice with a solution containing 135 mM NaCl, 5 mM KCl, 5 mM glucose and 15 mM Tris-Cl pH 7.5 and stored on ice as a thick suspension (30 mg protein/ml) in the same solution for not more than 6 hours.

Protein was measured by Lowry's procedure and K^+ by flame photometry. The release reaction was estimated by spectrophotometric determination of adenine nucleotides in the supernatant after spinning down the platelets. Changes of the membrane potential were monitored fluorometrically by using the potential indicating dye 3,3'-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] (4) in the Hitachi-Perkin Elmer 204 spectrofluorometer at a constant temperature of 25°C.

Crystalline trypsin was from Lachema, trypsin inhibitor from Calbiochem, thrombin (15 U/mg) from Imuna, phenylmethyl sulphonyl fluoride and valinomycin from Serva. diS-C_3-(5) was kindly donated by Dr. A.S.Waggoner (Amherst College, Amherst, Mass., U.S.A.).

Results

As found with other cells (5) the fluorescence of the dye diS-C_3-(5), when introduced to platelets suspended in a solution containing 135 mM NaCl and 5 mM KCl, was declining and finally stabilized at a low constant value. When then trypsin was added at concentrations which, in a parallel experiment, set off the release reaction, the fluorescence of the suspension dropped again and an additional slight drop took place after the addition of Ca^{2+} ions or of valinomycin (Fig.1). If, instead of trypsin, Ca^{2+} ions were added first, the pattern of fluorescence decrease was similar and so was it if valinomycin was the first addition followed by the other two substances.

The effect of trypsin on fluorescence was prevented by trypsin inhibitor or by the proteolytic inhibitor phenylmethyl sulphonyl fluoride indicating that the effect of trypsin was contingent upon its proteolytic action on the cells. Trypsin could be substituted by thrombin and the effect of thrombin was prevented by heparin.

As further shown in Fig. 1, the effect of trypsin was also inhibited by the Ca^{2+} chelator EGTA and by La^{3+}. As expected,

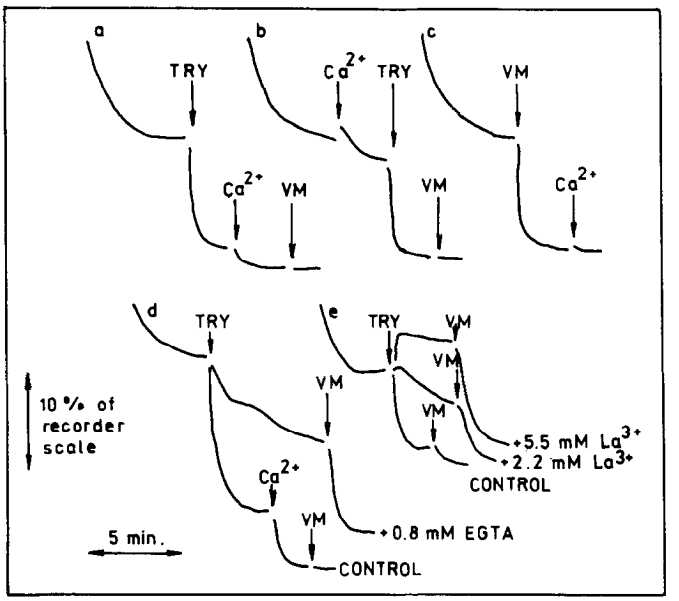

Figure 1. Changes of fluorescence of diS-C_3-(5) in the presence of pig platelets. diS-C_3-(5) (0.7 µM) was added to platelets (0.3 mg protein/ml) suspended in 135 mM NaCl, 5 mM KCl, 5 mM glucose, 15 mM Tris-Cl pH 7.5, 85 U heparin/ml. When indicated at the curves, EGTA or $La(NO_3)_3$ were also included into the suspensions. The suspension was constantly stirred at 25° C and the fluorescence measured by excitation at 620 nm and emission at 670 nm. The fluorescence is expressed in arbitrary units. At arrows, trypsin (TRY) (40 µg/ml), $CaCl_2$ (2.4 mM), valinomycin (VM) (1 µM, with 0.2 % ethanol) were added as indicated.

EGTA and La^{3+} also blocked the effect of exogenous Ca^{2+} (not shown in the Figure) but not that of valinomycin. It can be inferred that trypsin may have exerted its effect on fluorescence by intermediary of Ca^{2+} ions.

It has been shown previously with various kinds of cells that the fluorescence of diS-C_3-(5) and of similar cyanine dyes, once the dye had equilibrated with the cells, was monitoring the membrane potential (4). In order to prove that the changes of fluorescence observed in experiments with platelets corresponded to changes of the membrane potential and not to some unrelated events, the effects of trypsin, Ca^{2+} and valinomycin on fluorescence was examined with platelets suspended in media of different ionic composition. Gradual substitution of Na^+ for choline cation, or of Cl^- for gluconate anion did not substantially modify the fluorescence response. On the other hand,

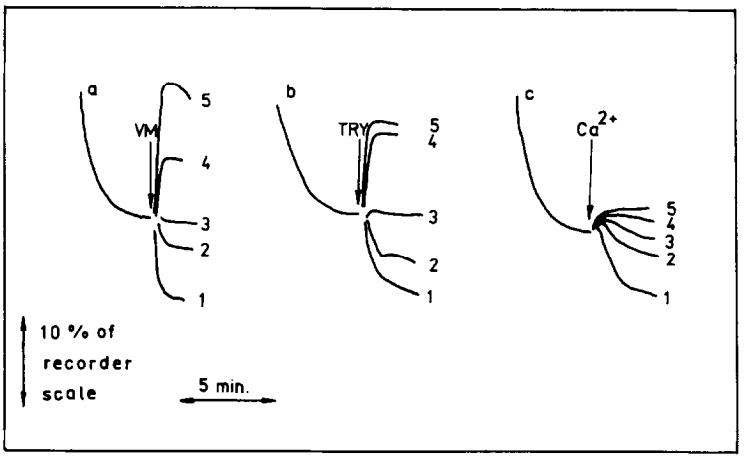

Figure 2. Changes of fluorescence of diS-C_3-(5) in the presence of pig platelets as a function of K^+ concentration. Conditions as in Fig. 1 except that concentrations of KCl (complemented with NaCl to keep constant molarity of 140 mM) varied as follows: 1, 4.25 mM; 2, 8.50 mM; 3, 17.70 mM; 4, 34.20 mM; 5, 68.40 mM. The fluorescence tracings were normalized to the same steady level of fluorescence prior to the additions of trypsin, Ca^{2+}, or valinomycin.

the direction and extent of the fluorescence changes were dependent on concentrations of K^+ ions in the medium: Fluorescence dropped upon the addition of either trypsin, or Ca^{2+}, or valinomycin at concentrations of K^+ lower than approximately 20 mM and rose at higher concentrations (Fig.2). This observation is consistent with the notion that the fluorescence changes reflected changes of the membrane potential and that in the presence of either of the three substances the membrane potential was determined by the distribution of K^+ ions across the platelet membrane. This means that upon treatment with trypsin or Ca^{2+} ions the permeability of the platelet surface membrane for K^+ ions became enhanced and may have been as high as in the presence of the K^+ -specific ionophore valinomycin. In fact, upon addition of either trypsin or valinomycin to platelets suspended in a medium with 135 mM Na^+ and 5 mM K^+ an efflux of K^+ was detected. Such an efflux had already been previously observed with human blood platelets (6).

Discussion

It can be taken as firmly established by these experiments that trypsin- or thrombin-induced exocytosis in pig platelets is

accompanied by changes of the membrane potential. This does not mean, however, that these changes are a causative factor of exocytosis in platelets - as indeed may be the case in neurons and in chromaffin cells (1)- and not even that they need be an obligatory phenomenon of platelet exocytosis. We have confirmed that valinomycin and gramicidin, which also affect the membrane potential, do not set off exocytosis in platelets (7,8). In addition, we found in experiments not detailed here that trypsin-induced exocytosis proceeded equally well in a low-K^+ medium and in a high K^+ medium even though the addition of trypsin brought about a hyperpolarization of the surface membrane in the former case and a depolarization in the latter case. What is common to the two cases is a highly raised permeability of the surface membrane for K^+ ions elicited by trypsin.

Ca^{2+} ions caused similar changes of the membrane potential of platelets as did trypsin, although they did not induce exocytosis. It is conceivable that when a steep concentration gradient of Ca^{2+} across the surface membrane is established by adding Ca^{2+} ions into suspension medium, some Ca^{2+} ions enter the platelet, bind to specific sites on the inner side of the surface membrane and open the gates of K^+- specific channels. Such an effect of internal Ca^{2+} had been previously demonstrated in energy-depleted erythrocytes (9) and in a number of other cells and may be responsible for the K^+ efflux phase of the action potential in neurons (reviewed in ref. 10).

The trypsin effect on the membrane potential of pig platelets seems to be mediated by Ca^{2+} ions, being prevented by EGTA and La^{3+}. Accordingly, the following course of events may be proposed to take place in trypsin- (or thrombin-) induced exocytosis in pig platelets: The proteolytic enzyme binds to specific receptors on the external side of the platelet membrane, (11), modifies membrane components by proteolysis (12), and this allows liberation of Ca^{2+} from some internal stores (13) or translocation of membrane Ca^{2+} from the external side to the internal side of the surface membrane. The translocation to the first class of sites, which can also be reached by exogenously added Ca^{2+}, may be responsible for opening of K^+ -specific channels and subsequent changes of the membrane potential, while the translocation to the second class of sites may trigger a

mechanism enabling the fusion of exocytotic vesicles with the surface membrane.

The function of the opening of K^+ -specific channels in the pig platelet membrane, either during exocytosis or by the action of Ca^{2+} ions, remains unknown. According to our preliminary experiments, in human blood platelets an increase in permeability of the platelet membrane for Na^+ is elicited by trypsin and is superimposed upon the increase in K^+ permeability observed in the present study with pig platelets. Changes of the membrane permeability may either generally reflect membrane rearrangement in exocytosis or participate specifically, along with numerous other factors, in tuning hemostasis.

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