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Staphylococcal alpha-toxin elicits hypertension in isolated rabbit lungs. Evidence for thromboxane formation and the role of extracellular calcium.

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Research Article

Staphylococcal alpha-toxin is known to damage mammalian cell membranes. Studies of erythrocytes indicate that the native toxin generates a discrete transmembrane channel with an effective diameter of 2-3 nm. (Füssle, R., S. Bhakdi, A. Szeigoleit, J. Tranum-Jensen, T. Kranz, and H.J. Wellensiek. 1981. J. Cell Biol. 91:83-94.) In isolated rabbit lungs, perfused with recirculating blood- and plasma-free perfusion fluid, the mediation of a toxin-provoked vascular pressor response by the triggering of the arachidonic acid cascade and its dependence on extracellular calcium were investigated. Dose-dependent pulmonary artery pressor responses were elicited by the injection of 0.5-5 micrograms staphylococcal alpha-toxin into the pulmonary artery. The pressor responses were completely abolished by preincubation of the toxin with neutralizing antibodies or by preformation of alpha-toxin hexamers in vitro. They were accompanied by the release of the arachidonic acid metabolites thromboxane B2 and 6-keto-prostaglandin F1 alpha (stable metabolites of thromboxane A2 and prostaglandin I2, respectively) into the perfusion fluid. They were blocked by inhibitors of thromboxane synthetase, cyclooxygenase, and phospholipase, as well as by substances that interfere with calcium-calmodulin function. alpha-Toxin induced selective release of potassium, but not lactatedehydrogenase into the medium. Calcium depletion of the intravascular space did not suppress the toxin-dependent potassium release but did abrogate the pressor response and the release of the arachidonic acid metabolites. When calcium was reintroduced into the […]

Staphylococcal α-Toxin Elicits Hypertension in Isolated Rabbit Lungs

Evidence for Thromboxane Formation and the Role of Extracellular Calcium

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Abstract. Staphylococcal α-toxin is known to damage mammalian cell membranes. Studies of erythrocytes indicate that the native toxin generates a discrete transmembrane channel with an effective diameter of 2–3 nm. (Füssle, R., S. Bhakdi, A. Szeigoleit, J. Tranum-Jensen, T. Kranz, and H. J. Wellensiek. 1981. *J. Cell Biol.* 91:83–94.) In isolated rabbit lungs, perfused with recirculating blood- and plasma-free perfusion fluid, the mediation of a toxin-provoked vascular pressor response by the triggering of the arachidonic acid cascade and its dependence on extracellular calcium were investigated.

Dose-dependent pulmonary artery pressor responses were elicited by the injection of 0.5–5 μg staphylococcal α-toxin into the pulmonary artery. The pressor responses were completely abolished by preincubation of the toxin with neutralizing antibodies or by preformation of α-toxin hexamers in vitro. They were accompanied by the release of the arachidonic acid metabolites thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ (stable metabolites of thromboxane A_2 and prostaglandin I_2, respectively) into the perfusion fluid. They were blocked by inhibitors of thromboxane synthetase, cyclooxygenase, and phospholipase, as well as by substances that interfere with calcium-calmodulin function. α-Toxin induced selective release of potassium, but not lactatedehydrogenase into the medium. Calcium depletion of the intravascular space did not suppress the toxin-dependent potassium release but did abrogate the pressor response and the release of the arachidonic acid metabolites. When calcium was reintroduced into the circulation without the application of a second toxin stimulus, marked pressor responses paralleled by the release of arachidonic acid metabolites occurred.

The conclusion drawn from these studies is that staphylococcal α-toxin provokes pulmonary vascular hypertension which is apparently mediated by thromboxane A_2 formation, which surpasses the biological effect of the simultaneously formed prostaglandin I_2. The triggering of the arachidonic acid cascade is strictly dependent on extracellular calcium and may be mediated by a nonphysiological calcium bypass through transmembrane toxin channels with subsequent stimulation of phospholipase activity.

Introduction

Staphylococcal α-toxin has been considered one of the essential factors associated with staphylococcal pathogenicity (1–3). Cytotoxic activities, and probably also the lethal effect of α-toxin, appear to be linked to its membrane-damaging action (4). Studies in the erythrocyte membrane model indicate that the native toxin oligomerizes to form a hexameric annular complex which, through its partial embedment within the lipid bilayer, generates a discrete transmembrane channel (5). When toxin is injected intravenously into rabbits, the most is recovered from the kidneys and lungs (6).

With isolated rabbit lungs as a model system, a variety of stimuli have recently been shown to provoke a constriction of the pulmonary vascular bed via activation of phospholipase, arachidonic acid release from membrane phospholipids, and subsequent formation of the pressor substance thromboxane

Parts of this work were presented at the Workshop Conference on Bacterial Protein Toxins, Seillac, France, June 1983. This work has been published in abstract. (1984. *In* Bacterial Protein Toxins. J. E. Alouf, J. H. Freer, F. Fehrenbach, and J. Jeljaszewicz, editors. Academic Press, New York. 353.)

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A_2 (TxA_2)[1] (7–10). In this communication we report that α-toxin provokes a dose-dependent pressor response which is apparently mediated by thromboxane formation in the pulmonary vascular bed. Thromboxane evidently surpasses the biological effect of the simultaneously formed prostaglandin I_2 (PGI_2). Pressor response and mediator release are strictly dependent on extracellular calcium and may initially be triggered by calcium flux through transmembrane toxin channels.

Methods

The investigations were performed with a model of isolated rabbit lungs which has been previously described (8). Briefly, lungs were removed from deeply anesthetized rabbits (body weight 2.2–2.6 kg) without interruption of ventilation and perfusion, and suspended freely from a force transducer in a chamber warmed to 38°C. They were ventilated (frequency, 45 strokes/min; tidal volume, 30 ml) with 4% CO_2, 17% O_2, and 79% N_2 and perfused with (calcium containing) Krebs Henseleit hydroxyethylamylopectine (200,000 mol wt, 4% wt/vol) buffer (KHHB) in a recirculating system (circulating volume, 125 ml) with a pulsatile constant flow of 200 ml/min. By the alternate use of two separate perfusion systems it was possible to perform numerous perfusion phases in the same isolated lung, each with fresh perfusion fluid with or without the addition of stimuli and/or inhibitors. The physical parameters—perfusion pressure, ventilation pressure, and the weight of the isolated lung—were registered continuously by pressure transducers and a force transducer. The reproducibility of these physical measurements showed a standard deviation of <3%. Only those lungs were selected that after a steady state period of at least 45 min were completely blanched and showed no spontaneous edema formation or changes of ventilation or perfusion pressure. Light microscopical examination of these lungs revealed no adherence of erythrocytes, platelets, or leucocytes to the vascular wall. α-Toxin, dissolved in 20 μl saline, was injected directly into the pulmonary artery (indicated by arrows in Figs 1, 2, and 4). Inhibitors were added to the perfusion fluid at the stated final concentrations before recirculation. Purified α-toxin and α-toxin antibodies were prepared as previously described (1). Thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable products of TxA_2 and PGI_2, were measured radioimmunologically with kits commercially available from New England Nuclear (Boston, MA). Lactatedehydrogenase (LDH) was measured photometrically and potassium was measured by flame photometry.

TMB 8 [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate] was a gift from Dr. P. W. O'Connell, and the endoperoxide analogue U-46619 [(15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid] was a gift from Dr. J. E. Pike (Upjohn Co., Kalamazoo, MI). OKY-046 was kindly supplied by Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Indomethacin was obtained from Merck Sharp & Dohme AG (München), trifluoperazine from Röhm Pharma (Darmstadt), mepacrine from Bayer AG (Leverkusen, Federal Republic of Germany), W 7 (*N*-6-aminohexyl-5-chloro-1-naphtalene sulfonamide) from Rikaken Co. (Nagoya, Japan), A 23187 from Calbiochem-Behring Corp. (Giessen, Federal Republic of Germany), bradykinin from Bachem (Bubendorf, Switzerland), and hydroxyethylamylopectine from Fresenius KG (Bad Homburg, Federal Republic of Germany). All other biochemicals were obtained from SERVA Feinbiochemica Gmbh & Co. (Heidelberg) and Merck AG (Darmstadt).

Statistical methods. Data were analyzed by the two-tailed *t* test for unpaired samples and by simple regression.

Results

α-Toxin causes a dose-dependent pulmonary artery pressor response

Low dosages (0.5–1.5 μg) of α-toxin caused a pressor response (Fig. 1), not during the injection phase, but during the subsequent rinsing phase. Higher dosages (5 μg) provoked an immediate steep pressor response (latent period 2.3±0.9 min) with a maximum rate of pressure increase of 19.4±7.6 mmHg/min (n = 8). 2 and 2.5 μg α-toxin showed an intermediate reaction with a modest pressure rise in the injection phase (1.7±0.7 mmHg/min after 2.5 μg; n = 19) and an additional postrinsing pressor response. After 2.5 μg α-toxin these postrinsing pressor responses were reproducible in at least four subsequent perfusion phases. There was a significant inverse correlation between the duration of the latent period (between injection and the beginning of the pressor response in the injection phase) and the maximum rate of pressure increase (r = 0.85; [y = a + b log x]; $P < 0.001$).

After the onset of the pressor response, α-toxin caused an increase in lung weight and, after the edema formation, an increase in ventilation pressure, neither of which has been further evaluated in this study.

Release of TxB_2 and 6-keto-$PGF_{1\alpha}$ into the perfusion fluid after stimulation with α-toxin

Compared with 15-min control phases (TxB_2, 65±21 pg/ml; 6-keto-$PGF_{1\alpha}$, 182±80 pg/ml; n = 18), the concentrations of these arachidonic acid metabolites were markedly higher after stimulation with 2.5 μg α-toxin (TxB_2, 256±215 pg/ml, $P < 0.05$; 6-keto-$PGF_{1\alpha}$, 880±455 pg/ml, $P < 0.001$; n = 7) and with 5 μg toxin (Fig. 2) (TxB_2, 1,092±764 pg/ml, $P < 0.001$; 6-keto-$PGF_{1\alpha}$, 2,240±1,380 pg/ml, $P < 0.001$; n = 7).[2] After the application of 2.5 μg α-toxin, the arachidonic acid metabolites continued to be released in the absence of a second toxin stimulus (TxB_2, 239±177 pg/ml, $P < 0.001$; 6-keto-$PGF_{1\alpha}$, 1,020±519 pg/ml, $P < 0.001$; n = 4; measured in the subsequent 15-min rinsing phase).

1. *Abbreviations used in this paper:* KHHB, Krebs Henseleit hydroxyethylamylopectine buffer; LDH, lactatedehydrogenase; $PGF_{1\alpha}$ and PGI_2, prostaglandins $F_{1\alpha}$ and I_2; TMB 8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TxA_2 and TxB_2, thromboxanes A_2 and B_2.

2. As a rule, the concentrations of TxB_2 and 6-keto-$PGF_{1\alpha}$ in the recirculating perfusion fluid were determined from a sample taken 15 min after application of the stimulus. However, experiments were stopped when the edema formation exceeded 15 g weight gain. Thus, after 5 μg α-toxin, the period between stimulus application and removal of sample for arachidonic acid metabolite determination varied between 7.5 and 15 min.

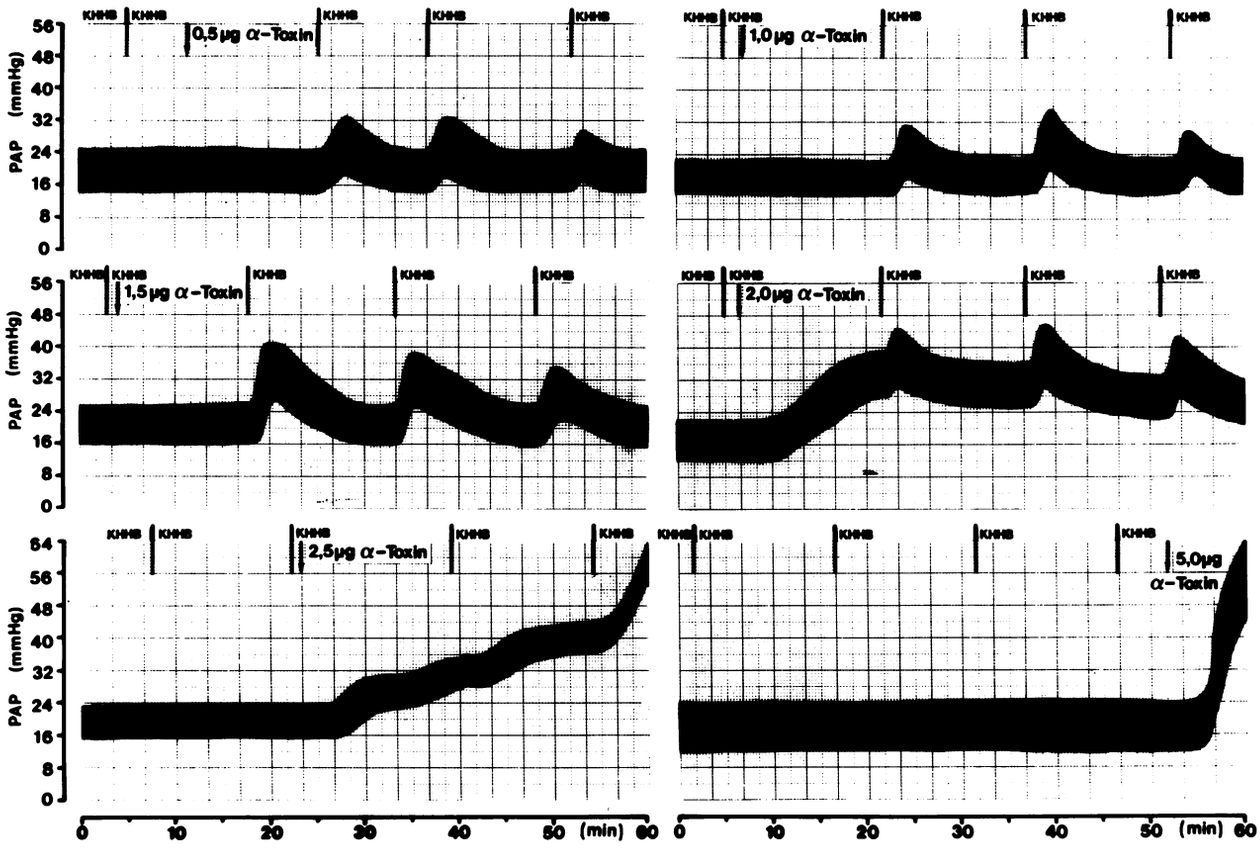


Figure 1. Pulmonary artery pressure (PAP) rise after increasing doses of α-toxin were administered in different isolated lungs. The dashes without arrows indicate the changes of perfusion fluid.

Inhibitors of cyclooxygenase, thromboxane synthetase, phospholipase, and calcium-calmodulin block the pressor response

Inhibition of arachidonic acid metabolism. Indomethacin (cyclooxygenase inhibitor) blocked, in a dose-dependent manner, the pressor response as well as the release of TxB_2 and 6-keto-$PGF_{1\alpha}$, yielding superimposable dose-inhibition curves (Fig. 3). OKY-046 (thromboxane synthetase inhibitor) caused a dose dependent and parallel inhibition of both the pressor response and TxB_2 release, whereas the release of 6-keto-$PGF_{1\alpha}$ even increased (Fig. 3). Both inhibitors as well as imidazole (thromboxane synthetase inhibitor) blocked the pressor responses after the administration of α-toxin to the same extent as the pressor responses after the direct application of arachidonic acid or after stimulation of the arachidonic acid cascade by the known calcium-ionophore A 23187 (8, 11) (Fig. 4, Table I).

Phospholipase inhibition. The pressor response after the administration of 5 µg α-toxin was markedly reduced by 100 µM mepacrine. This concentration also inhibited the pressor response after stimulation with the ionophore A 23187 but not after direct application of arachidonic acid (Fig. 4, Table I).

Inhibitors of intracellular calcium and of calcium-calmodulin. W 7 (calmodulin inhibitor), trifluoperazine (calcium-calmodulin inhibitor), and TMB 8 (intracellular calcium antagonist) were used in concentrations that block A 23187-induced pressor responses but not the response that follows direct application of arachidonic acid. All substances markedly blocked the pressor response after stimulation with 5 µg α-toxin. In addition, the remaining pressor response of <10–25% after the application of the different inhibitors appeared after a latent period of 5.1±1.9 min after injection of 5 µg α-toxin (control without inhibitor, 2.3±0.9 min; $P < 0.001$). The postrinsing pressor responses provoked by the lower α-toxin dose of 2.5 µg α-toxin were completely suppressed by all inhibitors in the above given concentrations ($n = 3$ for each substance; $n = 1$ for TMB 8).[3]

3. Due to a lack of material only a few experiments could be performed with TMB 8: because a concentration of 100 µM reduced the pressor response provoked by A 23187 by only 50%, the reported high concentration of 500 µM was selected (27–31).

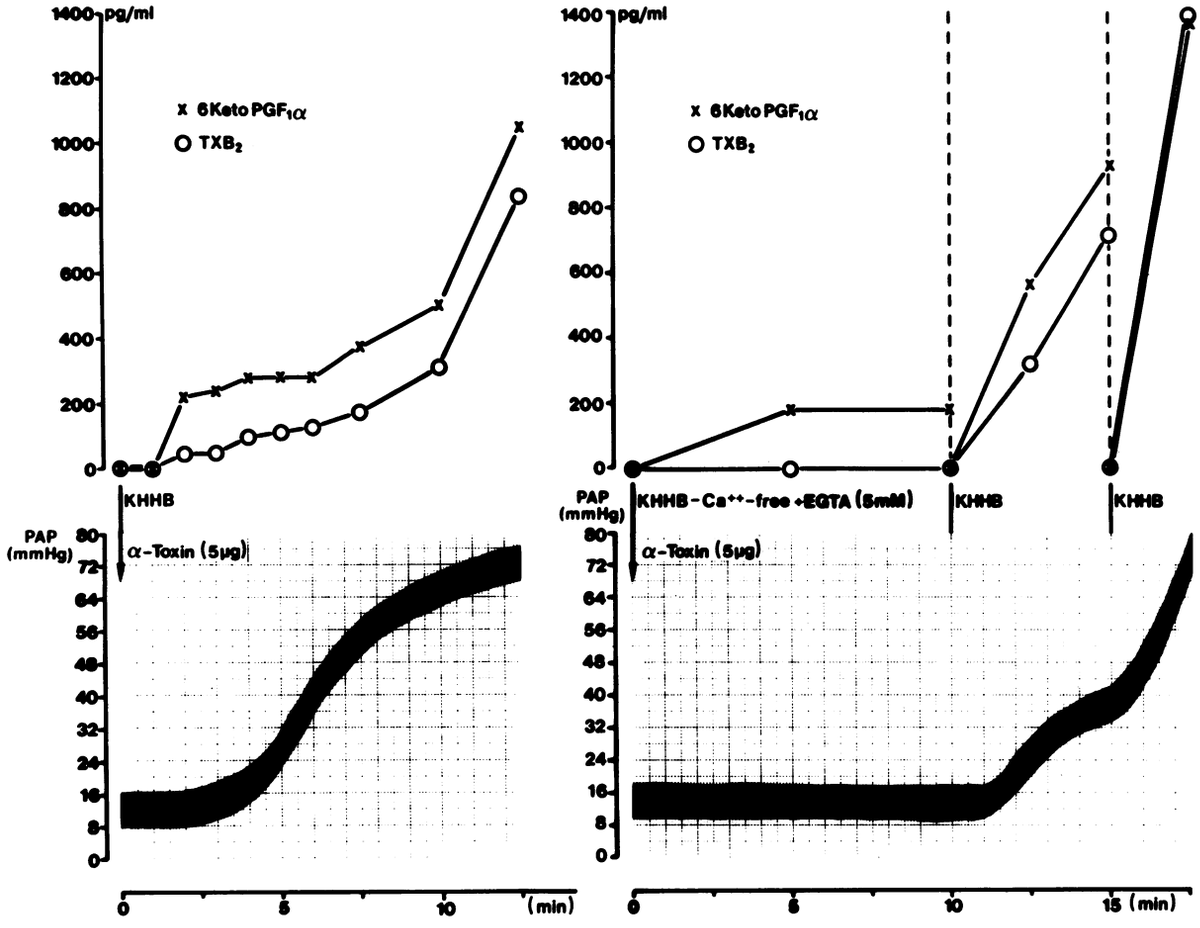


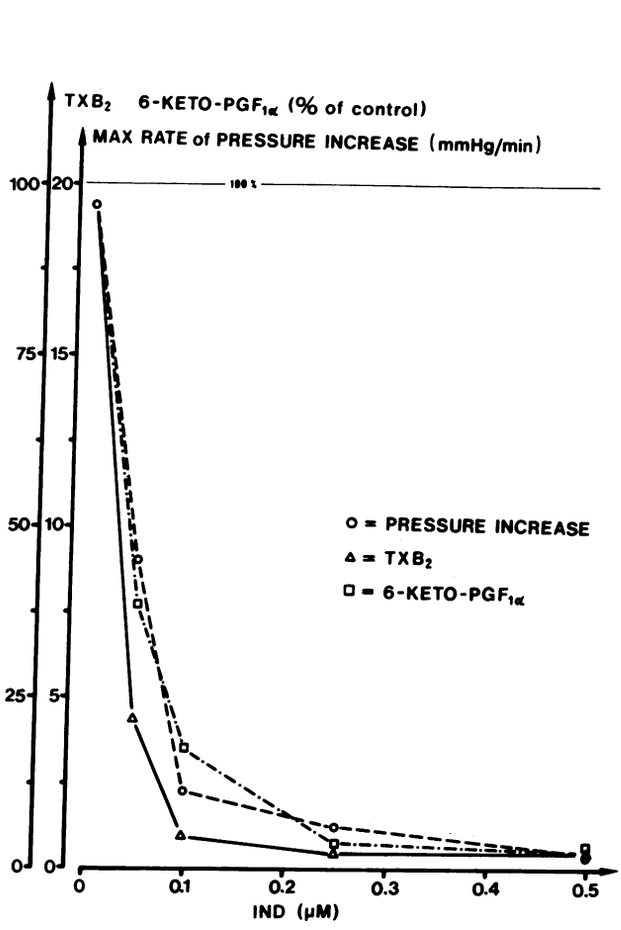
Figure 2. Course of the pulmonary artery pressure (PAP) and the concentrations of TxB_2 and 6-keto-$PGF_{1\alpha}$ in the recirculating perfusion fluid after application of 5 μg α-toxin in two different lungs in the presence and absence of intravascular calcium. The dashed lines in the righthand picture indicate the change of perfusion fluid from calcium-free (EGTA-containing) KHHB buffer to normal calcium containing KHHB. (Compared with Figs. 1 and 4 the time scale is extended.)

Pulmonary artery pressure rise after injection of the endoperoxide analogue U-46619

The endoperoxide analogue U-46619 is thought to mimic the actions of TxA_2 (12–14). Direct application of U-46619 in the concentration range in which TxB_2 was detected in the perfusion fluid after α-toxin stimulation caused an acute rise in pulmonary artery pressure: 4–16 mmHg (8.8±3.8 mmHg, n = 8) after 1 nM U-46619 and 6–24 mmHg (14.6±6.4 mmHg, n = 12) after 2 nM U-46619.

Calcium depletion in the intravascular space blocks pressor response and mediator release

After five rinsing phases with calcium-free KHHB the various stimuli were injected into the calcium-free fluid. Pressor responses due to arachidonic acid and prostaglandin $F_{2\alpha}$, neither of which depends on arachidonic acid release from phospholipid membranes, were only partly reduced, and the height of the pressure peaks was almost completely restored when the dosage was increased fourfold (Table II). In contrast, the pressure peaks after A 23187 and bradykinin, both of which depend on calcium shift and arachidonic acid release (7, 8), were markedly blocked and could barely be restored by a fourfold increase in dosage (<30%). The behavior of α-toxin mimicked the latter group (Table II, 5 and 20 μg toxin). In addition, there was no postrinsing pressor response after 2.5 μg toxin was added to calcium-free medium (n = 5). The described mode of calcium depletion did not completely suppress the reaction chain evoked by the toxin. This, however, could be achieved by the addition of EGTA (5 mM) to calcium-free KHHB (n = 3; example given in Fig. 2). Changing the perfusion fluid to calcium-containing KHHB, without a second application of the toxin, evoked a steep pressure increase without any latent period (<10 s), paralleled by an instantaneous and more than compensatory release of TxB_2 and 6-keto-$PGF_{1\alpha}$



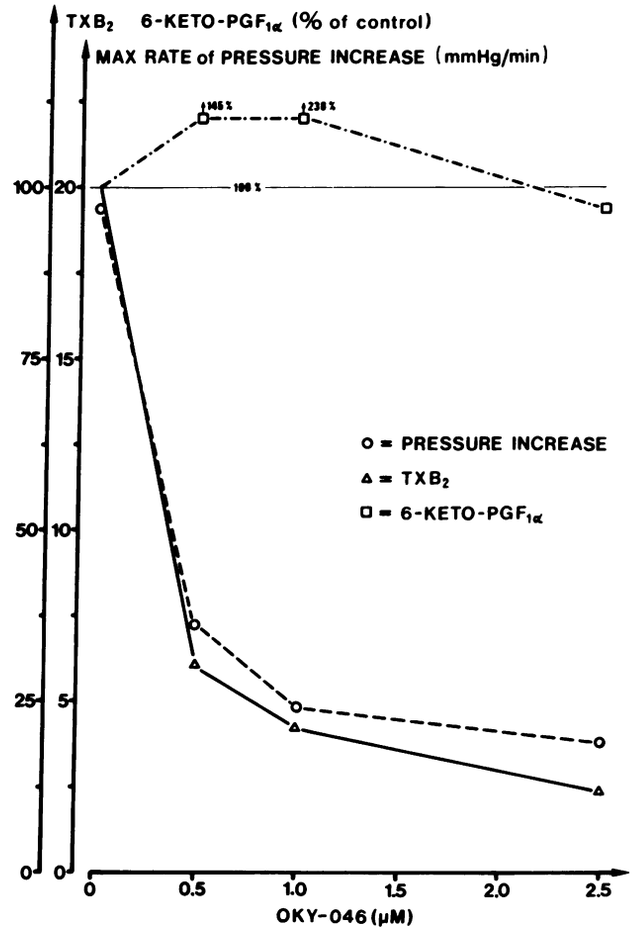


Figure 3. Dose-dependent effect of indomethacin (IND) and OKY-046 on pressure increase and mediator release after application of 5 μg α-toxin in different isolated lungs. The pressure increase is given in absolute data; the concentration of TxB_2 and 6-keto-$PGF_{1\alpha}$ in the recirculating perfusion fluid is in percentage of the control lung data (TxB_2: 1,092 pg/ml = 100%; 6-keto-$PGF_{1\alpha}$: 2,240 pg/ml = 100%). MAX, maximum.

into the recirculating perfusion fluid. It was thus the formation of these arachidonic acid metabolites in the vascular wall and not their effect on the vascular smooth muscles that was inhibited by calcium depletion in these experiments.

α-Toxin provokes potassium release, but not LDH release, from the lungs

α-Toxin provoked a dose-dependent release of potassium, but not of LDH, into the recirculating perfusion fluid (Table III). This contrasted with A 23187 which, being an ionophore specific for divalent cations, did not cause potassium release. The potassium release after the addition of α-toxin was not suppressed by the simultaneous application of indomethacin or by the use of calcium-free perfusion fluid, suggesting that these two maneuvers did not interfere with the primary toxin attack. The selective potassium release was characteristic of α-toxin (e.g., in contrast to the cytotoxin from *Pseudomonas aeruginosa,* which provokes a parallel release of potassium and LDH [15]). This was especially evident after the application of 20 μg α-toxin (in calcium-free buffer to allow a 15-min perfusion phase): a tremendous increase in the potassium concentration in the perfusion fluid ensued (0.98 mmol/liter) which was not accompanied by any increase of LDH release.

Ineffectiveness of preformed α-toxin hexamer or of toxin preincubated with specific antibodies

5 μg α-toxin self-associated to hexamers by deoxycholate contact (16) before application to the isolated lungs showed no effect on perfusion pressure, ventilation pressure, or organ weight (n = 3). The release of TxB_2 (92±30 pg/ml in 15 min), 6-keto-$PGF_{1\alpha}$ (212±58 pg/ml), and potassium (<0.05 mmol/liter) did not exceed that of the control phases. Correspondingly,

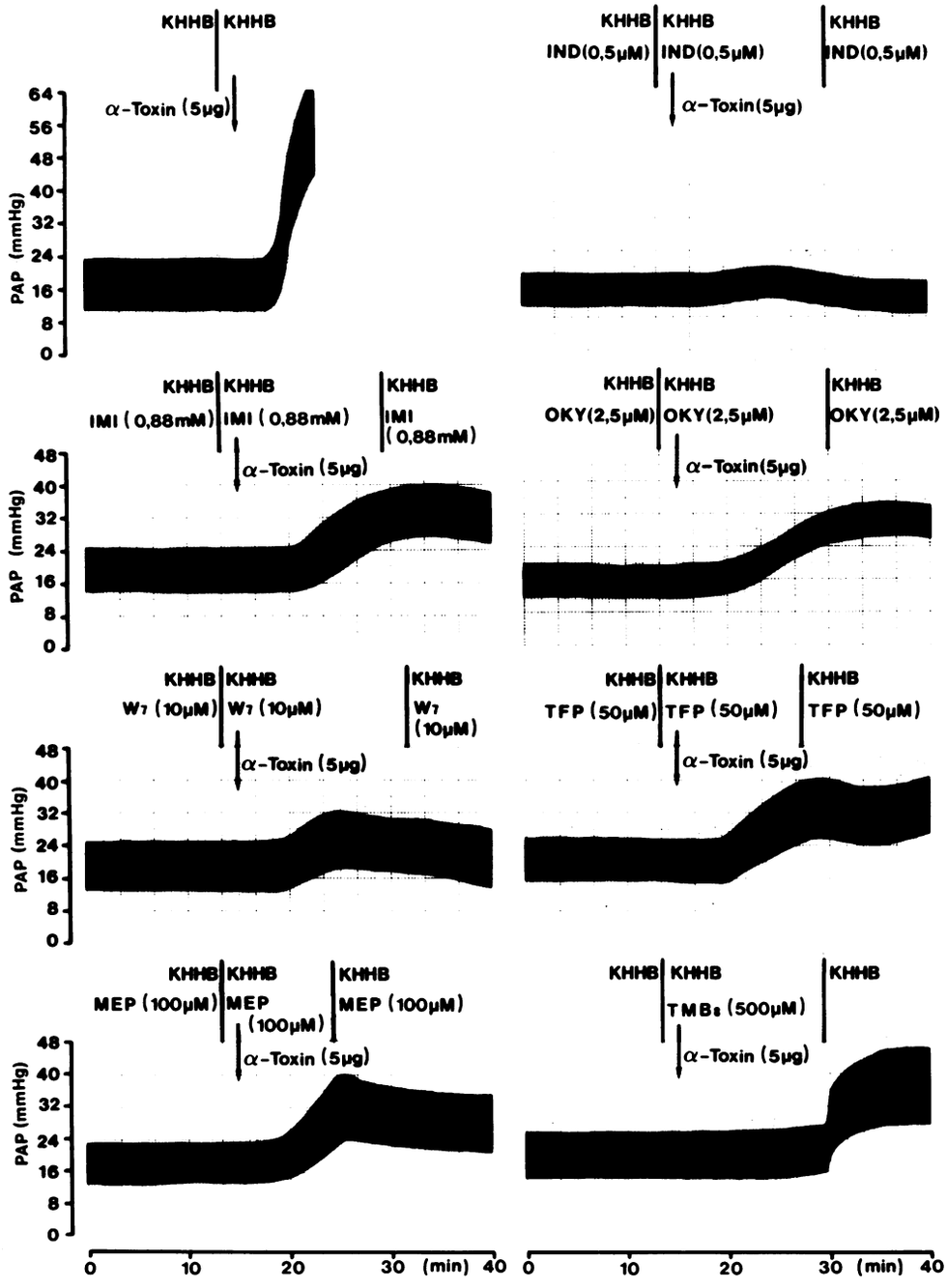


Figure 4. Blocking of the pulmonary artery pressure (PAP) increase after the administration of 5 μg α-toxin by different inhibitors. The inhibitors were applied to the perfusion fluid in the injection phase and in the preceding and following perfusion phases (exception: TMB 8). IND, indomethacin; IMI, imidazole; TFP, trifluoperazine; MEP, mepacrine.

preincubation of the α-toxin with specific antibodies completely suppressed pressor response, potassium release, and mediator release (n = 3 for 5 μg α-toxin).

Discussion

Staphylococcal α-toxin causes a pulmonary artery pressor response primarily mediated by TxA_2, which surpasses the biological effect of simultaneously formed PGI_2. In intact animals it is known that endotoxins from Gram-Negative bacteria provoke pulmonary artery hypertension, which appears to be mediated by TxA_2 (17–20). Endotoxins are, however, ineffective in isolated lungs perfused with blood- and plasma-free perfusion fluid (21 and Seeger, W., unpublished results). Here we show that a bacterial protein toxin, the α-toxin from *Staphylococcus aureus,* can provoke a fulminant pulmonary

Table I. Blocking of the Pressor Responses Induced by Arachidonic Acid, A 23187, and Staphylococcal α-Toxin by Various Inhibitors

	Arachidonic acid*		A 23187‡		
	KHHB	KHAB	KHHB	KHAB	α-Toxin§
Control	17.4±3.2 (n = 9)	16.4±7.4 (n = 15)	18.5±6.8 (n = 18)	17.2±5.5 (n = 15)	19.4±7.6 (n = 8)
Inhibitor					
Indomethacin (0.5 μM)	<1 (n = 5; $P < 0.001$)		< 1 (n = 5; $P < 0.001$)		< 1 (n = 3; $P < 0.001$)
Imidazole (0.88 mM)		2.8±2.2‖ (n = 6; $P < 0.001$)‖		3.4±1.3¶ (n = 5; $P < 0.001$)	3.0±0.5 (n = 3; $P < 0.01$)
Mepacrine (100 μM)		20.5±10.3¶ (n = 5)		2.0±2.8¶ (n = 5; $P < 0.001$)	4.5±1.7 (n = 3; $P < 0.05$)
W 7 (10 μM)	12.9±2.3 (n = 4)		<1 (n = 4; $P < 0.001$)		2.3±2.1 (n = 4; $P < 0.01$)
Trifluoperazine (50 μM)		13.7±3.5¶ (n = 4)		1.2±0.8¶ (n = 5; $P < 0.001$)	2.5±1.9 (n = 4; $P < 0.01$)
OKY-046 (1 μM)			4.4±1.6 (n = 8; $P < 0.001$)		4.9±2.3 (n = 4; $P < 0.01$)
TMB 8 (500 μM)			1.2 (n = 1)		0.8±1.8 (n = 2)

The Table gives the height (mmHg) of the initial pressor responses of the different isolated lungs (Control) and the height of one subsequent pressor response in each experiment in the presence of an inhibitor. All values are given as mean±SD. * Arachidonic acid, 1 μM in KHHB and 150 μM in Krebs Henseleit albumin (4%) buffer (KHAB) was applied. ‡ A 23187, 1 nM in KHHB and 2 μM in KHAB, was applied. These two stimuli provoked steep pressure peaks, which could be repeated several times within the same lung (8, 9). § For α-toxin the maximum rate of pressure increase (mmHg/min) is given for the control group and for each inhibitor group. ‖ and ¶ indicate values taken from previously published investigations (references 8 and 9, respectively).

artery pressor response in a concentration as low as 0.04 μg/ml perfusion fluid, and that this response occurs in the absence of plasma and circulating cells. It is apparently mediated by stimulation of the pulmonary vascular arachidonic acid cascade, and among the different arachidonic acid metabolites the cyclooxygenase product TxA_2 appears to be primarily responsible: the reaction is accompanied by the release of the stable TxA_2 metabolite TxB_2; it is dose dependent and, in higher concentrations, almost completely suppressed by inhibitors of cyclooxygenase (indomethacin [22]) and thromboxane synthetase (OKY-046 [23] and imidazole [24]). TxA_2 mimicking substance U-46619 (12–14), used in the same range of concentration in which TxB_2 is detected in the perfusion fluid after α-toxin stimulation, can provoke an acute pressure rise in the isolated lung model. The vasoconstrictive effect of TxA_2 apparently surpasses the biological effect of PGI_2, which is known to cause pulmonary vasodilatation (8) and which is released in the same or even in higher concentrations as thromboxane. This is in accordance with various other pathophysiological stimuli provoking pulmonary artery pressor responses mediated by thromboxane in isolated rabbit lungs, e.g., bradykinin and kallidin (9), fibrinmonomers (25), and phosphatidic acid (26).

In contrast to these stimuli, the calcium-ionophore A 23187 (8), and toxins such as eledoisin (9) and mellitin (Seeger, W., unpublished results), the pressor response provoked by 0.04 μg/ml α-toxin is not reversible by rinsing the lungs with fresh perfusion fluid. The cellular origin of the arachidonic acid metabolites has not been established in the isolated lung model. Endothelial cells, however, are very likely candidates for the noted PGI_2 release, since this arachidonic acid metabolite is generated by α-toxin-incubated pulmonary endothelial cells in vitro.[4] The simultaneous formation of arachidonic acid cyclooxygenase products with antagonistic effects, different kinetics of formation and turnover, and possibly different cellular sources may explain why subthreshold dosages of α-toxin provoked pressor responses not immediately, in the injection phase, but in the subsequent rinsing phase.

Triggering of the arachidonic acid cascade is strictly dependent on extracellular calcium and may be mediated by calcium-calmodulin. Calcium depletion of the vascular space markedly suppressed the toxin-induced pressor response. The addition

4. Suttorp, N., W. Seeger, S. Bhakdi, E. Dewein, and L. Roka. Submitted for publication.

Table II. Pressor Responses after Application of Various Stimuli in Calcium-free Perfusion Fluid

Stimulus	Control	Calcium-free medium	Calcium-free medium (fourfold dosage)
A 23187*	11.0±3.9 (n = 6)	<1 (n = 3; P < 0.01)	1.1±1.2 (n = 3; P < 0.01)
Bradykinin*	12.1±4.0 (n = 6)	1.2±1.1 (n = 3; P < 0.01)	2.1±1.8 (n = 3; P < 0.01)
Arachidonic acid*	14.1±2.4 (n = 7)	9.3±6.0 (n = 5)	10.6±12.8 (n = 2)
$PGF_{2\alpha}$*	14.5±16.5 (n = 2)	5.8 (n = 1)	16.0 (n = 1)
α-Toxin‡	19.4±7.6 (n = 8)	0.49±0.36 (n = 4; P < 0.001)	2.3±1.1 (n = 4; P < 0.001)

The values are given as mean±SD.
* A 23187 (1 nM in KHHB), arachidonic acid (1 μM in KHHB), bradykinin (1 μM in KHHB), and $PGF_{2\alpha}$ (100 nM in KHHB) provoked steep pressure peaks, which could be repeated several times within the same lung (8, 9). The Table gives the height (mmHg) of the initial pressor responses provoked by the stimuli in different isolated lungs (Control) and the height of one subsequent pressor response in each experiment in absence of calcium (partly with a fourfold stimulus dosage).
‡ For α-toxin the maximum rate of pressure increase (mmHg/min) is given for the Control group and for the pressor responses after stimulation with 5 or 20 μg α-toxin (fourfold dosage) in calcium-free medium.

Table III. Potassium and LDH Release into the Recirculating Perfusion Fluid after Stimulation with α-Toxin, A 23187, and Cytotoxin from Pseudomonas Aeruginosa

Stimulus	Potassium	LDH
	mmol/liter	*U/liter*
Control (KHHB, 15 min)	<0.05 (n = 30)	8.2±2.3 (n = 10)
α-Toxin (5 μg) (KHHB, 15 min)	0.12±0.06 (n = 8; P < 0.001)	5.9±3.5 (n = 8)
α-Toxin (5 μg) Indomethacin (35 μM) (KHHB, 15 min)	0.16±0.04 (n = 5; P < 0.001)	
α-Toxin (5 μg) (KHHB/Ca^{++} free, 15 min)	0.33±0.14 (n = 4; P < 0.001)	
α-Toxin (20 μg) (KHHB/Ca^{++} free, 15 min)	0.98±0.1 (n = 3; P < 0.001)	7.2±1.0 (n = 3)
A 23187 (1 nM) (KHHB, 10 min)	<0.05 (n = 5)	<4.0 (n = 5)
A 23187 (2 μM) (KHAB, 10 min)	<0.05 (n = 6)	4.5±3.2 (n = 5)
Control (KHAB, 30 min)	<0.1 (n = 5)	13±0.8 (n = 5)
Cytotoxin (13 μg/ml)* (KHAB, 30 min)	0.94±0.20 (n = 10; P < 0.001)	50±8.8 (n = 4; P < 0.001)

The different perfusion fluids (KHHB or Krebs Henseleit buffer with 4% albumin [KHAB]) and the duration of the perfusion phases are indicated. Note the selective potassium release characteristic of α-toxin, which was not effected by the presence of indomethacin or by calcium depletion. The values are given as mean±SD.
* indicates values taken from previously published investigations (15).

of EGTA to the perfusion fluid rendered the lungs refractory to the toxin stimulus, and no arachidonic acid metabolites were released. In all of these experiments, potassium efflux still occurred. This was expected since toxin-mediated membrane damage is not a calcium-dependent process (1). The results were very striking when calcium was offered again to such toxin-treated calcium-depleted lungs without a second toxin stimulus; fulminant pressor responses without any latent period ensued, paralleled in each case by an instantaneous release of the arachidonic acid metabolites TxB_2 and 6-keto-$PGF_{1\alpha}$ into the perfusion fluid (Fig. 2). In the studies with isolated endothelial cells, a dose-dependent uptake of 45-calcium after α-toxin incubation could be shown.[4] The necessity of an increase of intracellular calcium to trigger the arachidonic acid cascade after α-toxin stimulation in the isolated lung model was supported by the inhibitory effect of TMB 8, supposedly an antagonist of intracellular calcium (27–31). Since the cyclooxygenase, thromboxane synthetase, and PGI_2 synthetase steps are apparently calcium independent (32–34), it remains the release of the precursor arachidonic acid that must be inhibited by extracellular calcium depletion and by the application of TMB 8.

Phospholipase (A2/C)-dependent release of arachidonic acid from cell membranes appears to be the general rate-limiting step in arachidonic acid metabolism (35, 36). In accordance with this notion, mepacrine, reportedly an inhibitor of phospholipase A2 and possibly phospholipase C (37–40), blocked the pressor responses in the isolated lung. Does inflowing calcium stimulate phospholipase activity directly (the great majority of phospholipases being strictly calcium-dependent enzymes [41]) or via the formation of a stimulatory calcium-calmodulin complex (42–44)? Our experiments that use W 7 (31, 39) and trifluoperazine (31, 42, 45) favor the latter assumption: these substances, which interact with calmodulin and calcium-calmodulin complex, both inhibited the toxin-induced pressor response. It is clear, however, that none of these inhibitors is monospecific. More specific probes are required to provide direct evidence for the proposed role of calmodulin and phospholipase activity.

Nonphysiological calcium bypass through transmembrane toxin channels may initiate the triggering of the arachidonic acid cascade. Previous evidence obtained in the erythrocyte model indicated that α-toxin hexamerizes on target membranes to create small hydrophilic pores with a functional diameter

of 2–3 nm (1). Studies with isolated pulmonary endothelial cells showed compatible molecular sieving data with a restricted diffusion of large molecules (molecular weight > 5,000) and a passive uptake of 45-calcium after α-toxin incubation.[4] α-Toxin also caused the selective release of potassium, but not of LDH, in the isolated lung model. This differential release was characteristic: the calcium-ionophore A 23187 induced the release of neither, whereas the *Pseudomonas aeruginosa* cytotoxin released both markers (15). The potassium release was not a secondary phenomenon induced by calcium flux or arachidonic acid metabolites since it also took place in the absence of calcium and without the release of arachidonic acid metabolites. Thus, there is cause to assume that α-toxin forms transmembrane pores also in the isolated lung model. This idea is corroborated by the finding that the reintroduction of calcium into the circulation after previous toxin application in absence of calcium is followed by an immediate and more than compensatory pressure rise and by mediator release without any latent period. As expected, the injection of preformed toxin hexamers unable to attack the membrane (16) or of α-toxin preincubated with neutralizing antibodies provoked neither potassium release nor the pressor response.

Conclusion. Staphylococcal α-toxin causes a pulmonary artery pressor response in the absence of plasma and circulating cells, which is irreversible in higher concentrations. It appears to be primarily mediated by the arachidonic acid cyclooxygenase product TxA_2 which surpasses the biological effect of the simultaneously formed PGI_2. The triggering of the arachidonic acid cascade is strictly dependent on extracellular calcium and is markedly reduced by inhibitors of calcium-calmodulin complex and phospholipase. The selective release of potassium, but not of LDH, is compatible with the notion that also in the isolated lung model, α-toxin forms transmembrane pores. Calcium flux through these nonphysiological bypass channels according to the steep gradient of extracellular-to-intracellular calcium concentration may be the primary signal that triggers the arachidonic acid cascade below the threshold of overt cell damage.

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