EFFECT OF BACTERIAL TOXINS OF MITOGEN-INDUCED Ca^{++} CONCENTRATION RISE IN RAT THYMOCYTE CYTOPLASM. ROLE OF N-PROTEINS

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There are at least two ways of activating physiological processes developing in response to interaction between a stimulus and plasma membrane receptor. During triggering of processes developing in the first way, cAMP acts as secondary messenger: interaction between stimulus and receptor leads to activation of adenylate cyclase and to elevation of the intracellular cAMP level [15]. In recent years the second way of triggering physiological processes, namely by phosphoinositides, has been discovered and intensively research [6, 14]. Interaction between stimulus and receptor leads to activation in these processes of phospholipase C, which catalyzes the breakdown of polyphosphoinositides, with the formation of two products: inositol triphosphate and diacylglycerol, both of which are involved in generation of the cell response. Coupling of the receptor with the catalytic subunit of adenylate cyclase is brought about through two regulatory GTP-binding proteins: stimulating (N_S) and inhibitory (N_i) [9, 10]. The problem of how the signal is transmitted from the receptor to phospholipase C has not yet been explained. Recent data suggest that in some cells, coupling of receptor to phospholipase C also is effected by N-proteins [7, 11].

Bacterial toxins provide a convenient tool with which to study the role of N-proteins in activation processes. Cholera enterotoxin and the cholera-like thermolabile (TL) toxin of *Escherichia coli*, in the presence of ADP, ribosylate N_S-protein [8]; the target of pertussis toxin (PT), produced by *Bordetella pertussis*, is the N_i-protein of the adenylate cyclase complex. Ribosylation of N_S-protein leads to constant and receptor-independent stimulation of adenylate cyclase [8, 13]. Modification of N_i-protein by PT inhibits coupling between the catalytic subunit of adenylate cyclase and the receptor [5]. In some cells PT also ribosylates the N-protein involved in transmitting the signal from receptor to phospholipase C, thus leading to inhibition of hydrolysis of polyphosphoinositides [5].

In this investigation the possibility that N-proteins are involved in activation of rat thymocytes under the influence of the mitogen concanavalin A (con A) was studied. The effect of cholera, TL- and pertussis toxins was examined on one of the key activation processes [14]: con A-induced elevation of the Ca^{++} concentration in thymocyte cytoplasm ($[Ca^{++}]_i$). Changes in $[Ca^{++}]_i$ were monitored with the aid of the fluorescent Ca^{++}-probe quin-2.

EXPERIMENTAL METHOD

Thymocytes were isolated from the thymus of decapitated Wistar rats weighing 150 g by pressing the gland through a nylon sieve, as described in [4], and transferred into medium containing 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$ 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1mM Na_2HPO_4, 4 mM $NaHCO_3$, 10 mM HEPES, and 6 mM glucose, pH 7.1. The cells were incubated for 30-90 min at 37°C with different doses of the toxins (control cells were kept at the same temperature). The acetoxyethyl ester of quin-2 (Calbiochem), in a concentration of 15 μM, was added to the

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TABLE 1. Effect of Cholera (CT), TL-, and Pertussis (PT) Toxin on Changes in Thymocyte $[Ca^{++}]_i$ Level under the Influence of 15 μg/ml con A

Concentration of toxin, μg/ml	$[Ca^{2+}]_{i0}$, nM	$[Ca^{2+}]_i$ con A, nm
—	100	280
1 TL	100	160
10 TL	100	100
—	115	330
1 CT	115	180
1 PT	100	100
10 PT	100	100

Legend. $[Ca^{++}]_{i0}$ – $[Ca^{++}]_i$ level before addition of con A; $[Ca^{++}]_i$ con A – $[Ca^{++}]_i$ level established 5 min after addition of con A. Cells were incubated for 30 min with CT, 60 min with TL, and 90 min with PT. Error of measurement of $[Ca^{++}]_i$ does not exceed ±15 nM.

suspension 30 min before the end of incubation with the toxins. After the end of incubation the cells were washed, transferred to medium not containing quin or toxins, and poured into the cell of a Shimadzu RF-510 spectrofluorometer, kept at a constant temperature of 37°C. The wavelength of excitation was 339 nm and of recording 495 nm. The value of $[Ca^{++}]_i$ was calculated by the equation:

$$[Ca^{2+}]_i = K\frac{F - F_{min}}{F_{max} - F},$$

where F is the observed level of fluorescence; F_{max} the intensity of fluorescence of calcium-saturated quin (determined by addition of 50 μM digitonin to the thymocyte suspension); F_{min} the fluorescence of quin in calcium-free medium (obtained by adding 0.1 mM $MnCl_2$; K = 115 nM to the suspension at 37°C). The cholera toxin was obtained from "Calbiochem," PT was obtained by the method described in [3], and TL-toxin as described in [4].

EXPERIMENTAL RESULTS

As was shown previously [2], con A increased $[Ca^{++}]_i$ of the thymocytes to about 3 times the basal level (100 ± 7 nM, mean of 9 repetitions). Incubation with the test toxins did not affect the basal level of $[Ca^{++}]_i$, but caused dose-dependent suppression of the Ca-response to con A (Table 1). For instance, 1 μg/ml of TL-toxin or 1 μg/ml of cholera toxin reduced the con A-induced rise of $[Ca^{++}]_i$ by about 70% (Fig. 1, Table 1). After incubation with 10 μg/ml of TL-toxin, the Ca-response to con A could not be observed at all.

This inhibitory action of TL and cholera toxins can be explained by their effect on adenylate cylase, leading to elevation of the intracellular cAMP level [9, 10]. Treatment of the cells with other agents elevating cAMP, such as the cyclic nucleotide phosphodiesterase blockers theophylline or dibutyryl-cAMP, also weakened the Ca-response to con A. Lowering of the free Ca^{++} ion concentration with an increase in the cAMP level also is observed in other inexcitable cells, such as platelets [1]. However, besides their effect through elevation of the cAMP level, the toxins may also act in another way. Activity of N-proteins is controlled by equilibrium between association and dissociation of their component subunits, some of which are common to all N-proteins [5, 9]. ADP-ribosylation of α-subunits of N-proteins, catalyzed by toxins, shifts this equilibrium so that cholera toxin, for example, by modifying the structure of NS-protein, may also affect activity of other N-proteins [12].

Treatment of cells with CT, as Table 1 shows, also prevented the increase in $[Ca^{++}]_i$ due to the action of con A. The toxin does not abolish Ca-responses taking place without interaction between stimulus and receptor, such as, for example, elevation of $[Ca^{++}]_i$ due to the action of the Ca^{++}-ionophore A23187 (Fig. 3). The inhibitory action of CT on the Ca^{++}-signal may be due to two causes. Binding of con A with the receptor may cause activation of the N-protein of the adenylate cyclase system, as occurs in other physiological processes. In

that case the toxin, by blocking transmission of the signal from receptors to N-protein, may raise the cAMP level and thereby inhibit the Ca-response to the mitogen. Another possibility is that the target for CT in thymocytes is the N-protein coupling the receptor with phospholipase C, and for that reason the toxin suppresses the rise of $[Ca^{++}]_i$ by inhibiting hydrolysis of polyphosphoinositides. Irrespective of how this is brought about, our results are evidence that N-proteins are involved in transmission of the signal from the receptor inside the cell and in generation of the Ca-response during mitogenic activation of thymocytes.

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HEMATOPOIETIC PRECURSOR CELLS IN THE INTIMA OF THE ATHEROMATOUS HUMAN AORTA

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The presence of blood cells in the intima of human arterial walls affected with atherosclerosis has been noted in several investigations [2, 7, 8, 10, 15]. The appearance of leukocytes in the intima of atheromatous vessels is associated with their penetration from the circulating blood through the endothelium, after preliminary adhesion to its surface, but the possibility has never been envisaged that colony-forming units (CFU), cells capable of forming colonies in culture, can proliferate and differentiate in the intima. It is with this possibility that our data, some of which were published previously in abstract form [14], are concerned. In this paper we present data indicating that precursor cells of granulocytes and macrophages (CFU-GM) may be present in the intima of the atheromatous human aorta.

EXPERIMENTAL METHOD

The aortas from 22 persons dying accidentally or suddenly between the ages of 40 and 80 years were investigated. The thoracic part of the aorta was taken not later than 6 h after death. After mechanical separation of the adventitia the luminal surface of the vessel was carefully washed with phosphate-buffered saline to remove blood. The endothelium was taken from the surface of the vessel with 0.1% collagenase solution in medium 199 for 15 min at 37°C.

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